

## COMMENTARY

# Calcium confusion – is the variability in calcium response by Sertoli cells to specific hormones meaningful or simply redundant?

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### Abstract

When results of more than ten different studies on hormone-induced calcium signals in Sertoli cells are taken together, a wide variety of responses emerges. The reported changes range from increased concentrations, via no response at all, to decreased calcium concentrations. Minor variations in cell isolation techniques, culture conditions, or techniques for measuring the intracellular calcium could explain some of these differences. However, erratic variations in response are also observed within research groups under very similar experimental conditions. Such 'negative' findings are mainly reported orally and do not further penetrate the scientific community. As hormone-dependent calcium responses evidently may depend very much on the context of the cells, calcium

transients would appear to be unreliable bioassay principles with which to detect the primary actions of FSH and effectors such as androgens on Sertoli cells. A more important biological question is whether these sometimes opposed calcium transients are connected with a particular cellular response. To date there is no evidence for such a tight coupling in Sertoli cells, implying that, at least under *in vitro* conditions, calcium signals might even be redundant altogether. Such calcium variability is probably not unique to Sertoli cells, and the aim of this commentary is to promote an open debate that may help to transform the current state of 'calcium confusion' into a better understanding of the intracellular calcium language.

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### Introduction

Measuring the concentration of molecules in biological fluids using bioassays has been and continues to be a difficult task. Although the quality of the cells and the hormone preparations, antibodies, culture media, instrumentation and analytical techniques have improved vastly, discussions on the best conditions for bioassays have not stopped. On the one hand responsive cells have the advantage that they can amplify weak hormonal signals and generate a measurable response, whereas on the

other hand, the same cells can amplify non-specific or unknown specific signals and so mask the specific hormone response. Even the response of well-characterised cell-lines in chemically defined media is not always consistent, because properties of the cells can be modified during the culture period. Thus responses of cells may vary slightly between laboratories and even within one laboratory, when cells are used during different periods. This heterogeneity of responses between different experiments has been accepted as an inherent property of biological systems. However, heterogeneity in responses also seems

to occur within a population of identical cells, even when influences of the cell cycle have been minimised. From studying hormone responses in terms of changes in intracellular calcium in individual cells, it has become apparent that not all cells respond at the same time or in the same fashion. The origin and the implications of this heterogeneity in calcium signalling, which acts probably as the most universal second messenger system (Berridge *et al.* 1998), is difficult to understand. With our knowledge of paracrine and autocrine regulation of cell function, we can explain why density or position of individual cells in a population could have an influence on cellular properties. Yet it is difficult to explain the observed diversity of responses in apparently similar cells in the same environment. This could arise from the non-linear and chaotic properties of complex biological systems that operate inside the cell (Coffey 1998). It is therefore possibly understandable that similar cells will not always give identical calcium responses when exposed to specific hormones. However, recent observations with calcium responses in primary non-dividing rat Sertoli cells, and in cells from the human prostatic cell line LNCaP, appear to go beyond this generally accepted variability.

### Variability in calcium responses

Responses of intracellular calcium in Sertoli cells (and also in LNCaP cells) after addition of protein or steroid hormones are presented in Table 1. The data show that the responses of cells under very similar *in vitro* conditions can be very different, not only in regard to the kinetics or specificity of the response, but even between being stimulatory or inhibitory.

There are several technical possibilities to explain such variations. Differences in labelling or sub-cellular distribution of the calcium-sensitive fluorescent dye, back-transport or leakage of the dye after stimulation (especially in freshly isolated cells) and photobleaching could mask the authentic calcium response. However, in several instances cells did not show a calcium response to the specific hormone applied whereas, in the same cells, calcium responses could be shown after addition of potassium, foetal calf serum or ATP (D'Agostino *et al.* 1993, Lalevee *et al.* 1999). Thus technical problems seem to be of less importance and the variations in hormone-dependent calcium responses appear to depend more on the properties of particular receptors or cellular set points.

