Biochem. J. (1989) 259, 397-406 (Printed in Great Britain)

Polyphosphoinositide breakdown and subsequent exocytosis in the Ca^{2+}/ionophore-induced acrosome reaction of mammalian spermatozoa

Eduardo R. S. ROLDAN and Robin A. P. HARRISON

Department of Molecular Embryology, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, U.K.

An investigation was made of the modifications in phospholipids that occur during the exocytotic event known as the 'sperm acrosome reaction'. Phospholipids were prelabelled with ^{32}P, and exocytosis was induced with Ca^{2+} and the ionophore A23187. When incubated with [^{32}P]P_i in various media suitable for supporting sperm survival or fertilization *in vitro*, spermatozoa from all five species examined (ram, boar, guinea pig, mouse and human) incorporated ^{32}P rapidly into the components of the phosphoinositide cycle. There were differences both between species and between media with respect to the actual rate of incorporation of label, and also between species with respect to other phospholipids labelled. Treatment of spermatozoa with Ca^{2+} and A23187 to induce the acrosome reaction resulted in a rapid breakdown of phosphatidylinositol 4, 5-bisphosphate and phosphatidylinositol 4-phosphate, which was complete within 3 min; there was also a great increase in labelling of phosphatidate. Occurrence of acrosome reactions in the sperm population was only observed after 5–10 min and reached a maximum response of >90% after more than 30 min. The phosphoinositide breakdown was related to subsequent exocytosis: after EGTA/ionophore treatment, neither inositide breakdown nor exocytosis took place; however, later addition of Ca^{2+} resulted in immediate inositide breakdown, and exocytosis followed, with a delay relative to Ca^{2+} addition exactly similar to that following standard Ca^{2+}/ionophore treatment. Neomycin inhibited both inositide breakdown and subsequent exocytosis provided it was added together with Ca^{2+} and ionophore; however, if the drug was added 3 min after Ca^{2+} and ionophore (by which time inositide breakdown was already complete), exocytosis was not inhibited. Ca^{2+} seemed to have several consecutive roles in the acrosome reaction. Low (micromolar) levels of free Ca^{2+} were needed both for phosphoinositide breakdown and for an event downstream of this breakdown; no other bivalent cation could substitute for Ca^{2+} in either event, and inositide breakdown was actually inhibited by Mg^{2+}. In addition, millimolar levels of Ca^{2+} were needed for later stages of exocytosis, although this requirement could be satisfied by Sr^{2+}. We conclude that breakdown of polyphosphoinositides is an essential early process after Ca^{2+} entry in the chain of events that lead to exocytosis in the mammalian sperm acrosome reaction.

INTRODUCTION

The mammalian sperm acrosome is a secretory granule that overlies the anterior part of the nucleus; it contains hydrolytic enzymes that aid the spermatozoon to penetrate the egg vestments at fertilization. The acrosome reaction is an exocytotic event in which membrane fusion takes place between the outer acrosomal membrane and the overlying plasma membrane to form transmembrane pores and allow release or exposure of the acrosomal contents (Harrison, 1983; Yanagimachi, 1988). Under natural conditions, the reaction takes place in the vicinity of the egg in response to specific egg-associated stimuli (Wassarman *et al.*, 1986).

At present, rather little is known of the molecular events that underlie the acrosome reaction. On release from the male reproductive tract, the mature spermatozoon is unable to undergo the acrosome reaction in response to natural triggers; a prior period of residence in the female tract is required ('capacitation'; Yanagimachi, 1988); presumably during this time potential receptor sites on the sperm surface are unmasked or modified to become active. In accord with exocytosis in many other cell systems, external Ca^{2+} is an essential requirement for the acrosome reaction (Yanagimachi & Usui, 1974), and changes in Ca^{2+} permeability such as those induced by ionophore treatment will trigger the reaction (Talbot *et al.*, 1976; Green, 1978; Singh *et al.*, 1978; Shams-Borhan & Harrison, 1981). Lipid changes are clearly involved, for modifications of sperm lipid configuration or content have profound effects upon the reaction (Fleming & Yanagimachi, 1981; Fleming *et al.*, 1982). Recently, specific changes after Ca^{2+} influx have been observed in the distribution of intramembranous particles over the acrosomal region; these take place before fusion begins, and it has been suggested that

Abbreviations used: PIP_2, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol 4,5-bisphosphate; PIP, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol 4-phosphate; PI, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol; PA, phosphatidic acid; CDP-DAG, cytidine diphosphate diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; BSA, bovine serum albumin; PVA, poly(vinyl alcohol); PVP, polyvinylpyrrolidone; DTT, dithiothreitol.

modifications of cytoskeletal elements may be involved (Fléchon *et al.*, 1986).

Investigations of exocytosis in other systems have revealed a complex interplay between Ca^{2+}, lipid and protein components (Nishizuka, 1984; De Lisle & Williams, 1986); in particular, the breakdown of polyphosphoinositides to release inositol phosphates and diacylglycerol as second messengers is frequently found to be an early event in the process (Berridge & Irvine, 1984; Berridge, 1987). As yet, little advantage has been taken of these new discoveries in studies of the molecular events of the mammalian sperm acrosome reaction. One of the difficulties has been the establishing of a suitable model system; in many animal species, even in so-called 'capacitated' sperm populations, rather low numbers of cells undergo an acrosome reaction synchronously in response to physiological or pharmacological stimuli *in vitro* [mouse (Bleil & Wassarman, 1983); hamster (Meizel & Turner, 1984); ram (Thompson & Cummins, 1986); bull (Lenz *et al.*, 1983); man (Tesarik, 1985)]. However, treatment with Ca^{2+} and a bivalent-cation ionophore such as A23187 will induce a relatively rapid and synchronous acrosome reaction in a very large proportion of spermatozoa from many species (e.g. Shams-Borhan & Harrison, 1981). Although this model has the disadvantage that physiological events leading to initial Ca^{2+} influx are by-passed, morphologically the ionophore-induced reaction resembles closely that induced by more physiological means; moreover, treated spermatozoa will subsequently fuse with eggs.

We have therefore employed the Ca^{2+}/ionophore-induction system as a model to investigate molecular events downstream of Ca^{2+} entry. Here, we describe the rapid phosphoinositide breakdown that takes place after Ca^{2+} entry, its relationship to exocytosis, and the relative bivalent-cation requirements of the two processes.

MATERIALS AND METHODS

Reagents

[^{32}P]P_i (carrier-free; 10 mCi/ml on day 0) was purchased from Amersham International, Amersham, Bucks., U.K. The ionophore A23187 was a gift from Eli Lilly, Indianapolis, IN, U.S.A. BSA (fraction V), lactic acid (sodium salt), PVA (average M_r 10000), neomycin sulphate and kanamycin sulphate were from Sigma Chemical Co., Poole, Dorset, U.K. Hepes, pyruvic acid (sodium salt) and PVP (average M_r 44 000) were from BDH, Poole, Dorset, U.K. Before use, PVP was dialysed thoroughly against water and freeze-dried. Lipid standards were kindly provided by Dr. R. F. Irvine of this Institute.

