MECHANICAL PROPERTIES
OF
MAMMALIAN SINGLE SMOOTH MUSCLE CELLS

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MECHANICAL PROPERTIES
OF
MAMMALIAN SINGLE SMOOTH MUSCLE CELLS

MECHANISCHE EIGENSCHAPPEN
VAN
GEISOLEERDE GLADDE SPIERCELLEN VAN ZOOGDIEREN

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
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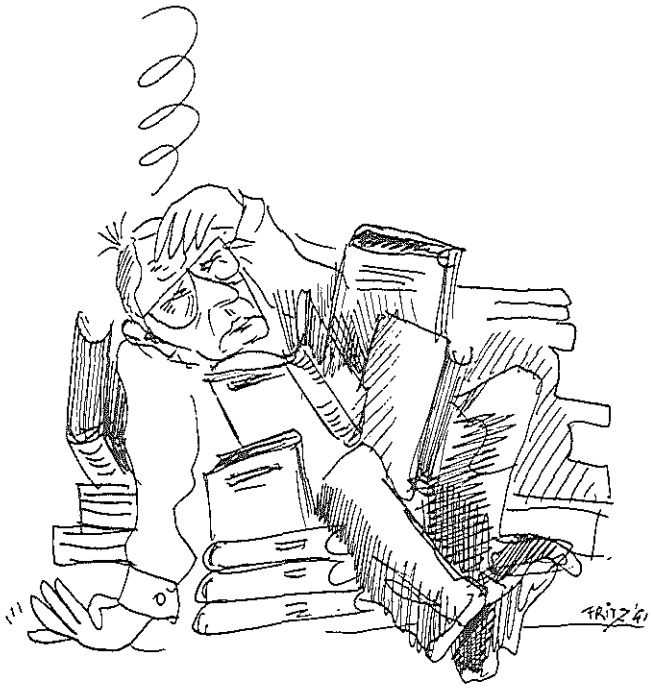
Aan mijn vader die mij de beginselen van de electronica
en het knutselen leerde welke beide zo onontbeerlijk waren
voor het tot stand komen van dit proefschrift.

Contents

Introduction

Inleiding



Introduction

A short historical review

Smooth muscle plays an important role both in human and animal life. This was recognized only rather late in the history of medicine, as primarily attention was directed towards skeletal and heart muscle. During the first half of the 20th century, for obvious reasons of knowledge and research techniques available, smooth muscle was viewed and treated in muscle research as more or less comparable to the other two muscle types.
It was not until the 1950's and 1960's that smooth muscle became fully recognized as an entity in itself with its own specific properties. Since that time a growing number of researchers became interested in it.
In the early 1970's it became apparent that smooth muscle properties could not be fully investigated in whole tissue or tissue preparations containing many cells, because the methods by which these measurements of functional properties were performed did not correspond to the morphological and histological arrangements that had already been elucidated.

At that time Fay (1975) was the first to report on a method for performing mechanical measurements on isolated single smooth muscle cells. For almost two decades the work of Fay and his co-workers was referred to as the most important on the subject of mechanical measurements on isolated single smooth muscle cells and this still is the case as far as experiments on smooth muscle cells isolated from the stomach of the toad Buffo Marinus are concerned.

Drawbacks of his method and of several of its variations were that it was restricted to rather large single smooth muscle cells, and that only cells of amphibian origin could withstand all adverse effects of isolation, preparation and cell attachment.

During the first half of the 1980's publications began to appear reporting on smooth muscle cells isolated from other types of tissues e.g. the Taenia Coli, now obtained from mammalian species such as rabbits or guinea pigs. In that same period in The Netherlands Van Dijk-Looyaard (1984) reported on mechanical measurements on single smooth muscle cells isolated from bovine coronary arteries.

In 1985 the authors of the articles in this thesis started to pursue some of their ideas on the subject of single smooth muscle cells in relation to the field of urology and urodynamics.

In urology and gynaecology and obstetrics many of the functions of the involved organs depend upon the contractions of smooth muscle cells. In these fields considerable research had already been done, in vivo and in vitro, on the function of urinary bladder and uterine tissues. Contrary to the small and large intestines, where smooth muscle cells can clearly be differentiated to run all in one direction within one layer, in the ureters, bladder and uterus, smooth muscle cells seem to be organized in a very random manner throughout the organ and, in addition, are richly embodied in connective tissue elements, such as collagen, elastine and reticuline.

This random orientation of muscle cells and muscle bundles greatly impedes the further understanding of both the passive and the active properties of the smooth muscle cells of these organs. Furthermore in whole tissue preparations the activities and properties of these cells are influenced by their innervation, by the mutual influence of smooth muscle cells on each other within the tissue and by the abundant connective tissue elements.
Therefore it seemed necessary to us to develop a method for isolation, attachment and mechanical measurement of smooth muscle cells obtained from these mammalian tissues.

The studies presented in this thesis concentrate on the isolation of single smooth muscle cells and on measurements of passive and active properties of these cells in vitro.

The structure of this thesis

In order to obtain single smooth muscle cells from the urinary bladder and uterine tissues, a method for their isolation had to be developed, as in the literature no methods for these specific tissues had been previously reported and methods intended for other tissues failed to yield acceptable results.
While developing the isolation procedure it seemed that

the only available standard for testing the quality of the isolated smooth muscle cells would be the assessment of their actual contractility. Therefore, methods and equipment were developed to assess the contractile function of smooth muscle cells in suspension without the necessity of attachment to mechanical measuring devices. These steps are described in **Chapters 2** and 3.

Once cells became routinely available, the development of an ultrasensitive microforce transducer was initiated, as no such measuring device was commercially available at that time. A study of the literature on devices for the measurement of muscle contractility showed that a virtually new transducer had to be developed in order to meet the requirements. In addition the technique of cell attachment to the transducer had to be given much attention, as these two problems cannot be separated. The related developments are described in **Chapters** 4 and 5.

In addition to the development of equipment and to the experiments mentioned above, a multitude of electrical, electronic and mechanical devices were designed and manufactured, in order to construct the complicated experimental set-up yielding the results reported in **Chapters** 6 and 7. In these chapters (6 and 7), passive and active or contractile properties of pig urinary bladder and human uterus single smooth muscle cells are reported. In this part of the thesis it is shown that the developed methods and instruments performed well and could be used to gain further knowledge about the properties and functioning of mammalian smooth muscle cells.

Still, in the future new techniques and methods will have to be developed in order to improve the reproducibility of the measurements, to simplify the methods of attachment and to enhance the sensitivity and stability of the total measuring device.

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Muscle cell isolation techniques

Spiercel isolatiemethoden

Isolation and individual electrical stimulation of single smooth-muscle cells from the urinary bladder of the pig

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Summary

In contrast to striated muscle, measurements on strips of smooth muscle cannot be uniquely interpreted in terms of an array of contractile units. Therefore scaling down to the single-cell level is necessary to gain detailed understanding of the contractile process in this type of muscle. The present study describes the development of a method for isolating contractile single smooth muscle cells from pig urinary bladders. Contractile responses evoked by individual electrical stimulation were used as a measure of cell quality during development of the method. Responses were evaluated by measuring latency, contraction and relaxation times, as indicated by visible length changes, and stored on-line in a computer. Initial length, relative shortening and shortening speed were determined by measuring cell lengths in previously timed still video frames using a computer-controlled crosshair device. Increase of stimulus pulse duration resulted in improved responses, indicating that the observed shortening represented a physiological contractile response. Ultimately this method of evaluation was applied to two sets of cell preparations obtained by two different methods, one using only collagenase digestion, the other using mechanical manipulation as well. Both sets showed two main patterns of response to electrical stimulation: a pattern of contraction upon stimulation followed by enhanced contraction when stimulation was switched off (CK), and a pattern of contraction upon stimulation followed by relaxation when the stimulus was switched off (CR).

The set of preparations containing the highest percentage of CR cells was found to be superior (i.e. greater initial length, shorter latency and contraction times, increased shortening and higher shortening speed). The method of isolation used for this set gives a high yield of contractile cells available for experimental use over a long span of time.

Introduction

Much research has been done on the contractility of skeletal and heart muscle. More recently smooth muscle and its contractile properties have become a field of interest (Murphy, 1976; Hellstrand, 1979; Van Mastrigt & Griffiths, 1979). As yet, however, little is known about the basic contractile units of the smooth muscle of specific human organs. In the field of urology, all knowledge of bladder contractility has been deduced from *in vitro* experiments on bladder strips (Van Mastrigt & Glerum, 1985; Van Mastrigt & Griffiths, 1986). Since, in contast to skeletal muscle, the organization of, and the interaction between, the smallest contractile units in smooth muscle is not immediately evident, it is impossible to interpret the measurements uniquely in terms of such units (Meiss, 1975; van Duyl, 1985). The scaling down of experiments to the single-cell level thus seems inevitable (Fay *et al.*, 1976; Fay, 1977). Experiments on single cells are promising because unwanted and/or unknown influences from neighbouring cells, connective tissue elements such as collagen and elastin, innervation and vascularization can be avoided, so making it possible to carry out very direct mechanical, pharmacological and electrophysiological experiments. It is expected that these experiments will open up new and interesting ways of evaluating biopsies from smooth-muscle organs such as the human bladder.

In the present study a method is described for obtaining viable, contractile, single smooth-muscle cells from pig bladders. Pig bladders were chosen, because they proved both anatomically and mechanically to be a good model of the human bladder (Douglas, 1972) and are easily obtainable. To evaluate the quality of the cells isolated using this empirically designed method, electrical stimulation experiments were performed, in which initial length, latency, contraction time, percentage shortening and unstrained speed of shortening were measured. The effects of variations in stimulus parameters were also investigated (Van Mastrigt & Glerum, 1985).

Material and methods

Pig bladders were collected from the local abattoir approximately 15 minutes after slaughter. After opening

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and emptying, two or three pieces measuring about 2 × 3 cm were cut out of the anterior wall, immersed in cold (4° C, pH 7.35) Krebs–Hepes buffer (fluid B, see Appendix, Table 1) in an insulated vessel and transported to the laboratory.

Krebs–Hepes buffer is a modification of the original Krebs buffer (fluid A). To ensure adequate buffering without gas bubbling, Hepes was added to fluid A (the stock solution) and volume corrections were made so as to restore the correct osmolarity.

Approximately 1 h after death of the animal, one of the strips was taken out and pinned to the bottom of a shallow container in preoxygenated (95% O_2/5% CO_2) Krebs–Hepes buffer (fluid B) at room temperature and its mucosa was carefully stripped away. About 25 pieces of muscle of about 2 × 4 mm were cut and immediately immersed in 4 ml of fluid B, contained in a 60 mm Petri dish. They were minced carefully with scissors until the largest dimension was about 1.5 mm. The collagenase and other constituents of the isolation solution (see Appendix, Table 2) were dissolved in 6 ml of fluid B and added to the contents of the Petri dish.

The complete contents were poured into a plastic vessel of diameter 65 mm, containing a magnetic stirring rod of length 15 mm. The lid was tightly closed and the vessel was placed in a heated (37° C) water bath and stirred at 1 to 2 rev. s^{-1} for 50 min (phase 1). Next 10 mg of DNAase, dissolved in 2 ml of fluid B, was added and stirring was continued for another 15 min (phase 2). Following this incubation period, 6 ml of MEM containing 10% FCS (fluid C), was added to the cell suspension.

The cell suspension was filtered with a 400 µm Cellector tissue sieve, after which the flocculent remnants were resuspended in fluid D, preoxygenated with 95% O_2/5% CO_2. The filtrate was divided into three portions which were centrifuged at 1000 rev. min^{-1} for 5 min. After centrifugation the supernatant was sucked away with a narrow-bore Pasteur pipette and the sediment was carefully resuspended in 3 ml of fluid D. This preparation is referred to as Fraction I.

Fraction II was prepared mechanically from the resuspended flocculent remnants, by sucking in and blowing out the partly digested tissue pieces through a series of specially made pipettes with successively smaller internal diameters in the range 5.0 to 0.5 mm, so as to loosen partly attached cells, continuing until the suspension became clearly opalescent. The remaining tissue parts were then sieved out with a 400 µm sieve, the filtrate thus forming Fraction II. Fraction II was immediately used for experiments, whilst Fraction I was kept in a vibration-free refrigerator for 6 to 8 h.

For the incubation of the cells during the electrical stimulation experiments, a modified version of a 35 mm culture dish incubator (Ince *et al.*, 1983) with built-in temperature regulation was used, mounted on a Zeiss IM inverted microscope. Temperature was kept at 37.0 ± 0.1° C. The atmosphere above the preparation, immersed in fluid D, was kept at approximately pO_2 150 mmHg and pCO_2 38 mmHg by gently blowing an air/5% CO_2 mixture over the fluid surface, so as to provide proper equilibration. Thus a pH of 7.35 was attained. To prevent evaporation a thin layer of Klearol was spread over the fluid surface (Van Dijk *et al.*, 1984). Prior to the electrical stimulation experiments cells were left for 1 h to accommodate and settle on the bottom of the culture dish. All procedures were conducted as far as possible aseptically to prevent bacterial contamination.

Electrical stimulation was performed using a 50 µm platinum wire electrode, drawn into a glass micropipette, which was mounted on a motor-driven micromanipulator. A 1 mm diameter silver wire lying on the bottom near the side of the culture dish was used as an earth electrode.

In each experiment 48 structures presumed to be smooth muscle cells were stimulated individually, by placing the micro-electrode near the chosen cell and applying an electrical pulse train until a few seconds after maximum shortening had been attained. Cells were picked out randomly over the whole bottom surface of the culture dish. The cell concentration was kept low so as to allow individual stimulation.

Stimulation pulses of 1.8 V amplitude and 5.0, 10.0 or 15.0 ms duration, and with a repetition frequency of 50.0 Hz, were generated by a standard pulse generator and amplified by a Kepco operational power amplifier. The pulse train was switched on and off by a relay controlled by a push button.

A black and white video camera was mounted via a beam-splitting device upon the IM microscope. Microscope and micromanipulator were placed upon a vibration-damped table. The video camera was driven by sync signals from the video-monitor output of a DEC PC 350 computer. The video signals from the computer and the camera were mixed and displayed on a high-resolution black and white monitor and recorded on a U-matic video recorder. The computer displayed a real-time clock, an event-marker clock and stimulus on/off and event-marker indicators, as well as additional information about the preparation and stimulus parameters in use.

The indicators were controlled by the stimulus control relay and by push buttons, which were depressed at the beginning and end of contraction and relaxation of the cell under observation. The times of activation of the indicators were stored on-line in pairs of records, one pair for each contraction–relaxation cycle and up to 48 pairs in one experiment file. Whenever a cell responded properly to electrical stimulation and timing was considered to be accurate, a quality character was added to the record pair.

Following the experiments measurements of length and calculations of length change and contraction velocity were performed off-line in still video frames, using a specially developed computer-controlled cross-hair device (Glerum *et al.*, 1986). By taking the exact frames in which the chosen events had been timed, the lengths before, during and after contraction and relaxation were measured. The measured values, together with one character describing the reaction pattern of the cell during stimulation and one character describing the pattern after switching off the stimulus, were added to the corresponding record pairs, which in this way ultimately contained experiment number, day, time, stimulus on and off times, start and end of contraction times, start and end of relaxation times, initial length, length at maximum contraction, length before relaxation and length at maximum relaxation and a character pair describing the reaction pattern of a specific cell.

The data obtained in this way were placed in groups corresponding to the reaction patterns found and to the various fractions and the different stimulus parameters used. Initially analysis of variance was performed in order to judge whether data from separate experiments could be combined, but as not all groups of data showed a normal distribution, further tests were performed non-parametrically. Thus correlations were investigated using Spearman's rank correlation coefficient and differences between groups were tested for significance by the Mann-Whitney U test. A significance level of 0.05 was used in all cases.

Results

In order to investigate the contractile responses quantitatively, 23 electrical stimulation experiments were performed on 13 preparations from eight successive isolation procedures. Each experiment consisted of 48 attempts to stimulate a cell individually, a different cell being used for every attempt. After every isolation Fraction II was tested for contractile behaviour with 10.0, 5.0 or 15.0 ms pulses. In five cases Fraction I, which had been stored in the refrigerator during the Fraction II tests, was tested with 10.0 ms pulses, once followed by a test with 5.0 ms pulses and once followed by a test with 15.0 ms pulses. Thus in total 1104 structures resembling smooth muscle cells were individually tested for a contractile response to electrical stimulation. A typical example of a cell from Fraction I before, during and after stimulation is shown in Figs 1a–1c.

The appearance of the smooth-muscle cells ranged from rather short (80–150 μm) cigar-shaped bodies with variable diameters (10–20 μm) to long (150–350 μm) more thread-like (approx. 10 μm wide) structures. In phase contrast the shorter cells displayed more contrast relative to the background than the longer cells. Some of the longer cells showed fine longitudinal striation. The shorter cells more often had irregularities in the shape of the cell wall (invaginations, evaginations, bends, etc.) than did the longer cells, but these features were not

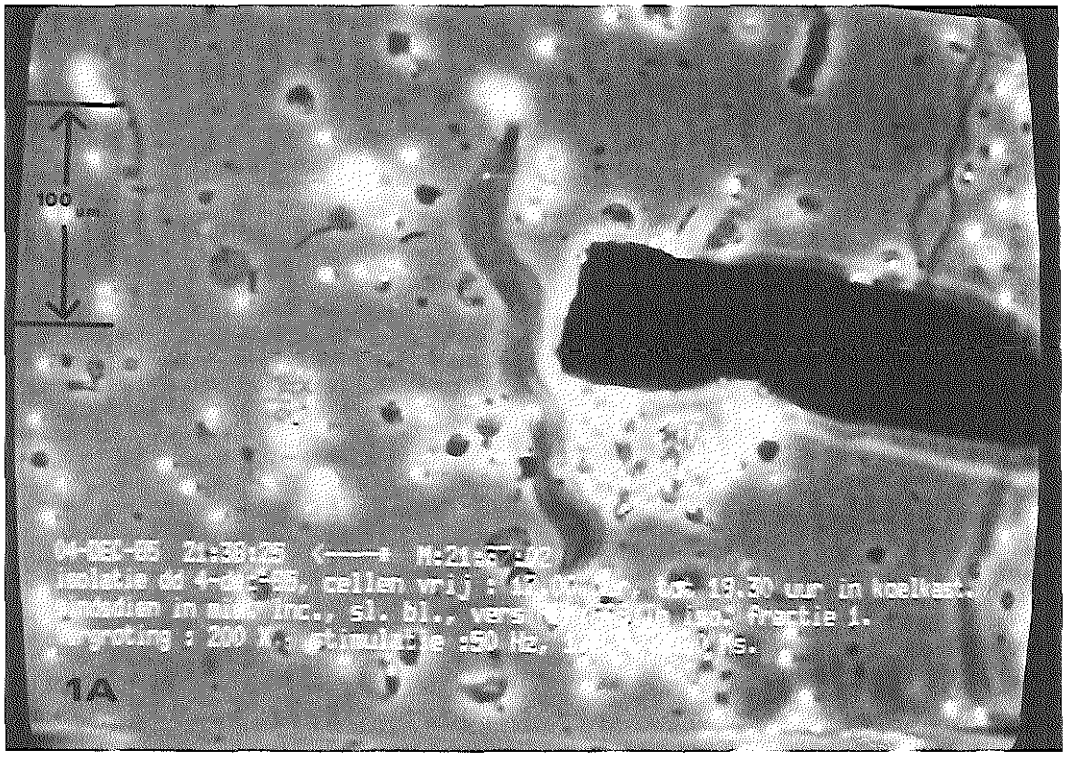
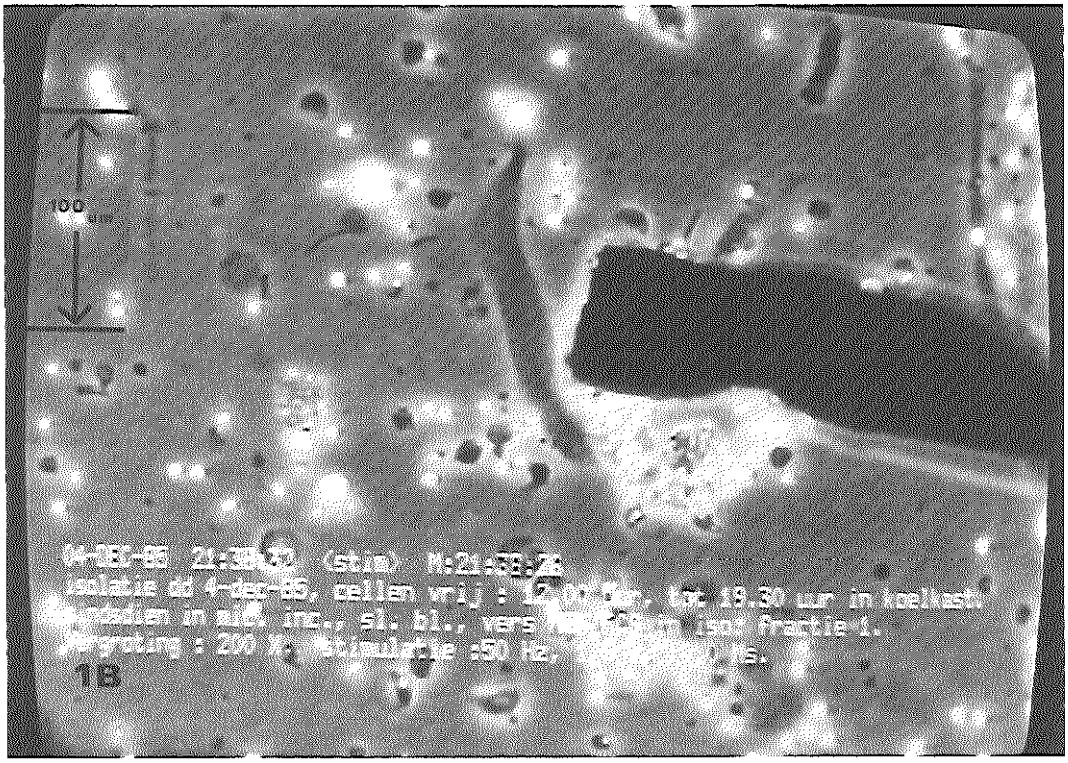


Fig. 1. Typical example of a single smooth muscle cell, from Fraction I, isolated from the bladder of the pig: (a) before, (b) during and (c) after electrical stimulation with a train of pulses of 10.0 ms duration and 1.8 V amplitude at a repetition frequency of 50.0 Hz. Photographs were made from a mixed video display of the phase contrast microscope image and the computer screen. The stimulation electrode (50 μm platinum wire) can be seen in the right half of each picture.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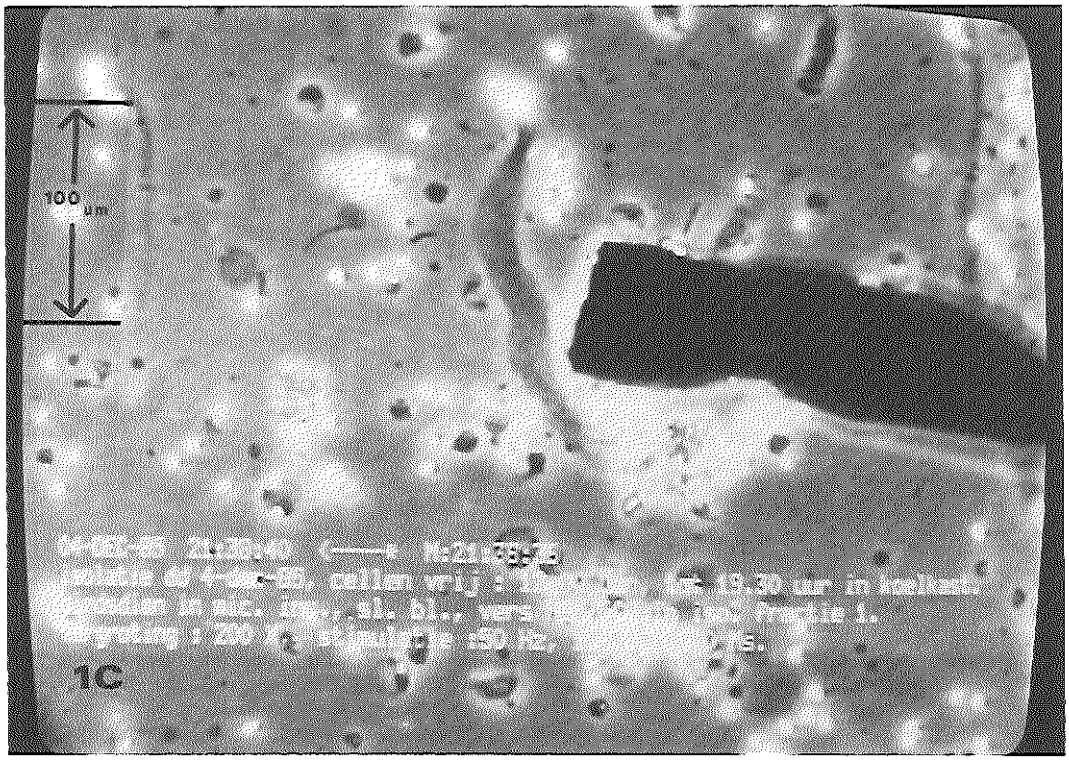
cellen in elch. inc.: sl. bl., vers [illegible] isol. fractie 1.
Bepaling : 200 Hz, Stimulatie 850 Hz, [illegible] µs.

1B


100 µm
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isolatie dd 4-dec-86, cellen vrij : 1[illegible], kwt 19.30 uur in koelkast.
cellen in elch. inc.: sl. bl., vers [illegible] isol. fractie 1.
Bepaling : 200 Hz, Stimulatie 850 Hz, [illegible] µs.

1C

Table 1. The major constituents of the fluids used during transport, cell isolation and electrical stimulation.

Constituent ions and molecules	*Fluid A (Krebs buffer)* (mmol litre^{-1})	*Fluid B (Krebs–Hepes buffer)* (mmol litre^{-1})	*Fluid C (MEM + 10% FCS)* (mmol litre^{-1})	*Fluid D* (2/3 B + 1/3 C) (mmol litre^{-1})
NaCl	118.0	104.8	116.3	108.6
KCl	4.7	4.2	5.4	4.6
$NaHCO_3$	25.0	22.2	26.2	23.5
KH_2PO_4	1.2	1.1	—	0.7
NaH_2PO_4	—	—	1.0	0.3
$CaCl_2$	1.8	1.6	1.8	1.7
$MgSO_4$	1.2	1.1	0.8	1.0
D-Glucose	11.0	9.8	5.6	8.4
Hepes	—	22.2	—	14.8
NaOH	—	13.3	—	8.9
Osmolarity (mOsmol litre^{-1})	316.6	316.6	310.4*	314.5*

The Krebs–Hepes buffer is made out of stock solution A by adding 25 mmol Hepes per litre, adjusting the pH to 7.35 with 1 mmol litre^{-1} NaOH and correcting the osmolarity by addition of distilled water to a total volume of 1.126.1. Fluid D is the result of adding one part of fluid C to two parts of fluid B. *The non-listed organic constituents of MEM were not taken into account in calculating the osmolarity. Measurements of osmolarity showed only minor differences from the calculated values.

Table 2. The constituents, with their respective concentrations, of the isolation solutions used in the first and second phases of the enzymatic incubation period.

	Concentration in fluid B			
	Phase 1 isolation solution		*Phase 2 isolation solution*	
	(mg 10 ml^{-1})	(%)	(mg 10 ml^{-1})	(%)
Albumin fraction 5	100	10.0	83.3	8.3
Antitrypsin II-O	60	6.0	50	5.0
Collagenase CLS IV	30	3.0	25	2.5
DNAase DN-25	—	—	8.3	0.8

The solution for phase 1 is made by dissolving albumin, antitrypsin and collagenase in 6 ml of Krebs–Hepes buffer and adding this to 4 ml of the same fluid containing the minced tissue. The phase 2 solution is obtained by adding 10 mg of DNAase dissolved in 2 ml of Krebs–Hepes buffer to the phase 1 solution.

consistently observed in a particular phase of the contraction cycle and also appeared in the resting state in varying degrees.

The two most frequently observed patterns of response were contraction upon electrical stimulation, followed by an enhanced contraction when the stimulus was switched off (code CK), and contraction upon electrical stimulation, followed by relaxation when the stimulus was switched off (code CR). Other, less frequently observed patterns were irreversible contraction into a globular form, loss of cell contents during contraction and explosion during the course of contraction.

Table 3 shows the total number of cells stimulated in both fractions and their distribution over the main types of reaction patterns.

Table 3. Numbers and percentages of smooth-muscle cells in two differently isolated fractions, showing various types of reactions upon individual electrical stimulation.

	Total number of attempts	*CK pattern*		*CR pattern*		*Miscellaneous patterns*		*Shortening, but by less than 5% of initial length*	
	(*n*)	(*n*)	(%)	(*n*)	(%)	(*n*)	(%)	(*n*)	(%)
Fraction I	336	58	17	192	57	58	17	22	6.5
Fraction II	768	369	48	55	7	265	35	51	6.6
Total	1104	Overall response: 1070 (97%).							

Fraction I = cells obtained using only enzymatic digestion. Fraction II = cells obtained by enzymatic pretreatment followed by mechanical loosening. CK pattern = contraction upon stimulation, enhanced contraction after stimulus is switched off; CR pattern = contraction upon stimulation, relaxation after stimulus is switched off; miscellaneous patterns = cells showing loss of internal material or explosion during contraction, contraction into a globular form or elongation upon stimulation. Shortening, but by less than 5% of initial length = cells responding according to the CK or CR pattern but shortening less than 5% of initial length.

In all responding cells the following variables were measured: *initial length*, before the start of the stimulation; *latency time*, from the beginning of stimulation until the first visible movement; *contraction time*, from the moment of first movement until maximum shortening; *relative shortened length*, i.e. the length at maximum shortening, shown as a percentage of the initial length and *shortening velocity*, the quotient of absolute shortening and contraction time.

To avoid large errors in variables such as the relative shortening and the unloaded shortening velocity, cells that showed a relative change in length

Table 4. Differences between variables from cells showing the same reaction pattern (CK or CR) and originating from the same fraction (I or II), but stimulated with pulses of different duration (5.0 ms, 10.0 ms or 15.0 ms).

Groups analysed by U tests ( ) = S.D.	*Number*	*Initial length* (μm)	*Latency time* (s)	*Contraction time* (s)	*Relative shortened length* (%)	*Shortening velocity* (μm s^{-1})
Fraction I CR						
5.0 ms	36	263 (90)	0.8 (0.4)	13.4 (8.0)	83 (12)	3.9 (2.9)
Significance		>	>	>	>	<
10.0 ms	118	196 (81)	0.8 (0.8)	8.1 (5.2)	66 (20)	10.4 (9.1)
Significance		=	=	>	>	<
15.0 ms	38	219 (74)	0.7 (0.2)	5.9 (3.1)	57 (14)	19.0 (11.0)
Fraction II CK						
5.0 ms	86	91 (34)	6.1 (9.2)	11. (7.0)	82 (12)	1.9 (1.7)
Significance		=	>	=	>	<
10.0 ms	194	97 (36)	3.5 (4.0)	9.8 (6.5)	77 (15)	3.7 (11.0)
Significance		=	=	>	>	<
15.0 ms	89	93 (31)	2.6 (2.6)	8.3 (4.6)	71 (16)	3.9 (2.9)

In every case the repetition frequency was 50 Hz and the voltage was 1.8 V. Significance according to the Mann-Whitney U-test at the 5% level is indicated with < or > sign; = indicates no significant difference. Values of variables are shown as averages (standard deviation).

of less than 5% on stimulation were excluded from further analysis (see Table 3). Also the cells showing miscellaneous reaction patterns were excluded from further analysis.

Analysis of variance showed that, in most cases, particularly those from Fraction I experiments, there was no significant variation between variables measured in corresponding groups from different experiments. Therefore corresponding data were combined and jointly analysed.

Cells from Fraction I showed a rather flat distribution of initial lengths, whereas the distribution of initial length of the Fraction II cells was almost normal, though somewhat skewed to the shorter lengths.

Non-parametric correlation tests demonstrated a strong correlation (Spearman's rank correlation coefficient about 0.8) between initial length and length at maximum shortening, but the correlation between initial length and absolute shortening, although significant, was only about 0.4. The correlation between relative shortening and initial length was marginal ($0.05 < P < 0.10$), suggesting that the relative shortening is more or less constant over the total initial length range.

