Membrane Potential Regulates Sea Urchin Sperm Adenylylcyclase†

Carmen Beltrán, Otilia Zapata, and Alberto Darszon*

Departamento de Genética y Fisiología Molecular, Instituto de Biotecnologia, Universidad Nacional Autónoma de México, Apdo. 510-3, Cuernavaca, Morelos 62271, México

Received November 27, 1995; Revised Manuscript Received April 1, 1996⊗

ABSTRACT: Adenylylcyclase (AC) from sea urchin sperm does not appear to be regulated by G proteins [Hildebrandt, J. D., Tash, J. S., Kirchick, H. J., Lipschunits, L., Secra, R. D., & Birmbaumer, L. (1985) *Endocrinology 116*, 1357−1366]. During sperm activation and the acrosome reaction, membrane potential changes and cAMP increases. Here we explore if membrane potential can modulate the sperm AC. Hyperpolarization of *Lytechinus pictus* sea urchin sperm either with valinomycin in artificial sea water (ASW) without K^+ or with dilution in ASW without Na^+ increased the [c-AMP] (2.2- and 5.8-fold, respectively). This increase also occurred in the absence of extracellular Ca^{2+} (1.9- and 3.1-fold, respectively) and was enhanced by 100 μM IBMX, a phosphodiesterase inhibitor. It has been suggested that sea urchin sperm AC is stimulated by increases in intracellular Ca^{2+} and intracellular pH. In ASW without Na^+ and Ca^{2+} (0Na0CaASW), sea urchin sperm intracellular pH decreases, and intracellular Ca^{2+} cannot increase. Therefore, these observations taken together indicate that AC in these cells is modulated by membrane potential. Dilution of *Strogylocentrotus purpuratus* sperm in 0Na0CaASW hyperpolarized them and increased their cAMP levels (1.3-fold). This stimulation was enhanced by IBMX (1.6-fold). Addition of the egg peptide speract under this condition further hyperpolarized *S. purpuratus* sperm and synergistically increased [cAMP] above 0Na0CaASW. This stimulation became larger in the presence of IBMX (from 1.6- to 5.2-fold). Since speract cannot elevate intracellular pH or $[Ca^{2+}]$ in 0Na0CaASW, the increase in [cAMP] it causes must be due to sperm hyperpolarization.

Adenylylcyclase (EC 4.6.1.1) is the enzyme that converts ATP into cAMP,[1] an important second messenger in signal transduction. This enzyme is part of a large family of proteins. In mammals, there are at least six distinct ACs, and all of them are stimulated by forskolin and by 5′-guanyl imidodiphosphate but differ in their sensitivity to Ca^{2+}/calmodulin (CaM) and G proteins (Choi et al., 1993). It is generally believed that AC regulation involves hormone binding to a cell surface receptor; the hormone−receptor complex activates a G protein which in turn modulates AC activity. Increases in [cAMP] stimulate cAMP-dependent protein kinases and regulate a variety of cell functions [for reviews see Federman et al. (1992) and Choi et al. (1993)]; they can also directly modulate ion channels (Zufall et al., 1994; Labarca et al., 1995).

Sea urchin sperm have an unusually high concentration of AC and cyclic nucleotide (cNMP) phosphodiesterases, as compared with somatic cells [reviewed in Garbers and Kopf (1980)]. The sea urchin sperm AC is membrane-bound and found preferentially in the flagella (Bookbinder et al., 1990). Unlike the AC from somatic cells, it is not activated by guanine nucleotides, fluoride, or forskolin and requires Mn^{2+} for its activity; thus, it seems not to be regulated by G proteins (Hildebrandt et al., 1985). In this sense, it is similar to the *Paramecium* enzyme which appears to contain a channel and can be regulated by membrane potential (Schultz et al., 1992).

In sea urchin sperm, cAMP has been implicated in the initiation and maintenance of motility. Although there is evidence indicating that the sperm AC is stimulated by increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$), its regulation in sperm is not well-understood. The rise in [cAMP] activates a cAMP-dependent protein kinase that phosphorylates axonemal proteins necessary to initiate motility (Bookbinder et al., 1990, and references therein).

Another important process in spermatozoa intimately related with a rise in [cAMP] is the acrosome reaction (AR) (Garbers, 1989). Sea urchin sperm must undergo the AR to fertilize the homologous egg. In this species, the AR occurs when sperm encounter a fucose sulfate glycoprotein, the Factor, from the egg jelly. This Factor induces acrosomal vesicle exocytosis, exposure of material required for sperm−egg binding, and extension of the acrosomal tubule which is surrounded by the membrane destined to fuse with the egg (Trimmer & Vacquier, 1986). In addition, binding of Factor to its receptor on the surface of the sperm triggers an

† This work was partially supported by the Mexican Council for Science and Technology (CONACyT) and DGAPA from the Universidad Nacional Autónoma de México. A.D. is an International Scholar of the Howard Hughes Medical Institute.

* Corresponding author: Alberto Darszon, Departamento de Genética y Fisiologia Molecular, Instituto de Biotecnologia, Universidad Nacional Autónoma de México, Apdo. 510-3, Cuernavaca, Morelos 62271, México. E-mail: darszon@ibt.unam.mx.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1996.

[1] Abbreviations: AR, acrosome reaction; ASW, artificial sea water; Factor (F), fucose sulfate glycoprotein factor; $[K^+]_e$, external K^+ concentration; 0CaASW, artificial sea water without $CaCl_2$; 0KASW, artificial sea water without KCl; 0K0CaASW, artificial sea water without KCl and $CaCl_2$; 0NaASW, artificial sea water without NaCl; 0Na0CaASW, artificial sea water without NaCl and $CaCl_2$; AC, adenylylcyclase; CaM, calmodulin; cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cNMP, cyclic nucleotide; [cAMP], concentration of cAMP; $[Ca^{2+}]_e$, extracellular Ca^{2+} concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; pH_i, intracellular pH; V_m, membrane potential; PDE, phosphodiesterases; J, egg jelly; S, speract; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; DiS-C_3-(5), dipropylthiodicarbocyanine; IBMX, 3-isobutyl-1-methylxantine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

S0006-2960(95)02806-6 CCC: $12.00

influx of Na^+ and Ca^{2+} and an efflux of K^+ and H^+. These ionic fluxes are associated with an increase in $[Ca^{2+}]_i$ and intracellular pH (pH_i), as well as with changes in membrane potential. The Factor also elevates the levels of inositol 1,4,5-triphosphate, cGMP, and cAMP [reviewed in Ward and Kopf (1993) and Darszon et al. (1994)].

All artificial treatments that induce AR (Talbot et al., 1976; Collins & Epel, 1977; Schackmann et al., 1978) raise cAMP levels in intact cells as well as in isolated sperm heads (Garbers, 1981). In both cases, the absence of external Ca^{2+} ($[Ca^{2+}]_e$) blocks AR and the increase in cAMP (Talbot et al., 1976; Collins & Epel, 1977; Schackmann et al., 1978; Garbers, 1981). Although the accumulation of cAMP is associated with the occurrence of AR, there is no direct evidence that it is required for its induction. Additionally, much as sea urchin sperm AC binds to CaM and is inhibited by high concentrations of CaM inhibitors that block the AR, there is no clear demonstration that CaM regulates sperm AC (Bookbinder et al., 1990).

