Alteration of Intracellular \( [\text{Ca}^{2+}] \) in Sea Urchin Sperm by the Egg Peptide Speract

EVIDENCE THAT INCREASED INTRACELLULAR \( [\text{Ca}^{2+}] \) IS COUPLED TO \( \text{Na}^+ \) ENTRY AND INCREASED INTRACELLULAR pH

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The egg peptide speract increases intracellular pH (pH) and cyclic nucleotides in sperm of the sea urchin Strongylocentrotus purpuratus by a mechanism dependent on seawater Na\(^+\) but not Ca\(^{2+}\) (Hansbrough, J. R., and Garbers, D. L. (1981) J. Biol. Chem. 256, 2233–2241; Repaske, D. R., and Garbers, D. L. (1983) J. Biol. Chem. 258, 6025–6029). Using the Ca\(^{2+}\) indicators quin\(^2\) and indo-1, we show that speract stimulates a transient rise in intracellular \( [\text{Ca}^{2+}] \) \(([\text{Ca}^{2+}]_i)\) when millimolar Ca\(^{2+}\) is present in seawater. The rise is increased and extended by the phophodiesterase inhibitor, 1-methyl-3-isobutylxanthine (MIX), which also enhances \( ^{22}\text{Na}^+ \) uptake with or without Ca\(^{2+}\). Without MIX, speract initiates a rise in \( [\text{Ca}^{2+}] \) that peaks within \( \sim 5 s \) and decreases with a \( t_\text{m} \) of \( \sim 9 s \). Activation of Na\(^+\):H\(^+\) exchange without speract by either Na\(^+\) addition to sperm in Na\(^+\)-free seawater (NaFASW) or by monensin also increases \( [\text{Ca}^{2+}]_i \), but neither change is transient. Inhibition of Na\(^+\):H\(^+\) exchange by increased seawater [K\(^+\)] prevents the rise in \( [\text{Ca}^{2+}]_i \), initiated by either speract or Na\(^+\) addition to sperm in NaFASW. Increasing pH, by adding 10 mM NH\(_4\)\(^+\) or by addition of Li\(^+\) to sperm in NaFASW does not increase \([\text{Ca}^{2+}]_i \). The data suggest that speract binding leads to rapid activation of Na\(^+\):H\(^+\) exchange and, as a consequence, \( [\text{Ca}^{2+}]_i \) entry increases transiently through either Na\(^+\):Ca\(^{2+}\) exchange or else through a verapamil-insensitive Ca\(^{2+}\) channel. MIX prevents the inactivation of this entry mechanism.

Increases in pH, \(^{22}\text{Na}^+ \), and \([\text{Ca}^{2+}]_i \), accompany a variety of eukar-

\( \text{N}^_,\text{N}^-,\text{bis}(2\text{-ethanesulfonic acid}) \); EGTA, \([\text{ethylenebis(oxy-}
\text{ethylenenitri1o)ltetraacetic acid}] \); DMCF, 5-\,(and 6-)\text{carboxy-4,5-di-}
\text{methylfluorescein}; TPP\(^*\), tetraphenylphosphonium bromide; Hepes, 4-\,(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Et, ethyl. 8719
by these peptides and suggests a relationship between the pH, increase and cyclic nucleotide changes (21).

Recently, the Arbacia peptide resect was found to alter swimming behavior of the Arbacia (24) sperm and to function as a sperm attractant. Sperm attraction, unlike the pH, increase and cyclic nucleotide changes by the peptide, requires millimolar levels of Ca++ in the seawater.

In this paper, we have used the recently developed intracellular Ca++ indicators quin2 and indo-1 (25, 26) to show that the S. purpuratus egg peptide speract leads to a transient rise in [Ca++]i, which is dependent upon millimolar seawater Ca++. The [Ca++]i increase is enhanced and extended by the phosphodiesterase inhibitor MIX, a change which can induce the acrosome reaction. These data indicate that the modifications of the sperm's biochemistry by the speract:receptor interaction are important not only to regulation of pH, but also serve as a means of modulating [Ca++]i.

**MATERIALS AND METHODS**

Sperm were obtained from S. purpuratus sea urchins by intracolonic injection of 0.5 M KCl and collected weekly (without dilution). Seawaters used were described previously (5). Seawaters basically contained 50 mM MgCl2, 10 mM KCl, 360 mM MCI (M = Na, Ca) (NaFASW) or choline (NaFASW), 1.0 mM EGTA, 10 mM Tris, 10 mM Hepes, pH 7.8, or as specified. If the pH was to be lowered to 7.4 or less, also included were Na2CO3 and NaHCO3 added as required to give total [Ca++] as specified in the figure legends. Standard ASW contained 10 mM total Ca++ and 360 mM Na+. If sperm were to be used in a Na+-free medium, dry sperm was diluted 500-fold into NaFASW, spun down (10 min at 1000 g), and resuspended to the original volume in NaFASW. This maintained seawater [Na+] sufficiently low to keep the sperm quiescent (5).

Na+ Uptake—Sperm were diluted directly from "dry" suspensions into the appropriate seawater containing radioactive 32P Na+ as indicated in the figure legends. Following incubation under the experimental conditions, 0.2-m1 aliquots were layered onto 300 ml of silicone fluid and centrifuged for 30 min. The supernatant and silicone fluid were removed by aspiration, and the tip of the centrifuge tube containing the sperm pellet was cut off and counted in a Beckman 4000 γ counter. Intracellular water space measurements and correction for 32P Na+ in the extracellular space was performed as previously described using [3C] sucrose as an extracellular marker (8). Filtration was not used because rapid isotopic exchange or efflux at normal seawater pH yields Na+ uptake levels substantially lower than by centrifugation (27).

