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Membrane potential depolarization and increased intracellular pH accompany the acrosome reaction of sea urchin sperm

(amine accumulation/tetraphenylphosphonium accumulation/thiocyanate exclusion/fluorescent tracers/fertilization)

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ABSTRACT **The intracellular pH and membrane potential in sperm of the sea urchin *Strongylocentrotus purpuratus* were investigated by using fluorescent and radiolabeled probes. The weak bases [^{14}C]methylamine, [^{14}C]diethylamine, and 9-aminoacridine were concentrated within sperm 5-fold or greater. The weak acid [^{14}C]dimethyloxazolidine-2,4-dione (DMO) was excluded from sperm. These data suggested that the apparent intracellular pH is acidic with respect to seawater (pH 8.0). Induction of the acrosome reaction caused efflux of the amines and uptake of DMO, consistent with an increase in apparent intracellular pH of 0.1–0.2 pH unit. The presence of an internally negative membrane potential was indicated by estimating the distribution of [^{3}H]tetraphenylphosphonium (Ph_4P^+) and [^{14}C]SCN^-. From SCN^- exclusion we estimated a value of about −30 mV for the nonmitochondrial membrane potential, whereas from Ph_4P^+ accumulation an apparent potential of −90 to −150 mV was demonstrated. The membrane potentials obtained with Ph_4P^+ and SCN^- were dependent upon the external K^+ concentration, with increasing K^+ leading to depolarization. Induction of the acrosome reaction led to efflux of Ph_4P^+ and uptake of SCN^- for an approximate depolarization of about 30 mV, primarily due to the collapse of the plasma membrane potential.**

In many cell types intracellular activities are regulated by environmental effectors. The acrosome reaction of sea urchin sperm is an interesting example of such a response: within seconds after encountering egg "jelly" (a component of the egg coat) sperm undergo exocytosis from an apical vesicle and assemble an actin-containing process (reviewed in refs. 1 and 2). That these prerequisites for sperm–egg fusion are modulated by ions of the extracellular milieu, seawater, has been recognized since the pioneering work of Dan (3, 4).

External Ca^{2+} is required for the acrosome reaction (4, 5) and Ca^{2+} influx accompanies it (6). Inhibitors of Ca^{2+} channels or antagonists of Ca^{2+} binding inhibit the reaction (6–8). Addition of egg jelly to sperm induces Na^+ influx and acid efflux, changes that could increase the intracellular pH and thereby control the acrosome reaction (9, 10) and other aspects of sperm activation, such as respiration and motility. Egg jelly also causes K^+ efflux (9) and might thereby alter the plasma membrane potential. That changes in membrane potential might regulate the acrosome reaction was suggested by the observations that either tetraethylammonium (Et_4N^+), a drug that blocks K^+ channels, or slight increases in the seawater K^+ concentration inhibit the reaction (6, 9).

We examined the possibility that egg jelly induces the acrosome reaction by altering the intracellular pH and membrane potential. Because sea urchin sperm are too small to be analyzed by insertion of microelectrodes, we used pH- and membrane potential-dependent radiolabeled and fluorescent tracers, as employed in other cellular and vesicular systems (11–17). For estimates of intracellular pH we used the weak bases [^{14}C]methylamine ($MeNH_2$), [^{14}C]diethylamine (Et_2NH), and 9-aminoacridine (9AA) and the weak acid [^{14}C]dimethyloxazolidine-2,4-dione (DMO). The membrane potential was estimated with the lipid-soluble ions [^{3}H]tetraphenylphosophonium (Ph_4P^+) and [^{14}C]SCN^-. We found that the acrosome reaction is associated with a decrease in a K^+-dependent membrane potential and an increase of the sperm internal pH.*

MATERIALS AND METHODS

The preparation of artificial seawater (ASW) and egg jelly, the isolation of sperm from *Strongylocentrotus purpuratus*, and the induction of the acrosome reaction were as described (6, 9) except where changes are indicated in the figure legends. The ASW was composed of 50 mM $MgCl_2$, 10 mM KCl, 10 mM $CaCl_2$, 360 mM NaCl, and either 10 mM Tris with 5 mM Hepes or 30 mM Hepes at pH 8.0. Experiments were performed at 10°C unless otherwise noted.

