Ion fluxes through the progesterone-activated channel of the sperm plasma membrane

Carlo FORESTA,* Marco ROSSATO* and Francesco DI VIRGILIO†‡

*University of Padova, III Cattedra di Patologia Medica, Via Ospedale Civile 105, I-35121 Padova, and †University of Ferrara, Institute of General Pathology, Via Borsari 46, I-44100 Ferrara, Italy

We have characterized ionic changes triggered by progesterone in human spermatozoa. This steroid, which is a fast-acting stimulator of the acrosome reaction, triggered a rapid increase in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) which was entirely due to influx across the plasma membrane, as it was obliterated by chelation of extracellular Ca^{2+}. Ca^{2+} fluxes were insensitive to verapamil and pertussis toxin, thus suggesting that they did not occur via voltage-gated channels and did not involve a pertussis toxin-sensitive G protein, and were potentiated in Na^+-free, choline-containing or methylglucamine-containing medium. Progesterone also caused a depolarization of the plasma membrane in Na^+-containing as well as in choline- or methylglucamine-containing saline; depolarization was larger in the absence of extracellular Ca^{2+}, suggesting that Na^+ and Ca^{2+} fluxes occurred through the same channel. Progesterone was able to trigger the acrosome reaction in the three media investigated (Na^+, choline and methylglucamine), provided that extracellular Ca^{2+} was also present. We conclude that progesterone activates a membrane ion channel that is permeable to monovalent cations as well as to Ca^{2+}.

INTRODUCTION

Mammalian spermatozoa need to undergo the acrosome reaction in order to penetrate the oocyte (Yanagimachi, 1988). The acrosome reaction is an exocytotic event involving multiple fusions of the sperm plasma membrane and outer acrosomal membrane. *In vivo*, the acrosome reaction takes place in the female genital tract and is preceded by a complex process of maturation known as capacitation. Only sperm that have undergone capacitation and the acrosome reaction can fertilize the egg. The biochemical and morphological changes in the sperm cell that precede fertilization can be described with great accuracy, but the identity of the physiological inducers and second messengers that are involved is still obscure (Wassarman, 1987). By analogy to exocytosis in other cellular systems, Ca^{2+} plays a central role in the acrosome reaction, although a few exceptions are documented (Foresta et al., 1992a). Several studies have shown that progesterone is able to induce Ca^{2+} influx in either capacitated or non-capacitated human sperm and to trigger the acrosome reaction *in vitro* in capacitated sperm (Osman et al., 1989; Blackmore et al., 1990). The mechanism of sperm activation by progesterone is presently unknown, but it clearly requires Ca^{2+} and seems to involve direct stimulation of a membrane receptor (an ion channel?), since BSA-conjugated progesterone is as effective as the soluble hormone. Thus progesterone receptors, distinct from the known intracellular genomic receptors, are likely to be present on the sperm plasma membrane (Meizel and Turner, 1991). Ion fluxes through the putative progesterone-activated channel are clearly not amenable to study with the patch–clamp technique in sperm cells; thus other experimental approaches have to be sought.

In the present paper we have investigated the kinetics and the ion selectivity of the progesterone-stimulated transmembrane ion fluxes. Our results suggest that progesterone activates an ion channel that is permeable to Ca^{2+} as well as to Na^+, choline and methylglucamine, thus resulting in not only an increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) but also plasma membrane depolarization.

MATERIALS AND METHODS

Preparation of spermatozoa

Samples of human semen were obtained from healthy donors after 3 days of sexual abstinence and allowed to liquefy at room temperature for 30 min. After liquefaction, all ejaculates were analysed for semen volume, pH, and sperm concentration, motility, viability and morphology. Samples with at least 60% motility, 80% viability and 60% normal morphology were utilized. Motile spermatozoa were isolated by the swim-up technique as previously described (Foresta et al., 1992a). Sperm suspensions were collected and centrifuged for 10 min at 800 *g* and the final pellets were resuspended in each specific medium, as described below.