Sea urchin sperm undergo membrane potential changes in response to their environment and to components from the outer layer of the egg. Using *Lytechinus pictus* and *Strogylocentrotus purpuratus* sperm, this work explored if membrane potential can modulate their cAMP levels independently of pH_i and $[Ca^{2+}]_i$. Sperm membrane potential was hyperpolarized by diluting washed cells in ASW of different ionic compositions. We show that hyperpolarization of intact sea urchin spermatozoa can increase cAMP levels independently of pH, $[Ca^{2+}]_e$, and the AR. This response is enhanced by the phosphodiesterase (PDE) inhibitor IBMX. We also investigated the effect of the hyperpolarization induced by the egg peptide speract on *S. purpuratus* sperm already exposed to hyperpolarizing conditions.

MATERIALS AND METHODS

S. purpuratus and *L. pictus* sea urchins were obtained from Marinus (Long Beach, CA). Spawning was induced by intracoelomic injection of 0.5 M KCl, and semen was kept undiluted on ice until use. IBMX, valinomycin, and *N*-methyl-D-glucamine were from Sigma (St. Louis, MO). Speract was from Peninsula Laboratories (Belmont, CA). Dipropylthiodicarbocyanine (DiS-C_3-(5)] and Fura-2 were from Molecular Probes (Eugene, OR). The cAMP ^3H assay system (TRK 432) was from Amersham (England) and DMSO from J. T. Baker.

Egg jelly (J) (García-Soto & Darszon, 1985) and Factor (Collins & Epel, 1977) were prepared as described. Both were quantified by their fucose content (Dische & Shettles, 1948). AR was determined by phase contrast microscopy as described (García-Soto & Darszon, 1985) and protein by the Bradford method modified for membrane proteins (Fanger, 1987), using BSA as standard.

Solutions. Artificial sea water (ASW) contained the following in millimolar: 486 NaCl, 10 KCl, 26 $MgCl_2$, 30 $MgSO_4$, 10 $CaCl_2$, 2.5 $NaHCO_3$, 0.1 EDTA, and 10 HEPES (pH 8.0). ASW without added Ca^{2+} (0CaASW) or K^+ (0KASW), or both (0K0CaASW), was also used. Na^+-free sea water (0NaASW) was ASW where NaCl and $NaHCO_3$ were substituted by *N*-methyl-D-glucamine and $KHCO_3$, respectively; pH was adjusted with 0.1 N KOH. Na^+- and Ca^{2+}-free sea water (0Na0CaASW) was prepared as 0NaASW without Ca^{2+}.

cAMP Determinations. cAMP was measured with the cyclic AMP ^3H assay system TRK 432. Briefly, 100 μL of 100-fold-diluted sperm (50−100 μg of protein) in 1 mM $CaCl_2$ ASW (pH 7.0) was centrifuged (1000*g* for 5 min). The pellet was resuspended in 200 μL of the indicated ASW at 14 °C. A 20 μL aliquot was transferred to the same volume of 12% glutaraldehyde to measure AR at determined times. Simultaneously, the rest of the volume was boiled for 3−5 min. The sperm suspension was cooled down in an ice bath, and cAMP was extracted with 2 volumes of ethanol by vortexing 1 min and allowing the suspension to stand for 5 min at room temperature. The particulate material was spun down (16000*g* for 20 min) and the supernatant containing cAMP evaporated to dryness in a Speed Vac at 55 °C. The residue was resuspended in 50 μL of the Kit buffer [50 mM Tris and 4 mM EDTA (pH 7.5)] to quantify cAMP as described in the Kit pamphlet TRK 432.

Membrane Potential (V_m), pH_i, and $[Ca^{2+}]_i$ Measurements. V_m was determined with the fluorescent membrane potential-sensitive dye DiS-C_3-(5) at 500 nM ($\lambda_{exc} = 620$, $\lambda_{emi} = 670$) (González-Martínez & Darszon, 1987; Reynaud et al., 1993). BCECF ($\lambda_{exc} = 500$, $\lambda_{emi} = 540$) was used to measure pH_i in *S. purpuratus* and DMCF ($\lambda_{exc} = 500$, $\lambda_{emi} = 540$) in *L. pictus* sea urchin sperm. The cells were loaded and calibrated as reported (Guerrero & Darszon, 1989; González-Martínez et al., 1992). Sperm $[Ca^{2+}]_i$ measurements were done using 10−20 μM Fura-2 ($\lambda_{exc} = 340$, $\lambda_{emi} = 490$) for *S. purpuratus* or 100 μM Quin-2 ($\lambda_{exc} = 340$, $\lambda_{emi} = 490$) for *L. pictus* as described (Guerrero & Darszon, 1989; González-Martínez et al., 1992).

Statistical Analysis. Where indicated, differences between conditions were analyzed using the unpaired *t* test.

RESULTS

Recently, it was reported that cell membrane hyperpolarization in *Paramecium* stimulates cAMP formation (Schultz et al., 1992). The sea urchin sperm adenylylcyclase shares several properties with the one from *Paramecium*. In *L. pictus* sea urchin sperm, egg jelly or the purified Factor, the natural inductors of the AR, triggers a K^+-dependent hyperpolarization (González-Martínez & Darszon, 1987), followed by a depolarization (Schackmann et al., 1981; García-Soto et al., 1987). Blocking the hyperpolarization inhibits the subsequent depolarization and the AR, suggesting that it is essential for this process (González-Martínez & Darszon, 1987). Therefore, as in *Paramecium*, membrane potential could regulate the sea urchin sperm AC.

Artificial Hyperpolarization of L. pictus Sperm with 0KASW Increases Their cAMP Levels. Figure 1 shows that the addition of egg jelly to *L. pictus* sea urchin sperm increases their cAMP levels and induces AR (see inset). In the experiments where cAMP levels were determined, AR is lower than usual because the cells were centrifuged. When sperm suspended in 0KASW were artificially hyperpolarized with valinomycin [−130.4 ± 6.7 mV; see inset of Figure 1A and González-Martínez et al. (1992), [cAMP] was raised ∼2-fold within 15 s (Figure 1). Under these hyperpolarizing conditions, sperm reach a new stable membrane potential value in ∼15 s, pH_i rises (from ∼7.2 to ∼7.5), there is a small increase in $[Ca^{2+}]_i$, and the AR is not induced (inset of Figure 1B) (González-Martínez et al., 1992).

