CHLORIDE TRANSPORT IN NORMAL AND CYSTIC FIBROSIS EPITHELIAL CELLS

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Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. C.J. Rijnvos
en volgens besluit van het College van Dekanen.
De openbare verdediging zal plaatsvinden op
woensdag 29 januari 1992 om 15.45 uur

door

Simon Maarten Kansen

geboren te Heemskerk.

universiteits
Erasmus
DRUKKERIJ

PROMOTIECOMISSIE:

Promotor:	Prof. Dr. H. Galjaard.
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Overige leden:	Dr. H.R. de Jonge. Prof. Dr. H.J. Neijens. Prof. Dr. P.R. Saxena

Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetika van de Fakulteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam en mede financieel gesteund door de Stichting Klinische Genetika regio Rotterdam.

Thoughts in time and out of season
The Hitchhiker
Stood by the side of the road
And leveled his thumb
In the calm calculus of reason.

Jim Morrison "An American prayer"

CONTENTS Pages

ABBREVIATIONS.

9-AC	anthracene-9-carboxylic acid
ATP	adenosine 5'-triphosphate
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	adenosine cyclic 3',5'-monophosphate
CF	cystic fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGMP	guanosine cyclic 3',5'-monophosphate
DIDS	4',4'-diisothiocyanato stilbene-2',2' -disulfonic acid
DPC	diphenylamine-2-carboxylic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	[ethylenebis(oxyethylenenitrilo)]tetraacetic acid
GTP	guanosine 5'-triphosphate
GTPßS	guanosine 5'-O-(2-thiophosphate)
GTP[S]	guanosine 5'-O-(3-thiophosphate)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I_{sc}	short-circuit current
IAA	indanyloxyacetic acid
IC_{50}	concentration giving half-maximal inhibition
IP_3	inositol triphosphate
I/V	current (I) to voltage (V)-relationship
kD	kilo Dalton
LT	leukotriene
MDR	multi-drug resistance
N	normal
NPPB	5-nitro-2-(3-phenylpropylamino)-benzoic acid
PG	prostaglandin
PKA	cAMP-dependent protein kinase A
PKC	Ca^{2+}/phospholipid-dependent protein kinase
PKG	cGMP-dependent protein kinase
PMA	phorbol 12-myristate 13-acetate
Po	open probability
pS	pico Siemens
RVD	regulatory volume decrease
SITS	4-aceto-4'isothiocyanatostilbene-2',2' disulfonate
SPQ	6-methoxy-N-(3-sulfopropyl)-quinolinium
TRIS	tris(hydroxymethyl)aminoethane

CHAPTER I.

GENERAL INTRODUCTION.

Cystic fibrosis (CF) was first described in 1938 [Anderson, 1938] and recognized as an autosomal recessive disease only a few years later [Anderson and Hodges, 1946], but was in detail defined after the development of the sweat test [di Sant'Agnese et al., 1953, Gibson and Cook, 1959]. CF is now known as a complex disorder affecting children having a mean life expectation of about 25 years. The disease is clinically heterogenous and characterized by chronic obstruction and infection of the respiratory tract, exocrine pancreas insufficiency and its nutritional consequences, obstruction of the intestine and elevated salt levels in sweat [Taussig, 1984, De Jongste et al., 1986, Boat et al., 1989, Neijens et al., 1990, Halley et al., 1990]. The diagnosis for CF is mainly based on the increased Cl^- levels in sweat [Sato and Sato, 1990]. Cystic fibrosis is the most frequent life-threatening recessive genetic disorder in the Caucasian population with an incidence of about 1 in 2000 to 1 in 4000 live births [Boat et al., 1989]. For the Netherlands the prevalence is 1 in 3600 [Ten Kate, 1975]. Being an autosomal recessive disorder, about 1 in 30 (Dutch population) individuals is a healthy carrier.

The CF-gene has been localized on chromosome 7 [Eiberg et al., 1985] and has only recently been identified [Rommens et al., 1989, Riordan et al., 1989, Kerem et al., 1989]. It encodes a 1480 amino acid protein named Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) with a predicted molecular mass of about 170 kD. From its amino acid sequence it was suggested that CFTR is a membrane protein spanning the membrane several times. The most frequent mutation in 70 % of the CF chromosomes is a 3 basepair deletion resulting in the absence of a phenylalanine at position 508. The identification of the CF-gene and its mutations allows prenatal diagnosis and carrier testing [Galjaard, 1989].

Among the numerous biochemical abnormalities reported for CF cells, a defect in the cAMP-regulated Cl^- transport in secretory epithelial cells is the most consistent observation. Therefore, much electrophysiological research, as described in this thesis, has been done in order to explain the defect in CF.

The function of the CF-gene product CFTR is at this moment not completely elucidated, but certainly plays a crucial role in cAMP-mediated chloride transport in the apical membrane of epithelial cells. Recent transfection and electrophysiological experiments indicate that the CF-gene encodes a Cl^- channel [Kartner et al., 1991]. Intergration with other biochemical observations and attempts for possible therapies have to be resolved.

The 1 in about 2500 incidence of CF raises the question why this frequency is so high. Various hypotheses have been proposed to explain this phenomenon, such as genetic heterogeneity, high mutation rate, meiotic drive, heterozygote advantage and genetic drift [Romeo et al., 1989]. A heterozygous advantage may be a satisfying alternative since carriers might have an increased resistance to Cl^--secreting diarrhoeas [Quinton, 1982] which, in contrast to the Cl^--undersecretion in CF epithelia, are characterized by Cl^--oversecretion in the intestine [de Jonge et al., 1987, Bijman et al., 1988, Hansson, 1988, Baxter et al., 1988]. However, hypotheses about heterozygote advantage are difficult to verify.

A. CLINICAL ASPECTS OF CYSTIC FIBROSIS.

1. Tissues involved.

1.1. The respiratory tract.

Mucus obstruction caused by an increased viscosity and bacterial infection by Staphylococcus aureus and Pseudomonas aeruginosa [Hioby and Koch, 1990, Elborn and Shale, 1990, Horrevorts et al., 1990] are the major pathologic events in the lung. The increased mucus production is responsible for an alternative name for CF i.e. "mucoviscidosis". Once established, infections in the lungs are nearly impossible to eracidate. More than 90% of the CF patients die of respiratory failure associated with pulmonary hypertension and cor pulmonale. Increased coughing and a loss of weight are two of the symptoms which accompany respiratory exacerbations. Treatment of CF respiratory disease includes postural drainage with chest percussion. This approach to clearance of mucus is based on the idea that cough clears mucus from large airways, but that vibrations are necessary to remove secretions from small airways. Because lung infection is the major source of death in CF, antimicrobial therapy is essential, but resistance against antibiotics is an increasing problem and combinations of

antibiotics may be helpfull. The mucus secretions can be hydrated with aerosols, using inhibitors of sodium resorption [Knowles et al., 1990] may be in combination with ATP [Mason et al., 1991] or with bronchodilators [Hordvik et al., 1985]. Finally physiotherapy may improve the impaired mucociliary clearance [Waltner et al., 1987]. Since a number of years heart and lung transplantation has been demonstrated to improve the condition of CF patients [Scott et al., 1988, Geddes and Hodson, 1989, Smith et al., 1991] but the number of donor lungs is too low to help every CF patients and selection is therefore necessary.

1.2. The pancreas.

The earliest characteristic morphological features of the CF pancreas are the accumulation of secretory material within the ducts resulting in ductal obstruction. Subsequent tissue damage leads to the progressive loss of acinar function and fibrosis [Anderson, 1938, Oppenheimer and Esterly, 1975]. Pancreatic enzyme deficiency causes fat and protein maldigestion, producing a distended abdomen and abnormal stools which can contain up to 70% of the total fat intake. Approximately 85% of the CF patients have pancreatic insufficiency requiring oral pancreatic enzyme replacement therapy with meals. In the remaining 15% there is sufficient exocrine function present and no special treatment is necessary [Park and Grand, 1981, Kopelman, 1991]. CF patients with pancreatic sufficiency are diagnosed at a later age. Moreover, they show milder symptoms such as lower sweat chloride levels, better pulmonary function with age and a lower risk for Pseudomonas infections. In general they have better prospects for survival than their counterparts with pancreatic insufficiency [Durie and Forstner, 1989]. Tests have been developed to monitor pancreas function as one tool in the diagnosis of CF [Durie and Forstner, 1989]. Another important feature of CF is an essential fatty acid deficiency, which is independent of pancreas insufficiency [Strandvik et al., 1988]. There is some evidence that the turnover of essential fatty acids is increased in CF patients [Rogiers et al., 1984]. As yet, the relationship of this symptom to the basis defect in CF remains unclear (see Section 2).

1.3. The intestine.

The major intestinal manifestations of CF are the result of partial or complete obstruction of the intestinal lumen. Obstruction may occur in utero or at any time

during the patient's life. One of the earliest symptoms, occurring in 10-15% of CF patients, is meconium ileus, generally ascribed to failure of pancreatic enzyme secretion and digestion and dehydration of intraluminal contents in utero. After the neonatal period the distal intestinal obstruction syndrome occurs in about 20% of the patients. The pathophysiological features probably arise from inspissation of intestinal secretions secondary to a combination of pancreatic insufficiency with diminished degradation of mucoproteinaceous secretions and poor clearance of concentrated dehydrated intestinal contents.

1.4. The sweat gland.

The most consistent functional alteration in CF are elevated levels (up to 3-5 fold) of NaCl in eccrine sweat. Based on this observation a diagnostic test has been developed [Denning et al., 1980]. In contrast to the respiratory tract and the pancreas, no morphological abnormalities of CF sweat glands have been observed [Munger et al., 1969]. Sweat glands cause only infrequently clinical problems.

1.5. Other tissues.

Other features of CF pathophysiology include infertility especially of male patients caused by obstruction of the genital tract with inspissated secretions [Taussig et al., 1972], liver cirrhosis [Gaskin et al., 1988] and focal biliary fibrosis, probably due to progressive obstruction of the bile ducts in the liver [Sinaasappel, 1989].

2. Diagnosis.

A diagnosis for CF is mainly based on the increased sweat Cl levels in sweat. The sweat test is up to now the diagnostic standard and involves the collection of sweat by pilocarpine iontophoresis coupled with the chemical determination of the chloride concentration [Denning et al., 1980, Sato and Sato, 1990]. Chloride concentrations of 60 meq/liter or more are characteristic for CF patients. Studies among normal and CF patients as well as heterozygotes revealed, on average, a significant difference in the ß-adrenergical secretory response not only between N and CF but also between normal patients and heterozygotes [Sato and Sato, 1984, Behm et al., 1987]. This gene-dosage effect is important evidence for a direct relation between salt transport and the CF gene.

A second diagnostic test is based on the measurement of the bioelectric potential

across nasal epithelial cells, which is significantly higher in CF patients as compared to controls [Knowles et al., 1981a, Knowles et al., 1983, Alton et al., 1990]. An alternative in vitro test developed in our laboratory is based on Ussing chamber measurements of secretagogue-induced transepithelial potentials and short-circuit currents in ileal mucosa and rectal biopsies (see Chapter 2), which, in CF, appeared to be drastically reduced or even reversed [de Jonge et al., 1987, Veeze et al., 1991]. A less reliable test measures the elevated levels of immunoreactive trypsin in the blood of CF patients [Crossley et al., 1979] thereby offering an opportunity to newborn screening [Wilcken et al., 1983]. However, the imunoreactive trysinogen screening procedure has had an unanticipated frequency of false positives and false negatives [Roberts et al., 1988, Wesley et al., 1989, Rock et al., in press].

3. Prenatal diagnosis and carrier testing.

The identification of the CF gene has important implications for genetic counseling and prenatal diagnosis. Before the CF gene had been identified and polymorhic markers were available, prenatal diagnosis was possible by an indirect method only using microvillar enzymes. The activities of the intestinal isoenzym of alkaline phosphatase, leucine aminopeptidase and intestinal disacharidases are decreased in amniotic fluid observed at the 16th-18th week of pregnancy if the fetus is affected with CF [Claass et al., 1986, Brock et al., 1988, Muller et al., 1988]. The accuracy of this type of fetal diagnosis was 90-98% [Boué et al., 1986, Brock et al., 1988]. After the identification of DNA markers closely linked to the CF mutation, in nearly all couples with a 1:4 recurrence risk seeking prenatal monitoring of the next pregnancy, a fully informative pattern of polymorphic alleles could be established on the condition that information of an index patient was available. The reliability of prenatal prediction is over 99%. Another advantage over the enzyme assay is that DNA analysis allows a much earlier fetal diagnosis at 9 to 10 weeks of pregnancy using chorionic villus sampling. The limitation of linkage analysis is that this form of diagnosis is restricted to families where DNA of an index patient is available. Identification of the CF gene and the major mutation, for which 50% of the patients are homozygous indicates that 50% of the pregnancies at a 1:4 risk for CF can be analyzed by mutation analysis instead of linkage analysis. The first studies on prenatal diagnosis have been

published [Halley et al., 1989, Lemna et al., 1990].

Apart from prenatal diagnosis, the recently obtained information about the CF gene and the various mutations allows screening of CF carriers. However, population screening is not only a technical issue (and with the large number of mutations a technical problem [Roberts, 1990]), but has a lot of social, economical and ethical consequences and is still a topic for intense discussion between those who are convinced of the advantages and those who emphasize the disadvantages [Goodfellow, 1989, Colton, 1990, Gilbert, 1990, Brock, 1990, Beaudet, 1990, Wilfond and Fost, 1990].

The identification of the CF gene has not only implications for the reliability of prenatal diagnosis, but offers new model systems to test possible CF therapies. Attempts are being made to establish an animal model for which the mouse is the best option. The identification of the mouse homologue of the CF-gene has recently been published [Tata et al., 1991]. A mouse model will complement studies with cultured human normal and cystic fibrosis cells and provide a basis for physiological and pharmacological testing. In order to establish such an animal model, it is necessary to derive pluripotent embryonal stem cells from mouse blastocysts, which can be maintained and manipulated in culture. After reintroduction into mouse blastocysts, genetically manipulated stem cells can contribute to both the somatic and germ line tissues of the resultant chimeric mouse. The second requirement is to mutate, in the stem cells, the mouse equivalent of the CFTR gene by homologous recombination or gene targeting [Capecchi, 1989]. Of special interest will be to mimic the 508 mutation.

Somatic gene complementation is a direct approach to the management of CF. One possibility would be the use of (retro)viral vectors to transfer a functional CF gene to secretory epithelial cells. The airway cells are of special interest because of its contribution to the mortality in CF patients. CF-mice will be an important test model for this kind of therapy to CF patients.

Since the latest evidence suggests that the CF gene encodes a chloride channel [Kartner et al., 1991, Tabcharani et al., 1991] and not a regulatory component of the channel, it may be a difficult task to bypass the defect in the cAMP activated chloride secretion using a pharmacological approach.

B. BIOCHEMICAL OBSERVATIONS.

In the search for the primary defect in CF, different biochemical approaches have been used and numerous hypotheses have been postulated to explain the symptoms observed in CF. It is worthwile paying attention to some of them.
One hypothesis focussed on the essential fatty acid deficiency frequently observed in CF patients. Arachidonic acid is one of the esssential fatty acids affected. Carlstedt-Duke et al. (1986) have studied the regulation of arachidonic acid release from membrane phospholipids in normal and CF lymphocytes. It appeared that the glucocorticoid dexamethasone, fetal calf serum, or both were able to inhibit arachidonic acid release in normal but not in CF lymphocytes. Therefore the primary defect in CF was thought to be at the level of the glucocorticoid-dependent phospholipase inhibitory protein lipomodulin (lipocortin) or phospholipase A_2 itself. It was claimed that fibroblasts showed comparable effects upon dexamethasone treatment. However, attempts to confirm these results were unsuccessful due to the large variation in the basal release of arachidonic acid observed in control samples [M. Kansen, unpublished observations].
Of particular interest is the so-called "CF-antigen" (CFA), since it was found in increased amounts in the blood of both homozygotes and heterozygotes and was used to distinguish between controls, CF patients and carriers [Bullock et al., 1982, Hayward et al., 1986, Dorin et al., 1987, Brüggen et al., 1988]. The gene coding for CFA was mapped to chromosome 1 [Van Heyningen et al., 1985], therefore the relation with the basic gene defect in CF, localized on chromosome 7 is only secondary. CFA is synthesised in chronic myeloid leukemia cells and in different types of cultured epithelial cells. In the latter cell types it was found to reach higher steady state levels in CF as compared to controls and to increase during cell aging [Keulemans et al., 1991]. CFA appears to be identical to the MRP-14 protein isolated from peripheral leukocyte cultures [Odink et al., 1987, Brüggen et al., 1988] and belongs to a group of low molecular weight calcium binding proteins including the heavy chain of the calcium binding protein L1 [Brandtzaeg et al., 1987], likewise expressed in epithelia [Andersson et al., 1988], and the leukocyte protein MRP-8 [Odink et al., 1987, Lagasse and Clerc, 1988].
Because they are putative determinants of the viscoelastic properties of mucus, emphasis has been placed on the so-called mucins, wich are high molecular weight

glycoconjugates (HMG). These mucins are secreted into airways and sulphated to a greater degree than the mucins of control subjects. With primary cell cultures it has been demonstrated that the difference is not caused by systemic factors, but that the CF cells secrete a range of oversulfated glycoconjugates, including mucin-like glycoconjugates [Cheng et al., 1988]. It has been speculated that these oversulfated HMG's play a key role in the colonization of specific Pseudomonas aeruginosa and Staphyloccus aureus strains in the lungs of CF patients [Cheng et al., 1989].

Many of the clinical features of CF can be explained by a defect in the ß-adrenergic stimulation of transepithelial Cl^--transport in CF affected tissues as first described by Quinton and Bijman for the sweat gland [Quinton and Bijman, 1983]. An essential step in salt transport most plausibly defective in CF, is the opening of chloride channels present in the apical membrane of secretory epithelial cells. An impaired salt- (and accompanying water) transport would explain the presence of viscous mucus in the lung and in the intestine as well as the increased protein concentrations in the pancreas. Very recently it has been found that the decreased acidification in intracellular organelles in CF airway cells results from a defective Cl^- conductance [Barasch et al., 1991, see Welsh, 1991 for comment]. Thus the defective Cl^- transport in CF manifests both at the apical membrane and in intracellular organelles. The vacuolar alkalinization may be an explanation for the increased sulphation and the abnormal glycosylation of mucus glycoproteins observed in CF. To understand the role of chloride transport and its implications for CF, an introduction to the mechanisms of electrolyte transport responsible for salt secretion and absorption in epithelial cells is inevitable.

C. ELECTROLYTE TRANSPORT IN NORMAL AND CYSTIC FIBROSIS EPITHELIA.

1. Defective Cl^- transport as the common defect in different CF-tissues.

Because the organs classically involved in CF, i.e. sweat glands, airways, pancreas and intestine, are composed of epithelial cells and because secretions of several organs are thick and dehydrated, research on the primary defect in CF has been

focussed on possible abnormalities in electrolyte transport epithelia (see [Quinton, 1990] for review).

The first organ studied was the sweat gland. The sweat gland is a single tubule separated morphologically into two compartments possessing distinct functions. When stimulated, the proximal half of the tubule (the secretory coil) actively secretes an isoosmotic fluid to the distal half (the reabsortive duct) which is not permeable for water. In the duct, electrolytes are reabsorbed in excess of water, and sweat becomes hypotonic. The duct can be separated from the coil by microdissection and studied by microperfusion [Sato and Sato, 1977]. These studies showed that the transepithelial potential was one order of magnitude more negative (lumen with respect to serosal fluid) in CF than in normal ducts when perfused with symmetrical Ringer's solution. A reduction of luminal Cl^- in N ducts resulted in a more negative luminal voltage, indicating that Cl^- transport is electrically conductive. In contrast, when luminal Cl^- was decreased in CF ducts, the transepithelial voltage became more positive [Quinton, 1983, Quinton and Bijman, 1983, Reddy and Quinton, 1989a]. In the absence of Cl^-, the Na^+ conductance, taken as the amiloride-sensitive conductance, was not different in N and CF ducts [Bijman and Frömter, 1986, Quinton, 1986]. It appeared that the apical membrane potentials of CF and normal ducts were about equal in magnitude but opposite in polarity, while the basolateral membrane was only hyperpolarized in CF by about 15 mV [Reddy and Quinton, 1989a]. Together these observations led to the conclusion that the defect in salt absorption in CF ducts was due to a Cl^- impermeability localized in the apical membrane. The permeability for Cl^- was not completely absent but considerably reduced in the membrane of CF cells [Reddy and Quinton, 1989b].

While sweating in response to cholinergic and α-adrenergic agonists was not affected in CF coils, injection of ß-adrenergic agonists stimulated fluid secretion in normal but not in CF sweat gland secretory coils [Quinton and Bijman, 1983, Sato and Sato, 1984]. Defective ß-adrenergic receptor activation or reduced adenylate cyclase activity did not explain this loss of secretory activity since the levels of cAMP in N and CF coils were equal upon stimulation. The defect in CF lies therefore downstream the cAMP signal, although involvement of other second messenger routes interacting with the cAMP-mediated pathway could not be excluded. A reduced Cl^- permeability was confirmed in sweat duct cells [Pedersen,

1989, Ram, 1989, Ram et al., 1990].

The results of the sweat gland studies stimulated others investigators to analyze Cl^- transport in airway epithelia. It is still not established which cells in airway epithelia are responsible for electrolyte and fluid secretion. Secretion can be induced by more than one stimulus-secretion pathway [see Welsh, 1987 for a review on electrolyte transport in epithelia and Boucher and Larse, 1988]. The short-circuit current (I_{sc}, see for technical aspects Chapter 2) of primary cultures of tracheal cells from a CF patient failed to show an enhancement in response to the ß-adrenergic agonist isoproterenol [Widdicombe et al., 1985, Cotton, 1987]. Isoproterenol however, acts not only through ß-adrenergic receptors, since it also caused a transient increase in intracellular Ca^{2+} followed by the stimulation of a Cl^- current in both normal and CF nasal polyp cells [Verbeek et al., 1990]. In addition, a number of arachidonic acid metabolites stimulated the I_{sc} in N but not in CF preparations [Boucher et al., 1986, Boucher et al., 1989]. Similar to the sweat gland, the inability of CF airways to respond to ß-adrenergic stimulation is not due to a defect in the receptor activation. Stimulation with isoproterenol increased cytoplasmic levels of cAMP in N and CF tracheal and nasal polyp cells to a similar extent [Boucher et al., 1986, Widdicombe, 1986, Barthelson and Widdicombe, 1987, Boucher et al., 1989]. The lack of response is also not due to abnormal cAMP-dependent protein kinase A (PKA) [Boucher et al., 1986, Barthelson and Widdicombe, 1987].

A second stimulation pathway involves acivation by protein kinase C (PKC). Phorbol ester, known to acivate PKC, increased the I_{sc} in N but not in CF tissues. As in case of PKA, the defective step lies beyond the expression and activation of PKC [Boucher et al., 1989].

The role of Ca^{2+} in Cl^- secretion has been mainly studied by the use of the Ca^{2+} ionophore A23187. Addition of A23187 to monolayers of primary cultures of trachea [Widdicombe, 1986] and of amiloride-inhibited nasal polyp cells [Boucher et al., 1989, Willumsen and Boucher, 1989] stimulated the I_{sc} in both N and CF preparations. It was therefore concluded that the Ca^{2+}-mediated Cl^- secretion was not affected in CF patients.

Airways perform not only secretion but absorption as well, probably within the same celltype. Some results have indicated an increased Na^+ absorption in CF airway epithelia (see [Boucher et al., 1986] and Section 4 for details).

To interprete the decreased apical Cl^- permeability in combination with increased NaCl absorption, one has to realize that the loss of Cl^- conductance in the apical CF membrane reduces the driving force for Na^+ entry at the luminal side. The increased Na^+ permeability in CF must compensate for its smaller electrochemical driving force so that the apical entry is enhanced over normal [Boucher et al., 1988, Willumsen et al., 1989].

There is less detailed information about Cl^- transport in the pancreas. The exocrine pancreas consists of two types of epithelial cells: acinar cells, which secrete digestive enzymes and duct cells which secrete a bicarbonate-rich isotonic fluid. Patients with CF characteristically produce a pancreatic secretion with abnormally high protein concentrations [Kopelman et al., 1985]. This phenomenon appeared to be related to a significant defect in net fluid secretion. The fluid secretory defect in CF was demonstrated to be independent of pancreatic protein secretion and can be accounted for by a reduced secretion of both chloride and bicarbonate in the duct cells [Kopelman et al., 1988]. The reduction in ion transport may block the ducts and initiate the characteristic pancreas fibrosis.

Like in the sweat gland, the airways and the pancreas, the CF small intestine, colon and rectum fails to secrete electrolytes and fluid in response to cAMP-linked agonists. The small intestine appeared insensitive to cAMP analogs, cholera toxin, theophylline, forskolin, prostaglandin E_2, Ca^{2+} ionophore A23187, a cGMP analog, heat stable E.coli toxin and phorbol ester. In contrast to the findings in the sweat gland coil and airway epithelial cells, cholinergic stimulation failed to activate secretion in CF intestine [de Jonge et al., 1987, Taylor et al., 1988, Berschneider et al., 1988, Baxter et al., 1989]. In most CF patients, cAMP-mediated and cholinergic agonists were reported to evoke inversed I_{sc}-responses, probably due to a the activation of apical K^+ channels [de Jonge et al., 1989, Bijman et al., 1990, Veeze et al., 1991]. Similar to the findings in airway epithelium, no differences in intracellular cAMP levels or cAMP-binding proteins following isoproterenol stimulation could be demonstrated between N and CF rectal mucosa [Goldstein et al., 1988]. In contrast to airways, no abnormalities in the amiloride-sensitive Na^+ absorption were found in the colon and rectum of CF patients [Orlando et al., 1988, Goldstein et al., 1988, Bijman et al., 1990,, Veeze et al., 1991].

2. A general cell model for secretion and absorption.

From the electrophysiological experiments described above, we may conclude that the principal abnormalities in CF is a Cl^- transport defect localized in the apical membrane of epithelial cells at a step distal to the activation of protein kinases, i.e. PKA, PKC and PKG. Since this Cl^--transport has been proven to be electroconductive, it is most likely that chloride channel proteins are involved. In order to understand the role of chloride channels in secretion and absorption, it is necessary to have a clear picture of the ion transporters involved in transepithelial Cl^- transport as illustrated in a general cell model (Fig. 1) [Frizzell, 1979].

In order to carry out vectorial transport, epithelial cells are polarized and linked together at their luminal borders by tight junctions which separate the cell membrane into apical (luminal) and basolateral (serosal) compartments. Consequently there are two routes for ion movement across epithelia: (1) by passive diffusion through the tight junctions (the paracellular pathway) or (2) by movement accross the cell and by crossing the apical and basolateral membranes in series (the transcellular pathway).

Cl^- secreting cells are present in the exocrine pancreas, sweat gland coil, intestinal crypts, salivary gland, lacrimal gland, trachea, shark rectal gland, cornea and choroid plexus. Although tissue specific differences exist a general cell model may account for Cl^- secretion in most cell types (Fig. 1 right). The driving force for transepithelial Na^+ and Cl^- transport is delivered by the $3Na^+/2K^+$-ATPase in the basolateral membrane which generates an inwardly-directed electrochemical Na^+ gradient across this membrane. Excess of K^+ leaves the cell through basolateral K^+-channels [Dawson and Richards, 1990] thereby generating an intracellular electrical potential of -50 to -70 mV. These K^+ channels serve two important functions. First they prevent accumulation of K^+ that would cause cell swelling and secondly, the opening of K^+ channels leads to hyperpolarization of the cellmembrane, thereby maintaining the driving force for Cl^- exit at the apical membrane. Similar to other tissues the intracellular Cl^- and Na^+ concentrations are lower while the intracellullar concentration K^+ is higher than in the extracellular fluid. Na^+ enters the cell down this gradient on a cotransporter which couples 1 Na^+, 1K^+ and 2 Cl^- [O'Grady et al., 1987] resulting in the accumulation of intracellular Cl^- above its equilibrium. On stimulation of the cell,

a Cl^--conductive channel in the apical membrane is opened allowing electrogenic secretion of Cl^- down the electrochemical gradient. Na^+ follows Cl^- by a paracellular route in response to the negatively charged lumen. Water follows the ions by osmosis.

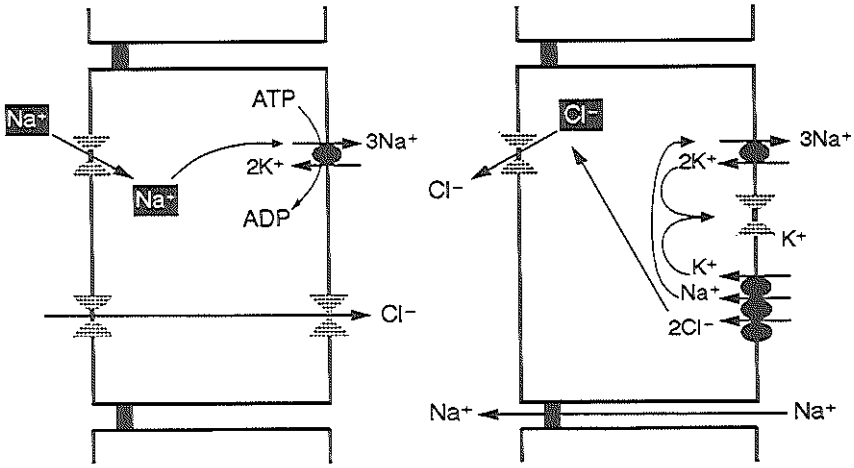

Figure 1.

General cell model of transepithelial ion transport pathways in salt absorbing (left) and secreting (right) tissue. The transporters involved include Na^+-, Cl^-- and K^+- channels, a Na^+-K^+-$2Cl^-$ cotransporter and the $3Na^+$-$2K^+$ ATPase.

Typical salt absorptive epithelia are the amphibian skin, urinary bladder, gallbladder, intestinal villus cells, renal proximal tubule, the thick ascending limb of Henle's loop and the collecting duct. Again celltype specific differences exist. In general both Na^+ and Cl^- enter the cell through channels or through electroneutral cotransporters, while in the basolateral membrane Cl^- channels or KCl-cotransporters carry Cl^- ions to the serosal side (Fig. 1 left).

3. Chloride channels.

3.1. General properties.

Chloride channels have been found in many epithelial and non-epithelial cells

[see Franciolini and Petris, 1990 for a review]. Following the development of the patch-clamp technique (see for a description Chapter 2), they can be investigated at the molecular level. It was this powerful technique which opened the way to an operational classification of ionic channels in general [Jan and Jan, 1989]. For an understanding of structure and function of ionic channels, the reader may consult some excellent reviews [Catterall, 1988, Maelicke, 1988, Krueger, 1989, Eisenberg, 1990, Montal, 1990].

Focusing on epithelial chloride channels [see Gögelein, 1988 for a review], they can, according to prevailing knowledge, be divided into 2 groups with respect to their gating mechanism:

(a). voltage activation

(b). second messenger activation (e.g. Ca^{2+}, cAMP and G-protein, see 3.2)

Furthermore, on the basis of the single channel conductance g, at least three categories of chloride channels can be defined:

(1). Maxi channels, g=200-450 pS

(2). Channels with an intermediate conductance of 20-60 pS

(3). Small chloride channels, g= 2-20 pS

We focus at the process of Cl^--secretion from the chloride channel point of view with special attention to results obtained with patch-clamp experiments. Data were not only derived from tissues or cell lines with control and CF equivalents, but also from well established epithelial cell lines as the human colon carcinoma cell lines T84 [McRoberts and Barrett, 1989] and HT29 [Augeron et al., 1984, Augeron et al., 1986], shark rectal gland cells [Valentich and Forrest, 1991, Moran and Valentich, 1991] and several other mammalian cell lines.

3.2. Regulation.

In most tissues, chloride transport is subjected to several regulatory mechanisms (Fig. 2.).

(1). Phosphorylation by the protein kinases A, C, G (occasionally) and calcium/calmodulin kinase.

(2). Interaction with Ca^{2+}.

(3). Direct or indirect interaction with G-proteins.

(4). Activation and inhibition by arachidonic acid metabolites.

(5). Opening by cell swelling.

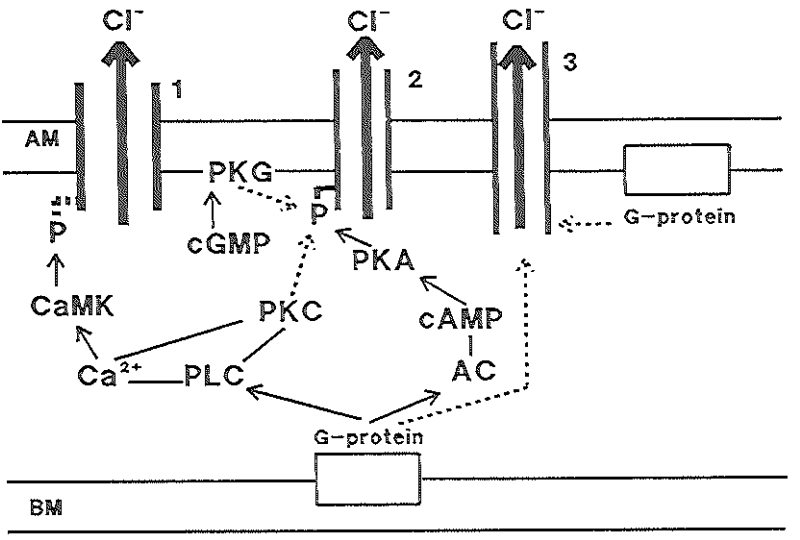

Figure 2.