Incubation of spermatozoa

For induction of the acrosome reaction, sperms that were more than 90% motile were adjusted at a concentration of 20×10^6/ml in BWW medium containing 95 mM NaCl, 4,.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.6 mM fructose, 0.25 mM sodium pyruvate, 20 mM sodium lactate, 100000 i.u./litre penicillin, 100 mg/litre streptomycin and 20 mM Hepes, pH 7.4, at 37 °C. Samples were incubated at 37 °C in a humidified atmosphere containing 5% CO_2, and aliquots were collected after 60 and 180 min for evaluation of motility, viability and the acrosome reaction. For measurement of $[Ca^{2+}]_i$ and plasma membrane potential changes, sperms were incubated in modified Krebs–Ringer solution (modified KRS) containing 154 mM NaCl, 5 mM KCl, 1.7 mM $CaCl_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 5 mM glucose and 20 mM Hepes, pH 7.4 (37 °C). This medium was used for all experiments, unless otherwise indicated. In some

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; KRS, Krebs–Ringer solution.

‡ To whom correspondence should be addressed.

experiments NaCl was replaced by an iso-osmotic concentration of choline chloride or methylglucamine. In these sodium-free buffers, KCl was omitted, Na_2HPO_4 and $NaHCO_3$ were replaced with K_2HPO_4 and $KHCO_3$ respectively, and penicillin sodium salt was replaced with penicillin potassium salt. Progesterone, dissolved in dimethyl sulphoxide, was added at a final concentration of 1 μg/ml from a stock solution containing 1 mg/ml. An equal volume of dimethyl sulphoxide was added to control samples. In all conditions the final dimethyl sulphoxide concentration never exceeded 0.1 % (v/v).

Evaluation of acrosome reaction

The sperm acrosome reaction was evaluated by the triple stain technique as proposed by Talbot and Chacon (1981). Spermatozoa were layered on microscope glass slides and observed at 1000 × magnification under bright-field illumination. At least 400 from each sample were scored and analysed for viability and the presence or absence of the acrosome reaction. The acrosome reaction was also evaluated by means of an indirect immunofluorescence technique using a monoclonal antibody against sperm cytokeratin, as described by Wolf et al. (1985). Aliquots of each sample were layered on microscope glass slides, allowed to dry for a few minutes and stored at −80°C until analysed. The presence or absence of the acrosome reaction was evaluated by adding the monoclonal antibody T6 at a 1:30 dilution; the reaction was revealed by the subsequent addition of biotin/avidin at a 1:30 dilution. Slides were then mounted in buffered glycerol and examined with a Leitz Orthoplan fluorescence microscope (Wild Leitz, Wetzlar, Germany). Cells were scored as positive or negative on the basis of the presence (intact acrosome) or absence (reacted acrosome) of uniform fluorescence restricted to the sperm acrosomal cap. The percentages of live acrosome-reacted spermatozoa obtained by the two methods showed a good positive correlation ($r = 0.94$).

Measurement of sperm $[Ca^{2+}]_i$ and membrane potential

Sperm $[Ca^{2+}]_i$ was measured with the fluorescent probe fura-2/AM, as previously described (Di Virgilio et al., 1987). Spermatozoa isolated by the swim-up technique were centrifuged at 800 *g* for 10 min, resuspended in BWW at a concentration of 20×10^6 cells/ml and incubated for 30 min at 37 °C in the presence of 2 μM fura-2/AM. After loading of the dye, sperm cells were washed free of extracellular fura-2 by centrifugation at 800 *g* for 10 min and resuspended in KRS. $[Ca^{2+}]_i$ levels were determined with the 340/380 nm excitation ratio, at an emission wavelength of 490 nm. Changes in the plasma membrane potential were monitored using the lipophilic, membrane potential-sensitive fluorescent dye bisoxonol (De Togni et al., 1984). Bisoxonol fluorescence and $[Ca^{2+}]_i$ were measured in an LS50 Perkin–Elmer fluorimeter equipped with a thermostatted and magnetically stirred cuvette holder.

