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Ionic bases of the membrane potential and intracellular pH changes induced by speract in swollen sea urchin sperm

Enrique Reynaud[a], Lucía De de La Torre[b], Otilia Zapata[a], Arturo Liévano[a] and Alberto Darszon[a,b]

[a]*Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca, MO., México* and [b]*Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional. Apdo. Postal 14-740, México DF, México*

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Signal transduction initiated by the egg peptide, speract, in sea urchin sperm is not fully understood. Hypotonically swollen sperm are a suitable model to study peptide signal transduction. Ion substitution experiments now indicate (i) that the permeability to Na^+, Ca^{2+}, and Mg^{2+} contributes to the sperm resting membrane potential; (ii) the repolarization induced by nM concentrations of speract is Na^+ dependent and mediated by an as yet unidentified channel; (iii) the depolarization triggered by nM concentrations of speract involves Ca^{2+} channels since it is Ca^{2+}-dependent and blocked by Co^{2+} and Ni^{2+}, two Ca^{2+} channel blockers; (iv) hyperpolarizing swollen sperm with valinomycin increases intracellular pH (pH_i) in the same way as speract, thus the speract-induced hyperpolarization may be responsible for the pH_i increase.

Speract; Sperm pH_i; Sperm membrane potential; Ion channel

1. INTRODUCTION

Resact, a decapeptide from the outer layer of *Arbacia punctulata* sea urchin eggs, is chemotactic [1]. However, the significance and the molecular mechanisms that mediate sperm response to speract, an analogous egg peptide from *Strongylocentrotus purpuratus*, are not fully understood. In sea water at pH 6–6.5, 1–10 pM speract stimulates respiration, but at the physiological pH 8, this activation requires two or three orders of magnitude more peptide [2–4]. At this higher concentration, as in the case of resact, speract also increases intracellular pH (pH_i), intracellular Ca^{2+} $[Ca^{2+}]_i$ and the cyclic nucleotide content of sperm [2–8].

Recently [9,10] it was demonstrated that *S. purpuratus* sea urchin sperm can be swollen in 10-fold diluted sea water containing 20 mM $MgSO_4$ (DASW). The swollen cells are nearly spherical (diameter ~ 4 μM), can be loaded with fluorescent probes for pH_i and $[Ca^{2+}]_i$ and their membrane potential, pH_i and $[Ca^{2+}]_i$ regulated. One of their main attractions is that they can be patch clamped.

Patch-clamp experiments have shown that K^+ channels activate when swollen sperm are exposed to ~ pM concentrations of speract. The cells undergo a sustained K^+-dependent hyperpolarization [10]. Since > 25 pM speract is required to detect increases in pH_i, it was suggested that the hyperpolarization and the change in pH_i may not be linked [10]. In the presence of > 100 pM speract, a repolarization, involving an as yet unidentified transport system, is observed. In addition to the previous responses, speract concentrations above 10 nM elicit a Ca^{2+}-dependent depolarization which surpasses the swollen cell's resting potential. This depolarization is associated with an increase in $[Ca^{2+}]_i$, as it occurs in normal sperm [8–10].

Here, ion substitution experiments were performed to further determine the nature of the permeability changes triggered by nM concentrations of speract in swollen sperm. It was found that Na^+ uptake is mainly responsible for the repolarization, which is Ca^{2+} independent. External Mg^{2+} regulates $[Ca^{2+}]_i$ and alters the response to speract. Also, it is shown that a valinomycin-induced hyperpolarization increases pH_i, which indicates the presence of a voltage-dependent regulation of pH_i in swollen *S. purpuratus* sperm.

2. MATERIALS AND METHODS

S. purpuratus sea urchins were obtained from Pacific Bio-Marine Laboratories (Venice, CA) or from Marinus (Long Beach, CA). Ionomycin and valinomycin were from Sigma (St. Louis, MO). Fura-2, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF) and dipropylthiodicarbocyanine (Dis-C_3-(5)) were from Molecular Probes (Eugene, OR). Speract was from Peninsula Laboratories (Belmont, CA).