It has been suggested that sea urchin sperm AC is regulated by Ca^{2+} (Watkins et al., 1978; Kopf & Garbers, 1980;

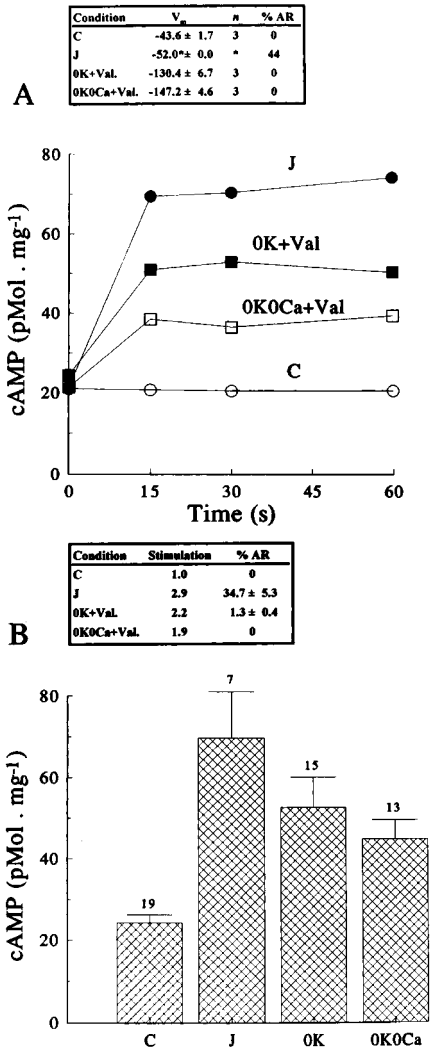

FIGURE 1: Increase of cAMP levels in *L. pictus* sperm artificially hyperpolarized with valinomycin in the absence of K^+. (A) Time course of the levels of cAMP. Washed sperm (52 μg of protein) were resuspended in 200 μL of ASW [control (C) and egg jelly (J)] or in 0K or 0K0CaASW and incubated at 14 °C. At different times, 20 μL aliquots were taken to measure the percent of AR, and the rest of the sperm suspension was used to assay cAMP as described in Materials and Methods. For C, incubation time (t_0) started with the resuspension of the cells with ASW at 4 °C. In the case of J, 0K, and 0K0Ca, t_0 was the time of addition of egg jelly (20 μL containing 4 μg of fucose/μL) or 2 μM valinomycin (0.8 μL of 0.5 mM in DMSO), respectively. Experiments where t_0 was when the cells were resuspended in ASW + J, 0K + valinomycin, 0K0Ca + valinomycin, or X condition in other figures gave similar results. Each data point is the mean of duplicate samples, and all time courses in the figures are representative of at least three experiments. The inset shows the membrane potential (V_m) values measured with the fluorescent dye DiS-C_3-(5) as previously described (González-Martínez & Darszon, 1987; Reynaud et al., 1993). The V_m value for J corresponds to the transient hyperpolarization induced by the addition of jelly to spermatozoa diluted in ASW and was taken from González-Martínez and Darszon (1987); the percent of AR was measured after 1 min of incubation. (B) Average changes in [cAMP] under various conditions after a 15 s incubation. Error bars indicate standard error (SE), and the number of experiments (n) is on top of them. The inset shows the stimulation (cAMP picomoles per milligram in X condition divided by the control) and the percent of AR ± SE. The difference between C and 0K0Ca is significant ($P < 0.01$). Experimental conditions were as in (A). Each experiment was performed in duplicate or triplicate with spermatozoa from one sea urchin.

Table 1: Stimulation of the AC in *L. pictus* Sperm by Ammonia[a]

condition	cAMP[b]	stimulation[c]	% AR	n[d]
C	24.2 ± 2.0	1.0	0	19
+ Factor	45.3 ± 5.3	1.9	54.6 ± 11.0	8
0Ca + Factor	19.2 ± 3.3	0.8	1.6 ± 0.9	4
NH_4^+	46.2 ± 2.4	1.9	0	6
0Ca + NH_4^+	38.6 ± 1.4	1.6	0	4

[a] Washed sperm (1 μL, see Materials and Methods) were incubated for 15 s in 200 μL of ASW (C, F, NH_4^+) or 0CaASW (0CaF, 0CaNH_4) at 14 °C. The time of incubation started with the addition of 5 μL of ASW (C), factor (factor and 0Ca + factor), or 10 mM NH_4Cl [2 μL of a 1 M stock solution in 10 mM Hepes (pH 8.0)]. [b] cAMP concentration units are picomoles per milligram, and the values represent averages ± standard error. All conditions were significantly different ($P < 0.01$). [c] Stimulation was calculated as described in Figure 1. [d] n represents the number of experiments each done in duplicate or triplicate with sperm from different sea urchins.

Mourelle et al., 1984; Bookbinder et al., 1990); therefore, spermatozoa were hyperpolarized in the absence of Ca^{2+}. It is worth noting that no increase in $[Ca^{2+}]_i$ is detected in Quin-2- or Fura-2-loaded *L. pictus* or *S. purpuratus* sperm after addition of valinomycin in 0K0CaASW (González-Martínez et al., 1992) or when exposed to egg jelly in 0CaASW (Guerrero & Darszon, 1989; Darszon et al., 1994; our unpublished results). Figure 1A illustrates that cAMP levels of sperm hyperpolarized in 0K0CaASW were elevated. Under this condition, cAMP accumulation is smaller, implying that, although not required for the membrane potential response, Ca^{2+} participates in AC activation (Watkins et al., 1978; Kopf & Garbers, 1980; Mourelle et al., 1984; Bookbinder et al., 1990). The cAMP levels, percent of AR, and degree of stimulation obtained by exposing sperm to egg jelly in ASW or hyperpolarizing them with valinomycin in 0K- or 0K0CaASW during 15 s are depicted in Figure 1B. These results also point out that increases in [cAMP] can be achieved by hyperpolarization in the absence of AR (Figure 1B inset) and hint that AC activation may precede the AR, as was observed with sperm heads (Garbers, 1981). Recently, it was suggested that elevation of pH_i in *S. purpuratus* sea urchin sperm was necessary and sufficient to activate AC (Cook & Babcock, 1993a). Since the hyperpolarizing conditions used to raise the sperm cAMP levels (0K and 0K0Ca plus valinomycin) also increase pH_i, it seemed important to dissect the effects of pH_i and membrane potential on sperm AC.

An Increase in pH_i Raises the cAMP Levels of L. pictus Sea Urchin Sperm. Addition of 10 mM NH^+_4 to *L. pictus* spermatozoa suspended in ASW increases their pH_i and [cAMP] (1.9-fold, Table 1), inducing modest changes in $[Ca^{2+}]_i$ (Schackmann & Chock, 1986; González-Martínez et al., 1992). In the absence of external Ca^{2+}, the stimulation persisted although to a lesser extent (1.6-fold), confirming the participation of Ca^{2+} in AC regulation. These data indicate that a rise in the pH_i of *L. pictus* sea urchin sperm does stimulate the AC as described for *S. purpuratus* (Cook & Babcock, 1993a). As controls, Table 1 shows that exposure of *L. pictus* sperm to Factor increases their cAMP levels and AR when suspended in ASW but not in 0CaASW, two conditions that have been studied only in *S. purpuratus* sperm (Kopf & Garbers, 1980).

