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Egg jelly triggers a calcium influx which inactivates and is inhibited by calmodulin antagonists in the sea urchin sperm

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Sea urchin sperm must undergo the acrosome reaction to fertilize eggs. The natural inducer of this reaction is the most external coat of the egg, named 'jelly'. The ionic composition of the extracellular and intracellular media and the permeability properties of the sperm plasma membrane are fundamental in this reaction. As Ca^{2+} is required for the acrosome reaction to occur, its intracellular concentration ($[Ca^{2+}]_i$) was measured with fura-2. In 10 mM Ca^{2+}, egg jelly induced the acrosome reaction and an increase in $[Ca^{2+}]_i$ that lasted for several minutes. However, at 0.5 or 2 mM Ca^{2+}, it became evident that the Ca^{2+}-influx pathway activated by jelly opened only for a few seconds; this prevented both the full increase in $[Ca^{2+}]_i$ and the acrosome reaction even after the concentration of Ca^{2+} was raised to 10 mM. In the presence of jelly, the time this permeability pathway remained open was inversely related to the extracellular concentration of Ca^{2+} ($[Ca^{2+}]_e$). Using Bisoxonol (a permeant fluorescent membrane potential probe), it was found that the jelly-induced depolarization depended on $[Ca^{2+}]_e$ and was proportional to the increase in $[Ca^{2+}]_i$. Since $[Ca^{2+}]_i$ could affect the jelly-induced Ca^{2+} influx through calmodulin, two of its antagonists, trifluoperazine and W-7, were tested. Both compounds blocked the acrosome reaction by inhibiting the jelly-induced increase in $[Ca^{2+}]_i$. W-5 at the same concentration had no effect. The results suggest that one of the jelly-activated Ca^{2+}-influx pathways, probably a channel, is the target of the calmodulin antagonists.

Introduction

In many species the acrosome reaction (AR) is a requisite for sperm to fertilize the egg. In sea urchin sperm the AR is characterized by the exocytosis of the acrosomal vesicle and the extension of an actin-containing filament, the acrosomal tubule [1]. This reaction is induced by the egg jelly (the most external coat of the egg), and is modulated by the composition of the ionic media and ion movements across the sperm plasma membrane. When jelly is added to sperm, there is an influx of Ca^{2+} and Na^+ and an efflux of K^+ and H^+ [2,3]. These ionic movements lead to an increase in both internal pH (pH_i) [4] and $[Ca^{2+}]_i$ [5], and to a 30 mV depolarization [4]. All these changes as well as the AR are inhibited by Ca^{2+}-channel antagonists such as D-600, verapamil and dihydropyridines [6,7], and by K^+-channel blockers, i.e., TEA^+ [2].

Among the ions in sea water, Ca^{2+} plays a central role in sperm physiology. For instance, egg jelly is unable to induce the sperm AR in the absence of Ca^{2+} [8]. All other modifications of the ionic conditions, i.e., absence of Na^+, high $[K^+]_e$ or low pH_e, can be overcome by ammonia [9] or ionophores like nigericin [2]. It has been shown that transiently permeant fluorescent Ca^{2+} indicators [10] can be used to study variations of $[Ca^{2+}]_i$ in the sea urchin sperm [11].

Here it is shown, with the use of fura-2 [10], that a pathway for Ca^{2+} uptake activated by egg jelly is only transiently opened, and that its closing depends on $[Ca^{2+}]_e$. The explanation of this may be the effect of $[Ca^{2+}]_e$ on $[Ca^{2+}]_i$. It was also found, in agreement with previous results [12], that the uptake of Ca^{2+} causes a nisoldipine-sensitive depolarization as monitored with the potential-sensitive dye Bisoxonol [13].

Sea urchin sperm contains high amounts of calmodulin [14] and its role during the AR is unknown.

Abbreviations: $[Ca^{2+}]_i$ and $[Ca^{2+}]_e$, intracellular and extracellular $[Ca^{2+}]$; W-7 and W-5, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and its chlorine-deficient analog; DMSO, dimethylsulfoxide; TFP, trifluoperazine; TEA^+, tetraethylammonium; pH_i, intracellular pH; ASW, artificial sea water; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AR, acrosome reaction.