Media

The standard saline-based medium used for labelling and incubation of spermatozoa consisted of 142 mM-NaCl, 10 mM-glucose and 20 mM-Hepes buffered with 2.5 mM-KOH and NaOH to pH 7.55 at 20 °C (Fléchon *et al.*, 1986); the sucrose-based washing medium contained 222 mM-sucrose in place of the NaCl. Both these media also contained 1 mg of PVA/ml, 1 mg of PVP/ml and 0.1 mM-DTT and had a final osmolality of 305 mOsM/kg.

Other media were also used for labelling of spermatozoa; details are given in Table 1 (below).

Preparation of spermatozoa

Ejaculated ram and boar spermatozoa were separated from seminal plasma by dilution and washing through sucrose medium as described by Harrison *et al.* (1982). Ejaculated human spermatozoa were washed as described by Bennet *et al.* (1987). Epididymal guinea-pig and mouse spermatozoa were collected by puncturing cauda epididymides and allowing spermatozoa to swim out into 0.16 M-NaCl (guinea pig) or selected labelling medium (mouse); spermatozoa were then washed through sucrose medium (guinea pig) or simply diluted further in labelling medium (mouse). Sperm concentrations were estimated by using a haemocytometer.

^{32}P-labelling of spermatozoa

Spermatozoa [$(0.2–1.0) \times 10^8$/ml] were incubated in various media (Table 1 below) for different times (0–3 h) at 37 °C under air in the presence of 100–500 μCi/ml of [^{32}P]P_i. In the case of the standard saline medium, compensating adjustments were made both for the acidity and for the volume of the added labelled-phosphate solution so as to maintain medium composition as constant as possible. In the case of the other media, only compensation for acidity was made because the labelled phosphate was used within 10 days of delivery and the slight (<8%) dilution of the medium resulting from labelled-phosphate addition was therefore ignored.

Induction of the acrosome reaction

The acrosome reaction was induced by treatment with Ca^{2+} and the bivalent-cation ionophore A23187 at 37 °C, as described by Shams-Borhan & Harrison (1981); the occurrence of the reaction was monitored by phase-contrast microscopy. The treatment was initiated by exposure of the cells to ionophore; for spermatozoa incubated in media without BSA, a final concentration of 1 μM-A23187 was used, whereas for spermatozoa incubated in media containing BSA, concentrations of 10–40 μM-A23187 were used. The concentration of Ca^{2+} varied between 1.7 mM and 3.0 mM according to the medium; in the case of the standard saline medium, the Ca^{2+} was added with the ionophore to a final concentration of 3 mM.

Lipid analysis

At various intervals after the beginning of Ca^{2+}/ionophore treatment, lipids were extracted and separated by using modified versions of the methods described by Mitchell *et al.* (1986). For each millilitre of sperm suspension, an equal volume of 15% (w/v) trichloroacetic acid was added. The resulting suspension was then vortex-mixed and centrifuged at 1000 $g_{max.}$ for 5 min. After resuspension in 2.5 ml of 5% (w/v) trichloroacetic acid and re-centrifugation, the pellet was resuspended in 3.75 ml of chloroform/methanol/conc. HCl (500:1000:6, by vol.) and centrifuged once more. The supernatant (lipid extract) was transferred to another centrifuge tube, and 1 ml of water, 0.25 ml of 100 mM-EDTA pH 7.4, 1.25 ml of 0.16 M-NaCl and 1.25 ml of chloroform were added. After vortex-mixing and centrifugation, the upper aqueous phase was discarded, and the lower phase was re-vortex-mixed with 2.5 ml of theoretical upper phase (chloroform/methanol/1 M-HCl; 3:48:47; by vol.) (Sheltawy & Dawson, 1969). After further centrifugation, the upper phase was again

discarded, and the lipid-containing bottom phase ('total lipid extract') was finally blown dry with a stream of N_2.

The lipids were separated by t.l.c. on silica-gel 60 F_{254}-coated plates (0.25 mm thickness; E. Merck, Darmstadt, Germany) that had been pretreated by spraying with 1% (w/v) potassium oxalate and activated by heating at 110 °C for 1 h. Two different systems were used: (1) a one-dimensional system in which 20 cm × 20 cm plates were developed in chloroform/methanol/water/conc. NH_3 (48:40:7:5, by vol.); (2) a two-dimensional system in which 10 cm × 10 cm plates were first developed as in the one-dimensional system, then dried to evaporate the residual NH_3, and finally developed in the second dimension in chloroform/methanol/formic acid (11:5:1, by vol.). The plates were air-dried briefly and the various spots were detected either by staining in an iodine tank or by autoradiography using Fuji RX film. By using the developed autoradiographs as a template, the individual spots were scraped off and the radioactivity in each determined by liquid scintillation counting. Autoradiographs were also analysed with the aid of an image analyser (Magiscan 2A, Joyce–Loebl, Gateshead, Tyne and Wear, U.K.), using a program originally developed for two-dimensional polyacrylamide-gel electrophoresis by the Center for Image Analysis, University of Cincinnati Medical Center, Cincinnati, OH, U.S.A. The two methods yielded similar data with respect to relative labelling intensity of the individual spots.

RESULTS

Labelling of phospholipids

Some previous reports have implied that living spermatozoa do not incorporate $[^{32}P]P_i$ readily (Babcock *et al.* 1975; Noland *et al.*, 1987). Such has not been our experience: considerable labelling of both phospholipid (see Table 1) and protein (results not shown) was achieved in the presence of reasonable levels of labelled phosphate (100–500 μCi/ml). The rate of incorporation of $[^{32}P]P_i$ into the total lipid fraction was both concentration-dependent and linear with time over 180 min. It differed between species when the spermatozoa were incubated for labelling in the same medium, but it also varied within a species when different media were used (Table 1); in both guinea pig and mouse there was some 6-fold difference between the best and the worst medium tested. Similar findings have been made with other cell types [see, for example, Cohen *et al.* (1971)]. Incorporation of $[^{32}P]P_i$ takes place essentially through the ATP pool, and it has been suggested that, at physiological pH values, the more important route of entry into this pool is a direct one via 1,3-diphosphoglycerate formed by membrane-associated glyceraldehyde-3-phosphate dehydrogenase (Niehaus & Hammerstedt, 1976). Obviously such a system requires the presence of a glycolysable substrate (cf. Noland *et al.*, 1987), but is also likely to be greatly affected by the cell's environment.