Uptake of Radiolabeled Compounds. Undiluted "dry" sperm (2–6 × 10^{10} sperm per ml) were diluted 1:100 into ASW and incubated with 2–4 μM [^{14}C]Et_2HN, [^{14}C]$MeNH_2$, or [^{14}C]DMO or 1–20 μM [^{3}H]Ph_4P^+ as described in each figure legend. For each time point, sperm were separated from the incubation medium by either of two protocols. In one, duplicate samples (200 μl) were centrifuged through silicone oil [General Electric Versilube F-50 or Dow Corning 704 with 5–10% (vol/vol) cyclohexane added to adjust the density] in an Eppendorf microcentrifuge at full speed for 30–45 sec (19). The supernatant solution was then removed, the bottom of the tube was cut off, the pellet was disrupted with 25 μl of concentrated formic acid, and this sample was placed in 10 ml of Aquasol (New England Nuclear) for liquid scintillation counting. Alternatively, for analysis of the distribution of Ph_4P^+ and $MeNH_2$, duplicate samples were filtered through glass fiber filters (Whatman GF/C). The filters were rapidly washed twice with cold seawater (4 ml) and placed in 10 ml of Aquasol (New England Nuclear). The filtration technique gave lower absolute results for uptake.

Uptake and Efflux of 9AA. 9AA is a fluorescent amine that is quenched when it accumulates in cells (20). Fluorescence as monitored on a Perkin–Elmer MPF44A fluorescence spectrophotometer (382-nm excitation, 454-nm emission) was nearly proportional to 9AA concentration between 0.25 and 12.5 μM.

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Abbreviations: $MeNH_2$, methylamine; Et_2NH, diethylamine; 9AA, 9-aminoacridine; DMO, dimethyloxazolidine-2,4-dione; Ph_4P^+, tetraphenylphosphonium; ASW, artificial seawater; Et_4N^+, tetraethylammonium.

* Some of these data have been presented at the meeting of the American Society for Cell Biology, November 1980 (ref. 18).

It was determined that nearly complete quenching of fluorescence occurred with accumulation of the dye, because removal of the cells (by centrifugation) caused less than 10% change in the signal.

Data Analysis. Weak acids and bases used as probes of intracellular pH are thought to equilibrate rapidly across membranes in their uncharged form (20). The total accumulation is then related to the pH differential across a membrane according to:

$$\frac{[H^+]_{ext}}{[H^+]_{int}} = \frac{[amine^+]_{ext}}{[amine^+]_{int}} = \frac{[DMO^-]_{int}}{[DMO^-]_{ext}},$$

in which ext and int indicate external and internal, provided that the pH of the incubation medium is at least two pH units lower than the pK of the amines or two units higher than the pK for DMO (14, 21), as in these experiments. Lipophilic probe molecules used to measure membrane potentials have a fixed charge and accumulate across membranes in accord with the potential, as calculated by:

$$\Delta\psi = \frac{-RT}{F}\ln\left(\frac{[TPP^+]_{int}}{[TPP^+]_{ext}}\right) = \frac{RT}{F}\ln\left(\frac{[SCN^-]_{int}}{[SCN^-]_{ext}}\right),$$

in which R is the gas constant, T is absolute temperature, and F is the Faraday constant.

For determining the internal concentration of isotopes, the total and extracellular H_2O spaces in the cell pellet were estimated for identical sperm suspensions with ^3H_2O or [^{14}C]inulin, respectively. As an alternative to inulin, [^{14}C]sucrose was used in several experiments and gave similar values. The intracellular water space (V_i) was calculated as the difference between the total water space in the pellet (V_t) and the external water space (V_e), as follows:

$$V_e = \frac{\text{cpm } [^{14}\text{C}]\text{inulin in pellet}}{\text{cpm } [^{14}\text{C}]\text{inulin}/\mu\text{l supernatant}}$$

$$V_t = \frac{\text{cpm } ^3H_2O \text{ in pellet}}{\text{cpm } ^3H_2O/\mu\text{l supernatant.}}$$

Typical values were $V_e = 0.2\ \mu l$, $V_t = 0.9\ \mu l$, $V_i = 0.7\ \mu l$ when the pellets contained 10^8 sperm and the external K^+ concentration was 10 mM. The values for V_i increased from 1.0 to 1.4 μl as the external K^+ increased from 60 to 180 mM.