L. pictus Sperm Increase Their cAMP Levels in 0NaASW. Since the accumulation of cAMP observed by hyperpolarization with valinomycin in 0K- and 0K0CaASW (Figure

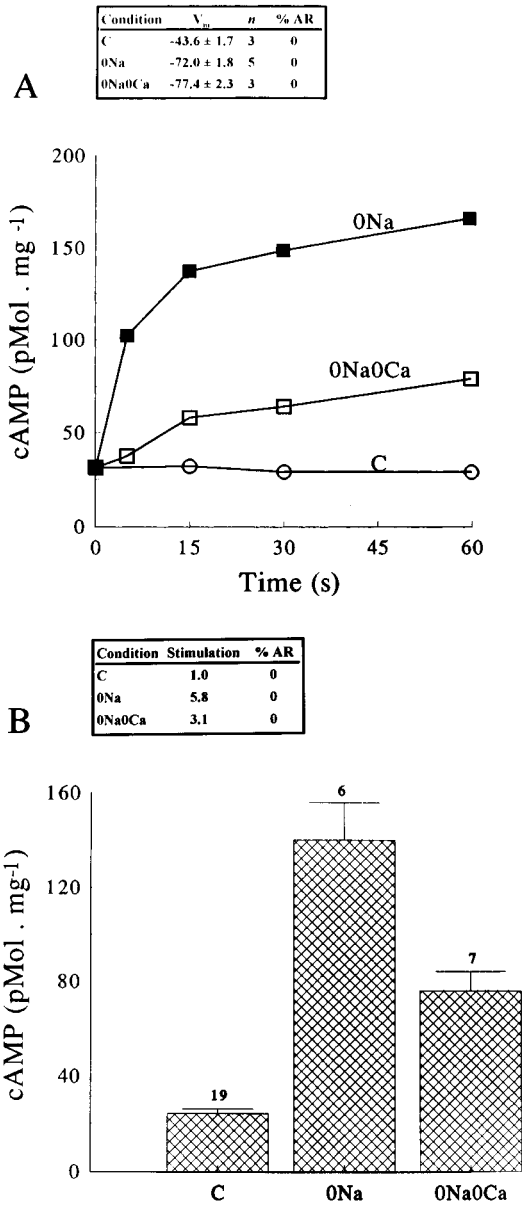

FIGURE 2: Hyperpolarization of *L. pictus* sperm by exposure to 0NaASW also increases cAMP levels. (A) Time course of the accumulation of cAMP in *L. pictus* sperm diluted in 0NaASW or 0Na0CaASW. The inset shows the values of the resting membrane potential obtained as described in Materials and Methods. (B) Averages of cAMP levels after 15 s of incubation under the indicated condition. The numbers on top of the error bars are *n*, and the inset shows the stimulation (X/C, see Figure 1) and the percent of AR. The difference between C and 0Na0Ca is significant ($P < 0.01$). Experimental conditions are as described in Figure 1.

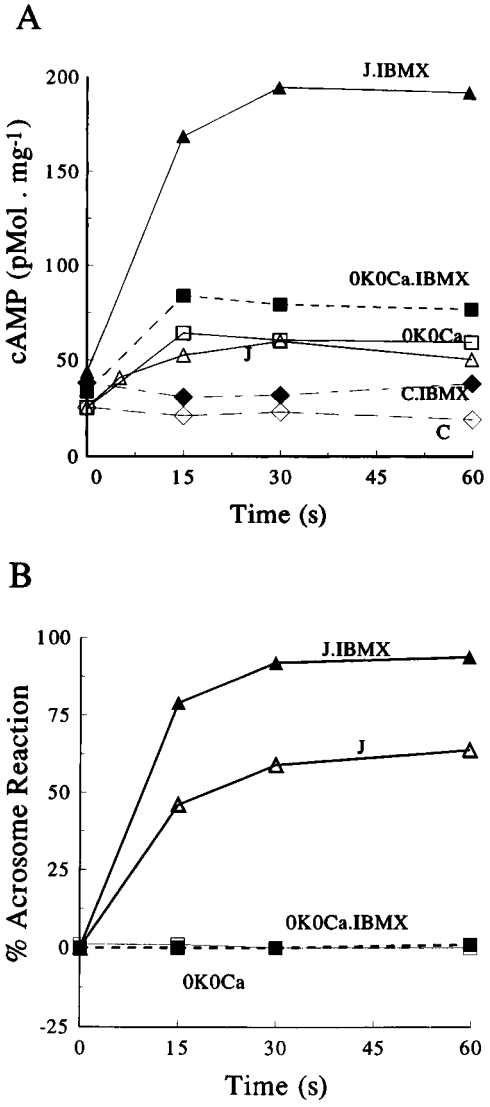

FIGURE 3: IBMX enhances the increase in cAMP levels induced by a hyperpolarization. (A) Temporal course of *L. pictus* sperm [cAMP] variations. The cells were incubated at 14 °C with J or in hyperpolarizing ASW (0K0Ca + Valinomycin) with or without 100 μM IBMX. At the indicated times, aliquots were taken to measure [cAMP] or percent of AR (B). Experimental conditions were as in Figure 1.

1) could be due to a rise in pH_i, we exposed sperm to a hyperpolarizing condition that does not increase pH_i. The 0NaASW condition was chosen since it hyperpolarizes sperm ($V_m = -72 \pm 1.8$; inset of Figure 2A), does not induce AR (Schackmann & Shapiro, 1981; Christen et al., 1986), and inhibits Na^+/H^+ exchange acidifying pH_i (~6.9) (Lee et al., 1983; Schackmann & Chock, 1986). When the washed cells were diluted in 0NaASW, by replacement of Na^+ with *N*-methyl-D-glucamine, the cAMP levels increased with time (Figure 2A), almost reaching their maximum within 15 s (5.8-fold; Figure 2B). An additional consequence of removing Na^+ from ASW is that the Na^+/Ca^{2+} exchanger cannot keep $[Ca^{2+}]_i$ low (Schackmann & Chock, 1986). Incubation of sperm in Ca^{2+}-free 0NaASW still elevated [cAMP] to the value reached by adding egg jelly (Figures 2B and 1B). These data indicate that in *L. pictus* sea urchin sperm membrane potential can regulate the AC, as reported for *Paramecium* (Schultz et al., 1992), independently of pH_i and $[Ca^{2+}]_i$.

The Phosphodiesterase Inhibitor IBMX Enhances the Increase in [cAMP] Induced by Hyperpolarization in L. pictus Sperm. The concentration of cAMP depends on the activity of two enzymes, AC and cAMP PDE. Since sea urchin sperm have a high content of cNMP PDE's, we used IBMX to monitor the cAMP accumulation resulting from AC activity. This phosphodiesterase inhibitor (100 μM) enhanced (~4-fold) the [cAMP] increase triggered by J and potentiated AR induction (Figure 3). A similar result was described earlier in *S. purpuratus* with theophylline (1.5 mM) (Garbers & Hardman, 1975). Hyperpolarization with valinomycin of sperm in 0K0CaASW increased [cAMP] ~1.5-fold more in the presence than in the absence of IBMX (0K0CaASW·IBMX; Figure 3A). As expected, no AR was seen in the absence of Ca^{2+} either with or without IBMX

(Figure 3B). These results indicate that hyperpolarization of *L. pictus* sperm stimulates AC.