Regulation pathways of Cl^- channels in a Cl^- secretory epithelial cell. Be aware of the fact that the regulatory pathways shown are from different cell types and do not exist in every cell. Regulation involves phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase (CaMK), cAMP-dependent protein kinase A (PKA), cGMP-dependent protein kinase (PKG) and protein kinase C (PKC). AC: adenylate cyclase. P: phoshorylation site. PLC: phospholipase C. AM: apical membrane. BM: basolateral membrane. The numbers 1, 2 and 3 refer to different types of Cl^- channels; channel 2 may be CFTR. Dashed lines indicate relations without experimental proof.

3.2.1. Regulation by protein kinases.

The characteristics and functions of protein kinases in epithelial tissues have recently been reviewed in detail [Rao and De Jonge, 1990, De Jonge and Rao, 1990].

From the experiments indicating a defect in the cAMP activated fluid secretion in CF, we know already that protein kinase A (PKA) can stimulate Cl^- secretion in a number of epithelia with only a few exceptions [Cook et al., 1988]. Phosphorylation of ion channels is a well known phenomenon [Levitan, 1985]. Whole cell recordings showed an increase in the outward (Cl^-) current after forskolin stimulation in canine tracheal epithelial cells [Shoppa et al., 1989], T84

colonocytes [Cliff and Frizzell, 1990] and in human tracheal cells when the catalytic subunit of PKA was included in the pipette solution [McCann et al., 1989a]. Transfection of T84 cells with a mutant form of the regulatory subunit of PKA resulted in a rise of the cAMP concentration after forskolin treatment, but in the inability of cAMP to activate Cl^- channels [Rogers et al., 1990]. cAMP stimulation of Cl^- channels in reabsorbing cells [Paulais and Teulon], in the shark rectal gland [La et al., 1991] and in amphibian gastric oxyntic cells [Demarest et al., 1989] has been reported as well. Inhibition of Cl^- transport by PKA in apical membrane vesicles of human placenta [Placchi et al., 1991] has also been published.

Like PKA, protein kinase C (PKC) plays an important role in the regulation of ion secretion and modulation of ion channel activity. In airway cells, the agonists bradykinin, carbachol and isoproterenol stimulate Cl^- secretion and increased the amount of diacyglycerol which serves as an activator of PKC [Anderson and Welsh, 1990]. PMA, a tumor-promoting phorbol ester, acting as a membrane permeant activator of PKC, was also found to modulate Cl^- secretion [Welsh, 1987a, Barthelson, 1987, Boucher et al., 1989]. This response, however, was complex: PMA alone caused a transient stimulation of secretion, but it also inhibited the cAMP-activated Cl^- secretion. This dual effect occurred, at least in part, at the level of the chloride channel as concluded from $^{125}I^-$ efflux experiments [Li et al., 1989]. In HT29.cl19A cells PMA provoked Cl^- secretion independently of a cAMP-mediated pathway, but prolonged incubation suppressed net Cl^- secretion by inhibiting K^+ transport pathways, probably by an inhibition of the Na^+-K^+-$2Cl^-$ cotransporter [Franklin et al., 1989] and K^+-channels [Vaandrager et al., 1991b, in press]. The inability of kinases to activate Cl^- channels has led to the analysis of a defect in the protein phosphorylation and dephosphorylation of specific membrane proteins as a possible basis to explain the defect in CF [Brautigan, 1988].

Whole-cell patch-clamp experiments showed no differences in the stimulation of a Cl^- current in response to Ca^{2+}/calmodulin-dependent protein kinase between control and CF airway epithelial cells [Wagner et al., 1991], suggesting that Ca^{2+} activated Cl^- channels were not affected by the CF-mutation.

In the intestine, Cl^- secretion can be uniquely stimulated by cGMP wich acts through a specific isoenzyme of protein kinase G (PKG II) [De Jonge, 1984, De

Jonge and Lohmann, 1985]. Ussing chamber measurements in intestinal mucosa from CF patients with meconium ileus have demonstrated a defect in cGMP actvation of the Cl^- channel at a site distal to PKG [De Jonge et al., 1987, Baxter et al., 1988].

The outwardly rectifying chloride channel.

In the search for the single channel characteristics of the chloride channel thought to be responsible for the defect in CF, more than one type of Cl^- channel has been identified, but most attention has been paid to one specific chloride channel. This channel has an outwardly rectifying current-to-voltage (I/V) characteristic with a slope conductance of 25-40 pS at 0 mV and is about 10 times more selective for Cl^- than for Na^+ [see Welsh, 1990 for a review]. A unique feature of this channel is that it can be activated in excised patches by large depolarizing voltages. Although the mechanisms involved in voltage-activation are unknown, this property has been proven to be an independent way of activating channels following more physiological manipulations. Activation was reported in cell-attached patches after Ca^{2+} ionophore addition in both control and CF cells. In response to elevated intracellular levels of cAMP however activation was observed in normal but not in CF cells. In excised inside-out patches this channel became active after addition of protein kinase A and C (PKA and PKC, the latter in the presence of < 10 nM Ca^{2+}) to the bath solution in N but not in CF cells. PKC in the presence of > 1 μM Ca^{2+} inhibited the channel both in N and CF cells. More recently however, the reported observations could not be reproduced by other investigators (see Chapter 5). Furthermore this channel was present in fibroblasts [Bear, 1988] in which CF was not expressed [Riordan et al., 1989]. In general no correlation between the presence of CFTR mRNA and the outward rectifyer could be demonstrated [Krouse et al., 1990, Ward et al., 1990, Ward et al., 1991]. Moreover, one study reported that not the outward rectifyer was the most prominent chloride channel in airway cells, but a 20 pS linear Cl^- channel [Duszyk et al., 1990]. This channel could be activated by PKA in N but not in CF cells, while the number of these channels as well as their open time probablity in CF was reduced. Investigators nevertheless thought that the outwardly rectifying chloride channel was the channel the electrophysiologists in CF research were looking for. Identification of this particular Cl^-channel and a study of its

regulation in different epithelial cell lines was therefore the major objective of this thesis. For details and references with respect to this channel see the Chapters 3,4,5 and 7. However, after introduction of the CF-gene (see 'GENETICS') into heterologous expression systems, the sole channel activated upon cAMP-stimulation was a linear low-conductance channel (about 8 pS) [Kartner et al., 1991, Tabcharani et al., 1991], indicating that the outwardly rectifying channel may not be the channel involved in CF. The different response to phosphorylation of the OR in N and CF cells reported in the literature are up to now difficult to explain. It is unlikely that CFTR embodies both the 8 pS Cl^- channel and the outwardly rectifying Cl^- channel [Frizzell and Cliff, 1991].

Only recently data were reported about the occurrence of cAMP activated small linear chloride channels in non-CFTR transfected tissues. Channels showing a linear I/V-plot and a low conductance (5-10 pS) were found in T84 cells [Tabcharani et al., 1990], thyroid cells [Champigny et al., 1990b], Necturus choroid plexus [Christensen et al., 1989], brushed nasal epithelial cells [Grygorczik et al., 1990], human fetal epididymis duct cells [Pollard et al., 1990] and human and rat pancreas [Gray et al., 1988, Gray et al., 1989, Gray et al., 1990] and even smaller channels were present in the nephron A6 cell line [Marunaka and Eaton, 1990]. Channels with a conductance of about 10 pS have also been found in excised patches of N and CF cultured sweat gland secretory coil cells [Krouse et al., 1989], in the shark rectal gland [Gögelein et al., 1987] and in canine tracheal epithelial cells [Shoemaker et al., 1986], but no information about their regulation is yet available.

Results of CFTR-transfection experiments suggest that CFTR may itself be a linear 8 pS chloride channel [Kartner et al., 1991, Tabcharani et al., 1991]. Alternatively, CFTR may act as a regulator of a cryptic Cl^- channel universally expressed in the plasma membrane of epithelial and non epithelial cells including the Sf9 insect cell line used for CFTR transfection experiments. In contrast, patch-clamp studies with lymphocytes suggest that the outward rectifyer is activated by CAM kinase and may therefore be identical to the Ca^{2+} activated channel which in most tissues is not defective in CF [Gardner et al., 1990].

3.2.2. Regulation of Cl^- transport by Ca^{2+}.

An increase in the intracellular Ca^{2+} concentration can be provoked by Ca^{2+} entry into the cell through Ca^{2+} channels present in the plasma membrane or via release of Ca^{2+} from intracellular stores in particular the endoplasmatic reticulum. The second messenger inositol 1,4,5-triphosphate (IP_3) regulates the Ca^{2+} release from intracellular stores and may indirectly stimulate Ca^{2+} entry. The signal transduction system involving IP_3 includes a receptor, a G-protein (see Section 3.2.3.) and phosphoinositidase C. Apart from IP_3 another second messenger is formed i.e. diacylglycerol (DAG) which activates PKC indicating that a single hormone by binding to this receptor may activate both Ca^{2+}-dependent processes as well as protein kinases [Berridge and Irvine, 1989].

As already mentioned before an increase in the intracellular Ca^{2+} concentration can stimulate transepithelial Cl^- secretion [Ziyadeh and Agus, 1988] even in CF cells [Quinton, 1990]. In the colon carcinoma cell line T84 both Ca^{2+} and cAMP stimulate Cl^- secretion [Cartwright, 1985]. However, the Cl^- conductance stimulated by Ca^{2+} is different from the one activated by cAMP with different halide permselectivities [Cliff and Frizzell, 1990, Anderson and Welsh, 1991]. Very recently it appeared that at least some of the Ca^{2+} effects were indirect and that Ca^{2+} acts via Ca/calmodulin dependent kinase II [Worrell and Frizzell, 1991]. In another colon carcinoma cell line, HT29.cl19A, isotopic efflux measurements showed a cAMP activated Cl^- current in the apical membrane and a separate Ca^{2+}-activated Cl^- conductance expressed in both the apical and the basolateral membrane [Vaandrager et al., 1991a, in the press]. A similar increase in Ca^{2+} was found upon apical or basolateral addition of ATP (acting through purinergic receptors), followed by an increase in the Cl^- current in both N and CF airway cells [Mason et al., 1991]. In airway cells a transient increase in intracellular Ca^{2+} brought about by the calcium ionophore A23187 produced a prolonged activation of Cl^- channels, measured as $^{125}I^-$ efflux [Clancy et al., 1990a]. Kinase dependent phosphorylation was suggested not to be involved in Ca^{2+} dependent Cl^- secretion because ATP depletion and the presence of kinase inhibitors had no effect [Clancy et al., 1989, Clancy et al., 1990b].

Most data from experiments in which the effect of Ca^{2+} was investigated were obtained by addition of the Ca^{2+} ionophore A23187. However, through the elevation of Ca^{2+}, A23187 may elicit numerous other effects e.g. the activation of

phospholipase A_2 with the subsequent release of archidonic acid and its metabolites, the prostaglandins and leukotrienes. It is therefore not unlikely that part of the Ca^{2+} effects on Cl^- channel activity may occur through a possible interaction of arachidonic acid or its metabolites with epithelial Cl^- channels.

3.2.3. Ion channel regulation by G-proteins.

GTP-binding proteins (G-groteins) have been identified in virtually all celltypes and mammalian tissues. Their major role is to transduce signals by interacting between receptor molecules on the cell surface and effector enzymes or ion channels in the cell membrane. The "classical" G-proteins are heterotrimers composed of alpha-, beta- and gamma-subunit. The alpha-subunits are different among the various G-proteins while the beta- and the gamma-subunits are less divers and exist as complexes which, in contrast to alpha-subunits are functionally interchangeable and are shared by different alpha-subunits. In the active form, GDP is bound to the catalytic domain of the alpha-subunit involved in GTP hydrolysis (GTPase). After binding of an agonist to its receptor, the conformation of the receptor changes, which urges the G-protein to release its bound GDP. Subsequently intracellular GTP binds to the same binding site on the alpha-subunit, resulting in the activation of the G-protein, dissociation of alpha-GTP from the beta/gamma-subunits and interaction with its effector. Hydrolysis of GTP by the intrinsic GTPase activity of the alpha-subunit leads to G-protein inactivation untill a new round of GDP/GTP exchange is initiated (see [Gilman, 1987 and Birnbaumer et al., 1990] for refs.).

A number of different G-proteins have been identified: G_s is the stimulatory and G_i the inhibitory regularory component of adenylate cyclase, G_o is involved in the regulation of Ca^{2+} channels, G_t (transducin) is linked to the photoactivated rhodopsin in the visual system, G_{plc} and G_{pla} activate phospholipase C and A_2 respectively, G_{olf} stimulates adenylate cyclase in olfactory cilia while G_k is involved in the regulation of K^+ channels [Birnbaumer et al., 1990].

Important tools in studying the role of G-proteins are GTP[S] and the bacterial toxins cholera (CT) and pertussis toxin (PTX). GTP[S] is a non-hydrolysable GTP-analogue which activates the G-protein in an irreversibly manner. Cholera toxin irreversibly ADP-ribosylates the alpha-subunit of G_s in the presence of nicotamide adenine dinucleotide resulting in inhibition of GTPase activity and

leading to the irreversible activation of G_s and permanently high intracellular levels of cAMP. Pertussis toxin ADP-ribosylates the alpha-subunits of G_i and G_o and uncouples the G-protein from the receptor, but it does not affect the GTPase activity.

Focusing on the involvement of G-proteins in ion channel regulation, there are at least two different ways of regulation [Birnbaumer et al., 1989]. Indirectly by stimulating enzymes that alter the levels of second messengers such as protein kinases, inositol triphosphate and Ca^{2+} which in turn act on ion channels. An example is the activation of G_s by forskolin leading to an increase the intracellular cAMP level and the subsequent activation of Cl^- channels. In contrast, direct gating of ion channels by G-proteins for K^+ and Ca^{2+} channels has been demonstrated by patch-clamp analysis (excised inside-out patches and whole cell recording) and the planar lipid bilayer technique [Birnbaumer et al., 1989, Fargon et al., 1990], for the amiloride sensitive Na^+ channel [Light et al., 1989, Garty et al., 1989, Light et al., 1990] and for a 305 pS Cl^- channel in rat kidney cortical collecting duct cells [Schwiebert et al., 1990] as well as for chloride channels in the rat lacrimal gland [Llano et al., 1987]. Most data point to a role of the alpha-subunit in ion channel activation [Birnbaumer et al., 1989, Light et al., 1989 Schwiebert et al., 1990, Cantiello et al., 1990], causing an increase in the channel open time, although a more active role for the beta/gamma-subunit has also been advocated (see [Bourne, 1989] for discussion).

3.2.4. Regulation by arachidonic acid and arachidonic metabolites.

Fatty acids have been shown to regulate ion channels either directly [Ordway et al., 1991] or indirectly following their metabolic conversion to the prostaglandins and leukotrienes or directly .

Arachidonic acid (AA) and its cyclooxygenase metabolites, the prostaglandins (PG) as well as its lipoxygenase metabolites, the leukotrienes (LT) [Sigal, 1991] have been implicated in epithelial Cl^- transport e.g. in rabbit colon and ileum [Musch et al., 1982], rabbit tracheal but not Clara cells [Van Scott et al., 1990], airway cells [Welsh, 1987] and canine tracheal epithelial cells [Eling et al., 1986, Leikauf et al., 1986]. Some of the prostaglandins like PGE_1 and PGE_2 stimulate Cl^- secretion by increasing intracellular cAMP and activating PKA [Lazarus, 1984].

The outwardly rectifying Cl^- channel in both control and CF airway cells was inhibited by arachidonic acid when added to the cytosolic side of the membrane. Unsaturated fatty acid were more potent than saturated fatty acids [Anderson and Welsh, 1990b] and cis unsaturated more potent than trans unsaturated acids [Hwang et al., 1990]. Inhibition was achieved in the presence of cyclooxygenase and lipoxygenase pathway blockers. The paradoxal stimulatory effect of AA on Cl^- transport in intact cells but its inhibition of outwardly rectifying Cl^- channels in excised patches argues against a physiological role of the outward rectifyer in AA-stimulated Cl^- transport in intact epithelial cells.

3.2.5. Activation of Cl^- currents by volume regulation.

Agonist stimulation of fluid secretion and absorption leads to an initial lowering of the cellular Cl^- content ($[Cl^-]_i$) in absorbtive as well as secretory epithelial tissues. A lower $[Cl^-]_i$ may be nessary to enhance secretion by promoting Cl^- influx into the cell across the basolateral membrane. This change in intracellular Cl^- reduces cell volume [Foskett, 1990]. Cell shrinkage would therefore be a consequence of the need for developing appropriate ion gradients necessary for performing vectorial salt (and fluid) transport. Because alterations of cell volume can activate volume sensitive transport pathways [Eveloff and Warnock, 1987, Rothstein and Bear, 1989] and because the level of intracellular Cl^- may affect various signal transduction pathways, e.g. G-proteins [Higashijima, et al., 1987], adenylate cyclase [Deterre et al., 1983], release of Ca^{2+} from inositol-triphosphate sensitive intracellular stores [Joseph and Williamson, 1986] and plasma membrane Ca^{2+} influx [Penner et al., 1988], it would be of interest to determine the role of alterations in cell volume in Cl^- channel modulation, as such a volume change may serve as a signal transduction mechanism itself.

Volume regulation is often studied by cell swelling in a hypotonic environment. The physiological response to cell swelling, the so-called regulatory volume decrease (RVD), involves transient stimulation of normally dormant pathways to decrease cell solute content and, via osmotic equilibration, return cell volume toward normal. RVD usually includes loss of KCl, often via separate conductive pathways for K^+ and Cl^- exit [Lewis and Donaldson, 1990].

As shown recently in T84 colonocytes, a volume sensitive G_{Cl} with outward rectification and currents which inactivate during depolarizing voltage pulses was

clearly different from the Ca^{2+}- and cAMP-sensitive Cl^- current [Worrell and Frizzell, 1989]. The application of lipoxygenase inhibitors suggested that leukotrienes may be involved in G_{cell} activation [Worrell and Frizzell, 1990]. At the single channel level this volume sensitive G_{Cl} appeared to be a 75 pS Cl^--channel showing outward rectification and anion selectivity. In contrast to the 25-40 pS outwardly rectifying channel, the volume sensitive channel inactivates upon depolarization.

Whole-cell recordings in airway cells following the reduction of the bath osmolarity revealed the activation of a Cl^- current, which, at the single channel level, was similar or identical to the outwardly rectifying Cl^- channel observed in excised patches of the same cell line. From the persistence of its activation in the presence of a PKA inhibitor and 10 nM Ca^{2+}, it was concluded that the reduced bath osmolarity was the trigger for activation [McCann et al., 1989b]. With transformed human tracheal epithelium comparable results as with T84 cells were obtained. The osmotically activated Cl^- current appeared to be independent of extracellular Ca^{2+} and cAMP [Galietta et al., 1990].

3.3. Chloride channel blockers.

Inhibitors can serve as an important tool in the identification, classification and purification of ion channels.

Blockers of ion channels can be classified in three categories according to their binding kinetics:

(1). 'Slow' blockers dissociate from the channel protein at a lower rate than the rate of channel closing. These blockers increase the closed time of the channel without inducing additional closing events.

(2). 'Intermediate' blockers have faster binding and dissociating rates than the normal closing rates af the channel and induce 'flickering'.

(3). 'Fast' blockers bind and dissociate so fast that single channel experiments can not resolve their kinetics. They decrease the single channel amplitude.

These phenomenologically different types of channel blockade are due to differences in the relative on and off rates of the blocker and the time resolution of the recording system.

Most epithelial chloride channel blockers [Gögelein, 1988, Greger, 1990] used, may roughly be divided structurally into three different groups:

(1). Stilbene disulfonates, like SITS, DIDS and DNDS.

(2). Diphenylamine-2 carboxylate (DPC) and related compounds, especially NPPB.

(3). Anthracene-9 carboxylic acid (9-AC).

No detailed information on the mechanism of action of chloride channel blockers, comparable to present knowledge about amiloride effects on the Na^+ channel or Ba^+ effects on K^+ channels, is yet available. DNDS and NPPB induce flickering [Dreinhöfer et al., 1988, Bridges et al., 1989, Singh et al., 1991] and are therefore intermediate blockers, while DPC and 9-AC are fast blockers because they decreased the single channel conductance [Welsh, 1986]. They do not affect every type of Cl^- channel e.g. NPPB inhibits the outwardly rectifying channel but not the linear 11 pS Cl^- channel in the shark rectal gland [Greger et al., 1987, Gögelein et al., 1987]. Likewise, the 10 pS channel which appeared after introduction of CFTR into Sf9 cells was relatively insensitive to DIDS [Kartner et al., 1991], while the outwardly rectifying channel in HT29.cl19A cells was inhibited by DIDS (see Chapter 7). Many aspects are still unresolved, such as the (ir)reversibility of the blockers, whether they block from the cytoplasmic side, the extracellular side or both, their IC_{50}-values and most importantly, their specificity. Stilbene disulfonates inhibit other transport systems like the Cl^-/HCO_3 anion-exchanger in red blood cells and even cation channels in the basolateral membrane of the rat exocrine pancreas [Gögelein and Pfanmüller, 1988]. NPPB blocks the basolateral K^+ conductance in bullfrog cornea [Reinach and Schoen, 1990], while the K^+ channel blocker quinine blocks Cl^- channels in the rat distal colon [Gögelein and Capek, 1990]. DPC inhibited both the Cl^- conductance and cyclooxygenase in canine tracheal epithelium [Stutts et al., 1990]. Finally Ca^{2+} channel blockers, like verapamil inhibited the outwardly rectifying channel in HT29 cells [Champigny et al., 1990]. From these few examples (see also chapter 7) we may conclude that we should be careful in the interpretation of chloride channel blocker data especially if obtained in intact epithelia.

3.4. Isolation of chloride channels.

Unlike other types of ion channels that have been purified, such as the gamma-aminobutyric acid and the glycine gated chloride channels [Stephenson, 1988, Langosch et al., 1990], no specific ligands were available for the isolation of

epithelial Cl^- channels. Therefore compounds known to inhibit Cl^- transport had to be screened for their possible use in purification by affinity chromatography. A derivative of the indanyloxyacetic acids (IAA), IAA-94, was considered the most potent with a K_i of 1 μM [Landry et al., 1987]. Isolation of chloride channels from kidney and tracheal microsomal membranes by affinity chromatography using IAA-94, resulted in the isolation of 4 proteins of 97, 64, 40 and 27 kD [Landry et al., 1989]. Reconstitution of the 64 kD protein into planar lipid bilayers demonstrated the presence of 3 different Cl^- channel activities with conductances of 26, 100 and 400 pS. Whether they are equal to the channels observed in intact cells has still to be determined. By using an antibody against the 64 kD protein it was shown that the active protein is most probably a homomultimer of four 64 kD subunits but an expression library showed no homology with CFTR [Al-Awqati et al., 1990].

After fusion of purified apical membrane vesicles of bovine tracheal epithelium with planar lipid bilayers, 5 different channels appeared [Valdivia et al., 1988]. Three of them were anion channels. Special attention has been paid to a 71 pS Cl^- channel which showed slight rectification at positive potentials. This channel inactivated after 2-3 min, but could be reactivated after addition of a phosphorylation cocktail containing PKA. The possible role of this channel in secretion has to be determined as well as its relation with a 38 kD Cl^- channel protein found in the same membranes [Ran and Benos, 1991]. A similar procedure for sheep airway epithelia resulted in a 80 pS, outwardly rectifying Cl^- channel that could be activated by PKA [Alton et al., 1990].

4. Possible role of other ion channels in CF.

In CF airway epithelia the transepithelial Na^+ transport is increased compared to control [Boucher et al., 1986]. Na^+ enters the cell through amiloride inhibitable Na^+ channels in the apical membrane [Ling et al., 1990]. Abnormalities in the regulation of Na^+ channels in CF may be responsible for this increase. However, since very recent studies indicate that CFTR may itself be a Cl^- channel [Kartner et al., 1991], the increased Na^+ absorption is most likely a secondary effect. Various explanations for the change in the Na^+ transport in CF airway cells have been given. Duszyk et al., (1989) have suggested an increase in the number of cation channels, whereas Disser and Frömter (1990) have attributed the increase

in the Na^+ channel activity to a doubling in the open probability of an amiloride-sensitive 8 pS Na^+ channel. Another study with airway epithelia concluded that 6-10 pS Na^+ and 20-25 pS nonselective cation channels were present in N and CF cells but that their low frequency would make it difficult to analyse their contribution to an increased Na^+ absorption [Boucher et al., 1990]. Amiloride- and voltage-sensitive linear Na^+ channels of 15 pS are also present in N human sweat duct cells [Joris et al., 1989] but unfortunately were not investigated in CF cells.

No differences were observed between the amiloride-insensitive Ca^{2+}-sensitive 20 pS linear non-selective cation channel in N and CF nasal epithelial cells [Jorissen et al., 1991].

K^+ channels with an inwardly rectifying I/V-plot and a conductance of approximately 25 pS were present in N and CF airway cells and showed the same ion selectivity properties; furthermore their regulation by Ca^{2+} in N and CF excised inside-out patches were equal [Welsh and McCann, 1985]. The presence of a cAMP-regulated Cl^- channel in the apical and a Ca^{2+}-regulated K^+ channel in the basolateral membrane in combination with the requirement of K^+ channels for Cl^- secretion, raises the question of how the two were regulated coordinately. It appeared that at least in airway cells, cAMP can cause a transient increase in Ca^{2+} from intracellular stores [McCann et al., 1989] and that this rise is sufficient to activate K^+ channels [McCann et al., 1990]. A second basolateral Ca^{2+}-insensitive K^+ channel is responsible for the sustained cAMP-stimulated Cl^- secretion [McCann et al., 1990a, McCann and Welsh, 1990b].

Several of the observations discussed above were confirmed in a second study showing that no differences were observed in K^+ channels between N and CF respiratory cells with respect to their general properties [Kunzelmann et al., 1989a], neither with respect to their regulation [Kunzelmann et al., 1989b].

D. GENETICS.

For inherited disorders in which the biochemical defect is unknown, molecular genetics allows the isolation of the gene without any prior knowledge of the nature of the gene product, the so-called "reverse genetics" approach. The search for an unknown gene is performed in two steps. In the first step, linkage analysis

of families is used to determine the approximate localization of the gene involved on a chromosome by showing that it cosegregates with a marker of known location. More markers can be generated by studying somatic cell hybrids that contain various portions of the particular chromosome to which the gene has been mapped. In the second step, molecular cloning techniques are subsequently used to isolate genes in this region and to show that one of them is responsible for the disease.

The demonstration of linkage of the CF mutation to known DNA markers led to the assignment of the CF locus to chromosome 7q31 [Eiberg et al., 1985, Knowlton et al., 1985, Wainwright, et al., 1985, White et al., 1985, Spence et al., 1987]. The identification of the closely linked flanking markers MET and D7S8 [Beaudet et al., 1986, Lathrop et al., 1988] used as a starting point for the chromosome walking and jumping techniques, has finally led to the isolation of the CF gene from lung and sweat duct cDNA libraries [Rommens et al., 1989, Riordan et al., 1989, Kerem et al., 1989a]. Only recently the full genomic sequence was published [Zielenski et al., 1991b].

The CF gene appeared to be 250 kilobase long, has 27 exons and encodes a 1480 amino acid protein called Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) with a predicted molecular mass of about 170 kD. It contains two repeated motifs, each of which consists of a domain capable of spanning the membrane 6 times and sequences resembling consensus nucleotide (ATP)-binding folds (NBF's); both these halves are linked by a R domain containing multiple potential phosphorylation sites for protein kinase A and C sites and apparently located at the cytoplasmic side of the membrane [Riordan et al., 1989] (Fig. 3). The expression of the CF gene is tissue specific and has so far been demonstrated in lung, colon, sweat glands, placenta, liver, parotid gland, pancreas, keratinocytes, lymphoblasts, kidney and nasal polyps, but not in the brain or adrenal gland, nor in skin fibroblasts [Riordan et al., 1989, Hoogeveen et al., 1991].

The molecular structure of CFTR shows resemblance with members of the so-called ATP-binding cassette (ABC) superfamily of transport systems. These transporters generally pump molecules into or out of cells [Hyde et al., 1990], like the P-glycoprotein responsible for multidrug resistance (MDR) in mammalian tumors [Ford and Hait, 1990] and the yeast STE6 gene product that mediates export of yeast a-factor pheromone [McGrath and Varshavsky, 1989].

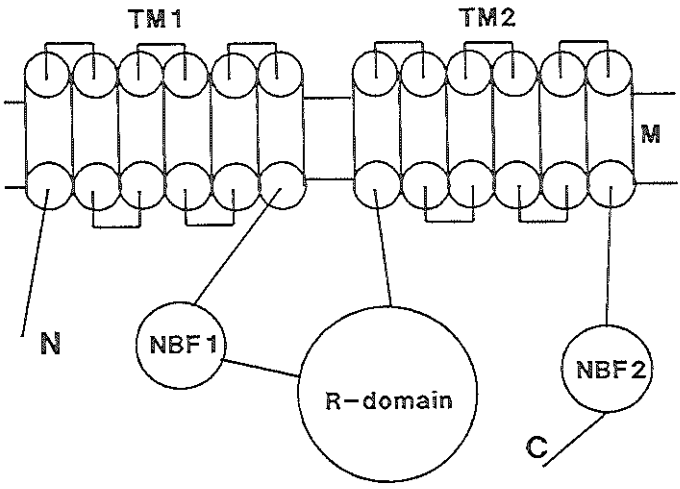

Figure 3.

Schematic structural model of the cystic fibrosis gene product, CFTR, based on sequence analysis. The two transmembrane parts (TM1 and TM2) are linked by the R-domain containing phosphorylation sites for PKA, PKC. Two nucleotide-binding fold domains (NBF1 and NBF2) are present. The Phe 508 deletion occur in NBF1. N: N-terminal end. C: C-terminal end.

This resemblance suggests that CFTR is involved in transport of chloride ions and thus is the chloride channel itself or is the transporter of molecules which act on separate chloride channel proteins. Studies with CFTR-directed antibodies have shown that CFTR is a membrane associated glycoprotein, localized in part but not exclusively at the apical membrane [Gregory et al., 1990, Hoogeveen et al., 1991]. Furthermore, the phosphorylation of immunoprecipitated CFTR in vitro by cAMP-dependent protein kinase A has been demonstrated [Gregory et al.,1990, Cheng et al., 1991]. Correction of the impaired chloride transport in CF airway and pancreatic duct cells by introducing the wild type CF gene into CF cells restored a cAMP-stimulated chloride current [Rich et al., 1990, Drumm et al., 1990] thereby relating CFTR expression directly to chloride transport. As already mentioned, CFTR pobably codes for a chloride channel [Anderson et al., 1991, Rich et al., 1991] with a linear I/V-characteristic and a conductance of 8 pS [Kartner et al., 1991, Tabcharani et al., 1991]. The resemblance of the molecular structure of CFTR to transport systems having a pump function, while CFTR is

probably an ion channel itself may be an example of a less clear distinction between carriers, ion pumps and ion channels than was realized up to now. A recently developed model suggests that the R-domain functions as a plug that occludes the channel pore and that this inhibition may be released by phosphorylation with PKA resulting in a conformational change of the R-domain, thereby opening the channel and allowing Cl^- to flow through the pore [Rich et al., 1991]. The definite proof that CFTR constitutes the chloride channel itself, awaits the incorporation of the purified protein into artificial lipid membranes. But CFTR seems not only to be involved in Cl^- transport. After introduction of wild type CFTR in CF pancreatic cells, forskolin induced an increase in the Cl^--permeability but a reduced rate of endocytosis and an increased rate in exocytosis as well [Jilling et al., in press]. The CFTR-dependent regulation appeared not to be a consequence of Cl^- activation and thus it was concluded that CFTR plays a role in the recruitment of cell surface proteins. Increasing evidence exists that Cl^- conductances are important in endo- and exocytotic processes in T84 cells [Huflejt et al., in press, Barber et al., in press], proximal tubule cells of the kidney [Bae and Verkman, 1990] and exocrine pancreas and parotid gland cells [Thévenod et al., 1991]. PKA has been reported to be involved in Cl^- channel activation in endocytotic vesicles [Bae and Verkman, 1990].

The most frequent mutation in CF patients is a 3 bp deletion which results in the loss of a phenylalanine at position 508 in the tenth exon of the amino acid sequence which is in the first NBF [Kerem et al., 1989]. This mutation is present in 70% of the CF chromosomes, although a European study demonstrated that the frequency can be as low as 30% or as high as 80% in different areas [Romeo and Devoto, eds., 1990]. Although an increasing number of mutations is found, no reliable correlation between genotypes and clinical symptoms has been observed [Kerem et al., 1989, Ferrari et al., 1990, Struhrman et al., 1990, Santis et al., 1990a, Santis, 1990b]. In model experiments, the 508 deletion and several other CFTR-mutations, introduced by site-directed mutagenesis, prevented maturation of CFTR upon its expression in COS-7 cells, suggesting that CF is caused mainly by missorting of CFTR rather than loss of its function. The data indicate that nascent CFTR first interacts with the endoplasmatic reticulum, in agreement with antibody-studies [Hoogeveen et al., 1991], and is glycosylated. The native protein is then transported to the Golgi where carbohydrate processing to

complex-type glycosylation occurs; subsequently some of the mature molecule is transported to the plasma membrane [Cheng et al., 1990, Gregory et al., 1991]. Deletion of a phenylalanine in a chemically synthesized 67 amino acid peptide is predicted to induce a significant change in the ß strand of the NBF and thereby to result in an altered nucleotide binding or a defective coupling between the R domain and the channel forming domain, eventually leading to the defect in chloride channel opening in CF [Thomas et al., 1991, Riordan, personal communication].

Other mutations include missense, nonsense, frameshift and RNA splicing mutations [White et al., 1990, Cheng et al., 1990, Cutting et al., 1990, Dean et al., 1990, Kerem et al., in press, Cutting et al., in press, Zielenski et al., 1991, Ivaschenko et al., 1991]. This list is far from complete and still growing. The total number of mutations in May 1991 was 108 of which 24 were summarized by Davies [Davies, 1990]. Most mutations map in the first half of the protein, in particular in the first NBF (exon 9-12).