The variability does not appear to have anything to do with expression of specific receptors as, for LNCaP cells for example, there is always a typical bell-shaped growth response to cognate effectors. Also, the Sertoli cells always respond to FSH with increased cAMP production and show characteristic changes in cell shape. Remarkable is the apparent age-dependent dissociation between the highly reproducible cAMP response and the variable

calcium response. Authors claim that at least part of the calcium responses in 21-day old rats are mediated via cAMP (Gorczyńska *et al.* 1994), but this is less in 9-day (Sharma *et al.* 1994), and does not occur in 13-day old rats (Grasso & Reichert 1990). Very recently it was suggested that the calcium response is only mediated by a particular, truncated FSH receptor, and not by the regular 7-transmembrane form (Touyz *et al.* 2000). On the other hand, the calcium response in a homogenous population may depend completely on contact with matrix components or interactions with homotypic cells (Ravindranath *et al.* 1996, Taranta *et al.* 2000). Variability in the kinetics, the dose-response characteristics and the subcellular localisation of calcium responses in single cells has also been reported for smooth muscle and endothelial cells (Wehling *et al.* 1994). Thus cells may show much more individual behaviour than we currently assume.

Although some variations in calcium responses can be understood, it is very difficult to explain how calcium responses can go in opposite directions under comparable *in vitro* conditions. The suggestion has been made that the basal calcium concentration could be an important factor for the direction of the response after hormonal stimulation (Lalevee *et al.* 1999), but it is hard to understand why basal calcium concentrations vary between similar experiments within a single laboratory. We have made many attempts to understand the origin of these calcium variations by making numerous alterations to the experimental conditions. However, despite these many efforts, the problems remain. In the original publications (see Table 1), details can be found about techniques, experimental conditions and results, in addition to attempts to understand the apparent discrepancies in experimental findings. However, all these published and unpublished observations, together with the derived (more verbal than printed) discussions do not give rise to concurrent views, but rather create confusion, with some investigators even describing their research as 'living in the Sertoli-cell-Hell'. Negative or unexpected observations in individual experiments do not necessarily indicate poorly executed experiments or a faulty experimental design that should be ignored. On the contrary, most of them may reflect genuine cellular properties that demand attention. However, because negative or unexplainable findings are difficult to publish, the current literature does not represent a true reflection of the 'calcium-reality'.

### Consequences of variability in calcium responses

If it is true that the context or origin of the cells can sometimes contribute more to the calcium response than the hormone of interest, then we must ask the question 'are bioassays using calcium responses reliable?' For FSH action, this is less of a problem, because the receptor has been cloned and many observations from *in vivo* and *in vitro*

**Table 1** Calcium responses in Sertoli cells and LNCaP cells after addition of protein and steroid hormones\*

	<b>Hormone</b>	<b>Age of rat (days)</b>	<b>Cellular calcium response</b>	<b>Remarks</b>	<b>Reference</b>
<b>Cell type</b>					
Sertoli	FSH	15	3-fold stimulation during 2 min	2 days culture	Grasso & Reichert 1990
Sertoli	FSH	21	3-fold stimulation during 3 min	Freshly isolated cell aggregates	Gorczyńska & Handelsman 1991
Sertoli	FSH Potassium serum	21	No response 3-fold stimulation within a few seconds	4 days culture	D'Agostino <i>et al.</i> 1993
Sertoli	FSH	9	2-fold stimulation during 2 min	3 days culture all or nothing responses in individual cells	Sharma <i>et al.</i> 1994
Sertoli	FSH or dbcAMP	21	2–3-fold stimulation Much less (no?) stimulation	Fresh aggregates Cultured cells	Gorczyńska <i>et al.</i> 1994
Sertoli	FSH	10	2–3-fold inhibition within seconds	4 days culture	Ravindranath <i>et al.</i> 1996
Sertoli	FSH	13	No response (20%) Inhibition during 6 min (20%) Rapid stimulation (few seconds), later, slow inhibition (60%)	4 days culture	Lalevee <i>et al.</i> 1999
	ATP	13	Stimulation (100%) within a few seconds		
Sertoli	FSH EGF	21–25	4-fold transient stimulation followed by plateau at lower level	4 days culture	Lyng <i>et al.</i> 2000
Sertoli	FSH	18–21	In isolated cells 2-fold stimulation during 3 min In aggregated cells no response	5 days culture	Taranta <i>et al.</i> 2000
HEK 293	FSH	—	Only one from three alternatively spliced FSH receptors stimulates calcium	Transfected cells	Touyz <i>et al.</i> 2000
LNCaP	Androgens	—	3-fold stimulation within 1 min	—	Steinsapir <i>et al.</i> 1991
Sertoli	Androgens	21	2-fold stimulation during 3 min	Freshly isolated cell aggregates	Gorczyńska & Handelsman 1991
LNCaP	Androgens Oestradiol	—	4-fold stimulation during 6 min 3-fold stimulation during 6 min	Inhibited by anti-androgens Not inhibited by anti-androgens	Audy <i>et al.</i> 1996
LNCaP and Sertoli	Androgens	—	Low doses: rapid transient response High doses: 5-fold stimulation with plateau after 2 min	Inhibited by anti-androgens	Lyng <i>et al.</i> 2000
	Oestradiol	—	No response	—	