The experiments with pulses of different duration (5.0, 10.0 and 15.0 ms) showed that pulses of longer duration yielded shorter latency and contraction times, greater relative shortening and a higher velocity of shortening. In most cases these differences were statistically significant (see Table 4).

The further analyses presented in this paper are based on the experiments using a pulse duration of 10.0 ms, which form the majority of all experiments performed.

Table 5 shows that cells showing the CK pattern tended to shorten to a lesser degree and more slowly than cells showing the CR pattern.

Table 6 shows that cells in Fraction I preparations generally shortened more quickly, with shorter latency and contraction times, than cells from Fraction II preparations. The differences in latency and contraction times were more pronounced in CK cells, whereas CR cells showed a larger difference in shortening velocity and also showed a much larger initial length in Fraction I preparations.

Discussion

Since the pig bladder contains substantial amounts of dense connective tissue structures in the form of collagen, elastin and reticulin, it makes severe demands on any method for isolating viable single cells. Rather aggressive enzymes and/or long incubation times are necessary and this means that the cells may be damaged by toxic remnants in the enzyme preparation, or by deviations of the pH from the physiological range caused by the products of enzymatic digestion. To minimize such damage an efficient enzyme system and a high-capacity buffer system are needed. These were ultimately found in the form of Worthington's Collagenase · CLS IV, which is not a very highly purified enzyme preparation, and a high-concentration Hepes buffer. The Hepes buffer eliminated the need for inconvenient pH adjustments (Obara, 1984) or for vigorous bubbling with CO_2, which damages the cells. During the development of the method, a number of published isolation methods (Chamley-Campbell *et al.*, 1979; Ishii & Takahashi, 1982; Thüroff *et al.*, 1982,

Table 5. Differences between variables estimated for cells reacting with either the CK or the CR pattern, as a response to stimulation with 1.8 V, 10.0 ms pulses at a repetition frequency of 50 Hz, for both Fractions I and II.

Groups analysed by U test ( ) = S.D.	*Number*	*Initial length* (μm)	*Latency time* (s)	*Contraction time* (s)	*Relative shortened length* (%)	*Shortening velocity* (μm s^{-1})
Fraction I						
10.0 ms CK	49	104 (51)	2.5 (3.9)	7.3 (4.5)	79 (11)	3.5 (2.4)
Significance		<	>	=	>	<
10.0 ms CR	118	196 (81)	0.8 (0.8)	8.1 (5.2)	66 (20)	10.4 (9.1)
Fraction II						
10.0 ms CK	194	97 (36)	3.5 (4.0)	9.8 (6.5)	77 (15)	3.7 (11)
Significance		=	=	=	>	<
10.0 ms CR	26	116 (26)	2.4 (3.0)	9.0 (4.4)	68 (15)	5.4 (5.1)

Significance according to the Mann-Whitney U-test at the 5% level is indicated with < or > sign; = indicates no significant difference. Values of variables shown as averages (standard deviation).

Table 6. Differences between variables from cells showing the same reaction pattern as a result of the same stimulus, but originating from the two differently isolated fractions.

Groups analysed by U test ( ) = S.D. 10.0 ms, CK	*Number*	*Initial length* (μm)	*Latency time* (s)	*Contraction time* (s)	*Relative shortened length* (%)	*Shortening velocity* (μm s^{-1})
Fraction I	49	104 (51)	2.5 (3.9)	7.3 (4.5)	79 (11)	3.5 (2.4)
Significance		=	<	<	=	>
Fraction II	194	97 (36)	3.5 (4.0)	9.8 (6.5)	77 (15)	3.7 (11)
10.0 ms, CR						
Fraction I	118	196 (81)	0.8 (0.8)	8.1 (5.2)	66 (20)	10.4 (9.1)
Significance		>	=	=	=	>
Fraction II	26	116 (72)	2.4 (3.0)	9.0 (4.4)	68 (15)	5.4 (5.1)

Significance according to the Mann-Whitney U-test at the 5% level is indicated with < or > sign; = indicates no significant difference. Values of variables shown as averages (standard deviation).

1983; van Dijk *et al.*, 1984) were tried without success. Although some of these methods resulted in reasonable cell yields, with Trypan Blue tests showing up to 95% exclusion, none of the preparations showed any form of contractile reaction on pharmacological or electrical stimulation. Contractility thus seems to be a more stringent requirement than Trypan Blue exclusion, and so it was used as a measure of the quality of the preparation during the further development of the method.

In order to test the contractility of individual cells in a reproducible manner, electrical stimulation was used. During the first trials fouling of the electrode by organic materials attracted by electrophoretic forces, posed a troublesome problem, which was overcome by diluting the original incubation fluid (MEM with 10% FCS) with twice its volume of Krebs–Hepes buffer (fluid D in Table 1). This resulted in a lower organic content, without removing all nourishment for the cells. A voltage of 1.8 V proved to be the maximum level at which, with the combination of platinum and silver electrodes used in these studies, effective stimulation was possible without eliciting electrolysis near the electrodes. Cells could be stimulated repeatedly to contract, thus showing that the shortening observed represented a physiological contractile response.

The isolation method described in this paper yields contractile cells which can be used continuously for at least 12 hours without loss of contractility or viability. In pilot experiments, contractile cells isolated in this way were cultured successfully, but the contractile properties were lost within the first few culture generations (Chamley-Campbell & Campbell, 1981). However, preparations can easily be preserved for use in later experiments, e.g. on the next day, without noticeable loss of contractile properties, by storage in closed plastic tubes or vessels in a vibration-free refrigerator at 4° C. Cooling may even have a relaxing effect on the cells, as is indicated by the significantly longer initial length found in the Fraction I preparations (see Table 6), which were kept in the refrigerator for at least 6 hours before testing. A possible alternative explanation for the different initial cell lengths observed in preparations from Fractions I and II is that Fraction II cells may already be in a partially contracted state induced by the relatively large forces used to loosen them mechanically.

Inspection of slides of Bouin-fixed Gomori-stained unstretched and unstimulated pig bladder material (the same as used for the isolations) suggested that, in adequately isolated preparations, the minimum cell length should be at least 80–100 μm. This criterion was met by both our cell preparations (see Table 4).

Comparison of all aspects of Fractions I and II suggests that Fraction I preparations are, for both CK and CR groups, superior to Fraction II preparations for the following reasons:

(1) In general Fraction I shows significantly longer initial cell lengths than Fraction II (see Table 6 for 10.0 ms groups).

(2) Fraction I tends to show shorter latency and contraction times (see Table 6).

(3) Fraction I cells contract with higher velocities (see Table 6), and the values are closer to those

published in the literature for other types of smooth-muscle cells (Bagby, 1974; Murphy, 1976; Small, 1977).

(4) Fraction I preparations contain a far higher percentage of cells responding to stimulation with the CR pattern than Fraction II preparations (see Table 3).

The CK or off-response pattern has been stated to occur in various kinds of smooth-muscle preparations, usually involving nerve stimulation (strips or organs) (De Carle *et al.*, 1977; Fox & Daniel, 1979; Papasova *et al.*, 1981; Hong *et al.* 1985). We believe, however, that for single cells it is a less physiological response than the CR pattern for the following reasons:

(1) Cells showing the CK pattern have a significantly smaller initial length than cells showing the CR pattern, possibly indicating contraction caused by mechanical manipulation and/or by ionic leakage following cell wall damage.

(2) Cells responding with the CK pattern show longer latency and contraction times, a smaller degree of shortening and a lower contraction velocity than those responding with the CR pattern (see Table 5).

In the literature dealing with the smooth muscle of the urinary bladder, there is an ongoing discussion about the pathways via which electrical field stimulation of bladder strips *in vitro* leads to contraction and whether direct stimulation of the cells is possible (Cowan & Daniel, 1982; Sjogren *et al.*, 1982; Sibley, 1984; Kinder & Mundy, 1985). In this series of experiments, we have shown that single cells contract in response to direct electrical stimulation, provided that the pulse duration is 5.0 ms or longer, and this implies that direct stimulation of bladder strips should be possible.

The dependence of responses on pulse duration (see Table 4) shows a general pattern of improvement in the latency time, the contraction time, the relative shortened length and the shortening velocity as the pulse duration is increased from 5.0 to 15.0 ms. This indicates that the responses obtained represent a physiological contraction and not a destructive electrocoagulation phenomenon. Moreover, repeated stimulation leads to repetition of the response.

Our conclusions may be summarized as follows.

The method that has been described for isolating smooth-muscle cells from pig bladders is simple and suitable for routine experimental use. It gives a satisfactory yield of cells with reproducible properties, especially when the Fraction I preparation is used.

Cells obtained with this method are ready for use in mechanical, electrophysiological or pharmacological experiments, and a sufficient number of viable cells is available for at least 12 hours after isolation. Furthermore, the cells can be stored without deterioration.

Factors influencing the viability and ultimately the contractile properties of the isolated cells are: the toxic contaminants remaining in the enzyme preparations, the pH and the amount of mechanical manipulations carried out in isolating the cells.

Direct electrical stimulation of single smooth-muscle cells from pig bladders has been shown to be possible. The consistent dependence of the contractile responses on the stimulus parameters demonstrates the validity of the stimulation experiments and the results obtained. If bladder strips are stimulated electrically with adequate stimuli, at least part of the response must be due to direct stimulation of the cells.

Acknowledgements

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Appendix

List of chemicals, enzymes and additives.

Albumin, Bovine, Fraction V, No. A-4503, Sigma Chemical Company.

Antitrypsin, from chicken egg white, type II-O, No. T-9253, Sigma Chemical Company.

Collagenase, CLS IV, 160 U mg^{-1}, Worthington Diagnostic Systems Inc.

Deoxyribonuclease 1, from Beef Pancreas, 410 Kunitz Units/mg, No. DN-25, Sigma Chemical Company.

FCS, Foetal Calf Serum, B 97140-308-3, Commonwealth Serum Laboratories, Melbourne, Australia.

Hepes, 4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid, No. H-3375, Sigma Chemical Company.

Klearol, Witco Chemical B.V. (Sonneborn Division), Haarlem, The Netherlands.

Krebs buffer: standard stock solution derived without D-glucose, made out of first-grade components, pharmaceutical department, Dykzigt Academic Hospital, Rotterdam, The Netherlands.

MEM, Earle's Salts, dry powdered medium, Cat. No. 072-1100, Gibco Europe BV, The Netherlands.

Tables 1 and 2 show the composition of the fluids and reagents used for transport, isolation and experiments. All fluids shown in Table 1 were sterilized by filtration and stored at 4° C. Foetal calf serum was added sterilely to the MEM solution in a 10% (v/v) concentration.

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Cross-hair device

Kruisdraadapparaat

Technical note

Low-cost computer-controlled asynchronous-video cross-hair device

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Keywords—*Computer control, Cross-hair device, Length measurements, Nonsynchronisable video source, Still video frames*

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1 Introduction

CLINICIANS and medical research workers often wish to measure simple variables, e.g. the length or diameter of objects such as cells, tissue structures or blood vessels, on previously recorded video tapes. In contrast to on-line measurements made in live video pictures directly produced by a video camera, there is no easy way to synchronise video signals from a measuring computer with signals from a video recorder, except by using very expensive time-base corrector units. If, however, the variable to be measured (e.g. length) is based on a series of two or more x–y coordinates, a movable cursor or cross-hair in the selected still video frame may be adequate. Relatively simple hardware can then be used to overcome all the synchronisation problems that are encountered when working with non-synchronisable video sources.

If the measuring device is controlled by an on-line computer, measured positions and resulting variables can be directly stored and/or processed. A design for a device of this kind is presented in this paper. It forms an accurate, versatile and useful instrument, and can be built at relatively low cost.

2 Requirements and practical design

(1) The electronic device should add a moveable cross-hair with computer-controlled position to any type of standard video source.
(2) No synchronisation between controlling computer and cross-hair device or video source should be necessary.
(3) The presentation of the cross-hair and its position should be jitter-free, no matter how irregular the timing of the incoming signal may be.

In order to meet these requirements, the cross-hair is added to the displayed video frame as follows. In every frame the frame sync pulse is detected and used to load a set of line counters (Y1 and Y2, see Fig. 1) with a vertical position value from the computer. The subsequent line sync pulses are used to clock these counters until the loaded value is reached, whereupon one line is set to a white video level, thus forming the horizontal part of the cross-hair.

The vertical part of the cross-hair is made in a similar way, using the line sync pulses as data-loading signals for the dot or pixel counters (X1 and X2). These counters are clocked by a 4·8 MHz dot clock signal until the loaded value is reached, whereupon one pixel is set to a white video level. In this way, with just a single white pixel per line, all lines together form the vertical line of the cross-hair.

Sync pulses of the incoming video signal are detected with a TBA 311 (IC 9) and modified to exact durations and TTL levels with an LS 221 (IC 10).

The dot frequency of 4·8 MHz is generated with an Am2925 dot clock generator (IC 11), whose output circuitry is started on the trailing edge of each line sync pulse and stopped on the leading edge of the following line sync pulse, so as to synchronise the dot clock with the tracing of the lines. Both line and dot counters load on the trailing edges of the respective frame and line sync pulses, so as to time the loading and counting processes exactly.

(4) Position and presentation resolution should meet the pixel and line resolution of the video source used.

Most video recorders in current use have a bandwidth which limits the resolution to not much more than 256 pixels per line or about 300 lines in one frame. Therefore a matrix of 256 × 256 position points displayed in the pre-recorded video frame is adequate, and so 8-bit digital words are used to represent the horizontal and vertical cross-hair positions.

(5) In order to be able to control the cross-hair device with most types of computers, parallel data input is preferred.
(6) When the controlling computer is not sending data, the cross-hair position should be maintained and only completely positioned cross-hairs should be visible, without any disturbing side-effects caused by the asynchronous updating of the position data.

To meet requirements (5) and (6), position data have to be saved as long as no (new) data are being transferred from the computer to the device. For this purpose latches H1 and H2, and V1 and V2 (IC 13 and 14), for horizontal

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and vertical position data respectively, are used. The combined horizontal and vertical latches contain two 8-bit words for the actual cross-hair position being displayed.

As in our particular case only six free parallel binary outputs were available on the computer, the 8-bit position data words were split up into 4-bit words. The remaining two binary outputs were used for synchronisation of data transfer, one for a data sync pulse, occurring only once in every complete set of position data, the other for an enable pulse, indicating the validity of the 4-bit data word presented.

Fig. 2 shows the Fortran 77 subroutine and communications protocol used to position the cross-hair by means of a DEC 350 personal computer. Fig. 3 shows how the data are transferred from the controlling computer to the cross-hair device.

Data transfer proceeds as follows. First the lower significant bits of the x position are fed into the data inputs together with the sync pulse which resets the data sequence counter (IC 16). Next the enable pulse is given. On its trailing edge the data sequence counter is clocked to its next position. Meanwhile, the enable pulse has latched

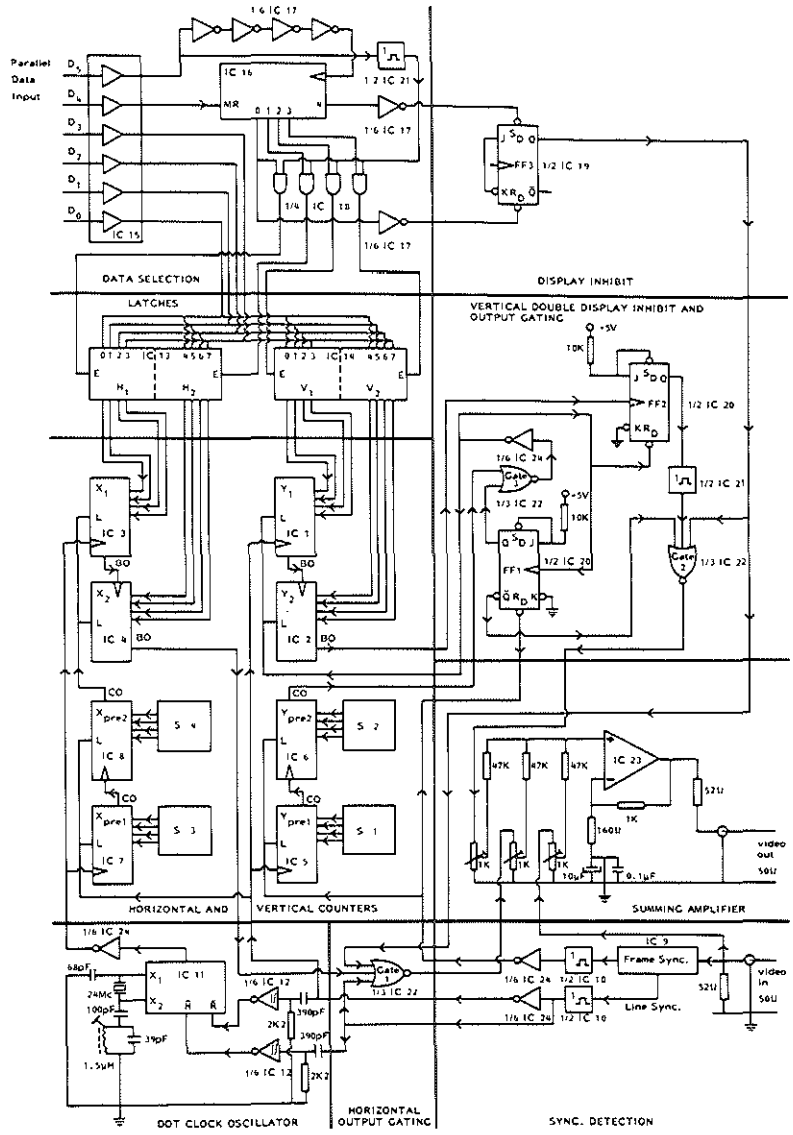

Fig. 1 *Circuit diagram of cross-hair device: IC 1–IC 8: LS 193, IC 9: TBA 311, IC10 and IC 21: LS 221, IC 11: AM 2925, IC 12: LS 14, IC 13 and IC 14: 4508, IC15: 4050, IC 16: 4017, IC 17: 4069, IC 18: 4081, IC 19 and IC 20: LS 109, IC 22: LS 27, IC 23: HA 5195 + LH 0002, IC 24: LS 04; S1–S 4: hexadecimal switch*

the data in the lower significant x position latches (H1). Then the upper significant bits of the x position are fed into the data input, followed by the corresponding enable pulse (H2). The transfer sequence is completed in a similar way with the lower and upper significant bits of the y position (V1 and V2).

The data sequence counter allows a second similar device to be addressed in order to have two cross-hairs in one video frame.

To meet requirement (6) completely, cross-hair display is

```
      SUBROUTINE VIDCUR(X, Y)
C
C     THIS SUBROUTINE CONTROLS A VIDEO CROSS-HAIR CURSOR USING HARDWARE
C     ATTACHED TO THE DIGITAL OUTPUTS ON THE PC350 REALTIME INTERFACE.
C     X AND Y ARE IN CM.
C     VIDCUR CALLS DOUTM, FROM THE DEC REALTIME INTERFACE LIBRARY, FOR
C     ACTUAL OUTPUT OF EIGHTBIT WORDS:
C     BITS 0-3: DATA BITS, 0 = LS, 3 = MS, CONTAIN POSITION DATA
C     BIT    4: SYNC
C     BIT    5: ENABLE (CLOCK)
C     BITS 6-7: FOR ADDRESSING AND NOT AVAILABLE TO CROSS-HAIR DEVICE
C
      BYTE IBYT(9)
      DIMENSION ISTAT(2), IOUT(9)
      EQUIVALENCE (IOUT(1), IBYT(1))
      IXCNT=X*255./24.
      IYCNT = 255-Y*255./16.
      IOUT(2)=IAND(IXCNT,'F'X)                ! LOW  FOUR X BITS
      IOUT (4) = IAND ( ISHFT ( IXCNT,-4) , 'F'X)   ! HIGH FOUR X BITS
      IOUT(6)=IAND(IYCNT,'F'X)                ! LOW  FOUR Y BITS
      IOUT(8)=IAND(ISHFT(IYCNT,-4),'F'X)      ! HIGH FOUR Y BITS
C
C                                       !     ES
C                                       !     NY
C                                       !     AN
C                                       !     BC
C                                       !     L
C                                       !     E DATA
C
      IOUT(1)=IOR('90'X,IOUT(2))        ! 10    01XL  O W
      IOUT(2)=IOR('A0'X,IOUT(2))        ! 10    10XL  O W
      IOUT(3)=IOR('80'X,IOUT(4))        ! 10    00XH  I G
      IOUT(4)=IOR('A0'X,IOUT(4))        ! 10    10XH  I G
      IOUT(5)=IOR('80'X,IOUT(6))        ! 10    00YL  O W
      IOUT(6)=IOR('A0'X,IOUT(6))        ! 10    10YL  O W
      IOUT(7)=IOR('80'X,IOUT(8))        ! 10    00YH  I G
      IOUT(8)=IOR('A0'X,IOUT(8))        ! 10    10YH  I G
      IOUT(9)=IOUT(7)                   ! 10    00YH  I G
      DO 10 I=1,9
   10 IBYT(I)=IOUT(I)
      CALL DOUTM(ISTAT,8, IBYT,9,'B',1,1)
      RETURN
      END
```

Fig. 2 *Fortran-77 routine for transferring position data to the cross-hair device*

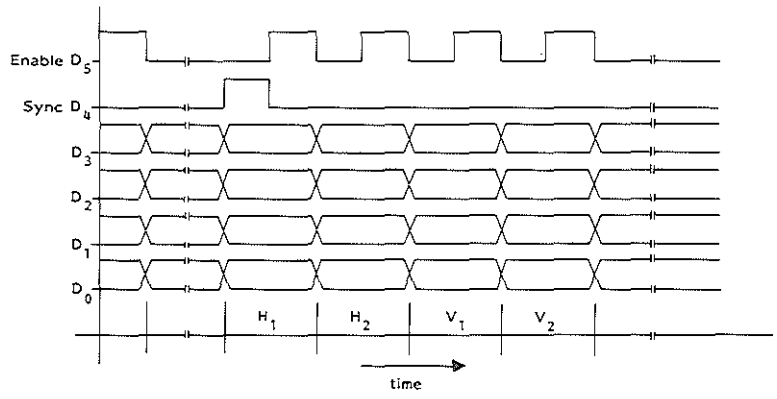

Fig. 3 *Timing diagram of the data transfer sequence: D_0 to D_3 : position data bits, D_4 : sync pulse, D_5 : enable pulse, H_1 : transfer of lower four bits for horizontal position, H_2 : transfer of upper four bits for horizontal position, V_1 : transfer of lower four bits for vertical position, V_2 : transfer of upper four bits for vertical position. Time scale depends upon the data transfer rate*

inhibited by setting a D-type flip-flop (FF 3) with the zero output of the data sequence counter and resetting it with the 4 output of the same counter, so gating the x and y video outputs through the triple-input NOR gates 1 and 2.

(7) The number of lines displayed in one frame is 312·5 for a 625-line video system. As this number exceeds 256 and as particular monitors might not display some lines of a frame or parts of lines, offsetting should be possible in order to position the range of cross-hair movement centrally in the displayed area.
(8) No double display of horizontal or vertical parts of the cross-hair should occur.

Position range alignment and offsets are provided by two sets of LS 193 counters (Xpre1 and 2 and Ypre1 and 2), one set for each direction, which delay the timing of the respective counter loading pulses by a number of dots or lines that can be preset with hexadecimal switches.

Double display of the vertical part of the cross-hair normally does not occur because the counting process is restarted in time by the next line sync pulse, which reloads the dot counters with data from the latches. Double display of the horizontal part of the cross-hair is prevented by two additional flip-flops, FF 1 and FF 2 and NOR gates 3 and 2, which prevent a second loading of the y position counters or further line pulses from occurring in the same frame.

In order to make the horizontal line white over its whole visible trajectory, and also to prevent disturbance of line sync pulses delivered to the display monitor, an LS221 (1/2 IC 21) one shot is employed to time the length of the white line. Ultimately, the x and y video outputs are combined with the original video signal in a video summing amplifier (IC 23) and fed into the display monitor.

3 Results and discussion

The hardware of the device was built on one Eurocard, the high-frequency parts such as the dot clock generator being soldered and carefully decoupled, the rest of the circuit being wire wrapped. The apparatus gives a very stable, well defined cross-hair, even when the incoming video signal is of bad quality with irregular sync pulses caused by poor video recording techniques.

Although the vertical range of the device is limited to 256 of the 312 lines theoretically displayed, there is no shrinkage of the effective area, as the first 25 lines following the frame sync pulse are used for vertical flyback, and also about 5 lines in the upper and lower parts of the frame are not actually displayed by most monitors. Thus only 10 lines in the upper and lower parts of the displayed area cannot be reached without changing the vertical offset. As the object of interest is normally situated in the middle part of the screen and seldom reaches the top or bottom, adding more operational lines to the vertical range seems unnecessary.

The cross-hair moves without flicker, the smoothness of its movement depending on the magnitude of the position changes sent by the computer (in our case this was selectable: 1, 10 or 50 lines or pixels per position step). If the controlling computer is programmed to send both horizontal and vertical position changes within one data transfer block, simultaneous movement in both directions is possible.

The minimum time in which the screen can be traversed is one frame period, but the actual time depends on the data transfer rate and the size of the position steps. With our DEC PC 350 and a step size of 10 pixels or lines, a full screen traverse takes 2 s.

To obtain maximum accuracy, the device was calibrated as follows: with the same microscope, objective (20×), camera and video recorder as used during the experiments, recordings were made of a microscope calibration slide, showing a graticule with a division in microns. The slide was placed in the vertical, horizontal and diagonal directions and also at intermediate angles. The vertical and horizontal recordings were replayed and measured with the cross-hair device, thus yielding scale factors in the vertical and horizontal directions. These scale factors were then incorporated in the software and all the graticule recordings were replayed and measured. Even in measurements in oblique directions the greatest errors encountered were only approximately 2 μm, thus showing that the overall precision was 2 μm or 1 per cent, which is close to the theoretical value of $1/256 \times 2 \times 100 = 0{\cdot}8$ per cent (the factor 2 represents the 50 per cent duty cycle of the dot clock or the non-scanned area between two lines for the two parts of the cross-hair, respectively)

More than 4000 length measurements have been made with this device in video recordings of contracting single smooth muscle cells isolated from pig urinary bladders (GLERUM *et al.*, 1987). During the measurements the data obtained were processed and stored in files together with other data concerning the cells being measured.

In our opinion the device described here solves the problem of making length measurements in pre-recorded video pictures. In applications which require only measurement of simple position data, its low cost (material: approximately US $ 50) and ease of construction (approximately 25 hours, including debugging) outweigh the advantages of more expensive systems embodying time-base correctors (approximately US $ 5000) and/or frame grabbers (approximately US $ 5000) or even more expensive dedicated video computers. Only if direct manipulation of the video information, such as image subtraction or contrast enhancement, is desired, is it worth considering such costly machines.

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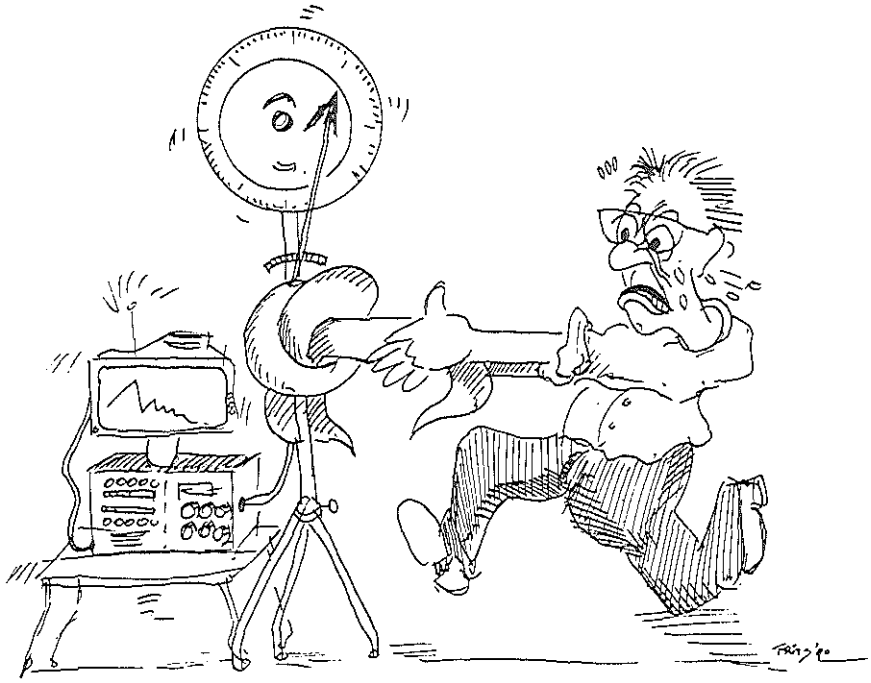
Microforce transducer

Krachtopnemer



Mechanical properties of mammalian single smooth muscle cells
I. A low cost large range microforce transducer

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Summary

A transducer has been developed for measuring the minute forces generated during isometric contractions (1.0–10.0 μN) of single smooth muscle cells from the pig urinary bladder and the human uterus.

In addition to its high sensitivity, resolution and stability (100 mV μN^{-1}, < 0.1 μN and < 2.0 $\mu N\ h^{-1}$), the transducer features a very wide range (100–140 μN) with good linearity, enabling measurement of contractions as well as passive force–length characteristics within one uninterrupted measurement session.

Since the transducer features an independent and interchangeable force to displacement conversion system, different force ranges can be realized by inserting force conversion systems with different compliances.

Introduction

To measure passive force–length characteristics of single smooth muscle cells from pig urinary bladders or human uteruses over the whole length range of these cells, 100–200 μm, one needs a force transducer with: (1) a large linear range; (2) a small compliance; (3) a longitudinal pull on the cell; (4) a horizontal working axis (so as to keep the cell in focus while stretching more and more) and (5) a good long term stability. For contractility measurements, the transducer should also: (6) have a high sensitivity; (7) show a good short term stability, and (8) be insensitive to electrical currents or fields (resulting from electrical stimulation) and, as mammalian cells are to be investigated at 37° C, it should, (9) be insensitive to external heat sources. Because measurements are conducted under a microscope, the transducer should (10) not be sensitive to external light sources, and (11) be sufficiently small to fit easily on the object stage without occupying to much space necessary for other instruments and manipulators. For electrophysiological measurements on the same cell, (12) the transducer should not emit any stray magnetic fields or (13) cause electric currents in the incubation bath. To avoid unnecessary loss of valuable experimentation time the transducer should be (14) repairable instantaneously at low cost.

A considerable number of papers on ultra sensitive force transducers have recently been published (Meiss, 1971, 1974; Minns & Franz, 1972; Canaday & Fay 1976; etc). The most commonly known designs are the transducers according to Canaday, Warshaw and Fay (Canaday & Fay, 1976; Warshaw & Fay, 1983), and the variations to the design of Meiss (1971, 1974). Other transducer types were published by Ishii (Ishii & Takahashi, 1982) and Wieringa *et al.*, (1984) and more recently Iwazumi and Tung introduced two (Iwazumi, 1982; Tung, 1986) very sensitive designs. None of these meets all the above design criteria. The design proposed by Fay and coworkers is very sensitive, and has a high resonant frequency and a good long term stability, but it has a rather limited force range and is very large in physical dimensions, owing to its cold light source and light conduction cables, which make it sensitive to mechanical noise. The Meiss type transducers and the design by Minns & Franz, according to the literature, (Canaday & Fay, 1976) lack the necessary stability and sensitivity. For the Meiss transducers this is probably owing to the spring or pivot suspension of a lever beam, whereas both designs do need considerable alterations to permit single cell attachment. The design by Ishii employs a very long light path, compromising long term stability, and uses suction as a method of cell attachment with a non axial pull on the cell. The design by Wieringa *et al*, (1984) involves an insufficient method of cell attachment showing slip at a force level of approximately 1.5 μN (Van Dijk *et al.*, 1984; Glerum & Van Mastrigt, 1990) and has a poor bandwidth

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due to its video method for position detection. Also the maximum length to which the cell can be stretched is rather limited. In Iwazumi's design stretching of the cells is limited to a few micrometers. The transducer system by Tung also seems to be rather impractical as far as the application of length changes is concerned and lacks a truly longitudinal pull on the cell, making recurrent calculations of the cell's contact region with the probe tip necessary as its diameter changes with increasing length.