Within a given species, the differences in the rate of $[^{32}P]P_i$ incorporation seen when spermatozoa were incubated in various media did not appear to affect the pattern of incorporation into the phospholipids, and in all five species examined the basic pattern was consistent: $[^{32}P]P_i$ was incorporated rapidly into the components of the phosphatidylinositol cycle. PA was labelled first, then PIP and PIP_2, and after 45 min incubation PI and CDP-DAG were also clearly labelled (see Fig. 1). In ram and boar spermatozoa, other phospholipids were only labelled after longer periods (180 min) of incubation: e.g. LPC, LPI, LPS and PC in ram spermatozoa (Fig. 1), and PE and PC in boar spermatozoa (results not shown); in guinea-pig and mouse spermatozoa, on the other hand, PS, PE and PC were already labelled by 45 min. The rapid labelling of PA and the phosphoinositides in relation to other phospholipid families has not previously

Table 1. $[^{32}P]P_i$ incorporation into phospholipids of mammalian spermatozoa incubated in different media

Spermatozoa were incubated with $[^{32}P]P_i$ in different media at 37 °C for 45 min under air (see the Materials and methods section). The media used were: (1) the standard Hepes-buffered saline medium containing 1 mg of PVP/ml and 1 mg of PVA/ml (see the Materials and methods section); (2) a Hepes/Tyrode's medium (HT) containing 3.5 mg of BSA/ml (Bennet *et al.*, 1987); (3) a modified Krebs–Ringer bicarbonate (KRB) medium with 4 mg of PVP or BSA/ml (Florman & Storey, 1982); (4) a modified Tyrode's medium (T/M) with 4 mg of BSA/ml (Fraser, 1983); (5) a modified minimal capacitation medium (MCM), with or without 3 mg of BSA/ml (Singh *et al.*, 1980); (6) a modified Tyrode's medium (T/GP) with 3 mg of BSA/ml (Roldan *et al.*, 1986). Kanamycin sulphate (50 μg/ml) was added to all media except the standard saline medium. The bicarbonate-containing media (2, 3, 4 and 6) were pre-equilibrated with CO_2/air (1:19), and the pH of all the media was adjusted to 7.4–7.5 with NaOH before use.

Species	Origin of spermatozoa	Medium	$[^{32}P]P_i$ (μCi/ml)	Radioactivity in total lipid extract (c.p.m./10^8 spermatozoa)
Ram	Ejaculated	Saline (1)	250	172913
Boar	Ejaculated	Saline (1)	500	16323
Human	Ejaculated	HT + BSA (2)	500	26866
Mouse	Epididymal	Saline (1)	500	471716
		KRB + PVP (3)	500	111710
		KRB + BSA (3)	500	86205
		T/M + BSA (4)	500	306654
Guinea pig	Epididymal	Saline (1)	500	949733
		MCM (5)	500	147350
		MCM + BSA (5)	500	233400
		T/GP + BSA (6)	500	534033

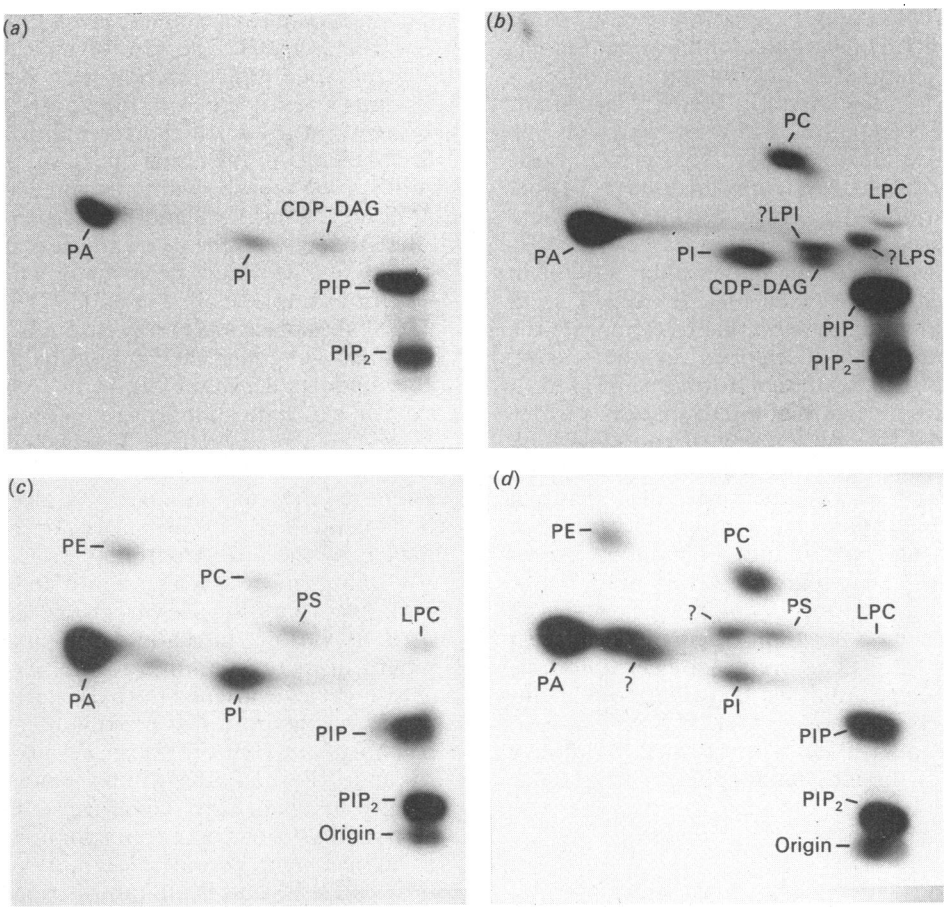

Fig. 1. Incorporation of [^{32}P]P_i into different phospholipids of ram, guinea pig and mouse spermatozoa

Spermatozoa were incubated in saline medium at 37 °C with [^{32}P]P_i. Ram spermatozoa were incubated with 250 μCi/ml for 45 min (*a*) or 180 min (*b*); guinea pig (*c*) and mouse (*d*) spermatozoa were incubated with 500 μCi/ml for 45 min. After the stated times, samples were precipitated with trichloroacetic acid, and lipids were extracted and separated by two-dimensional t.l.c., as described in the Materials and methods section. Autoradiograms of the plates are shown.

been described in mammalian spermatozoa, though it is, of course, a well-known feature of many other cell types [e.g. Cohen *et al.* (1971); see also Irvine *et al.* (1982)]. It is noteworthy that Bennet *et al.* (1987) did not report ^{32}P labelling of sperm phosphoinositides, though they detected labelling of PA, PE, PS (or PI) and PC. As they did not extract their lipids from an acidified system, it is probable that the phosphoinositides remained in the aqueous phase (Dawson & Eichberg, 1965).

Phospholipid changes after Ca^{2+}/ionophore treatment

When spermatozoa were incubated at 37 °C with A23187 and Ca^{2+}, acrosome reactions occurred in a rather fast and synchronous fashion with a lag of 5–10 min between initiation of ionophore treatment and visible occurrence of exocytosis (Fig. 4 below; cf. Shams-Borhan & Harrison, 1981; Fléchon *et al.* 1986).