E. SCOPE OF THE THESIS.

The experimental work described in this thesis was aimed at a better understanding of the pathogenesis of CF with the emphasis on electrophysiological studies on Cl^- transport. Chloride transport was studied with Ussing chamber, isotopic ($^{125}I^-$)-efflux and fluorescent (SPQ) measurements as well as with the patch-clamp technique. In search for a suitable cell culturing model, we studied Cl^- transport in CF keratinocytes, which are easy to obtain and would provide us with sufficient cell material (Chapter 3). However, the use of keratinocytes appeared to be limited to primary cell cultures which can only be passaged for maximal 10 times, necessatating the use of many different and often heterogeneous N and CF cell lines necessary. The availibility of a continuously growing cell line with CF genotype and phenotype would provide a more homogeneous model for biochemical, electrophysiological and genetic studies. We have therefore immortalized a CF nasal polyp cell line with a hybrid SV40/Ad12 virus, and have characterized this cell line biochemically and electrophysiologically as discussed in Chapter 4. A continuously growing homogeneous cell line selected for its chloride secreting characteristics would also be of much interest for CF

research. The subclone 19A of the human colon carcinoma cell line HT29 is such a cell line, easy to maintain in culture without the multiple additions to the medium required for culturing keratinocytes and primary nasal polyp cells and without the use of feeder cells. These colonocytes can be considered as a model for the study of Cl^- transport in normal cells. In Chapter 5 we describe the patch-clamp experiments with HT29.cl19A cells performed to characterize the cAMP-dependent regulation of the outwardly rectifying chloride channel which was thought to be defectively regulated in CF. Apart from cAMP regulation of chloride channels, we also found that G-proteins are involved in the regulation of a different type of chloride channel in intestinal membranes. Chapter 6 describes the vesicle and patch-clamp experiments leading to the identification of a novel potential signal transduction pathway for chloride transport in intestinal cells.

The homology between CFTR and different types of pump proteins like MDR led us to verify the hypothesis that CFTR could function as a pump with substrates exerting secondary effects on chloride channels. In Chapter 7 MDR-substrates and -blockers were tested on epithelial chloride transport and on the outwardly rectifying chloride channel in particular.

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CHAPTER II.

INTRODUCTION TO THE EXPERIMENTAL WORK: HOW TO MEASURE CHLORIDE TRANSPORT.

For the measurement of chloride transport biochemical as well as electrophysiological methods can be used [Wills and Zweifach, 1987, Gögelein, 1988, Widdicombe, 1990]. Cl^- transport techniques include nuclear magnetic resonance (NMR), the Scanning ion-conductance microscope (SCIM) [Hansma et al., 1989], X-ray probe analysis [Takemura et al., 1991], optical measurements of cell volume and plasma membrane potential, micro-electrode measurements of membrane potentials, the use of Ussing chambers, a radioactive $^{125}I^-$ tracer assay, experiments with Cl^--sensitive fluoresence indicators and the patch-clamp technique. An introduction into the methods used in this thesis may be helpfull in understanding the phenomenon of defectively regulated chloride transport in CF.
These methods may be divided into techniques measuring Cl^- transport at the macroscopic and at the microscopic level.

1. THE MACROSCOPIC CHLORIDE CURRENT.

1.1. The Ussing chamber.

The overall transport properties of flat sheets of epithelia such as urinary bladder or intestinal epithelia are usually investigated by mounting the tissue between two fluid-filled half chambers and measuring isotope fluxes, transepithelial potentials or electric currents between both fluid compartments. Such chambers were introduced by Ussing more than 40 years ago [Ussing, 1948] and are referred to as Ussing chambers (Fig. 1). With two sets of calomel and platinum electrodes one is able to measure both the potential difference (PD) and the voltage clamp current (the short circuit current or I_{sc}), respectively.

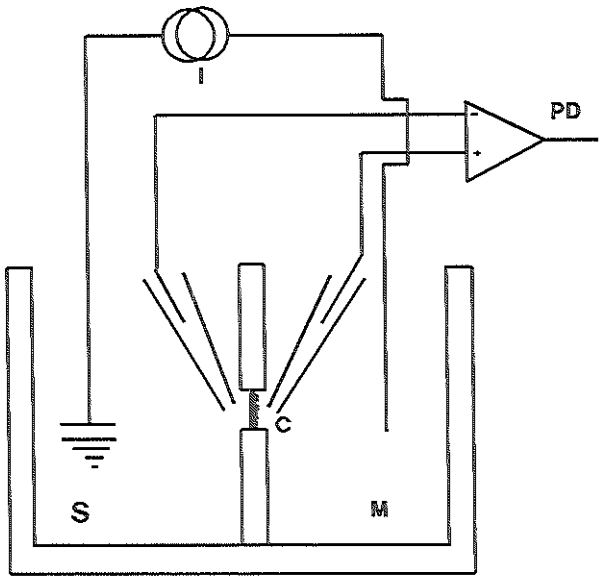

Figure 1.

Schematic presentation of the Ussing chamber. The two compartments of the Ussing chamber are divided by a tissue sheet or by a monolayer of cultured cells (C) on a permeable support. The potential difference (PD) across the tissue is measured through calomel electrodes and KCl agar bridges. The short-circuit current (I_{sc}) is applied by platinum electrodes in the mucosal (M) and serosal (S) baths.

In addition to freshly isolated tissues, epithelial cells cultured to confluency on permeable supports may also be used, on the condition that tight junctions have been formed. Measurements of changes in the I_{sc} after hormonal stimulation have been described for cultured sweat glands, airway and colon carcinoma cells.

A problem in Ussing chamber experiments may be the persistence of a leak pathway between the bath fluid compartments which results in augmented "transepithelial" flux rates and reduced "transepithelial" potential and resistance values, although some correction can be applied [Kottra et al., 1989]. Furthermore, in some cases where no increase in the I_{sc} was measured after addition of the calcium ionophore A23187, isotopic efflux measurements showed simultaneous opening of apical as well as basolateral membrane chloride channels resulting in the nullification of net chloride transport [Vaandrager et al., 1991, in

the press]. Thus effects of agonists on the Cl^- channel, although present, sometimes escape detection by the Ussing chamber technique.

1.2. The isotopic efflux assay.

Recently a convenient method has been developed to simultaneously measure K^+ and Cl^- conductive pathways in Cl^--secreting epithelia [Venglarik et al., 1990]. Cells cultured on plastic petri dishes were preloaded with $^{125}I^-$ and $^{86}Rb^+$ (as a tracer for Cl^- and K^+ respectively) after which the cells were washed and isotope-free solution was added. At different time intervals samples were taken and replaced by a fresh isotope-free solution. Agonists or antagonists were added in the preloading or at the sampling period. At the end of the experiment, tracer remaining in the cells was extracted with NaOH. All samples were counted in a liquid scintillation counter. The efflux can be expressed as the fractional efflux per minute i.e. the amount of isotope lost from the cells in the interval preceding a time point as a percentage of the amount of isotope associated with the cell layer at the beginning of that interval divided by the length of that interval. This simple efflux assay makes a rapid screening of different drugs or agonist affecting K^+ and Cl^- transport possible. Because no confluent monolayers are necessary, this assay can be applied to cells that do not form tight junctions. When they do, confluent monolayers cultured on filters instead of dishes can be monitored separately for their transport processes at the apical and basolateral membrane, respectively [Vaandrager et al., 1991, in press]. Not only cells can be used for this efflux assay, but vesicles and liposomes as well [Breuer, 1990; Bayliss et al., 1990]. In particular vesicles made from the apical membrane where the Cl^- conductance has been localized provide a better defined experimental source than intact cells (Fig 2). The use of $^{125}I^-$ instead of $^{36}Cl^-$ has several advantages: $^{125}I^-$ is available at higher specific activity than $^{36}Cl^-$, some epithelial chloride channels prefer I^- over Cl^- ([Halm et al., 1988]) and I^- is a poor substrate for the bumetanide sensitive Na^+-K^+-$2Cl^-$- cotransporter [O'Grady et al., 1987].

However with this efflux assay no direct information can be obtained about mechanisms responsible for stimulatory or inhibitory effects of agonists and antagonists, driving forces are inadequately controlled and the method is less suitable for establishing dose-response relationships.

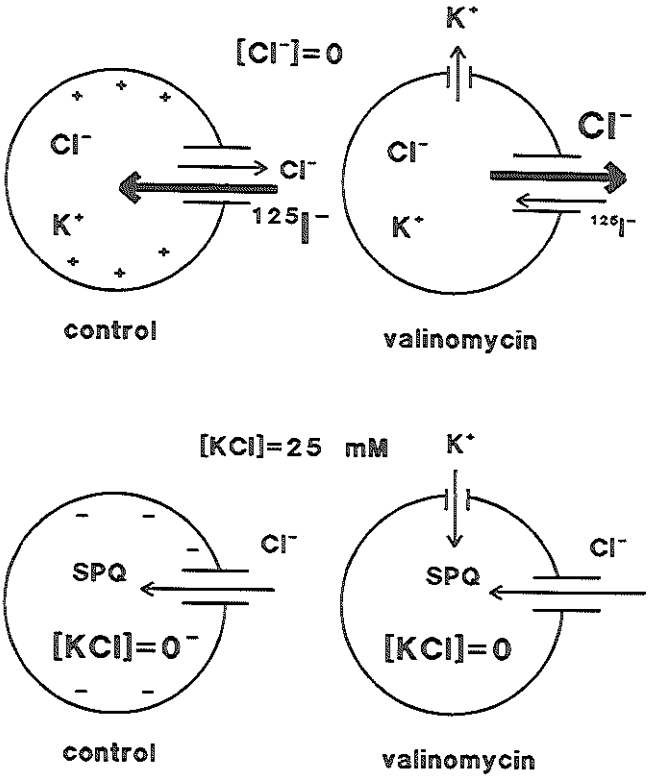

Figure 2.

Measurement of Cl^- transport in apical membrane vesicles by the radioactive $^{125}I^-$ efflux assay (upper part) and by the use of the Cl^--sensitive fluorescent dye SPQ (lower part). Upper part: after having removed the extravesicular Cl^-, the assay is started by mixing the vesicle suspension with tracer amounts of radioactive iodine in the absence or the presence of the K^+-ionophore valinomycin. The uptake is terminated by rapid removement of external $^{125}I^-$, followed by gamma-scintillation counting of the eluate. Lower part: first, the extravesicular SPQ is removed. Second, the vesicles are added to the cuvette and the fluorescence is monitored. Chloride uptake is started by adding KCl. After the experiment, trapped SPQ is released by the addition of Triton. The rate of salt-induced SPQ quenching is finally determined from the change in the fluorescence.

1.3. The fluorescent indicator.

Monitoring of transport by fluorescence is based on compartment-dependent variations in fluorescence associated with translocation of substrate [Eidelman et al., 1989]. Fluorescent indicators for measurement of intracellular calcium, sodium, potassium, magnesium and pH have been described (see [Verkman, 1990] for refs.) and contain a cation specific chelation site or titrable group coupled to a fluorescent moiety. Cell-permeant ester derivatives are loaded by passive diffusion and breakage by intracellular esterases results in a more polar molecule which remains entrapped at the cell interior. A well known example is the calcium indicator fura-2 [Minta and Tsien, 1989] for Ca^{2+} measurements.

For the biological application of (Cl^--sensitive)-fluorescent indicators a number of criteria are important. The indicator should be sensitive to changes in the intracellular Cl^--activity in the physiological range of 0-60 mM, should be selective for Cl^-, should not interact with biological systems, should have bright fluorescence with a high quantum yield (>0.5), should be loaded into cells by a non-invasive method and finally should give the absolute intracellular Cl^--activity without the knowledge of the local indicator concentration [Verkman, 1990; Chao et al., 1991].

Because no selective chloride chelators have been described, an indicator based on the sensitivity of fluorescence of quinoline-related molecules to quenching by halides has been developed: 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ). This compound is a zwitterionic salt of which the fluorescence is excited at ultraviolet wavelengths with maxima at 318 and 350 nm. SPQ is quenched with a 50% decline in fluorescence at 17 mM chloride. Maximal fluorescence is seen in Cl^--free medium, and on addition of Cl^-, there is a rapid decline in fluorescence as Cl^- enters the cell and quenches SPQ. Quenching can be calculated according to $F_0/F = 1 + K_{Cl}[Cl^-]$ where F_o is the fluorescence in the absence of Cl, F in the absence of Cl^- and K_{Cl} the Stern-Volmer quenching constant (in M^{-1}). In the presence of slowly permeating ions, conductive chloride transport is slow because the movement of a counterion required to maintain electroneutrality is rate limiting. To make transport of Cl^--ions rate limiting the membrane potential should be clamped by use of an ion-ionophore combination, of which potassium-valinomycin is the most effective. Like the radioactive tracer technique, the SPQ method can also be applied to apical membrane vesicles and liposomes (Fig 2).

Because SPQ is highly polar and poorly membrane permeant, it can be entrapped in a sealed membrane preparation by a single freeze-thaw cycle or by a sudden hypotonic shock and can function at the interior as a quantitative indicator of intravesicular Cl^- concentration.

Advantages of the use of a fluorescent indicator is its noninvasiveness, high time resolution for measurement of fast transport processes and high spatial resolution of intracellular chloride gradients en compartmentation. However, loading of SPQ into living cells requires a permeabilization procedure.

With overlapping and complementary parameters that have been measured in canine airway epithelial cells including short-circuit current, isotope ion fluxes, open-circuit and short circuit membrane potentials and intracellular ion concentrations using microelectrodes and fluorescent indicators a kinetic model has been described [Hartmann and Verkman, 1990].

2. THE MICROSCOPIC CHLORIDE CURRENT.

The patch-clamp technique.

In contrast to the already mentioned techniques where Cl^--transport in general or the macroscopic Cl^--current is measured without giving information about the identity of the ion channels involved, the patch-clamp technique allows biophysical characterization of single ion channels as well as the biochemical description of their regulation.

In the patch-clamp-technique (Fig 3.) [Hamill et al., 1981; Sakmann and Neher, 1983; Sakmann and Neher, 1984; Franciolini, 1986; Lindau and Neher, 1988] a fire-polished micro-pipette with a tip diameter of about 1 μm is connected to a current-to-voltage converter and mounted on a micromanipulator. The pipette, filled with a salt solution, is carefully pressed onto the membrane of cells or native tissue or onto cells cultured on petri dishes or filters kept at room temperature or 37°C placed on the stage of an inverted microscope. After applying gentle suction to the interior of the patch pipette, part of the membrane is pulled into the pipette forming a Ω-shaped patch in close contact with the glass wall. This contact between cellmembrane and pipette glass results frequently in a high electrical resistance seal ranging from about 3-10 Mega-ohms (pipette input resistance) to about 50-100 Giga-ohms. Hydrogen bonds, salt bridges and

van der Waals forces are probably involved in seal formation [Corey and Stevens, 1983; Franciolini, 1986]. This high resistance has two implications: (1) a noise reduction so that curents as low as 0.2 pA can be resolved and (2) a command voltage applied to the patch pipette by the patch-clamp amplifyer leads to a voltage drop across the membrane patch on the tip of the electrode, while the voltage drop across the electrical resistance of the pipette and the bath are negligible.

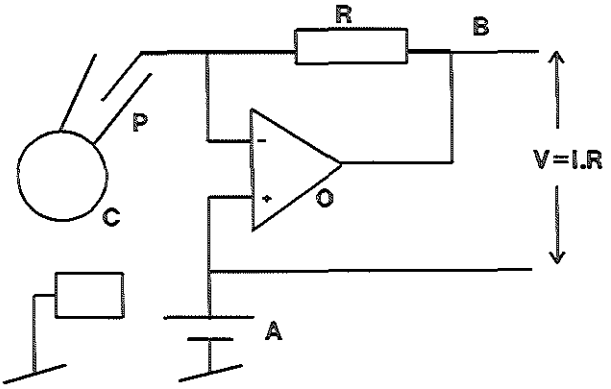

Figure 3.

General principle of patch-clamping. The operational amplifyer O can be thought of as a voltage controlled voltage source; the output voltage changes in response to differences in the voltages V_+ and V_- to keep the pipette potential at the reference voltage A. This means that for practical purposes the pipette potential can be assumed to be precisely A. This in turn allows us to measure B-A. The current I is measured across the resistance R. B is the voltage source, P the patch pipette attached to the cell C.

Patch-clamp experiments can be performed in a number of different configurations. These include the cell-attached, the whole cell and the excised inside-out and outside-out configuration. The loose patch-clamp technique

[Stühmer et al., 1983] is less frequently used and the perforated patch [Levitan and Kramer, 1991] has only recently been developed. With the loose and the whole cell confuguration only macroscopic currents can be measured but the cell-attached and the excised inside-out have a sufficient high resolution to measure microscopic Cl^--currents through one single ion channel (Fig 4). In the on-cell or cell-attached configuration the patch pipette remains attached to the cellmembrane keeping the intracellular second messenger machinery necessary for activation of single ion channels intact. Simply withdrawal of the pipette turns the cell-attached patch into an excised inside-out patch facing with its cytoplasmic side to the bath.

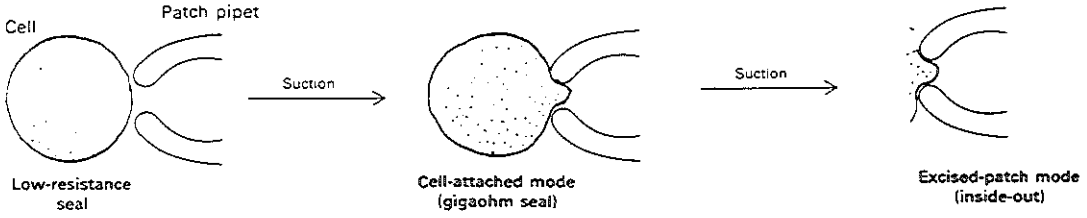

Figure 4.

Schematic presentation of the formation of a gigaseal and 2 patch-clamp configurations: the on-cell or cell-attached and the excised inside-out configuration.

After having established the desired patch-clamp configuration, the experiment is followed on an oscilloscope and the data can, after digitalization with a pulse code modulator, be stored on a video recorder for further analysis [Bezanilla, 1985] or can directly be registrated on a computer hard disc.

When an ion channel is present in the patch, the identity and the presence or absence of one or more subconductance states [Fox, 1987] must be characterized as well as a number of other parameters like the single channel conductance g, the open state probability Po and kinetic parameters, such as the time constants obtained from open- (t_o) and closed- (t_c) time histograms, while qualitative

characteristics should not be neglected.

1. The identity of an ion channel.

Identification of the nature of the channel requires the ion selectivity of the channel expressed as the permeability ratio. For Cl^--channels the P_{Cl}/P_X, where P_{Cl} and P_X are the permeabilities for Cl^- and ion X (cation or anion), has to be calculated from the shift in the reversal potential (the potential at which no net current is passing through the channel) after changing the ion composition in the bath solution of excised inside-out patches.

2. The single channel conductance.

The single channel conductance is generally calculated from the current-voltage (I/V) relationship. These relationships are linear or show rectification in one current direction. For some chloride channels the single channel amplitude for outward current is larger (outward rectification) or smaller (inward rectification) than for inward curents. In non-linear I/V plots the conductances are calculated separately for positive and negative voltages or alternatively at 0 mV.

3. The open state probability.

Another important parameter is the dependence of the single channel open state probability on the potential which reflects a possible voltage dependence of the macroscopic current. Po is defined as the time during which the channel is open, divided by the total observed time and calculated with the half-amplitude threshold method [Colquhoun and Sigworth, 1983]: every crossing of the threshold, set at half of the amplitude at a particular voltage is interpreted as an opening or closing of the channel.

4. Time constants.

The transition from the open to the closed state of a channel or vice versa is called an event. The time course of channel opening and closing can be observed at 5-10 kHz resolution. The number of events within a period of time (called a bin, e.g. a bin of 2 ms) is usually presented as function of the time in the form of histograms. These distributions of open and closed times can be fitted by

exponential functions with one or more time constants expressing mean opening (t_o) and closing (t_c) times (usually in the order of msec) each representing an open and closed state of the channel.

5. Qualitative aspects.

Some qualitative features include the distribution of the channels (in clusters or at random), opening in bursts or a stationairy activity throughout the time, inactivation within limited time, flickering, voltage activation or inactivation upon excision.

Although more theoretical aspects of ion channels and single channel recording are outside the scope of this thesis, it is worthwile mentioning some reports on this topic [Sachs et al., 1982; Colquhoun and Sigworth, 1983; Blatz and Magleby, 1986; Bauer et al., 1987; Sigworth and Sine, 1987; Cooper et al., 1988; Croxton, 1988; Franciolini and Petris, 1988; Ball and Sansom, 1989; Gates et al., 1989]. Introduction of single channel data in the secretion model [Hartman and Verkman, 1990] would be very helpfull in obtaining a more detailed picture of Cl^- secretion.

Despite its advantages, patch-clamp recording also has some problems. Vesicle formation at the tip of the pipette is not an unusual phenomenon, but breaking the vesicle by a brief air exposure can overcome this problem. Although theoretically all cell types can be used, a freely accesible cell surface is necessary, which sometimes can only be obtained after enzymatic treatment. Furthermore in polarized epithelial cells the patch pipette can only be placed onto the apical membrane because the basolateral membrane is in contact with the support. The nature of the membrane isolated by seal formation and the process of seal formation itself is unknown. The question arise then whether the membrane patch is representative of normal membrane surface. Application of suction to the pipette interior may cause bleb formation. The blebs may contain freely mobile lipids in a much higher content than the normal surface membrane; they may also contain much lower concentrations of proteins because of thei release from the cytoskeleton or extracellular matrix as has been demonstrated for Na^+ channels [Milton and Caldwell, 1990a]. Thus channel properties that depend on cytoskeletal attachments or other membrane proteins like G-proteins could be

altered drastically [Milton and Caldwell, 1990b].

A two electrode analysis revealed that the apparent seal resistance determined at the tip of the cell-attached patch electrode by changing the electrode potential may be a poor estimate of the true seal resistance, that the membrane resistance is often much lower than is usually assumed and that single channel conductance in the on-cell mode may be inaccurate because the membrane potential does not remain constant as the electrode potential is varied [Fischmeister et al., 1986]. Strong ionic currents across a patch membrane changes the driving force on the current by altering the ionic concentration near the surface membrane [Zimmerman et al., 1988].

It is therefore not surprising that the patch-clamp technique is still developing as is demonstrated by reports focusing on seal formation [Sokabe and Sachs, 1990; Ruknudin et al., 1991], new applications [Lindau and Neher, 1988; Poronnik et al., 1988], a new patch-clamp amplifyer [Strickholm, 1989; Prakash et al., 1989; Rohlicek et al., 1989], methods for temperature control and fast exchange of the bath solution [Pusch and Neher, 1988; Delbridge et al., 1990; Vassilev, 1990], a new patch-clamp configuration [Levitan and Kramer, 1990], new tissue preparation methods [Criado and Keller, 1987; Edwards et al., 1989; Wehner et al, 1990; Nicolson et al., 1991], effects of patch pipette glass [Cota and Armstrong, 1988; Furman and Tanaka, 1988; Zuazaga and Steinacker, 1990], fire-polishing methods [Fox, 1985] and more detailed analysis procedures [Ball and Sansom, 1989].

Patch-clamping is not only restricted to the use of tissues or cultured cells, but can also be applied to liposomes [Miller, 1984; Schmid et al., 1988; Keller et al., 1988; Riquelme et al., 1990] or vesicles that have been prepared in vitro [Criado and Keller, 1987].

A limitation of the patch-clamp technique is that the composition of only the bath solution can be changed during an experiment and therefore more attention is now paid to techniques in which isolated vesicles are fused with or purified channel proteins are reconstituted in artificial planar lipid bilayers [Montal, 1987; Reinhardt et al., 1987; Rousseau, 1989; Woodbury and Miller, 1990]. In contrast to patch-clamp recording, in the bilayer technique both sides of the channel are accessible to manipulation. Moreover, because the channel is not present in its

native membrane, its characteristics can be investigated in better defined conditions in particular when synthetic peptides are used [Lear et al., 1988; Tosteson et al., 1989, Tosteson et al., 1990].
Since single ion channel analysis is now possible by means of the patch-clamp recording and the related lipid bilayer technique, studies on the functional effects of genetic modifications by site-directed mutagenesis has become is an intruiging new field of research [White, 1985; Claudio, 1986; Dani, 1989]. Single channel characteristics of isolated ion channels can be investigated after expression of mRNA or cDNA in Xenopus laevis oocytes [Dascal, 1987; Sigel, 1990] or, when sufficient protein is available, in lipid bilayers. Site-directed mutagenesis of part of the channel can provide information about which part or subunit of the channel molecule is responsible of the conductance, the ion selectivity and the binding of drugs or modulators [Stühmer et al., 1989; Hoshi et al., 1990].

The patch-clamp technique not only allows the biophysical manipulation and characterization of ion channels but also the description of their biochemical regulation [Neher, 1988, Neher and Penner, 1989, Birnbaumer et al., 1989]. The presence of cAMP-, cGMP-, diacylglycerol- or Ca^{2+}-increasing hormones in the bath solution while recording in the cell-attached configuration or in the patch pipette in whole-cell patch-clamping can provide important information about the possible role of second messenger systems in ion channel regulation. Addition of cAMP-dependent protein kinase A, protein kinase C, Ca^{2+} and GTP[S] have been shown to activate channels in excised inside-out patches (see chapter 5 for refs., chapter 6). Patch-clamp experiments combined with the simultaneous measurement of changes in intracellular Ca^{2+} [Morris et al., 1990] or cAMP [Adams et al., 1991] by fluorescence imaging would be extremely powerfull in studying the regulation of single ion channels e.g. the separate Ca^{2+} and cAMP activated Cl^- conductances in T84 cells [Cliff and Frizzell, 1990].

In the present studies, Cl^- transport in normal and CF epithelial cells has been studied in the Ussing chamber (Chapters 3 and 4), by SPQ-measurements in apical membrane vesicles (Chapter 6), by isotopic efflux assays in intact cells (Chapters 3 and 7) and apical membrane vesicles (Chapter 6) and by the patch-clamp technique (cell-attached and excised inside-out configuration, Chapters 3

to 7).

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CHAPTER III.

REGULATION OF CHLORIDE TRANSPORT IN CULTURED NORMAL AND CYSTIC FIBROSIS KERATINOCYTES.

ABSTRACT.

Cultured normal (N) and cystic fibrosis (CF) keratinocytes were evaluated for their Cl^--transport properties by patch-clamp-, Ussing chamber- and isotopic efflux-measurements. Special attention was paid to a 32 pS outwardly rectifying Cl^- channel which has been reported to be activated upon activation of cAMP-dependent pathways in N but not in CF cells. This depolarization-induced Cl^- channel was found with a similar incidence in N and CF apical keratininocyte membranes. However, activation of this channel in excised patches by protein kinase (PK) A or PKC was not successfull in either N or CF keratinocytes. Forskolin was not able to activate Cl^- channels in N and CF cell-attached patches. The Ca^{2+}-ionophore A23187 activated in cell-attached patches a linear 17 pS Cl^- channel in both N and CF cells. This channel inactivated upon excision. No relationship between the cell-attached 17 pS and the excised 32 pS channel could be demonstrated. Returning to the measurement of Cl^- transport at the macroscopic level, we found that a drastic rise in intracellular cAMP induced by forskolin did not result, in N as well as CF cells, in a change in the short-circuit current (I_{sc}) or the fractional efflux rates of $^{36}Cl^-$ and $^{125}I^-$. In contrast, addition of A23187 resulted in an increase of the I_{sc} and in the isotopic anion efflux rates in N and CF cells. We conclude that Cl^--transport in cultured human keratinocytes can be activated by Ca^{2+}-but not by cAMP-dependent pathways.

INTRODUCTION.

In this study the membrane permeability for Cl^- of normal (N) and cystic fibrosis (CF) keratinocytes in culture was evaluated by Ussing-chamber-, patch-clamp- and isotopic efflux measurements. At present little is known about the electrophysiological response of cultured keratinocytes to neuro hormonal agonists. Recent immunological studies suggested that the CF-encoded protein, CFTR [1], is expressed at moderate levels in

cultured keratinocytes [2]. Therefore, as the keratinocytes are readily available, these cells in culture were considered as an interesting new model to study the possible expression of the chloride permeability defect that characterizes CF epithelial cells from distal airways [3], sweat duct [4, 5, 6], small intestine [7, 8] and pancreas [9].

Electrogenic Cl^--secretion in the keratinocyte appeared to be activated in response to Ca^{2+}-ionophore A23187 but not to ß-adrenergic stimuli or the cAMP agonist forskolin. Surprisingly, in excised patches but not in cell-attached patches of N and CF keratinocytes an outwardly rectifying Cl^--channel with 32 pS conductance was identified. A channel with similar biophysical properties has been shown following the exposure of airway cells to ß-adrenergic stimuli in cell-attached patches [10, 11]. In excised membrane patches of a variety of (non)-epithelial cells this channel was reported to be activated by purified catalytic subunit of protein kinase A (PK-A) or PK-C and ATP at physiological membrane potential [11-15]. In contrast, the outward rectifyer in the keratinocyte membrane could neither be activated by cAMP in intact cells nor by PK-A or PK-C in excised patches. We identified a linear 17 pS Cl^--channel which, as suggested by isotopic efflux- and Ussing chamber measurements, may be at least in part responsible for Ca^{2+}-mediated Cl^--current.

MATERIAL AND METHODS.

Cell culture.

Skin preparations, stripped of connective tissue, were chopped and incubated under sterile conditions in Dulbecco's modified Eagle's medium (DMEM) with the following additions; fetal calf serum (5%), penicillin (10^5 IU), streptomycin (0.1 g/l), epidermal growth factor (12.5 μg/l) and hydrocortisone (0.5 mg/ml). The skin pieces were maintained in 25 cm^2 plastic (Falcon, Heidelberg). After 24 hrs, the proliferating keratinocytes were co-cultured with irradiated fibroblasts feeder cells [16] to suppress fibroblast outgrowth. Before passaging the keratinocytes the feeder cells were eliminated with trypsin (2.5 g/l) in phosphate-buffered saline. The keratinocytes were harvested in phosphate buffer containing 1 mmol/l EDTA and 2.5 g/l trypsin. After washing, the cells were either grown on 0.4 μm mesh Costar (Cambridge) filters for Ussing chamber measurements or on petri-dishes (Falcon) for patch-clamp analysis. The filters were

confluent within 10-12 days, indicated by steady state transepithelial potential and resistance. The cells could be distinguished from sweat gland coil or duct cells, nasal polyp cells and fibroblasts on the basis of keratin content and pattern [17, 18]. The keratinocytes could be maintained in culture for several passages. We used cells of primary outgrowth, or cells up to passage number 4, 24 hrs or later after seeding.

Patch clamp experiments.

Heat polished pipettes (Corning, GC150TF-15) with 4-10 Mohm input resistance (List EPC-7) were used to monitor single channel current (filter frequency 500 Hz) in inside-out patches of cells in the centre of confluent cell layers. Successfull seals (2-10 Gohm) were obtained with approximately 80% of the pipettes. The patch-pipettes were filled with KCl solution (Table 1) and amiloride (5.10^{-5} M). Low Ca^{2+} solutions were prepared by replacing NaCl for KCl in the NaCl solution (Table 1) and by adding 5 mM EGTA according to a method described by van Heeschwijk et al [19]. Experiments were performed at room temperature. Clamp potential (V_c) is given with respect to the potential of the pipette (exterior) solution. Positive (upward) currents denote Cl^- ions flowing out of the pipette into the bath. To study the effect of protein kinases and trypsin on channel activity we developed the following protocol; patches were excised at physiological membrane potential (V_c=-40mV) and transferred within two minutes to a solution exchange compartment (Fig.1). After solution replacement, patch activity at physiological holding potential (V_c=-40mV) was studied for 10 min. Hereafter the membrane was depolarized to V_c=70mV and patch activity was studied during a second 10 min interval. By use of this protocol we obtained information about i) spontaneous activation of channels in the excised patch at physiological membrane potential (i.e. appr.-40 mV); ii) activation of channels by protein kinases and ATP at V_c=-40mV and iii) voltage activation of channels at V_c=70mV that were not activated previously at physiological membrane potential.

Ussing-chamber experiments.

The filters and support were mounted in an Ussing-chamber (37°C) to monitor short circuit current (I_{sc}) as described previously [18].

Table 1.

Composition of Ringer's solutions (mmol/l).

	Na^+	K^+	Mg^+	Ca^{2+}	Cl^-	Glu^-	F^-	Br^-	I^-	NO_3	HEPES
NaCl	140	5	1	1.5	150	-	-	-	-	-	5
KCl	95	50	1	1.5	150	-	-	-	-	-	5
NaGlu	140	5	1	1.5	10	140	-	-	-	-	5
NaF	140	5	1	1.5	10	-	140	-	-	-	5
NaBr	140	5	1	1.5	10	-	-	140	-	-	5
NaI	140	5	1	1.5	10	-	-	-	140	-	5
$NaNO_3$	140	5	1	1.5	10	-	-	-	-	140	5

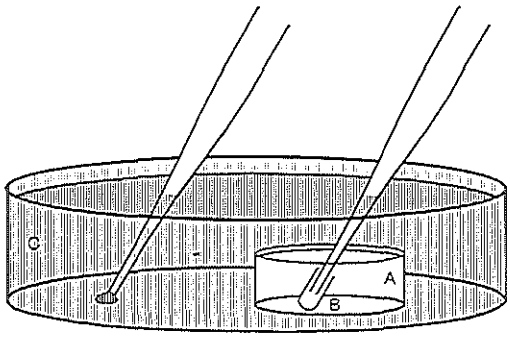

Figure 1.