Spermatozoa were obtained as described in [14] and stored undiluted on ice. The composition of artificial sea water (ASW) was (mM): 486 NaCl, 27 $MgCl_2$, 29 $MgSO_4$, 10 $CaCl_2$, 10 KCl, 2.5 $NaHCO_3$, 0.1 EDTA, 10 HEPES, pH 8.0. The cells were swollen in 10-fold diluted ASW, pH 6.8, containing 20 mM $MgSO_4$ (DASW), as in [10]. Cation substitutions in DASW were made by replacing the indicated salt(s) (of the cation(s) (mole per mole)) by choline chloride, approximately maintaining osmolarity (~ 120–160 mOsm). Cl^- was substituted with

Correspondence address: E. Reynaud, Univ. Nacional Autonoma de Mexico, Inst. de Biotecnología, Apdo. Postal 510-3, Cuernavaca, MO, Mexico.

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methane–sulfonic acid, titrated with the hydroxide of each of the cations in ASW.

$[Ca^{2+}]_i$ and pH_i measurements were made using the intracellular fluorescent probes, fura-2 and BCECF, monitored at 340/490 nm and 500/550 nm excitation/emission wavelength pairs, respectively, and loaded and calibrated as in [10,14]. The loaded cells were then diluted 10 times at 4°C, in 1 mM $CaCl_2$ ASW, pH 7.0, pelleted (6 min, 1,000 × g) and resuspended in the same media. Thereafter, 10–20 μl of this dilution was added to a round cuvette containing DASW (10^7 cells/ml final) or the indicated swelling solution for fluorescence measurements at 14°C.

Membrane potential was monitored with the fluorescent, positively charged, membrane potential-sensitive dye, DiS-C_3-(5) (2 μM final, 1 mM stock in DMSO), at the 620/670 excitation/emission wavelength pair, as described in [15]. The dye response was calibrated and the resting membrane potential determined after treatment with the mitochondrial uncoupler [16] bis(hexafluoroacetonyl)acetone (5 μM) as in [10], using valinomycin and K^+ additions with each of the swelling solutions employed.

Membrane potential and pH_i were simultaneously measured using a magnetically stirred, temperature-regulated Hansatech Mk II fluorescence cell (Norfolk, England) coupled to a Schott lamp (KL 1500) through a bifurcated optic fiber. Each arm of the fiber had the corresponding interference filter for excitation attached. The emission was determined with two Hansatech fluorescent detectors (FDC), each with its corresponding interference filter. In these experiments each dye was calibrated in the presence of the other, as described [14]. These experiments were confirmed by measuring membrane potential and pH_i separately, and also with a Perkin Elmer LS 3 spectrofluorometer, which was used to determine $[Ca^{2+}]_i$ with fura-2. All determinations were made at 14°C under constant stirring.

Membrane potential, $[Ca^{2+}]_i$ and pH_i experiments were performed at least 4 times with different batches of sperm.

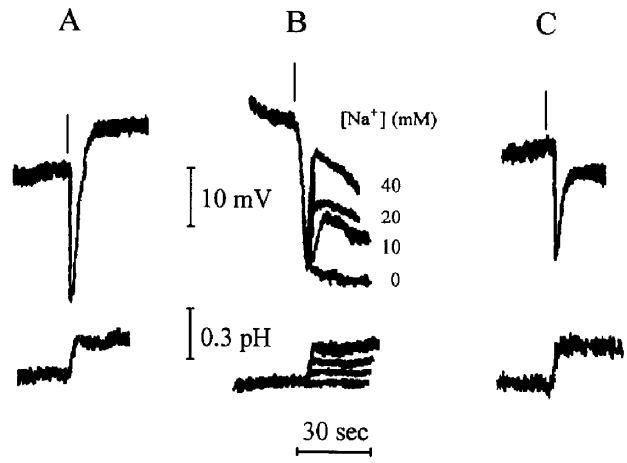

Fig. 1. Na^+ and Ca^{2+} dependence of the speract (100 nM)-induced changes in membrane potential (top traces and calibration bar) and pH_i (bottom traces and calibration bar) in swollen sea urchin sperm at 14°C. Loading of BCECF for pH_i and the simultaneous recording of membrane potential and pH_i were as described in section 2. Pre-equilibration with 2 μM DiS-C_3-(5) (10–20 s), the uncoupling effect of 5 μM bis(hexafluoroacetonyl)acetone (70–100 s) and the final calibration with valinomycin and K^+ (see section 2) are not shown. An upward deflection indicates a depolarization in the membrane potential changes, and an alkalinization in the pH_i traces. The bar on top of all traces in the figures corresponds to the speract addition. The time scale is in the middle of the figure. (A) Control responses to speract of swollen sperm in 1/10 diluted artificial sea water containing 20 mM $MgSO_4$ (DASW). (B) Speract responses in DASW with different Na^+ concentrations. (C) Speract response in DASW without Ca^{2+}. All experiments were repeated at least 4 times.