[Ca++]i measurements—[Ca++]i measurements were principally performed with two recently developed probes, quin2 and indo-1 (25, 26). Acetoxymethylesters of quin2 and indo-1 were incubated with sperm (10% dry sperm by volume) in Ca++/CaFASW or ASW at pH 6.8 or 7.4 at either 10°C or on ice for periods of 3–24 h. These ionic conditions lower pH, and preserve the fertilizing capacity of the sperm for tens of hours. The dyes were added following suspension of the sperm to a final concentration of 50 μM for quin2 and 9 μM for indo-1 in a series of 3–5 additions from stock solutions in dimethyl sulfoxide (10 or 3 mM, respectively) with a few minutes mixing between each addition. The esters were not completely soluble at the final concentrations in our seawaters in the absence of sperm. Following the incubation, sperm were usually centrifuged and washed once or twice in 5–10 volumes of the same seawater before being resuspended in the same seawater to 10% dry sperm loaded sperm were diluted to 2.0 ml in seawater, and fluorescence was recorded with a SLM 8000 spectrofluorimeter coupled to a Hewlett-Packard 8750 computer for data storage and manipulation. Alternatively, output was recorded directly using a Hewlett-Packard 7000B X-Y recorder. Quin2 and indo-1 fluorescence were monitored using an excitation wavelength of 339 and 355 nm, respectively. Slit widths were usually set at 4 nm. Fluorescence of the sperm suspensions was proportional to the sperm concentration over the range of sperm concentrations used. Experiments were at 11°C.

Determination of [Ca++]i were performed using the methods of Trien et al. (25, 26) and Hesketh et al. (28), and data presented here were based on emission measurements at a single wavelength (492 nm for quin2 and 405 nm for indo-1). At these wavelengths Ca++ binding increases emission intensity of the dyes. Ca++ binding also leads to a decrease in indo-1 fluorescence at longer wavelengths (e.g. 480 nm) which makes possible the more sensitive ratio method (26). Approach of this method, which compares either indo-1 or another 

\[
[Ca^{2+}] = K_0 F/F_{\text{Fmax}} - F
\]

where \( K_0 \) is the dye-Ca++ dissociation constant. In this manuscript, \([Ca^{2+}]\) is expressed in the figures as the dimensionless quantity \((F - F_0)/(F_{\text{Fmax}} - F)\) which must be multiplied by \( K_0 \) to give an absolute value for \([Ca^{2+}]\). Values for the dye, \( K_0 \), as determined from the Ca++ titrations using EGTA as a Ca++ buffer, may vary up to 4-fold depending on the association constant between EGTA and Ca++ (see Table 1).

**Measurements of [Ca++]i** was performed by determining the experimental fluorescence intensity, F, the maximum intensity, F_{\text{Fmax}}, and the minimum intensity, F_{\text{Fmin}}, and using the equation:

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[Ca^{2+}] = K_0(F - F_{\text{Fmin}})/(F_{\text{Fmax}} - F)
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\]
Sperm Intracellular [Ca\(^{2+}\)]

**Materials and Methods** For 10 h, 200 were incubated with 20 \(\mu\)M dimethylcarboxyfluorescein (DMCF) as indicated under "Materials and Methods." To achieve this we have incorporated dimethylcarboxyfluorescein (33) into the sperm by incubating the sperm with the diacetate derivative (at 10 or 20 \(\mu\)M) under conditions identical to those for the Ca\(^{2+}\) indicators. In some cases, both dyes were loaded into the identical sperm. Because indo-1 and dimethylcarboxyfluorescein excitation and emission spectra are sufficiently separate, pH, and [Ca\(^{2+}\)], can then be monitored separately in sperm loaded identically. To initiate measurements, sperm were diluted 10-fold (final concentration 4 \(\times\) 10\(^5\) sperm/ml) into ASW, and fluorescence intensity was measured (500-nm excitation, 530-nm emission with slit widths at 4 nm). To ascertain that fluorescence intensity changes corresponded to changes in pH, either NH\(_4\)Cl or nigericin was added to vary pH. 10 mM NH\(_4\)Cl rapidly increased the fluorescence (not shown) indicating a pH increase. Since both NH\(_4\)Cl in the 1 mM stock solution and seawater were at pH 7.7, changes did not result from alteration of extracellular pH. Additionally, the ionophore nigericin, when added to sperm in seawater with 1 mM K\(^+\) (enhanced [K\(^+\)]/[K\(^+\)], gradient), causes the fluorescence change shown in Fig. 1, first a decrease, followed by a slower increase. We have previously demonstrated by following H\(^+\) efflux induced by this ionophore (7) that it first collapses the cell K\(^+\) gradient (K\(^+\)/H\(^+\) exchange) and actually causes H\(^+\) uptake followed by a slow Na\(^+\)-dependent H\(^+\) efflux and pH increase. The data in Fig. 1 are readily interpreted only if fluorescence intensity follows pH. A pH decrease (fluorescence decrease) is followed by a pH increase (fluorescence increase). The extent of the initial pH decrease is inversely proportional to [K\(^+\)], or is proportional to the initial [K\(^+\)]/[K\(^+\)], gradient. Thus, dimethylcarboxyfluorescein is a useful qualitative indicator of pH. Calibration of fluorescence intensity against pH was approximated either by collapsing the pH gradient with the ionophores monensin and nigericin or by making sperm permeant with digitonin (250 \(\mu\)g/ml) to release the dye and then varying the pH. The values in Table I show some difference in pH measurements from those determined by weak base accumulation or \(^{31}\)P NMR, but are satisfactory close to allow for use of DMCF to record the time course of the pH changes. pH tracings are presented as a fractional change of the total fluorescence signal after digitonin lysis or collapse of 3pH by ionophores. Babcock (44) has previously shown nigericin to decrease fluorescence of 6-carboxyfluorescein in bovine sperm. We have used the diyethyl derivative because it has a higher pK\(_{a}\), and pH in sea urchin sperm is higher than that of bovine sperm.

**Chemicals**-Quin2, quin2/AM, and ionomycin were obtained from Calbiochem. \[^{3}H\]Quin2/AM was obtained from Amersham Corp. Indo-1, indo-1/AM, fura-2, fura-2/AM, and 5-(and 6-)carboxy-4,5-dimethylfluorescein diacetate were from Molecular Probes. All other chemicals were standard reagent grade as previously described (5).