Membrane Potential Also Regulates AC in S. purpuratus Spermatozoa. In 0NaASW, pH_i acidifies, and the Na^+/Ca^{2+} exchanger is unable to keep $[Ca^{2+}]_i$ low (Schackmann & Chock, 1986); thus, $[Ca^{2+}]_e$ was removed to eliminate its contribution to the response. When Na^+ is substituted by choline or *N*-methyl-D-glucamine in Ca^{2+}-free ASW, *S. purpuratus* sperm hyperpolarize from -47.7 ± 1.5 to -75.1 ± 3.8 mV (inset of Figure 4A) and pH_i acidifies to 6.8−6.9 (Schackmann et al., 1984). Under this condition, incubation of the cells for 15 s increased [cAMP] 1.3-fold and there was no AR (inset of Figure 4A) (Garcia-Soto et al., 1987). IBMX increased the sperm response to 0Na0CaASW (1.6-fold; Figure 4B lower inset). In 0Na0CaASW + IBMX, the increase in the cAMP levels cannot be due to pH_i or an increase in $[Ca^{2+}]_i$; thus, in *S. purpuratus* sperm, as in those from *L. pictus*, AC can be stimulated by hyperpolarization.

In ASW, IBMX (100 μM) increased the basal sperm [cAMP] from 16.4 ± 0.8 to 21.3 ± 1.5 pmol/mg and, as reported (Garbers & Hardman, 1975; Kopf & Garbers, 1980), enhanced the Factor-induced elevation of [cAMP] (12.2-fold) and the AR (not shown).

Figure 4A also illustrates that raising pH_i of sperm in ASW with 10 mM NH_4Cl elevated their cAMP levels (1.7-fold) without inducing AR (Schackmann & Chock, 1986) and increased $[Ca^{2+}]_i$ by 86.5 ± 4.9 nM ($n = 3$) (bottom panel). Under this condition, IBMX by itself raised $[Ca^{2+}]_i$ (182 ± 9.5 nM, $n = 4$) and stimulated the NH_4-induced $[Ca^{2+}]_i$ increase (133.3 ± 14 nM, $n = 4$) but not the rise in [cAMP] (Figure 4B) or the AR. Rising pH_i in 0CaASW caused a smaller (1.4-fold) [cAMP] elevation than in normal ASW (Figure 4A), which was enhanced by IBMX (1.6-fold, Figure 4B).

Speract Can Increase [cAMP] Independently of pH_i and $[Ca^{2+}]_i$ in S. purpuratus. Speract, a decapeptide purified from the egg jelly coat of *S. purpuratus*, binds at a nanomolar concentration to its receptor in homologous sperm, momentarily increasing the levels of cGMP and cAMP. This peptide induces a K^+-dependent transient hyperpolarization, an intracellular alkalinization, and a temporary rise in $[Ca^{2+}]$, (Garbers, 1989; Suzuki, 1990; Labarca et al., 1995). Control experiments with sperm suspended in ASW showed that speract increased cAMP levels at 15 s (1.3-fold; Figure 5A). As described (Hansbrough & Garbers, 1981; Garbers et al., 1982; Harumi et al., 1992), IBMX enhanced the accumulation of cAMP induced by speract (from 1.3- to 2.0-fold; Figure 5B) and partially triggered the AR ($54 \pm 6\%$; $n = 4$; Figure 5B legend) (Schackmann & Chock, 1986). The contribution of the speract-induced changes in pH_i and $[Ca^{2+}]_i$ to the increase in cAMP levels was eliminated by suspending sperm in 0Na0CaASW. Figure 5C illustrates the membrane potential changes induced by 100 nM speract in ASW and 0Na0CaASW ± IBMX. As in swollen sperm (Cook & Babcock, 1993a), IBMX enhanced the speract-induced hyperpolarization of normal sperm in ASW (from -7.4 ± 1.5 to -15.4 ± 2 mV; $n = 7$) without significantly affecting the magnitude of the repolarization (22.5 ± 1.6 mV without IBMX and 26.5 ± 2 mV with IBMX; $n = 7$). Speract further hyperpolarized sperm suspended in 0Na0CaASW (-13.1 ± 1.4 mV, $n = 6$; Figure 5C) and enhanced the accumulation of cAMP from 1.3- in ASW to 1.7-fold (Figure 5A). IBMX stimulated this response from