Therefore a new type of sensitive, rugged, but also small transducer system, fit for isometric contraction- and stress relaxation measurements, with a longitudinal, horizontal working axis, was developed.

Principals of operation

Mechanical

The system is principally formed by a stainless steel rod suspended at two points by quartz cantilever beams. The width (1.0 mm) of the quartz cantilever beams is large compared to their thickness (80 μm), so that bending takes place in one preferred plane. As the rod is suspended at two, rather distant, points, no up–down or sideways movement or inclination can take place, rendering a system moving according to one horizontal axis.

As the bending of *one* beam is given by:

$$d' = 4FL^3/(Ebh^3) \quad (1)$$

where: d' = displacement (m), F = force (N), L = length of the beam (m), E = elastic modulus of quartz (Nm^{-2}), b = width of the beam (m), and h = thickness of the beam (m), (McLaughlin, 1977), the displacement of the rod due to bending of *two* quartz suspension beams is given by:

$$d = 2FL^3/(Ebh^3) \quad (2)$$

where: d = displacement of the rod (m).

The compliance of the moving system thus is given by:

$$C = d/F = 2L^3/(Ebh^3) \quad (3)$$

where: C = compliance (m/N).

The resonant frequency is determined by:

$$f = 1/2\pi \times \sqrt{1/(C \times m)} \quad (4)$$

where: f = resonant frequency (Hz), m = moving mass (kg), (McLaughlin, 1977).

Optical

A vane, containing a tiny slit, is connected to the rear end of the rod and interrupts a homogeneous infra red light bundle. Displacement of the rod causes a small displacement of the resultant light spot projected on a dual planar photo diode pair. The difference in the photo voltages from both diodes forms a measure for the position of the light spot and thus for the position of the rod.

Electrical

A position dependent output signal is obtained by amplifying the difference signal from the photo diodes by a differential amplifier. Since both the sensitivity and the capacitance of the photo cells are dependent on the width of the P–N border layer, an inverse bias voltage is applied to enhance cell sensitivity and to obtain a maximum cut off frequency.

To avoid DC drifting, resulting from the high gain input stages and, even more important, from infra red light emission of surrounding structures, a carrier system, involving a chopper phase detection technique is used. In this way $1/f$ noise of the input stages is also converted to a frequency band above the low-pass frequency of the output filter, thus effectively increasing the signal to noise ratio.

Construction details

Mechanical

The transducer consists of 4 major mechanical parts, (Fig. 1A, B): (1) a main body, (2) a light path tube, (3) an active element suspension cylinder and (4) a motor driven mechanical zero adjustment mechanism.

The main body is formed by a brass cylinder with an external diameter of 40 mm, and a length of 60 mm. Its front end is open and has an inner diameter of 20 mm, its rear end is closed and accommodates the light path tube through an 18 mm diameter cross hole, perpendicular to the main shaft. The light path tube is also a brass cylinder, into whose ends the infra red power LED and the bi-cellular photo cell, with their respective holders and fastening screws, exactly fit.

The active element suspension cylinder is machined from stainless steel, and fits like a brass-steel bearing into the main body. The rod runs through a 2 mm diameter longitudinal hole in the suspension cylinder. It is made of the same type of stainless steel, in order to minimise expansion differences between the rod and the suspension cylinder caused by temperature changes. At the rear end of the rod a stainless steel vane, with a 0.8 mm wide, 5 mm high vertical slit is point welded. The quartz suspension beams are glued to the front and back rims of the suspension cylinder, and to the rod, with ultra violet light bounding glue, (type: Loctite U.V. glue 358, Loctite Limited, Dublin, Ireland).

The front of the suspension cylinder is covered with a perspex cover through which the rod tip extends approximately 3 mm, in order to prevent temperature differences between the two quartz suspension beams.

The suspension cylinder can be shifted in and out of the transducer main body and set to its mechanical zero position by a DC motor driven screw spindel.

Optical

The bi-cellular photo diode is soldered with its back to a small brass cylinder; both fit exactly in a mounting hole in

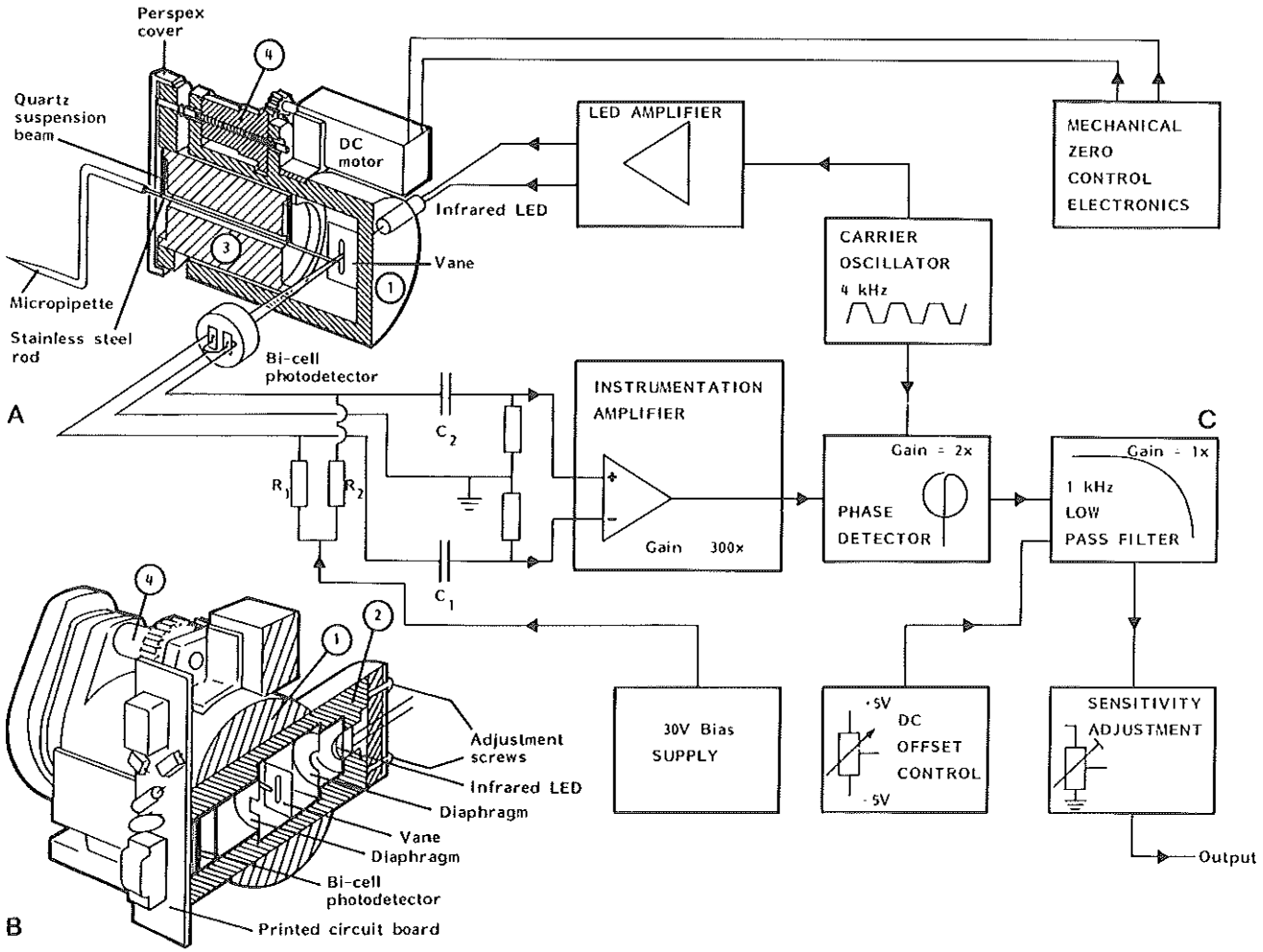

Fig. 1. Schematic diagram of the ultrasensitive force transducer. (A), longitudinal section in the vertical plane of the opto-mechanical parts of the transducer, illustrating how the stainless steel rod is suspended by quartz beams and how the infra red light bundle is intercepted by the vane attached to the rod: (1) = main body, (3) = active element suspension cylinder, (4) = mechanical zero adjustment mechanism. (B), cross section in the vertical plane through the light path tube: (2) = light path tube. It illustrates the arrangement of the infra red LED, the photo cell, the diaphragms and the vane in the light path tube. (C) shows a block diagram of the electronic circuitry and illustrates how the various subsystems are interconnected and related to the opto-mechanical parts of the transducer.

the light path tube. The back of this cylinder is soldered to a double sided glass-epoxy printed circuit board containing the primary amplifier stages. Three screws both align the photo cell and fix this combination to the light path tube.

The infra red power LED, also having a small printed circuit board at its back, is mounted in a similar way to the other end of the light path tube, but has three additional alignment screws to direct the light bundle exactly at the photo cells. To prevent undesirable stray light effects, two diaphragms are placed inside the light path tube, one in front of the LED and one in front of the bi-cell photo detector.

Electrical

Figure 1C shows a block diagram of the electronics and their connections to the opto-mechanical parts of the transducer system.

Central to the design is a 4 kHz, square wave, 50% duty cycle, carrier oscillator which injects its signal both to the LED driver amplifier and to the chopper phase detector.

The LED driver circuit sets the quiescent current of the LED and amplifies the carrier signal to the proper output power level.

The photo cells are supplied, through resistors 1 and 2, with a 30 V negative bias voltage to ensure optimum sensitivity, linearity and bandwidth.

The AC component of the photo voltages of both photo diodes is fed through capacitors 1 and 2 to the differential inputs of an instrumentation amplifier circuit with an overall differential gain of 300.

The differential photo signal is demodulated in a chopper type phase detector circuit by switching an operational amplifier from non-inverting to inverting gain in phase with the carrier signal, resulting in a position dependent DC voltage.

An elliptical type, 1 kHz low-pass output filter, removes all undesired high frequency signal components and provides optimum carrier frequency depression.

The DC offset control allows for accurate zeroing and subtraction of high passive force levels, using a ten turn precision potentiometer at a rate of 1 V per revolution to a maximum offset of $\pm$ 5 V.

At the final output of the electronic circuit the overall transducer sensitivity can be calibrated to a desired level, e.g. 10 μN V^{-1}.

A separate electronic system provides step-wise control of the DC motor which is driving the mechanical zero adjustment screw spindel, resulting in displacement steps of 0.2 um.

Calibration and performance

A prototype of the transducer was built using two quartz ($E = 7 \times 10^{10}$ N m^{-2}) suspension beams, each of 13.0 mm length, 1.0 mm width and 80 μm thickness. As the glue droplets immobilized the suspension beams over a length of 0.5 mm at each end, the effective length of the beams was approximately 12.0 mm. Equation (3) then predicts a compliance of 0.1 μm μN^{-1}. Since the weight of the rod with the vane welded to it is approximately 60 mg, this will yield, according to equation (4), a resonant frequency of 64 Hz. Adding the weight of the micro-pipette (30 mg) yields a resonant frequency of approximately 50 Hz.

To calibrate the transducer a force generator after Minns (Minns, 1971) was constructed, using a 100 μA moving coil meter with middle zero position. To drive the force generator, a dedicated wave form- and DC level generator was developed, producing 5 preset frequencies, each with a triangular, sinus or square wave output, and a regulated DC output, ranging in each case from 0 to 100 μN or 0 to 1000 μN effective force at the needle tip. This force generator was calibrated, up to 0.1 μN accuracy using an electronic micro-balance, type Sartorius 2004 MP (Sartorius GMBH, Goettingen, West Germany), of the electronic null compensation type.

Calibration was performed with the transducer mounted on the microscope stage and a micro-pipette mounted to the transducer rod tip. The force generator was mounted on a modified Zeiss IM object stage movement mechanism, with DC motor controls, so as to function as a 3-D micromanipulator, to position the calibrator accurately. The tip of the moving coil meter needle was placed behind the vertical part of the Z-bend micro-pipette, so that a pulling force could be exerted on the transducer's rod. Displacement of the tip of the micro-pipette was monitored simultaneously through the microscope, thus checking proper function and compliance at higher force levels. Using the sensitivity adjustment circuitry the transducer was trimmed to an output of 1 V per 10 μN. Figure 2A, B shows examples of the force signal resulting from calibration with a square wave and a triangular signal, Fig. 2A illustrates the pulse signal behaviour and the stability of the system at small forces, Fig. 2B illustrates the wide range linearity. Both signals were sampled with a PDP 11 type computer at 10 samples per second without any extra filtering or compensation networks. Table 1. gives an overall resume of the mechanical and electrical properties of the transducer system which are in good comparison with the above calculated values.

The linear response of the transducer is limited to plus or minus 12 V by the dynamic range of the output filter. As the preceding circuitry shows a linear response between plus and minus 14 V, the use of the DC-offset control allows a linear range of minus 140 to plus 140 μN.

Discussion

The design considerations set out at the beginning of this paper have almost completely been met at a very limited cost. A very rugged transducer has been constructed, with a large dynamic range, good linearity over this range, and

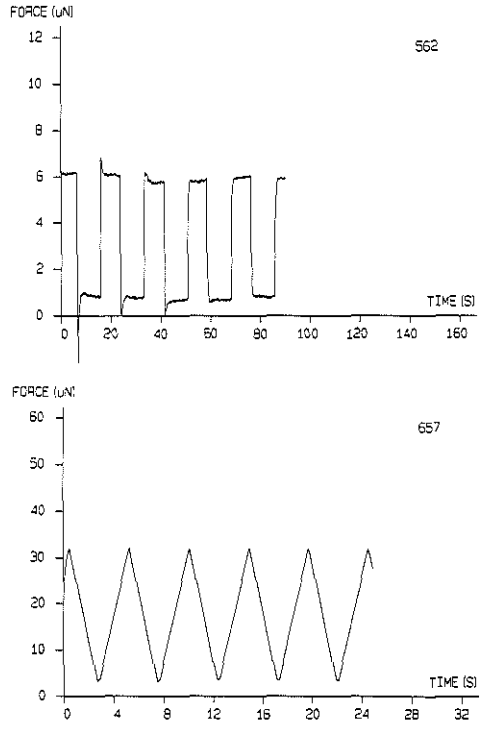

Fig. 2. (A) typical response of the force transducer to a square wave calibration force of 5 μN p-p amplitude with a repetition frequency of 0.05 Hz. The base line has been offset to approximately 1 μN in order to show negative overshoot. The recording was made under measuring circumstances with a micro-pipette mounted to the transducer rod and its tip submerged in the cell incubator bath. The signal was sampled at 10 Hz without filtering. (B) typical response of the force transducer to a triangular calibration force of 30 μN p-p amplitude with a repetition frequency of 0.2 Hz. The base line was offset to show clearly the lower parts of the signal. The recording was made under measuring circumstances with a micro-pipette mounted to the transducer rod and its tip submerged in the cell incubator bath. The signal was sampled at 10 Hz without filtering.

an acceptable sensitivity and signal to noise ratio. The costs of the electronic and mechanical parts of this transducer are in the order of $200.00 and with respect to the most delicate part of the system, a number of spares can be manufactured in advance. The construction of the whole system does not require any special machinery or testing equipment, and takes approximately 1 manweek for the electronics and 1 manweek for the mechanical parts, including spare cylinders.

More specifically demands (1) to (4), (7), (8) and (10) to (14) have completely been met. Long term stability, (5), is in the order of 1–2 μN h^{-1}, and is limited by temperature effects, (9), that influence the stability and position of

Table 1: Force transducer properties as measured under experimental conditions with the microscope illumination switched on and the object stage- and cell incubator heating at a working temperature of 37° C, after 4 h of initial warm up in a acrylic sheet cabinet (unless otherwise specified). Electrical properties are expressed in μN for a transducer calibrated to a typical output signal of 100 mV μN^{-1}.

Sensitivity	100 mV μN^{-1}
Linear range	$\pm$120 μN*
Maximum DC offset	$\pm$ 50 μN
Noise level:	
—electrical, power LED off	0.001 μN p-p
—electrical, power LED on	0.01 μN p-p
—total system	<0.2 μN p-p†
Resolution	<0.1 μN^{+}
Long-term drift:	
—electrical, power LED off	<0.01 μN h^{-1}‡
—total system	0.3 nN/sec = 1 – 2 μN h^{-1}°
Compliance	0.1 μm μN^{-1}
Resonant frequency	60 Hz without pipette
Moving mass	60 mg without pipette

* without using DC offset capability. $^{+}$ depending on the mechanical noise conditions at the site of experimentation. ‡ immediately after switch on. ° after 4 hours warming up at working conditions.

the cantilever-rod system. An important factor in temperature stability is formed by differences in the material properties (thermal expansion coefficients) of both rod and suspension cylinder. Another factor is the possible difference in temperature between both quartz suspension beams. This was controlled to a satisfactory level by adding a perspex shield to the front of the transducer. A final factor is formed by the temperature dependent properties of the glue, with which the quartz suspension beams are fastened; dual component epoxy types of glue proved to have worse temperature and elastic properties than the ultra violet light bounding glue that was finally used. To minimise these temperature effects, the whole measuring apparatus, including microscope and micro manipulator controls, was encased in an acrylic sheet cabinet.

In order to realise further improvement of thermal stability, all parts of the cantilever-rod system and suspension cylinder could be made from quartz, whereas the other parts of the transducer could be machined from glass ceramics such as Zerodur (Brehm & Van Grootel, 1985), which has a temperature coefficient of almost zero in a temperature range of −30° C to +60° C.

Sensitivity, (6), is only limited by the average noise level, which is in our case set by the ambient mechanical noise level. Although the total measuring apparatus was mounted on a vibration isolating table at the quietest part of the university's premises, most of the noise level

measured at the transducer's output was due to environmental mechanical vibrations. This was confirmed by interchanging the cantilever system with a solid metal dummy, which reduced the noise output to the level of the electronic's noise with the LED excitation power switched on (see Table 1).

Comparing this transducer design to other micro force transducers described in the literature, it is found to perform in most aspects equally as well as the transducer of Warshaw and Fay (1983) but is much smaller in physical dimensions, due to its electronically more sophisticated displacement detection system. Also the maximum linear range is considerably larger, as the detection of displacement is not limited to the width of the grating in the Ronchi rulings as in Warshaw's transducer, but limited to the width of the photo diodes which are each 3.0 mm wide, resulting in a theoretical displacement range of plus and minus 1.5 mm. As high gain is needed to obtain sufficient sensitivity, already at the first amplifier stage maximum range is limited electronically. If the rod mass were equally low, the frequency response of both transducers would also be comparable. The present fairly low resonant frequency resulted from the use of rather long and heavy micro-pipettes, bent in a Z like fashion, so as to overcome differences in height and distance from the transducer to the position of the cell, which in its turn, to maintain mechanical stability, necessitated the use of a rod of considerable length and mass. Improving this would require major changes in the arrangement of the cell incubator and surrounding instruments.

Comparing the transducer with the series of transducers reported on by Meiss (1974), it is found to be by far better suited for single cell measurements, as it is more sensitive and shows better long term stability. Also an equivalent assortment of sensitivity or force ranges can be achieved, by exchanging the suspension cylinder for a type of cylinder containing thicker, less compliant, suspension beams. The same holds for the design of Minns & Franz (1972). Compared to the design by Ishii (1982, 1988), our transducer does not need recalibration before or after each experiment, as the compliant part of it remains the same for every following experiment. Also there is no need for exact optical alignment of the cell preparation in the position detection field and long optical pathways are avoided, whereas influences from external heat or light sources are eliminated by using the synchronous pulsed light of the carrier system instead of a constant light- or laser beam.

Considering the design of Wieringa *et al.*, (1984), the new transducer is found superior in frequency response and in the amplitude of stretch that can be applied to a smooth muscle cell.

In contrast to Iwazumi's design (1982), no currents or magnetic fields, possibly interfering with electro-physiological measurements, are conducted or induced within the experimentation bath, whereas compared to both the former and Tung's transducer (Tung, 1986) extended length changes are much more easily performed. With respect to these last two designs it should be considered that both transducers are extremely sensitive and possibly intended for another type and level of single cell measurements.

It is concluded that the described transducer is very suitable for measuring length–tension relationships and the length dependence of force development. Such measurements were performed on urinary bladder and uterine smooth muscle cells. The results are reported in part II and III of this publication.

Throughout a period of 6 months the transducer performed according to specifications, without any recalibration procedures being necessary, except for the only occasion a suspension cylinder had to be changed due to accidental mechanical damage.

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Cell attachment

Celknooptechniek

Mechanical properties of mammalian single smooth muscle cells
II. Evaluation of a modified technique for attachment of cells to the measurement apparatus

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Summary

A method is described for attaching isolated single smooth muscle cells to an apparatus designed for measuring the longitudinal forces developed passively and actively by the cell upon straining, electrical or pharmacological stimulation.

Primary attachment of the cell is based on its natural negative surface charge in combination with a positive surface charge on the micro-tools used for attaching. Definite attachment is obtained by a knotting technique. Results show that this method of attachment is reliable and strong enough to withhold forces exceeding those necessary to break or tear the cell.

Although this method allows relatively short cells to be attached ($L > 80$ μm.) alternative methods e.g. glueing, are necessary to attach the shortest smooth muscle cells.

Introduction

In the field of muscle contractility research, especially on smooth muscle, there is general agreement on the value and necessity of single cell contractility measurements (Fay *et al.*, 1976, Glerum *et al.*, 1987). Amongst the essentials needed for such measurements are methods for single cell isolation, a sensitive microforce transducer and a method for cell attachment. In the first paper on single cell measurements Fay (1977) introduced a method for knotting a single cell to micro-tools. A number of other methods of smooth muscle cell attachment have been published, e.g. a method of sucking the cell ends into micro-pipettes (Ishii & Takahashi, 1982). This introduces the problem of maintaining compliance and stability in the measurement apparatus as the necessary vacuum has to be created, transduced, maintained and controlled separately from the actual transducer. In the quoted references it was shown that this problem can be solved by optically measuring the degree of bending of the tip of the micro-pipette the cell is sucked into, but due to a relatively long optical path, the long term stability of such a measurement method is poor and calibration of each new pipette tip is necessary (Ishii, 1988, personal communication). Another disadvantage of methods involving the measurement of the bending of a pipette tip, is the non-optimal axial pull on the smooth muscle cell. The cell either has to bend sharply at both ends, as in Ishii's method (Ishii *et al.*, 1988), or has to be folded in the middle as in the methods of Tung (1986) or Van Dijk-Looyaard (Van Dijk *et al.*, 1984). In this last method cells are clamped to the bottom of the cell incubator so that the stability of the attachment depends on the friction between the cell and the bottom of the incubator and also between the cell and the micro-tips. As both levels of friction are generally low, slipping of the attachment does occur in this method.

The best way of attaching a muscle cell would simply be to glue it to the micro-tools in use, but except for the use of fibrin glue (Copelas *et al.*, 1987), almost all attempts to do so have failed thus far. The major problem of glueing, besides finding a bio-compatible glue, lies in the far more difficult control of glue solutions in micro-pipettes and injectors and the controlling and positioning of a significantly increased number of micro-manipulators.

In this article we report our experiences with the knotting technique as originally published by Fay (1977) and described in detail by Warshaw & Fay (1983), applied to pig urinary bladder and human a terme pregnant uterus smooth muscle cells. Several fundamental changes in this method were necessary, which led to significant improvements.

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Materials and methods

Single smooth muscle cells from pig urinary bladders were isolated by enzymatic digestion of strips of bladder tissue collected at the local slaughterhouse. Single smooth muscle cells from a terme human uteruses were obtained likewise from biopsies excised at Caesarean sections. Both procedures were carried out as described previously (Glerum *et al.*, 1987), except for an additional overnight incubation in collagenase at 4° C for both types of tissue and an additional trypsinisation of the pig bladder tissue.

To be able to select vital cells during the experiments a Fluorecine Di-Acetate vital staining technique was used: After isolation the cells were incubated in a freshly prepared diluted FDA. solution for 15 min., next the FDA. was removed by centrifugation and the cells were resuspended in 2 ml of Krebs–Hepes buffer and 1 ml of MEM./10% FCS. This cell suspension was immediately poured into the cell incubator, the vital cells thus showing a clear green intracellular fluorescence. Detailed description of the procedure is available on request.

During experiments the cells were incubated in a specially constructed cell incubator with a bottom of optical glass mounted on a Zeiss inverted microscope equipped with phase contrast and incident light fluorescence optics. Conditions of incubation were kept at temperature 37° C, P_{O_2} 150 mm Hg and P_{CO_2} 38 mm Hg approximately, thus resulting in pH 7.35 (Glerum *et al.*, 1987). Fluid evaporation was prevented with a thin layer of Klearol (Van Dijk *et al.*, 1984).

Cell attachment micro-tools were drawn as micro-pipettes from Clarck Medical capillairy glass tubes (outer diameter 3.0 mm, type: GC200–15), with a tip diameter of approximately 50 μm at the base of the tip and 1–2 μm at the end of the tip, the tip diameter thus conically declined over a length of approximately 5 mm. After drawing, the micro-pipettes were bent in a Z like manner (Fig. 1.), in order to avoid a non-horizontal working plane of the transducer and length displacement instrument.

Cells were attached between a fixed micro-pipette mounted to a Märzhauser three-dimensional electromechanical micro-manipulator (type STM 3), fixed onto the microscope object table, and a similar pipette connected to an opto-mechanical micro force transducer, developed in our own laboratory, with a sensitivity of 10 μN V^{-1}, a linear range of $\pm$ 140 μN, a resolution better than 0.1 μN and a long term drift of approximately 2 μN h^{-1} (Glerum & Van Mastrigt, 1990). This transducer was mounted on a specially designed vertical movement stage connected directly to the side of the cell incubator. Figure 1 shows a cross-section through the central parts of the measurement apparatus.

Prior to the attachment procedure the tips of the micro-pipettes were coated by dipping them for 5 min in a 0.1 mg ml^{-1} solution of Poly-L-Lysine (mol. weigth 100 000, Sigma, P 1274), and subsequent rinsing for a few seconds in destilled water.

The smooth muscle cell selected by means of the fluorescence technique was first, at one of its ends, approached by the

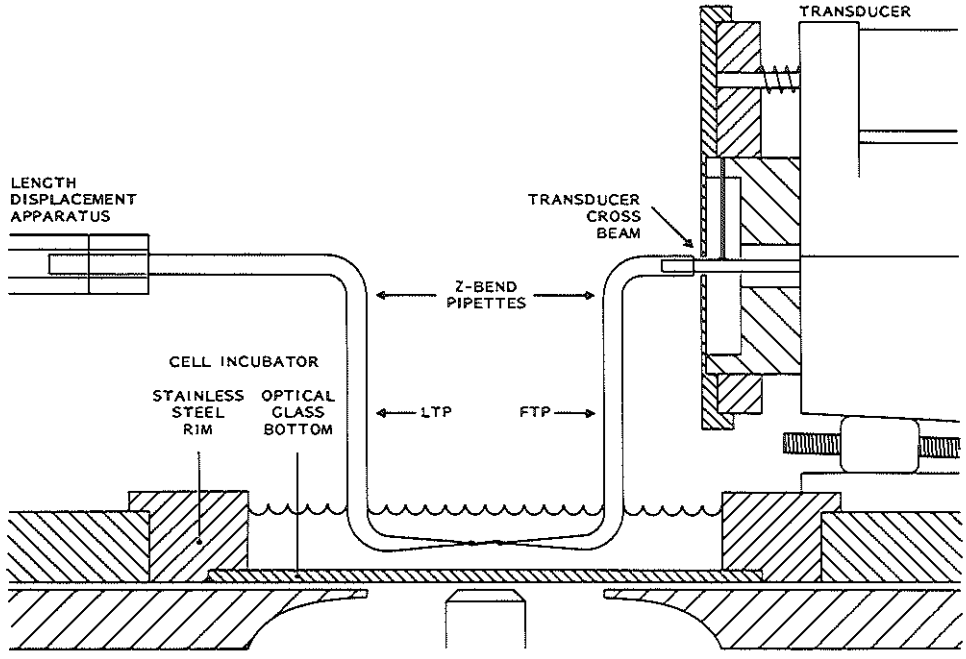

Fig. 1. Cross-section of the cell measurement apparatus. The cell is attached between two Z-bend micro-pipettes. The right pipette (FTP) is connected to an ultrasensitive force transducer, the left pipette (LTP) is connected to a length displacement apparatus. The cell is submerged in a temperature controlled cell incubator mounted on an inverted microscope.

tip of the micro-pipette mounted in the three-dimensional (left hand) micromanipulator. As soon as the tip met the cell, the cell would be attracted to the tip by static force. This binding was strong enough to lift the cell from the bottom of the incubator and bring it to the tip of the pipette attached to the transducer, which was brought into focus in the right part of the field of vision. At this point the right hand end of the cell was laid just under the tip of the transducer pipette and next this tip was lowered onto the cell by means of the transducer's elevation control. This was done in such a way that the cell was clamped between the tip and the bottom of the incubator. Next, the left hand end of the cell was brought alongside the transducer pipette (Fig. 2.1) and the first part of the knot was made by bringing the left hand manipulator clockwise over the transducer pipette (Fig. 2.2). To be able to complete the turn the transducer pipette was carefully elevated and the left hand pipette was successively turned clockwise underneath (Fig. 2.3) and again over the transducer pipette (Fig. 2.4). Next, another half clockwise turn was made by bringing the left hand pipette underneath and aside the transducer pipette again (Fig. 2.5). From this position the knot was tightened by carefully pulling the left hand manipulator to the left, thus stretching the cell between the two micro-pipettes until the first minimal rise in tension (approximately 1–2 μN) was observed (Fig. 2.5–2.7).

The cell was brought to its 'resting length' by applying one or more length steps of 10 μm. Resting length was defined as the free length between the knots that was optically measured through the microscope after the first length step of 10 μm that gave rise to a measureable tension increment directly followed by a visco-elastic relaxation.

In order to perform rampwise stretches on a single smooth muscle cell with the Märzhauser three-dimensional micromanipulator, its X-axis electronic control was modified to allow unidirectional pulse control by either an external timer or a computer, both via a TTL input. Velocity during the selected ramp duration could be adjusted by changing the motor drive voltage with a calibrated ten turn potentiometer. During all experiments ramp duration was kept at 0.2 s and velocity at 50 μm s^{-1}, resulting in length steps of 10 μm.

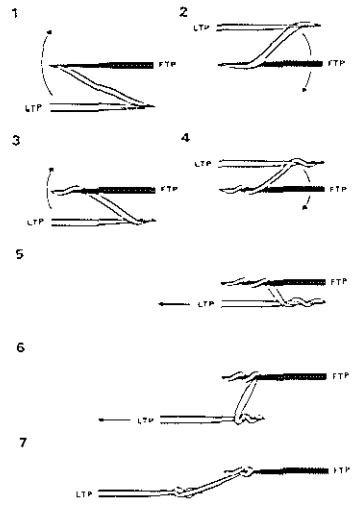

Fig. 2. Schematic representation of the knotting procedure used for attaching isolated mammalian single smooth muscle cells to two axially aligned micropipettes. See explanation of procedure in text.

Reliablility of the knotting technique was investigated by repeatedly stretching (at 15 min intervals) the cell by length increments of 10 μm, resulting in a number of force steps, directly followed by stress relaxation. Force steps and relaxation curves were stored online in a PDP 11 type computer and analysed to evaluate the cell attachment technique. Part of the analysis consisted of calculation and subsequent plotting of the force increment at the moment of the ramp stretch (dF), (Figs 4A, B), as a function of length. Three aspects of the stability of the knots were investigated: (1) whether the knots would hold or not at the moment of initial straining up to resting length; (2) whether the cell would break at/after the ultimate stretch ramp was applied or one of the knots would untie; (3) whether after any stretch ramp a decline in the amount of force increment (dF) had occurred and if so, whether these declines happened just before breakage of the cell, or in the middle of a series of stretch ramps.

Results

In a total of 17 experiments 11 pig urinary bladder smooth muscle cells and 6 smooth muscle cells from human a terme pregnant uteruses were successfully attached and the quality of attachment was investigated.