Solution exchange compartment (A) positioned in Petri dish (C) in which cells are grown. This compartment consists of a glass ring sealed off at the bottom with a glas cover slip. In the exchange compartment a glass capillary (B, inside diameter 0.3 mm) kept open at one end is glued to the bottom. After patch excision the pipette tip is positioned in the glass capillary.The fluid in the compartment is replaced by syringe suction and filling. The estimated dead volume in the device is < 4% due to dead space within the capillary and droplets sticking to the tip of the electrode salt bridge which is lodged in the compartment during the experiments. In our set-up exposing the patch to a fresh solution by raising the pipette is accomplished within 2 seconds.

Isotopic anion efflux studies.

The method to study the isotopic effluxes of $^{125}I^-$ and $^{36}Cl^-$ is a slight modification of the technique used by Venglarik et al. 1990 [20] as described elswhere [21]. In short, cells, grown to 70-80 % confluence in 6 well culture plates (34 mm ϕ; Nunc), were loaded for 90 min with $^{125}I^-$(3 μCi/ml) or $^{36}Cl^-$(5 μCi/ml) in 1.5 ml modified Meyler's solution at 37°C. After washing the cells, the basal rate of isotopic efflux was measured by replacing the supernatant by fresh solution at 1-2 min intervals and by liquid scintillation counting. At t= 4.5 min the solutions were replaced by a solution containing Ca^{2+}-ionophore or forskolin. Residual isotope was determined by adding 1 ml NaOH (1 mol/l). Data are expressed as fractional efflux/min.

cAMP Determination.

The accumulation of cAMP in reponse to forskolin was assessed as described previously [18].

Chemicals.

The chemicals used were applied in the following concentrations (mol/l); amiloride (5.10^{-5}), Ca^{2+}-ionophore A23187 (3.10^{-6}), forskolin (10^{-5}), indomethacin (10^{-5}),4-acetamido-4'-isothiocyanostilbene-2,4-beta-phorbol-12-beta-myristate-13-alpha-acetate (PMA), 51B (10^{-4}) , 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB, 10^{-6} M, 10^{-5} M, 10^{-4} M) (51B and NPPB were kindly provided by Prof R.Greger, Freiburg), 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS, 10^{-6} M, 10^{-5} M, 10^{-4} M). Homogenous preparations of purified rat lung PK-A (kindly provided by Dr U.Walter, University Clinic, Würzburg) and PK-C (kindly provided by Dr. P.Parker, U.C.R.F. London) were applied in concentrations of 200 and 160 nmol/l, respectively.

Statistics.

Results are presented as mean ± SEM. Statistical significance was evaluated using paired- or unpaired Student's t-tests with $p < 0.05$ considered as significant.

RESULTS.

Properties of the 32 pS Cl^--channel in excised patches of the keratinocyte membrane.

In excised patches a 31.7±1.2 pS (n=20) channel rectifying for outward current (Fig.2) was observed in 63% (512 in 809) succesful seals of N and 67% (315 in 467) of CF cells. The channel activated after prolonged depolarization at V_c=70 mV (see above). The relative anion permeability (p_x/p_{cl^-}; ref. 22) of the channel was $1.6NO3^- \geq 1.6I^- > 1.3Br^- > 1.0Cl^- > 0.6F^-$ (N; n=5). The 32 pS Cl^--channel appeared to be impermeable for large anions like gluconate (not shown) or for cations like K^+, Na^+ or Cs^+. With a cation gradient across the patch in the presence of symmetrical 150 mmol/l Cl^- ions, the current reversal potential and the channel conductance were unaffected (result not shown).

The open probability was in about 50% voltage dependent, ranging from about 0.2 at -70 mV to about 0.8 at 70 mV, but voltage independent in the other half of the channels (about 0.8 from -70 mV to 70 mV).

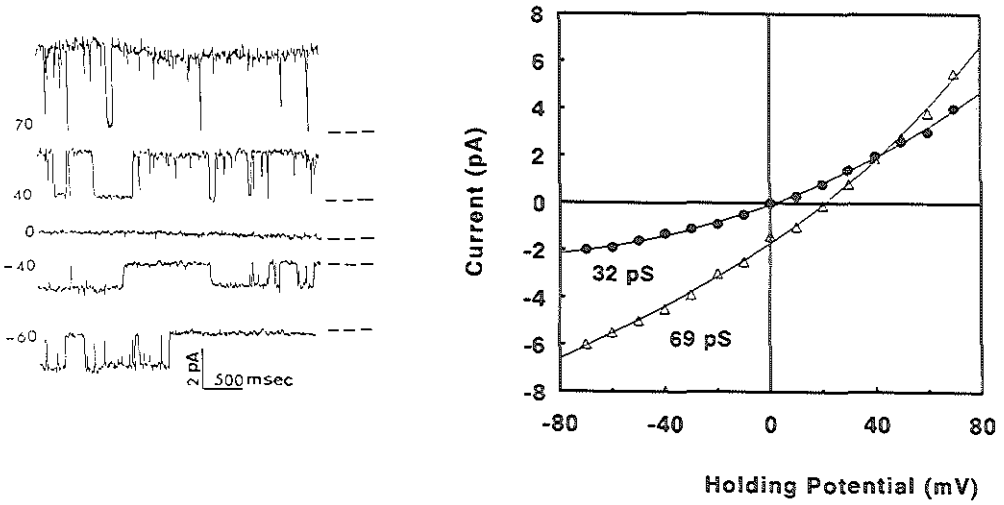

Figure 2.

Activity (left panel) and current to voltage relationship (I-V, right panel) of a 32 pS Cl^--channel rectifying for outward currents observed in excised patches of N and CF keratinocytes. Dashed line: closed state of the channel.●-●in symmetrical 150 mM NaCl solution,▲-▲with 450 mM NaCl in the bath solution.

The effects of the chloride channel blockers NPPB and DIDS were assessed by addition to the cytosolic face of the excised membrane. The 32 pS Cl-channel was reversibly inhibited by both blockers. NPPB and DIDS affected the open probability of the channel by inducing flickering. The NPPB and DIDS concentration with half maximal effect on the channel open probability were 9.10^{-6} mol/l and 5.10^{-6} mol/l respectively (M. Kansen et al., in preparation).

Spontaneous activation of the channel at physiological holding potential (V_c=-40mV).

In dog and N and CF human tracheal epithelial cells the outwardly rectifying Cl^--channel i) remained closed in excised membrane patches clamped at physiological holding potential [14, 15] and ii) is activated by applying large depolarizing potentials (V_c>50 mV). This finding offered a suitable experimental condition to study the activation of the channel by regulatory enzymes, like PK-A and PK-C, added to the solution of the excised patch. In setting-up conditions to study

phosphorylation-induced activation of the keratinocyte outwardly rectifying Cl^--channel at V_c=-40mV, we found that within a time span of 10 min upon excision about 30% of the Cl^--channels became spontaneously activated if the internal Ca^{2+} was raised above 10^{-7} mol/l (Table 2). With 10^{-8} mol/l Ca^{2+} in the internal solution, spontaneous activation of the channel was prevented (Table 2). These results suggested that the 32 pS channel activation in keratinocyte membranes is susceptible to alterations in internal Ca^{2+}, although the molecular mechanism involved is as yet unclear.

Activation of the outwardly rectifying channel by protein kinase A (PK-A), protein kinase C (PK-C) and trypsin.

The assays i) to activate the channel at physiological holding potential with PK-A or PK-C in the presence of 10^{-8} mmol/l Ca^{2+} and 1 mol/l ATP and PMA ii) to inactivate the channel by PK-C in the presence of 10^{-6} mmol/l Ca^{2+} and 1 mol/l ATP, as described previously [14, 15], were not successful when applied to the channel of the keratinocyte (Table 3). However, activation of the 32 pS channel was successful within one minute after exposing the internal site of the membrane to trypsin (0.05 %; Table 2). A similar activation of the outward rectifyer by trypsin has been reported earlier in excised patches from N and CF human airway cells [11], the channel was activated within one min after addition of trypsin.

Channel activity in cell-attached patches, effects of agonists.

In 7% of the N (5 in 57 seals) and 6% of CF cell-attached patches (5 in 85 seals) of resting cells a 17.1±3.6 pS (n=5) channel was identified with linear I-V characteristic (Fig.3). When present, the activity of this channel was apparent immediately after seal formation. At depolarized membrane potential the channel remained closed for long periods. The channel activity disappeared upon excision of the membrane patch. The reversal potential of cell-attached channel current was 15.0 ± 3.6mV (n=5). Since changing of the K^+-concentration in the patch-pipette did not result in the appearance of other channels, indicating that the channel is Cl^--selective. From the reversal potential it was calculated that in the cell Cl^--ions are accumulated approximately 1.8 fold above electrochemical equilibrium (as calculated according to ref. 23).

Table 2.

Keratinocyte chloride channel activation in excised patches by ambient internal Ca^{2+}-concentration or trypsin at V_c=-40mV, or voltage at V_c=70 mV. Ca^{2+}- or trypsin activation of the channel was studied for 10 min at -40 mV, whereafter the patch as depolarized to 70 mV holding potential.

Bath	Bath Calcium (mol/l)	n[i]	-40mV	70mV
Sham	10^{-3}	15	5	10
	10^{-5}	9	3	6
	10^{-7}	12	3	9
	10^{-8}	32	0	32
Trypsin 0.05%	10^{-8}	9	9	0

[i]number of patches containing channel activity.

Table 3.

Activation of the 32 pS Cl^--channel in excised patches by protein kinases and voltage depolarization. n=number of patches with channel activity. The Ca^{2+} concentration in the bath was 10^{-8} mol/l (see Table 5).

	number of patches	-40mV	-70mV
Activation:			
PKA + ATP	15	0	15
PKC* + ATP + PMA	11	0	11

*Inactivation of previously voltage activated channels by PK-C and ATP in 10^{-6} mol/l Ca^{2+} was not successfull (n=14).

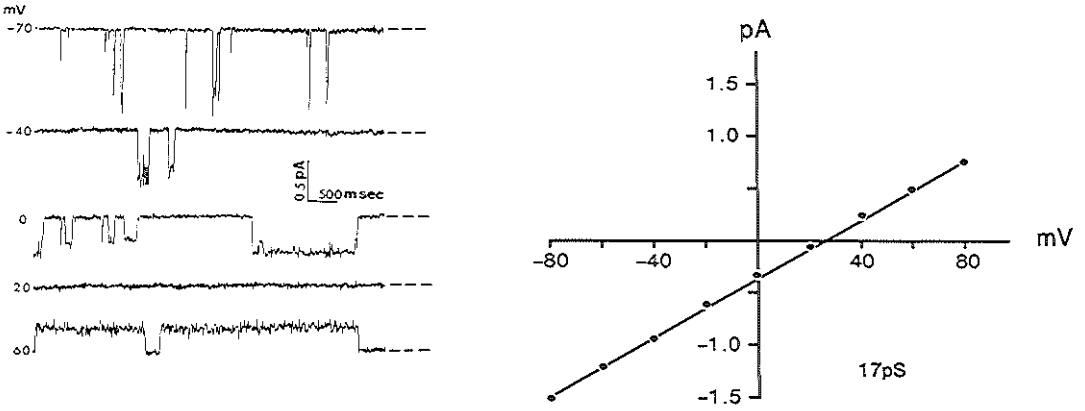

Figure 3.

Activity (left panel) and current to voltage relationship (I-V, right panel) of a 17 pS channel observed in N and CF cell-attached patches of keratinocytes exposed to A23187 (see Table 4). Channel activity disappeared upon excision of the patch. Dashed line: closed state of the channel.

In a second series of experiments we examined cell-attached activity (Table 4) in a 10 minute episode after preincubating the cells with foskolin or Ca^{2+}-ionophore. Addition of forskolin had no affect on cell-attached single channel activity and the channel incidence was about the same as in resting cells (appr. 12% and 7% respectively). However, we observed that in cell-attached patches of N cells pretreated with Ca^{2+}-ionophore the incidence of the 17 pS channel activity increased from aproximately 8% to 45% (9 in 20 patches). After excision the channel activity disappeared and the membrane patch was further analyzed for channel activity during a second period of 10 minutes at depolarized membrane potential (V_c=70mV). In 63% of these excised patches a 32 pS outwardly rectifying Cl-channel was apparent 0-8 min after depolarization independent of pre-stimulation with either forskolin (11 channels in 17 patches) or A23187 (14 channels in 20 patches; Table 4). The activity of the 32 pS outwardly rectifying Cl^- channel appeared 2.1±0.2 min (control; n=7), 2.3±0.4 min (forskolin; n=7) and 2.7±0.6 min (A23187; n=6) after the onset of depolarization for N and CF cells.

Table 4.

Number of cell-attached- and excised patches of N cells pre-treated with different agonists that contained 17- and/or 32 pS channel activity. Cell attached activity was monitored at -40 mV holding potential; thereafter channel activity was monitored for 10 min at 70 mV (see text).

	control	Forskolin	A23187
Cell-Attached Activity:			
17 pS channel[1]	5	2	9
32 pS channel	0	0	0
Excised Activivity:			
17 pS channel	0	0	0
32 pS channel[2]	34[3]	11[4]	14[5]
Total # of patches examined	57	17	20

[1] see fig. 3. [2] see fig. 5. One [3], zero [4] and five [5] of these patches contained previously cell-attached activity of the 17 pS channel.

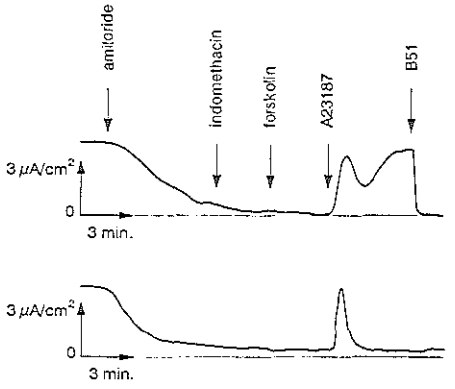

Figure 4.

Redraw of original recordings of Isc across N skin cells mounted in the Ussing chamber in chloride- (upper tracing) or gluconate containing solution (lower tracing). Similar responses were obtained for CF cells (not shown). Concentrations (mol/l): Amiloride (5.10^{-5}), indomethacin (10^{-5}), A23187 (3.10^{-6}), forskolin (10^{-5}), B51 (10^{-4}).

Ussing-chamber measurements and isotopic efflux studies.

The I_{sc} responses of filter-grown monolayers of keratinocytes are summarized in Table 5. A representative recording of the I_{sc} and the effect of agonists is given in Fig.4. The effects of secretagogues on I_{sc} were studied in the presence of amiloride and indomethacin. Mucosal amiloride reduced basal I_{sc} by 40-50% in N and CF cells, indicating the presence of an amiloride sensitive Na^+-conductance in the apical membrane of the cells (Table 5). The effect of indomethacin, an inhibitor of endogenous prostaglandin synthesis on I_{sc} was not significantly different between N and CF cells. The effect of secretagogues that raise intracellular Ca^{2+} or cAMP levels was studied by adding Ca^{2+}-ionophore A23187 and the adenylate cyclase activator forskolin, respectively. Forskolin did not affect basal I_{sc} (Table 5) although intracellular cAMP concentration showed a 50 fold increase, from 0.45±0.25 pmol cAMP/mg protein (n=2) to 28.3±3.5 pmol cAMP/mg protein after simulation with forskolin.

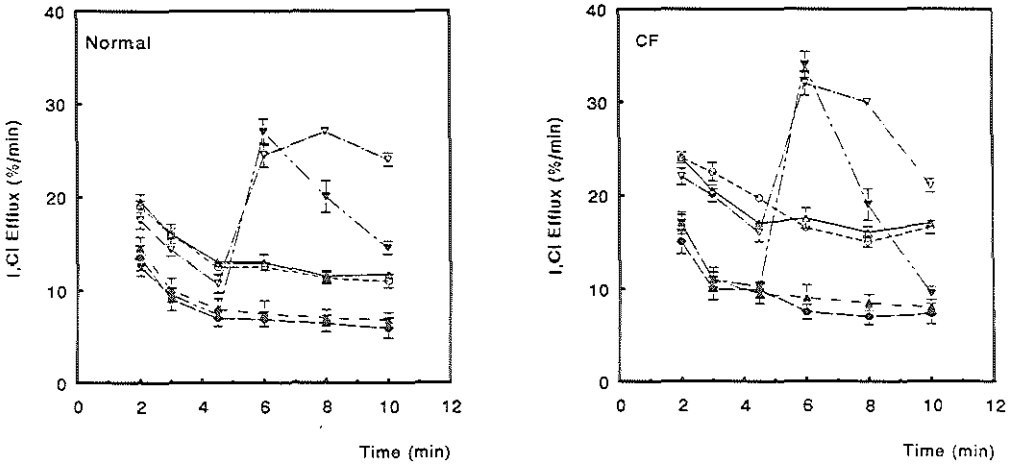

Figure 5.

$^{125}I^-$- (closed symbols) and $^{36}Cl^-$- (open symbols) efflux of N (left panel) and CF (right panel) keratinocytes in response to forskolin or A23187. Agonists and vehicle (0.1% ethanol) or vehicle alone (control) were added at t=4.5 min. The N (n=3) and CF (n=3) cells responded similarly to A23187. A forskolin response was absent in N and CF cells. -◆- $^{36}Cl^-$ Control , -O- $^{36}Cl^-$ Forskolin, -▽- $^{36}Cl^-$ A23187, -▲- $^{125}I^-$ Control, -◇- $^{125}I^-$ Forskolin, -▼- $^{125}I^-$ A23187.

Table 5.

I_{sc} ($\mu A/cm^2$) and change in I_{sc} (ΔI_{sc}) in response to agonists of N and CF keratinocytes mounted in the Ussing-chamber and exposed to NaCl or Na Gluconate (NaGlu) Ringer's solution. The cells were obtained from 7 N individuals and 7 CF patients. n=number of observations*.

	NaCl		NaGlu	
	N	CF	N	CF
n	21	23	8	5
I_{sc} (resting)	+1.23±0.37	+0.90±0.23	+1.44±0.49	+1.25±0.33
ΔI_{sc}				
Amiloride	-0.51±0.19	-0.51±0.11	-0.24±0.26	-0.77±0.24
Indomethacin	-0.15±0.09	-0.15±0.06	-0.16±0.09	-0.13±0.17
Forskolin	0.0	0.0	0.0	0.0
A23187[1]	+1.22±0.15	+0.70±0.23	+1.35±0.21	+1.22±0.26
A23187[2]	+1.32±0.17	+0.85±0.19	0.0	0.0

* The responses of amiloride and the A23187 (except the sustained A23187 response in NaGlu) were significantly different from the resting values. [1] peak response, [2] sustained response (see Fig.5).

The addition of Ca^{2+}-ionophore resulted in a sustained increase in I_{sc} which was not significantly different between N and CF cells (Table 5; Fig.5). The sustained A23187-provoked I_{sc} response was abolished by the Cl^--channel blocker 51B (Fig.5), and was inhibited in part following replacement of Cl^- in the bathing fluids by gluconate (Fig.5, Table 5: sustained response). The rapid and transient response observed in the gluconate solution is most probably due to the opening of K^+-channels. The Ca^{2+}-induced Cl^--permeability of the cell membrane was further assessed by measuring the $^{125}I^-$- and $^{36}Cl^-$-efflux rates in isotope-loaded N and CF keratinocytes (Fig.5). Addition of A23187 resulted in an acceleration of

$^{125}I^-$- or $^{36}Cl^-$-efflux in both N and CF cells. In contrast, the efflux of $^{125}I^-$ or $^{36}Cl^-$ was not affected by forskolin in N and CF cells. The estimated efflux ratio Cl^-/I^- was 0.7 in N and CF cells stimulated with A23187. These values agree well with resting and Ca^{2+}-provoked Cl^-/I^- efflux and anion channel selectivity ratio's of HT-29cl.19a- and T_{84} colonocytes [20, 21, 24].

Our observations suggest that at least part of the Ca^{2+}-mediated I_{sc}-increase in the Ussing-chamber experiments can be ascribed to activation of a Cl^--permeability. Also, the absence of a forskolin induced $^{125}I^-$- or $^{36}Cl^-$-efflux strongly suggest that cAMP-triggered electrogenic Cl^--secretion is absent in the keratinocyte.

DISCUSSION.

In this study we have evaluated the agonist dependency of Cl^--secretion in N and CF cultured human keratinocytes by three different methods, i.e. Ussing chamber, patch clamp- and isotope efflux measurements. The results of the three methods are in agreement and suggest that in both N and CF cells Cl^--secretion is stimulated by elevation of intracellular Ca^{2+} (Fig. 4, 5; Table 4, 5), whereas in both cell types Cl^--secretion in response to elevation of intracellular cAMP levels by forskolin is absent (Fig. 4, 5). The presence of ß-adrenergic control of Cl^--secretion is common to most exocrine epithelial cells, while lack of ß-adrenergic controlled Cl^--secretion is a common characteristic of CF exocrine epithelial cells. It has been reported that a 30-40 pS outwardly rectifying chloride channel in N but not in CF epithelial cells could be activated in excised patches by PKA [4, 12, 14]. Therefore we thought to bypass macroscopic Cl^- current measurements and start directly to activate this channel in N and CF keratinocytes by phosphorylation. Since this activation in N keratinocytes was unsucessfull, we were obliged to investigate the stimulation of Cl^- transport at the level of Ussing chamber and isotopic anion efflux measurements.

The I_{sc} response of CF cells was similar to that of normal cells. In both cell types the overall I_{sc} response ($< 2\ \mu A/cm^2$) was rather low as compared to other epithelial cells [25]. This may originate from culturing conditions, a cell cycle dependency of Cl^- channel expression [26] or, in analogy to HT29.cl19A colon carcinoma cells [20, 21], from a symmetrical distribution of Cl^- conductive

pathways among the apical- and basolateral keratinocyte membrane. The reversal potential of the 17 pS channel (15 mV; Fig.3) is expected for a channel that is Cl^--selective and suggests that in the keratinocyte Cl^--ions are accumulated above electrochemical equilibrium like in other Cl^--secreting epithelial cells [27]. The increase in the incidence upon Ca^{2+} ionophore treatment (Table 4) suggests, that this 17 pS channel might carry at least part of the Ca^{2+}-ionophore provoked I_{sc} and $^{36}Cl^-$ or $^{125}I^-$ efflux observed in cultured N and CF keratinocytes. Interestingly, Ca^{2+}-ionophore provoked Cl^--secretion is likewise unmodified in CF sweat gland coil cells and pancreas carcinoma cells [28, 29], CF nasal polyp and tracheal cells [18, 30, 32] but apparently defective in CF intestine and synchronized lymphocytes [23, 24, 26].

The 17 pS Cl^- channel was observed only in cell-attached patches, the 32 pS Cl^--channel only in excised patches. They are however not related. The number of 32 pS Cl^--channels in excised patches was similar in previously cell-attached patches of cells stimulated with Ca^{2+}-ionophore that contained 17 pS channel activity (65%, Table 4) as compared to non prestimulated cells (63%).

The absence of cAMP-triggered Cl^--secretion in the keratinocyte is of particular interest because a similar 32 pS rectifying Cl^--channel, identified in excised membrane patches of other epithelial cells, has been reported to have altered ß-adrenergic regulatory property in CF [10, 12, 14, 22, 15]. In human airway epithelial cells and lymphoblasts, this 32 pS Cl^--channel has been reported to be activated a) in cell-attached patches in response to ß-adrenergic agonists and 8-Br-cAMP and b) in excised membrane patches held at physiological membrane potentials by the addition of ATP plus PK-A and PK-C [11, 12, 14, 15]. In spite of extensive efforts, these agents failed to induce activation of the Cl^- channel under similar conditions in cultured human keratinocytes (Table 3). Nevertheless the channel characteristics and sensitivity to blocking agents are indistinguishable to the outwardly rectifying Cl^- channel which is expressed in many (non)-epithelial cells [10, 11, 13, 22, 32, 33, 34]. The apparent insensitivity of the keratinocyte 32 pS channel to phosphorylating enzymes may suggest that an unidentified regulatory component associated with the membrane, conferring protein kinase sensitivity to the Cl^--channel, is missing in this cell type. However, the importance of the outwardly rectifying channel in Cl^--secretion may be overestimated since a number of studies failed to detect a significant level of cell-attached activity of

the 32 pS Cl^--channel in cultured pancreatic duct cells [33], Necturus choroid plexus [32], colon carcinoma T84 cells [35], distal nephron [36] and thyroid cells [37]. In these cells cAMP/PKA activated Cl^--channel activity is non-voltage rectifying with approximately 5 pS channel conductance while activity of the 32 pS Cl^--channel in the cell-attached configuration is sporadically observed. Very recently this small Cl^--channel was shown to be present after cAMP stimulation in an insect cell line infected with a viral vector expressing CFTR [38].

The physiological role of the excised 32 pS rectifying channel is yet unclear. In a preliminary report it is suggested that the channel might be active in volume regulation [39] which may explain the finding that the channel is observed in many non-epithelial cells.

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CHAPTER IV.

IMMORTALIZATION OF NASAL POLYP EPITHELIAL CELLS FROM CYSTIC FIBROSIS PATIENTS.

ABSTRACT.

We have developed immortalized epithelial Cystic Fibrosis (CF) cell lines by infecting cultured nasal polyp cells with a SV40/Adeno12 hybrid virus. The cell lines obtained are epithelial in nature as shown by cytokeratin production and morphology, though cytokeratins 4 and 13 typical of primary nasal polyp cells are produced at a much reduced rate. Ussing chamber experiments showed that the pre-crisis CF cell line NCF3 was able to perform trans-cellular chloride transport when activated by agents which elevate intracellular calcium. cAMP agonists had no effect on chloride flux in NCF3 as expected for CF cells. The apical chloride channels found with the patch clamp technique in NCF3 and in the post-crisis cell line NCF3A have a conductance similar to chloride channels found earlier in normal and CF epithelial cells. The channels show a delay in the onset of activity in off-cell patches and are not activated by increased cAMP levels in the cell. This indicates that immortalized CF epithelial cells will provide a useful model for the study of Cystic Fibrosis.

INTRODUCTION.

Cystic fibrosis is a lethal inherited disease with a high incidence in the caucasian population. The primary defect of the disease is thought to be a defective regulation of apical chloride channels in epithelial cells [1,2,3,].The molecular basis of the disease has not been explained, however. It is clear from studies performed sofar that the chloride channels are regulated in a complex way [3,4,5,6,7,]. Which of the components involved is mutated in cystic fibrosis is unknown. Current research is aimed mainly at solving this problem.

The limited availability of suitable cell material presents a problem in cystic fibrosis research. Epithelial cells appear to be the material of choice and, indeed, cultured airway and sweat gland cells are frequently used [4,5,6,7,8,9,]. However, this material is available in small quantity only, has a limited proliferative capacity

in culture, and is often heterogeneous. Therefore, we felt the need to develop a continuously growing epithelial cell-line with CF genotype and phenotype. As spontaneously transformed epithelial cell lines from cystic fibrosis patients are not available we chose to use a protocol developed recently for immortalization of human epithelial cells using a hybrid SV40/Adeno12 virus [10,11]. In the present paper we describe the properties of cell lines obtained after infection of nasal polyp cells from a cystic fibrosis patient.

The widely accepted criterium for the CF phenotype is the absence of cAMP dependent activation of apical chloride channels. Electro-physiological characterisation of the immortalized CF cells suggests that they can be used as a model system of cystic fibrosis in future experiments.

MATERIALS AND METHODS.

Cell culture and isolation of clones.

Nasal epithelial cells were obtained from surgery. Epithelial cells were scraped off nasal polyps after pronase treatment (1 hr. 37 °C, 2.5 mg/ml pronase in RPMI medium plus 1% Fetal Calf Serum). The cells were seeded on feeder cells (3T3 J2, provided by Dr. Howard Green), irradiated with 30 Gy. The medium used was a mixture of DMEM and Ham's F12 (1:1) supplemented with 1% (V/V) Fetal Calf Serum, Epidermal Growth Factor 10 ng/ml, hydrocortisone 0.1 μg/ml, insulin 10 μg/ml, transferrin 10 μg/ml, Na_2SeO_3 50 nM, glutamine 2.4 mM, penicillin 100 I.E.units/ml, and streptomycin 100 mg/l. Cells were harvested with trypsin (0.05 % W/V) plus EDTA (0.02 % W/V) in NaCl 145 mM, 5 mM K_3P0_4 pH 7.2. Under these conditions the cells could be maintained for four to five passages (1:4) after which proliferation stopped. Approximately five million epithelial cells, passage three, from a CF patient were infected with a SV40/Adeno12 hybrid virus [10,11] at a multiplicity of 1:100. Following infection the cells were passaged on fresh feeder two more times until growth of primary cells stopped. Several weeks later 50-100 colonies of fast growing cells appeared which were subcloned by trypsinization from a cloning ring. The CF patient was a six year old boy with a clinical history of malabsorption and obstruction of upper airways. Repeated sweat tests confirmed the diagnosis of cystic fibrosis (sodium concentrations found in sweat were 93, 110 and 80 mmol/l). Pre-crisis cell lines like NCF3 were cultured on feeder as described. Postcrisis cell lines

were grown on either collagen or plastic. Collagen (human placenta Sigma type VI, calf skin Sigma type III) when used was applied to the surface of plastic culture dishes as an acid solution (3 mg/ml, 50 $\mu g/cm^2$) and dried. Virus infected cells were cultured in a laboratory equipped for virological and molecular biological techniques (CII level).

Cytokeratin pattern analysis.

Cytoskeletal proteins were isolated by suspending epithelial cells in a buffer containing Triton X-100 0.5%, KCl 1.5 M, Tris/HCl 10 mM, EDTA 5 mM pH 7.2, followed by centrifugation in an Eppendorf centrifuge [12]. Samples were analysed by SDS polyacrylamide gelelectrophoresis [13]. Proteins were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose paper electrophoretically. Immunoblotting was performed essentially as described by Towbin et al [14]. The monoclonal antibodies (kindly provided by Dr. Van Muijen, Leiden university, The Netherlands) used were 2D7 which binds to cytokeratin 13 (Mr 54000, pI 5.1), and 6B10 which binds to cytokeratin 4 (Mr 59000, pI 7.3) [15]. A polyclonal rabbit antikeratin serum was obtained from Eurodiagnostics (Holland). Bands on the immunoblots were visualised with alkaline phosphatase conjugated antibodies (Tago Inc., Burlinggame, Ca., USA)

Immunofluorescence.

Cells were seeded on glass cover slips, incubated overnight at 37° C, and fixed with methanol and acetone. Fixed cells were incubated with a monoconal antibody against SV40 large T antigen (L19 obtained from Dr. L. Crawford [16]) followed by a FITC labeled anti-mouse immunoglobulin serum. Cells were inspected with a fluorescence microscope equipped with a camera.

Electronmicroscopy.

Cells were grown on plastic dishes coated with collagen type IV (Sigma) and prepared for electronmicroscopy as described in[17]. Ultrathin sections (60 nm) were cut on a LKB III microtome and stained with uranyl acetate in water and lead citrate. The sections were photographed in a Phillips EM 400 microscope.

Ussing chamber experiments.

Cells were seeded at high density ($5.10^6/cm^2$) on uncoated 6.5 mm diameter polycarbonate membranes (Costar, Transwell (TM), 0.4 [m pore size). After four to nine days membranes were tested for electrical resistance and hormonal response in an Ussing chamber, essentially as described by Dharmsathaphorn et al [18]. Briefly, the membrane was mounted between two compartments so that both the potential difference and the voltage clamp current could be measured with two sets of platinum and calomel electrodes. Electrical resistance of the cell layer was calculated from the potential shift induced by a standard current pulse corrected for resistance of buffer and membrane. The two compartments were filled with Standard Ringer's solution: NaCl 140 mM, KCl 5 mM, HEPES 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.5 mM, glucose 1.5 mM, pH 7.4. The compartments were continuously stirred and saturated with oxygen gas, at 37 °C.

Patch clamp analysis.

For patch-clamp analysis the cells grown on plastic dishes were incubated at 25 °C in a Ringer's solution as described above. The pipette solution was identical to the bath, pipette resistance was 4 Mohm. Pipette current and potential were measured with a List EPC-7 device and stored digitally on a videorecorder for subsequent analysis [19]. After patch formation at least 4-6 min. of on-cell recording was performed before excision. Dibutyrilic-cyclic AMP (1 mM final concentration) or isoproterenol (0.01 mM final concentration) was added to the bath in 0.2 ml Ringer's solution either before or during on-cell recording. After excision the patch current was monitored at least 10 minutes at +70 mV (voltage with reference to pipette voltage). When a channel was activated the conductance and the chloride specificity of the channel was determined by clamp voltage stepping and changing the composition of the bath solution.

cAMP levels.

NCF3 or NCF3A cells were seeded at 70 % confluency on 35 mm petri dishes and incubated overnight. After 10 minutes preincubation in Ringer's solution at 25 °C isoproterenol in DMSO (final concentrations 0.01 mM and 0.1 % respectively) forskolin (final concentration 0.01 mM) or DMSO (final concentration 0.1%) was added. Cells were washed in Ringer's solution and

harvested in 5 % TCA. The pellet was used for a protein determination according to Lowry. The supernatant was extracted with ether to remove the TCA and cAMP was determined with a competetive binding assay kit (Amersham).

RESULTS.

Epithelial cells from nasal polyps were collected and cultured as described. Under these conditions we can culture nasal polyp cells for up to five passages. The feeder cells improve growth of epithelial cells and suppress outgrowth of fibroblasts [20].

