Introduction

Acrosomal exocytosis is triggered by oocyte-associated stimuli, such as progesterone or the zona pellucida, but it can also be stimulated by treatment with ionophores and Ca\(^{2+}\) (for review, see Roldan, 1995). Treatment with these agonists results in a rapid hydrolysis of polyphosphoinositides by a specific phosphoinositidase C (Roldan and Harrison, 1989; Thomas and Meizel, 1989; Roldan et al., 1994; O'Toole et al., 1996). A considerable increase in the mass of diacylglycerol (DAG) (Roldan and Murase, 1994; Roldan et al., 1994). The phospholipase D-phosphatidate phosphohydrolase pathway does not appear to be involved in the rapid DAG formation in mammalian spermatozoa (Roldan and Dawes, 1993; Roldan et al., 1994; O'Toole et al., 1996), and the direct action of phospholipase C (PLC) on phosphatidylcholine (PC) appears to be the major route for generating both DAG and alkyl-acyl-glycerol (Roldan and Murase, 1994).

During acrosomal exocytosis, DAG has important roles as a second messenger stimulating PC-specific PLC (Roldan and Murase, 1994), and in activating protein kinase C (PKC) (O'Toole et al., 1996; Naor and Breitbart, 1997) and phospholipase A\(_2\) (PLA\(_2\)) (Roldan and Fragio, 1994). It is not clear what type of PLA\(_2\) is present in spermatozoa, and whether PKC is involved in its activation, (discussed in Roldan and Fraser, 1998), but there is evidence indicating that DAG may have a direct role on PLA\(_2\) (Roldan and Fragio, 1994). Phospholipase A\(_2\) plays a fundamental role because it serves to generate an array of metabolites (lysophospholipids and fatty acids, and their derivatives) with distinct functions, including membrane perturbation (Lucy, 1970; Smith, 1989; Braquet et al., 1987).

Stimulation with natural agonists of exocytosis also causes activation of adenyl cyclase and formation of cAMP (Noland et al., 1988; Leclerc and Kopf, 1995; Parinaud and Milhet, 1996). However, although earlier reports suggested that cAMP and cAMP-activated protein kinase A (PKA) may play important roles during acrosomal exocytosis (Hyne and Garbers, 1979; Mrsny and Meizel, 1980; Fraser, 1981; De Jonge et al., 1991; Bielfeld et al., 1994), attention has moved towards the role of cAMP in capacitation (a physiological prerequisite to acrosomal exocytosis) as a modulator of tyrosine phosphorylation (Visconti et al., 1995, Galantino-Homer et al., 1997). Targets of the cAMP–PKA messenger system during acrosomal exocytosis have not been identified but one possible function of this pathway in exocytosis could...
be opening of ion channels, as seen in somatic cells (Sculptoreanu et al., 1993; Artalejo et al., 1994; Pedarzani and Storm, 1995). Therefore, it has been postulated that stimulation with natural agonists would lead to activation of this pathway and an increase in intracellular Ca\textsuperscript{2+} (either due to influx or mobilization from stores), and that this would set in train the sequence of events ending in membrane fusion (Naor and Breitbart, 1997). However, cAMP formation requires Ca\textsuperscript{2+} influx (Hyne and Garbers, 1979a; Parinaud and Milhet, 1996), so it is unclear how cAMP can have a role in increasing Ca\textsuperscript{2+}. It is also possible that the cAMP–PKA pathway has a role downstream of Ca\textsuperscript{2+} entry. Treatment of spermatozoa with A23187 results in the formation of cAMP (Garbers et al., 1982; Parinaud and Milhet, 1996), and stimulation with A23187 and Ca\textsuperscript{2+} (A23187/Ca\textsuperscript{2+}) in the presence of exogenous permeable cAMP leads to an enhancement of acrosomal exocytosis (Shams-Borhan and Harrison, 1981).

The present study was undertaken to examine potential targets of the cAMP–PKA pathway downstream of Ca\textsuperscript{2+} entry during acrosomal exocytosis. A model system in which exocytosis is stimulated with A23187 and Ca\textsuperscript{2+} was used to avoid any confounding effect of cAMP on early events, such as a possible modulation of ion channels (for example, see Weyand et al., 1994). In this way, mechanisms activated after Ca\textsuperscript{2+} entry, such as PLC and PLA\textsubscript{2} activation, and accompanying lipid changes, could be analysed directly.

**Materials and Methods**

**Reagents**

[9,10-\textsuperscript{3}H]Palmitic acid (specific activity 47–52 Ci mmol\textsuperscript{-1}) and [1-\textsuperscript{14}C]arachidonic acid (toluene solution; specific activity of 54–56 mCi mmol\textsuperscript{-1}) were from Amersham International (Amersham, Bucks). The ionophore A23187 was from Calbiochem (Nottingham). Poly(vinyl)alcohol (PVA)(type II) and polyvinylpyrrolidone (PVP) were from Sigma (Poole, Dorset). Hepes was from BDH (Poole, Dorset). Organic solvents were from BDH or Fisons (Loughborough, Leics). The following lipids were purchased from Sigma: 1,2-dioleoyl-sn-glycerol, 1,3-dioleoylglycerol, dimyristin (approximately 50\% 1,2-dimyrystoylgllycerol and 50\% 1,3-dimyrystoylgllycerol), arachidonic acid, and lysophosphatidylcholine (article L 4129). cAMP analogues, phosphodiesterase inhibitors and forskolin were from Sigma. The PKA inhibitor H-89 was from Biomol (Affiniti Research Products, Nottingham). Aristolochic acid was purchased from Aldrich (Milwaukee, WI) or Sigma. All other chemicals were of reagent grade and were purchased from BDH or Sigma. Stock solutions of reagents were prepared as follows: A23187 was dissolved in DMSO, cAMP analogues were dissolved in the saline medium (see below), phosphodiesterase inhibitors were dissolved in water, and forskolin, H-89 and aristolochic acid were dissolved in DMSO.