The accumulation ratios (A_r) were calculated as follows:

$$A_r = \frac{\text{cpm probe in pellet} - [V_e \times (\text{cpm probe}/\mu\text{l supernatant})]}{(\text{cpm probe}/\mu\text{l supernatant}) \times (V_i)}.$$

The apparent pH_{int} is then $pH_{ext} - \log A_r$ for amines, or $pH_{ext} + \log A_r$ for DMO. The apparent $\Delta\psi$ is $(-RT/F)\ln A_r$ for Ph_4P^+ or $(+RT/F)\ln A_r$ for SCN^-. Both acids and bases were used in estimates of internal pH, and cations and anions were used in estimates of $\Delta\psi$, in order to examine whether the data reflected binding of a specific ionic species rather than accumulation in response to the gradient of interest. All A_r values were calculated without attempts to correct for binding.

Chemicals. Radioisotopically labeled compounds other than [^{3}H]Ph_4P^+ were from New England Nuclear or ICN. [^{3}H]Ph_4P^+ was a gift from H. R. Kaback of the Roche Institute of Molecular Biology. 9AA was from Sigma. All other chemicals were commercially available reagent grade.

RESULTS

Internal pH of the Sperm. The results of incubating sperm in [^{14}C]$MeNH_2$, [^{14}C]Et_2NH, and [^{14}C]DMO are shown in Fig.

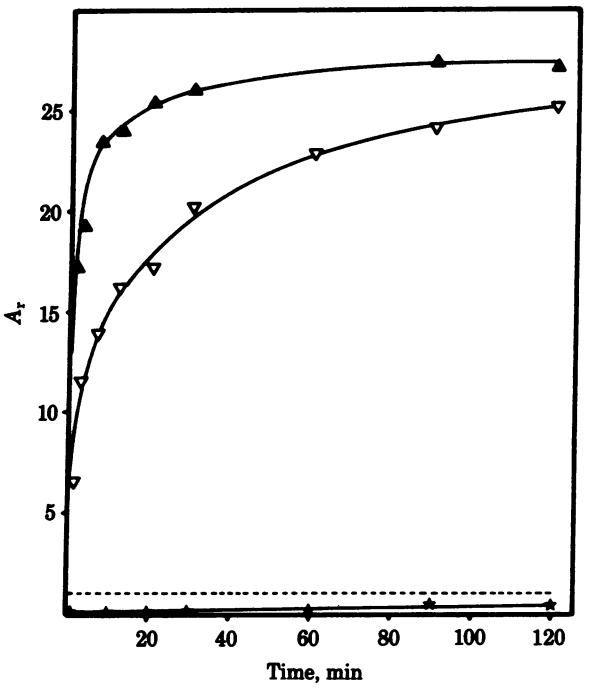

FIG. 1. A_r as a function of time. Dry sperm were diluted 1:100 in ASW on ice. [^{14}C]$MeNH_2$ (55 mCi/ml; ▽) and [^{14}C]Et_2NH (48 mCi/mmol; ▲) were added to give final radioactivities of 130 and 190 cpm/μl, respectively (1 Ci = 3.7×10^{10} becquerels). [^{14}C]DMO (45 mCi/ml; ★) was added to 140 cpm/μl. Accumulation of the radioisotopes was measured by centrifugation. The broken line indicates an A_r of 1 (i.e., the size of the intracellular water space).