Statistical analysis

Student's *t* test and the Wilcoxon signed rank test were used for statistical evaluation.

RESULTS

The mechanism whereby progesterone raises the $[Ca^{2+}]_i$ in sperm cells is obscure, but there is increasing evidence that the site of action is on the plasma membrane, probably a progesterone receptor localized on the sperm head (Blackmore et al., 1991). Figure 1(a) shows that this steroid hormone caused a biphasic $[Ca^{2+}]_i$ rise; the initial rapid increase was followed by a sustained plateau which lasted several minutes and was completely dependent on Ca^{2+} influx, as shown by the rapid return to basal levels upon chelation of extracellular Ca^{2+} with EGTA. Therefore it appears that the influx pathway activated by progesterone is not inactivated over several minutes. Lack of inactivation is also demonstrated by the experiment shown in Figure 1(b) where Ca^{2+} was added 3 min after progesterone to sperm incubated in the presence of EGTA. The challenge with the steroid in the absence of extracellular Ca^{2+} caused no increase in $[Ca^{2+}]_i$, thus confirming that progesterone is unable to mobilize Ca^{2+} from intracellular stores; however, addition of Ca^{2+} to progesterone-treated sperm caused a rise in $[Ca^{2+}]_i$ similar to that triggered by the addition of progesterone in the presence of Ca^{2+} (cf. Figures 1a and 1b). We also tested the effects of agents known to inhibit Ca^{2+} fluxes, such as blockers of voltage-gated Ca^{2+} channels, activators of protein kinase C or A, and G protein-specific reagents (results not shown). No inhibition of the progesterone-induced rise in $[Ca^{2+}]_i$ was observed when sperm cells were pre-incubated for 5 min at 37 °C under each of the following conditions before being stimulated: (a) 20 μM verapamil, (b) 10 μM phorbol myristate acetate, (c) 1 mM dibutyryl cyclic AMP plus 10 μM forskolin. Similarly, the progesterone-induced rise in $[Ca^{2+}]_i$ was not inhibited by pre-incubating sperm cells at 37 °C for 1 h in the presence of pertussis toxin (150 ng/ml).

It is known that ligand- or receptor-operated cation channels can admit both Ca^{2+} and Na^+, and even monovalent inorganic and organic cations including choline and methylglucamine (Benham and Tsien, 1987; Decker and Dani, 1990; Pizzo et al., 1991). It is difficult, for obvious reasons, to perform patch–clamp experiments in viable and motile spermatozoa. Thus we tested whether monovalent cations are also transported through the progesterone-activated channel by using the membrane-potential-sensitive dye bisoxonol as an indicator of

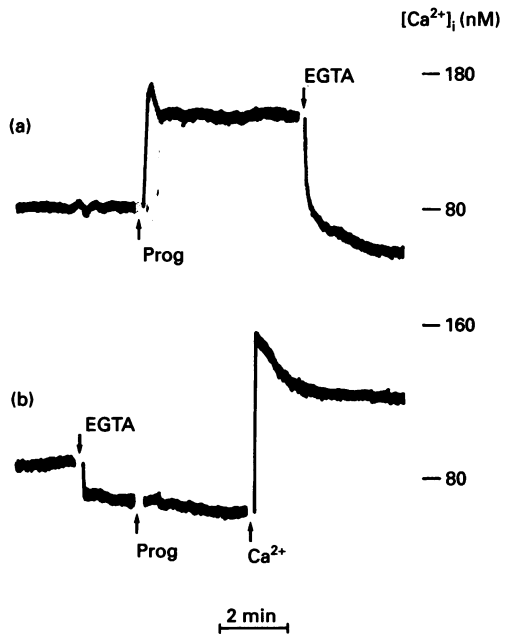

Figure 1 Progesterone causes a rapid Ca^{2+} influx that is not inactivated

Fura-2-loaded sperm (20×10^6/ml) were incubated in modified KRS (**a**) and stimulated with progesterone (Prog) at a concentration of 1 μg/ml. EGTA was added at 5 mM. In (**b**) the incubation was started in Ca^{2+}-free KRS and EGTA (100 μM) was added shortly before progesterone addition; finally 1 mM Ca^{2+} was added.