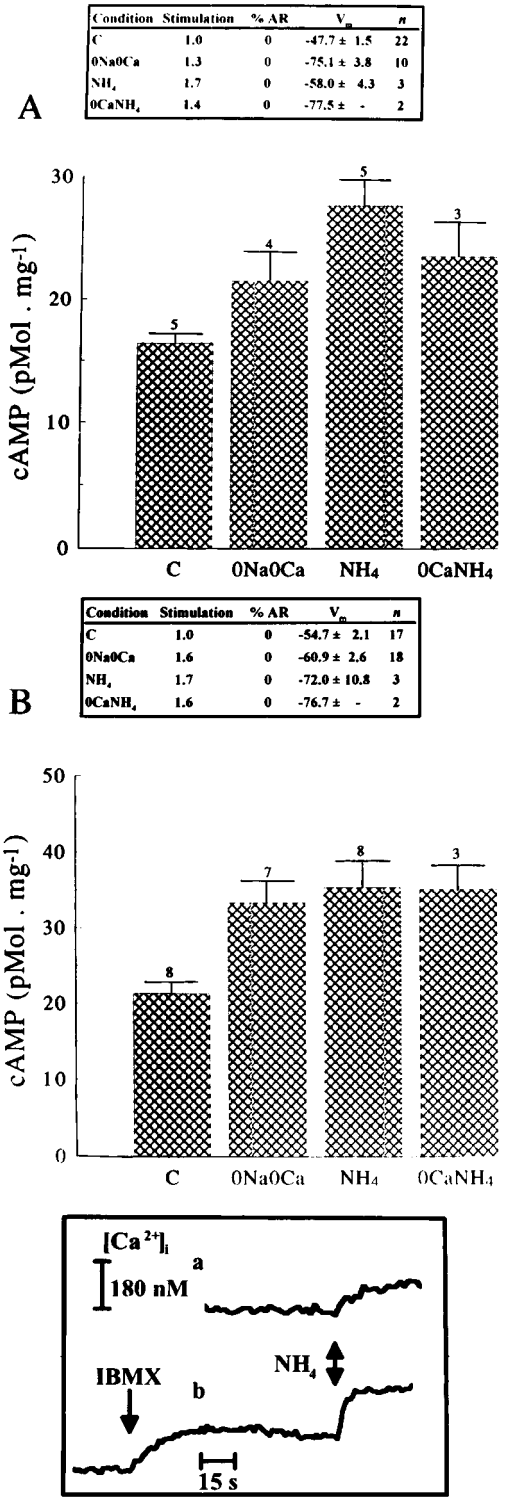

FIGURE 4: Stimulation of the *S. purpuratus* sperm AC by 0NaASW and pH_i. (A) Averages of cAMP concentrations obtained after a 15 s incubation in ASW with different compositions. The numbers above the error bars are *n*, and the inset shows the stimulation (X/C) and the percent of AR determined as in Figure 1. Experimental conditions are as in Figure 2 (for C and 0Na0Ca) and as in Table 1 (for NH_4 and $NH_4$0Ca). The difference between C and 0Na0Ca is significant ($P < 0.01$). (B) Same as in panel A except that ASW contained 100 μM IBMX. There is a significant difference ($P < 0.01$) in the stimulation between C and 0Na0Ca. (Bottom) Changes in $[Ca^{2+}]_i$ measured in Fura-2-loaded sperm (see Materials and Methods) induced by NH_4 in the absence (upper trace) or presence of IBMX (lower trace). The lower trace also shows that IBMX increased $[Ca^{2+}]_i$ by itself. The $[Ca^{2+}]_i$ scale is on the upper left and the time below the lower trace.

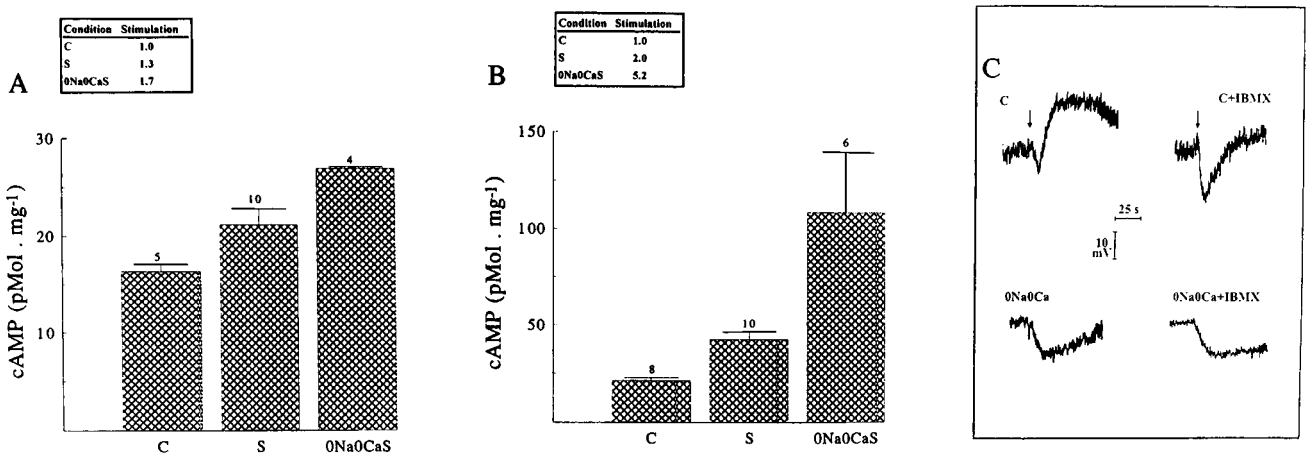

FIGURE 5: Speract (S) elevates cAMP in *S. purpuratus* sperm suspended in 0Na0CaASW. (A) cAMP levels obtained after a 15 s incubation of spermatozoa in ASW with speract or in 0Na0CaS. The numbers on error bars are *n*, and the inset shows the stimulation (X/C) determined as in Figure 1. Experimental conditions were as in Figure 1 except that the incubation time started with the addition of speract. (B) Same as in panel A except that the different ASW contained 100 μM IBMX. Under this condition, speract induced 54 ± 12% and $n = 4$ AR, as described (Schackmann & Chock, 1986). There is a significant difference ($P < 0.01$) in the stimulation between C and S and 0Na0CaS and between S and 0Na0CaS both in the absence (A) and in the presence (B) of IBMX. (C) Speract induced membrane potential changes, measured with the membrane potential sensitive fluorescent dye DiS-C_3-(5) (see Materials and Methods), in the absence or presence of IBMX. Pre-equilibration (3−4 min) with the dye, the effect of the mitochondrial uncoupler (~40 s), and the final calibration with valinomycin and K^+ are not shown. An upward deflection indicates a depolarization and a downward deflection a hyperpolarization. Sperm were suspended in ASW (C) and 0Na0CaASW (0Na0Ca). The arrows on the top traces indicate the addition of 100 nM speract for the traces below. The time and membrane potential scales are in the center of the inset.

1.7- to 5.2-fold (Figure 5B). This PDE inhibitor did not modify the speract-induced hyperpolarization of sperm in 0Na0CaASW (−10.4 ± 1.2 mV, $n = 3$) (Figure 5C). The fact that IBMX considerably increases the speract stimulation in sperm suspended in 0Na0CaASW indicates that under this condition the activity of the cAMP PDE's is high. These results show that speract can increase the cAMP levels independently of pH_i and $[Ca^{2+}]_i$ by hyperpolarizing *S. purpuratus* sperm. They further support the conclusion that membrane potential modulates AC in sea urchin sperm.

DISCUSSION

Adenylylcyclase is at the heart of many key cell transduction events (Federman et al., 1992; Choi et al., 1993). In somatic cells, this enzyme is regulated by many important messengers such as intracellular Ca^{2+}, hormones, and neurotransmitters (Tang & Gilman, 1992; Choi et al., 1993; Pieroni et al., 1993). On the basis of the transmembrane topographical homology between AC and various ion channels and transporters, it has been proposed, although not demonstrated, that this protein might have a dual life converting ATP to cAMP and operating as an ion channel itself (Krupinski et al., 1989). In this regard, the *Paramecium* AC was shown to be stimulated by cell-hyperpolarizing conditions and, when purified and reconstituted in lipid bilayers, to have an associated cationic pore-forming activity (Schultz et al., 1992). The *Paramecium* AC differs from the somatic cell enzyme and shares properties with the sea urchin sperm enzyme (Hildebrandt et al., 1985). Considering this, it seemed interesting to explore if the sperm AC could also be regulated by membrane potential (Beltrán et al., 1995). Recently, it was shown that AC activity from cerebellar neurons is synergistically stimulated by depolarizing agents and β-adrenergic receptor activation (Reddy et al., 1995).

Spermatozoa from many species are immotile in the seminal tract and become motile under fertilizing conditions. In many cases, including sea urchins (Schackmann et al., 1984), a high external K^+ concentration ($[K^+]_e$) is responsible for their low motility (Schlenk & Kahmann, 1938; Morisawa & Suzuki, 1980; Morisawa et al., 1983; Harumi et al., 1992). In rainbow trout, a decrease in $[K^+]_e$ causes an immediate transient increase in intracellular [cAMP] (Morisawa & Ishida, 1987) which initiates sperm motility (Morisawa & Okuno, 1982) through cAMP-dependent phosphorylation of axonemal proteins (Morisawa & Hayashi, 1985). The decrease in $[K^+]_e$, which increases motility, is associated with a pH-independent membrane hyperpolarization (Boitano & Omoto, 1991). However, how the hyperpolarization induces the synthesis of cAMP and stimulates motility is not well-understood.