**Fig. 2.** Speract causes a transient increase in [Ca\(^{2+}\)]. In A, sperm were loaded with indo-1/AM as described under "Materials and Methods." The experiment was initiated by 10-fold dilution of the 10% sperm stock into CaFASW containing 1.0 mM EGTA. Additional Ca\(^{2+}\) was added to bring the total [Ca\(^{2+}\)] of the seawater to a, 30 mM; b, 10 mM; c, 2 mM; d, 1.5 mM; or e, 1.0 mM, and fluorescence increases were followed for an additional 2 min until the steady-state levels were reached and the recordings began. Speract (30 nm) was added at the arrow, and fluorescence was followed. [Ca\(^{2+}\)] is given as \((F_F)/F_{(F)}\), a dimensionless value which must be multiplied by the \(K_a\) for the indo-1 Ca\(^{2+}\) complex to give absolute concentration (see Materials and Methods). In B, the data in A and that from additional fluorescence tracings (not shown) are plotted to show the [Ca\(^{2+}\)], dependence of the basal steady state (○) and speract-induced peak (△) [Ca\(^{2+}\)]. [Ca\(^{2+}\)] is given as a dimensionless multiplier of the Ca\(^{2+}\)indo-1 \(K_a\). Sperm were at \(~4 \times 10^5\) sperm/ml.

**Fig. 1.** Nigericin causes transient changes in pH. Sperm were incubated with 20 \(\mu\)M DMCF as indicated under Materials and Methods for 10 h. 200 \(\mu\)l were added to 1.8 ml in CaFASW at pH 7.8 containing 1 mM K\(^+\) (a), 10 mM K\(^+\) (b), or 30 mM K\(^+\) (c). Nigericin (20 \(\mu\)g/ml) was added at the arrow, and fluorescence (600-nm excitation, 530-nm emission) was followed as a function of time.

Calbiochem. [\(^{3}H\)]Quin2/AM was obtained from Amersham Corp. Indo-1, indo-1/AM, fura-2, fura-2/AM, and 5-(and 6-)carboxy-4,5-dimethylfluorescein diacetate were from Molecular Probes. All other chemicals were standard reagent grade as previously described (5).
Speract Initiates a Transient Rise in [Ca2+].—Fig. 2 shows that speract initiates a transient increase in the fluorescence of the Ca2+ indicator indo-1 when millimolar [Ca2+] is in the seawater. The speract-induced transient reaches a half-maximal value at -2 mM [Ca2+] (Fig. 2B). The steady state basal fluorescence also increased with increasing [Ca2+]. At 20 μM [Ca2+], (Fig. 2A, a) speract slightly decreases the indo-1 fluorescence. At 10 mM [Ca2+], (Fig. 2A, b) speract stimulates [Ca2+], ~2-fold over the basal concentration. The speract-induced transient peaks within 5 s after mixing and relaxes to a steady-state level via an apparent first-order process with a τobs = 9 s. The rate of the relaxation process is independent of [Ca2+], when this concentration is varied from 0.5 to 10 mM. The steady-state level reached after speract addition is always quite close to the original basal fluorescence but has on occasion remained either slightly elevated or depressed with respect to initial [Ca2+]. At 10 mM [Ca2+], [Ca2+]; approaches concentrations of up to ~2 μM estimated using 13°C. HCl was used to adjust the pH. The pH dependence effects were not corrected since the data necessary to modify each of the constants were unavailable.

Correction for temperature was performed as in Ref. 29, while ionic strength effects were not corrected since the data necessary to modify each of the constants were unavailable.

Table I

<table>
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<tr>
<th>pH</th>
<th>Kd</th>
<th>Kd</th>
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<tr>
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<td>1.62 x 10^-6</td>
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<tr>
<td>7.04</td>
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<td>1.46 x 10^-6</td>
</tr>
<tr>
<td>7.38</td>
<td>0.35 x 10^-6</td>
<td>1.40 x 10^-6</td>
</tr>
<tr>
<td>7.63</td>
<td>0.33 x 10^-6</td>
<td>1.40 x 10^-6</td>
</tr>
</tbody>
</table>

Equation 1 and the Kd value of 1.4 x 10^-6 M (see Table I) for the indo-1 Ca2+ complex (see "Materials and Methods"). At 30 mM [Ca2+], the basal [Ca2+] increases dramatically (Fig. 2A, a). The degree of speract stimulation is substantially reduced.

Fig. 3 shows that half-maximal stimulation by speract occurs at -0.3 ng/ml of speract or in the vicinity of 300 pM. This is higher than the concentrations necessary to half-maximally induce both acid efflux (20 pM) and stimulate respiration in sperm at low seawater pH (34). Furthermore, after an addition of speract at 30 nM subsequent addition of 10-fold more speract produces no change in fluorescence if added 2 min after the [Ca2+] transient has returned to its original level.

Fig. 4 shows that speract's ability to raise [Ca2+] is transient whether or not seawater Ca2+ is present. Speract added to sperm in seawater containing Ca2+ gives rise to the transient increase in [Ca2+] (Fig. 4d). If speract is added first, followed 3 s later by Ca2+ addition (Fig. 4b), the increase in [Ca2+] is not nearly as extensive but is again complete within 5 s and relaxes slightly. However, if Ca2+ is added 50 s after speract (Fig. 4c), the rise is much slower requiring over 20 s to reach a maximum. This time course is similar to that when Ca2+ is added without speract (Fig. 4d).

pH Dependence of the [Ca2+], Transient—Fig. 5 shows that when the pH of seawater is varied from 7.6 to 6.75, there is little change in either the amplitude or the time course of the [Ca2+], transient induced by speract. Higher pH values (up to 7.9) also did not substantially alter these parameters (not shown). However, by lowering the pH to 6.5, the fluorescence changes associated with increased [Ca2+], were substantially reduced, and at pH 5.3 the increase in [Ca2+] induced by speract is negligible. In contrast, speract (300 nM) is known to cause substantial acid efflux at pH 6.3 and also induces increased pH, at such a low extracellular pH (34). These data

Fig. 4. Speract effects on [Ca2+], are transient. Sperm were loaded with indo-1 as described under "Materials and Methods" in ASW. 200 μl of loaded sperm were diluted to 2 ml in CaFASW. In a, 10 mM Ca2+ was added prior to recording. Speract (30 nM) was then added (thick arrow). In b, 30 nM speract was added (thick arrow) followed -3 s later by 10 mM Ca2+ (thin arrow). In c, 30 nM speract was added (not shown), and 50 s later 10 mM Ca2+ was added (thin arrow). In d, no speract was added prior to addition of 10 mM Ca2+ (arrow).