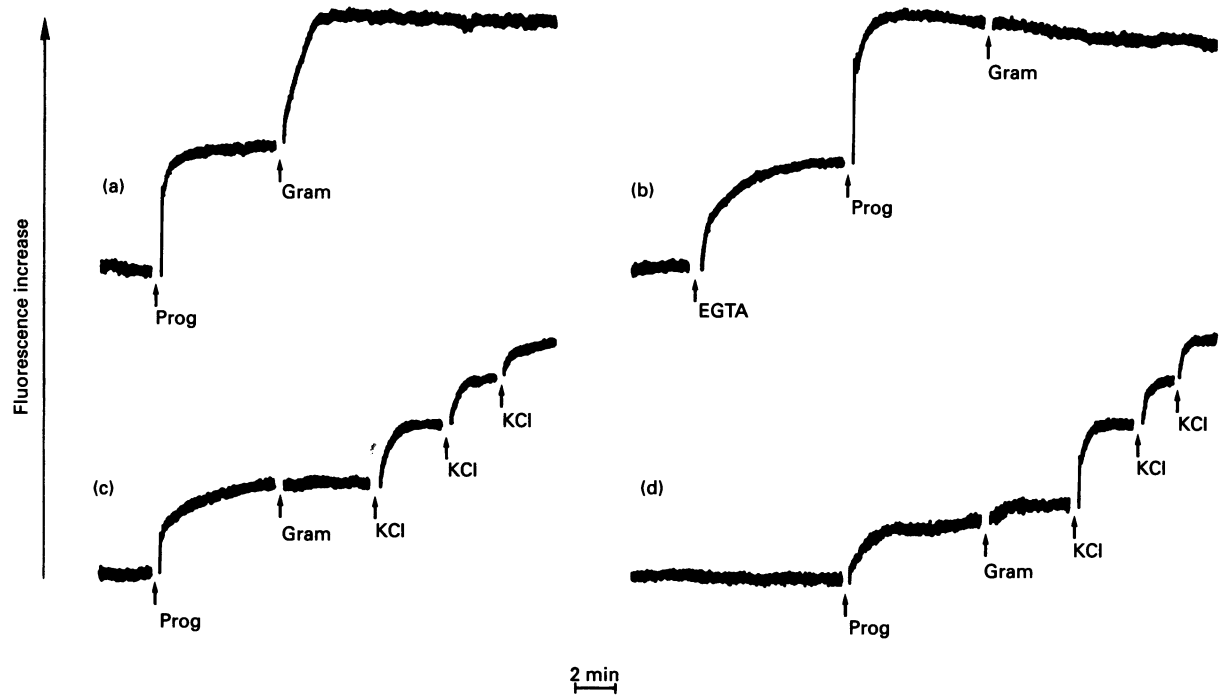

Figure 2 Progesterone causes plasma membrane depolarization

Sperms (20×10^6) were incubated in three different media in the presence of 200 nM bisoxonol: (**a**) and (**b**), modified KRS; (**c**) Na^+-free methylglucamine-containing medium; (**d**), Na^+-free choline-containing medium. Concentrations used: Progesterone (Prog), 1 μg/ml; EGTA, 2 mM; gramicidin D (Gram), 500 nM. Each KCl addition was 15 mM.

