Monoclonal antibodies increase intracellular Ca\(^{2+}\) in sea urchin spermatozoa  

(fertilization/acrosm reaction/ion fluxes/fura-2/sperm surface antigen)  

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ABSTRACT Changes in intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) of sea urchin (*Strongylocentrotus purpuratus*) spermatozoa were measured using the fluorescent Ca\(^{2+}\) indicators fura-2 and indo-1. The intracellular pH (pH\(_i\)) of sperm was also determined. The fucose sulfate-rich glycoconjugate component of egg jelly induced increases in [Ca\(^{2+}\)]\(_i\) and pH\(_i\) in sperm and induced the acrosome reaction. Monoclonal antibodies (mAbs) to external domains of a 210-kDa glycoprotein of the sperm plasma membrane induced a 23-fold increase in [Ca\(^{2+}\)]\(_i\) (vs. 9-fold for fucose sulfate-rich glycoconjugate), but the mAbs did not cause the pH\(_i\) to increase and did not induce the acrosome reaction. When the mAb treatment which induced an increase in [Ca\(^{2+}\)]\(_i\), was combined with an NH\(_4\)Cl treatment, which increased the pH\(_i\), the acrosome reaction was induced. mAb-induced increases in [Ca\(^{2+}\)]\(_i\) were dependent on millimolar concentrations of extracellular Ca\(^{2+}\) and were reversed by placing sperm in Ca\(^{2+}\)-free seawater or by chelating Ca\(^{2+}\) with EGTA. The mAb-induced [Ca\(^{2+}\)]\(_i\) increase was sensitive to the pH of the seawater, although mAb binding was not the data show that increased [Ca\(^{2+}\)]\(_i\) and pH\(_i\) are necessary for induction of the acrosome reaction and suggest that the 210-kDa protein may play a role in regulating Ca\(^{2+}\) entry into the spermatozoa. These mAbs make it possible to separate the increase in [Ca\(^{2+}\)]\(_i\) from the increase in pH\(_i\) and may be useful in the elucidation of the regulatory role of Ca\(^{2+}\) in sperm physiology.

Increases in both cytoplasmic free Ca\(^{2+}\) ([Ca\(^{2+}\)] \(_c\)) and pH (pH\(_c\)) underlie activation of cellular activities such as mitogenesis (1) and fertilization (2). The activation of sea urchin spermatozoa at fertilization consists of the following two major events: (i) initiation of motility and respiration resulting from an increase in pH\(_c\) of about 0.4 unit to pH\(_c\) 7.4 (3-7) and (ii) the exocytotic acrosome reaction (AR), occurring when the sperm encounters the jelly layer surrounding the egg (8). An ethanol-insoluble component of egg jelly, which is a large fucose sulfate-rich glycoconjugate (FSG), possesses the AR-inducing activity (9-11). Induction of the AR by FSG is dependent on millimolar concentrations of external Ca\(^{2+}\) ([Ca\(^{2+}\)] \(_e\)) and an additional increase in pH\(_c\) to \(\approx 7.6\) (5, 6, 12-15). The AR can also be induced by ionophores or alteration of the ion concentration of seawater to cause both the entrance of Ca\(^{2+}\) and the elevation of pH\(_c\) (12, 13).

The new fluorescent Ca\(^{2+}\) indicators indo-1 and fura-2 (16) make possible the continuous measurement of changes in [Ca\(^{2+}\)]\(_c\) during sperm activation (17). Before these probes were available the only method to measure Ca\(^{2+}\) influx in sperm was by 4\(^{2+}\)Ca\(^{2+}\) uptake using a filtration assay (18). After induction of the AR by FSG (or unfractionated egg jelly) 4\(^{2+}\)Ca\(^{2+}\) uptake is primarily (85%) into the sperm mitochondrion and may last for 20 min (12, 13). This long-duration 4\(^{2+}\)Ca\(^{2+}\) uptake is blocked by cyanide and carbonyl cyanide, p-trifluoromethylphenylhydrazulone; however, these mitochondrial poisons do not block the FSG-induced AR (12, 13). X-ray microanalysis localizes the long duration, post-AR accumulation of Ca\(^{2+}\) to the mitochondrion (19).