Very soon after the initiation of Ca^{2+}/ionophore treatment, ^{32}P label diminished in the region of the t.l.c. plates corresponding to PIP_2 and PIP, and increased in the region corresponding to PA; the changes occurred in a time-dependent manner (Fig. 2). The loss in PIP_2 and PIP labelling was almost complete by 3 min, whereas PA labelling continued to rise over several more minutes; labelling of PI, on the other hand, remained more or less constant. The same dramatic pattern of changes was observed in spermatozoa of all five species studied (Table 2), despite quantitative differences between species with respect to the relative changes detected. In the case of PI, whose response at first sight seemed the most variable, the changes always involved relatively little radioactivity.

Bivalent-cation requirements

Treatment with the ionophore A23187 alone, in the absence of added Ca^{2+}, induced changes in phospholipid labelling similar to those seen after standard Ca^{2+}/A23187 treatment (i.e. loss of polyphosphoinositide labelling and increase in PA labelling); we concluded that this was due to the presence of low amounts of Ca^{2+} in the system, since no changes were seen if 1 mM-EGTA was added with the ionophore (Fig. 3*a*). No acrosome reactions were induced in the absence of added millimolar Ca^{2+} (see Table 3). Thus, after EGTA/ionophore treatment, neither inositide breakdown nor exocytosis took place; however, later addition of Ca^{2+} resulted in polyphosphoinositide breakdown (Fig. 3*b*), and exocytosis followed, at a rate, relative to Ca^{2+} addition, almost identical with that following standard Ca^{2+}/ionophore treatment (Fig. 4). If spermatozoa were first exposed to the ionophore for 10 min without EGTA (during which time inositide breakdown occurred) and then Ca^{2+} was added, the onset of the acrosome reaction was accelerated (Table 4).

Other bivalent cations were tested for their ability to

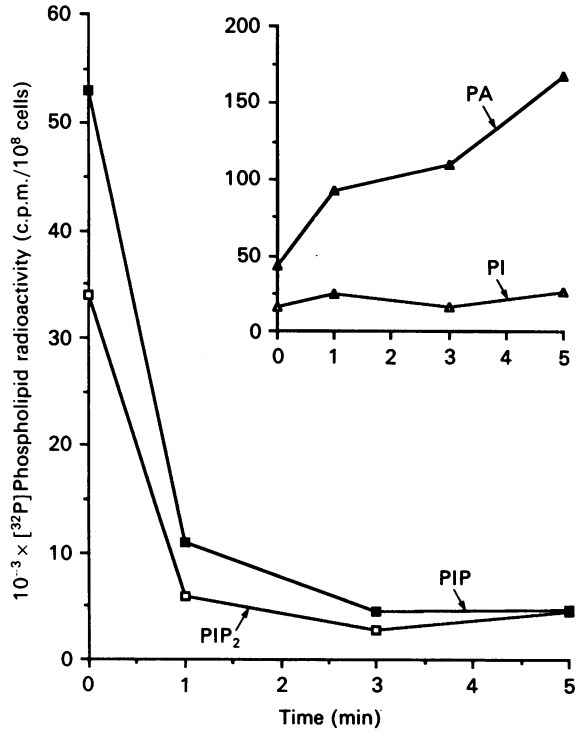

Fig. 2. Changes in ^{32}P-labelling of components of the inositide cycle following Ca^{2+}/ionophore treatment of ram spermatozoa

Spermatozoa in saline medium were labelled for 45 min with 250 μCi of [^{32}P]P_i/ml and then treated with 3 mM-Ca^{2+} and 1 μM-A23187 as described in the Materials and methods section. At various times after initiation of treatment, samples were precipitated with trichloroacetic acid; lipids were extracted, separated by two-dimensional t.l.c., the plates autoradiographed, and the detected spots scraped off and counted for radioactivity.

replace Ca^{2+} in our model system (Fig. 3 and Table 3). In the presence of A23187, Mg^{2+} did not induce polyphosphoinositide breakdown (Fig. 3*a*), nor did it induce the acrosome reaction (Table 3); since the low endogenous levels of Ca^{2+} in the system are sufficient to cause inositide breakdown in the presence of ionophore (see above), we concluded that added Mg^{2+} actually inhibits the Ca^{2+}-dependent PIP_2/PIP hydrolysis. Ba^{2+} had neither inhibitory nor stimulatory effects, because inositide breakdown occurred when endogenous free Ca^{2+} was available (Fig. 3*a*), but did not if EGTA was also included (Fig. 3*b*); no acrosome reactions were observed if spermatozoa were treated with Ba^{2+}/A23187 (Table 3).

The effects of Sr^{2+} were more complicated. Sr^{2+}/A23187 treatment resulted in both inositide breakdown (Fig. 3*a*) and subsequent exocytosis (Table 3). Thus Sr^{2+} did not inhibit Ca^{2+}-induced inositide breakdown, and, in contrast with Ba^{2+}, it was able to substitute for Ca^{2+} in a later stage of the acrosome reaction. But when no free Ca^{2+} was available (EGTA present), Sr^{2+} was unable to induce either inositide breakdown (Fig. 3*b*) or subsequent exocytosis (Table 3). Moreover, if spermatozoa were treated for 5 min with A23187 alone (during which time PIP_2/PIP breakdown takes place) and then EGTA + Sr^{2+} were added, no acrosome reactions occurred (Table 3). We concluded that low levels of Ca^{2+} are essential for a stage of exocytosis that follows PIP_2/PIP breakdown, although Sr^{2+} can replace Ca^{2+} in a third stage which needs millimolar levels of the ions.

Effect of neomycin on polyphosphoinositide breakdown and subsequent exocytosis

The loss of ^{32}P label from PIP_2 and PIP that occurs during the first 5 min of Ca^{2+}/ionophore treatment could be prevented by 10 mM-neomycin; neomycin is an aminoglycoside antibiotic that is known to bind strongly and

Table 2. Changes in ^{32}P-labelling of inositide cycle phospholipids in spermatozoa of different species after 5 min of Ca^{2+}/ionophore treatment

Spermatozoa of all species were labelled for 45 min at 37 °C in [^{32}P]P_i-containing saline medium, with the exception of human spermatozoa, which were labelled in Hepes/Tyrode medium (see Table 1). Concentrations of [^{32}P]P_i were 250 μCi of [^{32}P]P_i/ml for ram spermatozoa or 500 μCi of [^{32}P]P_i/ml for all other species. After labelling, the sperm suspensions were treated with Ca^{2+} (3 mM) and ionophore A23187 (1 μM; 10 μM for human spermatozoa). Treatment was stopped after 5 min by the addition of trichloroacetic acid, and lipids were extracted and separated by t.l.c. For all species except human, spots detected by autoradiography were scraped off and subjected to liquid-scintillation counting; results for human spermatozoa were obtained by analysing autoradiographs with the aid of an image analyser. For details of the procedures used, see the Materials and methods section. Results are given as percentages of control values (i.e. in the absence of Ca^{2+}/A23187 treatment); the actual control values are given in parentheses (c.p.m./10^8 spermatozoa). Results are means for two to four separate experiments; standard deviations were never higher than 10–20% of the mean values.