**Preparation, labelling and treatment of spermatozoa**

The saline incubation medium used in all experiments consisted of 142 mmol NaCl l\textsuperscript{-1}, 2.5 mmol KOH l\textsuperscript{-1}, 10 mmol glucose l\textsuperscript{-1} and 20 mmol Hepes l\textsuperscript{-1}, adjusted to pH 7.55 at 20°C with NaOH (Roldan and Harrison, 1989); a medium containing 222 mmol sucrose l\textsuperscript{-1} in place of the NaCl was used for washing spermatozoa. Both media also contained 1 mg PVA ml\textsuperscript{-1} and 1 mg PVP ml\textsuperscript{-1}, and had an osmolality of 305 mOsm kg\textsuperscript{-1}.

Ejaculated ram spermatozoa were separated from seminal plasma by dilution and washing through sucrose medium (400 g\textsubscript{max} for 5 min, and 1000 g\textsubscript{max} for 10 min), as described by Roldan and Harrison (1989).

Labelling was carried out routinely by incubating washed spermatozoa (about 1 x 10\textsuperscript{6} ml\textsuperscript{-1}) in 5–10 ml of saline medium containing either 0.5 µCi [\textsuperscript{3}H]palmitic acid ml\textsuperscript{-1} (Roldan and Murase, 1994) or 0.5 µCi [\textsuperscript{14}C]arachidonic acid ml\textsuperscript{-1} (Roldan and Frago, 1993) for 60 min at 37°C. Before stimulation, spermatozoa were washed through sucrose medium (400 g\textsubscript{max} for 5 min, and 1000 g\textsubscript{max} for 10 min) and resuspended in the saline medium containing 3 mmol Ca\textsuperscript{2+} l\textsuperscript{-1}.

Exocytosis of the sperm acrosome was induced by treating cells with Ca\textsuperscript{2+} (3 mmol l\textsuperscript{-1}) and the diveral cation ionophore A23187 (1 µmol l\textsuperscript{-1}) in saline medium at 37°C, and was monitored by phase-contrast microscopy of glutaraldehyde-fixed samples (Shams-Borhan and Harrison, 1981). Controls were included in all experiments and consisted of no additions, or addition of solvents used to prepare stock solutions. Analyses revealed that solvents did not affect sperm motility or integrity (that is, they did not induce acrosomal exocytosis or cause acrosomal damage).

**Lipid analyses**

Reactions were stopped at various intervals after the beginning of A23187/Ca\textsuperscript{2+} treatment for the quantification of changes in DAG or arachidonic acid, by the addition of chlorormoform–methanol (1:2 v/v), and lipids were then extracted according to Bligh and Dyer (1959). Lipids were separated by thin-layer chromatography on silica-gel 60-coated glass plates (E. Merck, Darmstadt).

The plates were developed twice in the solvent chlorormoform–methanol–acetic acid (98:2:1 v/v) (Roldan and Murase, 1994), using 1,2-dimyrystoyl-sn-glycerol, 1,3-dimyrystoylglycerol, 1,2-dioleoyl-sn-glycerol, and 1,3-dioleoylglycerol as internal lipid standards, for the quantification of labelled DAGs. After development, plates were allowed to dry and lipid spots were visualized by staining with iodine vapours, identified by comparison with the internal standards, scraped off, and the radioactivity in each was determined by liquid scintillation counting. This system allows a good separation of 1,2-DAGs containing saturated fatty acids in positions 1 and 2 (disaturated-DAG; DS-DAG) from 1,2-DAGs with a saturated fatty acid in position 1 and an unsaturated fatty acid in position 2 (saturated–unsaturated-DAG; SU-DAG) (Roldan and Murase, 1994).

Neutral lipids were separated by developing the plates twice using the solvent n-hexane–diethylether–acetic acid (70:30:1 v/v), for the quantification of arachidonic acid release. Lipids were visualized by staining in an iodine tank, identified by comparison with arachidonic acid, 1,2-dioleoyl-sn-glycerol and 1,3-dioleoylglycerol standards run on the
same plate, scraped off, and the radioactivity in each was determined by liquid scintillation counting.

Statistical analysis

Results are given as means ± SEM. The significance of results was examined after data transformation (arcsin \((\times 100)\) for percentages of exocytosis and \(\log_{10}\) for other variables), using ANOVA. Values of \(P < 0.05\) were regarded as statistically significant.

Results

Effects of dibutylryl-cAMP, phosphodiesterase inhibitors, forskolin and H-89 on exocytosis

Treatment of ram spermatozoa with 1 µmol A23187 \(l^{-1}\) and 3 mmol Ca\(^{2+}\) \(l^{-1}\) resulted in a time-dependent increase in exocytosis (Fig. 1). Exocytosis reached a maximum value (~90%) 30 min after the beginning of treatment (for comparison, see Roldan and Harrison, 1989). Exocytosis did not take place if cells were not exposed to A23187 or were treated with A23187 in the absence of Ca\(^{2+}\).

Addition of dibutylryl (db)-cAMP to spermatozoa stimulated with A23187/Ca\(^{2+}\) for 5, 10 or 15 min resulted in a concentration-dependent increase in exocytosis, with a maximal effect seen with 1 mmol db-cAMP \(l^{-1}\). In the presence of Ca\(^{2+}\), db-cAMP alone did not trigger exocytosis (Fig. 1). Furthermore, none of the treatments affected cell motility, which was ≥85% in all groups. Regardless of the concentration used, the stimulatory effect of db-cAMP was seen clearly at 5 and 10 min but was not observed at 15 min. This effect could be due to the action of phosphodiesterases catalyzing the added permeable cAMP.

Treatment of spermatozoa with the phosphodiesterase (PDE) inhibitors, caffeine, pentoxifylline or papaverine (Fig. 2), caused a concentration-dependent enhancement of exocytosis-induced by A23187/Ca\(^{2+}\), with maximal effects seen with 2 mmol caffeine \(l^{-1}\) (Fig. 2b), 1–2 mmol pentoxifylline \(l^{-1}\) (Fig. 2d), and 25–50 µmol papaverine \(l^{-1}\) (Fig. 2f). None of these reagents stimulated exocytosis or affected cell viability by themselves; that is, motility was the same (≥85%) in all treatment groups. Despite the concentration-dependence of the response to PDE inhibitors, the concentrations needed for minimal and maximal stimulation differed only by a factor of 2.0–2.5. Therefore, future work should address the effect of these reagents on isolated sperm PDEs, for which the concentrations needed for enzyme inhibition may span a wider range.