In this paper we show that the Ca\(^{2+}\)-indicating dyes detect increases in [Ca\(^{2+}\)], within seconds after FSG addition to sperm. These increases are insensitive to mitochondrial poisons and correlate temporally with occurrence of the AR. These new indicators of [Ca\(^{2+}\)], may thus be more accurate probes for measuring changes in [Ca\(^{2+}\)], during sperm activation.

Characterization of monoclonal antibodies (mAbs) to an integral plasma membrane glycoprotein (M, 210,000) of sea urchin spermatozoa has implicated this protein in the regulation of ionic changes associated with the AR (20). We have reported (20) that mAb J10/14 inhibits both the FSG-induced AR and the subsequent long-duration 4\(^{2+}\)Ca\(^{2+}\) accumulation. However, by using the Ca\(^{2+}\) indicators indo-1 and fura-2, we now report that both J10/14 and another mAb to the 210-kDa protein, J18/2, induce rapid increases in sperm [Ca\(^{2+}\)] to levels even greater than those induced by FSG. These mAb-induced increases in sperm [Ca\(^{2+}\)], occur without an increase in pH\(_c\), and the AR is not induced. When the mAb-induced increase in [Ca\(^{2+}\)], is combined with an NH\(_4\)Cl treatment to increase pH\(_c\), the AR is induced. These mAbs to the 210-kDa protein are thus valuable reagents for studying the physiological effects of increases in sperm [Ca\(^{2+}\)], in the absence of an elevation in pH\(_c\).

MATERIALS AND METHODS

Cells and Media. Gametes of*Strongylocentrotus purpuratus* were collected directly from the gonopore and stored undiluted ("dry" sperm); the AR was scored; and egg jelly was obtained as described (20). Externalization of the contents of the acrosome granule was assessed by anti-bindin immunofluorescence (21). The 70% (vol/vol) ethanol-insoluble fraction of egg jelly (FSG) was prepared as described (9, 22). Artificial sea water (ASW), calcium-free ASW, and ASW containing various concentrations of Ca\(^{2+}\), were formulated as described (23), and buffered to pH\(_c\) 7.8 with 10 mM Tris/10 mM Hepes. ASWs of differing pH were prepared and additionally buffered with 10 mM Pipes (23). EGTA was prepared as a 500 mM stock in H\(_2\)O and was adjusted with KOH so that upon addition the pH of ASW changed less than 0.1 pH unit.

Abbreviations: AR, acrosome reaction; ASW, artificial seawater; BCECF, 2',7'-bis-(2-carboxyethyl)-

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mAbs. mAbs J10/14, J4/4, and T8/40 were obtained as described (20). T8/40 is a mouse IgG mAb reacting with a cell surface antigen on the human CCRF CEM cell line (20). Hybridomas producing mAbs J16/18 and J18/2 were obtained by standard procedures (24, 25) from the fusion of spleen cells from a BALB/c mouse immunized with sperm and sperm plasma membranes (26) with the nonproducer myeloma line SP2/0. Hybridoma culture supernatants were screened for binding to intact sperm in ELISA assays and by indirect immunofluorescence as described (20). Biochemical characterization of mAb specificities was by immunoprecipitation of detergent lysates of T121-labeled sperm, followed by analysis on NaDodSO4/PAGE and autoradiography (20). Immunoglobulins were purified from ascites fluid, and Fab fragments were prepared and purified as described (20), except that the purified Fab fragments were passed over a column of goat anti-mouse IgG (Fc specific, Cappel Laboratories, Malvern, PA) conjugated to Sepharose CL-4B.