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However, it has been reported that anticalmodulin agents like TFP and W-7 [15,16] block the AR, and that TFP also inhibited the AR-related $^{45}Ca^{2+}$ uptake [15]. Therefore, these two calmodulin antagonists were studied in order to explore the possible relation that may exist between the uptake of Ca^{2+}, $[Ca^{2+}]_i$, and the AR by resolving Ca^{2+} influx in the time-range of seconds. It was found that TFP and W-7, but not W-5 (a 10-times less potent chlorine-deficient W-7 analog) inhibited the AR by directly blocking the Ca^{2+} influx induced by jelly.

Materials and Methods

Gametes, egg jelly components and reagents

Strongylocentrotus purpuratus sea urchins were obtained from Pacific Bio-Marine Laboratories (Venice, CA). Spawning was induced by intracoelomic injection of 0.5 M KCl. Sperm were collected from the gonopores with a Pasteur pipette and stored undiluted on ice until used.

The egg jelly was obtained at low pH as in Ref. 7, and the fucose-rich glycoconjugate that induces the AR, which we call factor, was purified from the jelly according to Ref. 15. The fucose content of both jelly and factor were quantitated as in Ref. 7. The presence of speract in the jelly and factor preparations was checked by measuring their capacity to increase sperm respiration at pH 6.6 [17]; according to this test, egg jelly, but not the factor, contained speract (M. González-Martínez, personal communication). The AR was determined by phase-contrast microscopy of an aliquot of sperm, obtained from the recording suspension, fixed with 12% formaldehyde as described previously [12].

Fura-2/AM was purchased from Molecular Probes (Eugene, OR) and W-7 and W-5 from Sigma (St. Louis, MO). Bisoxonol, ionomycin and trifluoperazine were gifts from Sergio Grinstein, Nancy Carrasco and Jaime Mass, respectively. The rest of the reagents employed were of the highest quality available commercially.

Loading of fura-2 into sperm cells

The loading procedure was carried out as in Ref. 11, with small modifications. Briefly, fresh sperm were diluted 1:10 in ASW at pH 7.0, containing 1 mM Ca^{2+}, and 8–10 μM of fura-2/AM. The suspension was kept on ice for 24 h to hydrolyze the ester. In these conditions fura-2 reached an internal concentration of about 0.6 mM.

Membrane potential measurements

Membrane potential changes were recorded with Bisoxonol as follows: 0.2 μM of Bisoxonol in DMSO (0.3% final concentration) was added to sperm incubated on ice as in the fura-2 loading procedure, but without fura-2/AM. The sperm were left to equilibrate with the probe for 2 min. After this time the recording was started.

Recording and analysis of the fluorescent signals

After the loading period, 20 μl of the suspension were added to a round cuvette with 3.0 ml of ASW that contained 486 mM NaCl, 10 mM KCl, 2.4 mM $NaHCO_3$, 10 mM $CaCl_2$, 56 mM $MgCl_2$, 0.1 mM EDTA and 10 mM Hepes (pH 8.0). The round cuvette was continuously stirred with a magnet and the temperature was kept at 15°C by means of a circulating bath. The fluorescence emission was recorded at 490 nm for fura-2 (excitation 340 nm) and 580 nm for Bisoxonol (excitation 540 nm) with a Perkin-Elmer LS-3 spectrofluorimeter connected to a chart recorder. Autofluorescence of sperm incubated with jelly was not significant. Also, loaded cells excited at the isosbestic wavelength, 357 nm, showed no detectable change in fluorescence despite the fact that jelly triggered the AR (data not shown).

Intracellular $[Ca^{2+}]$ was determined as in Ref. 18 using 3 μM ionomycin (0.1% ethanol) to attain the maximum fluorescence and 15 mM $MnCl_2$ to quench fura-2. All our $[Ca^{2+}]_i$ is indicated as the fraction of fura-2 bound to Ca^{2+}, namely f, due mainly to the fact that the precise intracellular conditions are unknown, and in consequence the K_d of the fluorophore in the sea urchin sperm is also unknown [11]. This fraction, f, and the $[Ca^{2+}]_i$ are related by: $[Ca^{2+}]_i = K_d\ (f/1-f)$, where $f = (F - F_{min})/(F_{max} - F_{min})$ and F = fluorescence.