1. Both radioactive amines accumulated in this experiment approximately 25-fold, although equilibrium was reached more rapidly with [^{14}C]Et_2NH. In contrast, the weak acid [^{14}C]DMO was excluded from sperm. These results suggest that sperm have an acidic intracellular pH. The accumulation ratio obtained in any experiment depended upon the batch of sperm that was used, and varied between 5 and 25 for the radioactive amines and between 0.3 and 0.9 for [^{14}C]DMO.

Addition of egg jelly to sperm triggers the acrosome reaction and acid efflux in less than 1 min (6, 9). When jelly was added to sperm after amine accumulation had nearly reached a steady state (Fig. 2), amine efflux ensued to reach a new level within 1 min. Thus, the apparent intracellular pH of the sperm became less acidic after induction of the acrosome reaction. The rate of efflux of weak bases from cells should be dependent upon the

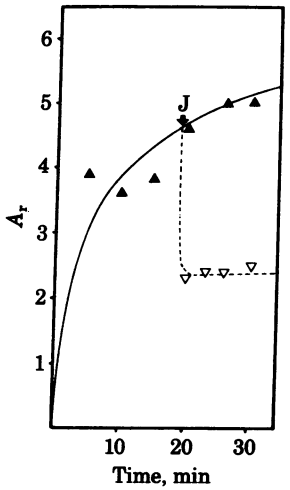

FIG. 2. Egg jelly-induced efflux of accumulated Et_2NH. Dry sperm were diluted 1:100 in ASW at 10°C. [^{14}C]Et_2NH was then added to give a final radioactivity of 100 cpm/μl, and uptake was followed with time (▲). After 19 min of incubation the solution was divided into two tubes and jelly was added to one (arrow) (200 μl of concentrated jelly to 3 ml of solution to give a final concentration of 2.9 μg of fucose equivalents/ml) (▽). Ninety-five percent of the sperm underwent the acrosome reaction.

concentration of the unprotonated species, which is a function of the intracellular pH (20). Egg jelly increased the rate of efflux of [^{14}C]$MeNH_2$ (Fig. 3) and induced the acrosome reaction. An alternative way of alkalinizing the sperm interior without triggering the acrosome reaction is to add a relatively high concentration of a weak base, such as ammonia. As expected, 25 mM NH_4^+ increased the rate of efflux of [^{14}C]$MeNH_2$ from sperm that had been preincubated with the labeled amine (Fig. 3).

Another means of estimating intracellular pH relies on quenching of the fluorescence of the weak base 9AA (20). Fig. 4 shows that a gradual decrease in 9AA fluorescence occurred when the dye was incubated with sperm. The steady state, or equilibrium level of fluorescence was the same whether sperm were preincubated with 9AA at higher concentrations before dilution or diluted immediately into 9AA. The steady-state level of 9AA accumulation decreased upon addition of jelly, as indicated by an increased fluorescence. This supports the inference of increasing alkalinization upon induction of the acrosome reaction. The A_r with 9AA was routinely greater than 1000, two orders of magnitude higher than for Et_2NH or $MeNH_2$. This discrepancy probably results from intracellular binding of the acridine (22). Consistent with this hypothesis is the finding (Fig. 4) that disruption of cell membranes with 0.04% Triton X-100 released only ≈35% of the accumulated 9AA. The same level of binding was reached immediately upon mixing sperm and 9AA in the presence of Triton X-100. Nigericin under conditions that should dissipate the pH gradient (23) caused efflux of 9AA to nearly the same steady-state level as did Triton X-100 (data not shown). Thus, 9AA accumulation is a complex function of transmembrane pH gradients and intracellular binding, and the two effects can be analyzed quantitatively (unpublished results).