Since the stimulatory effect of added db-cAMP was short-lived (see Fig. 1a), PDE inhibitors were included to examine whether they could enhance its effect. A clear additive effect was noticed when spermatozoa were treated with a combination of db-cAMP and any of the PDE inhibitors (Table 1); motility in all experimental groups was always high (≥85%). These results indicate that the increase in cAMP, by way of the addition of exogenous analogues and inhibition of its catabolism by inclusion of PDE inhibitors,
Fig. 2. Effect of phosphodiesterase inhibitors on A23187/Ca²⁺-induced acrosomal exocytosis. Ram spermatozoa in saline medium containing 3 mmol Ca²⁺ l⁻¹ were stimulated with 1 µmol A23187 l⁻¹ for various times in the absence or presence of phosphodiesterase inhibitors. Sperm subsamples were fixed with a glutaraldehyde fixative and examined by phase-contrast microscopy. Results are means ± SEM of three experiments. (a) Spermatozoa stimulated in the absence or the presence of caffeine for various times. △, control, solvent alone; ■, 2 mmol caffeine l⁻¹ alone; ○, A23187/Ca²⁺; ●, A23187/Ca²⁺ plus 0.5 mmol caffeine l⁻¹; □, A23187/Ca²⁺ plus 1 mmol caffeine l⁻¹; ■, A23187/Ca²⁺ plus 2 mmol caffeine l⁻¹. Two-factor ANOVA: treatment, P < 0.0001; time, P < 0.0001; control versus A23187/Ca²⁺, P < 0.0001; A23187/Ca²⁺ versus A23187/Ca²⁺ plus 0.5, 1, or 2 mmol caffeine l⁻¹, P < 0.0001; A23187/Ca²⁺ versus A23187/Ca²⁺ plus 0.5 mmol caffeine l⁻¹ versus A23187/Ca²⁺ plus 1 or 2 mmol caffeine l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 1 mmol caffeine l⁻¹ versus A23187/Ca²⁺ plus 2 mmol caffeine l⁻¹, not significant; control versus caffeine (2 mmol l⁻¹) alone, not significant. (b) Spermatozoa stimulated with A23187/Ca²⁺ for 10 min in the absence or the presence of different concentrations of caffeine. ANOVA: F (5,12) = 439.14, P < 0.0001. Fisher’s post-hoc tests: A23187/Ca²⁺ versus A23187/Ca²⁺ plus 0.5, 1 or...
has a marked effect on the occurrence of the acrosomal exocytosis. It should be mentioned that reagents such as caffeine may have other effects, such as mobilization of intracellular Ca²⁺ pools (Gamberucci et al., 1999; Holz et al., 1999) and, thus, that the effect seen here could be nonspecific. It is unlikely that this is the case in the present experiments because exocytosis was stimulated with the Ca²⁺ ionophore A23187 to avoid the confounding effects of upstream events, such as the regulation of ion mobilization and influx. Further support for a lack of effect of caffeine on the mobilization and increase of intracellular Ca²⁺ comes from the observation that treatment with caffeine alone, in the presence of Ca²⁺, did not induce exocytosis (Fig. 2a). If such an increase in intracellular Ca²⁺ were taking place, it should be sufficient to initiate exocytosis.

Stimulation of spermatozoa with A23187/Ca²⁺ in the presence of the adenylyl cyclase activator forskolin (Seamon et al., 1981) resulted in an enhancement of exocytosis (Fig. 3). The effect of forskolin was concentration-dependent, with maximal effect seen at 30 µmol l⁻¹. Stimulation of acrosomal exocytosis was not observed if cells were exposed to forskolin in the absence of A23187. Forskolin, at the concentrations used in the present study, did not affect sperm acrosome integrity (the amount of acrosomal loss was similar in control and groups treated with forskolin only) or motility (it was ≥ 85% in all treatment groups). As with db-cAMP, forskolin clearly stimulated exocytosis at 5 and 10 min but the effect decreased by 15 min. This effect may have been due to catabolism by phosphodiesterases of the endogenously generated cAMP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exocytosis (%) after 10 min&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Ca²⁺ alone)</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>A23187/Ca²⁺</td>
<td>38 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>A23187/Ca²⁺ + db-cAMP</td>
<td>52 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A23187/Ca²⁺ + caffeine</td>
<td>54 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A23187/Ca²⁺ + pentoxifylline</td>
<td>55 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>A23187/Ca²⁺ + papaverine</td>
<td>67 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A23187/Ca²⁺ + db-cAMP + caffeine</td>
<td>80 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A23187/Ca²⁺ + db-cAMP + pentoxifylline</td>
<td>75 ± 7&lt;sup&gt;c,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>A23187/Ca²⁺ + db-cAMP + papaverine</td>
<td>88 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ram spermatozoa in saline medium containing 3 mmol Ca²⁺ l⁻¹ were stimulated with 1 µmol A23187/Ca²⁺ for 10 min in the absence or presence of 1 mmol db-cAMP<sup>1</sup> and/or 1 mmol caffeine l⁻¹, 1 mmol pentoxifylline l⁻¹ or 25 µmol papaverine l⁻¹. Sperm subsamples were fixed with a glutaraldehyde fixative and examined by phase-contrast microscopy. Results are means ± SEM of three experiments. When spermatozoa were incubated with db-cAMP or phosphodiesterase inhibitors alone (without ionophore addition), values of exocytosis were not different from controls.

<sup>b</sup>ANOVA: F (8,18) = 26.55, P < 0.0001. Relevant Fisher’s post-hoc tests: different from control, P ≤ 0.0001; different from A23187/Ca²⁺, P ≤ 0.05; different from A23187/Ca²⁺ plus db-cAMP or A23187/Ca²⁺ plus caffeine, P ≤ 0.001; different from A23187/Ca²⁺ plus db-cAMP or A23187/Ca²⁺ plus pentoxifylline, P ≤ 0.006; different from A23187/Ca²⁺ plus db-cAMP or A23187/Ca²⁺ plus papaverine, P ≤ 0.001.