As pointed out in Refs. 19 and 20, the influx of Ca^{2+} at a given time is related to the concentration of the Ca^{2+}-indicator complex if: (a) the concentration of the indicator is higher than $[Ca^{2+}]_i$; (b) the internal buffering capacity is negligible compared to that of the indicator; (c) there are no internal stores of Ca^{2+} activated by the agonist and (d) the efflux is not decreased directly by the agonist. If (a) and (b) are true then the fraction of Ca^{2+}-indicator complex, f, is proportional to the total $[Ca^{2+}]$. Under these circumstances, the influx of Ca^{2+} through the sperm plasma membrane induced by the agonist can be defined as $\Delta f/\Delta t$, where $\Delta f = f_a - f_i$ and $f_a = f$ due to agonist (i.e., the egg jelly) at the time of the peak (5–10 s), and $f_i = f$ at rest. Although (a) and (b) are best accomplished with quin2, it has been shown that the high amounts of this indicator that are needed damp the increase in $[Ca^{2+}]_i$ [20,21]. Having considered this, fura-2 was chosen to resolve the kinetics of changes in $[Ca^{2+}]_i$ induced by egg jelly. The condition, (c), is met in sea urchin sperm, since the increase in $[Ca^{2+}]_i$ due to jelly is totally dependent on external Ca^{2+} (see below). It was assumed that (d) was acceptable due to the fact that there is no evidence suggesting that the egg jelly triggers a fast decrease (within 8 s) of Ca^{2+} efflux. On the other hand, it has

been shown that jelly induces an important increase of Ca^{2+} influx [2]. Taken together, these considerations indicate that fura-2 could be used to follow the changes in the Ca^{2+} influx at the plasma membrane of sea urchin sperm.

The Bisoxonol signals were analyzed using the following equation: $r = (F - F_0)/F_0$ where F_0 is the fluorescence value at rest and F its new value [12].

All the curves were fitted using a statistics program in which the correlation coefficients were obtained as indicated by Spiegel [22]: $r = \pm(1 - (\Sigma(y - y_{est})^2/\Sigma(y - \bar{y})^2))^{0.5}$, where y = the value experimentally obtained, y_{est} = the value of y obtained from the fitted curve and $\bar{y}$ = the average of y. All the 'r' values were larger than 0.98. The curves were drawn by hand, using the parameters obtained from the statistics program.

Results

Effects of egg jelly and $[Ca^{2+}]_e$ on $[Ca^{2+}]_i$

In agreement with previous results [5], egg jelly triggered 93.6% ± 5.4% ($n = 10$) of the AR and induced a 19.2 ± 7.5 ($n = 12$) fold increase in $[Ca^{2+}]_i$. This latter change peaked at around 8 s; thereafter, a small relaxation occurred (see Fig. 1a). All $[Ca^{2+}]_i$ values were taken at the peak. The increase in $[Ca^{2+}]_i$ induced by jelly was dose-dependent. Fig. 2 illustrates the relation between egg jelly, $[Ca^{2+}]_i$ and the AR. The increase in $[Ca^{2+}]_i$ due to jelly was adjusted by an equilateral hyperbola with a $K_m = 1.92$ nmol of fucose/ml and a maximum Ca^{2+} influx of 0.685 (fraction of Ca^{2+}-bound fura-2 (f); see 'Methods'). The dependence of the AR on the concentration of jelly was adjusted to a curve characteristic of positive cooperativity with a Hill coefficient of 1.75 and an EC_{50} of 5.8 nmol of fucose/ml.

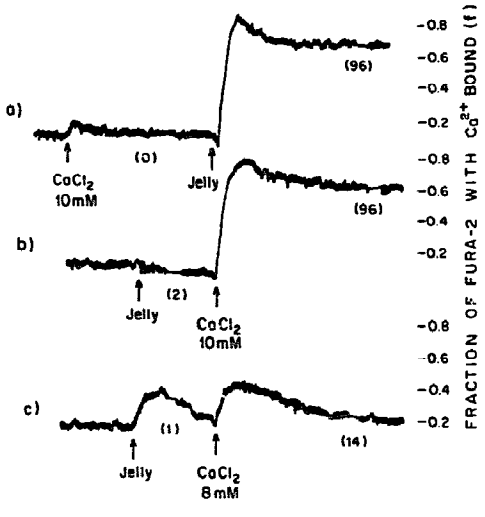

Fig. 1. Effect of egg jelly on $[Ca^{2+}]_i$ at different extracellular $[Ca^{2+}]$. Sperm cells (about $2 \cdot 10^7$) were resuspended in ASW 2 min before the recordings were started. The $[Ca^{2+}]_e$ was 0.5 mM for (a) and (b) and 2 mM for (c). All the additions were made through a hole at the top of the cell compartment with a microsyringe. The calibration is shown at the right side of the recordings as the fraction of Ca^{2+}-bound fura-2 (f, see Materials and Methods). The records are representative of at least five different batches of sea urchin sperm.