Determination of [Ca²⁺]i and pHₖ, [Ca²⁺], was determined by use of the fluorescent Ca²⁺ chelators indo-1 and fura-2 as described (17) and as modified below. Dry sperm were diluted 1:10 (4 × 10⁶ cells per ml, 100 μg of sperm protein per ml) in ASW (pH 6.8), and the acetoxyethyl esters of indo-1 or fura-2 were added to a final concentration of 9 μM. After mixing, the cells were incubated for 15 hr on ice (17). Sperm were centrifuged, resuspended to the same volume in fresh ASW (pH 6.8), stored on ice (17), and used from 24 to 48 hr after spawning (17). After this treatment a high percentage of the cells underwent the acrosome reaction when exposed to FSG. The sperm routinely contained ~200 μM dye. Very little unhydrolyzed dye remained in the cells as assessed by sensitivity to Mn²⁺ (27). Nonlabeled F-44 extracts of dye-loaded sperm (17) responded to <1 μM Ca²⁺ when titrated using EGTA buffers. Unhydrolyzed dye in the sperm would lead to lower estimates of [Ca²⁺], but would not affect the interpretation of the data. For the assays, the labeled sperm were diluted an additional 1:10 or 1:20 in ASW, pH 7.8. [Ca²⁺]i was determined from measurements of fluorescence emission for indo-1 at 405 nm (28) or by the method of Grynkiewicz et al. (16) using the ratio method for indo-1 or fura-2. The emission intensity ratio (I₄₄⁰/₃₈⁰) was used for indo-1 with excitation at 355 nm. For fura-2, emission intensity was monitored at 500 nm with excitation at 345 nm and 380 nm at the wavelength pair. Both the methods give qualitatively identical results, although numerical differences suggest that indo-1 and fura-2 may have different Kₐ values for Ca²⁺ in the sperm or that their fluorescent behavior is not identical inside these cells. Intracellular dye fluorescence in the absence of Ca²⁺ was determined by adding ionomycin to sperm in Ca²⁺-free ASW containing <10 nM [Ca²⁺], under conditions that collapse ΔpH and lower intracellular ATP (monensin, nigericin, and oligomycin; refs. 5 and 30). We assumed for our calculations that [Ca²⁺]i equaled [Ca²⁺] after a 10-min incubation in ionomycin, as fluorescence changes reflected a decrease in [Ca²⁺], to a new stable level. This technique must also be considered approximate, since we have no independent method for ascertaining that ionomycin equilibrates [Ca²⁺]i and [Ca²⁺]j. Dye fluorescence when saturated with Ca²⁺ was determined as described (17) by including 30 μM ionomycin in the presence of 10 mM [Ca²⁺],. Addition of the membrane permeant metal chelator tetrakis(2-pyridylmeth yl)ethylenediamine at concentrations up to 0.5 mM for 2 hr failed to substantially alter the fluorescence ratio for either indo-1 or fura-2, suggesting that the dyes are not binding metals inside the cells (28). In this paper, [Ca²⁺]j is expressed either as [Ca²⁺],/Kₐ where Kₐ is the dye-Ca²⁺ dissociation constant or as the fluorescence intensity ratio I₄₄⁰/₃₈⁰ for indo-1 or I₃₄₅/I₃₈₀ for fura-2 (16). As discussed (17), several factors determine the value chosen for Kₐ. The Kₐ value most closely approximating our conditions is 770 nM for fura-2 (29), and this value will be used for the molar values of [Ca²⁺], presented in the text.

pHₖ was determined by incorporation of the carboxyfluorescein derivative 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) as the tetracarboxyethyl ester, which allows for the continuous recording of pHₖ in sperm (17). pHₖ values are determined from a standard curve generated by varying extracellular pH (pHₖ) in the presence of 50 μM monensin and 50 μM nigericin to collapse the pH gradient across the plasma membrane (30). An apparent pKₐ of 6.2±1 was determined for BCECF. Indo-1, fura-2, BCECF, and their acetoxyethyl esters were obtained from Molecular Probes (Eugene, OR).