2 mmol caffeine l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 0.5 mmol caffeine l⁻¹ versus A23187/Ca²⁺ plus 1 or 2 mmol caffeine l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 1 mmol caffeine l⁻¹ versus A23187/Ca²⁺ plus 2 mmol caffeine l⁻¹, P = 0.04. (c) Spermatozoa stimulated in the absence or the presence of pentoxifylline for various times. Δ, control, solvent alone; ▲, 3.5 mmol pentoxifylline l⁻¹ alone; □, A23187/Ca²⁺; ●, A23187/Ca²⁺ plus 1 mmol pentoxifylline l⁻¹; ■, A23187/Ca²⁺ plus 2 mmol pentoxifylline l⁻¹; ▪, A23187/Ca²⁺ plus 3.5 mmol pentoxifylline l⁻¹. Two-factor ANOVA: treatment, P < 0.0001; time, P < 0.0001; control versus A23187/Ca²⁺, P < 0.0001; A23187/Ca²⁺ versus A23187/Ca²⁺ plus 1, 2, 3.5 mmol pentoxifylline l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 1 mmol pentoxifylline l⁻¹ versus A23187/Ca²⁺ plus 3.5 mmol pentoxifylline l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 2 mmol pentoxifylline l⁻¹ versus A23187/Ca²⁺ plus 3.5 mmol pentoxifylline l⁻¹, P = 0.004; control versus pentoxifylline (3.5 mmol l⁻¹) alone, not significant. (d) Spermatozoa stimulated with A23187/Ca²⁺ for 10 min in the absence or the presence of different concentrations of pentoxifylline. ANOVA: F (5,12) = 512.06, P < 0.0001. Fisher’s post-hoc tests: A23187/Ca²⁺ versus A23187/Ca²⁺ plus 1, 2 or 3.5 mmol pentoxifylline l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 1 mmol pentoxifylline l⁻¹ versus A23187/Ca²⁺ plus 2 or 3.5 mmol pentoxifylline l⁻¹, not significant; A23187/Ca²⁺ plus 2 mmol pentoxifylline l⁻¹ versus A23187/Ca²⁺ plus 3.5 mmol pentoxifylline l⁻¹, P = 0.02. (e) Spermatozoa stimulated in the absence or the presence of papaverine for various times. Δ, control, solvent alone; ▲, 50 µmol papaverine l⁻¹, alone; ○, A23187/Ca²⁺; ●, A23187/Ca²⁺ plus 10 µmol papaverine l⁻¹; □, A23187/Ca²⁺ plus 25 µmol papaverine l⁻¹; ■, A23187/Ca²⁺ plus 50 µmol papaverine l⁻¹. Two-factor ANOVA: treatment, P < 0.0001; time, P < 0.0001; control versus A23187/Ca²⁺, P < 0.0001; A23187/Ca²⁺ versus A23187/Ca²⁺ plus 10 µmol papaverine l⁻¹, not significant; A23187/Ca²⁺ versus A23187/Ca²⁺ plus 25 or 50 µmol papaverine l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 10 µmol papaverine l⁻¹ versus A23187/Ca²⁺ plus 25 or 50 µmol papaverine l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 25 µmol papaverine l⁻¹ versus A23187/Ca²⁺ plus 50 µmol papaverine l⁻¹, not significant; control versus 50 µmol papaverine l⁻¹ alone, not significant. (f) Spermatozoa stimulated with A23187/Ca²⁺ for 10 min in the absence or the presence of different concentrations of papaverine. ANOVA: F (5,12) = 555.72, P < 0.0001. Fisher’s post-hoc tests: A23187/Ca²⁺ versus A23187/Ca²⁺ plus 10 µmol papaverine l⁻¹, not significant; A23187/Ca²⁺ versus A23187/Ca²⁺ plus 25 or 50 µmol papaverine l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 10 µmol papaverine l⁻¹ versus A23187/Ca²⁺ plus 25 or 50 µmol papaverine l⁻¹, P < 0.0001; A23187/Ca²⁺ plus 25 µmol papaverine l⁻¹ versus A23187/Ca²⁺ plus 50 µmol papaverine l⁻¹, P = 0.003.
Forskolin (60 µmol l–1) alone; Fisher’s post-hoc tests: A23187/Ca2+ versus A23187/Ca2+ plus 3 or 10 concentrations of forskolin. ANOVA: A23187/Ca2+ for 10 min in the absence or the presence of different comparisons, not significant.

A23187/Ca2+ plus 60 µmol forskolin l–1, A23187/Ca2+ plus 30 µmol forskolin l–1, not significant; A23187/Ca2+ versus A23187/Ca2+ plus 30 or 60 µmol forskolin l–1, P ≤ 0.001; A23187/Ca2+ plus 3 µmol forskolin l–1 versus A23187/Ca2+ plus 10 or 30 µmol forskolin l–1, not significant; A23187/Ca2+ plus 30 or 60 µmol forskolin l–1 versus A23187/Ca2+ plus 60 µmol forskolin l–1, P < 0.02; A23187/Ca2+ plus 10 µmol forskolin l–1 versus A23187/Ca2+ plus 30 or 60 µmol forskolin l–1, P < 0.03; A23187/Ca2+ plus 30 µmol forskolin l–1 versus A23187/Ca2+ plus 60 µmol forskolin l–1, not significant.

The effect of H-89, a PKA-specific inhibitor (Chijiwa et al., 1990), was examined to test whether cAMP action is mediated by PKA. When spermatozoa were treated with A23187/Ca2+ and different concentrations of H-89, there was a time- and concentration-dependent effect of H-89 on exocytosis (Fig. 4). There was a clear inhibition of exocytosis, with concentrations in the range 0.1–1.0 µmol H-89 l–1 at 5 min, but such inhibition was not seen with further incubation (10 or 15 min). The effect of H-89 was, in fact, biphasic (Fig. 4b): treatment of spermatozoa for 5 min with A23187/Ca2+ and with up to 1 µmol l–1 of H-89 resulted in a clear inhibition of acrosomal exocytosis, whereas higher concentrations caused a concentration-dependent stimulation of exocytosis. None of the concentrations affected cell motility. The involvement of PKA was confirmed by additional experiments showing that H-89 (0.3 µmol l–1) blocked the ability of db-cAMP (1 mmol l–1), caffeine (1 mmol l–1) or forskolin (60 µmol l–1) to enhance exocytosis triggered by A23187/Ca2+ (Table 2).

Taken together, these results demonstrate that treatment with reagents known to result in an increase in cAMP stimulated exocytosis, whereas treatment with a PKA inhibitor diminished this response.