R. Schackmann, unpublished data.
also suggest that Ca\(^{2+}\) entry may be secondary to other changes stimulated by speract. Support for this relationship is demonstrated below.

**1-Methyl-3-isobutylxanthine Enhances the Speract-stimulated [Ca\(^{2+}\)]\textsubscript{i} Increase**—MIX, a cyclic nucleotide phosphodiesterase inhibitor, has been observed to increase \(^{45}\)Ca\(^ {2+}\) influx and induce the acrosome reaction in abalone sperm but not in *S. purpuratus* sperm (14). It does, however, increase both cAMP and cGMP in the latter sperm in conjunction with the peptide speract (20). Fig. 6A shows that addition of micro-molar MIX prevents the decrease in [Ca\(^{2+}\)], (following the speract-induced [Ca\(^{2+}\)], increase) and further increases [Ca\(^{2+}\)], to a value approximately 4 times the \(K_d\) for the indo-1 -Ca\(^{2+}\) complex. As shown in Fig. 6B, stimulation by speract appears to be half-maximal at approximately 20 \(\mu\)M MIX. MIX alone at 300 \(\mu\)M was able to substantially increase basal sperm [Ca\(^{2+}\)]. The nontransient increase in [Ca\(^{2+}\)], is quite similar to that initiated by egg jelly, the egg material responsible for inducing the acrosome reaction. Interestingly, in sperm treated with both the peptide speract and 0.3 mM MIX, 30–60% of the sperm were found to undergo the acrosome reaction. In the absence of MIX we have yet to observe any acrosome reactions induced by the peptide speract.

**Speract Increases pH\(_{i}\), More Rapidly than [Ca\(^{2+}\)\textsubscript{i}], Changes**—The sperm pH\(_{i}\) was monitored using dimethylcarboxyfluorescein as an indicator. Fig. 7A shows that the speract-induced increase in pH\(_{i}\) (increased fluorescence) in seawater containing Ca\(^{2+}\) is essentially complete in 1–2 sec. Considering the time required for mixing (1–2 sec) this time represents an upper limit for the actual time of the pH\(_{i}\) increase. The increase in DMCF fluorescence shown in Fig. 7A partially relaxes indicating that pH\(_{i}\) first rises and then partially decreases. In the absence of extracellular Ca\(^{2+}\) speract still stimulates a rapid increase in DMCF fluorescence. These data and those in Fig. 2 showing no increase in indo-1 fluorescence in the absence of extracellular Ca\(^{2+}\) demonstrate that DMCF is not responding to changes in [Ca\(^{2+}\)]. The time course for the [Ca\(^{2+}\)], increase is significantly slower, peaking in ~5 sec (Fig. 7B). Hence, the pH\(_{i}\) change appears to precede the increase in [Ca\(^{2+}\)]. These time courses have been confirmed in many samples in several different batches of sperm. In the presence of MIX, the difference between increased [Ca\(^{2+}\)], and increased pH\(_{i}\), is even more substantial. As shown in Fig. 7C, the pH\(_{i}\) increase is again complete within 2 sec. The increase in pH\(_{i}\), is also slightly greater (see Table II) as is H\(^{+}\) efflux (not shown). The speract-induced [Ca\(^{2+}\)], increase, which is approximately 3-fold larger than without MIX (Fig. 7D), requires ~40 sec to reach the final steady state. This time course nearly follows first-order rate
Sperm Intracellular $[\text{Ca}^{2+}]$

**Fig. 7.** The speract-induced $\text{pH}_i$ increases faster than the $[\text{Ca}^{2+}]_i$ increases. In A and C, sperm were loaded with 20 $\mu$M dimethylcarboxyfluorescein diacetate. In B and D, sperm were loaded with 9 $\mu$M indo-1/AM. In A and B, sperm were then diluted 10-fold into ASW, pH 7.8, with 10 mM $\text{Ca}^{2+}$. In C and D, sperm were diluted into ASW with 10 mM $\text{Ca}^{2+}$ and 30 $\mu$M $\text{MIX}$. Speract (30 nM) was added (arrows) and fluorescence followed as described under "Materials and Methods." Note that fluorescence scales are different in B and D.

**TABLE II**

**pH; measurements with dimethylcarboxyfluorescein**

Sperm were incubated as a 10% stock suspension in NaFSW with 20 $\mu$M dimethylcarboxyfluorescein diacetate for 10 h on ice as indicated under "Materials and Methods." 200 $\mu$l of the sperm stock was diluted to 2.0 ml in either ASW containing 10 mM $\text{Ca}^{2+}$ or NaFASW at the indicated pH, and fluorescence emission (530 nm) was monitored with time. To determine the approximate $\text{pH}_i$, fluorescence intensity was compared against a standard curve generated by varying the seawater pH after collapsing $\Delta\text{pH}$ with ionophores monensin (30 pg/ml) and nigericin (30 pg/ml).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>pH</th>
<th>DMCF</th>
<th>$^{14}\text{C-Et}_{2}\text{NH}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASW</td>
<td>7.8</td>
<td>7.25</td>
<td>7.4$^a$</td>
</tr>
<tr>
<td>ASW + 30 $\mu$M speract</td>
<td>7.8</td>
<td>7.34</td>
<td>7.5$^a$</td>
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<tr>
<td>ASW + 30 $\mu$M $\text{MIX}$ + 7.8</td>
<td>7.4</td>
<td>7.6$^a$</td>
<td></td>
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<td>CaFASW + 10 mM $\text{Ca}^{2+}$</td>
<td>6.2</td>
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<td>NaFASW</td>
<td>7.8</td>
<td>6.9</td>
<td>6.8$^a$</td>
</tr>
</tbody>
</table>

$^a$Values presented for pH, were determined by $^{14}\text{C-Et}_{2}\text{NH}$ accumulation as previously described (5).