3. RESULTS AND DISCUSSION

To further study the ionic basis for the permeability changes which occur when swollen sea urchin sperm are exposed to nM concentrations of speract, various ions were replaced in the swelling solution (1/10 diluted ASW supplemented with 20 mM $MgSO_4$, DASW). Membrane potential changes were monitored with a fluorescent cyanine dye, the response of which was calibrated (see section 2) under the various ionic conditions. At each condition the resting membrane potential was determined. Table I shows that the resting membrane potential hyperpolarizes towards the K^+ equilibrium potential (E_K = −130 mV, see [10]) as the main cations in DASW are replaced by choline. These results suggest that, in swollen sperm at rest, Na^+ and Ca^{2+} significantly contribute to the resting membrane potential, either through their respective permeabilities or by modulating other conductances that do so. Mg^{2+}, the main divalent cation present in DASW, in addition to the previous possibilities, may have a significant effect on surface membrane potential.

Fig. 1A illustrates a typical membrane potential (top) and pH_i response (bottom), recorded simultaneously from swollen *S. purpuratus* sea urchin sperm exposed to 100 nM speract. Experiments done at different Na^+ concentrations, substituting Na^+ for choline in DASW (Fig. 1B), show that the repolarization and the change in pH_i depend on the concentration of external Na^+. The clear contribution of Na^+ to the repolarization, only suggested previously [10], indicates that there is an electrogenic uptake of this cation into the cell.

Table I

Effect of cation substitution on the resting membrane potential of swollen sea urchin sperm

DASW	0 Na^+	0 Ca^{2+}	0 Mg^{2+}	0 Na^+, 0 Ca^{2+}	0 Na^+, 0 Mg^{2+}	0 Ca^{2+}, 0 Mg^{2+}	0 Na^+, 0 Ca^{2+}, 0 Mg^{2+}
−55 ± 7	−80 ± 9	−68 ± 7	−84 ± 10	−71 ± 7	−81 ± 5	−81 ± 2	−78 ± 2
(n = 7)	(n = 4)	(n = 4)	(n = 7)	(n = 4)	(n = 10)	(n = 5)	(n = 4)

The response of DiS-C_3-(5) was calibrated and the resting membrane potential calculated as described in section 2. The top row indicates the solution used (1/10 diluted artificial sea water containing 20 mM $MgSO_4$ (DASW); and the cations that were replaced by choline). The middle row shows the average ± S.D., and in the last, n = number of experiments.