Approximately 5 million nasal polyp cells from a normal individual and from a CF patient were infected with a hybrid SV40/Adeno12 virus at a multiplicity of 1:100. After infection the cells were passaged on feeder twice, until the epithelial cells stopped multiplying. Four to six weeks later colonies of cells with epithelial morphology, i.e. a typical cobblestone cell pattern, appeared (fig.1). Six clones from infected normal cells were subcultured and four of these showed high cloning efficiency and growth rate at first but were lost after a crisis at passage four. Of the fifteen clones from infected CF cells eight showed a cloning efficiency of over 50 % and a doubling time of approximately 48 hours. The morphology of the clones was epithelial in all cases (see fig 1) and resembled the original uninfected cells. One of these clones (NCF3) will be described in detail here. NCF3 grew at an apparently constant rate for more than fifteen passages without an obvious crisis. Proliferation appeared to be strongly dependent on a suitable extracellular matrix. NCF3 did not grow on plastic but several collagen preparations resulted in a doubling time of approximately 48 hrs (methods). In our hands optimal results were obtained when feeder cells were used.

About twenty passages after infection the cell line NCF3 went into crisis after which a new cell population, indicated as NCF3A, emerged. This cell line had properties similar to but different from the original clone. NCF3A did grow on uncoated dishes (doubling time 48 hrs). The morphology of NCF3A was similar to the primary cells (fig 1) but growing clones were much less flattened as the cells tended to heap up when reaching confluence. The Class I HLA antigens produced by NCF3A were identical to those produced by NCF3, as shown by isoelectric focusing followed by Western blotting with a serum which binds to all members of this class [21] (not shown). This strongly supports the common

genetic origin of NCF3 and NCF3A.

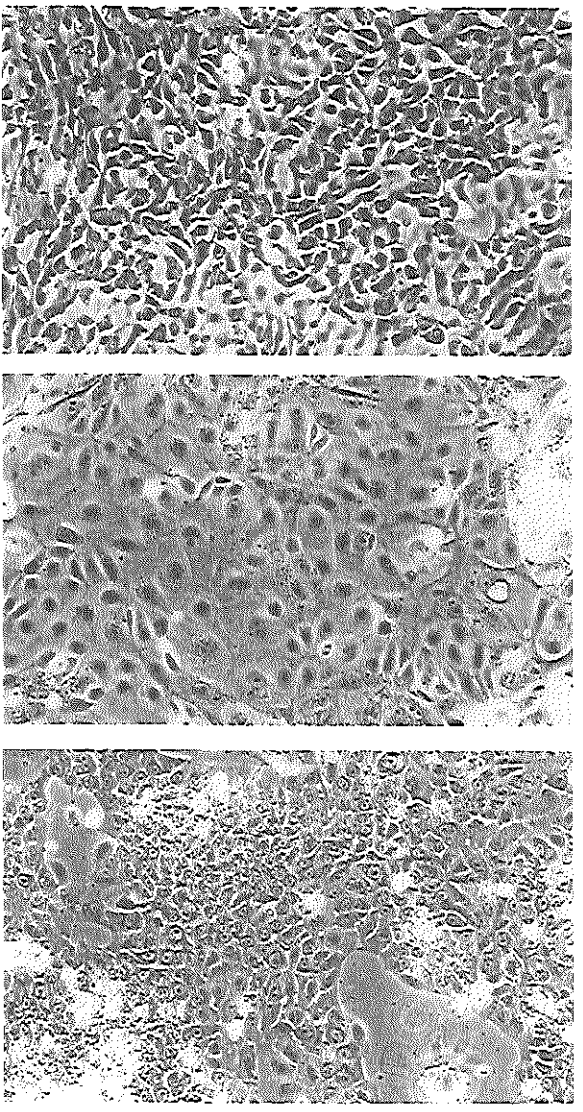

Figure 1.

Light microscopic pictures of unstained unfixed cells. A: CF primary nasal polyp cells in culture. B: NCF3 cells at passage 9. C: NCF3A cells 8 passages post crisis. In A and B cells were grown on irradiated feeder cells, in C cells were grown on on calf skin collagen (Sigma).

Both cell lines produced SV40 large T antigen as shown by immunofluorescence (fig.2), indicating efficient expression of viral DNA.

Electron microscopic analysis of NCF3 cells grown on collagen showed tight junctions and desmosomes as expected for epithelial cells (fig 3A). NCF3A cells lacked tight junctions in contrast to NCF3 cells (fig3B). Microvillar structures are visible on the apical membranes of both cell types but they appear to lack microfibrils (fig 3) .

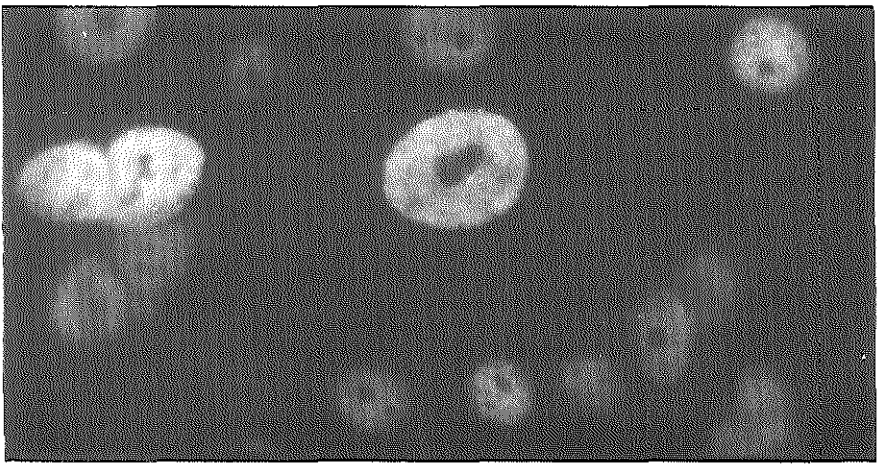

Figure 2.

Expression of large T antigen by NCF3 cells. Cells were prepared for immunofluorescence microscopy as described. The picture shows fluorescent nuclei of NCF3 cells incubated with an anti large T antiserum.

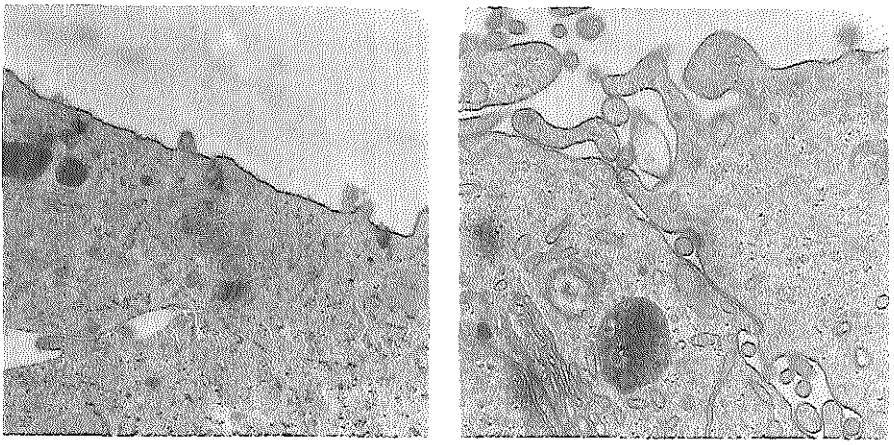

Figure 3.

Electron microscopic pictures of NCF3 and NCF3A cells. Cells were prepared for electronmicroscopy as described. Left: tight junction of NCF3 cells. Right: Cell-cell junction of NCF3A cells.(magnification on photoprints 53100x).

The karyotype of NCF3 at passage 11 obtained after Giemsa banding pattern analysis of spread chromosomes showed the cell line to be aneuploid male human (XXYY) with chromosome counts in the near tetraploid range. All normal chromosomes are represented, usually by three to four copies. Most cells contained some unassignable fragments of chromosomes. In contrast, cells of the NCF3A population were almost exclusively diploid, with all normal chromosomes represented. Band pattern analysis showed whole arm rearrangements resulting in a monosomy for 3p and 10q, a trisomy for 7p, 13q and 14q and a tetrasomy of 5p. Two out of seven cells showed additional changes (+13 -21, and -22 respectively).

An informative illustration of the state of differentiation of epithelial cells is the cytokeratin pattern [12,15,22]. We isolated cytoskeletal proteins from cultured nasal polyp cells and from the immortalized cell lines NCF3 and NCF3A. The protein patterns were analysed by SDS gelelectrophoresis and western blotting. The stained gels showed that NCF3 and NCF3A have a pattern similar to cultured nasal polyp cells but differences can be observed (fig.4A). Keratins k4 and k13 are produced by cultured nasal cells (fig 4B lanes 1-3) but not by other epithelial cells such as keratinocytes and cultured sweat gland cells [12,15,

Hoogeveen, unpublished results]. In contrast to uninfected nasal cells NCF3 and NCF3A produced no detectable keratin 13 and very small amounts of keratin 4 (fig 4B lanes 4-9).

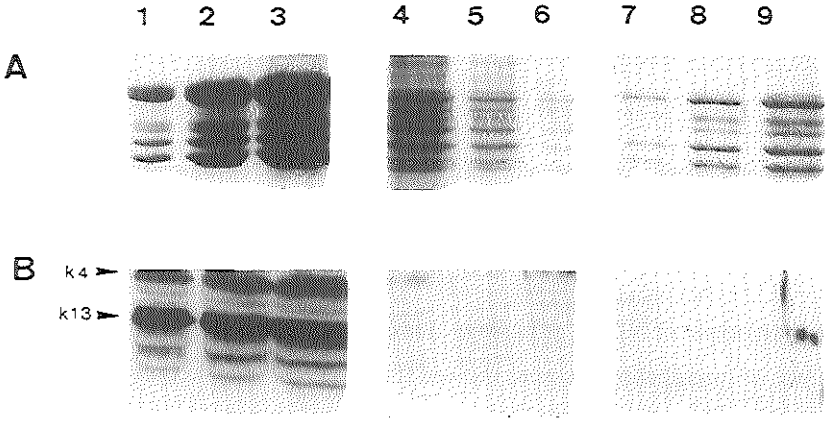

Figure 4.

Cytokeratins produced by primary and immortalized CF nasal polyp cells. Cytoskeletal proteins were prepared for SDS gelelectrophoresis and western blotting as described. The lanes of the Western blots contain the same amount of protein as the Coomassie stained lanes. A: Coomassie Brilliant Blue stain of cytoskeletal proteins B: western blot stained with monoclonal antibodies against keratins 4 and 13 (bands indicated in figure). Cytoskeletal protein samples used were 1,2,3: primary nasal CF polyp cells, 4,5,6,: NCF3 cells, 7,8,9: NCF3A cells.

NCF3 and NCF3A were cultured on permeable membranes, to study their electrophysiological properties. In the case of NCF3, confluent cell-layers were formed with a high electrical resistance (350 ± 100 Ohm.cm^2, N=20). As NCF3A was unable to form effective tight junctions (see fig 3) Ussing chamber experiments with these cells were impossible. Ion transport across the cells was monitored with an Ussing chamber as described in the methods section. Figure

5 shows a typical trace of short circuit current (Isc) versus time of NCF3 cells. The results of a series of experiments are summarised in Table 1. Positive values indicate either anion flow to the mucosal side or kation flow to the serosal side of the membrane.

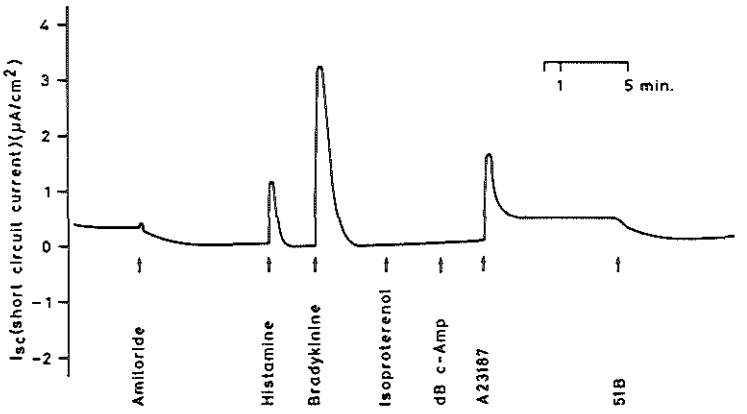

Figure 5.

Short Circuit Current (Isc) of NCF3 cells was measured in an Ussing chamber as described. The graph represents short circuit current versus time of a typical experiment. Experimental conditions were as in table 1.

Qualitatively the cells behave as expected for nasal epithelial cells with a cystic fibrosis genotype. The sodium channel inhibitor amiloride reduces the Isc to approximately zero values. This indicates the presence of apical sodium channels and the absence of active chloride current. In the presence of amiloride, transient increases of the Isc in response to the alpha-adrenergic agonists histamine and bradykinine were observed (fig. 5, table I). The changes in Isc correlated well with transient increases of intracellular calcium concentration as monitored by Indo-1-am fluorescence (data not shown). The chloride channel inhibitor 51B (kindly provided by Dr R. Greger, Freiburg, FRG) abolishes the histamine Isc response

and reduces the bradykinine response by 70% (table 1). The calcium ionophore A23187 causes a permanent increase of the Isc which is completely reversed by 51B (fig 5, table 1).

Table 1.

Effects of agonists and inhibitors on short circuit currents of NCF3 cells. Short circuit currents (Isc) were measured in an Ussing chamber as described. Averages of peak values (± S.D.) were used in the case of transient responses (see figure 5). Isc: $\mu A/cm^2$. mM: concentration in millimolar units, N: number of measurements per condition. *)The effects of all agonists were measured in the presence of 0.05 mM amiloride. +) The effect of 51B on the histamine response was tested on filters which did respond to bradykinine and vice versa. #) the effect of isoproterenol, forskolin and diBut.cAMP was tested on filters which showed a response to both histamine and bradykinine.

Addition	mM.	Isc	S.D.	N.
none		0.4	0.1	12
Amiloride	0.05	0.0	0.1	12
Histamine *)	0.05	2.0	0.3	9
Histamine				
plus 51B +)	0.02	0.0	0.1	2
Bradykinine	0.01	4.0	0.4	5
Bradykinine				
plus 51B +)	0.02	1.2	0.3	3
A23187	0.003	1.0	0.2	4
A23187				
plus 51B +)	0.02	0.0	0.1	4
Isoproterenol #)	0.01	0.0	0.1	5
diBut.cAMP #)	1.0	0.0	0.1	2
Forskolin #)	0.01	0.0	0.1	2

Addition of isoproterenol, forskolin or dibutiryl cAMP in the presence of amiloride had no effect on Isc (figure 5, table 1). Both NCF3 and NCF3A responded to forskolin and isoproterenol with a large increase of the intracellular cAMP levels (table 2). This shows that the absence of a response to these agonists

is not due to defective cAMP synthesis. Apparently, high intracellular calcium levels can trigger an increase of transcellular chloride transport in NCF3, whereas cAMP has no effect.

Table 2.

cAMP levels in NCF3 and NCF3A. cAMP levels were measured in NCF3 an NCF3A cells as described in the methods section and expressed as pmol/mg protein. Forskolin (0.01 mM) and Isoproterenol (0.01 mM) were added four minutes before harvesting the cells.

Agonist	NCF3	NCF3A
none	10	1
Forskolin	800	200
Isoproterenol	800	500

A more direct approach to measure chloride channel activity is the patch clamp technique. Here, microscopic patches of apical membrane of a cell are sealed to a glass-electrode after which single channel currents can be observed. Spontaneous chloride channel activity was not observed in either NCF3 or NCF3A while the patch was attached to the cell (on-cell recording). Excised patches were monitored for at least 10 minutes in standard Ringer's solution with 1.5 mM calcium at a clamp voltage of +70 mV (voltage relative to pipette, see methods section). In excised patches of unstimulated NCF3 (N=7) and NCF3A cells (N=12) chloride channels were observed only after on average 6 ± 2.5 minutes. The chloride channels observed in NCF3 and NCF3A had conductivity properties identical those of normal and CF human epithelial cells as described before [7,5,8]. The conductance of the chloride channel was 30 pS, it rectified for outward current (i.e. had a non-linear voltage to current relation) and responded to chloride gradients but not to sodium or potassium gradients with a change in current which identified it as a chloride channel (figure 6). In both NCF3 and NCF3A cells a chloride channel was found in about one out of ten patches, indicating a comparable rate of synthesis in both cell lines. In normal primary nasal cells one chloride channel per 6 excised patches was seen with an average

off cell opening time of 2.5 ± 2 minutes (N=13, M. Kansen unpublished results).

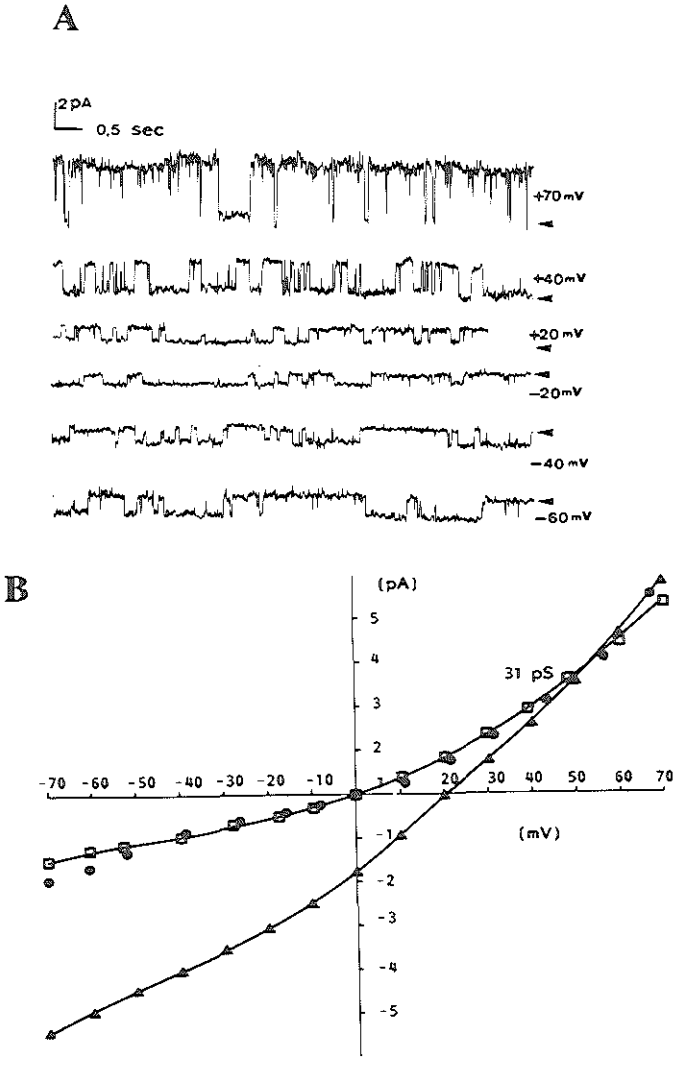

Figure 6.

Patch clamp analysis of chloride channels of NCF3 cells. NCF3 cells were seeded an cultured on plastic dishes for three days, patch clamp analysis was performed on off- cell patches of apical membranes, as described. A: A recording of pipette currents at different clamp potentials shows

characteristic changes from closed (arrows) to open state of the channel. B: Relationship of membrane clamp voltage and channel current of a typical chloride channel. The figure shows the channel current I (pA) as function of clamp voltage PD (mV). Δ -Δ standard Ringer's solution both in the pipette and bath. Nearly identical curves were obtained when the channel was exposed to tenfold sodium or potassium gradients (o-o).▲-▲ 150 mM NaCl in bath solution was replaced by 400 mM NaCl.

Activation of chloride channel activity by cAMP was not observed in NCF3A cells. Isoproterenol was added either during or before (5-60 minutes) a recording with the patch electrode attached to the cell. The patch was subsequently monitored for at least four minutes after which it was excised and monitored for ten more minutes. Chloride channels were observed only in excised patches after 6 ± 2.5 minutes at +70 mV (N=7 for addition during, N=3 for addition before on-cell recording). Also addition of dibutiryllic cAMP did not result in activation of chloride channels during on-cell recording (N=3). This strongly suggests that in NCF3A cells the CF phenotype, i.e. the abberant behaviour of chloride channels is retained.

DISCUSSION.

Human epithelial cells, including cells from nasal polyps, have a limited life span in culture. After a few passages, that is, after some ten to twenty cell divisions the cells enter a stage of terminal differentiation.

In the present study, nasal epithelial cells from cystic fibrosis patients were infected with a SV40/Adeno12 hybrid virus. The purpose of this experiment was to produce cell lines with an extended life span which can be used as a model to study the disease.

Immortalization of human epithelial cells by transforming viruses like SV40 and Adeno viruses has been described in literature [11,10,23]. Some infected cells obtain integrated copies of the viral genome expressing viral proteins including a DNA-binding factor called large T antigen, which is involved in the replication of the virus [23,24]. The T-antigen strongly promotes mitotic activity of the cells but generally does not induce a fully transformed phenotype, i.e. loss of contact inhibition, growth in soft agar and tumor growth in nude mice. Adeno viruses produce a protein of a similar nature [25]. Epithelial cells which express SV40

T-antigen have an extended but usually not unlimited lifespan. This results in the common observation of the "crisis" which signifies massive cell death sometimes followed by the appearance of one or several subclones with improved growth properties due to the activation of an oncogene. Only expression of an activated oncogene in cells previously activated by large T-antigen results in fully transformed cell lines. This has led to a two step model of transformation of human epithelial cells [10,11].

There is no clear definition of immortalization of human cell lines. Cell lines of human origin with unlimited lifespan in culture are rare. In this paper we will consider a cell line that survived the crisis and can be cultured for a considerable length of time to be immortalized. According to this definition only the cell line NCF3A is immortalized as it has been in culture for more than 30 passages post crisis.

The clinical symptoms of cystic fibrosis can be explained by a mutation which affects the activity of epithelial chloride channels. According to the current hypothesis activation by beta-adrenergic agonists, through a cAMP dependent pathway, of chloride channels in the apical membrane of epithelial cells is defective in cystic fibrosis [3]. Evidence for this hypothesis was found in electrophysiological studies with human sweatglands, and airway cells [3,4,5,6,7,26]. Several studies show that the regulation of the chloride channel is defective in cultured nasal epithelial cells from cystic fibrosis patients [5,6,7,26,27,28]. We may expect immortalized cells from this source to have similar characteristics.

Both the pre- and post-crisis CF cell lines we isolated seem to have the phenotypic properties expected. They express large T antigen (fig 2), have epithelial morphology (figs 1 and 3), and the production of cytokeratins is indicative of their epithelial nature. However, the synthesis of cytokeratins 4 and 13 appeares to be strongly diminished compared to uninfected nasal cells (fig.4). Changes in the relative and absolute amounts of cytokeratin after transformation of epithelial cells was observed by other authors also [23,29]. This illustrates that the process of immortalization, which is a multistep process that may be different for independent subclones, will affect the phenotype of cells.

NCF3 cells form cell layers with sufficient electrical resistance to allow Ussing chamber experiments (fig.5, table 1). The effect of the sodium channel inhibitor

amiloride on the short circuit current (Isc) suggests the presence of active apical sodium channels. In the presence of amiloride the Isc is reduced to zero, which can also be observed in primary CF nasal polyp cells in contrast to normal cells Moreover, Isoproterenol, forskolin and dibutyryl-cAMP do not elicit an electrical response (table I) while cAMP levels are increased dramatically by isoproterenol and forskolin (table 2). This indicates that the cAMP dependent signal transduction pathway needed to open chloride channels does not operate in NCF3. This pattern of responses is also seen with primary CF nasal epithelial cells whereas normal nasal cells do show a positive Isc in the presence of amiloride and a response to cAMP dependent agonists [27,28,Verbeek in preparation].

Histamine, bradykinine and A23187 cause a 51B sensitive response in the presence of amiloride (table 1). This can be interpreted as an increase of chloride transport, involving activation of chloride channels, caused by high intracellular calcium, as was also observed in normal and CF nasal epithelial cells [6,9,Verbeek in preparation].

It could be shown with patch clamp analysis that both NCF3 and NCF3A cells contain chloride channels with the characteristics previously described for channels of primary CF epithelial cells (fig.6). Activation of the chloride channels by an increase of intracellular cAMP was not observed in NCF3A cells (see results section). We suggest therefore that the behaviour of the chloride channels in NCF3A is conform to the current definition of CF phenotype: absence of activation by increased intracellular cAMP.

Sofar this is the only widely accepted phenotypic criterium of CF epithelial cells. It should be pointed out that cAMP dependent activation of chloride channels may involve a number of unknown components. Therefore further study is necessary to confirm and define the CF phenotype of NCF3A and other CF cells with CF genotype in molecular terms.

One relevant observation in this respect may be the voltage activation of chloride channels. All chloride channels observed in NCF3 and NCF3A were activated during prolonged off- cell depolarisation. We find this delayed onset of activation under the conditions used typical of chloride channels from CF cells. Chloride channels of normal epithelium are activated generally within two minutes whereas channels of CF cells are activated after on average six minutes (J. Bijman, in

preparation).

Attempts to clone the gene mutated in cystic fibrosis are in progress [30]. A crucial experiment, in fact one of the main reasons for isolating an immortalized CF cell line, would be to express a cloned candidate gene in CF3A or other cell lines suitable for isolation of stable transfectants. Correction of the CF phenotype after introduction of a candidate gene would be the final test to identify the candidate as the CF gene and open the way for further study of the encoded gene product.

The data obtained sofar lead us to believe that the cell lines isolated here provide a useful model system for the study of cystic fibrosis. Some relevant phenotypic parameters of immortalised and primary nasal polyp cells are summarised in table 3 for comparison. The availability of considerable amounts of characterised cystic fibrosis cellmaterial which can be manipulated genetically can be very helpful in the biochemical analysis of the disease.

Table 3.

Poperties of primary and immortalized cells.

phenotype	C	CF	NCF3	NCF3A
Growth for more than 30 passages	-	-	-	+
Tight junctions	+	+	+	-
Cytokeratin	+	+	+	+
Cytokeratin 4 and 13	+	+	-	-
Chloride channels	+	+	+	+
Stimulation of cAMP synthesis	+	+	+	+
Channel activation by cAMP (see ref. 5, 7).	+	-	-	-

Acknowledgements

We would like to thank Martina Wilke for the cAMP measurements, Dr Arnold Reuser for critically reading the manuscript, Dr Goos van Muijen for providing us with the monoclonal anti cytokeratin antibodies, Dr. Ward Peterson and Eric Kamst for the karyologic analysis, Joop Fengler for photography, Rob Willemse and Pim Visser for electronmicroscopy and Diana Heinsius for secretorial assistance.

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NOTE:

This chapter has been published as:

Immortalization of nasal polyp epithelial cells from cystic fibrosis patients.

Scholte, B.J., Kansen, M., Hoogeveen, A.T., Willemse, R., Rhim, J.S., Van der Kamp. A.W.M. and Bijman, J.

Exp. Cell Res. 182 (1989): 559-571.

CHAPTER V.

HT29.CL19A HUMAN COLON CARCINOMA CELLS AS A MODEL SYSTEM TO STUDY THE REGULATION OF CHLORIDE CHANNELS IN NORMAL CELLS.

ABSTRACT.

Chloride (Cl^-) channels are important in the regulation of salt and water transport in secretory epithelial cells. A disturbed Cl^- secretion is the most consistent characteristic in the genetic disease cystic fibrosis. An outwardly rectifying Cl-channel (OR) with a conductance of 25-50 pS has been proposed to play a major role in Cl^- secretion. Activation by Ca^{2+}, protein kinase (PK) A and C (at <10 nM Ca^{2+}) as well as inhibition by PKC (at 1 μM Ca^{2+}) has been reported. In the present study, we have identified and characterized the OR in HT29.cl19A human colon carcinoma cells as a model system for normal cells. The OR displayed a conductance of 31±4 pS (n=25). Its open probability in 10^{-8} M Ca^{2+} was voltage dependent in 50% of the patches, starting from 0.2 at -70 mV to 0.8 at 70 mV. The spontaneous activation in excised inside-out patches at -60 mV was Ca^{2+} dependent and decreased from 30% in 10^{-3} M Ca^{2+} to 3% in 10^{-8} M Ca^{2+}. Active OR's were found in i) 25% exposed to 10^{-8} M Ca^{2+}, ATP and cAMP only; ii) 40% of the patches exposed to 10^{-8} M Ca^{2+} , ATP and the catalytic subunit of PKA (C_{AK}); iii) 67% of the patches exposed to 10^{-3} M Ca^{2+}, ATP plus C_{AK}. Inhibition of voltage activated channels by addition of PKC in 10^{-6} M or 10^{-3} M Ca^{2+} was not observed. Attempts to activate the OR in cell-attached patches by increasing cAMP levels under different experimental conditions were unsuccessfull. However, a small 5 pS linear Cl^- channel was observed in on-cell patches, which inactivated upon excision. Our data suggest that the OR may not be as important in Cl^- secretion as has been thought. In contrast, the small linear channel may be a suitable candidate for carrying the cAMP-dependent Cl^- current in intact colonocytes.

INTRODUCTION.

The opening and closing of chloride channels present in the apical membrane is an essential event in the regulation of salt and water transport in secretory epithelia including airway, intestine and sweat glands [19, 29, 40, 61]. Chloride channels are regulated by multiple intracellular pathways acting through cAMP and cGMP, diacylglycerol, calcium and calcium/calmodulin [12, 58]. Disturbances in one or more regulatory pathways lead to hypersecretion in the intestine, which causes secretory diarrhoea [13, 14] and to hyposecretion in the genetic disease cystic fibrosis (CF) [62].

Much attention in the process of Cl^- secretion has been paid to a 30-50 pS voltage dependent outwardly rectifying chloride channel (OR). Opening of this channel by the cAMP-dependent pathway in cell-attached patches has been demonstrated in human colon carcinoma (T84), airway and gastric cells as well as in Necturus enterocytes [18, 29, 42, 51], but has failed in the Necturus choroid plexus, pancreatic cells and keratinocytes and in several other studies with T84 cells [9, 21, 22, 23, 56, Kansen et al., submitted]. In excised inside-out patches activation has been reported after addition of the catalytic subunit of protein kinase A (PKA) to the bath solution [4, 6, 8, 18, 35, 44, 45, 54]. However no activation could be shown in human lymphocytes and keratinocytes [28, Kansen et al., submitted].

The effect of calcium is even more complex. Calcium ionophore A23187 activates the channel in cell-attached patches [53] and Ca^{2+} increases the open probability in excised inside-out patches [15], although others find no differences in channel properties between 10^{-9} M and 10^{-3} M Ca^{2+} [10, 42]. In combination with protein kinase C low Ca^{2+} (< 10 nM) activates, but high Ca^{2+} (1 μM) inactivates chloride channels in airway cells [44].

Apart from regulation by phosphorylation and Ca^{2+}, channel activation by temperature, trypsin and depolarization has been demonstrated [63].

Using HT29.cl19A cells, a subclone of the human colon carcinoma cell line HT29 selected for its Cl^- secreting characteristics [1, 2] as a model system for the study of Cl^-channels in normal cells, we have studied the influence of Ca^{2+} in the bath solution on the incidence and kinetic properties of the OR in excised inside-out patches prior to its activation through depolarization. Spontaneous channel activation decreased from 30% to 2% after reduction of the Ca^{2+} concentration

from 10^{-3} M to 10^{-8} M, thereby creating conditions to test activation by cAMP and PKA. Increased channel activity could be shown in 40% or 25% of all attempts after addition of PKA plus ATP or cAMP plus ATP respectively. Attempts to activate the channel in cell-attached patches after incubation of intact cells with cholera toxin and forskolin (occasionally together with the cytoskeleton inhibitor cytochalasin B) were not successfull. However, in stimulated cells a 5 pS linear chloride channel was observed which was absent in unstimulated cells. Considering recent evidence for the possible identity of this channel as the product of the CF-gene, CFTR [39], we speculate that this small linear channel is probably not responsible for the cAMP-dependent chloride secretion in intact epithelium, leaving an as yet unknown physiological role for the outward rectifyer.

MATERIALS AND METHODS.

Materials.

Human HT29.cl19A colonocytes were generously donated by Dr.C.L. Laboisse (INSERM, Paris, France).

The catalytic subunit of rat lung cAMP-dependent protein kinase A (100 nM, specific activity 4.2 U/mg) was a gift from Dr. U. Walter (Würzburg, FRG). Protein kinase C (1 μg/ml) was provided by Dr. P. Parker, U.C.R.F., London. Forskolin, 8-Br-cAMP, cholera toxin, guanosine 5'-[gamma-thio] triphosphate (GTP[S]), 3-isobutyl-1-methylxanthine (IBMX), phorbol 12-myristate 13-acetate (PMA) and cytochalasin B were obtained from Sigma (St. Louis, U.S.A.).

Cell culture.

Human HT29.cl19A colonocytes were grown on petri dishes in Dulbecco's modified Eagles medium suppplemented with 10% fetal calf serum (Gibco), 0,19 mg/ml streptomycine and 10^5 I.E. units/l penicilline in 5% CO_2 at 37°C. Cells were harvested with trypsin (0,5 mg/ml) plus EDTA (1 mM).

Solutions.

Bath and patch-pipette solutions in mM, pH 7.4:

10^{-3} M Ca^{2+}: 140 NaCl, 5 KCl, 5 HEPES, 1 $MgCl_2$, 1 $CaCl_2$

10^{-6} M Ca^{2+}: as 10^{-3} Ca^{2+}, but with 0,95 $CaCl_2$, 1 EGTA

10^{-8} M Ca^{2+}: as 10^{-3} Ca^{2+}, but with 0,16 $CaCl_2$, 1 EGTA

In the cell-attached experiments the pipette solution contained 50 mM KCl, 100 mM NaCl, 5 mM HEPES, 1 mM $MgCl_2$, 1,5 mM Ca^{2+}, 1 mM EGTA (10^{-3} M Ca^{2+}).

Patch-clamp analysis.

Cell-attached and excised inside-out patches were made according to Hamill et al. [30]. Patch pipettes from borosilicate glass (Clark GC150-15TF) had resistances of 3-8 Mohm. Data were recorded with a LM-EPC 7 patch-clamp amplifier (List, Darmstadt, FRG), sampled at 3 kHz, filtered at 0,5 kHz, digitized with a pulse code modulator (Sony PCM-F1), stored on video tape (Sony SL-HF 950 E/ES) and analysed with a PDP 11/73 computer (Digital Equipment Corporation).