$^b$Designates values taken from Ref. 11.

laws with $t_0 \approx 8$ s. In the absence of MIX, the $[\text{Ca}^{2+}]_i$ increase reaches the peak in less time, but this presumably occurs because the initial $[\text{Ca}^{2+}]_i$ increase is opposed by an immediate $\text{Ca}^{2+}$ efflux. To evaluate properly the rate of $[\text{Ca}^{2+}]_i$ increase, it is necessary to solve two differential equations describing the $\text{Ca}^{2+}$ influx and efflux simultaneously. Currently, we do not have sufficient data to carry out this analysis; nevertheless, we plan to investigate this type of analysis in the near future. Preliminary experiments reveal little difference in the initial rate of the $[\text{Ca}^{2+}]_i$ increase with or without MIX (not shown). These data are most simply interpreted as speract acting through its receptor to enhance net $\text{Ca}^{2+}$ entry, but also allowing for rapid inactivation of the entry mechanism. Inclusion of MIX prevents inactivation.

Speract with MIX Stimulates a Large Permanent Increase in $[\text{Na}^{+}]$.—Hansbrough and Garbers (20) and Repaske and Garbers (34) showed that the peptide speract stimulates both $^{22}\text{Na}^{+}$ uptake and $\text{H}^+$ efflux qualitatively similar to the effects caused by the ionophore monensin. If the rapid pH; increase (Fig. 7A) is coupled to $\text{Na}^+$ influx, $\text{Na}^+$ entry also would precede the $[\text{Ca}^{2+}]_i$ increase. Either $\text{Na}^+$ entry, increased pH; or both, are, therefore, possible links to the $[\text{Ca}^{2+}]_i$ increase. A $\text{Na}^+$-dependent increase in $\text{Ca}^{2+}$ might be mediated through $\text{Na}^+/	ext{Ca}^{2+}$ exchange allowing for increased $\text{Ca}^{2+}$ entry as a consequence of partial collapse of the transmembrane $\text{Na}^+$ gradient.

Direct kinetic measurements of $\text{Na}^+$ uptake are difficult at normal seawater $[\text{Na}^+]$ with the temporal resolution necessary to follow changes during the first few seconds after speract addition. As shown in Fig. 8, however, MIX in conjunction with speract stimulates $^{22}\text{Na}^{+}$ uptake to a much larger degree. Uptake increases from ~5 nmol/10$^8$ sperm to 50–80 nmol/10$^8$ sperm (Fig. 8) as a function of the seawater pH. This extensive uptake, which is 4–5 times the $\text{H}^+$ efflux under similar conditions (not shown), is similar in magnitude to changes in $[\text{Na}^+]$, associated with egg jelly induction of the acrosome reaction (27). Speract-stimulated $^{22}\text{Na}^{+}$ uptake at seawater pH 7.8 was complete within 15 s and remained elevated for at least 8 min. $\text{Na}^+$ uptake is, therefore, at least as rapid as the $[\text{Ca}^{2+}]_i$ increase in Fig. 7D. The half-maximal concentration of MIX necessary for stimulation of $^{22}\text{Na}^{+}$ uptake is ~30 $\mu$M, similar to that necessary for stimulation of the permanent increase in $[\text{Ca}^{2+}]_i$.

The pH dependence of the speract plus MIX-stimulated
crease, or both, might be linked to the regulation of \([\text{Ca}^{2+}]_{\text{i}}\).

FIG. 8. MIX enhances sperm \(^{22}\text{Na}^+\) uptake. Sperm (4 \times 10^8 sperm/ml) were incubated in CaFASW (A) or in 10 mM \(\text{Ca}^{2+}\) ASW (B) with 5 \(\mu\)Ci/ml \(^{22}\text{Na}^+\) at the indicated \(pH\) values until a steady-state level was reached. In some batches of sperm this basal uptake at low \(pH_L\) (\(\leq 6.5\)) was slow and took tens of minutes; in others it was essentially complete within seconds. The origin of these differences is not understood. Under our conditions speract or speract and MIX-stimulated \(\text{Na}^+\) movements were rapid, reaching a maximum in less than 15 s. Samples were divided, and 3 \(\mu\)l/ml \(\text{H}_{2}\text{O}\) (•—•); 300 nM speract (•—•); or 300 \(\mu\)M MIX plus 300 nM speract (•—•) was added. Following an incubation of 2 min, duplicate 200-\(\mu\)l samples were centrifuged through silicone fluid and \(^{22}\text{Na}^+\) uptake was measured as described under “Materials and Methods.” MIX was added from a 0.15 M stock in dimethyl sulfoxide. Equivalent addition (2 \(\mu\)l/ml) of dimethyl sulfoxide alone was without effect on \(^{22}\text{Na}^+\) uptake. Speract was added from a 10^{-4} M stock in \(\text{H}_{2}\text{O}\).

### Table III

**Na\(^+\)/H\(^+\) exchange increases [Ca\(^{2+}\)].**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>([\text{Ca}^{2+}]<em>{\text{i}}) ((F - F</em>{\text{max}}) / F_{\text{max}} - F))</th>
</tr>
</thead>
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<td>NaFASW — Ca(^{2+})</td>
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<td>NaFASW + 4 mM (\text{Na}^+)</td>
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<tr>
<td>NaFASW + 4 mM Li(^+)</td>
<td>0.52</td>
</tr>
<tr>
<td>NaFASW + 10 mM (\text{NH}_4)</td>
<td>0.66</td>
</tr>
<tr>
<td>NaFASW + 10 mM Ca(^{2+})</td>
<td>3.2</td>
</tr>
<tr>
<td>NaFASW + 10 mM Ca(^{2+}) + 4 mM (\text{Na}^+)</td>
<td>7.1</td>
</tr>
<tr>
<td>NaFASW + 10 mM Ca(^{2+}) + 4 mM Li(^+)</td>
<td>1.7</td>
</tr>
<tr>
<td>NaFASW + 10 mM Ca(^{2+}) + 10 mM (\text{NH}_4)</td>
<td>2.6</td>
</tr>
</tbody>
</table>

increase in \([\text{Na}^+]\), is shown in Fig. 8A (without \(\text{Ca}^{2+}\)) and in Fig. 8B (10 mM \(\text{Ca}^{2+}\)). The data indicate that increased \([\text{Ca}^{2+}]_{\text{i}}\), which does not occur in the absence of external \(\text{Ca}^{2+}\) (Fig. 2), is not necessary for the increase in \([\text{Na}^+]\), by speract and MIX. This phenomenon has also been observed for speract alone (20) although the amount of \(^{22}\text{Na}^+\) uptake is smaller. These data show that speract causes changes in \([\text{Na}^+]\), and \(pH\), and suggest that either \(\text{Na}^+\) entry, \(pH\) increase, or both, might be linked to the regulation of \([\text{Ca}^{2+}]_{\text{i}}\).