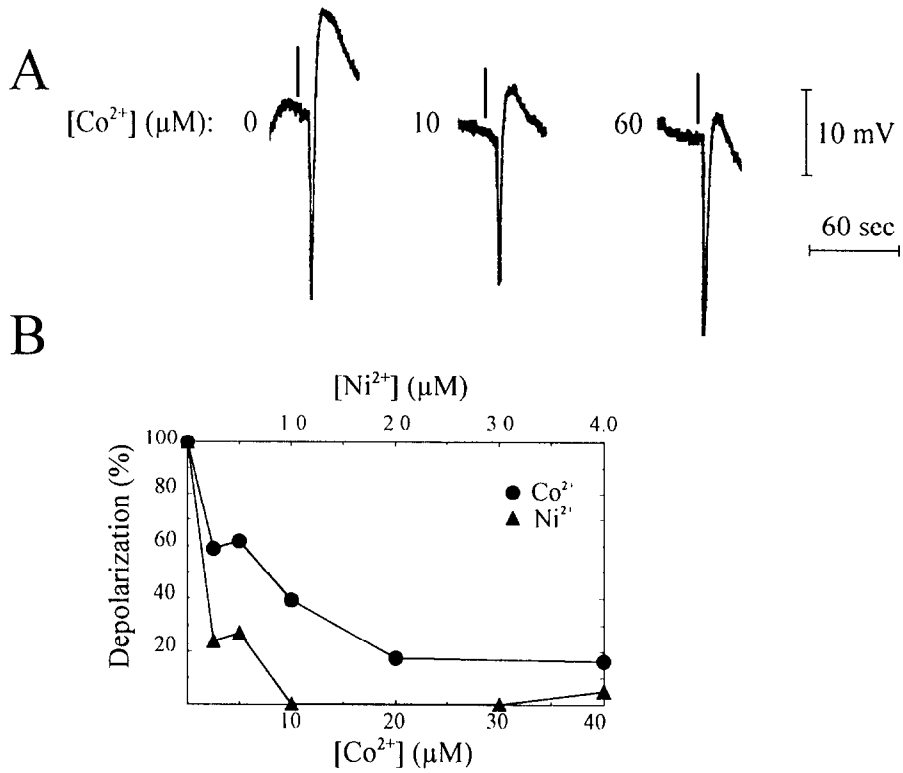

Fig. 2. Block by Co^{2+} and Ni^{2+} of the Ca^{2+}-dependent depolarization induced by speract (100 nM). (A) Swollen sperm in DASW were exposed to 0, 10 and 60 μM Co^{2+} and their membrane potential changes recorded as in Fig. 1 and section 2. Time and current scales are shown on the right. (B) Inhibition of the Ca^{2+}-dependent depolarization expressed as a percentage of the control, as a function of the concentration of Co^{2+} or Ni^{2+}. Results from a representative experiment out of 4, S.D. ~ 10%.

Before, it was shown that in normal sperm, the speract-induced change in pH_i was Na^+ dependent [8]. This finding was consistent with the presence of a putative electroneutral, voltage-dependent Na^+/H^+ exchanger in sperm flagella [11,12]. However, this electroneutral exchange can not explain the Na^+-dependent repolarization. Thus, in addition to Na^+/H^+ exchange, an electrogenic transport system for Na^+ is involved, possibly an exchanger, but more likely a channel. Experiments performed in the presence of ouabain, a known specific blocker of the Na^+/K^+ ATPase (5 mM in sea urchin sperm [17]) indicate that this pump is not involved in the speract response.

Fig. 1C illustrates the effect of removing Ca^{2+} from DASW on the response of swollen *S. purpuratus* sperm to nM concentrations of speract. As mentioned earlier, the net depolarization is Ca^{2+} dependent, but the repolarization and the increase in pH_i occur in the absence of this divalent cation. Therefore, in agreement with the previous result, the electrogenic uptake of Na^+ is mainly responsible for the repolarization.

The fact that the uptake of Ca^{2+} is associated with a depolarization discounts the possibility of a Na^+/Ca^{2+} exchanger proposed earlier [8] as the source for this uptake. The reverse operation of this transporter would hyperpolarize the cell, since it has a 3 Na^+/Ca^{2+} stoichiometry [13]. Fig. 2 shows that Co^{2+} (20–30 μM) and Ni^{2+} (2–3 μM), known antagonists of Ca^{2+} channels, also block this depolarization. Furthermore, Sr^{2+}, Mn^{2+} and Ba^{2+} may substitute Ca^{2+} in sustaining the depolarization which is also blocked by concentrations of μM Zn^{2+} [9]. Therefore, it is likely that, in response to nM concentrations of speract, Ca^{2+} can enter the cell through Ca^{2+} channels that are insensitive to verapamil and nimodipine [8]. Co^{2+} and Ni^{2+} at the concentrations tested did not significantly alter the pH_i change induced by 100 nM speract.

Fig. 3A displays the response of sperm swollen in DASW where Mg^{2+}, the most abundant divalent cation, was substituted by choline. Although the absence of Mg^{2+} in DASW does not change the overall pattern of membrane potential changes triggered by speract, it does modify it. The cells hyperpolarize less, the repolarization rate is slower and the depolarization is larger, slower and sustained. Initially, 20 mM Mg^{2+} was added to the 10-fold diluted ASW since preliminary experiments had indicated it improved the capacity of swollen sperm to regulate $[Ca^{2+}]_i$. Fig. 3B and C show that changes in external Mg^{2+} indeed regulate the speract-induced changes in $[Ca^{2+}]_i$ and its value at rest. In the absence of Mg^{2+} (0MgDASW), $[Ca^{2+}]_i$ becomes very high (~ 1.8 μM) and the fura-2 signal almost saturates.