Tables 1 and 2 show: (1) that in all cells the knots initially tightened correctly, as initial relaxation at 'resting length' could be attained; (2) that in all cells the strength of the attachment exceeded the force level necessary to break the cell, as all tested cells ultimately broke; (3) the total number of ramp stretches that could be applied to the cell, starting from rest length, including the ramp at which the cell broke; (4) the number of times a decline of force increment (dF) occurred per cell; (5) how many ramps could still be applied after the first decline of dF had occurred, including the ramp at which the cell ultimately broke.

Figure 3 shows an example of the force response resulting from application of a length ramp, figure 4A shows the dF length diagram of a bladder smooth muscle cell, without intermittent decrease in dF occurring. Figure 4B shows a cell displaying repeated decreases of dF, still ultimately leading to cell breakage.

Discussion

Four methods for attaching cells to measurement devices have been published: (1) *Clamping* seems to be the worst solution, inevitably leading to the two cell ends being clamped to a non–moving underlayer, which means indirect measurement of shortening or force on a somehow folded cell. Van Dijk and co-workers (1984) pointed out that with their clamping method slipping occurred at approximately 1.5 μN, probably long before maximum force was reached. These force levels are surpassed with a

Table 1. Pig urinary bladder smooth muscle cells; table shows in column (1) whether knots tightened correctly (+ or −), column (2) whether the cell ultimately broke or not (+ or −), column (3) the number of ramp stretches that could be applied to the cell, starting from rest length including the ramp at which the cell ultimately broke (n), column (4) the number of times a decline of force increment (dF) occurred per cell (n), and column (5) the number of ramp stretches that could still be applied, including the ultimate ramp at which the cell broke, after the first/last decline of dF had occurred (n/n). * = Standard Error of the Mean.

cell no	*initial knot tight* + / −	*ultimate breakage* + / −	*number of ramp stretches applied* (n)	*number of declines of dF* (n)	*number of ramps after first/last decline of dF* (n/n)
1	+	+	10	1	1
2	+	+	7	1	1
3	+	+	8	1	1
4	+	+	11	3	7/1
5	+	+	13	1	2
6	+	+	14	5	7/0
7	+	+	10	2	3/1
8	+	+	10	1	3
9	+	+	5	1	1
10	+	+	5	1	2
11	+	+	7	1	2
average	− · −	− · −	9.1	1.64	2.72
S.E.M.*			0.90	0.39	0.68

factor of thirty in our material. Also the pressure excerted on the cell by the micro-pipette tips used in this technique, might evoke depolarisation and/or contraction of the cell. (2) *Vacuum attachment* gives rise to the problem of transferring the vacuum to the right spot, without compromising transducer stability and compliance. Any tube or hose crossing to the active tip of a longitudinally working transducer to obtain vacuum at its end will influence the transducer's properties and stability, whereas working with a fixed base of the vacuum micro-pipettes attached to the cell ends leaves one with the opportunity to measure the bending of the tip in only one way or

Table 2. Human pregnant a terme uterus smooth muscle cells; table shows in column (1) whether the knots tightened correctly (+ or −), column (2) whether the cell ultimately broke or not (+ or −), column (3) the number of ramp stretches that could be applied to the cell, starting from rest length including the ramp at which the cell ultimately broke (n), column (4) the number of times a decline of force increment (dF) occurred per cell (n), and column (5) the number of ramp stretches that could still be applied, including the ultimate ramp at which the cell broke, after the first/last decline of dF had occurred (n/n). * = Standard Error of the Mean.

cell no	*initial knot tight* + / −	*ultimate breakage* + / −	*number of ramp stretches applied* (n)	*number of declines of dF* (n)	*number of ramps after first/last decline of dF* (n/n)
1	+	+	11	1	1
2	+	+	7	1	0
3	+	+	14	2	6/0
4	+	+	19	2	7/1
5	+	+	8	1	3
6	+	+	10	1	1
average	− · −	− · −	11.5	1.33	3.00
S.E.M.*			1.80	0.21	1.18

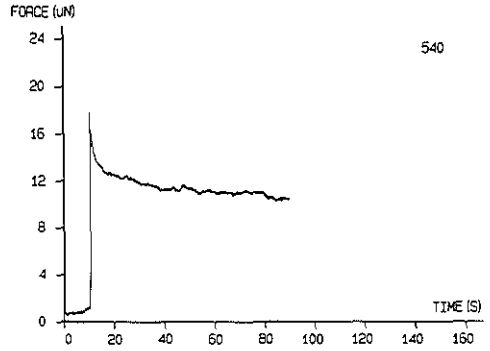

Fig. 3. An example of the force response resulting from application of a rampwise increment in length of an attached smooth muscle cell.

another, leading to inaccuracies and longterm instability (Ishii, 1988, personal communication) in the case of opto-electrical position detection, or, if the position detection is done by means of a video system, to a very low band width (Wieringa *et al.*, 1984).

Clamping neither vacuum attachment seem very attractive in view of the axial contractile and passive forces that are to be measured accurately, and require the introduction of various kinds of correction factors dealing with folding of the cell (Van Dijk., 1984), or cell to probe contact surface, (Tung, 1986), whereas the relatively sharp bends in the cell in Ishii's method (Ishii *et al.*, 1988) might cause other kinds of artefacts (e.g. premature depolarisation). (3) *Cell glueing* seems, theoretically, to be the most attractive method of cell attachment and even seems to be a *sine qua non* for cells other than smooth muscle cells (Copelas *et al.*, 1987). Of the few bio-compatible glues available, fibrin glue seems very promising, but in our limited experience it proved to be a very viscose fluid, rather difficult to apply and control at the desired microscopic scale. A few pilot experiments (Glerum, unpublished results), testing fibrin glue threads between micro-pipettes, showed results in accordance to Copelas (1987). (4) *Cell knotting* has for a decade or so proven to be a reliable method of cell attachment (Fay, 1977, Warshaw & Fay, 1983). The method has two principal prerequisites, a primary attachment procedure (static attraction between opposite charges) and cells that are long enough to permit the multiple knots (amphibian stomach cells, $L > 150\ \mu m$). If these conditions are met a stable attachment can be made, with an axial pull on the cell, without sharp bends at the cell ends.

The alternative knotting technique for shorter cells, as presented in this paper, involving a single knot and primary attachment to micro-pipettes without anion exchange resin beads, but with a coating of Poly-L-Lysine, shows very good stability and realiability of the knots obtained. All knots proved to be stronger than the cells themselves, as the cells broke or tore apart without any of the knots loosening.

The present technique was developed as a modification of the originally published technique (Warshaw & Fay, 1983), for the following reasons: (1) Glueing anion exchange resin beads to the micro-pipette tips was very time consuming, with a moderate to very low success rate, depending on the type of glue used. Even if successful, it seldom resulted in a stable binding resistant to salt water and activating bases and acids. (2) In those cases where reliably glued, activated beads had been obtained we found that either cells would not attach to the beads (which might have been a matter of cell vitality) or that even when a cell attached to one bead, it would seldom attach to a second bead (possibly because the charges of both beads interacted at the shorter distance due to the use of short cells). (3) In the few cases in which we succeeded to tie an inital knot we experienced that passing around the resin beads took up an unacceptable part of the cell's length.

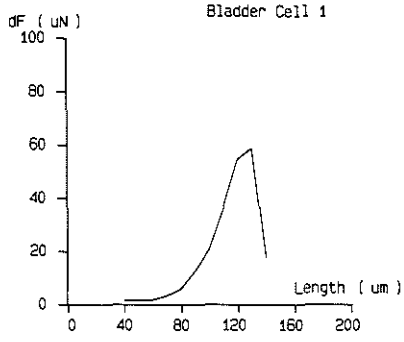

Fig. 4A. A typical example of the force increment (dF) resulting from a rampwise stretch of constant amplitude (10 μm) as a function of cell length, showing only a final discontinuity.

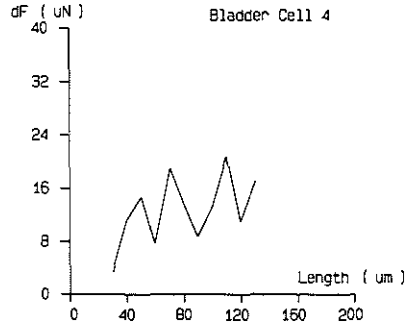

Fig. 4B. An example of the force increment (dF) resulting from a rampwise stretch of constant amplitude (10 μm) as a function of cell length, showing repeated discontinuities.

The method presented is also based upon attraction between oppositely charged surfaces, but the charge on the micro-tools is no longer concentrated in a hindering bead, but spread out over the surface of the micro-tool tips. In this way a smaller circumference has to be covered by the cell in making the turns for the knotting procedure and thus shorter cells can be measured. This also leads to a larger surface of statical attraction and contact between cell and micro-tool, thus increasing friction, and therefore enabling single knots which permit the investigation of shorter cells.

As the negative surface charge of the cell, which depends on the cell's metabolic processes, is crucial for the first attachment, only vital cells, showing a functional internal metabolism will demonstrate sufficient adherence to the coated micro-tips. It is therefore necessary to use an *in vitro* vitality test to select the best cells available. Only techniques that test for cytosol enzyme activity, such as FDA., or mitochondrial enzyme activity, such as Rhodamine 123, will yield proper information, whereas the Trypan Blue exclusion test proved insufficient (Glerum *et al.*, 1987). During the experiments it was observed that vital cells showed a double refracting cell membrane under phase contrast microscopy, so enabling limited cell selection without vital staining techniques.

The initial turn of the knot, before the cell overcrosses itself, is only possible because of friction between the cell and the micro-pipettes as a result of static attraction, so antagonising the rotatory pull on the cell while turning the knot. The larger the static attraction, the larger the normal force acting perpendicularly in the contact zone between cell and micro-tip, so enhancing friction. Also the longer the contact zone, due to the fact that the whole pipette tip is coated and thus stabilises the first knot turn over its entire circumference, the larger the resulting friction will be, making the tying of the knots easier. Once the cell body has crossed over itself, the normal force will increase due to the self bracing mechanism that is introduced in this way and the knots will tighten to such an extent that longitudinal pull is possible, even though the micro-tips become smaller in diameter towards the end.

The use of Poly-L-Lysine for cell attachment has been reported before (Tarr *et al.*, 1979), for attaching isolated cardiac myocytes with (contractile) forces not exceeding 1 μN. With our technique forces as high as 160 μN have been recorded without malfunction of the attachment.

The decreases in increment of dF seen in the majority of the dF vs length diagrams might be due to slipping of one or both knots. The dF decreases occurring halfway through the stretching cycle are especially suspect for knot slipping. As tables 1 and 2 show, most of the 'slips' occurred just prior to the breaking of the cell. If more than one 'slip' occurred, there was always, after a number of non 'slipping' ramp stretches, a final 'slipping' ramp stretch just prior to, or at the moment of, cell breakage, possibly indicating that it is not the knot stability that gives rise to the 'slip' phenomenon, but the yielding of an internal mechanism. This will be further discussed in part 3 of this publication.

It is concluded that our method of smooth muscle cell attachment is simple to perform and enables optimal longitudinal force measurements on such cells, with a minimal length of approximately 80 μm.

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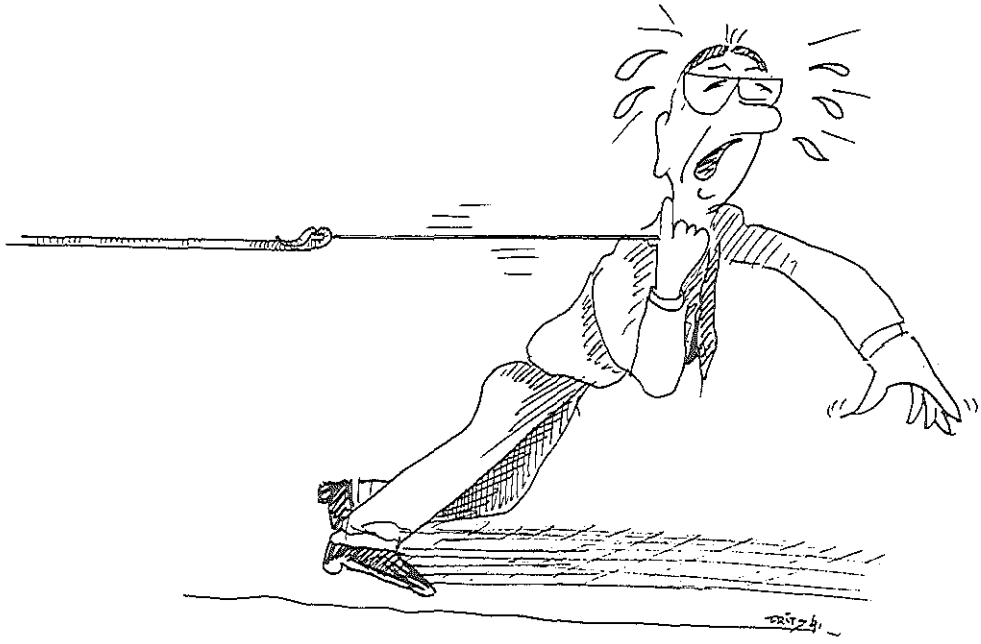
Passive properties

Passieve eigenschappen



Mechanical properties of mammalian single smooth muscle cells
III. Passive properties of pig detrusor and human a terme uterus cells

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Summary

Cells isolated from pig urinary bladders and pregnant full term human uteruses were attached longitudinally between a microforce transducer and a length displacement apparatus. Cells were stretched by applying a series of ramp-like length changes of 0.2 s duration and 10.0 μm amplitude at intervals of 15 min. Passive forces upon straining were as high as 70–100 μN. Following these peak forces stress relaxation occurred, levelling off approximately 50% of the maximum peak force. The maximum elastic modulus estimated for single cells was found to be at least a tenfold higher than was previously estimated from intact bladder strips. The relation between the increase in length and the increase in initial force increment was found to be approximately linear. An exponential equation was fitted to a selected number of stress relaxation curves. Relaxation curves of bladder cells show a clearly different time course as compared to bladder tissue strips, suggesting that a significant amount of relaxation in strips has to be contributed to the connective tissue components or to structural changes in these strips.

Introduction

The bladder in its filling phase and the growing uterus in the course of pregnancy show a remarkable increase in diameter and volume. For the bladder this increase in volume is solely attributed to passive stretching of connective and muscle tissue (Van Mastrigt *et al.*, 1978). The uterus not only shows passive elongation of its tissue components, but also growth, e.g. multiplication and hypertrophy of its smooth muscle cells (Ham, 1974). The time course of relaxation in bladder tissue has been shown to be clinically relevant (Coolsaet, 1977; Kondo & Susset, 1972; Van Mastrigt *et al.*, 1981; Susset & Regnier, 1981). Incompetence of the cervix in the first and second trimester of pregnancy seems to be related to a different passive and/or active behaviour of uterine smooth muscle (Drogendijk *et al.*, 1988; Van der Zon *et al.*, 1989).

In both cases it is difficult to relate measurements performed on large preparations to mechanisms at the cellular level. In this study the passive properties of single smooth muscle cells are reported. The pig urinary bladder was used as a well accepted model for the human urinary bladder (Douglas, 1972). The very small amounts of tissue necessary for single cell experiments with uterine muscle cells were obtained at Caesarean sections, without interference with either the procedure or with the mother's and child's interests.

Materials and methods

Single smooth muscle cells from pig urinary bladders were isolated by enzymatic digestion of strips of bladder tissue collected at the local slaughterhouse.

Single smooth muscle cells from a terme human uteruses were obtained likewise from biopsies excised at Caesarean sections. Both procedures were carried out as described previously (Glerum *et al.*, 1987; Glerum & Van Mastrigt, 1990b).

The cells were incubated in a cell incubator mounted on a Zeiss inverted microscope, equipped with phase contrast and incident light fluorescence optics. Conditions of incubation were kept at temperature 37° C; pO_2 150 mm Hg and pCO_2 38 mm Hg approximately, thus resulting in pH 7.35 (Glerum *et al.*, 1987).

Cells in suspension were checked for vitality by means of an FDA fluorescence vital staining technique and selected vital cells were knotted between two micropipettes attached to a length displacement apparatus and a micro force transducer (sensitivity 10 $\mu N\ V^{-1}$) as depicted before (Glerum & Van Mastrigt, 1990a, b).

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Subsequently cells were stretched to resting or initial length by applying one or more ramp-like length increments of 10μm amplitude and 0.2 s duration until a rise in force directly followed by visco-elastic relaxation was recorded. The free cell length between the knots was optically measured through the microscope at this moment and was defined and recorded as the initial length L_1.

After an initial resting period of 15 min, cells were stretched by applying further ramp stretches of 10 μm amplitude at intervals of 15 min until they broke.

The microforce transducer output signal, showing both the peak force and the stress-relaxation behaviour of the cell, was sampled at 10 Hz during 90 s by a PDP11 type computer, starting 10 s before the start of each length increment. The obtained data were stored on hard disk for analysis. Figure 1 shows a schematic diagram of the measurement apparatus.

The stored stress relaxation curves were analysed as follows: every curve, one at a time, was displayed at the computer screen and cursors were placed at the force levels corresponding to force before stretching (F_1), maximum peak force during the ramp (F_2) and force after 80 s of relaxation (F_3). F_2 minus F_1 was called dF, and the percentage of force (rF%) remaining after 80 s relaxation was calculated as:

$$rF\% = (F_3 - F_1)/(F_2 - F_1) \times 100\% \quad (1)$$

Plots of dF *versus* length were made for each cell, and the slope of the rising part of the curve (α = chord stiffness) was estimated:

$$\alpha = (dF_2 - dF_1)/(L_2 - L_1) \quad (2)$$

where

L_1 = cell length at the onset of the approximately linear rise of dF with L

L_2 = cell length at the end of the approximately linear rise of dF with L

dF_1 = dF value corresponding with L_1

dF_2 = dF value corresponding with L_2.

The definition of the elastic modulus E:

$$E = \Delta\sigma/\Delta\varepsilon \quad (3)$$

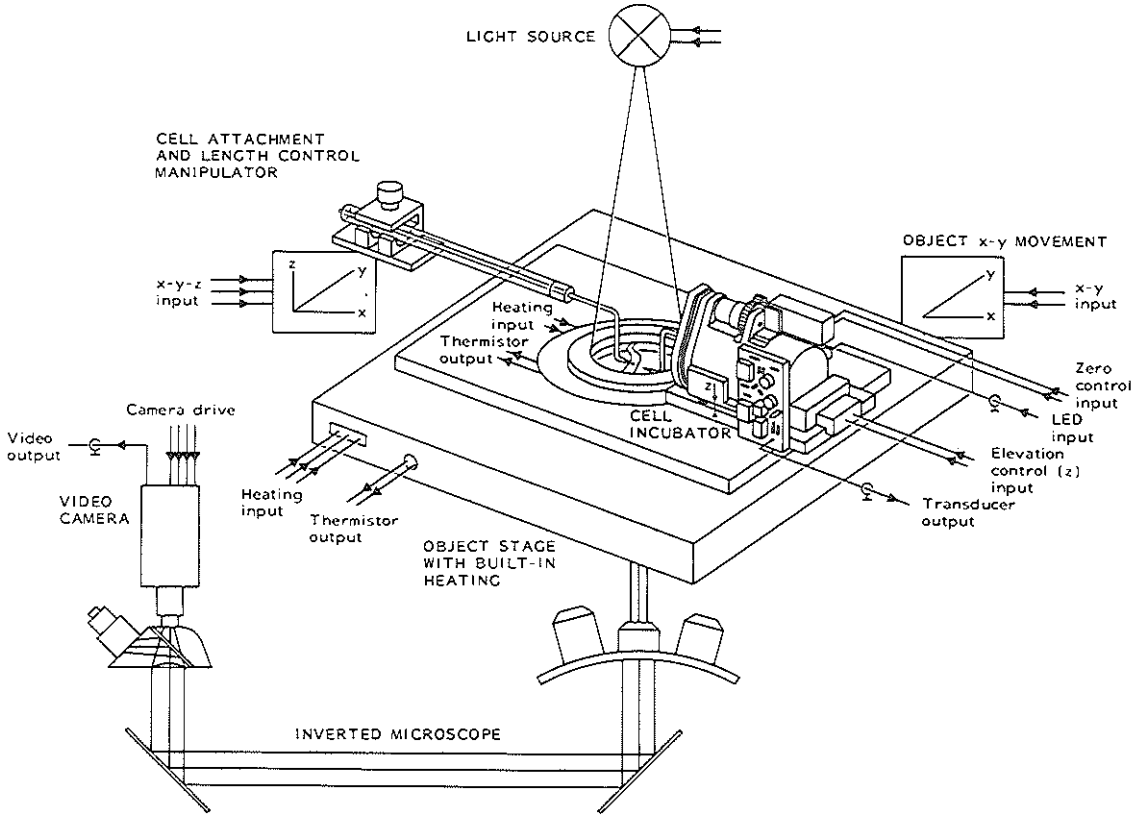

Fig. 1. Schematic diagram of the measurement apparatus. The diagram shows the cell incubation bath mounted on the object table of an inverted microscope. To the right side of the cell incubator an ultrasensitive force transducer is mounted on a specially designed elevation control mechanism. Left of the cell incubator a three-dimensional micromanipulator, serving as a length control apparatus, is attached to the object table. Micropipettes attached to the transducer and the micromanipulator are bent in a Z-like form to overcome the differences in height and distance from the transducer and the micromanipulator to the actual spot where the cell is situated in the incubation bath.

where
E = elastic modulus
$\Delta\sigma$ = increase in stress
$\Delta\varepsilon$ = increase in strain
was rewritten in the form:

$$E = (\Delta F/A) \times (L/\Delta L) \quad (4)$$

where
ΔF = increase in force
A = crossectional area
L = length
ΔL = change in length.
Assuming a cell to be a cylinder with a constant cell volume V_t:

$$V_t = \pi r_i^2 \times L_i = A \times L \quad (5)$$

where
r_i = the radius of the cell at resting length
L_i = resting length.
This yields:

$$E = \Delta F \times L^2/(\pi r_i^2 \times L_i \times \Delta L) \quad (6)$$

For comparison of elastic moduli determined in this way with moduli calculated from experiments on larger preparations the value of Equation (6) at maximum cell length (E_{max}) was taken:

$$E_{max} = dF_{max} \times L_{dF_{max}}{}^2/(\pi r_i^2 \times L_i \times \Delta L) \quad (7)$$

where
E_{max} = the maximum elastic modulus at maximum length
$L_{dF_{max}}$ = the cell's length obtained at the ultimate length increment where the cell still remained intact
ΔL = amplitude of the ramp stretch.
The radius of the cell at initial length, r_i, was estimated optically.

The following parameters were tested for reproducibility: the cell length prior to the (first) decrease in dF (L_{pd}), dF prior to decrease in dF (dF_{pd}) and the absolute force prior to decrease in dF (F_{apd}). Also the absolute force (F_{break}) and length (L_{break}) at the moment of breaking were estimated.

To quantify the process of stress relaxation to a number of curves an exponential function of the form:

$$F(t) = C_1 e^{B_1 t} + C_2 \quad (8)$$

was fitted using a Marquardt iterative procedure (Kirkegaard, 1970).

Only curves that showed a continuous decrease in force without intermediate dips or peaks greater than 1.5 μN deviation during at least 70 s, were considered correctly fitted curves.

Differences in variables were tested for significance using the Mann-Whitney U-test, at a significance level of 10%.

Results

In 19 experiments 12 pig urinary bladder smooth muscle cells and six human uterus smooth muscle cells were attached to the measuring apparatus and stepwise stretched until they broke. One pig urinary bladder cell accidentally broke at small length, due to wrong micromanipulations. Figure 2 shows a typical example of a stress relaxation curve, and the measured variables F_1, F_2 and F_3. Eighty-six such curves were obtained from the bladder cells and 53 from the uterus cells.

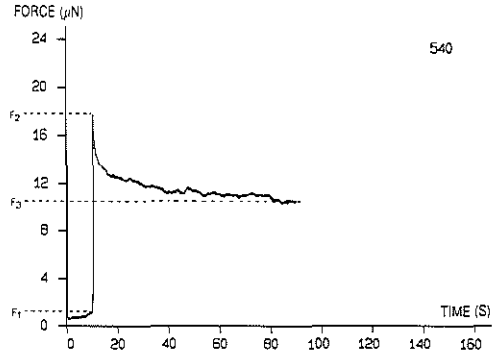

Fig. 2. A typical example of a stress relaxation curve measured in response to a 10 μm stepwise length increase of a human uterus smooth muscle cell. F_1 indicates the force level just prior to the length step, F_2 indicates the maximum force level at the moment of the length step and F_3 indicates the force level after 80 s of stress relaxation. Stretched cell length between the knots was 95 μm.

For all cells dF was plotted as a function of length. A total of 13 out of these 19 curves showed a continuous increase in dF with increasing length, except for a final discontinuity just before the cell broke. Figure 3 shows an example of this pattern. This same figure also illustrates how the slope of the curve was estimated from the linear part of the curve. One curve of a bladder cell was too

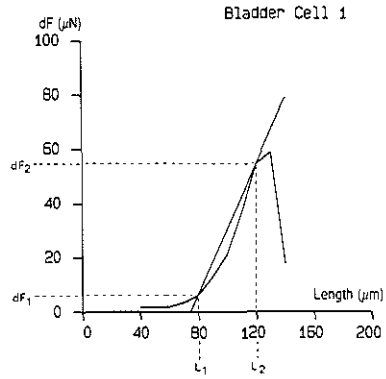

Fig. 3. A typical example of the change in the initial force increment (dF), resulting from stepwise straining of a bladder smooth muscle cell, as a function of cell length. L_1 indicates the onset of the linear segment of the curve and dF_1 is the corresponding force increment. L_2 indicates the end of the linear segment, with its corresponding force increment dF_2. The slope of the straight line (L_1, dF_1) to (L_2, dF_2) was calculated as α. The curve shows an abrupt decrease in dF, indicating the maximum range of cell lengthening. At a stretched length of 140 μm the cell broke.

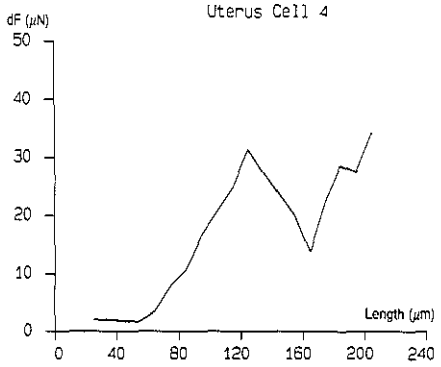

Fig. 4. An example of the change in dF as a function of length for a uterine smooth muscle cell showing two dips in the increment of dF before the cell broke.

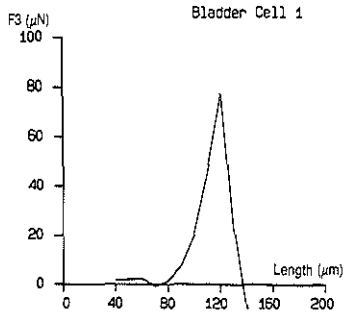

Fig. 5. A typical example of the change in F_3, remaining steady force after 80 s of relaxation, resulting from stepwise straining of a bladder smooth muscle cell, as a function of cell length. At a stretched length of 140 μm the cell broke.

short to allow further analysis (cell 12), two uterus cells showed one additional discontinuity in dF increment and three bladder cells showed two or more discontinuities in the increment of dF with length. Figure 4 shows dF *versus* length for one of the few cells that showed repeated dips in dF increment.

F_3 (remaining steady force after 80 s of relaxation) *versus* length was also plotted for the same cells. As this pattern grossly paralled that of dF *versus* length (see Fig. 5) further data on F_3 are not presented here and analysis was concentrated on dF and other variables.

As Tables 1 and 2 show, the average initial length (L_i) was 34 μm for bladder cells and 29 μm for uterus cells. This length is the free length between the knots on the micropipettes. A considerable part of the cellbody (20–30 μm for each knot) was used for the knots.

The average slope (α) of the approximately linear increase of dF with length was 0.52 $\mu N\ \mu m^{-1}$ for bladder cells and 0.28 $\mu N\ \mu m^{-1}$ for uterus cells.

Both types of cells showed a levelling off of relaxation at approximately the same average remaining strain level ($rF\%$) of 51 and 55% respectively. The average maximum elastic modulus (E_{max}) values were also similar, at 11.0 and 12.2×10^6 N m^{-2} respectively.

The moment at which a discontinuity in increase of dF with length occurred did not seem to be uniquely related to either length, dF or absolute force, as is shown in columns 1, 2 and 3 of Tables 3 and 4. These show, for the same cells as in Tables 1 and 2, length (L_{pd}), dF (dF_{pd}) and

Table 1. Passive properties of pig urinary bladder smooth muscle cells.

Cell no.	L_i *(μm)*	α $N\ m^{-1}$	$rF\%$ %	*SEM**	E_{max} $N\ m^{-2} \times 10^{-6}$
1	40	0.64	50.0	9.4	31.7
2	40	0.33	46.0	8.4	4.6
3	40	0.05	78.6	–.–	1.2
4	30	0.56	44.2	8.2	10.7
5	25	0.24	63.1	2.9	19.9
6	25	0.87	46.2	8.3	13.1
7	30	0.18	32.6	5.0	6.6
8	25	0.39	55.8	6.2	11.8
9	40	1.94	69.8	12.5	15.0
10	30	0.17	53.2	15.9	0.5
11	25	0.66	65.1	5.5	12.7
12	45	–.–	32.0	14.2	–.–
13	50	0.23	48.8	5.4	4.7
mean	34.2	0.52	50.9		11.0
SEM*	2.4	0.15	2.4		2.5

L_i = initial length; α = the slope of the increase of dF with length; $rF\%$ = the average remaining force after 80 s of relaxation; E_{max} = the maximum elastic modulus.

* Standard error of mean.

Table 2. Passive properties of human a terme uterine smooth muscle cells.

Cell no.	L_i (μm)	α $N\,m^{-1}$	$rF\%$ %	SEM*	E_{max} $N\,m^{-2} \times 10^{-6}$
1	30	0.22	67.0	2.7	12.7
2	30	0.39	51.3	3.0	8.4
3	50	0.15	49.9	2.8	10.3
4	25	0.42	54.1	3.7	25.0
5	20	0.22	42.8	4.9	6.1
6	20	0.29	57.8	5.6	10.8
mean	29.2	0.28	54.6		12.2
SEM*	4.5	0.04	1.9		2.7

L_i = initial length; α = the slope of the increase of dF with length; $rF\%$ = the average remaining force after 80 s of relaxation; E_{max} = the maximum elastic modulus.
* Standard error of mean.

Table 3. Passive properties of pig urinary bladder smooth muscle cells.

Cell no. (n)	L_{pd} (μm)	dF_{pd} (μN)	F_{apd} (μN)	L_{break} (μm)	F_{break} (μN)
1	130	58.9	122.0	140	90.7
2	90	17.7	21.6	100	22.2
3	100	3.7	9.0	110	5.2
4	50 (60/90/120)	14.7	14.0	130	23.7
5	125	25.0	60.6	135	63.2
6	75 (85/95) (115/125/135)	45.6	115.3	155	90.9
7	80 (90/110)	10.1	10.8	120	13.2
8	75	17.1	42.6	115	76.3
9	60	41.6	44.1	80	160.0
10	50	4.3	10.4	80	6.4
11	75	34.2	40.3	85	75.0
13	100	11.9	15.4	120	18.8
average	84.2	23.7	42.2	114.2	53.8
SEM*	7.5	5.1	11.4	7.0	13.6

L_{pd} = the cell length prior to the length at which the (first) discontinuity in increase of dF with increase in length occurred (figures in brackets indicate lengths at which such a discontinuity, if more than once, occurred); dF_{pd} = dF prior to the (first) discontinuity; F_{apd} = absolute force prior to the (first) discontinuity; L_{break} = length at which the cell broke; F_{break} = absolute force at which the cell broke.
* Standard error of mean.

absolute force (F_{apd}) as calculated from the stretch response prior to the first stretch at which such a discontinuity was observed. If any of the following stretches showed another discontinuity in dF increment, the lengths at which these occurred are printed in small print in column 1 of both Tables.

Graphical analysis of the data in Tables 1–6 showed that all parameter values could be considered as properly fitting in a continuum of values. Therefore, in all further analysis and statistics, cells of the same organ type were treated as one homogeneous group and differences in variables were only tested between bladder and uterus cells.