Here it is shown that artificially hyperpolarizing *L. pictus* sea urchin sperm in 0KASW + valinomycin (González-Martínez et al., 1992) increased cAMP levels 2.2-fold. This stimulation persisted in the absence of $[Ca^{2+}]_e$ (1.9-fold). Considering that the addition of valinomycin to sperm in 0K0CaASW does not increase $[Ca^{2+}]_i$ (González-Martínez et al., 1992), this result suggests that hyperpolarization increases sperm cAMP levels. The differential increase in [cAMP] in the presence and absence of Ca^{2+} in ASW (and in 0NaASW) shows that Ca^{2+} uptake enhances the accumulation of this nucleotide. It has been proposed that sea urchin sperm AC is regulated by $[Ca^{2+}]_i$, probably through calmodulin (Bookbinder et al., 1990). Making *L. pictus* sperm membrane potential more negative with 0K- or 0K0CaASW + valinomycin also raises pH_i, which could contribute to the observed increase in [cAMP] (Cook & Babcock, 1993a). In 0Na0CaASW, *L. pictus* sperm hyperpolarize, their $[Ca^{2+}]_i$ does not increase (not shown), and their pH_i is acidic relative to that of ASW, since the Na^+/H^+ exchanger cannot alkalinize (Christen et al., 1983). In spite of this, their cAMP levels elevate 3.1-fold. These results clearly suggest that the concentration of cAMP is modulated by membrane potential in *L. pictus* sperm (Figure 2).

To explore if the regulation of cAMP levels by sperm membrane potential is a general phenomenon, *S. purpuratus* spermatozoa were studied. Sperm from this species also responded with a 1.3-fold rise in cAMP levels when artificially hyperpolarized in 0Na0CaASW (Figure 4A). In Fura-2-loaded cells under this condition, $[Ca^{2+}]_i$ is not increased relative to that of normal ASW. To assay if the increase in [cAMP] caused by sperm hyperpolarization resulted from the activation of AC, IBMX was used to inhibit the phosphodiesterases. In the presence of this inhibitor, dilution in 0Na0CaASW raised [cAMP] 1.6-fold (Figure 4B), indicating that hyperpolarization activates *S. purpuratus* sperm AC. Since in 0Na0CaASW the changes in pH_i and $[Ca^{2+}]_i$ can be discarded as causes of AC activation, it is difficult to think of yet another variable that is modified when sperm are hyperpolarized by different procedures and which is responsible for the cyclase stimulation.

Speract, a decapeptide purified from the outer layer of *S. purpuratus* eggs, increases sperm [cGMP], [cAMP], pH_i, and $[Ca^{2+}]_i$. It also triggers a K^+-dependent hyperpolarization followed by a depolarization (Garbers, 1989; Suzuki, 1990; Harumi et al., 1992; Labarca et al., 1995) and has been proposed to influence motility (Cook et al., 1994). It is not known how this peptide increases [cAMP]; a sperm hyperpolarization could be, at least in part, the cause.

The speract-induced increase in pH_i is Na^+-dependent, and in 0CaASW, it does not increase $[Ca^{2+}]_i$ in Indo-1 (Schackmann & Chock, 1986)- or Fura-2-loaded sperm (our work, not shown). Thus, in 0Na0CaASW, neither pH_i nor $[Ca^{2+}]_i$ is increased by exposure of sperm to this peptide. Lee (1984) showed that sea urchin sperm flagellar membrane vesicles respond to speract with a hyperpolarization in the absence of external Na^+. In our experiments, speract hyperpolarized *S. purpuratus* whole sperm in 0Na0CaASW. Under this condition, this peptide elevated the cAMP levels 1.7-fold (Figure 5A) and 5.2-fold in the presence of IBMX (Figure 5B). These results suggest that speract can activate adenylylcyclase by hyperpolarizing sea urchin sperm independently of $[Ca^{2+}]_i$ and pH_i and support the conclusion that this enzyme is membrane potential-dependent.

The sperm AC has never been shown to be modulated by G proteins (Hildebrandt et al., 1985; Ward & Kopf, 1993). Therefore, it is likely that the catalytic region or subunit of the protein is voltage-sensitive and must contain charged or polar amino acids. Since the sperm enzyme has not been cloned, it is impossible to propose a specific region, which could have homology with voltage-dependent ion channels like the somatic AC (Reddy et al., 1995), as the voltage-dependent protein site. Voltage-induced conformational changes could allow specific phosphatases or kinases to change the phosphorylation state of the sperm AC. Alternatively, another protein could modulate the sperm AC activity; either of the two proteins could undergo voltage-dependent conformational changes that would regulate the affinity between them.

Our results demonstrate that, in two different sea urchin species (*L. pictus* and *S. purpuratus*), sperm AC responds to membrane hyperpolarization triggered by different methods independently of $[Ca^{2+}]_i$, pH_i, and the AR, suggesting that it is a common mechanism. Sperm hyperpolarization is one of the initial responses to peptides from the outer envelope of the egg involved in motility and chemotaxis. Since AC activation is temporally correlated with these events and with AR induction (Trimmer & Vacquier, 1986; Garbers, 1989), our observations suggest that a hyperpolarization might be one of the keys that "turn on" AC and raise cAMP levels to modulate sperm motility, chemotaxis, and AR, allowing sperm to reach its final goal, fertilization of the egg. One of the functions of this hyperpolarization-induced increase in cAMP levels is probably to phosphorylate different proteins through the activation of cAMP-dependent protein kinases (Porter & Vacquier, 1986; Vacquier et al., 1989). On the other hand, the group recently described a sea urchin sperm K^+-selective channel that is up-regulated directly by cAMP (Labarca et al., 1996), and the Ca^{2+} channels opened by speract have been proposed to be modulated by cAMP (Cook & Babcock, 1993b). These channels would also be targets for the membrane potential-regulated sperm AC. Although increases in [cAMP] are associated with mammalian sperm capacitation (Yanagimachi et al., 1988, and references therein; Ward & Kopf, 1993) and the AR, more studies are needed to clarify the relationship between AC activation, membrane potential, capacitation, and AR. As the sea urchin sperm AC has multiple forms of regulation, it could be working as a coincidence detector, as has been suggested for other cells (Anholt, 1994).

REFERENCES

Anholt, R. H. R. (1994) *Trends Neurosci. 17*, 37−41.

Beltrán, C., Zapata, O., & Darszon, A. (1995) *Biophys. J. 68*, A202.

Bookbinder, L. H., Moy, G. W., & Vacquier, V. D. (1990) *J. Cell Biol. 111*, 1859−1866.