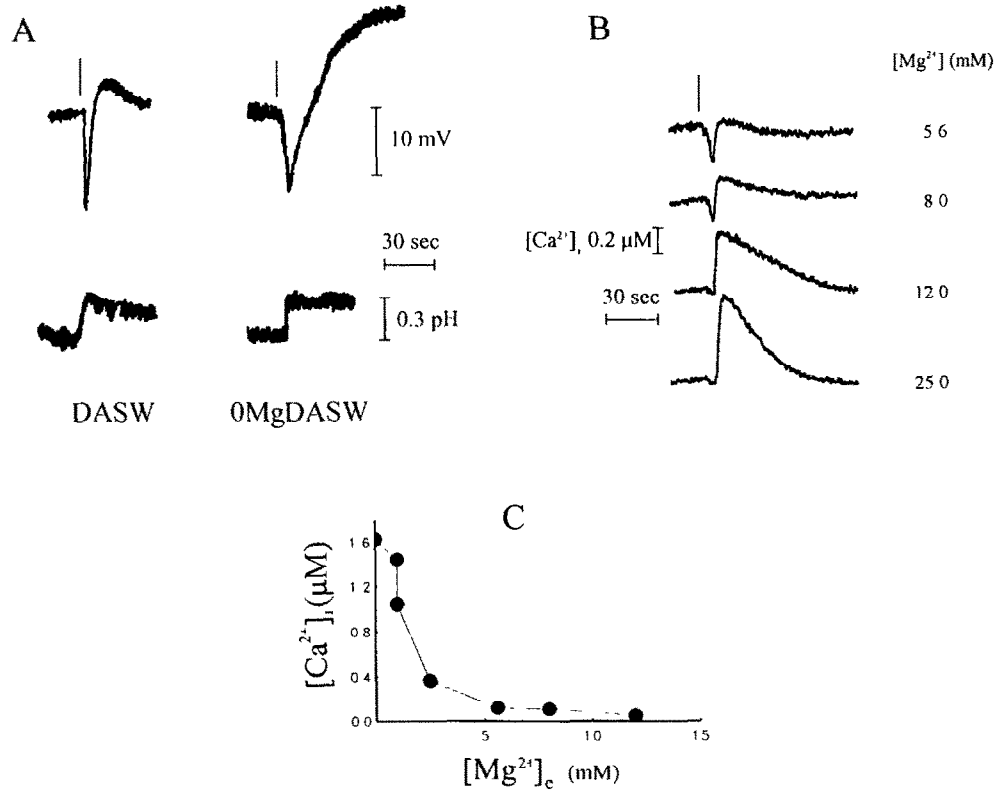

Fig. 3. External Mg^{2+} dependence of the speract (100 nM)-induced changes in membrane potential, pH_i and intracellular Ca^{2+} ($[Ca^{2+}]_i$) in swollen sperm. (A) Membrane potential (top) and pH_i changes induced by speract in DASW (left trace) and 0 Mg DASW (right). (B) Speract-induced changes in $[Ca^{2+}]_i$ at different external Mg^{2+} concentrations (right). Sperm were loaded with fura-2 and the signals calibrated as described in section 2. (C) Regulation of $[Ca^{2+}]_i$ as a function of the external Mg^{2+} concentration in swollen sperm. All other conditions were as in Fig. 1.

At 0–7 mM Mg^{2+}, 100 nM speract triggers a transient decrease in $[Ca^{2+}]_i$ (Fig. 3B). The activation of this interesting efflux mechanism should be further characterized. As external Mg^{2+} is increased, resting $[Ca^{2+}]_i$ decreases, reaching a value close to the one in non-swollen sperm (< 0.1 μM). At 8 mM Mg^{2+} speract first induces a decrease and then an increase in $[Ca^{2+}]_i$. At higher Mg^{2+} concentrations, 100 nM speract produces the nor-