RESULTS

mAb J10/14 Induces an Increase in Sperm [Ca²⁺], Either the IgG or the Fab of mAb J10/14 binds the 210-kDa protein and causes [Ca²⁺]j to increase within seconds after addition to sperm. In these fura-2-loaded cells, the [Ca²⁺]j increase is dependent on J10/14 concentration (Fig. 1). The IgG is half maximally effective at 2.5 μg/ml, whereas Fab fragments are half maximally effective at 5 μg/ml. Maximal increases in [Ca²⁺], occur at 5 μg of IgG per ml and 10 μg of Fab per ml. Higher concentrations of mAb produce no further increase in [Ca²⁺],. These maxima reflect saturation of the mAb response of the cells, and not the saturation of intracellular fura-2, as shown by the further 2.5-fold increase in the fluorescence ratio induced by the addition of ionomycin to sperm at the mAb-induced maxima. Another mAb, J4/4, which also reacts with external domains of the 210-kDa protein (20), does not induce increases in [Ca²⁺], at IgG concentrations of 260 μg/ml (Fig. 1, Table 1). At 120 μg/ml the binding of J4/4 Fab to cells is equal to that of J10/14 Fab at 10 μg/ml (20). Maximum increases in [Ca²⁺], of 23-fold were observed using fura-2 (Table 1). Depending on the value of Kₐ chosen, [Ca²⁺], may reach 1–2 μM in sperm treated with J10/14 at 10 μg/ml or greater. At saturating J10/14 Fab concentrations, peak increases in [Ca²⁺], are maintained for 1–3 min. After this time the [Ca²⁺], relaxes within 15 min by 50–90% (Fig. 2A). Both the initial rate of increase to maximum and the time before relaxation of fluorescence begins are dependent on antibody concentration. Microscopic examination of these cells showed that they had not undergone the AR (as judged

![Fig. 1](https://example.com/image)

**Fig. 1.** Effects of J10/14 and J4/4 on [Ca²⁺],. Sperm were loaded with fura-2 and diluted into ASW. IgG or Fab, dialyzed into ASW at 1–100 μg/ml, was added. [Ca²⁺], was plotted as the ratio of emission intensities (17) at excitation wavelengths of 345 nm and 380 nm. Data represent the peak increase in [Ca²⁺], that occurred 30–120 sec after IgG or Fab addition. ○, J10/14 IgG; △, J10/14 Fab; ●, J4/4 IgG; ▲, J4/4 Fab.
Values were determined using the fura-2 ratio method (16) with excitation at 345 nm/380 nm. [Ca2+]i values represent maximal increase and failure to bind (data not shown).

The increase in [Ca2+]i can be reversed by decreasing [Ca2+]i. If after adding J10/14 Fab in the presence of 5 mM [Ca2+]i, sufficient EGTA is added (final concentration 6 mM), the Indo-1 fluorescence relaxes to almost the original level (data not shown). Also, if J10/14 is added within 10 sec after diluting sperm into calcium-free ASW, no increase in [Ca2+]i occurs. Addition of FSG to sperm in calcium-free ASW does not cause [Ca2+]i to increase as detected by Indo-1 or fura-2. We feel the most likely explanation is that J10/14 and FSG cause [Ca2+]i to increase by inducing Ca2+ from ASW to enter.

[Ca2+]i increases induced by J10/14 are also sensitive to pH. The elevation of [Ca2+]i induced by J10/14 decreases below a pH of 7.0, and no change occurs below pH 6.3. This does not result from a decrease in binding of J10/14 to sperm, as no differences in binding exist between pH 6 and 8 (data not shown).

Effects of Other mAbs on [Ca2+]i and pH. In response to FSG, there are increases in [Ca2+]i and pH, and the AR occurs (3–7, 11–13). Table 1 characterizes the abilities of four mAbs reacting with external domains of the 210-kDa protein to increase [Ca2+]i. mAbs J10/14 and J18/2 inhibit the

![Diagram](image)

**Table 1. Effects of mAbs on [Ca2+]i and pH**

| Addition | Concentration | Inhibition of FSG-induced AR | [Ca2+]i/Kd | ΔpH
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>—</td>
<td>0.06</td>
<td>ND</td>
</tr>
<tr>
<td>FSG</td>
<td>374</td>
<td>—</td>
<td>0.55</td>
<td>0.40</td>
</tr>
<tr>
<td>T8/40 IgG (IgG2a)</td>
<td>78 µg/ml</td>
<td>No</td>
<td>0.06</td>
<td>0.00</td>
</tr>
<tr>
<td>J4/4 IgG (IgG2a)</td>
<td>260 µg/ml</td>
<td>No</td>
<td>0.06</td>
<td>0.00</td>
</tr>
<tr>
<td>J4/4 Fab (IgG2a)</td>
<td>120 µg/ml</td>
<td>No</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>J16/18 IgG (IgG1)</td>
<td>29 µg/ml</td>
<td>No</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>J10/14 IgG (IgG2a)</td>
<td>10 µg/ml</td>
<td>Yes</td>
<td>1.10</td>
<td>0.00</td>
</tr>
<tr>
<td>J10/14 Fab (IgG2a)</td>
<td>10 µg/ml</td>
<td>Yes</td>
<td>1.40</td>
<td>-0.02</td>
</tr>
<tr>
<td>J18/2 IgG (IgG3)</td>
<td>13 µg/ml</td>
<td>Yes</td>
<td>0.91</td>
<td>-0.04</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>5 mM</td>
<td>—</td>
<td>0.05</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>—</td>
<td>0.06</td>
<td>0.52</td>
</tr>
</tbody>
</table>