Although averages obtained for most variables in Tables 1–4 seem to show differences between bladder and uterus cells, none of these differences proved to be significant according to the Mann-Whitney U-test at a significance level of 10%. If, however, cells 2 and 3, which showed many wavelike force increases superimposed on their relaxation curves, and cell 10, which was very thin at one of its ends, are excluded from the bladder cell results, the difference in α between bladder and uterus cells (0.64 and 0.28 respectively) becomes significant ($p = 0.077$).

Figure 6 shows a typical example of a fitted stress relaxation curve describing the relaxation behaviour within the displayed time window. A significant number of the

Table 4. Passive properties of human a terme uterus smooth muscle cells.

Cell no. (*n*)	L_{pd} (*μm*)	dF_{pd} (*μN*)	F_{apd} (*μN*)	L_{break} (*μm*)	F_{break} (*μN*)
1	120	20.8	38.9	130	41.6
2	80	18.2	23.5	90	50.8
3	110 (120/170)	10.7	22.7	180	30.2
4	125 (135/185)	31.5	69.0	205	81.1
5	50	8.0	11.5	90	25.3
6	90	20.9	55.0	110	44.8
average	95.8	18.3	36.8	134.2	45.6
SEM*	11.6	3.4	8.9	19.7	8.1

L_{pd} = the cell length prior to the length at which the (first) discontinuity in increase of dF with increase in length occurred (figures in brackets indicate lengths at which such a discontinuity, if more than once, occurred); dF_{pd} = dF prior to the (first) discontinuity; F_{apd} = absolute force prior to the (first) discontinuity; L_{break} = length at which the cell broke; F_{break} = absolute force at which the cell broke.
* Standard error of mean.

relaxation curves, especially in bladder cells, showed increases of force superimposed on the relaxation patterns. In a small number of curves the initial force step was out of range, or accidental mechanical disturbances during the sampling of relaxation had occurred. As these curves could not be fitted with Equation 4 only a limited number of curves, 28 from bladder cells (i.e. 33%) and 34 from uterus cells (i.e. 64%), proved smooth and long enough to allow the exponential curve fitting. The resulting parameters are shown in Tables 5 and 6. In Table 5 no results are shown for bladder cells 2, 3, 4 and 9, as these cells displayed too many or too large superimposed force increases (see discussion) in all relaxation curves. The uterus cells, as shown in Table 6, showed hardly any of these force increases; a few curves showed short duration peaks smaller than 0.5 μN.

It was observed that in almost all cells of either type the last and sometimes also the last but one relaxation curve before cell breakage differed from the preceding ones, sometimes in such a way that a two exponential fitting was considerably better than the routinely applied one exponential fitting. The parameters from the most deviant of these curves were excluded from the averages per cell.

On average, bladder cells did not differ significantly from uterus cells in terms of the exponent (— 0.0094 *versus* — 0.011), and although average coefficients and constants seemed to be larger for bladder cells than for uterus cells (5.71 and 14.0 *versus* 4.50 and 9.4 respectively), these differences were not significant according to the Mann-Whitney U-test.

Discussion

Anatomically, the urinary bladder does not seem structurally organized: throughout the whole bladder circumference muscle cell bundles are seen to be diverting in all possible directions and converging again from all directions (Gosling *et al.*, 1983). There is no clear distinction between circular and/or longitudinal muscle layers, and the connective tissue components such as collagen, elastin and reticulin seem distributed randomly. The bladder wall must therefore be considered as homogeneous. In contrast, the uterus shows a rather organized structure. Muscle cells are organized in parallel and three ill-defined layers can be discerned. The outer and innermost layers are longitudinally and obliquely orientated; the thickest, middle layer shows a circular orientation and contains the larger blood vessels of the uterus (Ham, 1974).

Table 5. Results of fitting the stress relaxation curves of pig urinary bladder smooth muscle cells with an exponential function.

Cell no.	*N*	*Coefficient* (*μN*)	*SEM**	*Exponent* (s^{-1})	*SEM**	*Constant* (*μN*)	*SEM**
1	4	8.54	2.39	−0.017	0.0099	39.3	15.1
5	3	1.83	0.69	−0.0095	0.0081	14.08	8.6
6	7	13.19	5.11	−0.0078	0.0016	12.1	2.2
8	1	2.61	—.—	−0.0065	—.—	21.2	—.—
10	2	0.90	0.31	−0.0041	0.00007	3.5	0.4
11	3	3.48	1.72	−0.0076	0.00087	13.8	6.2
12	2	0.32	0.041	−0.012	0.0029	2.0	1.4
13	6	2.059	0.59	−0.010	0.0030	5.8	1.1
average		5.71		−0.0098		14.0	
SEM*		1.57		0.0017		3.1	

N = number of stress relaxation curves fitted per cell; columns 3, 4 and 5 show averages and SEM for respectively the coefficient, exponent and constant of the fitted curves per cell; bottom lines show overall averages and SEM for the same variables of all adequately fitted curves.
* Standard error of mean.

Table 6. Results of fitting the stress relaxation curves of human a terme uterine smooth muscle cells with an exponential function.

Cell no.	*N*	*Coefficient (μN)*	*SEM**	*Exponent (s^{-1})*	*SEM**	*Constant (μN)*	*SEM**
1	4	3.25	1.29	−0.0055	0.0021	12.3	2.6
2	3	2.68	1.14	−0.011	0.0033	8.1	2.0
3	6	2.30	0.62	−0.0070	0.0020	7.1	1.4
4	8	8.060	1.64	−0.013	0.0027	13.4	1.1
5	6	1.85	0.31	−0.0082	0.0032	6.4	1.1
6	7	6.067	2.52	−0.017	0.0045	8.5	1.2
average		4.50		−0.011		9.4	
SEM*		0.77		0.0014		0.7	

N = number of stress relaxation curves fitted per cell; columns 3, 4 and 5 show averages and SEM for respectively the coefficient, exponent and constant of the fitted curves per cell; bottom lines show overall averages and SEM for the same variables of all adequately fitted curves.
* Standard error of mean.

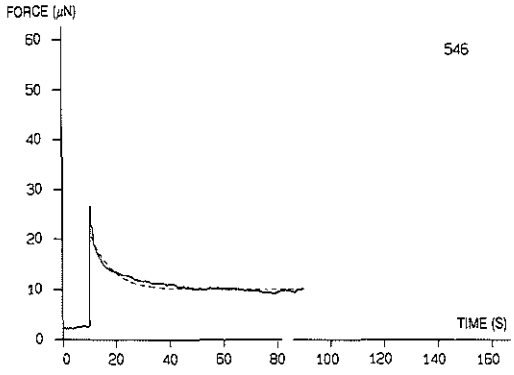

Fig. 6. A typical example of a stress relaxation curve measured in response to a 10 μm stepwise length increase of a human uterus smooth muscle cell to which a one exponential function was fitted. The dotted curve indicates the fitted exponential function. Stretched cell length between the knots was 115 μm.

The passive properties of smooth muscle cells play a significant role in a number of clinical problems. For instance in the filling phase of cystometry, where distended urinary bladders show low elasticity and large filling volumes. Regarding the uterus, passive properties of the cervix are clinically measured in relation to incompetence of the cervix. Although the cervix consists mainly of connective tissue components, it is also directly related to the lower segment of the uterus which consists mainly of smooth muscle. The latter might therefore play a role in the stable closure of the uterus until the moment of birth (Drogendijk *et al.*, 1988; Van der Zon *et al.*, 1989).

For a clinical evaluation of the relevance of the above outlined mechanisms, measurements of the passive properties of smooth muscle biopsies of these tissues are necessary. In the present study the passive properties of supposedly normal smooth muscle cells are discussed. As in both *in vivo* and *in vitro*, and even in single cells, there is the possibility of active processes underlying so called passive phenomena, we limit our definition of passive properties to those properties that can reasonably be described or explained by well known passive physical processes such as visco-elasticity.

Microanatomically a smooth muscle cell is not homogeneous but contains obliquely orientated contractile filaments anchored with dense bodies to the cell membrane. In the contractile filaments dense bodies are also seen which are thought to be transversely interconnected by intermediated filaments forming a cyto-skeleton (Squire, 1981; Stephens, 1984).

In a completely passive cell, therefore, the phenomena recorded in our experiments would reflect the properties of the cyto-skeleton in combination with those of the cell membrane. As, on the other hand, in these experiments no special measures were taken to exclude activation of the cell, passive and even active components of the contractile apparatus might contribute to the stress-relaxation recordings.

In the theoretical situation of a totally inactive contractile mechanism, repeated stepwise straining of the cell would result in successive uninterrupted steep rises in force upon stretching followed by a smooth exponential decay of force to a basic level. If, on the other hand, the cell actively maintains force by a continuous cycling of cross-bridges, it would show a sudden initial drop in force upon stretching it past the range of the cross-bridges (approximately 2–4% of muscle length [Van Mastrigt, 1988]). Force recovery would then occur as a result of cross-bridge cycling with a time constant in the order of 2.2 s (Van Mastrigt, 1989). The force relaxation curve in this case would not be convex towards the time axis, as is the case in passive stress relaxation.

An alternative that should be considered is that the cells maintain force through dephosphorylated cross-

bridges, in a low energy or 'latch' state (Marston, 1989). In such a case the stretch amplitudes applied in our experiments would also lead to detachment of these latch-bridges and any further influence of latch-bridges in the following stretches and relaxations would require re-cycled latch-bridges. Recent evidence (Hai & Murphy, 1988a,b) suggests that there is only one way to reattach latch-bridges after pulling them apart: by the normal sequence of actine myosine interaction, including the steps of phosphorylation and dephosphorylation. Therefore, latch-bridge reattachment would also result in rather steep rises in force or, as stated before, in case of stretch followed by relaxation, the relaxation curve would not be convex towards the time axis. This still leaves the possibility that the very first measurements made on each cell showed a considerable overestimation of dF, due to pre-existing latch-bridges, as possibly induced by, for example, depolarization during the isolation and knotting procedures. Since, however none of the dF *versus* length curves (see Fig. 3) showed a significantly higher dF value at the very first stretch, latch-bridge activity in these measurements could be ruled out.

For the data presented, typical stress relaxation curves were obtained in most cases as depicted in Fig. 2. In those cases where (small) wavelike increments of force were superimposed on the relaxation curves, the delay between the stretch and the onset of a superimposed force rise varied from at least 10–30 s, probably indicating a stretch induced cell depolarization, which gave rise to an additive active force component on the passive relaxation curve. In intact bladder muscle, and in bladder strips, the effects of stretch activated cell depolarization leading to force increases by cross-bridge cycling, were observed approximately 40–60 s after rapidly stretching the muscle (Levin *et al.*, 1986; Van Mastrigt, 1977). The resulting wave-like superimposed force increments on the relaxation curve did not affect the general form of the relaxation curve to a significant degree (Coolsaet, 1975b). In this study only bladder cells 2 and 3 showed superimposed force waves of such a magnitude (approximately 2–3 μN peak to peak) that this behaviour was reflected in the results of Tables 1 and 4.

In a limited number of pilot experiments, the contractile apparatus was inactivated by incubating the cells in a calcium-free solution containing EGTA after twice rinsing them in the same solution (unpublished data). In these calcium-free cells, wave-like force increments were not clearly observed but the stress-relaxation behaviour remained the same, resulting in comparable time constants.

We conclude that none, or very limited, contractile activity was present in the described measurements, so that Tables 1 to 6 correctly describe these in terms of passive mechanisms.

Apart from bladder cells 2 and 3, previously mentioned, the results in Tables 1 to 4 show a good reproducibility considering the range of forces measured. Bladder cell 10 yielded deviating data as it had only a very thin part of one of its ends knotted to a microtool. Uterine cells showed an even better reproducibility, which can be ascribed to the far less aggressive isolation method applied, using only collagenase, and the fact that almost none of these cells showed force increases superimposed on the relaxation curves within the observation time window.

Tables 3 and 4 describe the sudden decreases in increment of dF and the stretch limits of the cells. In the data presented all curves showed, at the final stretch or at the last but one, that a certain limit was reached as the increment of dF declined or reversed. These last stretches before the cells broke showed a different relaxation pattern which could not always adequately be described by a one exponential function. This indicates that physical changes did occur in the cells at the ultimate level of strain.

The levelling off in dF *versus* length curves occured at a cell length of 3–4 times the initial length, i.e. at the limit, or even beyond that, of the range of the contractile apparatus (Van Mastrigt, 1988, figure 6), so that actine-myosine overlap was minimal. This view is supported by the disappearance of superimposed contractions at these cell lengths, so that in our opinion the passive behaviour in the end range of stretching must be attributed to structural changes in the cytoskeleton.

In accordance with the length dependence of dF the variables in Tables 3 and 4 point to length as the most reproducible parameter for predicting the sudden force decreases and ultimate breaking of the cells, whereas absolute force is a likely second candidate.

From Equation 7, based on the definition of the cell as a homogeneous (visco) elastic body, it follows that E is proportional to L^2dF. As experiments show, dF is approximately proportional to length, so that E is approximately proportional to L^3. If on the other hand, it is the cyto-skeleton that bears the stress in the cells, this should probably be modelled as a bundle of thin wires, more or less orientated in parallel (Small *et al.*, 1986), so that a constant cross-section of the stress bearing filaments is a more likely assumption, leading to an elastic modulus proportional to LdF or to L^2. In both cases an elastic modulus increasing with length would result in agreement with our data.

Smooth muscle tissue, or more general soft tissue, has been generally known for a number of decades to exhibit increasing elastic moduli upon stretching (see for instance Remington, 1957; Fung, 1967; Ray, 1974). The urinary bladder wall forms no exception to this general rule (Coolsaet, 1975a; Susset & Regnier, 1981). Quantitatively, the dependence of the elastic modulus on strain was described by a mono-exponential function for strips of pig urinary bladder wall (Van Mastrigt *et al.*, 1978). The present data shows that the strain dependence in these tissues is not caused by connective tissue elements only, but that smooth muscle cells in isolation show comparable behaviour. In terms of magnitude, values calculated for E_{max} from pig bladder cells are found to be at least ten

times as high as E_{max} for pig bladder strips (Van Mastrigt, 1977). This could imply that, in the intact tissue, the cells are interconnected with structures of a lower elastic modulus, e.g. elastin and reticulin. Generally, urinary bladder wall tissue consists of five components: smooth muscle cells, collagen, elastin, reticulin and ground substance. According to Fung (Fung, 1981) elastin shows the lowest-but-one elastic modulus of all soft tissue components. Smooth muscle in the passive state shows the lowest elastic modulus, whereas in the active state it has a very high elastic modulus. Collagen is very rigid, but in the intact tissue it is structurally folded in such a way that it does not contribute to the stress-strain relationship until the elastin and smooth muscle structures are considerably strained. Ground substance cannot bear much stress but its hydration state seems to modulate the behaviour of the other components (Van Duyl *et al.*, 1987; Fung, 1981). It seems that at different lengths in the intact tissue stress is borne to varying degrees by the different tissue components, which implies also a variation in the tissue's effective cross-sectional area: the tissue behaves as a fibre enforced composite material of low elasticity (Regnier *et al.*, 1989).

The interconnection of the smooth muscle cells by components of relatively low elasticity forms a plausible explanation for the higher E_{max} found in single smooth muscle cells as compared to tissue strips. Another factor explaning the difference in E_{max} might be found in the difference in actual cross-sectional area for the tissue modelled as a homogeneous body or as a bundle of filaments.

In our results force in both types of cells does not decrease to zero, at least not within the observation window of 80 s (see Tables 1 and 2, column 3). In all measurements, taken with increasing lengths, it was also either necessary to introduce a negative offset, or to change the A/D converter input sensitivity, to keep the peak force and the baseline level within the limits of the computer's maximum input range, which again indicates that cells did not relax to a zero level even after 15 min. In intact pig bladder strips (Van Mastrigt, 1977; Van Mastrigt *et al.*, 1978) only 26% of the initial force remained after 80 s of relaxation, whereas in the present data the fitted curves show an average remaining force of 84%. If all the bladder cell relaxation curves, including the non-fitted ones are taken into account, average remaining force after 80 s still yields 51%. Based on extrapolation of the one exponentional model, the remaining force after 1000 s of relaxation would be 71%, which is considerably higher than the 17% measured after 1000 s of relaxation in intact bladder tissue strips (Van Mastrigt *et al.*, 1978). In the light of the complex structural arrangement of tissue components in the urinary bladder, as outlined previously, this difference can be explained by either a structural relaxation taking place, i.e. a rearrangement of the individual components (Alexander, 1957), or some of the non-muscular components showing a more viscous behaviour. These findings contrast with those of Van Dijk and coworkers (Van Dijk *et al.*, 1984) and Fay (1975), who find a stress relaxation to zero level within 30–120 s for bovine coronary and toad stomach smooth muscle cells respectively. As far as the findings of Van Dijk and coworkers are concerned, the decay to zero force level might be explained by slipping of the attachment which occurs, according to the same authors, at force levels above 1.5 μN.

Although based upon morphological findings, one would expect uterus cells to be longer, and therefore to show a different length dependent behaviour compared with bladder cells, no significant differences between both types of cells were demonstrated in the present data. To what extent the differences in the occurrence of contractile phenomena in the relaxation curves between bladder and uterus cell are of importance will be discussed in a separate study.

In conclusion we state that:

(1) Stress relaxation of urinary bladder single smooth muscle cells follows a pattern different from intact bladder tissue showing far less relaxation.

(2) Stress relaxation of single smooth muscle cells of the pig urinary bladder and the human uterus does not continue to zero force, but levels off at a definite constant force level.

(3) E_{max} for single urinary bladder cells is at least a tenfold higher than was previously estimated from intact bladder tissue.

(4) Passive properties of these cells are most likely determined by the cyto skeleton and the cell membrane.

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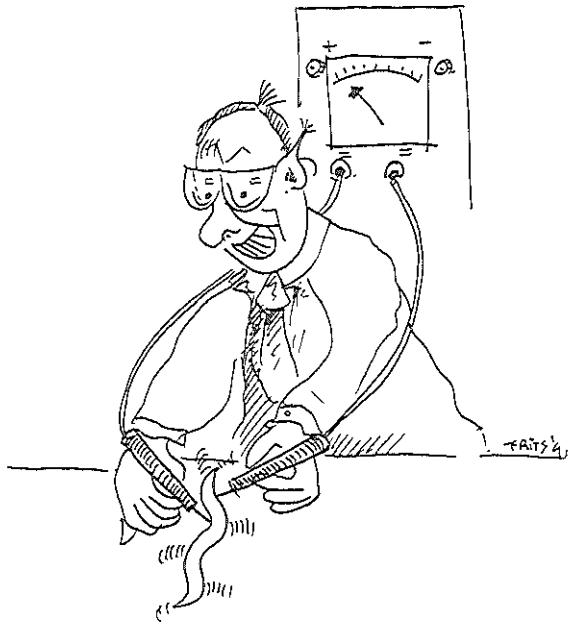
Active properties

Actieve eigenschappen



Mechanical properties of mammalian single smooth muscle cells
IV. Active properties of pig detrusor and human a terme uterus cells

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Summary

Smooth muscle cells were isolated from pig urinary bladders and human a terme pregnant uteruses. Cell suspensions were incubated in a specially designed cell incubator mounted on a Zeiss IM inverted microscope, keeping temperature at 37° C, and pO_2 and pCO_2 at tissue levels. Vital cells were selected using a Fluorescine Di-Acetate vital couloring technique and attached to a specially developed isometric microforce transducer and a length displacement apparatus.
Cells were stimulated to contract by electrical stimulation, using a platinum 50 µm diameter wire close to the cell and the stainless steel rim of the cell incubator bath as electrodes.
A rather small number of cells responded to stimulation with an increase in isometric force, whereas the majority of cells showed an electrically induced decrease in force level.
Stress relaxation curves, resulting from applied length increases, showed, particularly in the bladder cells, a number of wave like tension increases, suggesting stretch induced contractions, even in those cells that did not show electrically induced force increases.
It is concluded that a valuable method for contractility measurement and analysis of mammalian smooth muscle cells has been developed, ultimately leading to possibilities for evaluating muscle biopsies.

Introduction

Although smooth muscle has to an increasing extent become a subject of interest for contractility research, little is known about the properties of the basic contractile units of specific human smooth muscle organs such as the urinary bladder or the uterus. Especially for the bladder it is difficult to interpret the contractile behaviour of the whole bladder or bladder strips in terms of the smallest contractile elements, e.g. smooth muscle cells, as these contractile elements are not arranged in linear arrays similar to cardiac or skeletal muscle. Measurements on isolated smooth muscle cells are necessary to model the passive and active properties of these cells and their interaction, ultimately leading to a complete model of contractile behaviour and other properties of the complete organ.
Furthermore in experiments on single smooth muscle cells, unwanted and/or unknown influences from neighbouring cells, connective tissue elements such as collagen and elastin, innervation and vascularisation can be avoided, thus enabling very direct mechanical, pharmacological and electrophysiological experiments.

In this article a method is described for measuring and analysing isometric contractile responses from single mammalian smooth muscle cells isolated from the pig urinary bladder and the human a terme pregnant uterus.
Pig urinary bladders, which form a generally accepted and validated model for the human urinary bladder (Douglas, 1972), were used in order to avoid the need for human materials while developing research and analysis procedures.
Small specimens of human uterus could be obtained during planned Caesarean sectios without any interference with the mother's or child's interests or with the procedure itself. This type of smooth muscle cell was therefore evaluated directly on human uterine material.

Materials and methods

Pig urinary bladders were collected at the local abattoir. A small muscular part of the ventral bladder wall was minced into little fragments and cells were enzymatically isolated using collagenase, trypsine and D.N.A.'se, as described

before (Glerum *et al*, 1987 and Glerum *et al*, 1990). Uterus muscle biopsies were obtained at planned Caesarean sectios, prior to the start of labour. The obtained muscle strips were also minced and treated with enzymes, but only using collagenase and D.N.A.'se (Glerum *et al*, 1990).

Cells were incubated in a specially designed cell incubator mounted on a Zeiss IM inverted microscope, at 37° C, pO_2 150 mm Hg and pCO_2 38 mm Hg, with a pH of 7.35 (See Figure 1, Glerum et al, 1990).

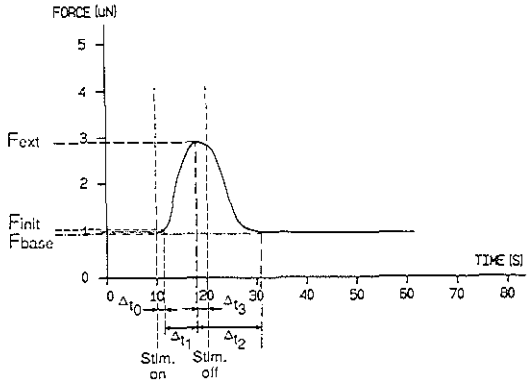

Fig. 1: Artist's impression of a typical Type II response of an isolated single pig urinary bladder smooth muscle cell to electrical stimulation. The following values were measured in such a response: 1) 'Stimulation on' latency time, Δt_0, i.e. the time from the start of the stimulation until the onset of the measured response, 2) The force level at the start of electrical stimulation, F_{init}, 3) The extreme of the change in force, F_{ext}, 4) The time lapsed during the change of force from F_{init} to F_{ext}, Δt_1, 5) The baseline level to which the force returned after the stimulation was switched off, F_{base}, 6) The time lapsed during the change of force from F_{ext} to F_{base}, Δt_2, and 7) The 'stimulus off' latency time, Δt_3, i.e. the time that lapses before a noticeable reversal of sign in the change of force occurs after the stimulation was switched off. Δt_3 is zero or positive in type I and type III responses and negative by definition in type II and type IV responses (see also Figure 3).

To check the obtained cells for vitality, the cell suspension was incubated with Fluorescine Di-Acetate, resulting in a clear green intracellular fluorescense in the vital cells.

Selected cells were attached between an ultra sensitive force transducer (resolution <0.1 μN , Glerum & Van Mastrigt, 1990a) and a length displacement apparatus using a knotting technique as described previously (Glerum & Van Mastrigt, 1990b). Next they were set at resting length by applying repetitive rampwise length increments of 10 μm until the first signs of stress relaxation were observed (Glerum *et al*, 1990c) and left to settle for five minutes.

At five minute intervals cells were stimulated to contract electrically, by applying positive, mono-phasic, rectangular pulses of 1.8 volt amplitude, 10.0 ms duration and a repetition frequency of 50 Hz (Glerum *et al*, 1987) to a 50 μm diameter platinum wire-electrode close to the centre of the cell and the cell incubator stainless steel rim. Electrical stimulation was switched on and off by a PDP 11 type computer, which simultaneously sampled the resulting force signal during 60 seconds at a 10 Hz sample rate. Sampling started 10 seconds before the switch on of the stimulation, which itself lasted 10 seconds, and continued for 40 seconds after the stimulus had been switched off.
Cell length was increased in steps of 10 μm; at each length cells were stimulated twice. This procedure was repeated until the cell finally broke.

The force responses measured during electrical stimulation were analysed as shown in Figure 1: Each curve was displayed at the computer screen and cursors were placed at the onset of the response (F_{init}), the maximum or minimum of the response (F_{extr}) and the point at which the curve had returned to its base line level (F_{base}). Values of the force levels were stored, together with the time elapsed between the start of stimulation and the beginning of the response (latency time or Δt_0), the time difference between the start of the response and the moment the extreme of the response was reached, or, if the change in force did not level off within the 10 s. of stimulation time, the time difference between the start of the response and the moment stimulation was switched off (Δt_1), the time elapsed between stimulus switch off and the change of direction of the response ('stimulus off' latency time or Δt_3) and the time it took the force to return from the extreme value to the base line level (Δt_2). From these data the slope of the initial force change (rise or decay), β_1 was calculated as follows:

$$\beta_1 = (F_{extr} - F_{init}) \Delta t_1 \qquad (1)$$

Also the slope of the stimulus off response (decay or rise), β_2 was calculated likewise:

$$\beta_2 = (F_{base} - F_{extr}) / t_2 \qquad (2)$$

(see Figure 2).
All values of the calculated variables were stored together with numbers indicating the cell type, cell number, stimulation number and the cell length at wich a specific stimulation was performed.
From the obtained data the average maximum force change in response to electrical stimulation, $dF_{act} = F_{extr} - F_{init}$ and the average change in force level after stimulus switch off, $dF_{return} = F_{extr} - F_{base}$, were calculated. Subsequently for all parameters averages and standard errors of the mean were calculated.

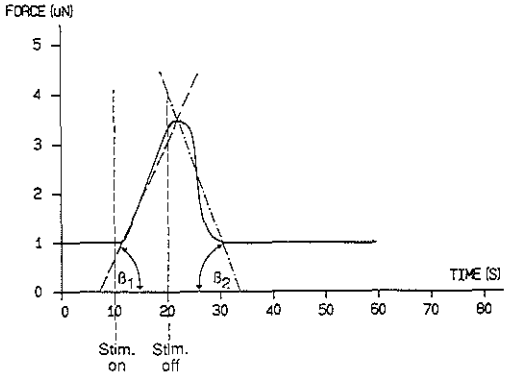

Fig. 2: Artist's impression of a typical Type I response of an isolated single pig urinary bladder smooth muscle cell to electrical stimulation, illustrating the approximation of the rising and descending slopes (β_1 and β_2) based on the values of F_{init}, F_{ext} and F_{base} as depicted in Figure 1.

The force signal was also sampled 10 seconds before, during and 80 seconds after each rampwise increase in cell length. The stress relaxation curves thus measured were analysed for stretch evoked contractile responses as follows: An exponential function of the form:

$$F(t) = C_1.e^{(\beta_1 \cdot t)} + C_2 \qquad (3)$$

was fitted to the measured data (Glerum *et al.* 1990c, Figure 6) and these were plotted together with the fitted function. The deviations of the measured curves from the fitted exponential function were inspected. Three categories of curves were discerned: 1) smooth curves with deviations <0.5 μN, 2) curves with deviations between 0.5 and 2.0 μN and 3) curves with deviations > 2.0 μN. If a curve could not be fitted by the computer because it deviated to much from the exponential form, number and magnitude of the deviations were estimated manually. For every cell the deviations from the exponential relaxation curve form were quantified both as a percentage of all relaxation curves per cell that showed either small (0.5 < dev. < 2.0 μN) or large (dev. > 2.0 μN) deviations (N_{devS} and N_{devL} respectively) and as the percentage of sampled curve time that was superimposed with either small or large deviations (t_{devS} and t_{devL} respectively).

The contractile responses due to electrical stimulation and in response to stretching were correlated (Spearman rank correlation coefficient). Differences in frequency of occurrence of the various responses were tested for significance (Chi-square test) in both bladder and uterus cell groups. Differences between absolute variables were tested for significance using the Mann-Whitney U-test, at a significance level of 5 %.

A limited number of curves, showing a positive response to electrical stimulation, were analysed by phase plot analysis (Van Mastrigt & Glerum, 1985), in order to compare the results on single cell preparations with similar data obtained from multicellular (strip) preparations of the same material (Van Koeveringe and Van Mastrigt, 1991). In a phase plot (see Figure 9) the rate of change of the variable 'force' is plotted as a function of that same variable. To the linear part of this curve a linear function of the form

$$dF/dt = 1/C.(F_{iso} - F) \qquad (4)$$

was fitted, where F = the measured force, t = time, C = the reciprocal value of the slope of the line and F_{iso} = the intercept of the line on the force axis.
In this model F_{iso} is the isometric force which would have been attained if the preparation had been stimulated infinitely long, as opposed to F_{max}, the maximal active force actually attained during an isometric contraction.
The second parameter, C, is the time constant, or the time it takes the force to attain 63 % of the value of F_{iso}.

The number of cells in cross-sections of smooth muscle strips studied in a separate project (Van Koeveringe and Van Mastrigt, 1991) were counted under a microscope in Gomöri stained slides. The average F_{iso} value of each strip was divided by the estimated number of cells in a cross-section and compared with F_{iso} values obtained from the single cell measurements.
The rate constant C is a parameter that is independent of the size of the preparation, so that values from different preparations could be directly compared.

Results

In 21 experiments 15 pig urinary bladder smooth muscle cells and 6 smooth muscle cells from pregnant a terme human uteruses were electrically stimulated.
All cells were tested at length intervals of 10 μm, starting at an average length of 34 μm for bladder cells and 29 μm for uterine cells, except for bladder cell 0, which was stretched in steps varying from approx. 5 until approx. 10 μm. Cells 12 and 14 were only stimulated at a limited number of lengths, as these cells broke prematurely due to unfortunate micro manipulations.

The 15 bladder cells were stimulated 210 times. In 92 cases (= 44 %) a response that could be discerned from ambient mechanical noise and spontaneous activity was observed. The 6 uterine cells were stimulated 108 times, resulting in 62 responses (= 57 %) above the noise level.

Both kinds of cells showed 6 types of response to electrical stimulation. The 4 most frequent types are illustrated in Figure 3:
Type 1: A steady force increase upon stimulation, followed by a force decrease shortly after stimulation was switched off (Fig. 3.1).