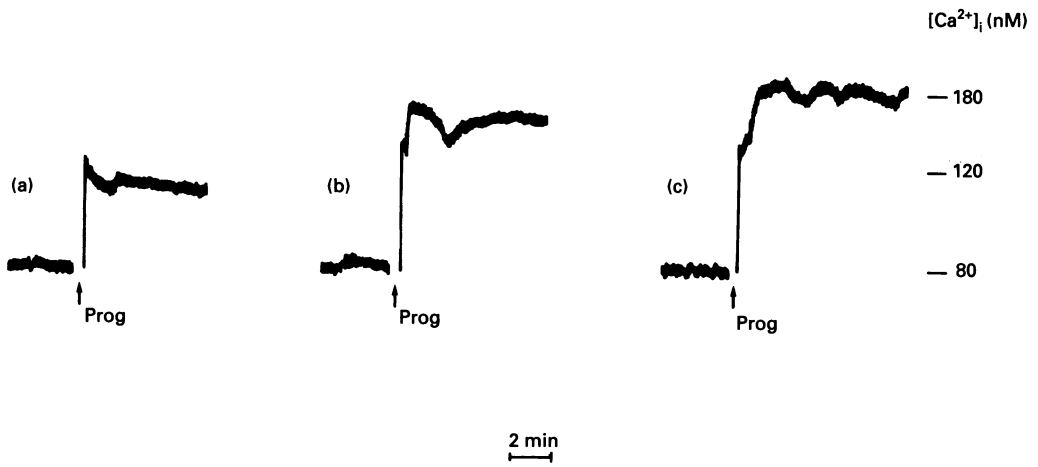

Figure 3 Progesterone triggers a larger rise in $[Ca^{2+}]_i$ in the absence of extracellular Na^+

Experimental conditions were as in Figure 1. Media: (**a**), Modified KRS; (**b**), methylglucamine; (**c**) choline. Abbreviation: Prog, progesterone.

transmembrane ion fluxes. Figure 2 shows that progesterone triggered a rapid plasma membrane depolarization which was observed in both the presence (Figure 2a) and absence (Figure 2b) of extracellular Ca^{2+}, indicating that the depolarizing current was not carried by Ca^{2+}, but very likely by Na^+. Depolarization triggered by progesterone in Ca^{2+}-containing medium was not complete, as shown by the further collapse in plasma membrane potential caused by the addition of gramicidin D (Figure 2a). Chelation of extracellular Ca^{2+} by EGTA (Figure 2b) was in itself depolarizing, probably as a consequence of a change in membrane ion conductivity due to removal of Ca^{2+}; under these conditions progesterone induced a complete depolarization. In the experiments shown in Figures 2(c) and 2(d), Na^+ was iso-osmotically replaced with methylglucamine and choline respectively. In these media, progesterone also triggered a depolarization, albeit smaller than that in Na^+ medium,

Table 1 Induction of the acrosome reaction by progesterone in media of different composition

Sperm were incubated as described in the Materials and methods section and stimulated with progesterone at a concentration of 1 μg/ml. *Significantly different from control ($P < 0.01$). Data are means ± S.D. of triplicate measurements from a single experiment representative of three others that gave similar results.

	Acrosome reaction (%)		
Medium Time (min)...	0	60	180
$+Ca^{2+}$			
BWW	4.3 ± 1.1	5.2 ± 0.9	5.4 ± 1.3
BWW + progesterone	4.2 ± 0.8	17.3 ± 2.5*	19.6 ± 2.3*
Choline	3.9 ± 1.0	4.0 ± 0.9	4.3 ± 1.2
Choline + progesterone	4.2 ± 1.2	14.5 ± 2.6*	16.8 ± 2.1*
Methylglucamine	4.3 ± 0.9	4.8 ± 1.0	5.0 ± 1.1
Methylglucamine + progesterone	4.0 ± 0.7	15.2 ± 2.3*	17.0 ± 2.0

indicating that other low-M_r monovalent cations besides Na^+ are permeant through the progesterone-activated channel. As expected, gramicidin D caused no (Figure 2c) or little (Figure 2d) depolarization in methylglucamine or choline media, since these cations are not permeant through the gramicidin D pore; however, sperm cells were depolarized by the addition of KCl.