ND, not determined.

* [Ca2+]i was determined using the fura-2 ratio method (16) with excitation at 345 nm/380 nm. [Ca2+]i values represent maximal increases determined 1–2 min after mixing.

† ΔpH values were determined using BCECF and are the difference between pH at the time of the maximum [Ca2+]i change and the original pH of 7.2. pH = 7.8.

‡ FSG was quantified as µg of fucose per ml.

**Fig. 2.** Induction of increased [Ca2+]i by J10/14 Fab or FSG. Sperm were loaded with fura-2 and diluted 1:20 into ASW. (A) J10/14 Fab was added at the arrow to 88 µg/ml (left curve) or 10 µg/ml (right curve). (B) Either equal volumes of ASW (thick arrow) or FSG (37 µg of fucose per ml of ASW, thin arrow) was added. In control sperm and J10/14 Fab-treated sperm, less than 5% underwent the AR. FSG addition resulted in 64% of the sperm undergoing the AR. [Ca2+]i values were determined as in Fig. 1.

**Fig. 3.** Dependence on [Ca2+]i. Sperm were loaded with indo-1 and diluted into ASW containing various [Ca2+]i. J10/14 Fab was added to 5 µg/ml at the arrow, and the fluorescence at 405 nm was monitored. The three tracings correspond to increases in [Ca2+]i, at 10 mM (top curve), 0.55 mM (middle curve), and 0.02 mM (bottom curve) [Ca2+]i.
FSG-induced AR, do not elevate pH, but do cause increases in [Ca²⁺]i greater than those induced by FSG. mAb J4/4 (as IgG or Fab) does not inhibit the FSG-induced AR nor cause changes in [Ca²⁺]i or in pH, mAb J16/18 is weaker than J10/14 or J18/2 at both inhibiting the FSG-induced AR and inducing increased [Ca²⁺]i. J16/18 (29 μg/ml) does not increase [Ca²⁺]i detectably; however, at 80 μg/ml, increases of 0.7/[Kₐ] occur. What is most interesting is that all mAbs that increase [Ca²⁺]i do so without an accompanying increase in pH.

Effects of mAb Plus NH₄Cl. Because FSG induces increases in [Ca²⁺]i and pH during induction of the AR, We tested the effect of artificially raising the pH on sperm that had been treated with J10/14 Fab, which increases [Ca²⁺]i. Combining the J10/14 Fab-induced increase in [Ca²⁺]i with the NH₄Cl treatment to elevate pH, results in the induction of the AR (Fig. 5). The maximal percentage of cells undergoing the AR occurs at 2.5 mM NH₄Cl, with the 50% of maximum occurring at 0.7 mM. NH₄Cl alone does not induce the AR, nor does NH₄Cl combined with J4/4 Fab at 200 μg/ml (Fig. 5). NH₄Cl at 10 mM has almost no effect on [Ca²⁺]i; however, 5 mM NH₄Cl causes an increase in pH similar to FSG (Table 1).