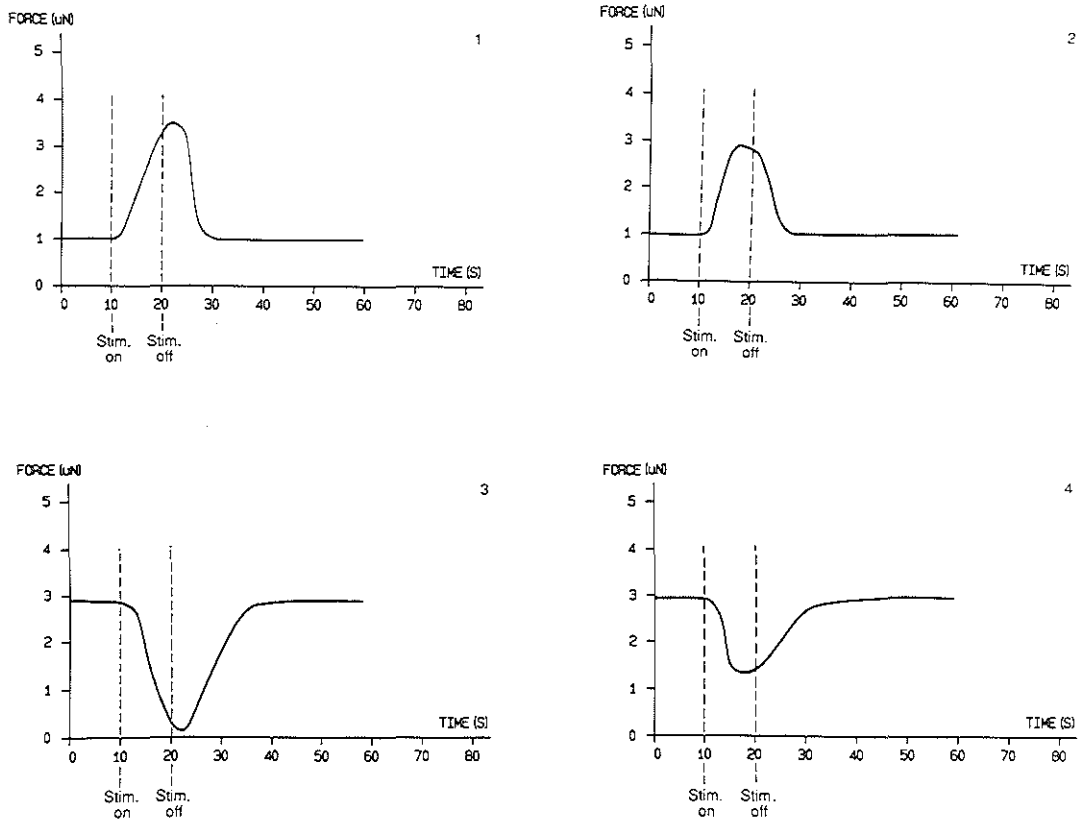

Fig. 3: Artist's impression of the four main types (I to IV) of responses to electrical stimulation of pig urinary bladder and human a terme uterus single smooth muscle cells.
Fig. 3.1: Type I response: After a short latency time the force starts to rise and continues to do so until shortly after stimulation has been switched off, then relaxation occurs and the force returns to the base line level.
Fig. 3.2: Type II response: After a short latency time the force starts to rise, but it levels off and declines before stimulation is switched off. After the stimulus is switched off a much steeper decline of force occurs and it returns to the base line level.
Fig. 3.3: Type III response: After a short latency time force starts to decline and continues to do so until shortly after stimulation has been switched off, then the force starts to rise until, or eventually beyond, the base line level.
Fig. 3.4: Type IV response: after a short latency time force starts to decline, but the decline already levels off and eventually reverses before stimulation is switched off. When stimulation is switched off the rise in force accelerates and it stops at or beyond the base line level.

Type 2: A force increase upon stimulation, levelling off (or even reversing into force decrease), before stimulation was switched off, followed by a (further) decrease in force after stimulation was switched off (Fig. 3.2).
Type 3: A force decrease upon stimulation, followed by a force increase shortly after stimulation was switched off (Fig. 3.3).
Type 4: A force decrease upon stimulation, levelling off (or even reversing into force increase), before stimulation was switched off, followed by a (further) increase in force after stimulation was switched off (Fig. 3.4).
Type 5: A force increase upon stimulation up to a plateau level, followed by a further increase in force shortly after stimulation was switched off (not illustrated).
Type 6: A force decrease upon stimulation to a plateau level, followed by a further decrease in force shortly after stimulation was switched off (not illustrated).

In all types of responses, in all cells, it was observed that simultaneously with the electrical stimulation and the measured rise or decay in force, changes in 'phase-contrast optical properties' occurred, which were always very similar no matter what kind of response was measured mechanically and which reversed after cessation of the stimulation.

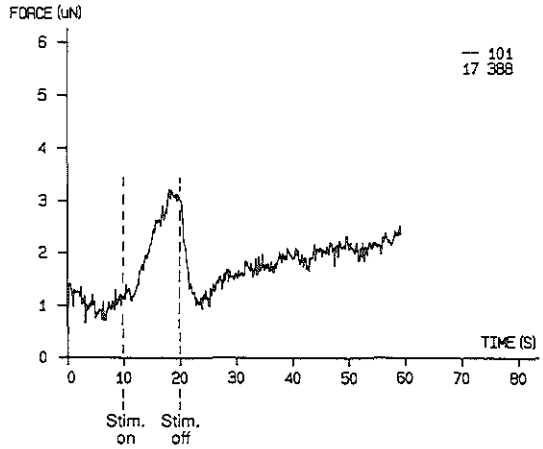

Fig. 4: A typical example of a type II response resulting from the electrical stimulation of a pig urinary bladder smooth muscle cell at a cell length between the knots of 90 µm. The dotted lines indicate the moments at which the stimulation was switched on and off.

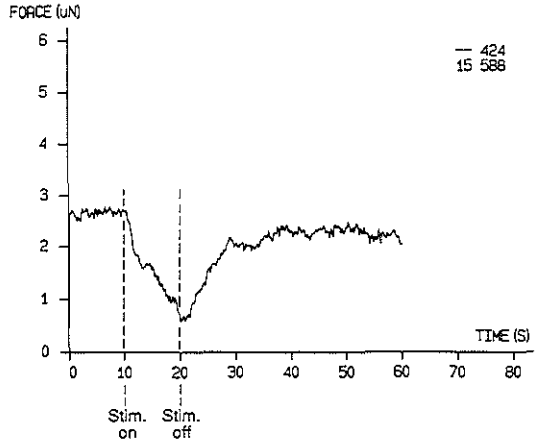

Fig. 5: A typical example of a type III response resulting from the electrical stimulation of a human a terme uterus smooth muscle cell at a cell length between the knots of 120 µm. The dotted lines indicate the moments at which the stimulation was switched on and off.

In the bladder cells 9 reactions according to type 1 were seen, 30 according to type 2, 16 of type 3 and 33 of type 4. Type 5 was observed once and type 6 three times.
In uterus cells a type 1 reaction was observed twice, type 2 once; type 3 reactions occured 32 times and type 4 was seen 22 times. Type 5 reactions were not seen in uterus cells and type 6 was observed 5 times (see Tables 2 and 3, column 1 and Table 1 for relative frequencies).

Figures 4 and 5 show typical examples of reaction types 1 and 3. As patterns 5 and 6 were only observed very infrequently in both kinds of cells, these observations were grouped with the non-response attempts and excluded from further analysis. Figures 6 and 7 show the absolute distribution of the four main response types for each cell, in bladder and uterus cells. The distribution of response types between bladder and uterus cells was significantly different according to a Chi-square test ($p < 0.0001$).

Table 1 shows which differences in average frequency of occurrence of the response types between bladder and uterus cells are significant according to Mann-Whitney U tests at a 5 % significance level. The table also shows the average frequencies of the estimated deviation types and the significance of differences between bladder and uterus cells.

Tables 2 and 3 show for the various response types in bladder and uterus cells the average values and standard errors of the mean of: The length of the cell, the latency time (Δt_0), the change in force upon stimulation (dF_{act}), the rate of stimulated change in force (β_1), stimulus-off latency time (Δt_3), the stimulus-off change in force (dF_{return}) and its corresponding rate of change ($\beta 2$).
In bladder cells the values found for dF_{return} in the response type groups 3 and 4, and for β_2 in response type group 3 (indicated by * in table 2) were significantly different from the corresponding values found for uterus cells (table 3).

Significant differences in parameter values between response types are indicated in tables 4 and 5. These tables also show a comparison of force increases (and the corresponding rates of change) in response to stimulation (type 1 and 2 responses) with the force increases (and corresponding rates of change) after stimulus switch off in response types 3 and 4. To this end dF_{act} and β_1 were interchanged with dF_{return} and β_2 in this second pair of response types.

Type 1 responses in bladder cells occurred at significantly shorter cell lengths than type 3 responses in the same cells, with a longer Δt_0 or latency time, equal positive change in force, a smaller rate of positive force change, equal stimulus off delay or Δt_3 times, equal negative change of force, but with a faster rate of change. Responses of type 2 and 4 in bladder cells occurred at equal lengths, with equal latencies and positive changes in force, but with differences in rates of positive force changes and in the amounts of negative change of force, whereas the rate of negative force change did not reveal significant differences.
In uterus cells significant differences in cell length and in amount and rate of positive change in force were detected between response types 1 and 3. Type 3 and 4 responses show significant differences for all the parameters listed in table 5.

Table 6. shows the dependence of the responses on the current cell length, in terms of Spearman rank correlation coefficients.

Table 1. The frequency of occurrence of the four main response types (illustrated in Figure 3.) for bladder and uterus cells, and the frequency of occurrence of the active response to stretching of the cells.

Cell type	Type 1	Type 2	Type 3	Type 4	N_{devS}	N_{devL}	t_{devS}	t_{devL}
bladder N = 15	0.051 (0.024)	0.142 (0.050)	0.057 (0.017)	0.142 (0.031)	0.464 (0.076)	0.195 (0.052)	0.176 (0.032)	0.120 (0.032)
	-	>>	<<	-	-	>>	-	>>
uterus N = 6	0.020 (0.012)	0.0075 (0.007)	0.265 (0.097)	0.226 (0.081)	0.284 (0.086)	0.0 (0.0)	0.110 (0.038)	0.0 (0.0)

Average values and Standard Errors of the Mean [numbers in ( )] are shown. A significant difference according to the Mann-Whitney U-test at the 5 % level between values estimated for bladder and uterus cells is indicated by a << or >> symbol. A value of 1.0 means that all responses were of the indicated type, a value of 0.0 means no such response type did occur in this cell type.

Table 2. Active properties of pig urinary bladder smooth muscle cells.

Reaction type N / n	Cell length (µm)	Latency stim. on (s)	Force change (µN)	β_1 (µN/s)	Latency stim. off (s)	Force change (µN)	β_2 (µN/s)
1 / 9 4.3 %	61.1 (6.17)	1.94 (0.47)	1.03 (0.22)	0.10 (0.019)	0.91 (0.33)	-1.26 (0.25)	-0.29 (0.065)
	-	>>	-	-	[>>]	-	-
2 / 30 14.3 %	78.0 (4.73)	1.12 (0.20)	1.10 (0.16)	0.20 (0.030)	-3.71 (0.58)	-1.62 (0.23)	-0.26 (0.030)
3 / 16 7.6 %	83.8 (5.84)	0.86 (0.09)	-1.43 (0.15)	-0.14 (0.015)	1.28 (0.29)	1.51* (0.21)	0.21* (0.021)
	-	-	>>	-	[>>]	>>	>>
4 / 33 15.7 %	78.8 (4.84)	0.90 (0.14)	-0.97 (0.11)	-0.33 (0.089)	-4.21 (0.46)	0.77* (0.083)	0.11 (0.012)

Averages and Standard Errors of the Mean [in parentheses] for: column 1; the type of response observed upon stimulation, the absolute number of such responses and the percentage of all stimulation attempts, column 2; the cell length, column 3; the average latency time after stimulus switch on, before the start of a change in force was observed, column 4; the average change in force upon stimulation, column 5; the average rate of change in force, column 6; the average time after or before stimulus switch off at which the change in force due to stimulation levelled off or reversed, column 7; the average change in force after stimulus switch off, column 8; the average rate of change in force after stimulus switch off. Significant differences according to the Mann-Whitney U-test at the 5 % level are indicated with >> or << symbols. An * indicates a value that differs significantly from the corresponding value for uterus cells, shown in table 3.

Table 3. Active properties of human a terme uterus smooth muscle cells.

Reaction type N / n	Cell length (μm)	Latency stim. on (s)	Force change (μN)	β_1 (μN/s)	Latency stim. off (s)	Force change (μN)	β_2 (μN/s)
1 / 2	50.0	0.71	0.33	0.028	1.64	-0.65	-0.16
1.9 %	(0.0)	(0.53)	(0.20)	(0.014)	(1.35)	(0.16)	(0.031)
	-	-	-	-	[-]	-	-
2 / 1	50.0	1.11	0.15	0.029	-3.64	-0.36	-0.064
0.9 %	(—)	(—)	(—)	(—)	(—)	(—)	(—)
3 / 32	98.3	1.12	-1.10	-0.11	1.49	0.85*	0.14*
29.6 %	(5.67)	(0.18)	(0.087)	(0.008)	(0.19)	(0.073)	(0.012)
	>>	>>	>>	<<	[>>]	>>	>>
4 / 22	69.5	0.54	-0.93	-0.32	-5.73	0.72*	0.13
20.3 %	(5.20)	(0.094)	(0.19)	(0.11)	(0.94)	(0.28)	(0.052)

Uterus cells. Averages and Standard Errors of the Mean [in parentheses] for: column 1; the type of response observed upon stimulation, the absolute number of such responses and the percentage of all stimulation attempts, column 2; the cell length, column 3; the average latency time after stimulus switch on, before the start of a change in force was observed, column 4; the average change in force upon stimulation, column 5; the average rate of change in force, column 6; the average time after or before stimulus switch off at which the change in force due to stimulation levelled off or reversed, column 7; the average change in force after stimulus switch off, column 8; the average rate of change in force after stimulus switch off. Significant differences according to the Mann-Whitney U-test at the 5 % level are indicated with >> or << symbols. An * indicates a value that differs significantly from the corresponding value for bladder cells, shown in table 2.

Table 4. Significance of differences in active properties (as listed in table 2) of pig urinary bladder smooth muscle cells.

Reaction type pair	Cell length	Latency stim. on	Force increase	Positive rate	Latency stim. off	Force decrease	Negative rate
1 - 2	-	>>	-	-	[>>]	-	-
1 - 3	<<	>>	-	<<	-	-	>>
1 - 4	-	>>	-	-	[>>]	-	-
2 - 3	-	-	-	-	[<<]	-	>>
2 - 4	-	-	-	>>	-	>>	-
3 - 4	-	-	>>	>>	[>>]	>>	-

Significant differences in values of the parameters listed in table 2 are indicated by << or >> sign according to the Mann-Whitney U-test at the 5 % level. [<<] or [>>] indicates that the significance is primarily based on inversion of sign in one member of the tested pair.

Table 5. Significance of differences in active properties (as listed in table 3) of human a terme uterus smooth muscle cells.

Reaction type pair	Cell length	Latency stim. on	Force increase	Positive rate	Latency stim. off	Force decrease	Negative rate
1 - 2	-	-	-	-	[-]	-	-
1 - 3	<<	-	<<	<<	-	-	-
1 - 4	-	-	-	-	[-]	-	-
2 - 3	-	-	-	-	[-]	-	-
2 - 4	-	-	-	-	-	-	-
3 - 4	>>	>>	>>	>>	[>>]	>>	<<

Significant differences in values of the parameters listed in table 3 are indicated by << or >> sign according to the Mann-Whitney U-test at the 5 % level. [<<] or [>>] indicates that the significance is primarily based on inversion of sign in one member of the tested pair.

Table 6. Correlation of parameters describing electrically stimulated force changes with cell length for the most frequent response types in bladder and uterus cells.

	Bladder Cells				Uterus Cells	
	Type 1	Type 2	Type 3	Type 4	Type 3	Type 4
No of responses:	9	30	16	33	32	22
Δt_0	-	-	-	-	-	-
Δt_1	-	-	-	-	-	-
Δt_2	0.672	0.353	-	-	-	-
Δt_3	-	-	-	-	-0.377	-
β_1	-	0.322	-	-	-0.340	-
β_2	-	-0.406	-	-	-	-
dF_1	-	0.414	-	-	-0.328	-0.389
dF_2	-	-0.562	-	-	-	-

Spearman rank correlation coefficients are shown for correlations with a significance of 5 %. For definition of parameters and response types: see Materials and Methods and also Figures 1, 2 and 3.
dF_1 is the absolute change in force from F_{init} to F_{ext}.
dF_2 is the absolute change in force from F_{ext} to F_{base}.

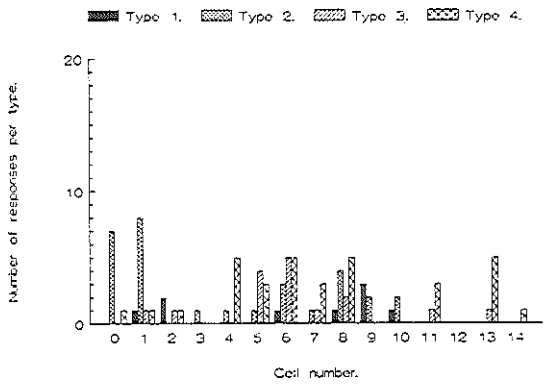

Fig.6: Histogram of the distribution (in absolute numbers) of the four most frequently observed response types in a series of 15 pig urinary bladder smooth muscle cells. For a description of the response types: see Figure 3 and text.

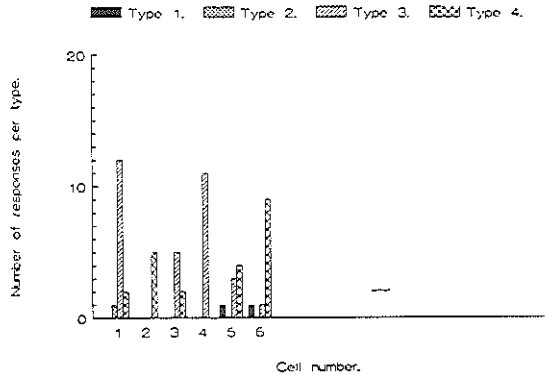

Fig. 7: Histogram of the distribution (in absolute numbers) of the four most frequently observed response types in a series of 6 human a terme uterus smooth muscle cells. For a description of the response types see Figure 3 and text.

Table 7. Correlation of parameters describing electrically stimulated force changes with Latency time (stim. on, Δt_0) for the most frequent response types in bladder and uterus cells.

	Bladder Cells				Uterus Cells	
	Type 1	Type 2	Type 3	Type 4	Type 3	Type 4
No of responses:	9	30	16	33	32	22
Cell length	-	-	-	-	-	-
Δt_1	-	-	-	-	-0.498	-
Δt_2	-0.678	-	-	-0.364	-	-
Δt_3	-	-	-	-	-	-
β_1	-	-	-	-	-	-
β_2	-	-	0.539	0.308	0.314	-
dF_1	-	-	-	-	-	-
dF_2	0.610	-	0.521	-	-	-

Spearman rank correlation coefficients are shown for correlations with a significance of 5 %. For definition of parameters and response types: see Materials and Methods and also Figures 1, 2 and 3.
dF_1 is the absolute change in force from F_{init} to F_{ext}.
dF_2 is the absolute change in force from F_{ext} to F_{base}.

Table 8. Active properties of pig urinary bladder smooth muscle cells. A comparison of latency times (all response types) and 'contraction times' (responses types 2 and 4) of bladder cells from the present data with values measured on freely shortening bladder cells in previous experiments.

	Type 1 Latency time (s)	Type 2 Latency time (s)	Contraction time (s)		Type 3 Latency time (s)	Type 4 Latency time (s)	Contraction time (s)
New data:	1.94 (0.47)	1.12 (0.20)	6.50 (0.65)		0.86 (0.091)	0.90 (0.13)	4.90 (0.45)
	>>	>>	-		-	<<	<<
Old data: CR Fract. I	0.77 (0.071)	0.77 (0.071)	8.11 (0.48)	CK	2.47 (0.56)	2.47 (0.56)	7.32 (0.65)
	-	-	<<		<<	<<	<<
Old data: CR Fract. II	2.39 (0.60)	2.39 (0.60)	8.96 (0.87)	CK	3.54 (0.29)	3.54 (0.29)	9.83 (0.47)

Fraction I: Cells isolated without mechanical manipulation.
Fraction II: Cells isolated with the help of mechanical manipulation.
CR: Cells responding to electrical stimulation with a contraction and relaxing after stimulus switch off.
CK: Cells responding to electrical stimulation with a contraction and showing an enhanced or 'off-contraction' after stimulus switch off.
<< or >> sign indicates significant difference at less than 5 % level. [numbers between ( ) = Standard Error of the Mean]

Table 7. similarly shows the correlation between latency (Δt_0) and the force variables. In bladder cells only in type 2 responses consistent correlations are found with cell length: dF_1 increases with increasing length, as also the rate of force change, β_1, does. Consequently the amount (dF_2) and rate of relaxation (β_2) increase with length. In uterus cells response types 3 and 4 both show an increase of stimulated force decay with increasing length, whereas in type 3 also β_1 is similarly correlated. Correlations based on latency time (Δt_0) as an indicator of cell performance only reveal that β_2 is correlated significantly with latency time in 3 out of 6 response and cell types.

The results of evaluating the 'contraction like' deviations superimposed on the stress relaxation curves that were measured in response to the increases in cell length are summarised in table 1, columns 5 and 6. Column five shows weighted average percentages of relaxation curves observed with these phenomena (N_{devS} and N_{devL}) and column six the average percentages of total sampled time during which the relaxation curves showed the deviations (t_{devS} and t_{devL}).

Although the data suggested that in uterus cells a smaller number of curves, and also a smaller percentage of total sampled curve time showed small deviations, both differences were not significant at the 5 % level according to Mann-Whitney U-tests. In contrast to bladder cells, deviations larger than 2.0 μN were not observed in uterus cells. This difference was significant. Figure 8 shows a typical example of a bladder cell relaxation curve upon which, after a delay time of approximately 20 seconds, a 'large contraction wave' is superimposed. Figures 2 and 6 in part III (Glerum *et al.*, 1990) demonstrate the smoothness of the uterine cell relaxation curves.
The relation between the cells' active responses to electrical stimulation and to stretch were studied by correlating the frequencies of the four response types and of the curve deviations shown in table 1. At the 5 % level in bladder cells only 3 significant correlations were found: type 3 responses with type 4 responses (Spearman's rho = 0.470), N_{devS} with N_{devL} ($\rho = 0.781$) and t_{devS} with t_{devL} ($\rho = 0.735$). In uterus cells only the correlation between response types 3 and 4 was found to be significant, but negative ($\rho = -0.771$).

Table 8 compares the values found in previous experiments for latency time and contraction time in bladder single smooth muscle cells that were electrically stimulated to shorten freely (Glerum *et al.* 1987), with those bladder cells in the present data reacting similarly: CR cells were compared to type 2 cells and CK cells to type 4 cells. In the last category it is assumed that the stimulus-off contraction found in CK cells is equivalent to the rise in force that occurred in type 3 and 4 responses after stimulation was switched off. In both type 1 and type 3 responses, force kept increasing during the whole period of stimulation, so that there was no comparable measure for contraction time in these cells.
As can be seen in the table the bladder cells in the present isometric measurements responded (according to latency)

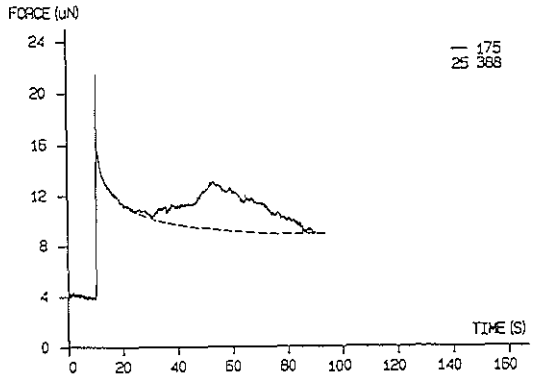

Fig. 8: A typical example of a stress relaxation curve measured in response to a 10 μm rampwise length increase starting at t = 10 s. At t = 30 s a wave like superimposed increase in force started, its maximum occurred at t = 50 s. The dotted line indicates how the initial stress relaxation curve was supposed to have continued if this rise in force had not occurred.

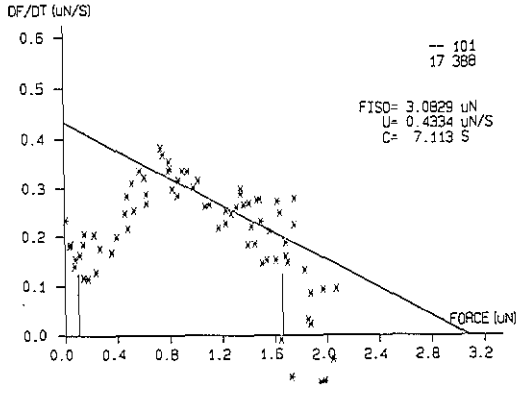

Fig. 9: A typical example of a phase plot of the single smooth muscle cell contraction shown in Figure 4. Asterisks depict the change in force as a function of force. The straight line was fitted to the linear phase of this change of force and intercepts the force axis at F_{iso}, the force that would have been attained if the cell had been stimulated infinitely long. The inverse reciprocal value of the slope of the line, C, indicates the time constant i.e. the time needed for the force to reach 63 % of the value of F_{iso}.

Table 9. Active properties of pig urinary bladder smooth muscle cells.

a: Isolated single pig urinary bladder smooth muscle cells.

	Cell number	Cell length (μm)	F_{iso} (μN)	C (s)
	0	90	1.89	5.53
	0	90	3.08	7.11
	0	90	2.31	7.52
	1	80	1.16	2.27
	6	55	2.25	3.91
Average		81.	2.11	5.27
S.E.M.*		(6.)	(0.31)	(0.88)

b: Small pig and human urinary bladder smooth muscle bundles.

Preparation number	F_{iso} (μN)	C (s)
Pig bladder 1	4.89	3.2
Pig bladder 2	2.12	2.76
Human bladder 1	6.80	7.03

a) Results of phase-plot analysis of a selected number of contractions measured on isolated single pig urinary bladder smooth muscle cells. F_{iso} = extrapolated isometric force, C = time constant of force development.
b) Estimates for F_{iso} and C per cell in micro strips of smooth muscle, calculated by dividing F_{iso} values by the number of cells counted under a microscope in Gomöri stained slides of a cross-section of the strip. C is independent of the geometry and number of cells and therefore values for cells and strips are directly comparable. From (Van Koeveringe and Van Mastrigt, 1991).
* = Standard Error of the Mean.

slower than Fraction I CR cells, but faster than Fraction II CR cells, whereas the type 3 and 4 responses were in most cases faster than the CK type bladder cells from either Fractions I or II. In bladder cells the responses, in which force increase levelled off before the end of stimulation, lasted shorter than similar responses in the cells from the previous experiments.

Table 9 shows the results of phase plot analysis of a limited number of cells that responded to electrical stimulation with a force increase (see also Figures 4 and 9) compared to normalized average values of the parameters F_{iso} and C obtained from measurements on small pig bladder muscle bundles (Van Koeveringe and Van Mastrigt, 1991).
As is shown in this table, the selected cells perform with a lower value for F_{iso} and a higher one for C, i.e. the isolated cells are weaker and slower when compared to cells in muscle bundles.

Discussion

In this study single smooth muscle cells isolated from the pig urinary bladder and from the human a terme uterus were electrically stimulated. In a limited number of attempts this gave rise to an increase in force, in a considerable number of other attempts it resulted in a reversible decrease in force. The possible origin and cause of these force changes and, more specifically, whether these might be ascribed to processes in the contractile apparatus of the cell, are discussed here.

Table 10 summarises data from contractility studies reported in literature to facilitate comparison with our present data (Tables 2 and 3). It shows values for physiological parameters measured in response to various methods of activation applied to single smooth muscle cells and micro-preparations of smooth muscle tissue.

Comparison shows that the parameters found in our type 1 and 2 responses fit well into the spectrum of values displayed.
In more detail, the pig urinary bladder cells we have isolated are shorter than the Buffo Marinus cells (Glerum *et al.*, 1987). Latency times could only be found for swine carotid artery rings and are shorter in this preparation than in our bladder cells, but differences in method of estimation may contribute to this.
In bladder cells the maximum force increase upon stimulation is slightly smaller than the low end of the range reported for Buffo Marinus. The average rate of force increase in type 1 and 2 responses concurs well with the values reported for Buffo Marinus.
The contraction time is by definition limited to approximately 9.5 s in type 1 responses and averages 5.5 s in type 2 responses, which agrees well with Buffo Marinus stomach, swine carotid artery and rabbit taenia coli.
Data on the dynamics of the relaxation phase were not published but were deducted from graphs depicting Buffo Marinus recordings, showing that our bladder cells relax more and at a higher rate than toad stomach cells.

Data on phenomena comparable to response types 3 and 4 were not found in literature, except for electrically stimulated bronchial smooth muscle strips that revealed equivalent relaxations upon stimulation (De Jongste, 1988).

Although the parameter values measured in type 1 and type 2 responses do quantitatively resemble those reported on other preparations, these positive responses represent only approximately 42 % of the measured responses (above the noise level) in bladder cells and even a lesser minority (approximately 5 %) in uterine cells, which means that most of our observations are deviant from those generally presented in literature.

One possible cause for such aberrations is the applied method of stimulation: In order to avoid temperature and/or osmolarity changes and mechanical disturbances and to be able to stimulate only one cell at a time, the attached single smooth muscle cells were stimulated electrically. This method of stimulation, which is generally accepted in muscle physiology, leads to reproducible intra- and inter-experimental results (Van Mastrigt and Glerum, 1984, Glerum *et al.* 1987, Harder, 1982) and has been shown to avoid rate limiting processes in the excitation contraction coupling (Singer and Murphy, 1987, Van Koeveringe & Van Mastrigt, 1991).

In the experiments now presented, variations in the net effective stimulation field may have occurred. The highly alinear electrical properties of the electrode did prove to be dependent on the degree of organic pollution caused by electrophoresis of protein molecules in the bathing solution. In former experiments (Glerum *et al.*, 1987) the electrodes were repeatedly cleaned after every 3 - 5 stimulation attempts. In the current experiments this was mechanically impossible as it would have endangered the stability of the measurement and in particular that of the attached cell.
Assuming that deterioration of the net electrical field did occur, this could have formed a possible source of variation in normal contractile responses. However, a critical, sub-minimal stimulating voltage/current that evokes stimulated force decays in single cells was, to our knowledge, never reported in literature. Moreover, all experiments were started with the same intensively cleaned electrode whilst most uterine cells showed force decays from the onset of an experiment on. It is therefore unlikely that sub-level stimulation played a key role in the force decay phenomena.

Because in our method of electrical stimulation voltages were kept as low as 1.8 V p.p. and currents near the cells were as small as 10 - 100 μA, we consider the possibility that free radicals, which are believed to exert in some way inhibitatory effects on the smooth muscle cells, were being formed rather unlikely (Harder, 1982).

Other possible sources of variability in overall performance of both types of cells open to discussion are differences in metabolic condition of the cells, possibly caused by uncontrolable impurities and variation in local enzyme activity during the isolation procedure, ultimately resulting in differences in excitability of the cells.
In this respect one would anticipate that the most mildly treated cells, i.e. the uterus cells that underwent a shorter and less agressive isolation procedure, would show the best contractile performance. This better performance is however only reflected in the percentage of vital cells, the uterus cells showing more fluorescent cells than the bladder cells (unpublished results).
In this same context the possible detrimental effects of Trypsine on bladder cells appear to have been moderate or even insignificant. Table 8 shows that the cells isolated in the present study, with the use of Trypsine, performed better in terms of latency and contraction time than the cells isolated in previous studies without the use of Trypsine but using vigorous mechanical manipulation (i.e. Fraction II cells), whereas they performed equally well or even better than the cells that only underwent collagenase treatment (Fraction I cells), which makes it less likely that the actions of Trypsine caused our contrasting findings.

One possible side effect of the method of isolation used for the bladder cells is the occurrence of a calcium paradox (Ruigrok, 1985), as these cells all went through a calcium free phase. However, as most cells showed several normal contractions, an evident calcium paradox seems not to have occurred and gross damage in terms of ion balances does not seem to have taken place. This view is supported by the observation that in pig urinary bladder strip preparations contractile activity returned back to normal with the restoration of the external calcium concentration (Van Mastrigt *et al.*, 1986), a mechanism which apparently was conserved in the isolated bladder cells.

Table 10: Comparison of literature values for parameters describing contractile properties of single cell and micro strip smooth muscle preparations of various species.