We have found that speract added to sperm in the absence of \(\text{Na}^+\) causes no effect on \([\text{Ca}^{2+}]_{\text{i}}\), (data not shown). However, this observation may be derived from the fact that the \([\text{Ca}^{2+}]_{\text{i}}\), is higher (see Table III and Fig. 12) in Na\(^+\)-free seawater than in ASW and at elevated \([\text{Ca}^{2+}]_{\text{i}}\), little speract stimulation is expected even in ASW (Fig. 2A, a). Additionally, speract binding is reduced in Na\(^+\)-free seawater (35). Nevertheless, we can alternatively increase \(pH\), without \(\text{Na}^+\) by addition of 10 mM \(\text{NH}_4\) to sperm in Na\(^+\)-free seawater. The result is a significant decrease in \([\text{Ca}^{2+}]_{\text{i}}\), (Table III) suggesting that a \(pH\) increase alone is not only insufficient to cause an increase in \([\text{Ca}^{2+}]_{\text{i}}\), but may also activate a regulatory mechanism for lowering \([\text{Ca}^{2+}]_{\text{i}}\). The data in Table III also show that \(\text{Li}^+\), which passes through the sperm \(\text{Na}^+/\text{H}^+\) exchange and can increase the intracellular \(pH\), also decreases \([\text{Ca}^{2+}]_{\text{i}}\). It is known that \(\text{Li}^+\) will not support \(\text{Ca}^{2+}\) movements through Na\(^+\)-Ca\(^{2+}\) exchange in other cells (36). Addition of \(\text{Na}^+\) to sperm in NaFASW leads to \(\text{Na}^+\) entry through the \(\text{Na}^+/\text{H}^+\) exchange (37-39), to a rapid rise in \(pH\), (\(t\)\(_{\text{a}}\) \(\approx 6\) s as monitored with DMCF), and to subsequent \(\text{Ca}^{2+}\) entry (Table III) that begins \(\sim 7\) s after \(\text{Na}^+\) addition, \(\text{NH}_4\) (10 mM), when added to sperm in ASW (10 mM \([\text{Ca}^{2+}]_{\text{i}}\)), rapidly increases \(pH\), to a level higher than that produced by speract but produced no increase in \([\text{Ca}^{2+}]_{\text{i}}\). These experiments suggest that \(\text{Ca}^{2+}\) entry results either directly from \(\text{Na}^+\) influx or else from the combination of \(\text{Na}^+\) entry and the rise in \(pH\).

**Monensin Stimulates Increased [Ca\(^{2+}\)]**—If \(\text{Na}^+\) entry and increased \(pH\), lead to conditions which allow for the increase in \([\text{Ca}^{2+}]_{\text{i}}\), the ionophore monensin, which catalyzes \(\text{Na}^+/\text{H}^+\) exchange, should also cause increase in \([\text{Ca}^{2+}]_{\text{i}}\). As shown in Fig. 9, monensin, at concentrations necessary to increase \(pH\), and \([\text{Na}^+]\), does increase \([\text{Ca}^{2+}]_{\text{i}}\). Monensin does not increase \([\text{Ca}^{2+}]_{\text{i}}\), if choline replaces \(\text{Na}^+\) in the seawater or if extracellular \([\text{Ca}^{2+}]_{\text{i}}\) is absent; it is, therefore, not transporting \(\text{Ca}^{2+}\) directly nor acting nonspecifically to disrupt plasma membrane integrity.

**K\(^+\) Inhibits Speract-induced pH, and [Na\(^+\)], Increases**—Increased seawater \([\text{K}^+]\) depolarizes the sperm plasma membrane potential (8, 40), inhibits \(\text{Na}^+/\text{H}^+\) exchange in whole sperm (37), isolated tails (38), and membrane vesicles (39), and also blocks ion fluxes associated with the egg jelly-mediated acrosome reaction (7). Fig. 10 shows that a modest increase in seawater \([\text{K}^+]\) blocks the \(\text{MIX}^+\) speract increases in \([\text{Na}^+]\), and the corresponding \(pH\), increase. In Fig. 10A, 30 mM \([\text{K}^+]\) is sufficient to completely inhibit the permanent increase in \([\text{Na}^+]\), induced by the combination of peptide and MIX. Fig. 10B shows the time course of \(pH\), changes induced by speract and MIX. Inhibition of the \(pH\), increase occurs over a narrow range of \([\text{K}^+]\) between 20 and 30 mM in

\[ [\text{Ca}^{2+}]_{\text{i}} \]
this case. The absolute range of [K\(^+\)]\(_\text{e}\), over which inhibition occurs varies somewhat in various batches of sperm between 20 and 30 mM for full inhibition. Inhibition of both Na\(^+\) uptake and increased pH also occurs in the absence of seawater Ca\(^{2+}\) (not shown). Additionally, the speract-stimulated pH\(_i\) increase is blocked by comparable [K\(^+\)]\(_\text{e}\) in the absence of MIX.