**Effects of dibutyryl-cAMP, caffeine and forskolin on generation of diacylglycerol**

Spermatozoa were labelled with [3H]palmitic acid, washed, resuspended in saline medium, and stimulated with A23187/Ca2+ in the absence or presence of compounds known to affect endogenous amounts of cAMP to test whether the stimulatory effect of cAMP on exocytosis (described above) is mediated by the activation of PC-specific PLC. A chromatography system was used that distinguished between DS-DAG and SU-DAG (the PC-derived DAG containing saturated fatty acids in position 1 and unsaturated fatty acids in position 2, respectively) (Roldan and Murase, 1994) to avoid the confounding effects of the high basal concentrations of disaturated DAG (DS-DAG) in spermatozoa (Mann and Lutwak-Mann, 1981).

Treatment of spermatozoa with A23187/Ca2+ resulted in a considerable increase in SU-DAG, which peaked at 2.5 min and decreased slowly thereafter. Inclusion of db-cAMP (1 mmol l–1) did not enhance SU-DAG formation. Treatment with A23187/Ca2+ plus caffeine (1 mmol l–1) or forskolin (60 µmol l–1) did not affect SU-DAG formation (Table 3).

**Effects of dibutyryl-cAMP, phosphodiesterase inhibitors, forskolin and H-89 on phospholipase A2 activity**

Spermatozoa pre-labelled with [14C]arachidonic acid, were washed, resuspended in saline medium and stimulated with A23187/Ca2+ in the absence or presence of the various compounds to test if the cAMP–PKA pathway affected PLA2 activation. Stimulation with A23187/Ca2+ resulted in a time-dependent increase in arachidonic acid release, which is indicative of PLA2 activity (Roldan and Fragio, 1993). The release of arachidonic acid observed when cells were exposed
to A23187/Ca\textsuperscript{2+} and db-cAMP, PDE inhibitors, forskolin, or H-89 is shown (Table 4). None of these compounds affected the release of arachidonic acid when they were added alone or together with A23187/Ca\textsuperscript{2+}. Therefore, these results indicate that PLA\textsubscript{2} activation is not modulated by cAMP.

**Action of cAMP downstream of phospholipase A\textsubscript{2} in A23187/Ca\textsuperscript{2+}-induced exocytosis**

Since compounds known to affect cAMP concentrations did not affect PLC or PLAC\textsubscript{2} activation, it was examined whether cAMP could act at a step downstream of PLAC\textsubscript{2} by testing whether a cAMP analogue would stimulate exocytosis in spermatzoa in which PLAC\textsubscript{2} was inhibited by aristolochic acid (Rosenthal et al., 1989, 1992). In ram spermatzoa, aristolochic acid caused a concentration-dependent inhibition of A23187/Ca\textsuperscript{2+}-stimulated exocytosis (Fig. 5). Inhibition reached a maximum at 400 \textmu mol l\textsuperscript{-1}, a concentration found to inhibit PLAC\textsubscript{2} in other cells (Rosenthal et al., 1989, 1992). Aristolochic acid alone did not affect cell integrity and did not affect sperm viability (all treatment groups exhibited motility \geq 85\%). Aristolochic acid also inhibits arachidonic acid utilization in eicosanoid synthesis (a step taking place immediately downstream of PLAC\textsubscript{2}), since this compound was found to inhibit lipo-oxygenase in vitro (Moreno, 1993). However, although this inhibition may have taken place in spermatzoa under the conditions used in the present study, the use of this inhibitor should still yield valid information on whether cAMP acts further downstream of arachidonic acid generation or utilization (via PLAC\textsubscript{2} or cyclo/lipo-oxygenase) at a point close to membrane fusion.

The results of the experiment designed to test whether cAMP would stimulate exocytosis when cells were treated with aristolochic acid are shown (Fig. 6). Inclusion of 1 mmol db-cAMP l\textsuperscript{-1} did not result in exocytosis when spermatzoa were treated with A23187/Ca\textsuperscript{2+} in the presence of 400 \textmu mol aristolochic acid l\textsuperscript{-1}. However, if a sub-maximal concentration of lysophosphatidylcholine (2.5 \textmu g ml\textsuperscript{-1}) (Roldan and Fragio, 1993) was included together with db-cAMP, exocytosis did take place, and to a greater extent than that seen after stimulation in the presence of significant. (b) Spermatozoa incubated for 5 min without or with different concentrations of H-89 and then stimulated for 5 min with A23187/Ca\textsuperscript{2+}. ANOVA: \(F(9,19) = 25.14\), \(P < 0.0001\). Fisher’s post-hoc tests: A23187/Ca\textsuperscript{2+} versus A23187/Ca\textsuperscript{2+} plus 0.1 or 3 \textmu mol H-89 l\textsuperscript{-1}, not significant; A23187/Ca\textsuperscript{2+} versus A23187/Ca\textsuperscript{2+} plus 0.3, 0.5, 1, 3 or 10 \textmu mol H-89 l\textsuperscript{-1}, \(P \leq 0.02\); A23187/Ca\textsuperscript{2+} plus 0.1 \textmu mol H-89 l\textsuperscript{-1} versus A23187/Ca\textsuperscript{2+} plus 0.3, 0.5 or 1 \textmu mol H-89 l\textsuperscript{-1}, not significant; A23187/Ca\textsuperscript{2+} plus 0.1 \textmu mol H-89 l\textsuperscript{-1} versus A23187/Ca\textsuperscript{2+} plus 3 or 10 \textmu mol H-89 l\textsuperscript{-1}, \(P \leq 0.02\); A23187/Ca\textsuperscript{2+} plus 0.3 \textmu mol H-89 l\textsuperscript{-1} versus A23187/Ca\textsuperscript{2+} plus 0.5 or 1 \textmu mol H-89 l\textsuperscript{-1}, not significant; A23187/Ca\textsuperscript{2+} plus 0.3 \textmu mol H-89 l\textsuperscript{-1} versus A23187/Ca\textsuperscript{2+} plus 3 or 10 \textmu mol H-89 l\textsuperscript{-1}, \(P = 0.0001\); A23187/Ca\textsuperscript{2+} plus 0.5 \textmu mol H-89 l\textsuperscript{-1} versus A23187/Ca\textsuperscript{2+} plus 1 \textmu mol H-89 l\textsuperscript{-1}, not significant; A23187/Ca\textsuperscript{2+} plus 0.5 \textmu mol H-89 l\textsuperscript{-1} versus A23187/Ca\textsuperscript{2+} plus 3 or 10 \textmu mol H-89 l\textsuperscript{-1}, \(P = 0.0001\); A23187/Ca\textsuperscript{2+} plus 3 \textmu mol H-89 l\textsuperscript{-1} versus A23187/Ca\textsuperscript{2+} plus 10 \textmu mol H-89 l\textsuperscript{-1}, \(P = 0.0001\); control versus 10 \textmu mol H-89 l\textsuperscript{-1} alone.
Table 2. Effect of the protein kinase A inhibitor H-89 on the A23187/Ca\textsuperscript{2+}-induced acrosomal exocytosis of ram spermatozoa\textsuperscript{*}