		Change in ^{32}P-labelling (% of control value) (actual control value before treatment)				
Phospholipid	Species...	Ram	Boar	Human	Mouse	Guinea pig
PIP_2		14.6 (34710)	20.3 (1156)	3.5 (2705)	15.4 (70848)	12.3 (69116)
PIP		7.9 (45185)	8.9 (2565)	58.5 (4191)	21.1 (51166)	7.0 (129768)
PI		135.3 (13218)	110.8 (793)	249.0 (774)	122.8 (11363)	108.0 (65613)
PA		265.3 (50041)	182.5 (4924)	276.0 (15017)	235.7 (165586)	138.0 (455138)

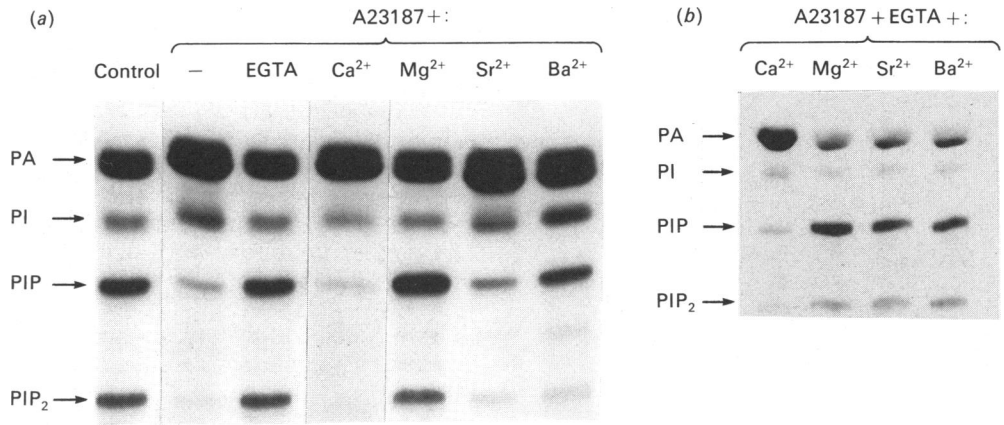

Fig. 3. Effects of different bivalent cations on polyphosphoinositide breakdown in ionophore-treated ram spermatozoa

Spermatozoa in saline medium were labelled with 250 μCi of [^{32}P]P_i/ml for 45 min at 37 °C, and then treated with 1 μM-A23187. In (*a*), either EGTA (1 mM) or various different bivalent cations (3 mM) were included with A23187; an untreated control sample was also processed. In (*b*), both EGTA (1 mM) and various different bivalent cations (3 mM) were included with A23187. After 5 min of treatment, samples were precipitated with trichloroacetic acid, and lipids were extracted and separated by one-dimensional t.l.c. Autoradiograms of the plates are shown.

Table 3. Ability of different bivalent cations to induce the acrosome reaction in ram spermatozoa in the presence of ionophore

Ram spermatozoa in saline medium were exposed to the ionophore A23187 at 37 °C as described in the Materials and methods section. Additions of 1 mM-EGTA and/or various different bivalent cations (3 mM) were made, either simultaneously with 1 μM-A23187 or 5 min later (concentrations given are final ones). After a total of 60 min of ionophore treatment, the sperm suspensions were analysed for the occurrence of acrosome reactions (means ± S.D.; three separate experiments).

Treatment	Bivalent cation...	Acrosome reactions after 60 min (%)				
		None	Ca^{2+}	Mg^{2+}	Sr^{2+}	Ba^{2+}
A23187		8.0±4.2	89.0±1.4	14.0±2.8	82.5±2.1	6.0±0.0
A23187+EGTA		3.0±1.4	97.0±5.2	10.0±3.5	18.3±8.7	6.0±3.6
A23187 for 5 min; then EGTA and the cation added		–	92.3±4.7	15.3±5.8	23.0±10.8	6.0±2.6

selectively to polyphosphoinositides (Schacht, 1978) and that has been used as a relatively specific inhibitor of phosphoinositide metabolism (Cockcroft & Gomperts, 1985; Whitaker & Aitchison, 1985; Carney *et al.*, 1985). If spermatozoa were incubated longer (30 min) in Ca^{2+}/A23187 + neomycin, loss of ^{32}P label did eventually occur (Fig. 5). Neomycin also modified the rate of onset of the ionophore-induced acrosome reaction. After 10–15 min, about 50% of ram spermatozoa treated with Ca^{2+}/A23187 alone showed acrosome reactions, whereas if neomycin was also present, similarly treated spermatozoa only showed 10–15% acrosome reactions (Fig. 5); after 60 min, however, the samples incubated with neomycin had reached values similar to those found in spermatozoa incubated in its absence. That the effect of neomycin was related to the breakdown of PIP_2 and PIP is strongly suggested by the fact that addition of neomycin 3 min after addition of Ca^{2+}/A23187 (by which time breakdown of inositides would already have occurred; see Fig. 2) did not inhibit the onset of the acrosome reaction (Fig. 5).

The effect of neomycin on inositide breakdown and subsequent exocytosis was similarly tested in boar spermatozoa, with essentially identical results (not shown). Neomycin also delayed inositide breakdown greatly in human, mouse and guinea pig (results not shown), though acrosome reactions were not investigated in these latter experiments.

DISCUSSION

Most previous biochemical studies of the mammalian sperm acrosome reaction have concentrated on overall ion requirements and mechanisms of ion entry, especially those relating to Ca^{2+}. Other lines of investigation have involved induction, acceleration or inhibition of the reaction with various substances, from which inferences have been made regarding the involvement of some specific event or metabolite in the process. In the vast majority of cases the acrosome reaction has not been considered as a chain of molecular events culminating in physical membrane fusion. Clearly, however, it must be so considered, and the aim of the present study was to initiate a search for this chain. For reasons outlined in the Introduction, we have used the Ca^{2+}/ionophore-

induction system to study the events downstream of Ca^{2+} entry.

The results described above show that a large-scale breakdown of polyphosphoinositides and a concomitant increase in PA take place very soon after Ca^{2+} entry in spermatozoa of all five species studied; in ram, breakdown of the polyphosphoinositides was completed within 3 min, although few exocytotic responses were observed before 10 min.