K\(^+\) Inhibits Speract-induced [Ca\(^{2+}\)]\(_i\). Increase—The data in Fig. 11 show that K\(^+\) inhibition of Na\(^+\) and pH changes prevents Ca\(^{2+}\) uptake and that increased [K\(^+\)]\(_\text{e}\) does not directly act at the level of the Ca\(^{2+}\) entry mechanism (Fig. 11). In Fig. 11a, speract is used to stimulate [Ca\(^{2+}\)]\(_i\), in sperm treated with MIX. In Fig. 11b, 10 mM Ca\(^{2+}\) was added 45 s after speract. [Ca\(^{2+}\)]\(_i\), reached nearly the same concentration as in Fig. 11a. If 30 mM K\(^+\) is added prior to speract and Ca\(^{2+}\) addition (Fig. 11c), the [Ca\(^{2+}\)]\(_i\), increase is largely inhibited. The slight increase is equivalent to that which occurred in this batch of sperm when 10 mM Ca\(^{2+}\) is added to sperm in Ca\(^{2+}\)-free seawater without speract or MIX. However, if speract is added, followed by 30 mM K\(^+\), and Ca\(^{2+}\) is subsequently added, no inhibition occurs (Fig. 11d), and the [Ca\(^{2+}\)]\(_i\), increase is comparable to that at the normal seawater [K\(^+\)] (10 mM). Additions of 20 mM K\(^+\) to sperm already in 10 mM Ca\(^{2+}\)-seawater also prevent speract stimulation of the Ca\(^{2+}\) transient or speract + MIX stimulation of the permanent [Ca\(^{2+}\)], increase (not shown). The data are consistent with K\(^+\) directly blocking the mechanism by which speract causes the Na\(^+\) and H\(^+\) movements. The data in Fig. 11 also show that with MIX, the ability of speract to increase [Ca\(^{2+}\)] is much less transient than without MIX (Fig. 4). Ca\(^{2+}\) may be added to the sperm suspension minutes after speract and still causes a rapid rise in [Ca\(^{2+}\)], K\(^+\) might either inhibit speract binding or block the initial reaction(s) triggered by speract. Since increased seawater K\(^+\) and membrane potential depolarization inhibit Na\(^+/\)H\(^+\) exchange activity directly when the exchange is initiated by Na\(^+\) addition to sperm in Na\(^+\)-free seawater (37-39), we consider it least likely that K\(^+\) prevents speract binding.

The data in Fig. 12 show that Na\(^+\) entry through the Na\(^+\)/H\(^+\) exchange triggered without speract allows for [Ca\(^{2+}\)], increase in sperm. Inhibition of Na\(^+\) entry by tetraphenylphosphonium (another inhibitor of the Na\(^+\)/H\(^+\) exchange (39)) also does not directly inhibit the Ca\(^{2+}\) entry mechanism but prevents Na\(^+\) entry and the pH\(_i\) increase necessary for Ca\(^{2+}\) entry. In Fig. 12a, 4 mM Na\(^+\) added to sperm in NaFASW with 10 mM Ca\(^{2+}\) increases [Ca\(^{2+}\)]. In Fig. 12b, 4 mM Na\(^+\) was
is added to sperm in Ca$^{2+}$-free NaFASW and to lead to suppression of [Ca$^{2+}$]. Subsequent addition of 10 mM Ca$^{2+}$ increases [Ca$^{2+}$]$_i$, to 75% of the level in Fig. 12a. If, as in Fig. 12c, the lipophilic cation TPP$^+$ (1 mM), an inhibitor with Na$^+/H^+$ exchange (39), is added to sperm in NaFASW with 10 mM Ca$^{2+}$ (arrow 2), subsequent 4 mM Na$^+$ addition (arrow 3) yields no further increase in [Ca$^{2+}$]$_i$ as Na$^+$ entry and the pH$_i$ increase are blocked (data not shown). However, if Na$^+$ is added first (Fig. 12d, arrow 1), followed by 1 mM TPP$^+$ (arrow 2), subsequent Ca$^{2+}$ (10 mM) addition (arrow 3) leads to a rapid increase in [Ca$^{2+}$]$_i$, indicating that TPP$^+$ does not inhibit Ca$^{2+}$ entry directly. This experiment also has been performed using K$^+$ instead of TPP$^+$ (not shown).

**DISCUSSION**

The data presented show that speract not only stimulates changes in [Na$^+$], pH$_i$, and cyclic nucleotide concentrations (20, 34) but also serves to alter [Ca$^{2+}$], if external Ca$^{2+}$ is available. Unlike stimulation of respiration (6, 20, 34), which is optimal at low pH$_i$, speract induces a transient rise in [Ca$^{2+}$], which may double [Ca$^{2+}$]$_i$ within 5 s at physiological pH$_i$. Such an increase may seem modest but readily provides possible regulatory control through intracellular Ca$^{2+}$-binding proteins with multiple sites like calmodulin (31). For example, it was shown by Huang et al. (31) that phosphodiesterase activation may be quite sensitive to small changes in [Ca$^{2+}$]$_i$ in the micromolar and submicromolar range. We have measured [Ca$^{2+}$]$_i$ of ~1 μM for sperm in seawater and up to ~2 μM briefly following speract stimulation (using $K_{sp}$, Table II). However, depending upon one’s choice for Ca$^{2+}$ affinity constants for EGTA, absolute concentrations could be 4-5-fold smaller but still in a range in which the activities of Ca$^{2+}$/calmodulin-dependent enzymes are modulated.

The metabolic changes allowing for increased [Ca$^{2+}$]$_i$ are transient for sperm in ASW. Within 50 s [Ca$^{2+}$]$_i$ returns to a level determined by [Ca$^{2+}$]$_e$ and the ability of the sperm to maintain a substantial Ca$^{2+}$ gradient across the plasma membrane. The requirement of millimolar [Ca$^{2+}$]$_e$, for the speract-stimulated [Ca$^{2+}$]$_i$ increase suggests that the additional intracellular Ca$^{2+}$ is derived from the external Ca$^{2+}$ source. This rationale is in accord with the fact that sperm contain no intracellular vesicular system from which Ca$^{2+}$ might be released, except the mitochondrion, and uncoupling drugs such as 2,4-dinitrophenol and carbonyl cyanide p-chlorophenylhydrazone do not significantly alter the [Ca$^{2+}$]$_i$ transient stimulated by speract if the incubation times are kept reasonably short. In Ca$^{2+}$-free seawater (CaFASW), speract causes a decrease in [Ca$^{2+}$]$_i$. Additionally, if sufficient EGTA is added to chelate nearly all seawater Ca$^{2+}$ (5 mM Ca$^{2+}$, 6 mM EGTA) within seconds after a large increase in indo-1 fluorescence has been initiated (e.g. egg jelly or speract + MIX), the fluorescence increase stops and relaxes back to the original level. Hence, our data suggest that speract stimulates rapid but transient Ca$^{2+}$ entry across the sperm plasma membrane.