The sign of the clamp voltage refers to the bath with respect to the patch pipette. Positive (upward) currents denote Cl^--ions flowing out of the pipette into the bath. The open probability (Po) of a channel is expressed as the ratio of open the time of the channel over the total time recorded (more than 1 minute per clamp voltage). Po was only calculated if one channel was present in the patch. Patch-clamp recordings were derived from cells in the center of confluent islands of 15 cells or more. Experiments were performed at room temperature and at 37°C when indicated.

Cell-attached experiments (only in 10^{-8} M Ca^{2+} bath-solution) were performed by addition of 10^{-5} M forskolin (0.5% v/v ethanol) to the bath solution when for 4 minutes no channel activity was observed. The clamp potential was held at 0 mV for 10 minutes in order to test chloride channel activation. When no channels appeared in the cell-attached patch, excised inside out patches were held at the physiological membrane voltage of -60 mV for 5 minutes followed by a 10 minute depolarization (70 mV). In pre-incubation experiments with IBMX (1 mM), forskolin (10^{-5} M) and cholera toxin (200 ng/ml), IBMX and forskolin were already present at the time of seal formation or cells were cultured for one day in the presents of cholera toxin.

When cytochalasin B (10 μg/ml, 0.02% v/v DMSO) was used, cells were incubated for 1 hour at 37°C prior to seal formation.

In order to test the activation of chloride channels by phosphorylation in excised

inside out patches, the NaCl-solution was removed and the same solution was added (control) or the same solution supplemented with the catalytic subunit of PKA (100 nM) + ATP (1 mM), cAMP (10 μM) + ATP or ATP alone. Activity was tested for 10 minutes at -60 mV, followed by depolarization (70 mV, 10 minutes).

We defined a complete protocol as one in which the clamp potential in excised inside out patches was maintained for 10 minutes at 70 mV without interruption by seal deformation.

The inhibitory effect of PKC was investigated by activation of the OR through voltage induction in normal 10^{-8} M Ca^{2+} Ringer's solution followed by exchanging the bath solution for a mix containing PKC (100 nM) + PMA (0.1 μM) + MgATP (1 mM) in 10^{-6} M or 10^{-3} M Ca^{2+}. Channel activity was observed for 10 min at 70 mV.

Data are presented as means ± SD.

RESULTS.

Incidence of the OR in excised inside-out patches.

About 70% of the attempts (n=1348) to patch clamp apical membranes of HT29.cl19A cells in culture were successfull and resulted in a gigaohm seal; in one third of the excised inside-out patches the clamp potential could be held for 10 minutes at 70 mV. In 30% of these complete protocols a chloride channel was present, with, in a symmetrical 150 mM NaCl solution, a larger amplitude at depolarizing, positive potentials than at the corresponding negative potentials, indicating outward rectification (Fig. 1). The number of channels in a patch varied from 1 to 4 with a mean of 1.9 ± 0.9 (n=54).

Single channel properties.

The conductance of the channel at 0 mV in a symmetrical 150 mM NaCl solution was 31 ± 4 pS (n=25).

The open probability in 10^{-8} M Ca^{2+} (n=11) in the range from -70 mV to 70 mV was voltage dependent in 50% of the patches, starting from 0,2 at -70 mV to 0,8 at 70 mV, but was voltage independent in the other experiments. Activation of the channel could be induced by the non-physiological manipulation of depolarization and showed an average lag phase of 3.5 ± 2.9 min (n=27). Once activated by

depolarization, in some cases, although not systematically investigated, inactivation occurred at depolarizing as well as physiological membrane (-60 mV) voltages. Re-activation of voltage-inactivated channels could be induced by switching between -70 mV and 70 mV every 5 seconds.

The ion-selectivity of the channel in HT29 cells has already been established [32, 41]. Since we were mainly interested in the regulation by Ca^{2+} and cAMP we did not re-investigate this characteristic. We only changed the bath Cl^--concentration to 33% and 300% of the pipette Cl^--concentration in order to identify this channel as a chloride channel by the shift of the reversal potential.

Spontaneous channel activation after excision.

Because activation could be induced by depolarizing voltages (70 mV), activation of the channel by phosphorylation with the catalytic subunit of PKA had to be performed in the physiological voltage range (-60 mV). In control experiments spontaneous activation was tested after excision in the 10^{-3} M Ca^{2+} NaCl-bath solution for 10 minutes followed by a 10 minute depolarization on order to verify whether the observed activity was the outwardly rectifying chloride channel as well as to test whether the channel was present, albeit silent under the test conditions. Activation at -60 mV was observed after 4.4 ± 2.8 min (n=4) in 4 out of 12 patches with channels (Table 1), which renders this Ca^{2+}-concentration unsuitable to perform phosphorylation experiments. Moreover, 3 or more Ca^{2+}-dependent non-selective cation channels (see [50] for review, [11, 20]) per patch were activated upon excision in more than 50% of the patches having an amplitude at -60 mV which is about equal to the amplitude of the OR, further complicating the analysis of the OR. In order to minimize spontaneous channel activity prior to depolarization, the Ca^{2+}-concentration was therefore reduced to 10^{-8} M. As a consequence, the percentage chloride channels active at -60 mV was diminished from 30% to 2%, allowing experiments in which channel activation with PKA could be tested. Increasing the temperature from about 23 °C to 37 °C did not induce channel activity at -60 mV in 10^{-8} M Ca^{2+}. Both Ca^{2+}-concentations are rather extremes and therefore we tested the effect of 10^{-6} M Ca^{2+} on the spontaneous activation after excision and found that 1 out of 8 channels was actived spontaneously prior to voltage induction, which is less than in 10^{-3} M but more than in 10^{-8} M Ca^{2+}.

Once activated by depolarizing voltages, a decrease in the Ca^{2+}-concentration from 10^{-3} M to 10^{-8} M did not inactivate channel activity, nor did it influence the number of channels per patch.

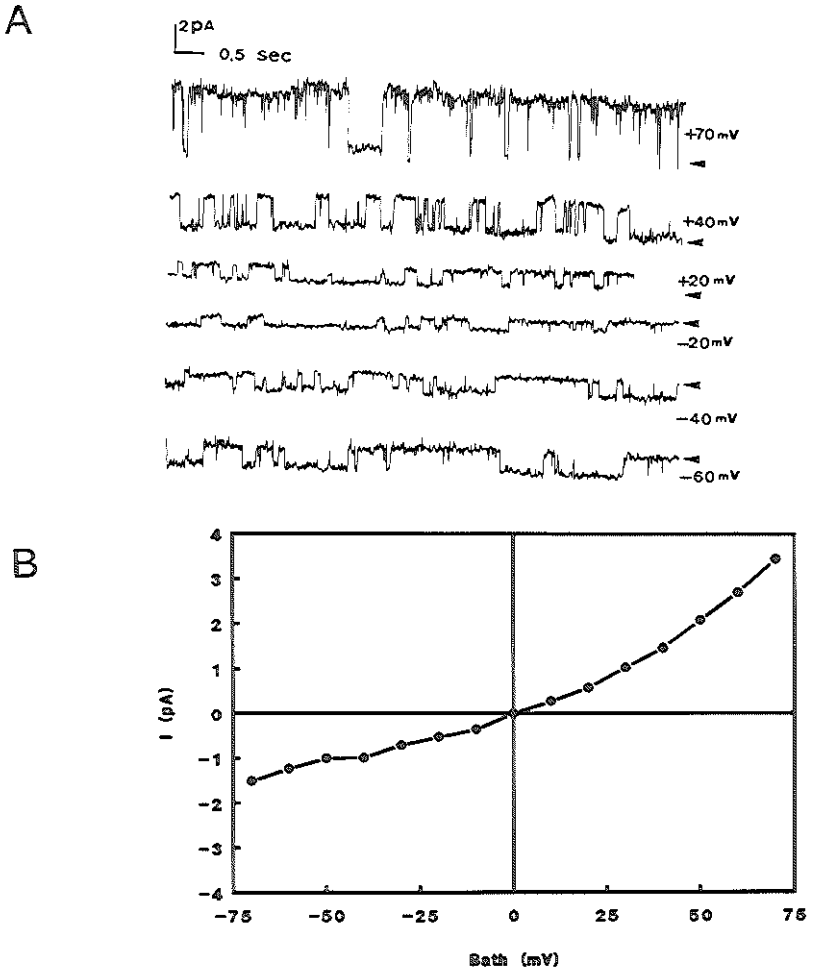

Figure 1.

(A) Recording and (B) I/V-characteristic of the outwardly rectifying chloride channel in an excised inside-out patch and symmetrical bath and pipette solutions. Arrows indicate the zero current level.

Table 1.

Effect of Ca^{2+}, phosphorylation and temperature on the activation of the outwardly rectifying chloride channel in excised inside-out patches from HT29.cl19A cells. Data are presented as the number of patches containing chloride channel activity with the total number of chloride channels present in those patches between brackets. Pipette solution in all experiments is 10^{-3} M Ca^{2+} standard Ringer's solution. Temperature, except when indicated otherwise, is 23°C. The potential was held for 10 min at -60 mV, followed by a 10 min period at 70 mV.

bath solution	$[Ca^{2+}]$ (M)	-60 mV	70 mV	total
	10^{-3}	4 (4)	8 (10)	12 (14)
	10^{-6}	1 (1)	7 (10)	8 (11)
23°C	10^{-8}	1 (1)	35 (40)	36 (41)
37°C	10^{-8}	0	7 (10)	7 (10)
PKA + ATP	10^{-3}	6 (6)	3 (3)	9 (9)
PKA + ATP	10^{-8}	8 (8)	11 (13)	19 (21)
cAMP + ATP	10^{-8}	3 (3)	9 (13)	12 (16)
ATP	10^{-8}	0	4 (4)	4 (4)

Activation by PKA in excised inside-out patches.

Obviously, experiments in which activation with PKA was tested had to be performed at -60 mV in 10^{-8} M Ca^{2+} NaCl bath solution to prevent spontaneous channel activation (the pipette solution could be either 10^{-3} M or 10^{-8} M Ca^{2+}). Addition of the catalytic subunit of PKA plus ATP resulted in an increase in the percentage of active channels from 3% to 42% (Fig. 2, Table 1).

On average, the activation was seen 3.7 ± 2.6 min (n=7) after addition. ATP alone did not activate channels (n=4), but in combination with cAMP the activity increased to 25% after 5.0 ± 1.0 min (n=3), indicating that an endogenous PKA may be present in the patch. No differences were observed in the number of

channels per patch activated at -60 mV in the presence of PKA + ATP or cAMP + ATP and the number of channels seen after depolarization, suggesting that all previously silent channels were activated by phosphorylation.

Experiments with PKA + ATP in a 10^{-3} M Ca^{2+} bath solution did activate chloride channels in 67% of all attempts after 1.8 ± 1.1 min (n=6) i.e. 25% more than in 10^{-8} M Ca^{2+}, but this difference is close to the difference between the percentage of spontaneous activation at 10^{-3} M and 10^{-8} M Ca^{2+} M (30%).

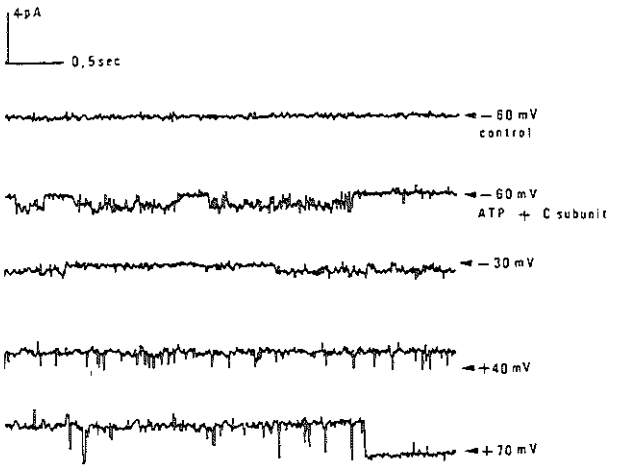

Figure 2.

Activation of the outwardly rectifying chloride channel at -50 mV in an excised inside-out patch after addition of the catalytic subunit of PKA and ATP at the cytosolic side. Arrows indicate the zero current level.

No activation of other (chloride)-channels was observed upon PKA/ATP/cAMP-stimulation in 10^{-8} M Ca^{2+}, although we know that a G-protein dependent Cl^--channel is expressed in the apical membrane of these cells [57] together with a 15 pS linear chloride channel [32] and a 300 pS anion channel [Kansen and Bijman, unpublished results].

In a series of experiments in which we tried to activate a G-protein dependent

chloride channel by addition of 10 μM GTP[S] to the cytosolic side [57], we did not find any effect on the OR with respect to its possible activation, its incidence (35% of the patches contained 1 or more OR) or its open probability at positive holding potentials (n=10).

Apart from acivation by PKA, activation as well as inactivation by PKC, depending on the Ca^{2+}-concentration has been reported in airway epithelial cells [44]. Because the percentage of channels activated by PKA was rather low and because blocking experiments with voltage induced channels are far more easy to perform than activation experiments, we started with attempts to inhibit the OR with PKC in a 10^{-6} M and 10^{-3} M Ca^{2+} bath solution. Among 11 patches containing 19 OR, only 2, each with 1 OR, could be blocked by PKC in 10^{-6} M Ca^{2+}, while in 4 patches 1 or 2 additional channels appeared during the 10 min blocking period. Experiments with 10^{-3} M Ca^{2+} in the bath solution did not inhibit voltage induced channels (n=3) and we therefore may conclude that the voltage activated OR is not reproducibly inhibited by PKC in Ca^{2+} concentrations of 10^{-6} M or more.

Cell-attached channel activation.

Although we observed an increase in the percentage of active channels after PKA stimulation, the success rate was only moderate (40%). It might be that the process of excision influences the efficiency of channel activation, and it seemed therefore usefull to examine the activation in cell-attached patches. Furthermore, it should be noted that, alhough cell-attached activation of the OR has been claimed, mainly recordings of channel openings were published but only a few, sometimes different I/V-characteristics [18, 42].

In unstimulated cells no cell-attached channel activity with an outwardly rectifying I/V-characteristic was identified, nor activity of a channel with a different I/V-curve, but with a reversal potential between 0 mV and 10 mV as expected for a chloride channel in combination with the outward rectifyer in the excised configuration.

We therefore tried to activate the chloride channel by stimulation with 10 μM forskolin in two different ways. First by addition of forskolin to the bath solution after a 4 minute period without any channel activity followed by monitoring

activation during a 10 minute period at $V_{pipette}$=0 mV, excision and depolarization in 10^{-8} M Ca^{2+}-solution and secondly by pre-incubation with forskolin or 8-Br-cAMP followed by the same procedure. However no activity revealing the activation of the outward rectifyer was observed (Table 2), nor activation of a channel which, after excision, always resulted in the appearance of the outwardly rectifying channel. Neither culturing the cells for at least one day in the presence of cholera toxin, known to increase the amount of cellular cAMP, nor preincubation with the phosphodiesterase inhibitor IBMX, result in on-cell stimulation of the chloride channel. Other approaches, like culturing the cells to confluency or growing them on collagen instead of plastic also failed to result in the on-cell activation of the OR by forskolin.

The cytoskeleton inhibitor cytochalasin B has been claimed to specifically induce chloride channels and block sodium channels in renal epithelial cells [Ausiello, personal communication] and to augment the ß-adrenergic responsiveness in mouse lymphoma cells [60]. However preincubation with cytochalasin B followed by stimulation with forskolin or cholera toxin did not result in cell-attached activity related with to OR.

Since all our attempts to show cell-attached channel activity or to observe an increase in the number of patches containing the OR after excision failed, it seems apparent that for unknown reasons the channel is always blocked in the on-cell configuration, despite the elevation of intracellular cAMP and activation of endogenous PKA.

However, in 9% of the cell-attached experiments, addition of forskolin or cholera toxin in the presence or absence of cytochalasin B resulted in the appearance of a 6 ± 1,5 pS (n=16) channel with a reversal potential of 5 ± 6 mV (n=14), indicating a small chloride channel (Fig. 3). Usually only one channel was present in the patch with an open probability of less than 0,1. This channel was never seen in unstimulated cells. After excision the channel inactivated which precluded examination of its single channel properties under those conditions. Therefore further analysis will be limited to the cell-attached configuration by using pipette solutions with different ionic compositions. This small channel had no relation with the OR because from the 16 on-cell channels 11 contained no OR in excised inside-out patches.

Table 2.

Attempts to activate the outwardly recifying chloride channel (OR) in cell-attached patches at 23°C by increasing cAMP levels under different experimental conditions (10^{-8} M Ca^{2+} bath-, 10^{-3} M Ca^{2+} pipette solution). Data between brackets represent the number of channels.

stimulation	number of patches	on cell active[1]	excised OR	on cell + excised OR[2]	complete protocol
none	27	7	6 (11)	2?	10
forskolin (direct)	25	9	5 (11)	2?	9
forskolin (pre-incubation)	57	14	11 (18)	3 (1?)	20
forskolin (pre-incubation)[3]	16	7	6 (9)	3 (1?)	12
cholera toxin	76	13	10 (20)	3?	47
cytochalasin B+ forskolin	24	14	4 (6)	2	9
cytochalasin B+ cholera toxin	35	15	3 (4)	1?	13
forskolin + IBMX	39	1	3 (4)	2?	11

[1]= any form of cell-attached channel activity
[2]= any form of cell-attached ion channel activity in combination with the OR in the excised configuration
[3]= support is collagen
?= channel activity without sufficient data to construct a I/V-plot

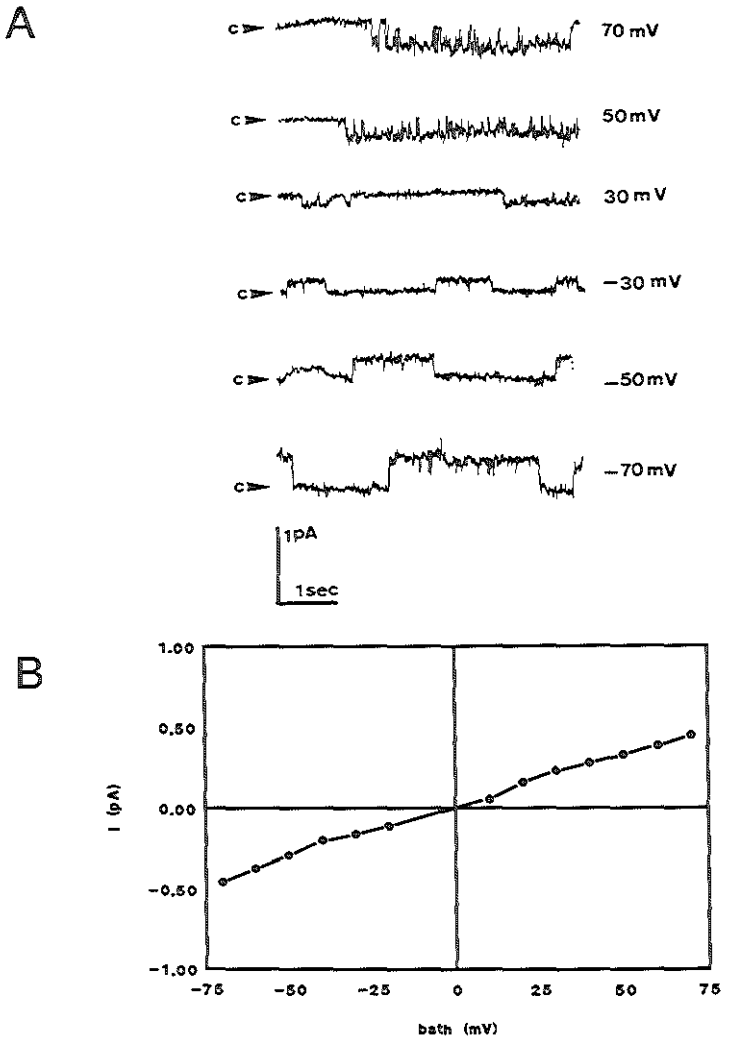

Figure 3.

(A) Recording and (B) I/V-characteristic of the small chloride channel in a cell-attached patch following preincubation with forskolin. Arrows indicate the closed state of the channel.

DISCUSSION.

Single channel characteristics.

The outwardly rectifying I/V-characteristic and the conductance of the chloride channel in HT29.cl19A cells examined were identical to those described in (immortalized) airway cells [16, 26, 64], the colon carcinoma cell lines T84 and HT29 [29, 32], sweat glands [6, 40], fibroblasts [4], pancreatic carcinoma cells [31, 53], nasal polyp cells [36, 37, 52], keratinocytes [17, Kansen et al., submitted], lymphocytes [8], shark rectal gland [25] and gastric cells [51]. The open probability Po in HT29.cl 19A cells was variable, which is consistent with the observation in airway cells [63] in which the Po is sometimes voltage dependent and increasing with depolarization but sometimes voltage independent. Activation of the channels could be induced upon depolarization but inactivation was, in contrast to airway cells [66], seen after hyperpolarization as well as depolarization. Like in the parental HT29 cell line [32] the 19A subclone shows cluster activity i.e. two or more current levels were seen in about 35% of the recordings.

Spontaneous channel activation after patch excision.

By the microelectrode technique it has been shown that the physiological membrane voltage in HT29.cl19A cells was about -60 mV [3]. Activation of the OR upon excision at this voltage is apparently Ca^{2+}-dependent and decreases at reduced concentrations, which is in agreement with the results obtained with keratinocytes [Kansen et al., submitted]. In contrast, airway cells show spontaneous activation after excision irrespective of the polarity of the clamp voltage and the Ca^{2+}-concentration [42]. The explanation may be methodological, although it is unlikely that the differences in temperature are important (Table 1). A second study about the role of Ca^{2+} reports neither effects on previously quiescent channels nor on the Po [10], while a third report demonstrates activation after an increased Ca^{2+}-concentration [15]. In better agreement with the results reported for airway cells is the Ca^{2+}-independence of channel activity in HT29.cl19A cells following voltage induction. The discrepancies between the various Ca^{2+}-effects on the Cl^--channel may be due in part to the possible a role of Ca^{2+}/calmodulin dependent kinase in the excised patch whose presence might be essential to detect Ca^{2+} dependent regulation [58].

Activation by cAMP, ATP and PKA; inhibition by PKC.

In the 10^{-8} M Ca^{2+} cytosolic solution the efficiency of activation of the OR in apical membrane patches of HT29.cl19A cells by phosphorylation catalyzed by the catalytic subunit of PKA and ATP is 40% i.e. much lower than the 70-100% reported for (immortalized) airway cells [33, 35, 44, 54], fibroblasts [4], lymphocytes [8], Necturus enterocytes [18], sweat glands [6] and in the shark rectal gland [24], although activation in lymphocytes could not be reproduced [28]. In these reports little detailed information is available about spontaneous channel activation which varied between 10 and 35% in airway cells and "varied from day to day and from culture to culture" [63], so the published activation efficiencies by PKA and ATP may have been overestimated.

Phosphorylation experiments performed in a 10^{-3} M Ca^{2+} bath solution increased the activation with about the same percentage (25%) as the difference between spontaneous activation in 10^{-3} M and 10^{-8} M Ca^{2+} at the cytosolic side (31%), suggesting no interrelationship between the Ca^{2+} and the PKA + ATP effects.

Cyclic AMP together with ATP was able to activate chloride channels in 25% of Cl^--channel containing patches, less than with PKA and ATP suggesting that an endogenous PKA is sometimes present in the patch membrane.

Similar to PKA, PKC has been reported to induce channel activity in 10^{-8} M Ca^{2+}; in contrast it has been shown to inactivate voltage activated channels in 10^{-6} M Ca^{2+} [34, 45]. Our inhibition experiments with PKC in 10^{-6} M and 10^{-3} M Ca^{2+} however did not confirm these results; even previously quiescent channels were activated at 70 mV in in the presence of PKC and high calcium. The different calculations of the free Ca^{2+} concentrations may be one of the explanations for the dual effect of Ca^{2+} in combination with PKC on the OR [27, 65, 66].

Chloride channels in cell-attached patches.

In studies reporting the OR in cell-attached patches, usually only channel recordings were presented but very rarely I/V-plots [16, 18, 25, 28, 31, 33, 41, 51, 52, 61]. However on-cell activation was not always successful [9, 56]. When I/V-plots were available, they varied in characteristics: outwardly rectifying in airway cells [42] or linear in Necturus enterocytes [18]. Our data confirm reports in which cell-attached activity of a channel corresponding to the OR in excised inside-out patches could not be detected. An explanation for this failure may be the

generation of "blebs" [48, 49] during seal formation or the lack of cell-attached channel activity may be the presence of a cytosolic inhibitor [39, 42, 43] which is released from the channel after excision leading to activation. The absence of active outward rectifyers in intact cell membranes even following stimulation with cAMP, and the data about activation by PKA and PKC in excised patches raise the question whether or not this channel is the physiologically relevant cAMP-dependent Cl^--channel in secretory epithelial cells. In various cell types, a 2-10 pS Cl^- channel has been identified as the one carrying the cAMP-dependent Cl^- current [7, 9, 21, 22, 23, 46, 56]. Expression of CFTR, the CF-gene product, in a non-epithelial cell line followed by cAMP stimulation resulted in the appearance of 5-10 pS linear Cl^--channel [38]. These data indicate that the physiological relevant cAMP activated chloride channel is probably a small linear channel. In cell-attached patches of HT29.cl19A cells we found a Cl^--channel which resembles the small channel described in T84 cells with respect to cAMP-acivation, its linear I/V-characteristic, its conductance and its inactivation after excision [56].

In conclusion, the function of the OR in the intact cell and the physiological relevance of its enhanced activity after stimulation with PKA under excised patch coditions remains uncertain. Moreover, its presence in non-epithelial cells [4, 8] may indicate that the OR is not involved in Cl^--secretion but in a more general physiological process such as volume regulation [47, 55, 67] or cell division [5]. Since no correlation was found between the cystic fibrosis gene expression and the presence of the OR, this channel is probably not involved in this genetic disease [59].

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CHAPTER VI.

G-PROTEINS MEDIATE INTESTINAL CHLORIDE CHANNEL ACTIVATION.

ABSTRACT.

The localisation of several GTP-binding regulatory proteins in the apical membrane of intestinal epithelial cells has prompted us to investigate a possible role for G-proteins as modulators of apical Cl^--channels. In membrane vesicles isolated from rat small intestine or human HT29-cl.19A colon carcinoma cells, the entrapment of GTP[S] led to a large increase in Cl^- conductance, as evidenced by an increased $^{125}I^-$-uptake and faster SPQ-quenching. The enhancement was observed in the presence, but not in the absence of the K^+-ionophore valinomycin, indicating that the increased Cl^- permeability is not secondary to the opening of K^+-channels. The effect of GTP[S] was counteracted by GDP[S] and appeared to be independent of cytosolic messengers, including ATP, cAMP and Ca^{2+}, suggesting that protein phosphorylation and/or phospholipase C activation is not involved. Patch clamp analysis of apical membrane patches of HT29-cl.19A colonocytes revealed a GTP[S] activated, inwardly rectifying anion selective channel with a unitary conductance of 20 ± 4 pS. No spontaneous channel openings were observed in the absence of GTP[S], while the open-time probability (P_o) increases dramatically to 0.81 ± 0.09 upon addition with GTP[S]. Since the electrophysiological characteristics and regulatory properties of this channel are markedly different from those of the more widely studied cAMP/A-kinase operated channel, we propose the existence of a separate Cl^- selective ion channel in the apical border of intestinal epithelial cells. Our results suggest an alternative regulatory pathway in transepithelial salt transport and a possible site for anomalous channel regulation as observed in cystic fibrosis patients.

INTRODUCTION.

Chloride fluxes across biological membranes are involved in a number of important physiological processes, including osmo-regulation, salt absorption and secretion, neurotransmission and intracellular pH regulation. Among these,

transepithelial salt secretion is of a particular interest, since anomalous regulation of the Cl^--channel involved leads to severe pathological conditions like secretory diarrhoea (cholera) and cystic fibrosis [1-3]. Although little is known of the physical structure of epithelial Cl^--channels [4], their mode of activation has been analyzed in more detail. On the basis of electrophysiological techniques (e.g. patch clamp, planar lipid bilayers, Ussing chamber) several intracellular regulatory mechanisms are emerging. These include; 1) channel activation through cAMP- and, in intestine, cGMP-dependent protein phosphorylation [1,2]; 2) Activation through Ca^{2+}-mobilizing hormones such as acetylcholine, bradykinin or histamine [1,2] and 3) modulation of channel activity by protein kinase C [5,6].

According to the classical concept, regulation is initiated by hormone-receptor activation of GTP-binding regulatory proteins (G_s, G_p) in the basolateral membrane, activation of adenylate cyclase or phospholipase C and subsequent generation of cytosolic messengers (cAMP, Ca^{2+}) capable of communicating with the apical membrane [1,2]. However, recent studies have demonstrated that G-proteins are also capable of modulating ion channel activities through a direct or lipid metabolite-derived mechanism, independent of cytosolic messengers. This has been demonstrated definitely for cardiac Ca^{2+}-, neuronal K^+-and epithelial Na^+- and Cl^--channels [7-11], but G-protein involvement in intestinal Cl^--channel regulation has not been reported previously. We have recently found that a number of GTP-binding proteins, including G_s, G_p and p21 *ras*, are abundant in apical membranes of intestinal epithelial cells, despite the absence of any known G-protein-coupled hormone receptors ([12] and footnote 1). Since intestinal Cl^--channels are localized preferentially in the same membranes [1,2], a possible role for apical G-proteins as modulators of intestinal Cl^- secretion was studied. In this report, we present data illustrating that activation of G-proteins with GTP or GTP[S][2] enhances the Cl^- conductance of vesiculated intestinal membranes and unmasks the existence of a novel type of Cl^--channels in apical membrane patches from human colonocytes.

MATERIALS AND METHODS.

Materials

Human HT-29cl.19A colonocytes were generously donated by Dr. Laboisse, Unite 239 INSERM, Paris, France. All nucleotides were purchased from Boehringer

Mannheim (FRG). Other reagents were from the following sources: [^{125}I]-iodine from Amersham Ltd. and SPQ from Calbiochem.

Cell culture

HT-29cl.19A colonocytes were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco). Cells were harvested 3-5 days after reaching confluency.

Vesicle preparation

Right-side out apical membrane vesicles from human HT-29cl.19A cells were prepared from cell homogenates by differential Mg^{2+}-precipitation in the presence of trypsin-inhibitor (50 μg/ml), PMSF and 2 mM EGTA as described [1]. Thereafter, the vesicles were resuspended in a buffer containing 20 mM Tris/HEPES (pH = 7.0), 300 mM mannitol, 150 mM KCl, 6 mM $MgSO_4$, 3 mM Ca/EGTA (pCa = 6) and 1 mM $NaVO_3$, supplemented with either 150 mM KCl (iodine uptake assays) or 20 mM SPQ (Cl^--influx assays). Various nucleotides were trapped into the vesicles through a single cycle of freeze-thawing in liquid nitrogen. Rat brushborder membrane vesicles ('Mg/EGTA-BBMV') were prepared as previously described [13] and guanine nucleotides were trapped as described above.

Iodine uptake assay

Chloride efflux was quantitated using a ^{125}I-uptake assay analogous to the $^{23}Na^+$-assay developed by Garty et al. [14]. In short, extra-vesicular Cl^- was removed by eluting the vesicles through a DOWEX 1X8 column (gluconate form, 700 μl bedvolume) with 150 mM potassium gluconate. Assays were started by mixing the vesicles suspension with tracer amounts of radioactive iodine (3 μCi/mg of protein) in the absence or presence of the K^+-ionophore valinomycin (10 μM). Uptake was terminated by rapid elution of the vesicle suspension through a second DOWEX 1X8 column (formate form, 400 μl bedvolume), to remove external $^{125}I^-$, followed by τ-scintillation counting of the eluate. Background radioactivity (usually less than 1000 c.p.m), as measured by the amount of $^{125}I^-$ in the eluate in the absence of membrane vesicles, was subtracted.

SPQ-quenching

Extravesicular fluorochrome was removed prior to the experiments, by washing three times with excess (> 10 volumes) buffer. Vesicles were added to the cuvette at a concentration of approx. 100 μg protein/ml and continously stirred. Fluorescence was monitored at 350 nm (excitation) and 450 nm (emission) as described [15], using a Perkin Elmer LS-3B fluorescence spectrometer. Chloride uptake was started by adding KCl (final concentration: 25 mM) to the cuvette. After 3 - 5 minutes, trapped SPQ was released by addition of 10 μl 10% (v:v) Triton X-100, in order to achieve maximal quenching. The rate of salt-induced SPQ quenching was determined by fitting the change of fluorescence to $F(t) = A_0 + A_1 / [1 + KC_\infty (1 - e^{-k1t})] + k_0 t$ [16]. In this equation, $F(t)$ represents fluorescence at time t, A_0 and A_1 the residual fluorescence and the amplitude of quenching, respectively. k_1 represents the rate constant and the term $k_0 t$ was used to correct for linear, Cl^--channel independent quenching [16]. KC_∞ is the product of the Stern-Volmer constant (K; 92 M^{-1}) and the final [Cl^-] (25 mM).

Patch clamp analysis

Single channel recordings from apically localized channels were obtained from confluent monolayers of HT-29cl.19A cells using a List EPC7 amplifier. Signals were filtered at 3 kHz, digitized and stored on video tape prior to data analysis. Pipette solution contains 140 mM NaCl, 5 mM KCl, 5 mM HEPES (pH = 7.4), 1.2 mM $MgCl_2$ and 1 mM Ca/EGTA (pCa = 8), whereas bathing solution was identical except that 10 μM GTP[S] was added. GTP[S]-activated Cl^--channels were identified by the expected shift in voltage-current relationship observed after increasing the NaCl concentration in the bath to 420 mM. The channel incidence was approx. 25% (n = 98).