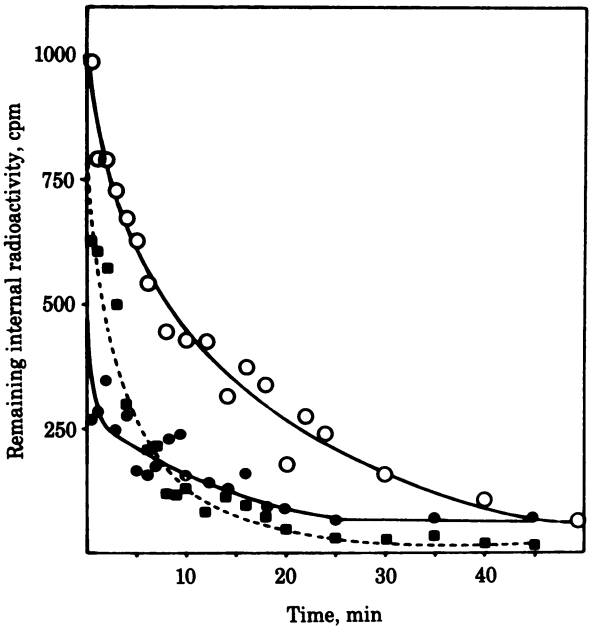

FIG. 3. Efflux of $MeNH_2$ induced by egg jelly or NH_4^+. "Dry" (50 μl) sperm was diluted with 150 μl of solution of [^{14}C]$MeNH_2$ in ASW. After 25 min of incubation on ice the sperm were diluted into 10 ml of ASW (○) or ASW containing 25 mM NH_4^+ (■), and efflux was measured by filtration. For initiation of the acrosome reaction, sperm were first diluted in 5 ml of ASW with jelly added at 4.7 μg of fucose equivalents/ml for 20 sec and then 10 ml of ASW was added (●); 80% of the sperm underwent the acrosome reaction. In all curves the first measurement was made 1 min after the initial dilution.

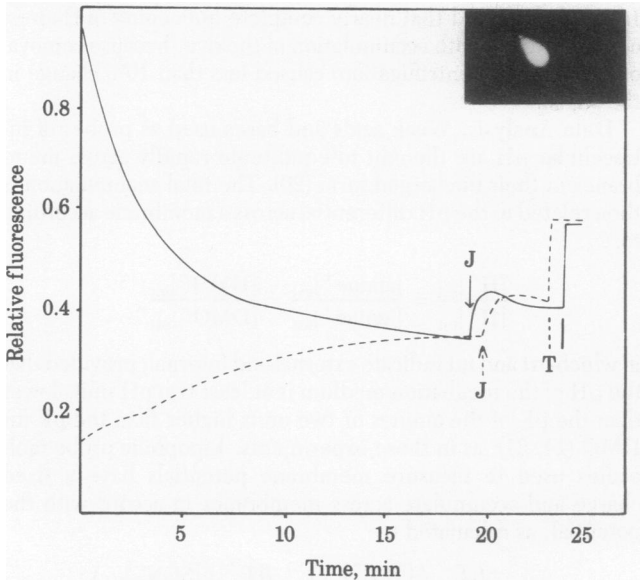

FIG. 4. Uptake and efflux of 9AA. For uptake, sperm (1.6×10^8 per ml, final concentration) were added at zero time to 0.25 μM 9AA in ASW. The fluorescence of the suspension decreased with time, to a plateau (——). Sperm preincubated on ice with 9AA at 100-fold higher concentrations of both sperm and 9AA and then diluted 1:100 released the fluorescent amine and reached nearly the same plateau value (----). J designates the addition of egg jelly (3.6 μg of fucose equivalents/ml, final concentration) to trigger the acrosome reaction. T designates addition of Triton X-100 to a final concentration of 0.04%. (*Inset*) Fluorescence micrograph of a sea urchin sperm after incubation in 25 μM 9AA, showing the localization of fluorescence in the head and acrosomal regions.

One advantage of 9AA is that the intracellular location of the reagent can be monitored by the residual fluorescence seen in fluorescence micrographs (Fig. 4 *Inset*). Both the region of the head over the nucleus and a zone around the acrosomal granule are prominently labeled. After induction of the acrosome reaction, fluorescence remained over the nucleus, but none was found in the acrosomal region.