\*Summary of effects of protein and steroid hormones on intracellular calcium measured with <sup>45</sup>calcium (Grasso & Reichert 1990) or with calcium sensitive fluorescent dyes (all except Grasso & Reichert 1990) in non-dividing primary rat Sertoli cells, or in cells from the human prostatic cell line LNCaP.

studies have shown the importance of this receptor for regulation of the function of the Sertoli cell, acting mainly via cAMP. The situation is completely different for complementary actions of androgens, possibly acting at the plasma membrane. Non-genomic actions of steroids have often been identified by measuring rapid calcium fluxes (see review by Christ *et al.* 1999). If steroid-activated membrane receptors on other cells, and other steroids besides androgens, all behave in a manner similar to what we observe with Sertoli and LNCaP cells, then it will be very difficult to prove the existence of membrane steroid receptors using changes in calcium concentrations as the response parameter. Other approaches such as binding studies and cloning strategies have also not been very successful. Thus the position of separate steroid membrane receptors in general is not very strong. This uncertainty may have contributed to the title of the previously cited review 'Nongenomic steroid actions: fact or fantasy?' (Christ *et al.* 1999). As negative findings on calcium responses have less weight than positive findings, the overall balance of published experimental evidence still supports the existence of separate membrane receptors for steroids. Clearly, other reliable indices of hormone action are required before these alternative receptor systems can be firmly accepted as important entities. It would be much more convincing if another specific biological response, rather than calcium, could be found that is triggered by these membrane receptors. However, most biological responses appear to develop slowly, and signals from slowly acting nuclear receptors may then become integrated into the same signal transduction pathway during the generation of the response to the membrane receptor. As specific ligands for separate activation of the membrane and nuclear receptors are often not available, and as the majority of the cells contain nuclear steroid receptors, it becomes very difficult to demonstrate a specific activation of a putative steroid membrane receptor, if we cannot accept rapid calcium transients as a reliable tool for identification of alternative steroid action.

Accepting for the moment that calcium transients can go in opposite directions, even when *in vitro* conditions are similar, the question then arises whether the opposed calcium transients are connected with a particular cellular response. From the limited number of experiments discussed here, we have found no evidence for such a connection, and we can only conclude that opposed calcium signals do not necessarily generate different biological responses. This speculative conclusion is clearly in contrast with the current view that even minor variations in calcium in space or time are important in specific signal transduction events (Berridge *et al.* 1998). If the variability in intracellular calcium concentrations occurs without measurable biological effects, then does this mean that calcium signals in specific cell types are redundant in a fashion analogous to certain genes ablated in transgenic animals?

Notwithstanding the fact that these observations are made with cells in culture and may not apply under physiological conditions when cells are organised in a tissue environment, most of our knowledge about hormone actions and calcium regulation comes from studies with isolated cells, especially after specific genetic manipulations have been conducted.

## Conclusion

Current studies on calcium responses to specific effectors in cultured Sertoli cells, despite careful experimentation, are highly contradictory. We have to find a way to characterise the origin and significance of this variation in calcium signalling. One way might be to use a single-cell approach, correlating calcium and cAMP responses, measured simultaneously with different fluorescent probes, in the same cell. Similarly, other parameters, e.g. pH, potassium concentration, oxidative activity, cytoskeleton or enzymatic activities could also be analysed at the single cell level. As the number of fluorescent probes available is increasing steadily, as also is the quality of instrumentation, it should eventually be possible to identify patterns of calcium individuality after exposure to hormones, and thus finally to throw some light onto the current confusing situation.

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