RESULTS.

In an initial series of experiments, Cl^--channel activation was studied in a suspension of KCl-loaded plasma membrane vesicles obtained from human HT-29cl.19A cells, a Cl^--secreting subclone isolated from the original HT-29 colon carcinoma cell line [17]. Conductive chloride efflux was measured by quantitating Cl^--diffusion potential-driven uptake of tracer amounts of radio-labelled iodine. We found this method superior to $^{36}Cl^-$-influx assays because: 1) epithelial anion

channels prefer I^- over Cl^- ($P_I/P_{Cl} > 1$) [17], 2) $^{125}I^-$ is available at higher specific radioactivity, and 3) iodine is accumulated exclusively into intact vesicles containing functional Cl^--channels (c.f. ref. [14]), resulting in diminished background labelling and increased sensitivity. Addition of trace amounts of radio-labelled iodine to KCl^--loaded vesicles results in a rapid increase in intravesicular $^{125}I^-$ (Fig.1). As compared to control preparations, $^{125}I^-$ accumulation is much larger (approx. 2-3 times control values) in GTP[S] containing vesicles, suggesting that GTP[S] induces a sustained increase in transmembrane chloride permeability. Disturbing the Cl^--diffusion gradient, by adding the K^+-ionophore valinomycin (10 μM), eliminates the GTP[S]-stimulation of $^{125}I^-$ uptake (Fig.1.), confirming its identification as conductive Cl^--efflux. In contrast, $^{125}I^-$-uptake in control preparations was not affected. In the presence of valinomycin, $^{125}I^-$ accumulation is somewhat lower in GTP[S]-containing relative to control vesicles (Fig.1). This phenomenon is probably an artifact of the experimental procedure used, since increased anion permeability, as occurs in GTP[S] containing vesicles, may facilitate exchange of iodine from the vesicle to the Dowex column during elution. Although considerable variation in basal $^{125}I^-$ uptake between the various vesicle preparations was noted (approx. 2000 - 7000 c.p.m.), the GTP[S]-induced increase in $^{125}I^-$ accumulation appeared highly reproducable (1.51 ± 0.12 times basal uptake at t = 30; mean ± S.E.M. for n = 3).

The accumulation of $^{125}I^-$ in GTP[S]-containing vesicles persists for at least 30 - 60 minutes. At least two phenomena may underly this sustained enhancement of intravesicular $^{125}I^-$ uptake. First, the electrogenic efflux of Cl^- generates a sustained inside-positive diffusion potential, which acts as a driving-force for prolonged $^{125}I^-$ accumulation (c.f. see: ref.[14]). Secondly, the contribution of an additional subpopulation of vesicles which accumulate $^{125}I^-$ solely in the presence of guanine nucleotides may give rise to a further increase in the equilibrium concentration of intravesicular $^{125}I^-$. As expected for right-side out vesicles, no effects of GTP[S] were observed when added after vesicle resealing (not shown), demonstrating that GTP[S] acts on the cytoplasmic side of the membrane.

To further substantiate our finding that GTP[S] increases Cl^--conductance, we measured Cl^--uptake using the chloride sensitive fluorescent probe SPQ. Recent studies have revealed that this water-soluble dye, whose fluorescence is quenched in the presence of Cl^-, is a suitable and sensitive probe for chloride transport

assays in biological systems [15]. As shown for rat small intestinal brushborder membrane vesicles (Fig.2), addition of KCl (25 mM final concentration) to SPQ-loaded vesicles rapidly quenches fluorescence, indicative of an increasing intravesicular Cl^--concentration. In the presence of valinomycin, i.e. when generation of a counteracting membrane potential is inhibited, SPQ quenching is much faster in GTP[S]-loaded relative to control vesicles (Fig.2 and Table 1). Apparently, the GTP[S]-sensitive Cl^- conductance is not confined to human colon carcinoma cells, but occurs also in the apical membranes of non-neoplastic enterocytes. Qualitatively similar results were obtained with vesicle preparations from cultured HT-29cl.19A cells (not shown). Importantly, no difference between control and GTP[S]-containing vesicles was observed in the absence of valinomycin (Fig.2), indicating that increased Cl^--conductance is not secondary to GTP-induced opening of K^+-channels. The notion that GTP-binding proteins may be involved in stimulating Cl^--conductance is further supported by our observation that GTP-induced acceleration of transmembrane Cl^--influxes is diminished in the presence of trapped GDP[S], a well known inhibitor of GTP-binding proteins (Table 1).

Table 1.

Effects of GDP[S] on GTP[S]-induced transmembrane Cl^- fluxes. SPQ-quenching experiments were performed as described in 'Materials and Methods'. Rate constants (k_1) were calculated and expressed as percentage relative to control preparations (mean ± SEM for 3 independent experiments). k_1 in control preparations was 0.6 ± 0.1 min^{-1}. Concentrations used: GDP[S], 5 mM and GTP[S], 100 μM. Asterisk indicates $P < 0.05$ relative to the control.

	k_1 of SPQ quenching (% of control)
Control	100
GDP[S]	150 ± 11*
GTP[S]	316 ± 15*
GDP[S] + GTP[S]	209 ± 8*

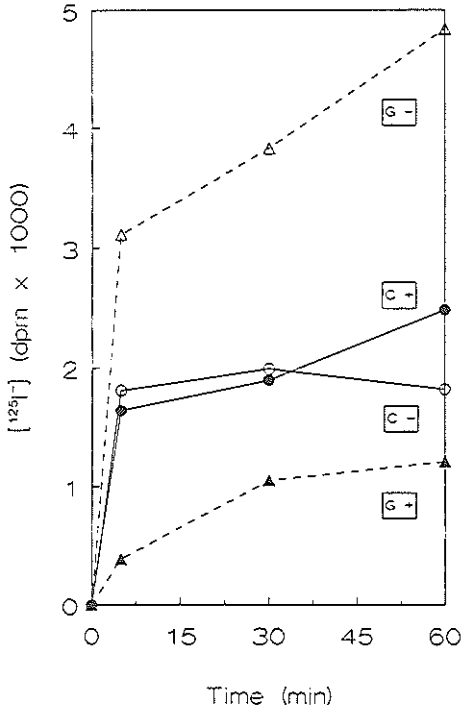


Figure 1.

[^{125}I]uptake in apical membrane vesicles from HT-29cl.19A colonocytes. Time-course of radiolabelled iodine uptake in control (O,●) and 100 μM GTP[S]-containing (△,▲) vesicles in the absence (open symbols) and presence (closed symbols) of valinomycin (10 μM). Open square represents inhibition of the GTP[S] response by GDP[S] (2 mM). Data points are all derived from one representative experiment (n = 3).

We have studied the effects of various nucleotides by trapping equimolar (100 μM) concentrations into rat intestinal brushborder membranes vesicles. As clearly shown in Table 2, non-hydrolysable GTP-analogues such as GTP[S] and GMP-PNP are potent activators of conductive Cl^--influx. GTP activates chloride transport also, but only when GTPase inhibitor vanadate [19] is present (Table 2). Other guanine nucleotides, including GMP and GDP, were always ineffective. A small increase in SPQ-quenching, however, was observed in GDP[S]-containing vesicles (Table 2), suggesting the possible existence of a channel inhibitory G-protein in brushborder membranes. ATP (1 mM) or ATP[S] (200 μM) alone did not induce Cl^--influx (Table 2), nor did it potentiate the response to GTP[S] (not shown). This suggests that protein phosphorylation and/or ATP-binding is not essential for a GTP-mediated increase in Cl^- conductance. In the presence of 1

mM ATP, cAMP was found to stimulate transmembrane ion fluxes additional to the response to GTP[S] (Table 2), most likely through protein kinase A mediated protein phosphorylation [1].

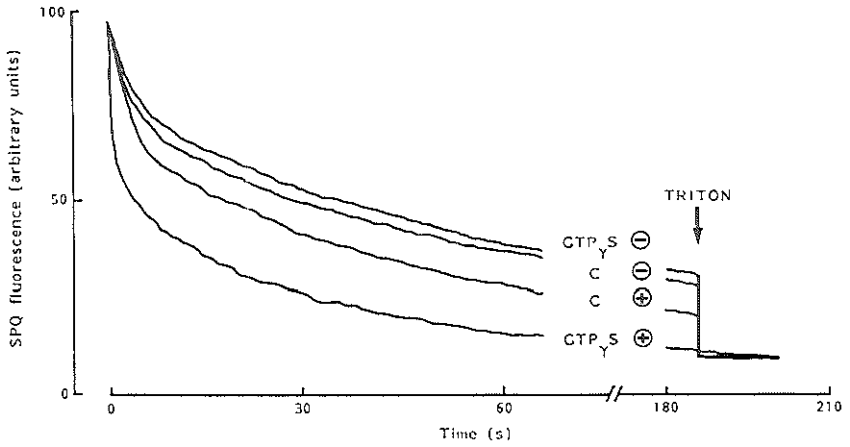


Figure 2.

Rate of SPQ quenching. Cl^--influx in control (C) and GTP[S]-containing rat brushborder membrane vesicles was determined in the absence (-) or presence (+) of valinomycin (10 μM) using the Cl^--sensitive probe SPQ.

Chloride selective anion channels have been previously studied in detail in various human tissues, including nasal polyps [20], tracheal cells [21, 22], sweat gland duct cells [3] and renal epithelium [11], as well as in a number of tumour cell lines such as T84 [23] and HT29 intestinal cells [24]. We have studied the biophysical characteristics of GTP[S]-activated Cl^--channels in HT-29cl.19A colonocytes using patch clamp analysis, enabling a comparison with the well documented cAMP-activated Cl^--channel.

Upon exposure of inside-out apical membrane patches to 10 μM GTP[S], highly frequent channel openings were observed within 2 - 20 minutes (Fig.3A). The channels were anion-selective ($P_{Cl}/P_{Na} > 8$), as determined by changing the ionic composition of bathing solutions, and showed the permeability sequence $NO_3^- >$

$Br^- \simeq I^- > Cl^- > F^-$. Since addition of GDP[S] (2 mM, Fig. 3A) blocks the channel rapidly, we suggest that they are identical to those involved in the GTP[S]-induced increase in transmembrane Cl^--flux (c.f. Fig. 1 and 2). GTP-activated Cl^--channels display an average single channel conductance of 20 ± 4 pS and exert a prominent, inward rectification (Fig.3B,C). At physiological membrane potential, the open-state probability (P_o) is 0.81 ± 0.09 (n = 10), which tends to decrease by depolarization (Fig. 3D). Interestingly, this particular channel was only found after stimulation with GTP[S] and, in contrast to the cAMP/protein kinase A activated channel, never occurred spontaneously or following membrane depolarization.

Table 2.

Effects of guanine nucleotides on SPQ quenching. Various guaninine nucleotides (100 μM), ATP[S] (200 μM) and cAMP (1 mM) were trapped into rat brushborder membrane vesicles as described in Figure 2. Effects of cAMP ± GTP[S] were tested in the presence of 1 mM ATP. Rate of SPQ quenching was determined as described under 'Materials and Methods' and expressed as mean ± SEM. Number of independent experiments is given between parentheses. Asterisk indicates $P < 0.05$ relative to the control.

	k_1 of SPQ quenching (min^{-1})
Control	0.64 ± 0.08 (10)
ATP (1 mM)	0.70 ± 0.09 (5)
ATP[S]	0.69 ± 0.17 (3)
cAMP	1.63 ± 0.10 (3)*
GDP	0.65 ± 0.02 (3)
GDP[S]	0.90 ± 0.05 (3)*
GTP	1.08 ± 0.18 (3)*
GTP - Vanadate	0.69 ± 0.17 (3)
GTP[S]	1.52 ± 0.20 (10)*
GTP[S] + cAMP	2.17 ± 0.12 (2)*
GMP-PNP	2.03 (1)

Electrical properties of the GTP[S]-activated channels differ markedly from the cAMP/protein kinase A activated Cl^--channels, which have a somewhat larger unitary conductance and, most importantly, were found to be outward rectifying [21-24]. This suggests that, in addition to cAMP-activated channels, a different class of Cl^--selective channels exists, which are coupled to G-proteins. This notion is supported by our observation that, in vesicle preparations, co-stimulation with GTP[S] (100 μM) and cAMP (1 mM), in the presence of ATP, results in an additional rather than synergistic, increase in the rate of SPQ quenching (Table 2).

DISCUSSION.

The co-existence of an activating G-protein and ion channel in the apical membrane of a polarized epithelium is not unprecedented: the activity of amiloride-sensitive Na^+-channels and of Cl^--channels in renal apical membranes has likewise shown to be regulated by GTP and GTP[S], presumably acting through $\alpha_{i,3}$ [9-11]. Although the renal Cl^--channel has characteristics very different from the GTP[S]-activated channel in intestinal epithelia (e.g. single channel conductance of 305 pS, activation by protein kinase C and Ca^{2+}, frequent spontaneous openings [11]), these findings suggest a more wide spread role for G-protein operated anion channels in transepithelial ion fluxes. Presently, little is known about the identity of the channel-associated G-protein or about its cellular activators. According to the classical concept of cholera toxin-provoked intestinal salt and water secretion, the A_1 subunit of the toxin is released at the apical membrane and, upon translocation to the basolateral membrane, activates adenylate cyclase through ADP-ribosylation of a basolateral pool of the stimulatory G-protein G_s [25]. Subsequently, cAMP triggers the opening of apical Cl^--channels through protein kinase A-mediated phosphorylation [1]. The recent demonstration of a distinct pool of G_s (G_s^{ap}) in the apical membranes [26] has led to the speculation that α_s^{ap}, rather than A_1, translocates to the basolateral membrane following its ADP-ribosylation at the apical border [25, 26].

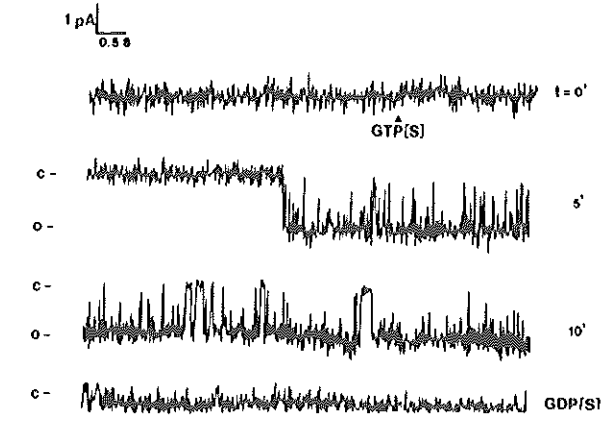
Alternatively, it remains possible that the inward-rectifyer functions as a stress- or swelling-activated Cl^--channel recently identifies in epithelial tissues, including intestine [27, 28].

Coupling of the G-protein to the Cl^--channel may either be direct, as shown

previously for the action of several hormones and neurotransmitters on cation channels [7-11], or indirect, possibly through locally produced autacoids, as demonstrated earlier for cardiac muscarinic K^+-channels [29, 30]. Our experimental conditions however, exclude the possibility that GTP[S] acts indirectly through the generation of cytosolic messengers such as cAMP and Ca^{2+}. Furthermore, the co-localization of the GTP-receptor and the Cl^--channel in the apical membrane patches and vesicles virtually rules out a channel recruitment model in which the G-protein, analogous to the G_E postulated for exocytosis [31], would trigger the fusion of channel-containing vesicles with the apical membrane (c.f. ref. [32]). As yet, no information is available about the molecular structure of GTP-activated Cl^--channels. A putative structure of the cAMP/protein kinase A-activated Cl^--channel has recently been proposed from the nucleotide sequence of CFTR, the cystic fibrosis transmembrane conductance regulator [33]. Because CFTR contains two nucleotide binding folds [33], capable of binding both ATP and GTP [34], we cannot exclude that GTP[S] increases Cl^--conductance through CFTR.

Figure 3.

Patch clamp analysis of GTP[S]-activated Cl^--channels. A) Activation of Cl^--channels by GTP[S] (10 μM) in apical membrane patches obtained from HT-29cl.19A cells. Closed (c) and open (o) states of the channel are indicated. GDP[S] represents a recording of a GTP[S] activated channel 2 minutes after addition of 2 mM GDP[S]. B) Patch clamp recordings of a GTP[S]-stimulated Cl^--channel at various holding potentials. C) Voltage/current relationship of GTP[S] activated Cl^--channels. D) Open state probability of GTP[S] activated Cl^--channels. Data are expressed either as mean ± S.D. for 4 determinations or as the mean of a duplicate observation.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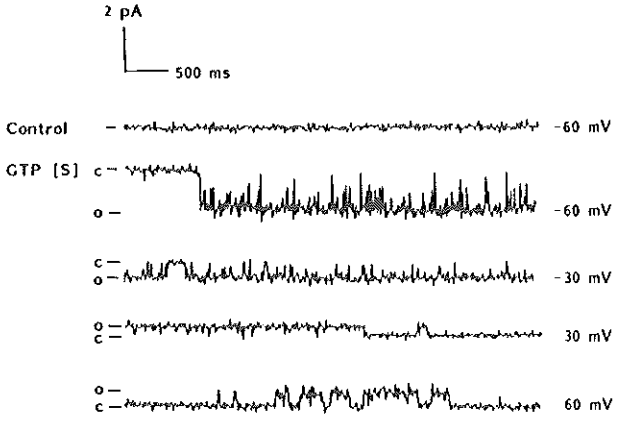
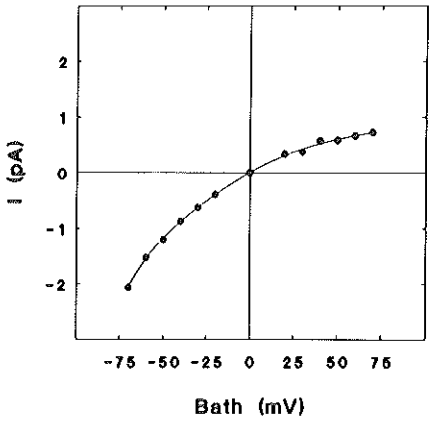
0.5 s
t = 0'
GTP[S]
c -
o -
5'
10'
GDP[S]

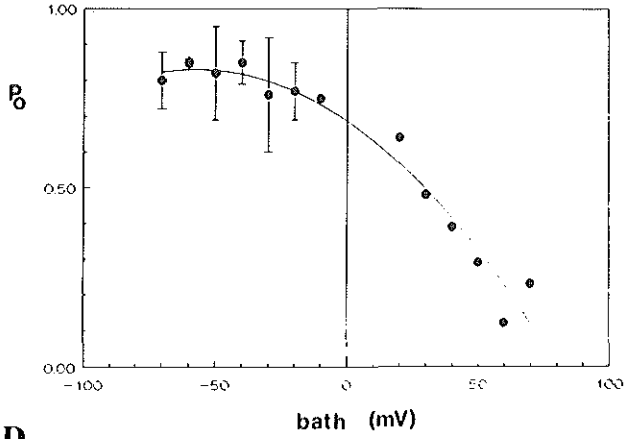
A


2 pA
500 ms
Control
GTP [S]
-60 mV
-30 mV
30 mV
60 mV

B


I (pA)
Bath (mV)

C


Po
bath (mV)

D

However, in view of the prominent differences between cAMP- and GTP[S]-sensitive Cl^--channels (I/V characteristic, voltage activation; c.f. ref [1]), existence of a different class of anion-selective channels seems more likely. Interestingly, an apically localized, inwardly rectifying Cl^--channel has recently been reported for gastric oxyntic cells in amphibians [35]. Although this particular channel has electrical characteristics very similar to the GTP[S]-activated channels, they are certainly not identical, since marked differences exist in sensitivity for cAMP-dependent protein phosphorylation and membrane depolarization. Therefore, it is important to establish whether or not G-protein coupled Cl^--channels are functional in cystic fibrosis patients, because the presence of functional GTP-activated channels may imply a possible route for bypassing the defect in cAMP-, cGMP and Ca^{2+} activation of Cl^- secretion [2, 36-38], most plausibly the primary lesion in this disease.

Acknowledgements: The authors wish to thank Dr.C.Laboisse, Unite 239, INSERM, Paris, France for his generous gift of the HT-29cl.19A human colon carcinoma cell line and L.G.J.Tertoolen, Hubrecht Laboratory, Utrecht, the Netherlands for help with the analysis of SPQ data. B.C.T. and P.G.M.G. were supported by the Cystic Fibrosis Foundation (U.S.A.). N.v.d.B. and J.B. were supported by N.W.O., the Netherlands and the Royal Dutch Academy of Sciences, respectively.

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FOOTNOTES

1. N.v.d.Berghe et al., in preparation.

2. Abbreviations used: ATP[S], adenosine 5'-O-(3-thiophosphate); CFTR, Cystic Fibrosis transmembrane conductance regulator; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GDP[S], guanosine 5'-O-(2-thiophosphate); GMP-PNP, guanylyl-imidodiphosphate; GTP[S], guanosine 5'-O-(3-thiophosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenyl-methyl-sulfonyl-fluoride; P_o, open time probability; P_{Cl}, channel permeability to Cl^--ions; P_I, channel permeability to I^--ions; P_{Na}, channel permeability to Na^+-ions; SPQ, 6-methoxy-N-(3-sulfopropyl)-quinolinium.

NOTE:

This chapter has been published as:

G-proteins mediate intestinal chloride channel activation.

Tilly, B.C., Kansen, M., Van Gageldonk, P.G.M., Van den Berghe, N., Galjaard, H., Bijman, J. and De Jonge, H.R.

J. Biol. Chem. 266 (1991): 2036-2040.

CHAPTER VII.

EFFECTS OF PUTATIVE TRANSPORT INHIBITORS ON INTESTINAL Cl^- AND K^+ TRANSPORT PATHWAYS.

ABSTRACT.

Several putative inhibitors on Cl^- and K^+ transport pathways and outwardly rectifying Cl^- channels were tested in HT29.cl19A human colon carcinoma cells. The multi-drug resistance (MDR) substrate (colchicine) and -blocker (verapamil) did neither inhibit the forskolin or the A23187 stimulated $^{125}I^-$- or the $^{86}Rb^+$ efflux, but reduced the open probability of single Cl^- channels without affecting the conductance. The organic anion transport blocker probenecid and the epithelial Cl^- channel blocker NPPB inhibited the $^{86}Rb^+$- but not the $^{125}I^-$ efflux and blocked the outwardly rectifying Cl^- channel in a similar way as the MDR-compounds. The inhibitory effects at the single ion channel level were reversible and dose-dependent with IC_{50}-values ranging from 5 to 250 μM.

INTRODUCTION.

Apical membrane chloride channels are involved in transepithelial chloride secretion in various epithelial tissues like sweat glands, airway and intestine. Activation of these channels may occur through a number of second messengers including Ca^{2+}, cAMP, cGMP, diacylglycerol and Ca^{2+}-calmodulin (7, 31 , 38, 39). Patch-clamp experiments showed that different types of chloride channels are expressed in epithelial cells (11). An outwardly rectifying chloride channel of 25-50 pS has been extensively studied for its possible role in the inhereted disease cystic fibrosis (40).

The glycoprotein P-gp is an ATP-driven pump capable of transporting cytostatic drugs out of the cell. An increased expression of P-gp in tumor cells can result in the phenomenon of multi-drug resistance (MDR) in cancer therapy (10). P-gp is, like the the outwardly rectifying chloride channel, expressed in the apical membrane of epithelial cells (34). Expression and localization of P-gp in normal cells suggests a role in transepithelial secretion of hydrophobic toxic substances and unknown cell metabolites or in an alternative pathway for secretion of

substances which have no formal signal sequence for secretion (22, 28).
In a number of cells including polarized epithelial cells (17, 18, 20, 26, 32) organic anion transporters have been identified which could be inhibited by probenecid. The physiological role of these transporters is not known, but they are probably involved in transport of prostaglandins and leukotrienes (24, 32), urate (20) and cholate or cAMP (17). Cyclic AMP and the arachidonic acid metabolites prostaglandins and leukotrienes are known for their role in epithelial Cl^--transport (9, 24, 36); arachidonic acid itself inhibits the outward rectifyer in excised patches (1, 19).
In order to examine whether the P-gp substrates colchicine and vinblastine and the MDR-inhibitor verapamil as well as the anion transport blocker probenecid would affect Cl^--transport and the outwardly rectifying Cl^- channel (OR) in particular, we tested the P-gp substrates vinblastine and colchicine, the MDR inhibitor verapamil and the organic anion transporter probenecid on the Cl^- and K^+ transport in HT29.cl19A colonocytes (2, 3) by the isotopic efflux- and patch-clamp technique. The results were compared with the effects of the classical epithelial chloride channel blocker NPPB (8, 25).

MATERIALS AND METHODS.

Materials.

HT29.cl19A cells were kindly donated by Dr. C.L. Laboisse (INSERM, Paris, France). Colchicine, vinblastine, verapamil, forskolin, carbachol and A23187 were obtained from Sigma (St Louis, USA]. NPPB [5-nitro-2-(3-phenylpropylamino)benzoate] was a gift of Dr. R. Greger (Freiburg, F.R.G.); probenecid [p-(dipropylsulfamoyl)benzoic acid] was from Merck, Sharp and Dohme. $^{86}RbCl$ (5 mCi/mg) and $Na^{125}I$ (14 Ci/mg) were purchased from Amersham (UK).

Cell culture.

HT29.cl19A human colon carcinoma cells were grown on plastic petri dishes in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum (Gibco), 40 mg/ml penicillin and 90 mg/ml streptomycin in 5% CO_2 in 37°C. Cells were harvested with 0,5 mg/ml trypsin and 1 mM EDTA.

Isotope efflux experiments.

HT29.cl19A cells were grown to confluency in 6 well cell culture plates (Nunc) and loaded for 90 minutes at 37°C in 0.5 ml modified Meyler solution (in mM, pH 7.4: 108 NaCL, 4.7 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 20 $NaHCO_3$, 0,8 Na_2HPO_4, 0.4 NaH_2PO_4, 20 Na-HEPES, 10 glucose) 1 μCi/ml $^{86}Rb^+$ and 3 μCi/ml $^{125}I^-$. The extracellular isotope was removed within 1 min with 3 washes of 3 ml modified Meyler solution at room temperature. The isotope efflux was measured at 37°C by addition and consecutive replacement of 1 ml modified Meyler at 1 to 2 min intervals. The residual isotope at the end of the experiment was determined after addition of 1 ml 1 M NaOH. $^{125}I^-$ and $^{86}Rb^+$ were measured simultaneously with by gamma-scintillation counting.

Forskolin (10^{-5} M) and the Ca^{2+}-ionophore A23187 (10^{-5} M) were added 4.5 min after the start of the efflux experiment. Drug effects were tested by pre-incubating for 30 min in 0.1 mM (colchicine and verapamil), 2.5 mM (probenecid) and 0.05 mM (NPPB).

Data were expressed as the fractional efflux per minute i.e. the amount of isotope lost from the cells in the interval preceding a time point expressed as a percentage of the amount of isotope associated with the cell layer at the beginning of that interval divided by the length of that interval (6).

Patch-clamp recordings.

Excised inside out patches were made according to Hamill et al. (15). Outwardly rectifying chloride channels were activated by depolarization i.e. switching from -70 mV to 70 mV every 5 sec., after which the tested drugs were added to the bath (cytoplasmic) solution. For monitoring their effect from the extracellular side, they were added to the pipette solution. Dilutions were made from 10^{-2} M stock solutions in DMSO (NPPB, probenecid) and ethanol (colchicine and verapamil). Ethanol (0.5% v/v) and DMSO (0.2% v/v) alone had no effect on the channel activity.

Kinetic analyses with sampling- and filter frequency 1 kHz and 0.5 kHz respectively were made off line with software developed in our labaratory on a Tulip 386SX computer. Drug effects were studied with respect to the single channel amplitude as well as to the open probability (Po), the latter defined as the ratio of open time of the channel over the total time recorded. Po was only

calculated if not more than one channel was present in the patch (in about 35% of the pathes). The sign of the clamp potential refers to the bath with respect to the patch pipette. Positive (upward) currents are Cl^--ions flowing out of the pipette into the bath.

Solutions in mM pH 7.4: 150 NaCl, 5 KCl, 5 HEPES, 1 $MgCl_2$, 1 $CaCl_2$ (pipette and bath-solution) or 1.2 $MgCl_2$ and 0.16 $CaCl_2$ (10^{-8} M Ca^{2+}, bath-solution). Experiments were performed at room temperature.

RESULTS.

MDR-substrates and an MDR-blocker inhibit chloride channels.

Pre-incubation with the MDR-substrate colchicine (10^{-4} M) did neither affect the forskolin- nor the A23187-stimulated $^{125}I^-$-and $^{86}Rb^+$-efflux (data not shown). However, colchicine in the bath solution of excised inside-out patches at 50 mV induced channel flickering (Fig. 1) followed by a complete inhibition at 1 mM with a half-maximal inhibitory concentration (IC_{50}) of 250 μM (Table 1). The amplitude (about 1.7 pA) was unaffected (Fig. 1). Colchicine present in the patch pipette decreased the open probability as well, but inhibitory effects started at a higher concentration (10^{-4} M) and were seen immediately with the appearance of the channel. The specificity of the blocking effect of colchicine is demonstrated by the absence of inhibitory effects on other channels like a 20 pS non-selective cation channel. Vinblastine (10^{-6} M to 10^{-3} M), vincristine (10^{-5} M to 10^{-3} M) and actinomycin D (10^{-6} M and 10^{-5} M) had comparable effects (data not shown).

Verapamil, a known inhibitor of MDR-1, was tested in a comparable way as colchicine. At concentrations up to 10^{-4} M no significant inhibition of the forskolin and Ca^{2+}-ionophore stimulate $^{125}I^-$ and $^{86}Rb^+$-efflux was found. Its inhibitory effects on the OR resembled those of colchicine with a IC_{50}= 90 μM. The inhibitory effects of verapamil as well as the MDR-substrates were reversible and did not show a measurable lag time.

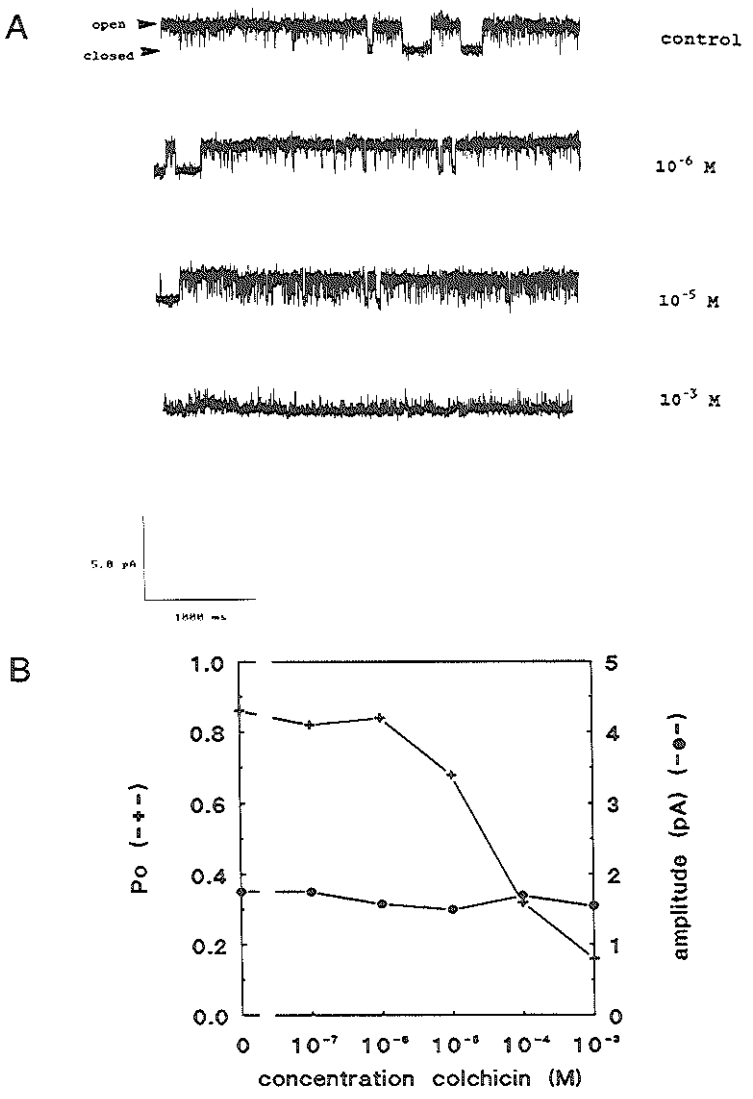

Figure 1.

Effect of colchicine on outwardly rectifying chloride channel in HT29.cl19A cells.

(A) Recording; (B) open probability (Po, -+-) and amplitude (-●-) at a holding potential of 30 mV at increasing concentrations of colchicine. Arrows indicate the open and closed state of the channel.

Table 1.

IC_{50}-values of the MDR-substrates colchicine and vinblastine, the MDR inhibitor verapamil, the organic anion transport blocker probenecid and the epithelial chloride channel blocker NPPB on the open probability of the outward rectifyer of HT29.cl19A cells following their addition to the cytoplasmic side of excised inside-out membrane patches.

Compound tested	IC_{50} (μM)
colchicine	250
vinblastine	>100
verapamil	90
probenecid	170
NPPB	9

Effect of probenecid on Cl^- and K^+ transport.

Pre-incubation for 20 min with 2.5 mM probenecid had no effect on the forskolin stimulated $^{125}I^-$-efflux but inhibited the efflux of $^{86}Rb^+$ almost completely (Fig. 2). In contrast, the same concentration of probenecid reduced the maximum carbachol and A23187 stimulated $^{125}I^-$-efflux by only 20%. The inhibition of the $^{86}Rb^+$-efflux at maximal stimulation with A23187 and forskolin was 57% and 65% respectively. Therefore, in spite of its known action as an anion transport blocker, probenecid seems to inhibit potassium efflux pathways rather than chloride channels in intestinal epithelial cells.