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Exocytosis (%) after 5 min\textsuperscript{a}</th>
<th>Exocytosis (%) after 10 min\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>– – – – –</td>
<td>11 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>+ – – + –</td>
<td>39 ± 1\textsuperscript{c}</td>
<td>53 ± 1\textsuperscript{b}</td>
</tr>
<tr>
<td>+ – – + –</td>
<td>19 ± 1\textsuperscript{cd}</td>
<td>58 ± 2\textsuperscript{b}</td>
</tr>
<tr>
<td>+ + – – –</td>
<td>61 ± 3\textsuperscript{cd}</td>
<td>82 ± 2\textsuperscript{h}</td>
</tr>
<tr>
<td>+ + – – +</td>
<td>23 ± 1\textsuperscript{cd}</td>
<td>59 ± 1\textsuperscript{h}</td>
</tr>
<tr>
<td>+ – + – –</td>
<td>65 ± 3\textsuperscript{cd}</td>
<td>83 ± 3\textsuperscript{h}</td>
</tr>
<tr>
<td>+ – + – +</td>
<td>21 ± 1\textsuperscript{cd}</td>
<td>77 ± 1\textsuperscript{h}</td>
</tr>
<tr>
<td>+ – – + +</td>
<td>57 ± 1\textsuperscript{cd}</td>
<td>75 ± 1\textsuperscript{h}</td>
</tr>
<tr>
<td>+ – – + +</td>
<td>24 ± 1\textsuperscript{cd}</td>
<td>56 ± 2\textsuperscript{h}</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Ram spermatozoa in saline medium containing 3 mmol Ca\textsuperscript{2+} l\textsuperscript{-1} were incubated for 5 min in the absence or presence of 0.3 µmol H-89 l\textsuperscript{-1} and were then stimulated with 1 µmol A23187 l\textsuperscript{-1} for 5 or 10 min in the absence or presence of 1 mmol dibutyryl (db)-cAMP l\textsuperscript{-1}, 1 mmol caffeine l\textsuperscript{-1}, or 60 µmol forskolin l\textsuperscript{-1}. Sperm subsamples were fixed with a glutaraldehyde fixative and examined by phase-contrast microscopy. Results are means ± SEM of three experiments. When spermatozoa were incubated with db-cAMP, caffeine, forskolin or H-89 alone (without ionophore addition), values of exocytosis were not different from controls.

\textsuperscript{a}ANOVA: F (8,9) = 106.96, \( P < 0.0001 \). Relevant Fisher’s post hoc tests: \( P \leq 0.005 \) for different from control;
\textsuperscript{b}ANOVA: F (8,9) = 104.66, \( P < 0.0001 \). Relevant Fisher’s post hoc tests: \( P < 0.04 \) for different from control.

Table 3. Effect of dibutyryl (db)-cAMP, caffeine or forskolin on the A23187/Ca\textsuperscript{2+}-induced generation of saturated–unsaturated diacylglycerol (SU-DAG) in ram spermatozoa\textsuperscript{*}

<table>
<thead>
<tr>
<th>Treatments</th>
<th>[3H]SU-DAG after 2.5 min (c.p.m. per 10\textsuperscript{8} spermatozoa)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Ca\textsuperscript{2+} alone)</td>
<td>144 818 ± 616</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+}</td>
<td>317 555 ± 1192\textsuperscript{b}</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+} + db-cAMP</td>
<td>318 809 ± 2739\textsuperscript{b}</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+} + caffeine</td>
<td>320 236 ± 1777\textsuperscript{b}</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+} + forskolin</td>
<td>323 157 ± 2780\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Ram spermatozoa were labelled with 0.5 µCi of [3H]palmitic acid ml\textsuperscript{-1} for 60 min at 37ºC, washed, and resuspended in saline medium with 3 mmol Ca\textsuperscript{2+} l\textsuperscript{-1}. Spermatozoa were then treated with 1 µmol A23187 l\textsuperscript{-1} in the absence or presence of 1 mmol dibutyryl (db)-cAMP l\textsuperscript{-1}, 1 mmol caffeine l\textsuperscript{-1} or 60 µmol forskolin l\textsuperscript{-1}. After incubation for 2.5 min, lipids were extracted, separated and diacylglycerol with saturated fatty acids in position 1 and unsaturated fatty acids in position 2 (SU-DAG) was quantified. Results are means ± SEM of four experiments.

\textsuperscript{a}ANOVA: F (4,10) = 18.20, \( P < 0.0001 \). Fisher’s post hoc tests: \( P < 0.0001 \) for different from control, \( P > 0.0001 \) for all other comparisons.