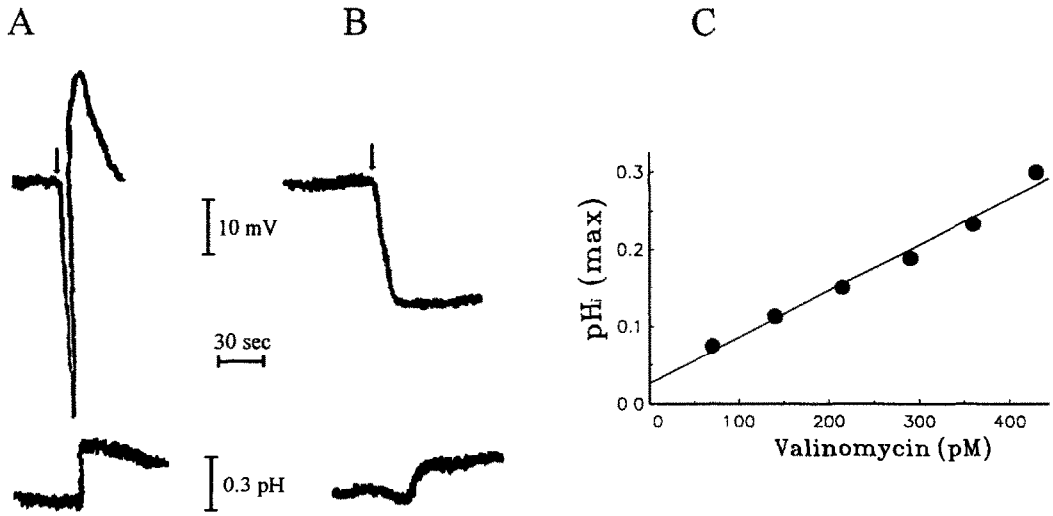

Fig. 4. A valinomycin-induced hyperpolarization alkalinizes swollen sperm. (A) Control membrane potential (top) and pH_i (bottom) changes induced by 100 nM speract. (B) Hyperpolarization (top) and increase in pH_i (bottom) induced by 400 pM valinomycin. (C) Concentration dependence of the valinomycin-induced alkalinization. Membrane potential, pH_i and time scales are between panels A and B. Other conditions are as in Fig. 1.

mal transient increase in $[Ca^{2+}]_i$ also observed in normal cells [8,10].

Mg^{2+} may modulate resting $[Ca^{2+}]_i$ and its speract-induced changes through surface potential, or directly, either from the outside or the inside, by modulating the extrusion of Ca^{2+} from the cytoplasm. It has been argued that intracellular Mg^{2+} may regulate Na^+/H^+ exchange and a Cl^- permeability [11]. Many questions remain to be answered regarding the regulating effects of Mg^{2+}.

The inactivation of K^+ channels could contribute to the repolarization, however, it has been shown that there is a long-lasting increase in K^+ permeability induced by speract, both in swollen and in normal cells [10]. Preliminary results indicate that the internal concentration of Cl^- in sea urchin sperm is high (~ 200 mM; García-Soto, De de La Torre and Darszon, unpublished). The opening of Cl^- channels would depolarize. Experiments done substituting SO_4^- for Cl^-, or all Cl^- from DASW with methanesulfonate (not shown), discount a possible significant contribution of anion permeability changes during the sperm response to speract.

Fig. 4A depicts a control record of membrane potential (top trace) and pH_i (bottom trace) during a 100 nM speract response. Fig. 4B demonstrates that an artificial hyperpolarization induced by valinomycin increases pH_i in swollen sperm. This result indicates the presence of a voltage-dependent system for the regulation of pH_i in swollen *S. purpuratus* sperm. Such a system had been reported in isolated flagella, in flagellar plasma membrane vesicles [11,12] and in *Lytechinus pictus* sperm [15]. In this context, it appears that the hyperpolarization triggered by speract does regulate a voltage-dependent system that modulates pH_i. Possibly, at pM concentrations of speract which hyperpolarize, the change in pH_i is simply not detected due to the buffering capacity of the cytoplasm, which is increased by the presence of the pH-sensitive fluorescent dye.

In summary, the ion substitution results indicate (i) that the permeability to Na^+, Ca^{2+}, and Mg^{2+} contribute to the resting membrane potential of swollen sperm; (ii) that the repolarization induced by nM concentrations of speract is Na^+ dependent, and is probably mediated by an as yet unidentified channel; (iii) it is likely that the Ca^{2+}-dependent depolarization triggered by nM concentrations of speract occurs through channels since it is blocked by Co^{2+} and Ni^{2+}, two Ca^{2+} channel blockers. In addition, the experiments showing that a valinomycin-induced hyperpolarization increases pH_i suggest that there is a link between membrane potential and pH_i in response to speract.

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