The experiments described so far did not allow us to determine whether progesterone induces fluxes of divalent and monovalent cations through the same or two different channels. If two ions permeate through the same transmembrane channel, it is to be expected that they also compete for entry; therefore Ca^{2+} influx should be larger in the absence of extracellular Na^+, and Na^+ influx should be larger in the absence of extracellular Ca^{2+}. As shown in Figure 2, progesterone caused a larger depolarization in the absence than in the presence of extracellular Ca^{2+}; this result, however, is difficult to interpret since Ca^{2+} chelation by EGTA also caused a partial plasma membrane depolarization. We thus examined the effect of replacement of Na^+ on increases in $[Ca^{2+}]_i$. As shown in Figure 3, the progesterone-induced rise in $[Ca^{2+}]_i$ was potentiated in the absence of extracellular Na^+: it was about 50% greater in methylglucamine-containing (Figure 3b) or choline-containing (Figure 3c) than in Na^+-containing (Figure 3a) medium.

We have recently shown that progesterone can induce the acrosome reaction in both capacitated and non-capacitated human sperm incubated in BWW medium (Foresta et al., 1992b). Ca^{2+} influx is known to be required for a progesterone-dependent acrosome reaction. However, the experiments reported in the present paper indicated that this steroid hormone also causes Na^+ fluxes, and thus we investigated whether Na^+ influx is also necessary for sperm activation. Sperm cells were stimulated in BWW medium in which Na^+ had been replaced by equimolar concentrations of choline or methylglucamine. As shown in Table 1, progesterone was able to trigger the acrosome reaction in Na^+-free medium, indicating that Na^+ influx was not required. As expected, the acrosome reaction was completely inhibited by chelation of extracellular Ca^{2+} in all three media tested (results not shown).

DISCUSSION

Progesterone is a fast-acting stimulator of the sperm acrosome reaction. It has been shown that the sperm-activating effects of this steroid are not due to the known interaction with cytoplasmic or nuclear steroid receptors but with a hitherto undescribed receptor which causes Ca^{2+} fluxes across the sperm plasma membrane (Thomas and Meizel, 1989; Blackmore et al., 1990). These extra-genomic effects are not restricted to spermatozoa, as progesterone rapidly decreases the activity of the ATPase of synaptosomal membranes (Delicostantinos, 1988), and induces the maturation of *Xenopus laevis* oocytes, a cell type lacking the cytoplasmic receptor for this steroid (Duval et al., 1983). The progesterone receptor is likely to be exposed on the outer surface of the sperm plasma membrane, since BSA-conjugated progesterone is as effective as unconjugated progesterone in triggering both $[Ca^{2+}]_i$ increases and the acrosome reaction (Blackmore et al., 1991; Meizel and Turner, 1991). The rapid increase in $[Ca^{2+}]_i$ induced by progesterone could be due to several mechanisms: (1) direct opening of an ion channel; (2) generation of an intracellular second messenger which in turn opens a channel; (3) depolarization of the plasma membrane and consequent opening of a voltage-gated Ca^{2+} channel; (4) activation of an antiport which exchanges Ca^{2+} with another intracellular cation or a symport which catalyses the co-transport of Ca^{2+} together with an accompanying anion; (5) release of Ca^{2+} from intracellular stores. Ca^{2+} mobilization from intracellular stores was ruled out by studies showing the inability of progesterone to increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Blackmore et al., 1990) (see also Figure 1 in the present paper). Our study provides further information which helps in discriminating between the above-mentioned mechanisms. Progesterone caused a rapid plasma membrane depolarization which was not inhibited, and in fact was increased, by chelation of extracellular Ca^{2+}; this was probably caused by Na^+ influx. Ca^{2+} and Na^+ fluxes might occur through separate progesterone-activated pathways; however, we do not think this to be the case, as Ca^{2+} and Na^+ appeared to compete for the same entry pathway ($[Ca^{2+}]_i$ increases were higher in the absence of extracellular Na^+; depolarization was greater in the absence of extracellular Ca^{2+}). Such competition, already described for other ligand-gated ion channels (Benham and Tsien, 1987; Pizzo et al., 1991), is highly suggestive of the influx of Na^+ and Ca^{2+} through a single pathway. Other monovalent cations besides Na^+, such as choline (M_r 100) and methylglucamine (M_r 190), were also permeant through the progesterone-activated channel (as shown by the depolarization effected by progesterone in the choline- or methylglucamine-containing media; Figure 2), a further similarity with other well known ligand-gated channels which also admit cations up to M_r 200.