Author	Year	Cell type	Temp (°C)	Cell length (μm)	Latency time (s)	Force increase (μN)	V_{max} unloaded (L_{cell}/s)	Rate of force increase (μN/s)	Contraction time (s)	Force decrease (μN)
Warshaw & Fay	'83	Buffo Marin.	20	137 (7)	-	2.26 (0.20)	-	0.32	7.0	-
Warshaw & Fay	'83	Buffo Marin.	20	-	-	1.25-2.14	-	-	-	-
Yagi, Becker, & Fay	'88	Buffo Marin.	25	-	-	1.69 (0.48)	-	0.10	11.07	1.11 β_2: 0.077 Δt_2: 4.29
Warshaw, Work, & McBride	'87	Buffo Marin.	20	99.7 (7.2)	-	2.13 (0.16)	Freely Shortening 0.20 Slack 0.58	-	-	-
Hai & Murphy	'88	Swine carotid artery	37	-	-	-	0.033 0.029	-	-	-
Singer & Murphy	'87	Swine carotid artery	37	-	0.34 (0.07)	-	0.067 0.043	-	10.0	-
Siegman *et al.*	'84	Rabbit taenia coli	18	-	-	-	0.050	-	13.33	-
				Fraction I CR type cells: (Freely shortening)						
Glerum *et al.*	'87	Pig urinary bladder	37	196 (81)	0.8 (0.8)	-	*0.053 (0.046)	-	8.1 (5.2)	-
				Fraction II CR type cells: (Freely shortening)						
				116 (26)	2.4 (3.0)	-	*0.046 (0.044)	-	9.0 (4.4)	-

For sources of information see References. Cell type: the kind of species/tissue the preparation was taken from. Temp: the temperature at which experiments were performed. Cell length: the average cell length of the single cells. Latency time: the time delay between start of the stimulus and measurement of the contractile response. Force increase: the average force increase measured as a maximum response. V_{max} unloaded: the maximum speed of shortening (calculated from experimental data, or measured on freely shortening cells or in slack test experiments). Rate of force increase: average rate of force increase as approximately measured for β_1. Contraction time: the average time needed to complete a contraction from the moment of stimulation until the moment of levelling off of the active increment of force. Force decrease: the average amount of decay in force after cessation of stimulation. * = Average speed of shortening during the full duration of the contraction. [Numbers between ( ) = Sandard Error of the Mean, when available.]

A much more mechanically inspired explanation for the observed negative force responses can be sought for in the relationship between cell length and optimum contractile response. In this study responses were measured at a range of lengths including and beyond L_{max}, i.e. the length at which maximum isometric output is recorded. In order to cover this wide range of cell lenghts, rather large length changes of 10 µm were applied, so as to avoid cell deterioration by a too long experiment duration.

Nowadays smooth muscle cells are believed to have a cyto-skeleton-like supportive system that runs from one end of the cell to the other and is situated in a micro-anatomically separate compartment of the cell (Small *et al.*, 1986). It therefore seems possible that the contractile system, once it has been overstretched beyond its momentary working range by a too large length increment, can rearrange or reorganise or even resynthesize contractile filaments along the lines of the supportive filaments. In the course of such a process various arrangements could be possible that result in a situation in which thick and thin filaments are arranged thus that a retrograde cycling of cross-bridges is favoured, or that a 90 degree strained state of the cross-bridges occurs (Eisenberg and Greene, 1980, Eisenberg and Hill, 1985) that gives (temporarily) rise to negative force generation.
In our results positive or negative force responses did not correlate significantly with absolute cell length and in the uterine cells negative responses occurred already at short cell lengths at the onset of the experiment. Therefore absolute cell length was not directly related to the variability of responses, but momentary overstretching might have been.

Table 1 shows two striking differences between bladder and uterus cells, both related to the excitability of the cells:

1) Bladder cells showed, both in terms of absolute numbers and of percentages of all recorded bladder cell responses, significantly more responses with increasing force upon stimulaton (Type 1 and 2 responses) than uterus cells. The distribution of response types in uterus cells is more than just complementary to the pattern in bladder cells, as in uterine cells the percentage of attempts resulting in measurable responses is higher (57 % as opposed to 44 %) and at the same time is dominated by responses with decreasing force upon stimulation (Type 3 and 4 responses).

2) There was a significant difference in the 'smoothness' of the relaxation curves. Especially the uterus cells did not show any deviations or superimposed contractions that exceded the 2.0 µN peak limit.

Inter experimental variability due to procedural variations in the process of isolation are very unlikely to have been the cause of these differences as both types of experiments were performed in a more or less random order, depending on the availability of the uterine material. It is more likely that these differences were caused by differences in excitatory and inhibitatory pathways, very commonly encountered in various types and species of smooth muscle cells (Brading, 1989) which are related to the specific functions of the smooth muscle.

In case of the urinary bladder, 'designed for' accommodating increasing, rather large, volumes of fluid to be expelled in one continuous contraction, a contractile apparatus is necessary that has the ability to act over a large length range and is instantaneously excitable.
The uterus in contrast adapts at a different time scale to an even larger volume change, but has to remain effectively silent until the moment of birth. At this particular time scale hypertrophia, growth and hormonally controlled changes in membrane excitability or even micro-structural changes in the smooth muscle cells and in the interconnecting junctions (Fuchs and Fuchs, 1984, Garfield *et al.*, 1977, Kao, 1989, Cole and Garfield, 1989, Soloff, 1989) play a role as important additional factors to simple stretching of connective tissue and smooth muscle elements.
Considering this with respect to the moment in pregnancy at which the uterine cells were obtained (all women were obviously not about to start labour for at least 24 hours), it is concluded that our data reflects the properties of uterine smooth muscle cells that are still in a kind of 'resting' or low excitability phase.

In single cell experiments two mechanisms have been proposed for stretch evoked contractions: Generally Ca^{2+} influx initiated by stretch induced membrane depolarisation (Tomita, 1981), or, specific for these kind of preparations, direct leakage of Ca^{2+} into the cells at sites of great membrane stress, such as the knots (Warshaw *et al.*, 1987).
In our attachment technique the stress at the locations where the membrane interacts with the pipette did not amount to levels comparable to those in other attachment techniques due to the Poly-L-Lysine coating of the micro-pipettes, spreading out the force over a larger surface area (Glerum and Van Mastrigt, 1990), so that the latter mechanism is less likely to have caused the superimposed waves on the stress relaxation curves.
Furthermore all curves showed a delay time of at least 10 s after a stretch (see Fig. 8) before the typical waxing and waining, that is so commonly known from smooth muscle preparations in vitro, did occur. Both facts exclude a considerable continuous inward leakage of Ca^{2+} and make a continuous cycling of cross-bridges very unlikely.

No significant (at the 5 % level) correlations were found between the frequencies of the occurrence of the different response types and the occurrence of stretch induced force changes in either kind of cells. This finding suggests that the electrically induced force changes and the stretch induced phenomena were mediated through different excitatory pathways.

Although the positive responses obtained in bladder cells fit nicely into the spectrum of data illustrated in table 10, comparison of the type 1 and 2 responses with our own findings in strip preparations of the same material shows that, in terms of F_{max} (dF_{act} in the present data) and average rate of force increase (β_1), force development in the single cells was rather small and slow.

Frequently, (continuous) variation of the rate of force development in smooth muscle cells, brought about by internal regulating systems controlling the cross-bridge cycling rate has been reported (eg. Hai and Murphy, 1988a and b, Butler *et al*, 1986).
Several reports indicate that in various kinds of smooth muscle cells there is a bi-phasic pattern of force development, starting with a high inital rate, then slowing down after 1 - 5 s to a much slower cross-bridge cycling rate (Singer and Murphy, 1987, Krisanda and Paul, 1984, Siegman *et al*., 1984), possibly related to different calcium mechanisms (Rasmussen, 1989).

Both effects might play a role in the findings reported here: Modulation of the cross-bridge cycling rate might be caused by external factors such as specific hormones or intermediates of metabolism missing in the bathing solution, or result from non-optimal electrical stimulation.
To what extend the modulation of the rate of force development truly is an effect of changes in cross-bridge cycling rates remains to be investigated as experiments on small smooth muscle bundles of similar pig urinary bladders indicated that the rate of isometric force development is limited by the rate of extra-cellular Ca^{2+} influx, whereas cross-bridge cycling is one order of magnitude faster ($t = 0.45$ s), (Van Koeveringe & Van Mastrigt, 1991).

As every contractile response of smooth muscle tissues or cells in tissues is probably the net result of excitatory and inhibitatory effects through different pathways (Brading, 1989, Dillon and Murphy, 1982, Johansson and Somlyo, 1980), it is defendable that this may also be the case at the cellular level (Brading, 1989, personal communication). A negative net balance between excitation and inhibition at the cellular level might then result from excitatory pathway regulating channels being more easily damaged by the cell isolation techniques than the inhibitatory pathway channels.

In disagreement with this hypothesis the most harmlessly treated cells (uterus cells) showed almost solely force decay responses, whereas the trypsinisated bladder cells showed rather good force increment responses too.
Furthermore, if the stimulated force decays resulted merely from inhibition overruling excitation, it is strange to experience that the 'optical phase-contrast changes' and the dynamics and time course of these decays are very much similar to those of the force increments, (as is also illustrated by the absence of significant differences in Tables 4 and 5), and also that after a stimulated decay force is regained up to at least the pre-stimulation level. To our opinion, if merely passive relaxation were taking place, different time courses are to be expected.

From the arguments discussed we conclude that both the stimulated force increases and the force decays observed are probably related to 'active' processes in the cells. Whether the negative responses represent a modified (unphysiological) state of the cells remains as yet unclear.

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Summary and General Discussion

Summary and General Discussion

General aspects

Smooth muscle plays a key role in many functions of the human body. In the field of urology, especially that of urodynamics, much effort has been made to analyse and model micturition. In gynaecology and obstetrics an even longer history exists of research dealing with the process of childbirth. Both are processes concerned with the regulated emptying of a hollow organ, preceeded by a filling phase, but they greatly differ in terms of time scale, force development and mechanisms of regulation and control.

The main and most important structural component of both, the urinary bladder and the uterus with respect to mechanical functions is smooth muscle. The other important components in this context are connective tissue materials such as collagen, elastin and reticulin, which determine to a considerable extent the passive properties of both types of tissues.

Both passive and active properties of the tissues can be measured in vitro and in vivo on whole organs and on tissue strip preparations, but all these types of experiments result in answers that cannot discriminate between properties of the muscle component and the connective tissue components. Moreover, much confusion is generated by the random manner in wich both smooth muscle cells and connective tissue elements are intermingled in the tissue, resulting in a lack of one preferential direction for force development.

With the studies presented in this thesis it was attempted to achieve a discrimination between properties of smooth muscle and of other components by measuring the passive and active properties of single smooth muscle cells isolated from both types of tissues.

At the onset of the study experiments were solely performed on cells isolated from pig urinary bladders, which are quite similar to those of the human urinary bladder. In a later phase the study was extended to human uterine tissue fragments obtained at planned Caesarean sections.

In order to achieve the goal of the study many practical and technical problems had to be solved. Some of the technical aspects have been published in international journals as a technical note or are embodied in the publications describing the results obtained. Many others, although of crucial importance for the success of these experiments, especially in the field of cell and muscle physiology, were considered to have more practical and too little scientific value to be described in separate publications or in this thesis.

Contents of the thesis

Chapter 1 contains the introduction into the thesis. A short review of the recent history of smooth muscle physiology with respect to contractility and single cell isolation is given. The goal of the thesis is explained. Next the structure of the thesis and the steps taken with the different sets of experiments are described.

Chapter 2 deals with the problem of isolating smooth muscle cells from tissues that have a high content of connective tissue components such as that of the urinary bladder. As no such methods had been published so far and as methods developed for tissue types less rich in connective tissue components in our hands failed to result in reasonable amounts of cells of acceptable quality, it was necessary to develop a new method of cell isolation.

During this process there was a need for better tests for assessing the quality of the resulting cells than those commonly used in the field of cell biology. Simple dye exclusion tests such as the Trypan Blue test proved to be of too little value. In search for a better viability test it seemed that the only good standard for muscle cell vitality and functionality is the assessment of contractility.
As it was intended to test relatively large numbers of cells in the suspensions obtained by the various isolation procedures performed, methods and equipment were developed to assess contractility on cells in suspension without the need for mechanical attachment. In short: Single smooth muscle cells in a relatively diluted suspension were individually stimulated electrically to contract. For every attempt of stimulation of a cell, the whole process from stimulation through contraction until relaxation was recorded on a videotape for later analysis. At the same time these events

were timed by pressing buttons that started and stopped timers that were generated and controlled by a computer program. These timers were also simultaneously projected on the video image.
During the analysis, guided by the timers, specific video frames showing the responses of the cells to electrical stimulation were projected as still video pictures on a monitor. In the same picture a computer controlled cross-hair was positioned along the cell of interest in order to accurately estimate the cell's length in relation to the contractile events. From these combined data on time and length, cell length, latency, time and speed of shortening and relaxation and percentages of shortening and of relaxation were estimated.

Using this method of contractility assessment two different versions of the new method of cell isolation were evaluated with respect to the quality of the single smooth muscle cells obtained. This evaluation showed that a smaller degree of mechanical manipulation resulted in a smaller number of cells in suspension, but that the cells were of a better functional quality. The isolation procedure that incorporated vigourous mechanical loosening of the cells resulted in shorter cells of which a smaller percentage responded in a classical manner to electrical stimulation. The cells obtained in this way showed longer latency and contraction times and a lower percentage of shortening than those found using the 'non-mechanical' method of isolation.

With the experiments reported in Chapter 2 it was proven that direct electrical stimulation of single smooth muscle cells from urinary bladder is actually possible and that this method of stimulation generates reproducible contractions of acceptable quality. From the analysis of the data it was concluded that mechanical manipulation increased the numbers of isolated cells but gave cells of lower functional quality.
Both methods proved appropriate for routine isolation of this type of cells. The best method was used during the further experiments described in this thesis with some small modifications.
Neither of both methods yielded a final answer to the question of how to deal efficiently with the gross amount of connective tissue elements greatly complicating and protracting smooth muscle cell isolation from pig urinary bladders. Also the problem of how to separate the cells at the level of the gap-junctions, which are so abundantly interconnecting the cells in bladder tissue, without damaging at least one of two interconnected cells, had to remain unsolved within this study. This last problem probably is the reason why the overall yield of good functional cells remained relatively low with respect to the amount of incubated tissue fragments. Based on these findings contractility assessment as the method of first choice for determining the quality of isolated smooth muscle cells is strongly advocated.

Chapter 3 reports on the design and features of the cross-hair apparatus employed in the measurements described in Chapter 2.

The essential property of this apparatus is the possibility to position a cross-hair in still and moving video pictures originating from any kind of video source without the need for synchronisation between the controlling computer and the video signal. Other specifications are its ease of software control by any type of computer that has a standard parallel digital output and its very low cost (f 200,—) in comparison to other solutions available at that time (f 10.000,— to f 100.000,—).

Chapter 4 describes the development of an ultra sensitive force transducer for isometric measurement of contractile forces generated by single smooth muscle cells in a range of 0.1 to 10.0 μN. With the same transducer and on the same cell also the measurement of passive forces as high as 100 μN, which result from stretching the cell by length ramps, had to be possible. These two main prerequisites led to the conclusion that no such transducer had been described in literature or was commercially available at that time. Therefore such a transducer had to be developed.

The basic principle of the transducer is the transformation of force into displacement by elastic deformation of a spring or beam with known properties. To realise a stable construction two beams, interconnected by a cross beam, were used, thus obtaining a system capable of moving only along one axis. High resolution detection of the displacement was achieved by an electrical-optical system sensing the position of a vane attached to the cross beam. The system uses the principles of synchronous carrier detection in order to avoid influences from other visible and infra red light sources.

The transducer performed to expectation for the duration of the study and in the proto-type all requirements formulated at the start of the development were met. However, to upgrade the overall performance, enhancement of the temperature stability is desirable and also the resultant overall signal to noise ratio needs improvement, although shortcomings of the last kind were not primairily attributable to the properties of the transducer but to the inevitable mechanical noise level at the site of experimentation.
The feature of instantaneous exchangeability of the cross beam element proved to be of great value in the practice of daily measurements. During the few experiments in which one of the beams became damaged, the measurements could still be performed after the cross beam element had been replaced. As yet this valuable property has not been realised in comparable transducers that are commercially available now.

Chapter 5 describes the evaluation of a method for attaching single smooth muscle cells to micro instruments developed as a modification of the procedure originally reported by Fay (1977, Warshaw and Fay, 1983).
Initially it was attempted to employ this method in an unmodified fashion but the technique was to complicated for routine use and did not frequently result in appropriate attachment of the type of cells used.

Both the original and the modified method are based on a combination of two principles of attachment: First the cell is losely attached to two micro pipettes on the basis of electrostatical attraction, next the attachment is secured by one or more knots that are fastened simultaneously at both ends of the cell.

The principle difference between the original method and our modification is that in the former one ion exchange raisin beads are used to create local sources of electrostatic attraction, whereas in the latter Poly-L-Lysine, evenly spread over the pipette tip surface creates a much larger area for such attraction. This results in three advantages: There are no longer rather large raisin beads that occupy valuable space, the area of electrical attraction is greatly enlarged and less critically located and, because of the first two advantages, shorter cells can be knotted with only one knot instead of three as was required in the original method.

Evaluation of the new method of attachment showed that urinary bladder and uterine smooth muscle cells could be routinely attached in a way that always proved to be stronger than the force a cell ultimately could withstand upon stretch. The method offers the possibility to perform force measurements in line with the cell's length axis and allows for fast variation of the cell's length during experiments. Both are important prerequisites for investigating the mechanism of cell contractility in relation to cell length.

Chapter 6 describes experiments in which the passive properties of pig urinary bladder and human a terme uterine smooth muscle cells were investigated.

In these experiments the cells were isolated according to the methods described in Chapter 2 and attached as described in Chapter 5. Following its attachment the length of a cell was increased until the first signs of stress relaxation appeared in the measured force signal. This length was optically determined and defined as 'initial length' or 'length in rest'.

Next the cell went through repeated cycles of two electrical stimulations, to evoke contractile responses, concluded with an increment in cell length. These cycles were continued until the cell finally broke as a result of the summation of the length increments.

The length increments were of a relatively fast ramp like shape and resulted in immediate rises in passive force followed by stress relaxation. The force curves that resulted were sampled and stored by a computer.

During off-line analysis the maximum rise in force resulting from the length ramp was estimated for all curves. From these peak forces as a function of cell length the average chord stiffness was calculated. Also the remaining force level after 80 seconds of stress relaxation was determined in all curves. By combining the cell's volume with the rise in force at the cell's greatest length the maximum modulus of elasticity at maximum length was estimated.

Selected curves were fitted with a single exponential function. Throughout the selection and fitting of the curves it turned out that many more relaxation curves of bladder cells showed deviations from the ideal exponential form than relaxation curves of uterine cells and that the bladder cell curves were much more heavily disturbed than the uterine ones.
In these experiments it was also demonstrated that single cells isolated from pig urinary bladders show a relaxation pattern that clearly differs from that of intact bladder tissue, as the first yield much less stress relaxation than the latter in the same period of time.
In a more general sense, neither the bladder cells nor the uterine cells showed a relaxation to the zero level such as reported by several authors for other types of cells, but all curves levelled off at a defined value which averaged to 50 % of the initial peak force after 80 seconds of relaxation. This means that passive force is sustained for a much longer period of time in these types of cells.
In our results we estimated the maximum elastic modulus for bladder single smooth muscle cells as at least a tenfold higher than in intact bladder tissue.
These three combined findings clearly demonstrate the value of measurements of passive properties on single cells versus those on whole tissues, offering an oppertunity to discriminate between properties of cells and other constituting elements.

Based on these findings we concluded that passive properties of these single smooth muscle cells are most likely determined by the properties of either the cytoskeleton or the cell-membrane, but most likely the combination of both.

With this method passive properties of single smooth muscle cells have been investigated at ranges of length and force far beyond those previously reported in literature.

Chapter 7 describes how some aspects of the active or contractile properties of smooth muscle cells isolated from pig urinary bladders and human uterusses were investigated.

In this Chapter the responses to electrical stimulation of the same cells that were described Chapter 6 have been analysed. In 44 % of the stimulation attempts on bladder cells a response to the applied electrical stimulus was obtained that rose above the noise level. In uterus cells this percentage was 57 %.
Four main types of responses were discerned. Two types started with an initial rise in force in response to the stimulation, next followed by a force decay. Two other types started with a force decay in response to stimulation, then followed by a rise in force upto or beyond the pre-stimulation level. In each two types there was one type where the stimulated response continued until the stimulation was switched off and one where the stimulated response already levelled off before stimulation was halted.

In bladder cells, responses with an initial force increase were almost as frequent as responses with an initial decay of force. The uterine cells almost solely responded to stimulation with force decays.

During both the initial force increase and the initial force decay responses, changes in phase-contrast were observed that were very similar and independent of the type of reaction that occurred in response to the stimulus. This falling together with the observation that the dynamics of the changes of force did not differ significantly in force decays and force rises and the fact that both types of responses could be repeatedly elicited by electrical stimulation of the same cell convinced us that both are results of activities of the contractile apparatus. The responses with initial force increase were considered to be contractions in the 'classical sense'.

For a selected number of the 'classic' contractions measured in single bladder cells, phase-plot analysis was performed. This method of analysis describes the change in force as a function of the force at that moment. It showed that single bladder cells contracted slower and attained lower maximum force levels than cells in small intact bundles of smooth muscle from the same organ and species. In our opinion the explanation for this difference must be found in a non-optimal method of cell isolation, reducing cell functionality, or in a non-optimal method of stimulation or both. Another possible explanation is that the interconnection of cells between each other plays an essential role in the regulation of contractility of these kinds of cells.

Further analysis of the force deviations observed on the stress relaxation curves as described in Chapter 6 showed that these were most likely stretch induced spontaneous contraction waves, so commonly known in smooth muscle tissues. It was shown that the uterine cells differed significantly from the bladder cells with respect to the height of the deviations and the frequency with which they occurred. The uterine cells showed only very small deviations on the stress relaxation curves.

The large observed variety of responses, especially the stimulated force decays, calls for extensive deliberation on the cause(s) of these effects. In general it seemed that at least a significant part of the possible reasons for such variability was cell type specific. The uterine cells almost always showed force decays after stimulation and in these same cells no large deviations superimposed on the stress relaxation curves were observed. Explanations such as major differences in methods of isolation and experimentation seemed unlikely.

In both types of cells similar unphysiologically large length steps together with a more or less uncontrolled method of electrical stimulation were used during the experiments. These two factors might explain the appearance of unusual responses to electrical stimulation, but do not explain the consistent difference in the frequencies of these phenomena. This leaves for explanation of the observed variety of phenomena factors that are closely related to cell type specificity such as the absence of hormones and prostaglandines in the incubation fluid of the uterine cells. The fact that none of the biopsies was obtained at a time that close to the start of labour that the cells could have undergone the 'priming' that seems so essential for full myogenic activity of the uterus may have played a role.

In general to our opinion none of the foregoing arguments is conclusive and further research will have to elucidate the true meaning of especially the electrically induced relaxations.

Final conclusions and directions for further research

In this thesis a method for measuring contractile and passive responses of single smooth muscle cells is described which can serve as a basis for future developments. The clinical implication of improving this technique may be that biopsies obtained from various types of smooth muscle can, in addition to cytological and histological tests, be screened for contractile function at the cellular level. This may lead to the possibility of better differential diagnosis between disturbances at either the cellular i.e. the muscular level or at the level of innervation and higher centres of neural control and elucidate whether any functional disorder is caused by abnormalities of the smooth muscle cells themselves.
In the field of experimental physiology and pharmacology the use of single smooth muscle cells offers the possibility to reduce the turnover of animals for experimental purposes, as large amounts of cells can be recovered from one (piece of the) organ. This also implies a more economic means of testing many pharmaca in parallel. The effects observed in this way should be much closer related to the actual and direct action of a pharmacon on a muscle cell.
There may be an advantage in measuring contractility related effects directly in single smooth muscle cells above receptor binding studies in tissue cultures. If a receptor is present on the cell and the molecule of interest has the ability to bind and exert an effect on contractility, this effect can directly be studied.

Future research will have to be directed towards improvement of the method of isolation. This may result in better cellular responses. Also the transducer needs more sophistication, in terms of dimensions, thermal stability and bandwidth. The whole measurement apparatus should be modified towards smaller and much more integrated instruments. A glueing technique for the method of cell attachment would form a considerable improvement. Finally the role of the stroma surrounding the cells and of the junctions interconnecting the cells in contractility should be studied.
It would be desirable to simultaneously study functional events of contraction on a mechanical and on a biochemical level. Unfortunately a multidisciplinary smooth muscle study group does not exist in Rotterdam.

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Samenvatting en Beschouwing

Samenvatting en Beschouwing

Algemeen

Glad spierweefsel speelt een sleutelrol in veel onderdelen en functies van het menselijk lichaam. Binnen het specialisme urologie is, speciaal vanuit de urodynamica, sinds enige tientallen jaren veel aandacht besteed aan de analyse en modelvorming van de mictie. Binnen de gynaecologie en obstetrie tekent zich historisch gezien een veel langere traditie af betreffende onderzoek van het proces van de geboorte.
In beide gevallen gaat het om het bestuderen van een gereguleerde ontlediging van een hol orgaan. Beide organen (de urineblaas en de uterus) kennen voorafgaand aan de ontlediging een fase die juist vulling toestaat. Hoewel in algemeen mechanische zin de functie van blaas en uterus sterk overeenkomen, bestaan er grote verschillen voor wat betreft de tijdschaal waarover de processen zich afspelen, de grootte van de kracht die ontwikkeld wordt, en de wijze waarop de gladde spierfunctie gereguleerd en gecontroleerd wordt.

Het meest voorkomende en voor het mechanisch functioneren meest belangrijke bouwelement van zowel de urineblaas als van de baarmoeder is glad spierweefsel. Naast deze zijn het, vanuit mechanische optiek gezien, vooral de bindweefselcomponenten zoals collageen, elastine en reticuline die voor een belangrijk deel de passieve eigenschappen van beide soorten weefsels bepalen.

Van deze gladde spierweefsels kunnen zowel de passieve als de actieve eigenschappen in vivo en in vitro gemeten worden. Deze metingen kunnen verricht worden aan complete organen of aan weefselstripjes, maar altijd blijft de (relatieve) onmogelijkheid bestaan om onderscheid te maken tussen de bijdragen in de meetresultaten van de bindweefsel- en de gladde spiercomponent. Deze onmogelijkheid tot het onderscheiden van de eigenschappen naar samenstellende componenten wordt merendeels veroorzaakt door het feit dat het weefsel van deze organen geen regelmatige verdeling van de structuren kent, maar dat juist contractiele en passieve componenten op onregelmatige wijze verdeeld en met elkaar verstrengeld zijn. Als gevolg hiervan kent het weefsel geen voorkeursrichting waarin de maximale contractiekracht ontwikkeld wordt.

Met het werk dat in dit proefschrift gepresenteerd wordt werd geprobeerd toch een onderscheid tussen de eigenschappen van enerzijds de spiercomponent en anderzijds de bindweefselcomponenten aan te brengen. Dit is gedaan door de gladde spiercellen uit de weefsels te isoleren en vervolgens aan individuele spiercellen de passieve en actieve eigenschappen experimenteel te meten.

In het begin van het onderzoek werd alleen gewerkt met spiercellen geïsoleerd uit de urineblaas van het varken. Deze cellen zijn een goed en representatief alternatief voor de spiercellen uit menselijke blaas. In een latere fase van het onderzoek werden ook spiercellen bestudeerd die geïsoleerd waren uit uterusweefsel. Dit was afkomstig van biopsieën verkregen tijdens electieve sectio's.

Gedurende het gehele onderzoek moesten vele praktische en technische problemen overwonnen worden om uiteindelijk tot de resultaten te komen zoals die nu gepubliceerd zijn. Enkele van de technische oplossingen/apparaten werden als 'technical note' gepubliceerd in internationale vakbladen, andere zijn opgenomen als onderdeel van een van de publikaties waarin de in dit onderzoek verkregen resultaten zijn beschreven. In veel gevallen waren wij echter van mening dat, hoewel de ontwikkelde technische methoden en apparatuur van cruciaal belang waren voor het kunnen verrichten van deze vorm van spiercelonderzoek, deze toch te zeer van praktische en te weinig van wetenschappelijke aard waren om wetenschappelijke publikatie te rechtvaardigen. Om diezelfde redenen werd besloten om ook in dit proefschrift beschrijving van vele kleine apparaten en methodieken achterwege te laten.

De inhoud van het proefschrift

Hoofdstuk 1 vormt de inleiding tot het proefschrift. Allereerst wordt een beknopt overzicht gegeven van de meer recente geschiedenis van het fysiologisch onderzoek van glad spierweefsel, met name betreffende het meten aan geïsoleerde cellen. Hierin wordt de aanleiding gevonden voor het onderzoek zoals dit in dit proefschrift is weergegeven. Aansluitend wordt de opbouw van het proefschrift besproken en aangegeven met behulp van welke experimenten

diverse stappen in het onderzoek werden gezet.

In Hoofdstuk 2 wordt, aan de hand van de voor de urineblaas van het varken ontwikkelde methode, behandeld hoe uit weefselsoorten die een hoog gehalte aan bindweefsel componenten bevatten gladde spiercellen geïsoleerd kunnen worden.
Aangezien de literatuur geen procedures vermeldde voor het isoleren van cellen uit de urineblaas van het varken of van de mens en de procedures beschreven voor minder collageenrijke vormen van glad spierweefsel niet in staat bleken uit de urineblaas voldoende cellen van acceptabele kwaliteit te isoleren, was het nodig een nieuwe methode voor de isolatie van deze cellen te ontwikkelen.

Tijdens het ontwikkelen van de isolatiemethode bleek dat de gangbare testen op vitaliteit van de resulterende cellen niet voldoende specifiek waren. De eenvoudige exclusietests, zoals die van Trypan-blauw, bleken ontoereikend om aan te geven in welke richting de isolatiemethode moest worden gemodificeerd. Al doende bleek dat in geval van gladde spiercellen de enig juiste maat voor de kwaliteit van de verkregen cellen de *functionaliteit*, d.w.z. "het met contractie reageren op de externe stimuli" was. Aangezien het noodzakelijk was om in de verkregen celsuspensies relatief grote aantallen cellen te testen, werden methoden en apparatuur ontwikkeld waarmee het mogelijk is de contractiele eigenschappen te inventariseren zonder dat de cellen mechanisch worden vastgemaakt.

In het kort: Losliggende gladde spiercellen bevonden zich in een betrekkelijk verdunde suspensie, zodat deze stuk voor stuk electrisch tot contraheren gestimuleerd konden worden. Tijdens iedere stimulatiepoging werd per cel het gehele verloop van de respons, vanaf even voor de start van de stimulatie tot even na de laatst zichtbare bewegingen, op videoband vastgelegd. Tevens werd handmatig een tweetal door een computerprogramma gegenereerde timers, die in het videobeeld te zien waren, op geleide van de observaties bediend.
Bij de analyse achteraf van de verkregen responsies werden, aan de hand van een in de computer automatisch opgeslagen lijst van de timingresultaten, precies die beelden geselecteerd die begin of einde van een bepaalde fase van de respons weergaven. Deze beelden werden stilstaand op een monitor geprojecteerd en in hetzelfde beeld werd een kruisdraad met behulp van computersturing gepositioneerd om op die manier de lengte van de cel op dat specifieke moment te kunnen bepalen. Vanuit de gecombineerde lengte- en tijdgegevens konden parameters zoals cellengte, latentie-tijd, duur en snelheid van de verkorting en van de relaxatie, en percentages verkorting en relaxatie worden bepaald.

Met behulp van deze techniek werden twee varianten van de inmiddels beschikbare isolatietechniek vergeleken op het punt van de functionele kwaliteit van de verkregen gladde spiercellen. Deze vergelijking toonde aan dat de methode waarbij het minst gebruik werd gemaakt van mechanische manipulaties weliswaar minder cellen opleverde per hoeveelheid bewerkt uitgangsmateriaal, maar dat deze cellen van een functioneel significant betere kwaliteit waren. Met andere woorden, mechanisch losmaken resulteerde wel in kwantiteit maar niet in kwaliteit. De op die manier verkregen cellen toonden in een geringere frequentie normale responsies op electrische stimulatie en bovendien was hierbij de latentie-tijd en de contractie-tijd langer en bleek het bereikte percentage verkorting kleiner.