Nevertheless, we also tested the possible effects of probenecid on the activity of the outwardly rectifying chloride channel after voltage activation by depolarization in excised inside-out patches. While the amplitude at 30 mV remained about 1.5 pA, the open probability decreased from 0.85 in the control experiment (no probenecid) to 0.32 in 0.25 mM probenecid (Fig. 3) This dose-dependent inhibition with IC_{50}=190 μM was instantaneous and reversible.

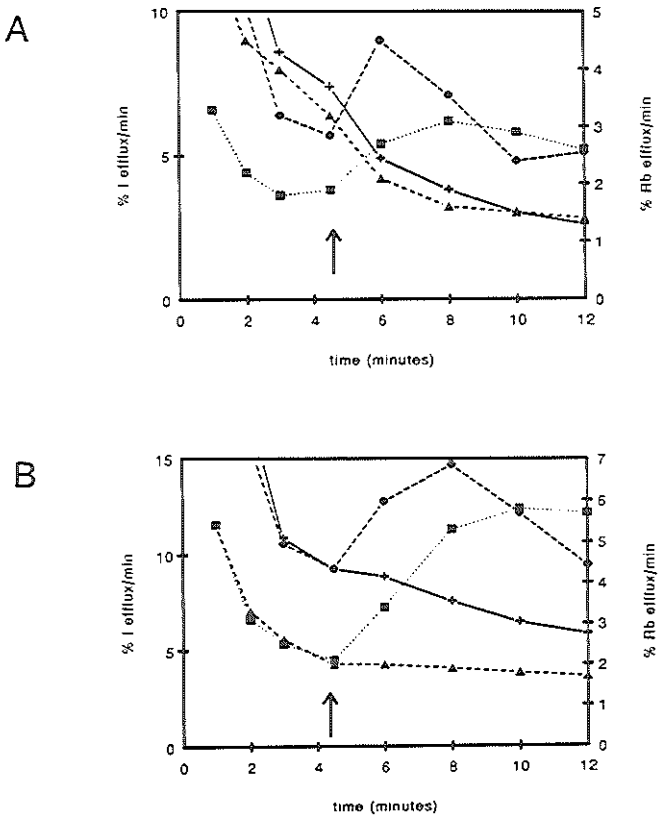

Figure 2.

Effect of the organic anion transport blocker probenecid on $^{125}I^-$ and $^{86}Rb^+$ efflux after stimulation with A23187 (A) and forskolin (B). HT29.cl19A colonocytes were cultured to confluency on plastic wells. Forskolin and A23187 were included in the efflux media 4.5 min after the start of the efflux experiment as indicated by the arrow. Probenecid (2.5 mM) was added to the isotope loading buffer 25 min before the start of the experiment and was included in the efflux media. (A): -+- $^{125}I^-$ A23187 (control), -●- $^{125}I^-$ A23187 + probenecid,-▲- $^{86}Rb^+$ A23187 (control), -■- Rb^+ A23187 + probenecid.(B): -+- $^{125}I^-$ forskolin (control), -●- $^{125}I^-$ forskolin + probenecid, -▲- $^{86}Rb^+$ forskolin (control), -■- $^{86}Rb^+$ forskolin + probenecid.

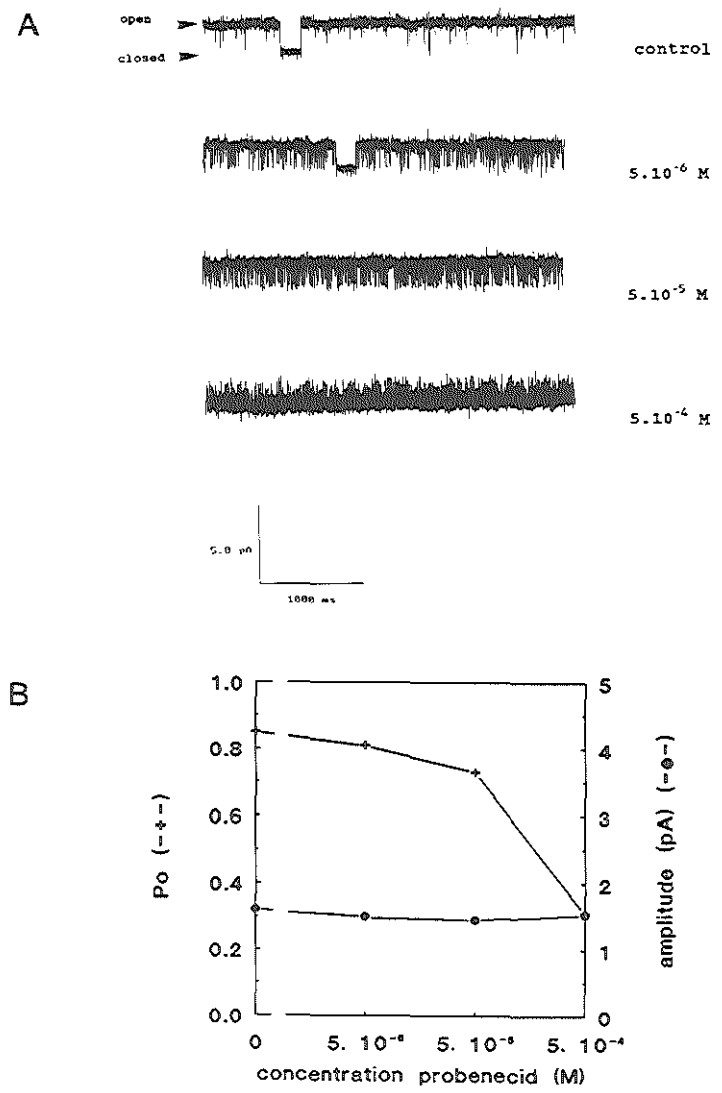

Figure 3.

Effect of probenecid on the outward rectifyer. (A) Recording; (B) open probability (Po, -+-) and amplitude (-●-) at a holding potential of 30 mV at increasing concentrations of probenecid in the bath solution. Arrows indicate the open and closed state of the channel.

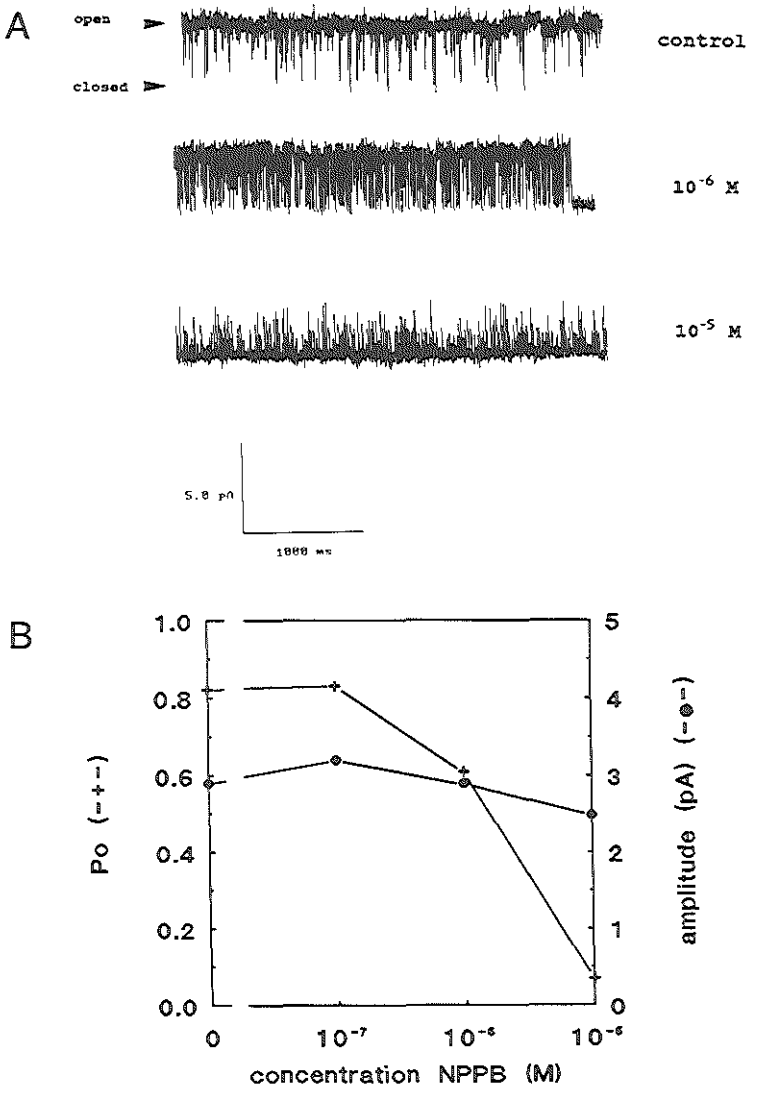

Figure 4.

Effect of NPPB on the outward rectifyer. (A) Recording; (B) open probability (Po, -+-) and the amplitude (-o-) at a holding potential of 30 mV at increasing concentrations of NPPB present at the cytosolic side. Arrows indicate the open and closed state of the channel.

Effect of NPPB on Cl^-- and K^+-transport.

The inhibitory effects of the MDR-substrates and -blockers as well as of probenecid were compared with the putative inhibitory effects of NPPB and DIDS on epithelial Cl^- channels. Pre-incubation with 50 μM NPPB for 30 min caused a decrease of the Ca^{2+}-ionophore stimulated $^{125}I^-$-efflux with 20%, but shared hardly any effect on the forskolin stimulated efflux. However, the forskolin and A23187 induced $^{86}Rb^+$-efflux was reduced by 50% and 65% respectively (53). In excised patches, NPPB induced a reversible inhibition of the OR with IC_{50}=7 μM without exerting a significant effect on the channel conductance (Fig. 4).

DISCUSSION.

The characteristics shared by all compounds tested in this study is that they show reversible inhibition of the outwardly rectifying Cl^- channel when applied to the cytoplasmic side of the membrane patch and that they have no effect on the single channel conductance but induce channel flickering and may therefore be classified as "intermediate" ion channel blockers (11). The absence of effects on a non-selective cation channel indicates to some degree of specificity in their action.

The IC_{50} of 250 μM for the colchicine inhibition is of the same order of magnitude as the inhibitory effects of colchicine on vinblastine transport sharing a IC_{50} of 160 μM (13).

The reversibility of the inhibition and the IC_{50} (90 μM) value of verapamil is in agreement with similar data reported for outside- out excised patches of the HT29D4 subclone (IC_{50}=100 μM, ref. 5).

Probenecid blocked the outward rectifyer with a IC_{50} of 170 μM, somewhat higher than the value reported for the inhibition of an anion pump sharing a IC_{50} of 110 μm (17), but much lower than the IC_{50} (1-2 mM) for the organic anion transporter in macrophages (33).

NPPB inhibited the Cl^--conductance in a number of epithelial tissues based on measurements of either single channel activity or intracellular electric parameters (9, 14, 16, 23, 25, 27). However, inhibition of a K^+-conductance and of the Ca^{2+}-dependent non-selective cation channel were reported as well (12, 29). Isotopic efflux experiments and micro electrode studies demonstrated that NPPB indeed inhibites a K^+-rather than a Cl^+-conductance (4, 35, 37). But addition of NPPB

to the bath solution of excised inside out patches showed a reversible inhibition of the outwardly rectifying chloride channel as has been reported earlier (5, 8, 16). The IC_{50} value was 7 μM, in agreement with airway epithelia (23, 25) but somewhat lower than in the HT29 subclone D4 (IC_{50}=20 μM, ref. 5).

HT29.cl19A colon carcinoma cells are a convenient experimental system for studying epithelial Cl^- secretion in intact epithelia monitored by means of the isotope efflux technique (35). However, neither the MDR substrate colchicine nor the MDR inhibitor verapamil had any effect on the Ca^{2+}- or the cAMP-stimulated $^{125}I^-$- and $^{86}Rb^+$-efflux, whereas probenecid and NPPB inhibited mainly the $^{86}Rb^+$-efflux in this cell line. The discrepancy with the inhibitory action of these compounds on Cl^- channels in excised patches is unlikely to be due to the inability of the drugs to accumulate to the cell interior: addition of colchicine and verapamil to the patch exterior in the same concentration as added to intact cells in the isotopic efflux assay (10^{-4} M), again resulted in acute inhibition of the OR sharing similar blocking kinetics as provoked by the drugs added in lower concentrations at the cytoplasmic side of the patch. According to a more likely explanation, the outwardly rectifying Cl^- channel does not participate in the Ca^{2+}- and cAMP-stimulated Cl^- secretion in intact cells. In accordance with this assumption, we were unable to observe the OR following cAMP stimulation in cell-attached patches (M. Kansen, submitted). In contrast, following introduction of the CF-gene in a non-epithelial cell line, cAMP stimulation resulted in the appearance of a 8 pS Cl^- channel (21). These observations suggest that a Cl^- channel different from the outward rectifyer is involved in Cl^- secretion. A possible role for the outward rectifyer in volume regulation has been suggested (41). It would be of interest to verify the effects of the blocker effects on the small Cl^- channel. The absence of any effect of colchicine and verapamil on the cAMP-activated $^{125}I^-$ efflux would suggest that these compounds do not affect the 5-10 pS Cl^- channel. MDR-compounds are are therefore unlikely to exert major effects on CFTR-activity, despite the fact that the MDR- and the CF-gene product share considerable structural homology (30).

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NOTE ADDED IN PROOF:

At the CF-conference in October 1991, Dallas, USA, Higgins and Sepúlveda reported that overexpression of MDR in 3T3 fibroblasts resulted in the appearance of a volume-regulated Cl^--conductance. In whole-cell patch clamp experiments, this conductance displayed an outwardly rectifying I/V-characteristic and could be blocked by verapamil. The possible identity of the OR in our intestinal cell lines as MDR-1 is presently verified by single channel patch clamp and isotopic efflux experiments using MDR-1 directed inhibitory monoclonal antibodies.

REFERENCE:

Higgins, Ch.F., Gill, D.R., Hyde, S.C., Gibson, M.M., Tucker, S., Valverde, M.A. and Sepúlveda, P. (1991). Chloride channel activity associated with the human multidrug resistance P-glycoprotein.
(abstract). Late-breaking science session at the North American Cystic Fibrosis Conference, Dallas, Texas, USA, October 2-5, 1991.

SUMMARY.

This thesis deals with the measurement of chloride transport in epthelial cells of normal (N) individuals and patients with the lethal, hereditary disease cystic fibrosis (CF). Chapter I describes the clinical, physiological and genetic characteristics of CF. CF is the most common fatal genetic disease among the Caucasian population with an incidence of about 1 in 2500 and a median age of survival of about 26 years.

The gene responsible for CF, localized on chromosome 7, has been identified in 1989. It encodes a transmembrane protein, called CFTR, resembling members of a family of transport molecules such as the multi-drug resistance (MDR) gene product. The most frequent mutation in CF is the deletion of a phenylalanine at position 508. The identification of the CF gene and its mutations allows prenatal diagnosis and carrier testing. CF is clinically characterized by functional disturbances in various organs such as the lungs, the pancreas, the intestine and the sweat glands, probably caused by abnormalities in fluid and electrolyte transport in exocrine epithelia. The most consistent biochemical observation to explain these abnormalities is a defect in the Cl^- transport across epithelial cells. Therefore much cell physiological research has focused on the topic of Cl^- transport. An important role in the secretion of fluid is played by chloride channel proteins present in the apical membrane of epithelial cells. In secretory epithelia, opening of these channels is necessary for the exit of Cl^- out of the cell. Sodium and water follow passively. In N cells, chloride channels are regulated by various second messengers such as Ca^{2+} and cAMP. Calcium, but not cAMP could stimulate Cl^- secretion in CF epithelial cells. Special attention has been paid to the biophysical description and the mode of regulation of a 30 pS outwardly rectifying chloride channel (OR). This channel has been extensively characterized in this study.

The function of CFTR still has to be elucidated, although recent transfection experiments suggest that CFTR may act as a chloride channel itself.

Chapter II is an introduction to the methods used to measure chloride transport. They include the detection of macroscopic Cl^- currents with the Ussing chamber, an isotopic efflux assay ($^{125}I^-$) and the use of a Cl^--sensitive fluorescent indicator

(SPQ) and of microscopic Cl^--currents with the patch-clamp technique. The (dis) advantages of the various techniques are discussed.

In our search for a epithelial cell type in culture suitable to analyse the defect in cAMP-mediated Cl^- secretion in CF at the molecular level, we first evaluated the potential value of cultured keratinocytes as a model for CF (Chapter III). But surprisingly, normal keratinocytes failed to express cAMP-dependent Cl^- transport pathways as concluded from short-circuit current measurements in Ussing chambers and $^{125}I^-$ and $^{36}Cl^-$ efflux studies. Activation of Cl^- channels, especially of the OR, in patch-clamp experiments could not be detected in cell-attached patches following ß-adrenergic stimulation nor in excised inside-out patches upon addition of protein kinase A and ATP. However Cl^- secretion was observed following a rise in intracellular Ca^{2+}. The absence of a cAMP-regulated Cl^- secretion in N keratinocytes made this cell type unsuitable as a a model to study the Cl^- transport defect in CF.

Also, keratinocytes can only be passaged to a limited extent and grown in limited quantities thereby urging us to use several cell batches to complete a study. An immortalized cell line would overcome this problem. In Chapter IV a SV40/Ad12 immortalized CF nasal polyp cell line is characterized with respect to its morphology, cytokeratin pattern and Cl^- transport. In Ussing-chambers, pre-crises cells showed a Ca^{2+}- but not a cAMP-activated Cl^- secretion as expected for CF cells. In the apical membrane of post-crises cells the outwardly rectifying Cl^- channel was present in excised inside-out patches, but could not be activated by the elevation of intracellular cAMP levels. These data indicate that this immortalized CF nasal polyp cell line is a useful model for the study of CF, both for electrophysiological and biochemical experiments aiming at the correction of the defect by introduction of the wild type CF-gene.

The human colon carcinoma cell line HT29.cl19A showed cAMP-dependent Cl^- currents in Ussing chamber experiments and was considered as a model system for Cl^- transport in normal epithelial cells. The outwardly rectifying Cl^- channel was studied in detail by means of the patch-clamp-technique (Chapter V). Spontaneous activation after excision was Ca^{2+} dependent and could be nullified by reducing the Ca^{2+} concentration at the cytoplasmic side from 10^{-3} M to 10^{-8} M. Protein kinase A, in the presence of ATP, was able to acivate the OR in about 40% of the excised patches. However, attempts to open this channel in cell-

attached patches under different experimental conditions were unsuccessful. Instead, an on-cell 6 pS linear Cl^- channel was detected in the presence, but not in the absence of the adenylate cyclase activator forskolin. This channel had no relation with the OR in excised patches and may be a suitable candidate as a physiological carrier of the cAMP dependent Cl^- current.

Isotopic ($^{125}I^-$)- and fluorescent (SPQ) measurements with plasma membrane vesicles from HT29.cl19A cells and rat small intestine showed that addition of GTP[S] induced a Cl^- current in the absence of other signalling molecules (Chapter VI). These results suggested that G-proteins are involved in the regulation of apical membrane chloride channels. To identify these channels at the molecular level, GTP[S] was also added to the cytoplasmic side of excised inside-out patches of HT29.cl19A colonocytes. A novel, inwardly rectifying (IR) Cl^- channel with a conductance of about 20 pS was observed. The presence of both the inwardly and the outwardly rectifying chloride channel in the same membrane patch suggests that they represent two different types of chloride channels. The physiological role and mode of activation of this IR, sofar found exclusively in intestinal epithelial cells, is presently unknown. Its presence and regulation in CF intestinal cells has to be elucidated.

The structural resemblance of CFTR to members of a family of transport molecules including the multi-drug resistance (MDR) gene product made it interesting to study whether substrates and blockers of MDR-1 could functionally interact with CFTR-dependent Cl^- transport pathway and in particular with the OR, considering its putative role in the Cl^--secretory defect in CF (Chapter VII). The MDR-substrate colchicine and the MDR-blocker verapamil were found not to affect the Ca^{2+}- or cAMP-stimulate Cl^- efflux in intact HT29.cl19A colonocytes, but reversibly inhibited the OR in excised inside-out patches both from the cytoplasmic and the extracellular side. The inhibitory effects of the tested MDR-substrates and blockers were reversible with a IC_{50} ranging from 5 to 250 μM. The outcome of these studies, together with the recent data from CFTR transfection studies indicating that CFTR may be a 5-10 pS Cl^- channel, argue strongly against a role of the OR in cAMP-provoked Cl^- secretion in epithelial cells and in the secretory defect in CF. Furthermore, because of the absence of any effect on the cAMP-activated Cl^- efflux, it is unlikely that MDR-compounds exert a major effect on CFTR-activity.

SAMENVATTING.

Dit proefschrift behandelt het meten van chloride transport in epitheel cellen van gezonde mensen (N) en van patienten met de lethale, erfelijke ziekte cystic fibrosis (CF). Hoofdstuk I beschrijft de klinische, fysiologische en genetische kenmerken van CF. Cystic fibrosis is de meest voorkomende fatale, erfelijke ziekte binnen de Kaukasische bevolking met een frekwentie van ongeveer 1 op 2500 nieuwgeborenen en een gemiddelde levensverwachting van plusminus 26 jaar.

Het gen dat verantwoordelijk is voor CF, gelegen op chromosoom 7, werd in 1989 gevonden. Het kodeert voor een transmembraan eiwit, CFTR genoemd, dat gelijkenis vertoont met leden van een familie van transport molekulen zoals het multi-drug resistance (MDR) genprodukt. De meest voorkomende mutatie in CF patienten is het ontbreken van het 508ste aminozuur, fenylalaline. Met de isolatie van het CF-gen en het vinden van de mutaties behoort prenatale diagnostiek zowel als het testen op dragerschap tot de mogelijkheden.

CF wordt klinisch gekarakteriseerd door verstoringen in verscheidene organen zoals de longen, de alvleesklier, de darm en de zweetklieren, veroorzaakt door afwijkingen in vloeistof- en elektroliet transport in exokriene epithelia. De meest konsistente biochemische observatie die deze afwijkingen zou kunnen verklaren is een defekt in het transport van Cl^--ionen in epitheelcellen. Daarom is veel celfysiologisch onderzoek gericht op het ontrafelen van Cl^--transport in N en CF epitheelcellen. Een belangrijke rol in de sekretie van vloeistof is weggelegd voor chloride kanalen: eiwitmolekulen die zich bevinden in de apikale membraan van epitheel cellen. In sekretoire epithelia zorgt de opening van deze kanalen ervoor dat Cl^- de cel verlaat. Natrium en water volgen dan passief. In N cellen worden chloride kanalen gereguleerd door verschillende second messengers zoals Ca^{2+} en cAMP. Het is nu gebleken dat Ca^{2+} wel, maar cAMP niet in staat is om Cl^- sekretie in CF epitheel cellen op gang te brengen. Speciale aandacht is in dit kader besteed aan de biofysische beschrijving en de wijze van regulatie van een 30 pS uitwaards rectificerend chloride kanaal (OR). Dit kanaal speelt dan ook een belangrijke rol in dit proefschrift.

De funktie van CFTR moet nog worden opgehelderd, maar recente transfektie experimenten suggereren dat CFTR wel eens het chloride kanaal zelf zou kunnen

zijn.

Hoofdstuk II is een inleiding in de technieken die in dit proefschrift gebruikt zijn om Cl^- transport te meten. Deze houden zowel het meten van makroskopische Cl^- stromen via de Ussing kamer, via een isotoop ($^{125}I^-$) efflux assay en via een Cl^--gevoelige fluorescente indikator (SPQ) in, als detektie van de mikroskopische Cl^- met behulp van de patch-clamp techniek. De voor- en nadelen van de verschillende methoden worden besproken.

In een poging om een geschikt celkweek model ter bestudering van het defekt in de cAMP-afhankelijke Cl^- sekretie in CF te vinden, werden gekweekte huid epitheel cellen (keratinocyten) op hun Cl^- sekreterend vermogen onderzocht (Hoofdstuk III). Normale keratinocyten vertoonden echter geen cAMP-afhankelijk Cl^- transport mechanisme zoals bleek uit Ussing-kamer en $^{125}I^-$ en $^{36}Cl^-$ efflux metingen. Chloride kanalen, speciaal de OR, konden niet worden geaktiveerd in patch-clamp experimenten in cell-attached patches na ß-adrenerge stimulering, noch in excised inside-out patches na toevoeging van protein kinase A en ATP. Cl^- sekretie kon daarentegen wel op gang gebracht worden na verhoging van de intracellulaire Ca^{2+} concentratie. De afwezigheid van een cAMP-gereguleerde Cl^- sekretie in N keratinocyten maakt dit celtype ongeschikt als model om het defect in het Cl^- transport in CF te bestuderen.

Keratinocyten kunnen slechts voor een beperkte periode gekweekt worden in geringe hoeveelheden, wat ons vaak dwingt om voor een enkele studie cellen van meerdere patienten te gebruiken. Met een geïmortaliseerde cellijn zou deze nadelen niet optreden. In hoofdstuk IV wordt een SV40\Ad12 geïmmortaliseerde CF neus poliep cellijn gekarakteriseerd aan de hand van de morfologie, het cytokeratine patroon en het Cl^- transport. In Ussing kamer experimenten bleken pre-crisis cellen wel een Ca^{2+}- maar geen cAMP-geaktiveerde Cl^- sekretie te bezitten, zoals verwacht mag worden voor CF cellen. In de apikale membraan van post-crisis neus poliep cellen konden we de OR detekteren, die niet te aktiveren bleek na verhoging van de intracellulaire hoeveelheid cAMP. Deze resultaten wijzen erop dat de geïmmortaliseerde CF neus poliep cellijn een geschikt model is om het defekt in CF elektrofysiologisch zowel als biochemisch te analyseren in experimenten die gericht zijn op het korrigeren van het CF-defekt na introduktie van het wild type CF-gen.

Uit Ussing-Kamer experimenten met de humane colon carcinoma cellijn

HT29.cl19A bleek dat cAMP Cl^- sekretie op gang bracht, waardoor deze cellijn als een model kon dienen voor het bestuderen van Cl^- transport in N cellen. Het uitwaards rectificerend chloride kanaal (OR) werd met behulp van de patch-clamp techniek gedetailleerd bestudeerd in (Hoofdstuk V). Spontane aktivatie na excisie was afhankelijk van de Ca^{2+} concentratie en kon worden voorkomen door deze aan de cytoplasmatische kant te verlagen van 10^{-3} M tot 10^{-8} M. Protein kinase A in de aanwezigheid van ATP was in staat om de OR in ongeveer 40% van de excised patches te aktiveren. Echter, pogingen om dit kanaal onder verschillende experimentele omstandigheden in cell-attached patches te aktiveren hadden geen sukses. In plaats daarvan bleek een klein lineair 6 pS chloride kanaal in cAMP-gestimuleerde cellen aanwezig te zijn dat in ongestimuleerde cellen afwezig was. Dit kleine kanaal vertoonde geen relatie met de OR en zou een goede kandidaat zijn voor de fysiologische drager van de cAMP-afhankelijke Cl^- stroom.

Uit isotoop ($^{125}I^-$)- en fluorescentie (SPQ)-metingen aan vesicles gemaakt van de plasmamembraan van HT29.cl19A cellen en van de dunne darm van de rat, bleek dat GTP[S] een Cl^- stroom teweeg bracht zonder dat andere signaalmolekulen aanwezig waren (Hoofdstuk VI). Deze resultaten suggereren dat G-eiwitten betrokken zijn bij de regulatie van chloride kanalen in de apikale membraan. Om dit kanaal op het molekulaire nivo te kunnen analyseren, voegden we GTP[S] toe aan de cytoplasmatische kant van excised inside-out patches van HT29.cl19A colonocyten. Een nieuw, inwaards rectificerend chloride kanaal (IR) met een konduktantie vam ongeveer 20 pS werd hierop aktief. De aanwezigheid van zowel het uitwaards als het inwaards rectificerend chloride kanaal in een en dezelfde membraan patch suggereert dat ze twee verschillende kanalen vertegenwoordigen. De fysiologische betekenis en de wijze van aktivatie van deze IR, tot dusver uitsluitend gevonden in darm epitheel cellen, is nog onbekend, evenals de aanwezigheid in CF cellen.

De strukturele gelijkenis tussen CFTR en leden van een familie van transport molekulen zoals het multi-drug resistance genprodukt deed ons afvragen of substraten en remmers van MDR-1 van invloed zouden kunnen zijn op de CFTR-afhankelijke Cl^- transport en op de OR in het bijzonder gezien de mogelijke rol voor dit kanaal in het CF defekt (Hoofdstuk VII). Het MDR-substraat colchicine en de MDR-remmer verapamil hadden geen effekt op de Ca^{2+}- en cAMP-

gestimuleerde Cl^--efflux in intakte HT29.cl19A colonocyten, maar remden de OR in excised inside-out patches zowel van de cytoplasmatische als de extracellulaire kant. De remmende effekten van colchicine, verapamil waren reversibel met IC_{50}-waarden variërend van 5 tot 250 μM. De resultaten van deze studie, tesamen met recente gegevens van CFTR-transfektie experimenten die erop wijzen dat CFTR een 5-10 pS Cl^- kanaal is, spreken ze een rol voor de OR in de cAMP-gestimuleerde Cl^- sekretie in epitheel cellen en dus in ook het sekretoire defekt in CF, tegen.

CURRICULUM VITAE.

De schrijver van dit proefschrift werd op 3 januari 1961 geboren te Heemskerk. Na het behalen van het VWO-B diploma aan het Ichthus College te Velsen in 1979 is hij begonnen met de studie biologie aan de Rijksuniversiteit Leiden. In februari 1983 werd het kandidaatsexamen B4 (biochemie) afgelegd. Het eerste bijvak biochemie werd gevolgd bij Prof. Dr. W. Möller van de vakgroep Medische Biochemie. Het tweede bijvak omvatte de botanische celmorfogenese bij de vakgroep Moleculaire Plantkunde onder leiding van Prof. Dr. K.R. Libbenga. Het hoofdvak moleculaire genetica vond plaats bij de vakgroep Biochemie en stond onder leiding van Prof. Dr. Ir. P. van de Putte. Het auteur behaalde het doctoraaldiploma in augustus 1986. Vanaf 1 januari 1987 was hij werkzaam bij de afdeling Celbiologie (Prof. Dr. H. Galjaard) aan de Erasmus Universiteit te Rotterdam waar het promotie onderzoek begeleid werd door Dr. J. Bijman and Dr. H.R. de Jonge (Afd. Biochemie I).

NAWOORD.

Een proefschrift schrijven doe je niet in je eentje. Hulp van diverse kanten werd me geboden. Een dankwoord lijkt me daarom op zijn plaats.
Allereerst mijn promotor, Prof. Galjaard, voor de geboden kansen (!) om dit onderzoek uit te voeren en de Stichting Klinische Genetika om het af te ronden tot een proefschrift.
De dagelijkse begeleiding werd echter uitgevoerd door Jan Bijman en Hugo de Jonge. Jan, van jouw praktische kennis op het gebied van de elektronika, maar ook van de komputerij heb ik veel geleerd en kan ik nog steeds veel leren. Beiden weten we inmiddels wat patch-clampen is: vallen en opstaan en doorgaan en vallen en doorgaan en opstaan en ....., maar gelukkig, we leven nog! Jouw literatuurkennis, Hugo, is allom bekend en reeds eerder geroemd en ik kan mij daar alleen maar bij aansluiten. Je celmodellen ter verklaring van het CF-defekt zijn beroemd (en berucht!) en na vele herhalingen zeer leerzaam geweest. Voor beiden geldt natuurlijk: mijn dank!
Bob, van jouw inventiviteit en je kritische maar altijd korrekte visie's op resultaten en experimenten heb ik altijd verbaasd gestaan. Ik hoop er in de afgelopen tijd wat van opgestoken te hebben.
André doet altijd duizend dingen tegelijk en is toch nog in staat je à la minute te helpen als je met een probleem bij hem komt. Hoe je dat voor elkaar krijgt is me nog steeds een raadsel, maar ik heb er baat bij gehad.
Joke, volgens mij ben je de efficiëntie zelve en is de snelheid van de TGV niets vergeleken bij die waarbij jij je experimenten uitvoert om maar te zwijgen over het aantal technieken dat je beheerst. Een eredoctoraat?
Rajesh, jij begon al over chloride kanalen te praten voor je goeiemorgen had gezegd. Dit kenmerkt je grote betrokkenheid bij het onderzoek. De periode in "het hok" heb ik als aangenaam ervaren.
Ben, jouw bijdrage aan dit boekje kan niet onder stoelen of banken gestoken worden. Proost!
Zonder Julius de Vries met zijn komputerprogramma zouden we onze opnamen van ionkanalen nooit hebben kunnen analyseren.
Genoemd moeten worden natuurlijk Elly, Martina, Bram en Hikke, de

lysosomenklub met Frans, Grazia en Cécile, alsmede Arnold Reuser met z'n meiden.
Wim Vermeulen leerde me het mikro-injekteren. Wim, hoewel de micro-injektie experimenten geen konkrete resultaten hebben opgeleverd, beheers ik dankzij jou ik echter wel de techniek. Thanks.
En laten we nu even wel wezen, zonder het werk van Jopie en Ellie, de heren fotografen Ruud, Mirko en Tom en zonder Piet Hartwijk en Rein Smid begin je als onderzoeker toch niets?
Wie blijven er nu nog over? Bach en Sjostakovitsj voor hun prachtige muziek, waar ik in de afgelopen jaren veel naar heb geluisterd. Ouders; vrienden en vriendinnen, teveel om op te noemen. En Jeanne natuurlijk. Jeanne, of ik woorden heb voor je steun de afgelopen jaren? Is zwijgen geen goud?