Two lines of evidence suggest that this breakdown is an essential preliminary in the build-up to exocytosis. In the presence of neomycin, a drug known to bind to polyphosphoinositides, both inositide breakdown and subsequent exocytosis was inhibited. However, the drug was only able to inhibit exocytosis if inositide breakdown had not occurred; when it was added 3 min after initiation of Ca^{2+}/ionophore treatment (by which time breakdown was complete), no delay of the onset of exocytosis was observed. Although there have been reports that neomycin can have non-specific effects (e.g. Polasick *et al.*, 1987), the speed of action of the drug in our system (no prior incubation was needed; Fig. 5) argues for its specific action on phosphoinositide breakdown, and hence our observations imply a close link between this breakdown and exocytosis. A similar conclusion can be drawn from the observations relating to Ca^{2+} requirements. When free Ca^{2+} was withheld from the ionophore-treated system (EGTA included with ionophore), neither phosphoinositide breakdown nor exocytosis occurred; if Ca^{2+} was added later, breakdown of phosphoinositides immediately ensued, and the time curve of subsequent exocytosis relative to Ca^{2+} addition was indistinguishable from that seen after normal Ca^{2+}/ionophore induction, implying that the breakdown initiated the necessary train of events. If spermatozoa were treated with ionophore alone, phosphoinositide breakdown occurred immediately (due to the presence of low levels of mobilized Ca^{2+} in the system), but exocytosis did not follow until millimolar levels of Ca^{2+} had been added to the system; then exocytosis ensued at an accelerated rate, as if inositide breakdown had resulted in a build-up of an intermediate whose subsequent metabolism/function was blocked in the absence of high Ca^{2+}.

That the disappearance of ^{32}P label from PIP_2 and PIP is due to phospholipase C action can be deduced from two pieces of evidence. Firstly, a considerable and very rapid increase in inositol trisphosphate, one of the two products of phospholipase C action on PIP_2, can be detected in sperm preparations after Ca^{2+}/ionophore treatment (E. R. S. Roldan, R. A. P. Harrison, D. Lander & R. F. Irvine, unpublished work). Secondly (E. R. S. Roldan & R. A. P. Harrison, unpublished work), the increase in PA that accompanies phosphoinositide breakdown can be inhibited by inclusion of the drug R-59022, which is known to inhibit diacylglycerol kinase (De Chaffoy de Courcelles *et al.*, 1985). It therefore appears that this PA is derived from diacylglycerol, the other product of phospholipase C action on phosphoinositides. A recent publication has described the presence, in human spermatozoa, of considerable quantities of phospholipase C specific for phosphoinositides (Ribbes *et al.* 1987). Moreover, Bennet *et al.* (1987) have found that human spermatozoa released diacylglycerol after treatment with Ca^{2+}/A23187.

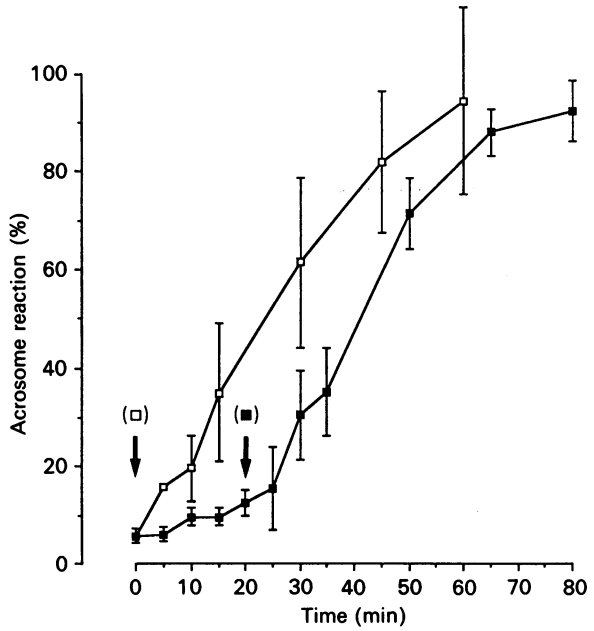

Fig. 4. Effect of delayed addition of Ca^{2+} on the time-course of the ionophore-induced acrosome reaction in ram spermatozoa

Spermatozoa in saline medium were treated with 1 μM-A23187; 1 mM-EGTA was included to prevent effects due to endogenous Ca^{2+} in the system. The arrows indicate the time at which Ca^{2+} (3 mM) was added: (□) 0 min (i.e. together with A23187) or (■) 20 min after A23187. At various time intervals, subsamples were analysed for the occurrence of acrosome reactions. Values are means ± S.D. for four separate experiments.

Table 4. Effect of late addition of Ca^{2+} on the onset of the ionophore-induced acrosome reaction in ram spermatozoa

Spermatozoa were exposed to 1 μM-A23187 in saline medium at 37 °C; Ca^{2+} (final concn. 3 mM) was added either with the ionophore or 10 min later. At intervals after addition of the cation, subsamples were analysed for occurrence of acrosome reactions. Results are means ± S.D. for four separate experiments. $*P < 0.05$, $**P < 0.01$, relative to results with Ca^{2+} added at start of treatment.

Treatment	Time after Ca^{2+} addition (min)...	Acrosome reaction (%)			
		5	10	15	30
A23187/Ca^{2+}		19.0 ± 4.8	23.5 ± 11.3	38.0 ± 19.2	67.5 ± 12.7
A23187; Ca^{2+} added 10 min later		16.5 ± 8.8	34.5 ± 17.2*	59.2 ± 15.6**	79.5 ± 11.1