Two plausible mechanisms by which Ca$^{2+}$ entry might be stimulated include: (a) activation of a Ca$^{2+}$ channel; or (b) increased [Na$^+$], coupled to activity of a Na$^+/Ca^{2+}$ exchange. Since neither verapamil nor nimodipine at 50 μM have any effect on the speract-induced transient, Ca$^{2+}$ entry does not occur through a channel sensitive to these antagonists. Likewise, the [Ca$^{2+}$]$_i$ increase here occurs over seconds, a much slower time frame than that associated with electrically active Ca$^{2+}$ channels blocked by these antagonists (41).

The alternative, that increased [Ca$^{2+}$]$_i$, is coupled to Na$^+$ entry, is supported by the following data: (i) monensin increases Na$^+$ entry (20) and [Ca$^{2+}$], (Fig. 9); (ii) MIX allows for a large permanent increase in [Na$^+$], and [Ca$^{2+}$], (Figs. 6 and 8); (iii) Na$^+$ (but not Li$^+$) entry into sperm in NaFASW through Na$^+/H^+$ exchange allows for increased [Ca$^{2+}$], (Table III; Fig. 12); and (iv) inhibition of Na$^+$ entry through the Na$^+/H^+$ exchange by membrane potential depolarization (increased seawater K$^+$) prevents Ca$^{2+}$ entry, but Ca$^{2+}$ entry is not inhibited when the inhibitor is added after Na$^+$ entry has already occurred (Figs. 11 and 12). Statement iv predicts that with each method of stimulating Ca$^{2+}$ entry, the rise in Na$^+$ should precede the change in [Ca$^{2+}$]. Although we cannot follow [Na$^+$] continuously, the pH$_i$ increase stimulated by speract, speract + MIX (Fig. 7), or by Na$^+$ addition to sperm in NaFASW (not shown) does precede the rise in [Ca$^{2+}$] as predicted. This hypothesis is further supported by the time course in Fig. 12 showing that Ca$^{2+}$ entry is more rapid in the presence of Na$^+$ (Fig. 12, b compared to c). Speract may initially activate Na$^+/H^+$ exchange and by virtue of partially collapsing the trans-plasma membrane Na$^+$ gradient increase cytosolic Ca$^{2+}$. In normal seawater, [Na$^+$]/[Na$^+$], ~3 (27), this gradient could partially be responsible for maintaining
low cytosolic [Ca\textsuperscript{2+}]. Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange has been reported in plasma membrane vesicles derived from bovine sperm (45).

However, in each of the above examples where Na\textsuperscript{+} entry can be initiated prior to Ca\textsuperscript{2+} addition (with monensin, speract + MIX, or with Na\textsuperscript{+} addition to sperm in NaFASW) both the [Na\textsuperscript{+}], increase and the [Ca\textsuperscript{2+}], increase are permanent. The temporal relationship between [Na\textsuperscript{+}] and [Ca\textsuperscript{2+}] has yet to be directly determined with speract in the absence of MIX because of the small increase in [Na\textsuperscript{+}] (Fig. 8) and the short time duration. It remains possible that speract activation of Na\textsuperscript{+}/H\textsuperscript{+} exchange and/or alteration of cyclic nucleotides activates a Ca\textsuperscript{2+} channel insensitive to dihydropyridines or verapamil. Such a channel might also transport Na\textsuperscript{+}.

Our data demonstrate that increased pH\textsubscript{i} alone is insufficient to allow for the [Ca\textsuperscript{2+}], increase and may actually activate a Ca\textsuperscript{2+} removal mechanism under some conditions. In the absence of substantial extracellular Ca\textsuperscript{2+} (~20 mM), speract increases pH\textsubscript{i}, but actually decreases [Ca\textsuperscript{2+}], (see Fig. 2A, e). Other methods of increasing pH\textsubscript{i} (Li\textsuperscript{+} or NH\textsubscript{4}\textsuperscript{+} addition to sperm in NaFASW) also decreased [Ca\textsuperscript{2+}],. The fact that Li\textsuperscript{+} increases pH\textsubscript{i} by passing through the Na\textsuperscript{+}/H\textsuperscript{+} exchange suggests that increased [Ca\textsuperscript{2+}], is not tightly linked to movement of ions through this exchange system.

The data of Ward et al. (24) show that the peptide resact causes Ca\textsuperscript{2+}-dependent chemotaxis of the sperm from the sea urchin A. punctulata. Our data suggest that, if speract and resact function analogously in their respective species, a transient rise in [Ca\textsuperscript{2+}], might act to mediate the swimming behavior to allow for the attraction response. Brokaw and Nagayama (16) have demonstrated in permeant sperm models that very low [Ca\textsuperscript{2+}], and calmodulin can affect flagellar bending, making the waveform more asymmetric, a means by which swimming can be localized.

Data presented here also show that speract with MIX stimulates large increases in [Na\textsuperscript{+}], and [Ca\textsuperscript{2+}], similar to those initiated by the high molecular weight fucosulfate polymer responsible for inducing the acrosome reaction (6, 46). Consistent with this similarity is the ability of ~30 mM K\textsuperscript{+} to block both the speract responses shown here and the egg jelly-induced acrosome reactions as previously reported (7). However, resact inhibits egg jelly triggering of the reaction and the corresponding increase in [Ca\textsuperscript{2+}], but is ineffective at inhibiting the changes stimulated by MIX plus speract. Also, Na\textsuperscript{+} entry induced by speract plus MIX, unlike that induced by egg jelly, is not dependent upon extracellular Ca\textsuperscript{2+}.

Thus, speract, acting through its receptor, is capable of producing a Ca\textsuperscript{2+} removal mechanism under some conditions. In the absence of substantial extracellular Ca\textsuperscript{2+} (~20 mM), Na\textsuperscript{+} entry and the pHi increase (20) which we have shown here to allow for activation of Ca\textsuperscript{2+} entry and removal.

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