Table 4. Effect of dibutyryl (db)-cAMP and phosphodiesterase inhibitors, forskolin and H-89 on the A23187/Ca\textsuperscript{2+}-induced release of arachidonic acid in ram spermatozoa\textsuperscript{*}

<table>
<thead>
<tr>
<th>Treatments</th>
<th>[14C]Arachidonic acid after 5 min (c.p.m. per 10\textsuperscript{8} spermatozoa)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Ca\textsuperscript{2+} alone)</td>
<td>501 ± 54</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+}</td>
<td>2186 ± 67\textsuperscript{b}</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+} + db-cAMP</td>
<td>2112 ± 23\textsuperscript{b}</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+} + caffeine</td>
<td>2379 ± 66\textsuperscript{b}</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+} + papaverine</td>
<td>2247 ± 72\textsuperscript{b}</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+} + db-cAMP + caffeine</td>
<td>1963 ± 78\textsuperscript{b}</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+} + db-cAMP + papaverine</td>
<td>1951 ± 141\textsuperscript{b}</td>
</tr>
<tr>
<td>A23187/Ca\textsuperscript{2+} + H-89</td>
<td>2282 ± 268\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Ram spermatozoa were labelled with 0.5 µCi of [14C]arachidonic acid ml\textsuperscript{-1} for 60 min at 37ºC, washed, and resuspended in saline medium with 3 mmol Ca\textsuperscript{2+} l\textsuperscript{-1}. Spermatozoa were then treated with 1 µmol A23187 l\textsuperscript{-1} in the absence or presence of 1 mmol dibutyryl (db)-cAMP l\textsuperscript{-1}, 1 mmol caffeine l\textsuperscript{-1} or 60 µmol forskolin l\textsuperscript{-1}. After incubation for 5 min, lipids were extracted, separated and quantified. Results are means ± SEM of four experiments.

\textsuperscript{a}ANOVA: F (12,19) = 48.20, \( P < 0.0001 \). Fisher’s post hoc tests: \( P < 0.0001 \) for all other comparisons.
PLA2 but, rather, seems to relate to the promotion of mechanisms leading to DAG formation or the activation of acrosome. The action of cAMP does not appear to be related to the promotion of membrane fusion itself.

**Discussion**

The results of the present study indicate that cAMP has an important role, downstream of Ca\(^{2+}\) entry, in the sequence ending in membrane fusion during exocytosis of the sperm acrosome. The action of cAMP does not appear to be related to mechanisms leading to DAG formation or the activation of PLA\(_2\), but, rather, seems to relate to the promotion of membrane fusion itself.

**Fig. 6.** Effect of aristolochic acid (arist. acid), dibutyryl cAMP (db-cAMP), lysophosphatidylcholine (lysoPC) and H-89 on A23187/Ca\(^{2+}\)-induced acrosomal exocytosis. Ram spermatozoa in saline medium containing 3 mmol Ca\(^{2+}\) l\(^{-1}\) were incubated for 5 min in the absence or the presence of aristolochic acid (400 µmol l\(^{-1}\)), db-cAMP (1 mmol l\(^{-1}\)), lysoPC (2.5 mg ml\(^{-1}\)) and–or H-89 (0.3 mmol l\(^{-1}\)) and were then stimulated with A23187 (1 µmol l\(^{-1}\)) for 15 min. Sperm samples were fixed with a glutaraldehyde fixative and examined by phase-contrast microscopy. Results are means ± SEM of three experiments. ANOVA: treatment, \(P < 0.0001\); time, \(P < 0.0001\); control versus A23187/Ca\(^{2+}\), \(P < 0.0001\); A23187/Ca\(^{2+}\) plus aristolochic acid, db-cAMP and lysoPC. "When compared with control, \(P < 0.001\).

Despite some early work dealing with the generation and involvement of cAMP in exocytosis in mammalian spermatozoa Hyne and Garbers, 1979; Garbers et al., 1982, the evidence for a role of the cAMP–PKA pathway in acrosomal exocytosis remains circumstantial (De Jonge et al., 1991; Bielfeld et al., 1994). So far, no targets for the cAMP–PKA pathway have been identified. Therefore, this study was designed to test whether pathways known to underlie exocytosis in mammalian spermatozoa were activated by the cAMP–PKA pathway.

Sperm adenylyl cyclase is regulated, among other factors, by Ca\(^{2+}\) ions (Hyne and Garbers, 1979a,b; Garbers et al., 1982; Rojas et al., 1992). Ca\(^{2+}\) is necessary for enzyme activation and cAMP generation during acrosomal exocytosis since experiments in which Ca\(^{2+}\) entry was prevented led to no enzyme activity (Hyne and Garbers, 1979a; Garbers et al., 1982; Parinaud and Milhet, 1996). Furthermore, stimulation with the Ca\(^{2+}\) ionophore A23187 resulted in adenylyl cyclase activation and cAMP formation (Garbers et al., 1982; Parinaud and Milhet, 1996). cAMP formation after Ca\(^{2+}\) entry may be important for the activation of events underlying exocytosis, as indicated by the present study. A series of experiments using a permeable analogue of cAMP, an
activator of adenyl cyclase, and PDE inhibitors, were coincident in that exocytosis was stimulated. Therefore, the present results are in agreement with earlier observations indicating that reagents known to increase endogenous concentrations of cAMP stimulate exocytosis (Mrsny and Meizel, 1980; Shams-Borham and Harrison, 1981; De Jonge et al., 1991; Tesarik et al., 1992; Leclerc and Kopf, 1995). The present study extends these findings by addressing which molecular mechanisms may be the target of the cAMP–PKA signalling pathway.

The present study concentrated on pathways activated downstream of Ca2+ entry by using a model system in which exocytosis is triggered with A23187/Ca2+, thus avoiding the possible confounding effect of the role of cAMP in the modulation of ion fluxes. Since previous work has revealed that generation of DAG, via activation of PC-specific PLC, and the activation of PLA2 are two important mechanisms in the sequence leading to membrane fusion (Roldan and Frago, 1993; Roldan and Murase, 1994), it was hypothesized that these mechanisms could be the targets of cAMP action.

It has been reported that cAMP may activate, inhibit or have no effect on phosphoinositide-specific phosphoinositidase C (Alava et al., 1992; Bold et al., 1995; Tsai et al., 1995) but, to the best of our knowledge, there is no clear evidence of cAMP-mediated regulation of PC-specific PLC. Treatment with A23187/Ca2+ and reagents known to enhance endogenous cAMP concentrations caused no alteration of PLC–PLC or PLA2, the generation of arachidonic acid was tested but it was found that db-cAMP, PDE inhibitors, and forskolin did not affect PLA2 activity. Even if combinations of db-cAMP and PDE inhibitors were used, no evidence for PLA2 stimulation was obtained, indicating that the stimulatory action of cAMP-related compounds on exocytosis is not on this locus. In this context, it is important to consider that the PLA2 isozyme that is activated by the cAMP–PKA–MAP kinase pathway is the 85 kDa cytosolic PLA2 (Lin et al., 1993). It is not known which PLA2 isoenzymes are present in spermatozoa and are, therefore, responsible for the PLA2 activity detected. So far, it has been shown that spermatozoa have a 14–16 kDa PLA2 (Langlais et al., 1992), and this isoform may not be a substrate for the cAMP–PKA–MAP kinase pathway (for further discussion, see Roldan and Fraser, 1998).