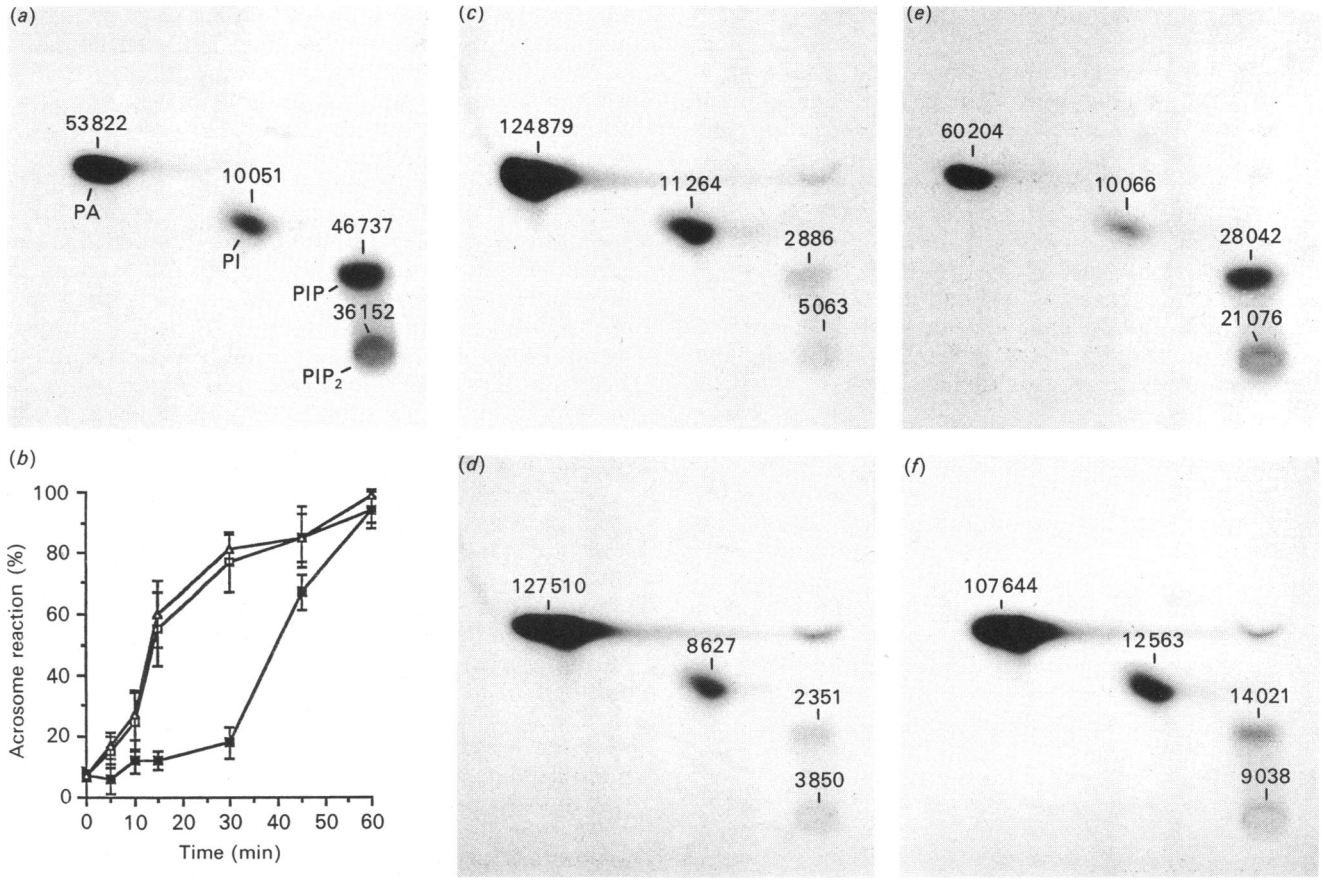

Fig. 5. Effect of neomycin on polyphosphoinositide breakdown and the acrosome reaction induced by Ca^{2+} and ionophore in ram spermatozoa

Spermatozoa in saline medium were labelled for 45 min with 250 μCi of [^{32}P]P_i/ml and then treated with 3 mM-Ca^{2+} and 1 μM-A23187. To one set of samples, 10 mM-neomycin was added at the same time as the ionophore. At various times after initiation of treatment labelled samples were precipitated with trichloroacetic acid. Lipids were extracted from the latter, separated by two-dimensional t.l.c., the plates autoradiographed, and the detected spots scraped off and counted for radioactivity. Autoradiograms are shown, together with the radioactivities associated with each spot (c.p.m./10^8 spermatozoa). (*a*) Control, no treatment; (*c*) Ca^{2+}/A23187 for 5 min; (*d*) Ca^{2+}/A23187 for 30 min; (*e*) Ca^{2+}/A23187/neomycin for 5 min; (*f*) Ca^{2+}/A23187/neomycin for 30 min. Parallel unlabelled samples were also treated with Ca^{2+} and ionophore. To one set of samples, 10 mM-neomycin was added with the ionophore or 3 min after ionophore addition. At various times after initiation of treatment, subsamples were analysed for the occurrence of acrosome reactions (*b*). □, Ca^{2+}/A23187; ■, Ca^{2+}/A23187/neomycin; △, Ca^{2+}/A23187; neomycin added 3 min later. Results are means ± S.D. for four separate experiments.

Given the breakdown of polyphosphoinositides to produce inositol phosphates and diacylglycerol, one can speculate as to their possible involvement in the processes leading to the acrosome reaction. In other cell systems, the role of inositol trisphosphate is to mobilize Ca^{2+}, principally from internal stores such as the endoplasmic reticulum (see Berridge, 1987), although recently it has been proposed that inositol 1,3,4,5-tetrakisphosphate, working together with inositol trisphosphate, may promote Ca^{2+} entry into the cell via plasma membrane-associated pathways (Irvine & Moor, 1987). In our particular model, Ca^{2+} entry and mobilization are triggered by the use of the ionophore A23187 and, therefore putative mechanisms involving inositol phosphates would have been over-ridden. However, in a more physiological situation, phosphoinositide breakdown might initially take place through receptor-mediated phospholipase C activation to bring about enhanced Ca^{2+} entry via inositol phosphate action; then, after further phospholipase C activation by newly entered Ca^{2+}, the breakdown might play a second role, that seen in the ionophore induction model.

The other product of phosphoinositide breakdown, diacylglycerol, has been ascribed a second-messenger role in other cell systems via its stimulation of protein kinase C (Nishizuka, 1984); we have searched for evidence of protein kinase C activity in ram spermatozoa and have been unable to detect any, either in unstimulated spermatozoa or in those undergoing the acrosome reaction (Roldan & Harrison, 1988). A protein kinase C-stimulating role for diacylglycerol in the acrosome reaction thus seems unlikely, at least downstream of Ca^{2+} entry. Both diacylglycerol and its product, PA, have been reported to be fusogenic (Sundler & Papahadjopoulos, 1981; Das & Rand, 1984), but the relatively slow exocytotic response relative to phosphoinositide breakdown tends to argue against such a direct role. On the other hand, diacylglycerol has been shown to increase the susceptibility of phospholipids to attack by phospholipases (Dawson *et al.*, 1984), whence fusability of

membranes could be enhanced via production of lysophosphatides; a role for phospholipase A_2 in the acrosome reaction has been proposed by others [see Meizel (1984) for a review].

Many studies have been carried out on the bivalent-cation requirements of the mammalian acrosome reaction. It is known to be a process requiring Ca^{2+}, for which Sr^{2+}, but not Mg^{2+} or Ba^{2+}, can substitute (Yanigimachi & Usui, 1974; Fraser, 1987); indeed, Rogers & Yanagimachi (1976) have reported that Mg^{2+} actually inhibits the acrosome reaction in guinea-pig spermatozoa. However, until now, no information has been available as to the molecular interactions in which the ions are involved. Our investigations described above have yielded several significant findings. Three Ca^{2+}-dependent events could be distinguished in the build-up to exocytosis, reinforcing the concept of the sequential nature of the process. The first event was the phosphoinositide breakdown, which showed an absolute requirement for low levels of Ca^{2+} and which could be inhibited by Mg^{2+}; clearly, this may be the stage at which Mg^{2+} inhibition (Rogers & Yanagimachi, 1976) is exerted. The two other Ca^{2+}-dependent events followed inositide breakdown; one again appeared to show an absolute requirement for low levels of Ca^{2+}, whereas the other needed millimolar levels of Ca^{2+} for which Sr^{2+} could substitute. So far, there is no indication as to what these two latter events may be. It may be noted, however, that Ca^{2+} is not only an activator of phospholipase A_2, but has also had a central role in many hypotheses relating to mechanisms of molecular rearrangement during the actual physical process of membrane fusion (Papahadjopoulos, 1978).