Met de experimenten zoals die in Hoofdstuk 2 worden beschreven werd bewezen dat rechtstreekse electrische stimulatie van gladde spiercellen geïsoleerd uit de urineblaas mogelijk is en dat dit reproduceerbare resultaten oplevert van acceptable kwaliteit.
Beide methoden voor het isoleren van deze cellen bleken geschikt voor routine gebruik, en de beste van de twee werd, behoudens enkele kleine wijzigingen, tijdens de verdere experimenten gebruikt.
Toch werd binnen het bestek van dit proefschrift met deze methoden geen definitief antwoord gevonden op het probleem van hoe de grote hoeveelheden bindweefsel componenten, die het isoleren van gladde spiercellen uit urineblazen sterk blijken te bemoeilijken en te vertragen, efficient te verwijderen. Een ander resterend probleem is het feit dat 'gap-junctions', die zo veelvuldig voorkomen tussen de gladde spiercellen onderling, tot op heden nog niet chemisch of mechanisch verbroken kunnen worden zonder dat op zijn minst de celwand van één van de twee aaneenliggende cellen beschadigd wordt. Vanwege deze laatste reden wordt vermoed dat de totaalopbrengst aan functioneel intacte cellen naar rato van de bewerkte hoeveelheid uitgangsmateriaal altijd zo laag is gebleven.
Gebaseerd op deze bevindingen stellen wij dat het meten van de contractiele functionaliteit de methode van eerste keus is om de kwaliteit van geïsoleerde gladde spiercellen te bepalen.

Hoofdstuk 3 beschrijft het apparaat waarmee de in Hoofdstuk 2 beschreven computergestuurde analyse van de eerder verkregen videobeelden werd verricht. Het meest essentiële kenmerk van dit apparaat is het feit dat het een door een computer gepositioneerde kruisdraad in een stilstaand videobeeld kan genereren, onafhankelijk van het feit uit welke bron dit videosignaal afkomstig is en of deze bron op enigerlei wijze gesynchroniseerd is met de besturingscomputer. Hiermee wordt zowel de noodzaak van het compatible zijn van de video-standaarden van computer en bron omzeild als ook de bij andere systemen gangbare verplichting één of andere vorm van vergaande synchronisatie tussen beide systemen tot stand te brengen.
Andere kenmerken zijn de mogelijkheid tot eenvoudige controle met behulp van een willekeurige computer die voorzien is van een acht-bits parallelle uitgangsbus en het feit dat het apparaatje voor een gering bedrag (f 200,-) te realiseren is, terwijl de toenmalig beschikbare oplossingen een veelvoud gekost zouden hebben (f 10.000,- tot f 100.000,-).

Hoofdstuk 4 beschrijft de ontwikkeling van een zeer gevoelige krachttransducer voor het isometrisch meten van de contractiekracht gegenereerd door één enkele gladde spiercel over een bereik van 0.1 - 10.0 μN. Tevens moest met dezelfde transducer aan dezelfde cel het meten van passieve krachten tot minimaal 100 μN mogelijk zijn. Dergelijke relatief grote krachten ontstaan als gevolg van het plotseling lineair (in de tijd) verlengen van de cel. Op basis van deze twee hoofdeigenschappen waaraan de transducer zou moeten voldoen, kwamen wij tot de conclusie dat een dergelijke transducer nog niet eerder in de literatuur beschreven, noch op dat moment commerciëel beschikbaar was. Om die reden werd een eigen ontwikkeling gestart.

Als uitgangspunt voor de transducer werd gekozen voor de omzetting van kracht in verplaatsing op basis van de buiging van een qua dimensies en gedrag bekende bladveer. Ter verkrijging van voldoende stabiliteit en een beweging in slechts één (as)richting werden een tweetal bladveren met elkaar verbonden door een dragersysteem. Voor de detectie van de beweging werd gekozen voor een elektro-optisch systeem dat nauwkeurig de positie van een spleetvaan, bevestigd aan de drager, bepaalde. Ten einde de invloed van uitwendige warmte- en lichtbronnen op het met infra-rood licht werkende detectie systeem te minimaliseren, werd hierin het principe van synchrone draaggolf-detectie toegepast.

Grosso modo kan gesteld worden dat vrijwel alle vooraf geformuleerde eisen gehaald werden. Op het punt van gevoeligheid en temperatuursstabiliteit zijn echter zeker nog verbeteringen wenselijk. Bij de gevoeligheid lag de beperking evenwel niet in de transducer of zijn signaal/ruis verhouding, maar in het hoge stoorniveau veroorzaakt door trillingen afkomstig uit de omgeving van de experimentele opstelling.
Terugkijkend kan worden geconstateerd dat het transducersysteem voor de duur van het onderzoek voldoende en op betrouwbare wijze heeft gefunctioneerd. Hieraan valt toe te voegen dat de mogelijkheid van het à la minute kunnen vervangen van het drager-veersysteem van niet te onderschatten waarde is gebleken. In de weinige gevallen dat het bewegende deel van de transducer door manipulaties werd beschadigd, kon na uitwisseling van dit transducerdeel de meting alsnog worden voortgezet. Deze laatste, belangrijke eigenschap is tot op heden in de inmiddels verkrijgbare commerciële transducers nog niet gerealiseerd.

Hoofdstuk 5 beschrijft de ontwikkeling en beproeving van een methode om individuele gladde spiercellen cellen te bevestigen. Deze methode betreft een modificatie van een bevestigingstechniek zoals die eerder door Fay (1977, Warshaw en Fay, 1983) is beschreven.
Aanvankelijk werd getracht deze zelfde methode in oorspronkelijke vorm voor onze soorten cellen te gebruiken, maar na een reeks van experimenten werd geconcludeerd dat deze voor routinematig gebruik te ingewikkeld was en niet vaak genoeg een goede bevestiging van de gladde spiercellen opleverde. Zowel de originele als de gemodificeerde bevestigingstechniek berusten op dezelfde principes: De cel wordt eerst op basis van electrostatische aantrekking tussen tegengestelde ladingen losjes aan twee micro-pipetten gehecht. Vervolgens wordt deze verbinding aan beide celuiteinden tegelijk door één of meerdere knopen gestabiliseerd.

Het grote verschil tussen de originele en de gemodificeerde methode is dat in de eerste geladen harsbolletjes[1)], gelijmd aan de pipetpunten, werden gebruikt om de lokale electrische lading op te wekken, terwijl in de tweede methode Poly-L-Lysine deze functie vervulde. Dit Poly-L-Lysine was in onze toepassing over een veel groter gedeelte van de pipetpunt veel gelijkmatiger verdeeld dan in geval van het enkele harsbolletje aan de pipetpunt, zodat een veel groter oppervlak ontstond waarover electrostatische aantrekking plaats kon vinden.
Deze wijziging leidt tot drie voordelen: De relatief grote harsbolletjes zitten niet langer de cel en het manipuleren hiervan in de weg en de electrische lading bevindt zich op een veel groter deel van de pipetpunt en daarmee is de plaats van eerste hechting veel minder kritisch. Als gevolg van deze twee eerste voordelen kunnen kortere soorten cellen worden vastgeknoopt, waarbij volstaan kan worden met slechts één knoop in plaats van de oorspronkelijke drie.

Evaluatie van de nieuwe methode toonde aan dat zowel gladde spiercellen uit de urineblaas als uit de uterus routinematig bevestigd konden worden. Dit resulteerde altijd in een hechting die sterker was dan de kracht die de cel over zijn dwarsdoorsnede kon weerstaan.
De methode biedt het grote voordeel krachtmetingen te kunnen verrichten in de richting van de lengteas van de cel en laat tijdens de meting het opleggen van snelle veranderingen in de lengte van de cel toe. Beide eigenschappen zijn belangrijk voor het experimenteel onderzoeken van het functioneren van het contractiel mechanisme in relatie tot de lengte van de spiercel.

Hoofdstuk 6 beschrijft hoe metingen verricht zijn aan de passieve eigenschappen van individuele gladde spiercellen, zoals deze door isolatie verkregen werden uit varkensblazen en uit à terme humane uterussen.

De cellen werden geïsoleerd zoals voorgaand beschreven in Hoofdstuk 2, vervolgens vastgehecht zoals beschreven in Hoofdstuk 5. Aansluitend aan de bevestiging werden de cellen op hun 'rustlengte' gebracht door de cellen in stapjes te verlengen totdat de eerste tekenen van stress-relaxatie zich presenteerden in het gemeten krachtsignaal. Ter bepaling van deze 'rust-' of 'aanvangslengte' werd deze optisch door de microscoop heen gemeten.

[1)] Bedoeld worden harsbolletjes zoals die gebruikt worden in ionen-wisselaar kolommen.

Vervolgens werd een meetcyclus herhaald doorlopen waarin de cel twee keer electrisch tot contraheren werd gestimuleerd en aansluitend op een grotere cellengte werd gezet. Deze meetcycli werden herhaald totdat de cel uiteindelijk brak als gevolg van het opeenstapelen van de celverlengingen.

De lengtestappen werden aangeboden in de vorm van relatief snelle lineaire verlengingen, welke een directe stijging van de passieve kracht die door de cel werd uitgeoefend, veroorzaakten. De zo ontstane krachttoename werd onmiddelijk gevolgd door stress-relaxatie. De op deze wijze verkregen kracht-tijdcurves werden door een computer bemonsterd en voor latere analyse opgeslagen.

Bij de analyse van de stress-relaxatiecurves werd ondermeer de maximale krachttoename die het gevolg was van de lengtestap bepaald. Hieruit werd de gemiddelde stijfheid als functie van de lengte bepaald. Ook werd voor alle curves het resterende krachtniveau na 80 seconden relaxatie bepaald. Door de maximale krachtstijging bij de hoogst bereikte lengte te relateren aan het geschatte celvolume kon de maximale elasticiteitsmodulus bij die lengte berekend worden.

Aan geselecteerde curves werd met behulp van een computerprogramma een eerste orde exponentiële functie gefit. Tijdens het selecteren en fitten van deze curves bleek dat in het relaxatie deel van de curves van de urineblaascellen veel vaker verstoringen op het krachtsignaal gesuperponeerd waren dan in de curves van de uteruscellen. Tevens waren de verstoringen in de blaascelcurves veel groter van amplitude dan in de uteruscelcurves.

Uit de resultaten van deze experimenten concluderen wij dat individuele cellen geïsoleerd uit de urineblaas van het varken een relaxatiepatroon vertonen wat duidelijk verschilt van dat van intakt varkensblaasweefsel. Daarbij trad in de cellen, gemeten over dezelfde tijdsperiode, veel minder relaxatie op dan in de intakte stripjes urineblaas.
Meer algemeen, noch de urineblaascellen, noch de uteruscellen toonden een stressrelaxatie tot het nulniveau, dit in tegenstelling tot wat over andere typen cellen eerder was gerapporteerd. In andere woorden, bij de door ons onderzochte cellen resteerde na 80 seconden relaxatie een krachtniveau dat gemiddeld gelijk was aan 50 % van de initiële piekwaarde op het moment van de lengtestap. Dit betekend dat in dit type cellen de passieve kracht langer aanhoudt dan eerder werd verondersteld.
Uit onze resultaten bleek dat de gevonden maximale elasticiteitsmodulus voor geïsoleerde gladde spiercellen uit de urineblaas op zijn minst een factor tien hoger is dan die welke eerder bepaald werd voor intakte stripjes varkensblaas.
Deze drie bevindingen illustreren duidelijk de waarde van metingen van de passieve eigenschappen aan geïsoleerde gladde spiercellen ten opzichte van die van metingen aan intakt weefsel, aangezien het daarmee mogelijk is onderscheid te maken tussen de eigenschappen van de diverse weefselcomponenten.

Gebaseerd op onze bevindingen concludeerden wij dat de passieve eigenschappen van deze typen gladde spiercellen naar alle waarschijnlijkheid bepaald worden door een combinatie van eigenschappen van zowel het cytoskelet als van de celmembraan.

Met deze methode zijn passieve eigenschappen van individuele gladde spiercellen gemeten ver buiten de ranges van lengte en kracht zoals die eerder op beperkte schaal in de literatuur zijn gerapporteerd.

Hoofdstuk 7 beschrijft hoe actieve eigenschappen van individuele gladde spiercellen, geïsoleerd uit varkensblaas en humane à terme uterussen, onderzocht werden.

In dit hoofdstuk worden de responsies geanalyseerd die resulteerden uit de electrische stimulaties zoals die begrepen zijn in de experimenten beschreven in Hoofdstuk 6.
Bij blaasspiercellen werd in 44 % van de stimulatiepogingen een respons verkregen die te onderscheiden was van de mechanische achtergrondruis. Bij de uteruscellen bedroeg dit percentage 57 %.
In deze responsies konden vier hoofdtypen onderscheiden worden. Twee typen begonnen met het oplopen van de kracht als reactie op de stimulus. Deze stijging werd aansluitend gevolgd door een daling van de kracht. Twee andere responsietypen begonnen met het dalen van de kracht als reactie op de stimulus. Aansluitend trad een krachtstijging op tot aan, of voorbij, het niveau van voor het begin van de stimulus. In ieder tweetal typen was er één waarbij de gestimuleerde respons aanhield totdat de stimulus werd uitgeschakeld, en één waarbij de gestimuleerde respons reeds begon af te vlakken voordat het stimuleren ophield.
Bij de groep gladde spiercellen geïsoleerd uit urineblazen kwamen responsies met een initiële krachtstijging bijna even vaak voor als responsies met een initiële krachtdaling. In de groep gladde spiercellen geïsoleerd uit uterussen kwamen vrijwel alleen maar initiële krachtdalingen voor.

Zowel tijdens de responsies met initiële krachtstijgingen als tijdens die met initiële krachtdalingen werden veranderingen in de fase-contrast beelden van de cellen waargenomen die grote gelijkenis met elkaar vertoonden, ongeacht welk type respons optrad. Dit verschijnsel, samen met de constatering dat de dynamiek van de krachtdalingen niet significant verschilde van die van de krachtstijgingen en dat beide verschijnselen herhaaldelijk als reactie op electrische stimulatie konden worden waargenomen, deed ons er van overtuigd raken dat beide soorten responsies de resultante waren van processen binnen het contractiel apparaat in de cel. Hier van uitgaande werd aangenomen dat de responsies met de initiële krachtstijgingen te beschouwen zijn als 'klassieke' contracties.

Van een geselecteerd aantal van de 'klassieke' contracties afkomstig van de urineblaascellen werd een fase-plot analyse verricht. Bij deze analyse wordt de verandering van de kracht als functie van de kracht op dat moment beschreven.

Deze analyses toonden aan dat de geïsoleerde gladde spiercellen uit de varkensblaas langzamer en met een kleinere maximum kracht contraheerden dan bepaald was voor de cellen in intakte mini-stripjes afkomstig uit hetzelfde soort orgaan. De verklaring van deze verschillen moet gezocht worden in een nog steeds niet optimale methode van isoleren van de cellen die de functionaliteit aantast, of in een niet optimale manier van stimuleren, of beiden. Een andere mogelijke verklaring zou kunnen zijn dat de verbindingen tussen de cellen onderling een essentiële rol spelen in de regulatie van de contractiliteit van deze soorten cellen.

Verdere analyse van de verstoringen in de stressrelaxatiecurves zoals beschreven in Hoofdstuk 6, toonde aan dat dit waarschijnlijk rek geïnduceerde spontane contractiegolven zijn, zoals deze zo algemeen aangetroffen worden bij metingen aan intakte stukjes glad spierweefsel. Daarbij werd aangetoond dat zowel de hoogte van de gemeten 'contractiegolfjes' als het voorkomen ervan significant verschilde tussen de blaas- en de uteruscellen: In de uteruscellen werden alleen heel kleine verstoringen geobserveerd.

Het voorkomen van een dergelijke variëteit aan responsies, speciaal die van de initiële krachtdalingen, riep heel wat vragen op naar de aard en oorzaak van deze responsies. In het algemeen leek op zijn minst een belangrijk deel van de mogelijke oorzaken celtype gebonden, aangezien in de uteruscellen praktisch alleen maar gestimuleerde krachtdalingen werden gemeten en dat in deze zelfde cellen geen grote 'contractie golven' in de stress-relaxatiecurves werden gezien.
Verklaringen in de zin van significante verschillen in de methoden van isolatie en van meten konden gevoeglijk worden uitgesloten. Een tweetal redenen voor het optreden van afwijkende responsies die beide typen cellen juist gemeen hadden waren de nogal onfysiologisch grote lengtestappen die aan de cellen werden opgelegd en de wat gebrekkig te beheersen methode van electrisch stimuleren. Hoewel hierin mogelijk de verklaringen schuilen van het kunnen optreden van afwijkende responsies in beide typen cellen, verklaart dit niet de consistente verschillen in de frequenties van geobserveerde fenomenen tussen uterus- en blaascellen onderling.
Wat dit laatste gegeven betreft resteerden alleen nog verklaringen die sterk gerelateerd zijn aan de specificiteit van een celtype, zoals het in de incubatievloeistof ontbreken van bepaalde hormonen en prostaglandines die van invloed zijn op de contractiliteit van de uteruscellen. Of ook het feit dat geen van de biopsieën zo kort voor de mogelijke start van de bevalling werden verkregen dat er sprake had kunnen zijn van voldoende natuurlijke 'priming' zoals die noodzakelijk wordt verondersteld voor het optreden van volledige contractiele activiteit van de uterus.

Samenvattend: Geen van de besproken redeneringen geeft een definitieve verklaring voor de gemeten fenomenen en toekomstig onderzoek zal moeten ophelderen wat met name de betekenis is van de gestimuleerde krachtdalingen.

Slotconclusies en aanwijzingen voor verder onderzoek

In dit proefschrift wordt een methode beschreven voor het meten van contractiele en passieve responsies aan individuele gladde spiercellen die als basis kan dienen voor verdere ontwikkelingen. Uitvloeisel van het beschikbaar komen van een techniek zoals verwoord in de hoofdstukken 6 en 7 zou kunnen zijn dat biopsiën van willekeurige soorten glad spierweefsel niet alleen maar cytologisch en histologisch onderzocht kunnen worden, maar dat juist ook de mogelijkheid geboden wordt tot het bepalen van het fysiologisch functioneren van het onderhavige spierweefsel op celniveau. Naast mogelijkheden tot vermeerdering van de inzichten in het functioneren van gladde spiercellen in empirisch-fysiologische zin, kan dit nieuwe wegen openen tot het onderzoeken van pathologische situaties bij de mens waarbij dan onderscheid gemaakt zou kunnen worden tussen diverse oorzaken van dysfunctie, met name of deze gelegen zijn op het niveau van de spier of op dat van de innervatie of in de hogere controlecentra. Hiermee zou opgehelderd kunnen worden of bepaalde functionele stoornissen inderdaad veroorzaakt worden door stoornissen in de gladde spiercellen zelf.
Daarnaast biedt het werken met individuele cellen mogelijkheden tot schaalvergroting en besparing op proefdieren/organen op het terrein van het fysiologisch en farmacologisch onderzoek, daar zowel grote aantallen cellen uit een enkel stukje orgaan geïsoleerd kunnen worden, als series van stoffen parallel getest kunnen worden.
Deze methode zou het voordeel kunnen hebben dat, in tegenstelling tot receptor-studies aan gekweekte cellen, een veel reëler en directer beeld verkregen wordt van de feitelijke invloed van farmaca op de beoogde functionaliteit van individuele gladde spiercellen. Dit omdat, wanneer een receptor feitelijk op de te onderzoeken cel vertegenwoordigd is en het te onderzoeken farmacon daadwerkelijk hierop kan binden en een invloed op de contractiliteit kan uitoefenen, dat direct in het gemeten krachtsignaal tot uitdrukking komt.

Toekomstig onderzoek zal gericht moeten worden op het verbeteren van de isolatiemethode, opdat zo mogelijk de responsies van de cellen verbeteren. Vervolgens is het nodig de transducer met name op de punten afmetingen, thermische stabiliteit en bandbreedte te verbeteren. Ook de onderdelen van de totale meetopstelling dienen zodanig te worden verbeterd en verkleind dat een veel stabieler en meer geïntegreerd geheel ontstaat. Verder moet gedacht worden aan het ontwikkelen van een betrouwbare lijmtechniek voor het bevestigen van gladde spiercellen aan de meetapparatuur. Tevens zou de mogelijke invloed die uitgaat van het materiaal dat de cellen omgeeft (stroma) op de contractiliteit moeten worden onderzocht, alsmede het belang van de verbindingen die tussen de cellen onderling bestaan.

Het zou wenselijk zijn om tegelijkertijd de functionele gebeurtenissen van de contractie zowel mechanisch als biochemisch te onderzoeken. Spijtig genoeg bestaat in Rotterdam nog geen multidisciplinaire onderzoeksgroep die dergelijk geïntegreerd onderzoek aan glad spierweefsel zou kunnen verrichten.

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Curriculum Vitae

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 14 november 1955 te Biezelinge, gemeente Kapelle.
Van 1960 tot 1968 genoot hij kleuter- en lager onderwijs in zijn geboorteplaats. Aansluitend volgde hij middelbaar onderwijs aan het Christelijk Lyceum voor Zeeland te Goes, alwaar hij op 27 juni 1974 het diploma Atheneum B behaalde.
Van 1975 tot 1977 volgde hij de opleiding tot klinisch-chemisch analist aan de Laboratorium School Zeeland, eveneens te Goes.
In 1977 starte hij met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam, welke op 19 oktober 1984 met het behalen van het arts-examen werd afgesloten.
Tijdens zijn studie was hij van 1980 tot en met 1984 werkzaam als student-assistent bij de Urodynamica groep van de Afdeling Urologie, dit binnen het verband van de Vakgroep Biomedische Natuurkunde & Technologie.
Op 1 januari 1985 werd op dezelfde afdeling een aanvang gemaakt met het promotie-onderzoek naar de mechanische eigenschappen van geisoleerde gladde spiercellen. Het experimentele deel van dit onderzoek werd in juni 1988 afgerond.
Aansluitend was de auteur werkzaam als assistent chirurgie in het Ikazia ziekenhuis te Rotterdam.
Van november 1989 tot april 1990 was hij docent Fysiologie en Pathologie aan de verpleegkundigen-opleiding van de M.D.G.O. school 't Raetsgoet te Ede.
In augustus 1990 kwam hij in dienst als wetenschappelijk medewerker bij de Hoofdafdeling Gezondheidsstatistieken van het Centraal Bureau voor de Statistiek te Voorburg.

Dankwoord

Dankwoord

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Dan is er die lange rij van mensen die op één of andere manier professioneel hebben bijgedragen:
Allereerst mijn promotor Prof. Dr. F.H. Schröder en mijn co-promotor Dr. Ir. R. Van Mastrigt die samen mij het groene licht gaven om aan dit gewaagde onderzoek te beginnen. U beiden bent in het onderzoek en zijn voortgang blijven geloven, op gezette tijden ondersteunend door zorg te dragen voor financiën en faciliteiten. In de eindfase is met name mijn co-promotor een grote steun geweest bij de analyse en het publiceren van de soms lastig te interpreteren bevindingen. Beiden nogmaals hartelijk dank.

Gedurende de onderzoeksperiode zelf hebben in het bijzonder de collega's van de vakgroep Biomedische Natuurkunde en Technologie vele hand- en spandiensten verleend. Prof Dr. Ir. C.J. Snijders stelde mij vele faciliteiten ter beschikking zoals een haast onbeperkt gebruik van de mechanische- en electronicawerkplaatsen van de afdeling B.M.T.. Vaak ging werk ten behoeve van mijn onderzoek voor op het maken van andere apparaten bedoeld voor het nieuwe 'Biomechanica-onderzoek'.
Zonder met name de faciliteiten van de mechanische werkplaats onder de bezielende leiding van instrumentmaker Cor Goedegebuur zou er van de opstelling niet veel terecht zijn gekomen. Cor jij hebt mij leren draaien, frezen en precisie bankwerken, zonder welke vaardigheden wij nooit samen al dit moois hadden kunnen maken.
Niet alle fijnmechanica konden wij zelf doen, omdat zowel de tijd als de beperkingen van 'onze' machines dat niet toelieten. Gelukkig waren er dan de mensen zoals Frans Schumacher en de zijnen van de CRW instrumentmakerij die, als zij tijd hadden en de afd. Urologie budget, menige helpende hand hebben toegestoken om met name de precisie-mechanica van de transducer te maken. Camiel Schellevis en later Alex Brouwer hebben hier kleine wondertjes van fijninstrumentmakerij verricht.

Terugkomend bij mijn collega's van de vakgroep Biomedische Natuurkunde en Technologie wil ik met name een aantal electronici noemen die mij vele fijne kneepjes van het bouwen van direct werkende proto-typen hebben bijgebracht: Jan van Deursen, Cees Kemink, Ronald Maas, Wim Groeneveld en Hans de Bakker. Jullie adviezen waren veelvuldig, en met name die van jou Hans ook altijd zeer uitgebreid en met redenen omkleed.

Dan waren er bij diezelfde vakgroep een aantal wetenschappers die mij hebben geleerd voor- en tegenspoed in dit métier in het juiste (relativerende) licht te plaatsen:
Om te beginnen Dr. Ir. W.A. Van Duyl: Wim jij hebt mijn eerste prille schreden op het gebied van de gladde spierfysiologie begeleid en in mijn latere carrière binnen de vakgroep altijd weer een andere kijk op de gladde spier en z'n functioneren laten zien dan die welke à priori geleerd wordt uit de syllabi en handboeken tijdens de Geneeskundestudie. Dit heeft mij later veel steun verschaft om niet bij de eerste paar afwijkende bevindingen de moed te laten zakken.
In deze zelfde rij wil ik noemen Dr. Ir. E.J. Van der Schee; Evert, hoewel jij maag en darmen trachtte te doorwrochten, hadden we de gladspierlijke wederwaardigheden gemeen en mocht ik samen met jouw in een aantal experimenten het electrisch en mechanisch gedrag van de caviamaag exploreren. Helaas kwam aan deze succesvolle pilot-experimenten door wederzijdse omstandigheden een voortijdig einde.
Ook wil ik hier Dr. P.J.J. Lamoré en Dr. G.J. van der Wildt noemen die met hun kennis op het gebied van 'horen en zien' in gesprekken juist die andere kijk op het wetenschappen lieten blijken die mijn eigen cirkelgang langs schijnbare

onmogelijkheden deed doorbreken en zodoende vaak ongemerkt een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift.
Ook andere leden van de toenmalige en huidige vakgroep B.M.T., waaronder Prof. Drs. J. Steketee, hebben her en der hun steentje bijgedragen aan mijn werk en aan dit proefschrift, maar het voert te ver iedereen met name te noemen, te meer daar anders de niet genoemden echt het idee zouden krijgen vergeten te zijn. Toch ben ik ook al deze mensen dankbaar voor hun bijdragen aan het geheel.

Om nog even op het technologische vlak te blijven, wederom een uitstapje naar de CRW, speciaal de afdeling glastechniek waar Jan Ekas en z'n compaan Toon Hoegee de vele specialité's voor menig onderzoeker op de M.F.R. maken en hebben gemaakt. Zo ook voor mijn promotieonderzoek. De metingen stonden of vielen door de micro-kunstwerken van jouw Jan, zoals de kwarts-balkjes voor de transducer of de Z-vormige micro-pipetten. Verder bracht jou praktische kijk op glastechnische en aanverwante zaken mij menigmaal sneller op weg dan wanneer ik zelf verder had geprutst. Ook de vele leuke gesprekken die we al werkend hadden zal ik lang blijven herinneren.

Nu een geheel ander terrein: Van weefselstrip tot geïsoleerde spiercel.

Op diverse plaatsen in dit proefschrift is de weerbarstigheid vermeld waarmee de gladde spiercellen uit de urineblaas zich hebben verzet tegen een vlotte bevrijding uit de hun omringende weefselstructuren. De strijd tegen dit 'gedrag' heb ik niet alleen gevoerd.
Allereerst waren er Dr. J.C. Romijn en Dr. G.J. van Steenbrugge van de tumorgroep van de afd. Urologie die mij, waar mogelijk te hulp gesneld door de vele anderen van de tumorgroep, wegwijs hebben gemaakt in de vele technieken en kunstgrepen van celisolatie en celkweek. Vele uren heb ik in hun laboratorium gastvrijheid genoten.
Na het isoleren was het zaak de verkregen cellen te identificeren en hun vitaliteit aan te tonen: Dhr. J.E. Josselin de Jong van de afdeling Celbiologie en Histologie 1 leende mij in die prille dagen een microscoop en aanverwante spullen en gaf later nog menig optisch advies. Dr. R.E. Ploemacher van Celbiologie en Histologie 2 wees mij de weg naar de mensen die bij de eerder genoemde afdelingen cytologie en electronenmicroscopie bedreven. Van hen mocht ik menige truc afkijken om uiteindelijk tot de conclusie te kunnen komen dat ik inderdaad gladde spiercellen te pakken had.
Toen in een later stadium de methode van isoleren nòg belangrijker bleek voor de functionaliteit van de cellen dan eerder bevroed, werd het tijd voor echt histologisch onderzoek door de afdeling Pathologische Anatomie 1, in de persoon van Dr. A.H. Mulder. Samen bekeken wij preparaten van isolaties in allerlei fasen, lieten hier alle denkbare technieken op los en kwamen uiteindelijk tot de conclusie dat wij op het einde van de rit nog niet wisten waarom die 'gladde jongens' aan elkaar bleven zitten. Toen gaf jij Andries het advies om dan maar selectie achteraf van de meest vitale gladde spiercellen toe te passen op basis van Fluorescine-Di-Acetaat vitaalkleuring vlak voor en tijdens het vastknopen van de spiercellen. Dit bleek een gouden greep.

Op dit punt aangeland past een woord van dank aan het adres van Carl Zeiss B.V. die altijd zeer in het geweer is geweest om aan mijn nogal exotische wensen op microscopisch gebied tegemoet te komen. Het meest bijzondere gebeurde toen op het moment dat een fluorescentieoptiek voor de IM microscoop nodig bleek, deze plots uit de hoge hoed werd getoverd, hoewel er in Nederland slechts 2 van zulke instrumenten bestonden. De firma Carl Zeiss vond één van haar andere afnemers bereid voor de duur van het onderzoek een dergelijke optiek ter beschikking te stellen: Het laboratorium van de Sectie Biologie/Biochemie van de Dienst Getijde Wateren te Middelburg zij nogmaals hartelijk bedankt.

Nog een aantal andere mensen wil ik met name noemen:-
Allereerst Dr. J. Bijman (afd. Celbiologie en Histologie 1) die mijn problemen met de mechanische trillingen met de hoogbouw van de faculteit aanhoorde en spontaan en belangeloos mij ruimte aanbood in de kelder van het zusterhuis van het Dykzigt ziekenhuis, alwaar hij ook nog maar net mechanische rust had gevonden. Mede op aanwijzingen van hem en zijn mederwerkers kwam ik tot een zodanig laag mechanisch stoorniveau dat er in ieder geval in de verkeersarme uren gemeten kon worden.

Voor het testen van de fysiologische milieukwaliteiten van de gerealiseerde opstelling mocht ik gebruik maken van gekweekte ratte hartspiercellen van Dr. A. Bom van de afdeling Farmacologie en van gekweekte menselijke skeletspiercellen van Dr. A. Van der Ploeg (afd. Celbiologie en Histologie 1).

Dan een speciaal woord van dank aan Dr. D.J. Griffiths: Derek jij was altijd het rustpunt binnen de Urodynamische groep. Door je 'Engelse' manier van benaderen en analyseren van praktische en theoretische problemen deed je een ander licht op zaken schijnen, die anders toch minder snel en harmonieus waren opgelost. Verder was je iedere keer weer bereid mijn gebrekkige Engels op te poetsen tot een publiceerbaar niveau, een 'faciliteit' die niet alleen ik heb gemist sinds je vertrek naar Canada.

Aan het slot van het dankwoord wil ik stil staan bij de leden van de leescommissie, Prof. Dr. Ir. N. Bom, Prof. Dr. Ir. J. A. E. Spaan en Prof. Dr. H.C.S. Wallenburg. U allen hartelijk dank voor het gedurende de vakantieperiode beoordelen van het proefschrift. Verder in het bijzonder nog een woord van dank aan Prof. Spaan die, samen met Dr. A.M. Van Dijk-Looyaard, mij de richting heeft gewezen voor het zetten van de eerste schreden op het pad van 'single cell' metingen aan glad spierweefsel. Ook Prof. Wallenburg nogmaals hartelijk dank voor uw medewerking bij het verkrijgen van de uterusbiopsieën.

Een aparte plaats ruim ik in voor de mederwerkers van Het Rotterdams Varkensslachthuis die al jarenlang de tijd nemen om ten dienste van de Urodynamicagroep blaas- en ureter-materiaal uit de varkens te snijden. Om dezelfde reden wil ik ook Prof. Dr. Ir. P. Verdouw en de zijnen van de afd. Experimentele Cardiovasculaire Research bedanken voor de vele keren dat jullie met de experimenten rekening hielden met de Uro-boys die weer een blaasje wilden verschalken.