Choi, E.-J., Xia, Z., Villacres, E. C., & Storm, D. R. (1993) *Curr. Biol. 5*, 269−273.

Christen, R., Schackmann, R. W., Dahlquist, F. W., & Shapiro, B. M. (1983) *Exp. Cell Res. 149*, 289−294.

Christen, R., Schackmann, R. W., & Shapiro, B. M. (1986) *J. Physiol. 379*, 347−365.

Collins, F., & Epel, D. (1977) *Exp. Cell Res. 106*, 211−222.

Cook, S. P., & Babcock, D. F. (1993a) *J. Biol. Chem. 268*, 22402−22407.

Cook, S. P., & Babcock, D. F. (1993b) *J. Biol. Chem. 268*, 22408−22413.

Cook, S. P., Brokaw, C. J., Muller, C. H., & Babcock, D. F. (1994) *Dev. Biol. 165*, 10−19.

Darszon, A., Labarca, P., Beltrán, C., García-Soto, J., & Liévano, A. (1994) *Methods: Companion Methods Enzymol. 6*, 37−50.

Dische, A., & Shettles, L. B. (1948) *J. Biol. Chem. 75*, 595−603.

Fanger, B. O. (1987) *Anal. Biochem. 162*, 11−17.

Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R., & Bourne, H. R. (1992) *Nature 356*, 159−161.

Garbers, D. L. (1981) *J. Biol. Chem. 256*, 620−624.

Garbers, D. L. (1989) *Annu. Rev. Biochem. 58*, 719−742.

Garbers, D. L., & Hardman, J. G. (1975) *Nature 257*, 677−678.

Garbers, D. L., & Kopf, G. S. (1980) *Adv. Cyclic Nucleotide Res. 13*, 251−306.

Garbers, D. L., Watkins, H. D., Hansbrough, J. R., Smith, A., & Misono, K. S. (1982) *J. Biol. Chem. 257*, 2734−2737.

García-Soto, J., & Darszon, A. (1985) *Dev. Biol. 110*, 338−345.

García-Soto, J., González-Martínez, M., De de Latorre, L., & Darszon, A. (1987) *Dev. Biol. 120*, 112−120.

González-Martínez, M., & Darszon, A. (1987) *FEBS Lett. 2*, 247−250.

González-Martínez, M. T., Guerrero, A., Morales, E., De de la Torre, L., & Darszon, A. (1992) *Dev. Biol. 150*, 193−202.

Guerrero, A., & Darszon, A. (1989) *J. Biol. Chem. 264*, 19593−19599.

Hansbrough, J. R., & Garbers, D. L. (1981) *J. Biol. Chem. 256*, 2235−2241.

Harumi, T., Hoshino, K., & Suzuki, N. (1992) *Dev. Growth Differ. 34*, 163−172.

Hildebrandt, J. D., Tash, J. S., Kirchick, H. J., Lipschunits, L., Secra, R. D., & Birmbaumer, L. (1985) *Endocrinology 116*, 1357−1366.
Kopf, G. S., & Garbers, D. L. (1980) *Biol. Reprod. 22*, 1118−1126.
Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W.-J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R., & Guilman, A. G. (1989) *Science 244*, 1558−1564.
Labarca, P., Santi, C., Morales, E., Zapata, O., Beltrán, C., Liévano, A., & Darszon, A. (1995) *Biophys. J. 68*, A224.
Labarca, P., Santi, C., Zapata, O., Morales, E., Beltrán, C., Liévano, A., & Darszon, A. (1996) *Dev. Biol. 174*, 271−280.
Lee, H. C. (1984) *J. Biol. Chem. 259*, 4957−4963.
Lee, H. C., Hohnson, C., & Epel, D. (1983) *Dev. Biol. 95*, 31−45.
Morisawa, M., & Suzuki, K. (1980) *Science 210*, 1145−1147.
Morisawa, M., & Okuno, M. (1982) *Nature 295*, 703−704.
Morisawa, M., & Hayashi, H. (1985) *Biomed. Res. 6*, 181−184.
Morisawa, M., & Ishida, K. (1987) *J. Exp. Zool. 242*, 199−204.
Morisawa, M., Suzuki, K., & Morisawa, S. (1983) *J. Exp. Biol. 107*, 105−113.
Mourelle, M., Vargas, I., & Darszon, A. (1984) *Gamete Res. 9*, 87−97.
Pieroni, J. P., Jacobowitz, O., Chen, J., & Iyergar, R. (1993) *Curr. Opin. Neurobiol. 3*, 345−351.
Porter, D. C., & Vacquier, V. D. (1986) *Dev. Biol. 116*, 203−212.
Reddy, R., Smith, D., Wayman, G., Wu, Z., Villacres, E. C., & Storm, D. R. (1995) *J. Biol. Chem. 270*, 14340−14346.
Reynaud, E., De de La Torre, L., Zapata, O., Liévano, A., & Darszon, A. (1993) *FEBS Lett. 329*, 210−214.
Schackmann, R. W., & Shapiro, B. M. (1981) *Dev. Biol. 81*, 145−154.
Schackmann, R. W., & Chock, P. B. (1986) *J. Biol. Chem. 261*, 8719−8728.
Schackmann, R. W., Eddy, E. M., & Shapiro, B. M. (1978) *Dev. Biol. 65*, 483−495.
Schackmann, R. W., Christen, R., & Shapiro, B. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6066−6070.
Schackmann, R. W., Christen, R., & Shapiro, B. M. (1984) *J. Biol. Chem. 259*, 13914−13922.
Schlenk, W., & Kahmann, H. (1938) *Biochem. Z. 295*, 283−301.
Schultz, J. E., Klump, S., Benz, R., Rchurhoff-Goters, W. J. CH., & Schmid, A. (1992) *Science 255*, 600−603.
Suzuki, N. (1990) *Zool. Sci. 7*, 355−370.
Talbot, P., Summers, R. G., Hylander, B. L., Keough, E. M., & Franklin, L. E. (1976) *J. Exp. Zool. 198*, 383−392.
Tang, W.-J., & Gilman, A. G. (1992) *Cell 70*, 869−872.
Trimmer, J. S., & Vacquier, V. D. (1986) *Annu. Rev. Cell Biol. 2*, 1−26.
Vacquier, V. D., Porter, D. C., Keller, S. H., & Aukerman, M. (1989) *Dev. Biol. 133*, 111−118.
Ward, C. R., & Kopf, G. (1993) *Dev. Biol. 158*, 9−34.
Watkins, H. D., Kopf, G. S., & Garbers, D. L. (1978) *Biol. Reprod. 19*, 890−894.
Yanagimachi, R. (1988) in *the Physiology of reproduction* (Knobil, E., Neil, J. D., Ewing, L. L., Greenwald, G. S., Markert, C. L., & Pfaff, D. W., Eds.) pp 135−185, Raven Press, Ltd., New York.
Zufall, F., Firestein, S., & Shepherd, G. M. (1994) *Annu. Rev. Biophys. Biomol. Struct. 23*, 577−607.

BI952806V