Finally, the characteristics of the Ca^{2+}/ionophore-induced acrosome reaction as a model for exocytosis may be compared with cortical-granule exocytosis in sea-urchin egg cortex preparations as elucidated by Whitaker & Aitchison (1985). In both systems, artificially introduced Ca^{2+} provokes phosphoinositide breakdown as well as exocytosis; inhibiting the phosphoinositide breakdown with neomycin prevents exocytosis, and the two processes are clearly linked. However, the speed of response of the egg model makes it very difficult to analyse the chain of events following inositide breakdown. The slower response of our sperm model may offer important advantages in this respect.

We thank Dr. R. F. Irvine for much helpful advice and discussion, and Dr. P. Smith, Mr. R. T. Lancaster and Mr. A. Tilley for their cooperation. E.R.S.R. is a recipient of Research Fellowships from Journals of Reproduction and Fertility Ltd. and from The Wellcome Trust.

REFERENCES

Babcock, D. F., First, N. L. & Lardy, H. A. (1975) J. Biol. Chem. **250**, 6488–6495

Bennet, P. J., Moatti, J. P., Mansat, A., Ribbes, H., Cayrac, J. C., Pontonnier, F., Chap, H. & Douste-Blazy, L. (1987) Biochim. Biophys. Acta **919**, 255–265

Berridge, M. J. (1987) Annu. Rev. Biochem. **56**, 159–193

Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321

Bleil, J. D. & Wassarman, P. M. (1983) Dev. Biol. **95**, 317–324

Carney, D. H., Scott, D. L., Gordon, E. A. & LaBelle, E. F. (1985) Cell (Cambridge, Mass.) **42**, 479–488

Cockcroft, S. & Gomperts, B. D. (1985) Nature (London) **314**, 534–536

Cohen, P., Broekman, M. J., Verkley, A., Lisman, J. W. W. & Derksen, A. (1971) J. Clin. Invest. **50**, 762–772

Das, S. & Rand, R. P. (1984) Biochem. Biophys. Res. Commun. **124**, 491–496

Dawson, R. M. C. & Eichberg, J. (1965) Biochem. J. **96**, 634–643

Dawson, R. M. C., Irvine, R. F., Bray, J. & Quinn, P. J. (1984) Biochem. Biophys. Res. Commun. **125**, 836–842

De Chaffoy de Courcelles, D., Roevens, P. & Van Belle, H. (1985) J. Biol. Chem. **260**, 15762–15770

De Lisle, R. C. & Williams, J. A. (1986) Annu. Rev. Physiol. **48**, 225–238

Fléchon, J. E., Harrison, R. A. P., Fléchon, B. & Escaig, J. (1986) J. Cell Sci. **81**, 43–63

Fleming, A. D. & Yanagimachi, R. (1981) Gamete Res. **4**, 253–273

Fleming, A. D., Kosower, N. S. & Yanagimachi, R. (1982) Gamete Res. **5**, 19–33

Florman, H. M. & Storey, B. T. (1982) Dev. Biol. **91**, 121–130

Fraser, L. R. (1983) J. Reprod. Fertil. **69**, 539–553

Fraser, L. R. (1987) Gamete Res. **18**, 363–374

Green, D. P. L. (1978) J. Cell Sci. **32**, 137–151

Harrison, R. A. P. (1983) in The Sperm Cell (André, J., ed.), pp. 259–273, Martinus Nijhoff, The Hague

Harrison, R. A. P., Dott, H. M. & Foster, G. C. (1982) J. Exp. Zool. **222**, 81–88

Irvine, R. F. & Moor, R. M. (1987) Biochem. Biophys. Res. Commun. **146**, 284–290

Irvine, R. F., Dawson, R. M. C. & Freinkel, N. (1982) Contemp. Metab. **2**, 301–342

Lenz, R. W., Ball, G. D., Lohse, J. K., First, N. L. & Ax, R. L. (1983) Biol. Reprod. **28**, 683–690

Meizel, S. (1984) Biol. Rev. **59**, 125–157

Meizel, S. & Turner, K. O. (1984) J. Exp. Zool. **231**, 283–288

Mitchell, K. T., Ferrell Jr., J. E. & Huestis, W. H. (1986) Anal. Biochem. **158**, 447–453

Niehaus, W. G. & Hammerstedt, R. H. (1976) Biochim. Biophys. Acta **443**, 515–524

Nishizuka, Y. (1984) Nature (London) **308**, 693–698

Noland, T. D., Abumrad, N. A., Beth, A. H. & Garbers, D. L. (1987) Biol. Reprod. **37**, 171–180

Papahadjopoulos, D. (1978) in Membrane Fusion (Poste, G. & Nicolson, G. L., eds.), pp. 765–790, Elsevier/North-Holland Biomedical Press, Amsterdam

Polascik, T., Godfrey, P. P. & Watson, S. P. (1987) Biochem. J. **243**, 815–819

Ribbes, H., Plantavid, M., Bennet, P. J., Chap, H. & Douste-Blazy, L. (1987) Biochim. Biophys. Acta **919**, 245–254

Rogers, B. J. & Yanagimachi, R. (1976) Biol. Reprod. **15**, 614–619

Roldan, E. R. S. & Harrison, R. A. P. (1988) Biochem. Biophys. Res. Commun. **155**, 901–906

Roldan, E. R. S., Shibata, S. & Yanagimachi, R. (1986) Gamete Res. **13**, 281–292

Schacht, J. (1978) J. Lipid Res. **19**, 1063–1067

Shams-Borhan, G. & Harrison, R. A. P. (1981) Gamete Res. **4**, 407–432

Sheltawy, A. & Dawson, R. M. C. (1969) in Chromatographic and Electrophoretic Techniques: Chromatography (Smith, I., ed.), 3rd edn., vol. 1, pp. 450–493, Heinemann, London

Singh, J. P., Babcock, D. F. & Lardy, H. A. (1978) Biochem. J. **172**, 549–556

Singh, J. P., Babcock, D. F. & Lardy, H. A. (1980) Biol. Reprod. **22**, 566–570

Sundler, R. & Papahadjopoulos, D. (1981) Biochim. Biophys. Acta **649**, 743–750

Talbot, P., Summers, R. G., Hylander, B. L., Keough, E. M. & Franklin, L. E. (1976) J. Exp. Zool. **198**, 383–392

Tesarik, J. (1985) J. Reprod. Fertil. **74**, 383–388

Thompson, J. G. E. & Cummins, J. M. (1986) Anim. Reprod. Sci. **12**, 151–155

Wassarman, P. M., Bleil, J. D., Florman, H. M., Greve, J. M., Roller, R. J. & Salzmann, G. S. (1986) in The Molecular and Cellular Biology of Fertilization (Hedrick, J. L., ed.), pp. 55–77, Plenum Press, New York

Whitaker, M. & Aitchison, M. (1985) FEBS Lett. **182**, 119–124

Yanagimachi, R. (1988) in The Physiology of Reproduction (Knobil, E. & Neill, J., eds.), pp. 135–185, Raven Press, New York

Yanagimachi, R. & Usui, N. (1974) Exp. Cell Res. **89**, 161–174

Received 14 July 1988/27 October 1988; accepted 3 November 1988