Despite the ability of progesterone to cause monovalent ion fluxes, no sperm-activating effect was detected in the absence of extracellular Ca^{2+} whether Na^+ was present or not, a further confirmation that monovalent ion influx and the accompanying depolarization are not sufficient by themselves to trigger the acrosome reaction.

In conclusion, our study describes novel features of progesterone-activated transmembrane ion fluxes which suggest that this steroid, at concentrations that are present in the cumulus matrix of the oocyte (Lindner et al., 1988; Osman et al., 1989), causes the opening of an ion channel that is permeable to cations of M_r up to 200.

This work was supported in part by grants from the National Research Council (Target Project BTBS; Special Project Biology and Pathology of Calcium) and the Ministry of Scientific Research (MURST 40% and 60%).

REFERENCES

Benham, C. D. and Tsien, R. W. (1987) Nature (London) **328**, 275–278

Blackmore, P. F., Beebe, S. J., Danforth, D. R. and Alexander, N. (1990) J. Biol. Chem. **265**, 1376–1380
Blackmore, P. F., Neulen, J., Lattanzio, F. and Beebe, S. J. (1991). J. Biol. Chem. **266**, 18655–18659
Decker, E. R. and Dani, J. A. (1990) J. Neurosci. **10**, 3413–3420
Delicostantinos, G. (1988) Comp. Biochem. Physiol. **89B**, 585–594
De Togni, P., Cabrini, G. and Di Virgilio, F. (1984). Biochem. J. **224**, 629–635
Di Virgilio, F., Milani, D., Leon, A., Meldolesi, J. and Pozzan, T. (1987) J. Biol. Chem. **262**, 9189–9195
Duval, D., Durant, S. and Homo-Delarche, F. (1983) Biochim. Biophys. Acta **737**, 409–442
Foresta, C., Rossato, M. and Di Virgilio, F. (1992a) J. Biol. Chem. **267**, 19443–19447
Foresta, A., Rossato, M., Mioni, R. and Zorzi, M. (1992b) Andrologia **24**, 33–35

Received 11 February 1993/1 April 1993; accepted 15 April 1993

Lindner, C., Lichtenberg, V., Westhof, G., Braendle, W. and Bettendorf, G. (1988) Horm. Metab. Res. **20**, 243–246
Meizel, S. and Turner, K. O. (1991) Mol. Cell. Endocrinol. **77**, 1–3
Osman, R. A., Andria, M. L., Jones, A. D. and Meizels, S. (1989) Biochem. Biophys. Res. Commun. **160**, 828–833
Pizzo, P., Zanovello, P., Bronte, V. and Di Virgilio, F. (1991) Biochem. J. **274**, 139–144
Talbot, P. and Chacon, R. S. (1981) J. Exp. Zool. **215**, 201–208
Thomas, P. and Meizel, S. (1989) Biochem. J. **264**, 539–546
Yanagimachi, Y. (1988) in Physiology of Reproduction (Knobil, E. and Neils, D., eds.), pp. 135–185, Raven Press, New York
Wassarman, P. M. (1987) Annu. Rev. Cell Biol. **3**, 109–142
Wolf, D. P., Boldt, J., Byrd, W. and Bechtol, K. B. (1985) Biol. Reprod. **32**, 1157–1162