Membrane Potential of Sea Urchin Sperm. The lipid-soluble cation [^{3}H]Ph_4P^+ accumulated in sperm slowly (Fig. 5), reaching equilibrium only after 3–6 hr. Addition of Ph_4P^+ to sperm that had been incubated in ASW for 3 hr showed the same uptake kinetics (data not shown), suggesting that the slow accumulation is not dependent upon a slowly developing membrane potential but reflects the slow diffusion of Ph_4P^+ across the sperm membrane. Addition of the lipid-soluble anion tetraphenylboron (1 μM) did not increase the rate of [^{3}H]Ph_4P^+ uptake. A_r values of 50–250 for [^{3}H]Ph_4P^+ were generally found in ASW containing 10 mM K^+ and varied with the batch of sperm. As the K^+ concentration was increased, accumulation of Ph_4P^+ was reduced (Fig. 5). In contrast, the lipid-soluble anion [^{14}C]SCN^- was excluded from sperm (Fig. 6). Increasing the extracellular K^+ concentration decreased the exclusion of [^{14}C]SCN^-. The results with both reagents suggest that a K^+-dependent, internally negative membrane potential exists in sperm. Addition of jelly to trigger the acrosome reaction caused a rapid depolarization of this potential, as measured either by an accumulation of [^{14}C]SCN^- (Fig. 6) or by a loss of [^{3}H]Ph_4P^+ (Table 1). The rapid change in SCN^- uptake (Fig. 6) and Ph_4P^+ efflux, which was completed by 10 min (data not shown), suggests that a general permeability change occurs upon induction of the acrosome reaction as previously suggested (6, 9).

Inhibitors of the Acrosome Reaction. Verapamil and Et_4N^+ inhibit the acrosome reaction (6, 9), yet partial alterations in the

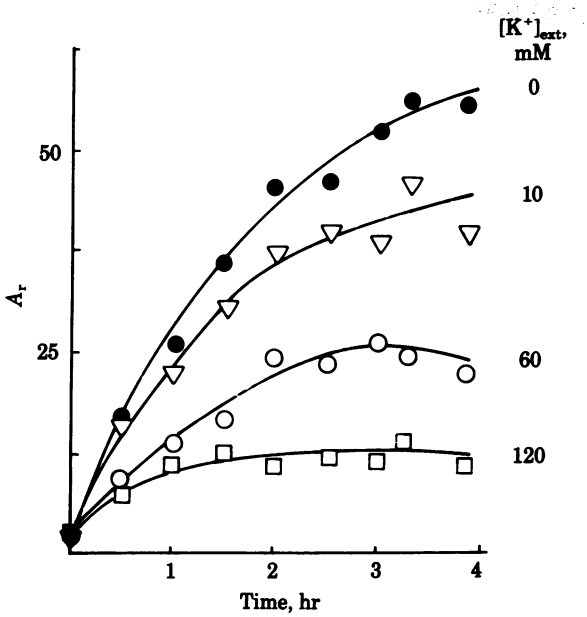

FIG. 5. Accumulation of Ph_4P^+ as a function of the extracellular K^+ concentration. Sperm (4×10^8 per ml) were incubated in ASW of various K^+ concentrations (K^+ substituted on a 1:1 molar basis for Na^+) containing 20 μM Ph_4P^+ with [^{3}H]Ph_4P^+ at 0.3 μCi/ml. At given times 200-μl aliquots were filtered on GF/C filters and rapidly washed two times with ice-cold seawater, and radioactivity was measured. A_r was calculated by using intracellular water space values determined by centrifugation on identically treated sperm samples.

apparent membrane potential and internal pH still occur. For example, when jelly was added to sperm, the A_r for [^{3}H]Ph_4P^+ went from 133 to 38 after 10 min; with jelly added in the presence of 5 mM Et_4N^+, the A_r went to 100, and in 40 μg/ml verapamil it was 110. Similarly, 5 min after jelly addition, the A_r for [^{14}C]Et_2NH went from 4.6 to 2.6; in 5 mM Et_4N^+ it decreased