DISCUSSION

The induction of increases in [Ca²⁺]i by mAbs to the 210-kDa sperm membrane protein was initially surprising to us, since it apparently contradicts our report (20) that J10/14 inhibits the long-term ⁴⁰Ca²⁺ influx occurring after the egg jelly induction of the AR. We believe the ⁴⁰Ca²⁺ uptake assay does not measure the [Ca²⁺]i increase required for induction of the AR (as discussed in ref. 13), because the influx detected by ⁴⁰Ca²⁺ lasts for minutes after mAb induction (12, 13), is primarily into the mitochondrion (19), and is blocked by mitochondrial poisons that do not block the induction of the AR by FSG. In contrast, the increase in [Ca²⁺]i, detected by indol-1 and fura-2, occurs in the same time frame as the induction of the AR (seconds) and is insensitive to mitochondrial poisons; and, furthermore, dye fluorescence is visually uniform throughout the cell. This suggests that the dyes are in fact measuring increases in [Ca²⁺]i. However, upon induction of the AR by FSG, the long-duration (>5 min) ⁴⁰Ca²⁺ uptake into the mitochondrion is not seen by dye fluorescence (Fig. 28). The internal volume of the sperm mitochondrion is 10–15% of the total cellular volume (23). If the dye partitions uniformly among intracellular compartments, dye within the mitochondrion would be rapidly saturated by the levels of Ca²⁺ accumulation estimated from ⁴⁰Ca²⁺ studies (12, 13). Once saturated, the dye would not detect the further increases in mitochondrial Ca²⁺ continuing for 20 min after induction of the AR.

If a sufficiently high concentration of J10/14 is used, the rate of increase in [Ca²⁺]i (as determined by these Ca²⁺ dyes) is equal to that induced by FSG. mAb J10/14 and FSG are also similar in their dependence on [Ca²⁺]i and pH in the induction of increases in [Ca²⁺]i. The differences between the two inducers are that J10/14 elevates [Ca²⁺]i to higher levels than does FSG and that the peak value induced by FSG remains high for minutes, whereas the J10/14 (Ca²⁺) peak is transitory (Fig. 2). The similarities suggest that J10/14 and FSG may induce increases in [Ca²⁺]i by the same mechanism. The fact that J10/14 induces increases in [Ca²⁺]i similar to those of FSG yet blocks the FSG-induced AR, suggests that the 210-kDa protein is involved in mediating the FSG-induced changes resulting in the AR. One hypothesis to explain these data assigns to the 210-kDa protein a role in regulating the entrance of Ca²⁺ and the exit of H⁺ from the sperm. The interaction of FSG with sperm activates fluxes of both Ca²⁺ and H⁺ and induces the AR. However, J10/14 reacts with only certain domains of the 210-kDa protein, activates Ca²⁺ influx, but not H⁺ efflux. In addition, J10/14 blocks FSG from inducing H⁺ efflux (and consequently increasing pH). Activation of the 210-kDa protein by J10/14 may thus interfere with the coordinated elevation of [Ca²⁺]i and of pH by FSG, thereby inhibiting the induction of the AR. Similar results have been reported in human T lymphocytes, where mAbs reacting with the T3 cell surface complex increase [Ca²⁺]i (31–33). However, these mAbs to T3 also block certain Ca²⁺-dependent T-cell functions, such as the generation of cytotoxic T lymphocytes in mixed lymphocyte culture and the proliferation of T cells in response to soluble antigen (34, 35). Weiss et al. (36) found that OKT3 treatment alone raised [Ca²⁺]i, but a mitogenic effect was
seen only when this treatment was combined with phorbol 12-myristate 13-acetate. Treatment of cells with phorbol 12-myristate 13-acetate may result in an elevation of pHj by activation of Na+/H+ exchange (37). Our results are similar in that elevation of [Ca2+]j, by mAb alone does not induce the AR. However, when mAb-increased [Ca2+] occurs together with an NH4Cl-induced increase in pHj, the AR is triggered. These data not only support the hypothesis that both increased [Ca2+]j and elevated pHj are necessary to induce the AR (12, 13, 15), but also show that an increase in pHj alone does not necessarily lead to increased [Ca2+]j.

Unlike other agents utilized in the study of fertilization, treatment of sperm with mAbs to the 210-kDa protein elevate [Ca2+]j without increasing pHj. Because FSG induces profound Ca2+-dependent changes in sperm cyclic nucleotide metabolism, protein kinase, and protein phosphatase activities, and phosphorylation state of sperm proteins (reviewed in ref. 38), these mAbs may be valuable reagents for the study of the relationships between increased [Ca2+]j and these events and of sperm functions such as motility and respiration.

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