The effects of cAMP in spermatozoa were mediated by PKA, as in other cells (Taylor et al., 1990; Walsh and van Patten, 1994). The finding that the specific PKA blocker H-89, reduced exocytosis triggered by A23187/Ca2+ and, also, exocytosis stimulated by db-cAMP, caffeine or forskolin is in agreement with this hypothesis. The effect of H-89 was biphasic (that is, concentrations up to 1 μmol l−1 were inhibitory, and higher concentrations were stimulatory). Similar results were obtained in parotid acini (Takuma and Ichida, 1994) in which 33 μmol H-89 l−1 inhibited exocytosis and higher concentrations markedly increased basal exocytosis. This finding indicates that high concentrations of H-89 may perturb the plasma or the secretory granule membranes. However, the effect of H-89 was reversed over time: when spermatozoa were treated with A23187/Ca2+ and low concentrations of H-89, the inhibition of exocytosis was seen at 5 min but not at 10 or 15 min. This finding indicates that there may be some degree of redundancy in the pathways involving cAMP–PKA, as Roldan and Harrison (1989) have suggested for other pathways.

Since no effect was seen on PC–PLC or PLA2, the cAMP–PKA system may activate events downstream of PLA2 activation. Support for this hypothesis comes from results showing that cAMP helped to restore exocytosis in sperm cells treated with the PLA2 antagonist aristolochic acid and that the effect was inhibited by inclusion of H-89. The single addition of cAMP did not result in exocytosis in spermatozoa exposed to aristolochic acid, whereas the inclusion of both lysophosphatidylcholine (a metabolite generated by PLA2) and cAMP did restore exocytosis. These findings indicate that the cAMP–PKA system alone does not drive the final stages of membrane fusion but, rather, that it acts together with other messenger systems or metabolites.

Since the substrates for PKA in spermatozoa are not known, the potential targets of the cAMP–PKA system in the final stages of fusion should be elucidated in future work. One possible substrate could be ralphilin-3A (Numata et al., 1994), a rab3A-binding protein that regulates secretion in chromaffin cells (Chung et al., 1995). Work by Garde and Roldan (1996) indicates that rab3A is indeed involved in exocytosis of the acrosome and, therefore, it is possible that the cAMP–PKA system is functionally coupled to rab3A–ralsphilin-3A during sperm membrane fusion.

In conclusion, the present study shows that the cAMP–PKA system has an important regulatory role in the sequence underlying exocytosis, acting on a step downstream of PLA2 activation. This signalling system may be involved in the phosphorylation of proteins that modulate membrane fusion.

References
Artalejo CR, Adams ME and Fox AP (1994) Three types of Ca2+ channel trigger secretion with different efficiencies in chromaffin cells Nature 367 72–76
Bligh EG and Dyer WJ (1959) A rapid method for total lipid extraction and purification Canadian Journal of Biochemistry and Physiology 37 911–917
Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T and Hidaka H (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective...
inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isooquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells Journal of Biological Chemistry 265 5267–5272
Garde J and Roldan ERS (1991) raf3-Peptide stimulates exocytosis of the ram sperm acrosome via interaction with cyclic AMP and phosphatidase A2 metabolites F6S Letters 291 263–268
Hyne RV and Garbers DL (1979a) Calcium-dependent increase in adenosine 3',5'-monophosphate and induction of the acrosome reaction in guinea pig spermatozoa Proceedings National Academy of Sciences of the USA 76 5699–5703
Hyne RV and Garbers DL (1979b) Regulation of guinea pig sperm adenyl cyclase by calcium Biology of Reproduction 23 1135–1142
Naor Z and Breibart H (1997) Protein kinase C and mammalian spermatozoa acrosome reaction Trends in Endocrinology and Metabolism 8 337–342
O’Toole CMB, Roldan ERS, Hampton P and Fraser LR (1996a) A role for diacylglycerol in human sperm acrosomal exocytosis Molecular Human Reproduction 2 117–124
O’Toole CMB, Roldan ERS and Fraser LR (1996b) Protein kinase C activation during progesterone-stimulated acrosomal exocytosis in human spermatozoon Molecular Human Reproduction 2 921–927
Roldan ERS and Dawes E (1993) Phosphatidase A2 and exocytosis of the ram sperm acrosome Biochemistry et Biophysica Acta 1210 48–54
Roldan ERS and Fragio C (1994) Dihydroyglycerol stimulates phosphatidase A2 and subsequent exocytosis in ram spermatozoa. Evidence that the effect is not mediated by protein kinase C Biochemical Journal 297 225–232
Roldan ERS and Fraser LR (1998) Protein kinase C and exocytosis in mammalian spermatozoa Trends in Endocrinology and Metabolism 9 296–297
Roldan ERS and Harrison RAP (1989) Polyphosphoinositide breakdown and subsequent exocytosis in the Ca2+/ionophore-induced acrosome reaction of mammalian spermatozoa Biochemical Journal 259 397–406
Roldan ERS and Murase T (1994) Polyphosphoinositide-derived diacylglycerol stimulates the hydrolysis of phosphatidylcholine by phosphatidylcholine C during exocytosis of the ram sperm acrosome Journal of Biological Chemistry 269 23 583–23 589
Rosenthal MD, Lattanzio KS and Franson RC (1992) The effects of the phosphatidase A2 inhibitors aristolochic acid and PGB2 on A23187-stimulated mobilization of arachidonate in human neutrophils are overcome by diacylglycerol or phosphol ester Biochimica et Biophysica Acta 1126 319–326
Scruporeau A, Sucheur T and Catterall WA (1993) Voltage-dependent potentiation of L-type Ca2+ channels due to phosphorylation by cAMP-dependent protein kinase Nature 364 240–243
Seamon KB, Padgett W and Daly JW (1981) Forskolin. Unique diterpene activator of adenylyl cyclase in membranes and in intact cells Proceedings National Academy of Sciences of the USA 78 3363–3367
Thomas P and Meisel S (1989) Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca2+ influx Biochemical Journal 264 539–546