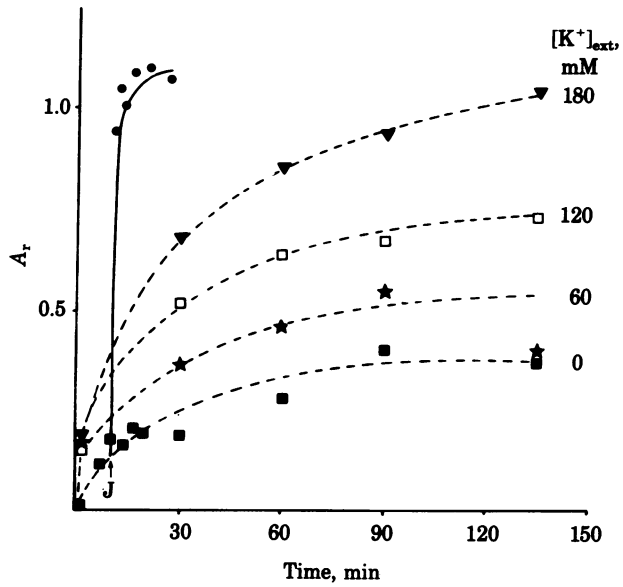

FIG. 6. Accumulation of [^{14}C]SCN^- as a function of extracellular K^+ concentration. Sperm (7×10^8 per ml, final concentration) were added to ASW of various K^+ concentrations (1:1 molar substitution for Na^+) in the presence of [^{14}C]SCN^- (72 μM, 1.1 μCi/ml). At various times 200-μl aliquots were centrifuged through silicone oil. Egg jelly was added (4.7 μg of fucose equivalents/ml) to the sperm in 0 mM K^+ at 10 min (J) to trigger the acrosome reaction (60% of sperm reacted) and [^{14}C]SCN^- uptake was measured (●).

Table 1. Changes in tracer accumulation upon triggering the acrosome reaction

Measurement	Tracer used	A_r Without jelly	A_r With jelly	$\Delta\psi$, mV or ΔpH
Membrane potential	[^{14}C]SCN^-	0.25	1.0	+34 ± 2
	[^{3}H]Ph_4P^+	220	55	+32 ± 4
pH	[^{14}C]$MeNH_2$	8.6	5.5	0.18 ± 0.02
	[^{14}C]Et_2NH	5.2	3.3	0.22 ± 0.06
	[^{14}C]DMO	0.63	0.87	0.12 ± 0.03
	9AA	2200	1500	0.15 ± 0.02

Sperm were added to ASW containing Ca^{2+} and incubated to allow for accumulation of the probes to an equilibrium level. Egg jelly (1–5 μg of fucose equivalents/ml, final concentration) was added to induce the acrosome reaction (over 70% of the cells reacted). Values given for with jelly were obtained for [^{14}C]Et_2NH, 9AA, [^{14}C]$MeNH_2$, and [^{14}C]DMO at 1 min and for [^{14}C]SCN^- and [^{3}H]Ph_4P^+ at 10 min. Each value represents the mean of two to four experiments on different sperm samples with points taken in duplicate or triplicate for each experiment. $\Delta\psi$ and ΔpH means are given ± SEM. Cells were isolated by centrifugation through silicone oil. In the case of [^{3}H]Ph_4P^+ 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was included in the ASW to maintain sperm viability.

to 3.2 and in 40 μg/ml verapamil, to 3.3. Thus, some of the depolarization and alkalinization of sperm after addition of jelly is not dependent upon exocytosis or acrosomal filament extension.

DISCUSSION

From the distribution of the radiolabeled and fluorescent probes used in this study, we conclude: (*i*) sperm have an internal compartment that is acidic with respect to seawater; (*ii*) sperm have a negative internal membrane potential that is regulated by the external K^+ concentration; and (*iii*) induction of the acrosomal reaction results in an increase in the apparent internal pH and in a net depolarization of the membrane potential.

Using our values for the internal water space (*Materials and Methods*), we can calculate the apparent intracellular pH and membrane potential for the sperm. Such calculations are dependent upon assumptions concerning the number and the sizes of the compartments involved in accumulating the probes. These quantitative estimates are nonetheless useful, for they provide a common basis for discussion of differences found in the accumulation of acids and bases and cations and anions. As discussed below, such considerations suggest that more than one internal compartment contributes to the results found with probes of both the pH and the membrane potential.

The uptake of the radiolabeled amines to A_r values of 5–25 suggests the average internal pH of the entire sperm to be between 7.3 and 6.6, in ASW of pH 8.0. The pH values determined with [^{14}C]DMO are somewhat higher, between pH 7.5 and 7.9. The estimate of the change in intracellular pH upon triggering the acrosome reaction was slightly greater with [^{14}C]Et_3NH or [^{14}C]$MeNH_2$ (Table 1) than with [^{14}C]DMO. These discrepancies could be caused by differential binding of the anion or cation to cellular sites. They might also reflect the existence of several intracellular compartments of different pH that respond differently to induction of the acrosome reaction. For example, the weak acid DMO would not accumulate in acidic regions that concentrate amines. One such acidic compartment is around the acrosomal vesicle (Fig. 4 *Inset*). Upon induction of exocytosis, this acidic compartment disappears, which could account for some of the decreased accumulation

of amines (Figs. 2 and 4) and the increased rate of [^{14}C]$MeNH_2$ efflux (Fig. 2). However, the slight increase in [^{14}C]DMO accumulation with induction of the reaction (Table 1) suggests that an internal compartment also increased in pH; likewise, partial alkalinization was seen even with inhibitors of exocytosis. This might in part occur by an increase in the pH of the mitochondrion, a typically basic intracellular compartment (17, 24).

The probes of membrane potential may also accumulate in different intracellular compartments, depending upon their charge and binding characteristics. [^{3}H]Ph_4P^+, a cation, should accumulate in response to both the negative plasma membrane and mitochondrial potentials, as discussed previously (11, 17). In contrast, [^{14}C]SCN^- should be almost totally excluded from regions with extremely negative potentials, such as the mitochondrion. Thus, [^{14}C]SCN^- should give an index of the nonmitochondrial potential (16, 17), which is probably dominated by that of the plasma membrane. Values for the apparent membrane potential determined by [^{14}C]SCN^- uptake ranged between -10 and -45 mV (internally negative), depending upon the batch of sperm. The values for the apparent membrane potential determined by [^{3}H]Ph_4P^+ accumulation were much higher, -90 to -150 mV. The difference probably represents the mitochondrial potential.

These data are of interest in that they indicate relatively large and K^+-dependent membrane potentials in sperm. Earlier examinations of mammalian sperm showed either a small negative potential of -8 mV in the bull (25) or a slightly positive potential in the guinea pig (26). A recent brief report using the probe triphenylmethylphosphonium on sperm of a different sea urchin found a K^+-dependent potential of -20 mV, but details of the methods were not given. (27). Lee *et al.* (28) have recently obtained values comparable to ours for uptake of Ph_4P^+. The addition of egg jelly to stimulate the acrosome reaction resulted in depolarization of the membrane potential (Fig. 6, Table 1) that can largely be accounted for by a decrease in the nonmitochondrial membrane potential, because the change found with [^{14}C]SCN^- was nearly as large as that for [^{3}H]Ph_4P^+ (Table 1).

Although problems exist in assigning specific compartments of a complex cell to different roles in accumulating the probes, certain results are clear. Sperm are excitable cells that respond to external stimuli with permeability changes (6, 9) that affect the intracellular pH and membrane potential. The regulation of cellular activities by ionic fluxes and gradients is a quite general phenomenon. It is central to chemosmotic mechanisms, in which proton gradients and membrane potentials are coupled to ATP synthesis, molecular transport, or both in mitochondria, bacteria, and chloroplasts (12, 29, 30). Other complex cellular phenomena such as responses to peptide hormones (31, 32), mitogenic stimulation of lymphocytes (33), and assembly of membrane proteins (34) may respond to altered membrane potentials or pH. Increased intracellular pH appears to activate protein synthesis after fertilization (35, 36). We have shown that a similar regulation of ionic permeability in response to a component of the egg surface accompanies the events that prepare the sperm for its biological function, fertilization.

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