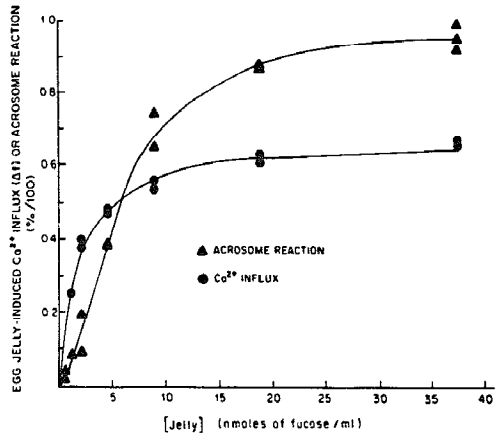

Fig. 2. Egg jelly dose-response curves for changes in $[Ca^{2+}]_i$ and AR. Ca^{2+} influx (●) and the AR (▲) values were obtained at 8 s and 1 min, respectively, after the egg jelly addition. The dialyzed egg jelly added was less than 2% of the total volume. The curve was fitted with the average of two experiments, on the basis of the experimental points shown.

In sperm suspended in 0.5 mM $[Ca^{2+}]_e$, jelly did not change $[Ca^{2+}]_i$ and there was no AR (Fig. 1b). However, in the presence of jelly, the addition of 10 mM $CaCl_2$ increased $[Ca^{2+}]_i$ and induced the AR. Under these conditions, it was possible to separate the addition of jelly from Ca^{2+} uptake. In contrast, when $[Ca^{2+}]_e$ was 2 mM (Fig. 1c), the addition of jelly produced a small increase in $[Ca^{2+}]_i$ and no AR. Under these conditions, an increase of $[Ca^{2+}]_e$ to 10 mM brought about a 70% inhibition of Ca^{2+} uptake and induced only a low percentage of the AR. It is interesting to note from Fig. 1a that the addition of 10 mM $CaCl_2$ before adding egg jelly produced a small increase in $[Ca^{2+}]_i$, but no AR.

At 0.5 mM $[Ca^{2+}]_e$, the increase in $[Ca^{2+}]_i$ depended on the time between the addition of egg jelly and an increase in $[Ca^{2+}]_e$ to 10 mM. The increase in $[Ca^{2+}]_i$ was inhibited with a $t_{1/2}$ of 67 s (Fig. 3). Thus, the level of $[Ca^{2+}]_e$ not only affects the AR and Ca^{2+} uptake, but also the time the jelly-induced Ca^{2+} pathway remains open. The dependence on $[Ca^{2+}]_e$ was also observed when the purified factor was used to induce an increase in $[Ca^{2+}]_i$ and the AR (Fig. 4).

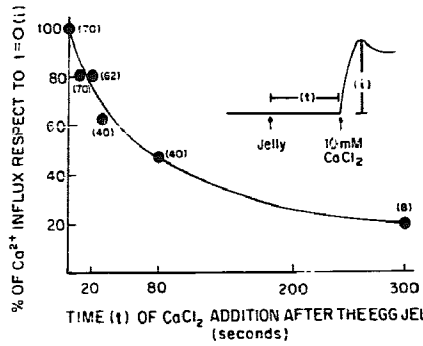

Fig. 3. Inactivation of the egg-jelly-induced Ca^{2+} entry mechanism. Egg jelly was added to sperm cells resuspended in ASW (0.5 mM Ca^{2+}) and thereafter, $CaCl_2$ was added at different times. The ordinate shows the value of f (at a given time) as the percentage of the f value at time zero, that is, when $[Ca^{2+}]$ was 10 mM before the egg jelly addition. The curve was fitted with a $t_{1/2}$ of 67 s. the inset shows a scheme of how the variables were measured (t = time; i = Ca^{2+} influx). Values shown in parentheses are the percentages of AR. The data are from one representative experiment of three.

$[Ca^{2+}]_i$ and membrane potential

It is known that the AR is accompanied by a membrane potential depolarization [4,23]. Recently, diS-C_3(5), a positive fluorescent potential-sensitive dye, was used to estimate the changes in sperm plasma membrane potential that occur during the AR. It was found that the jelly-induced depolarization was inhibited by nisoldipine, a Ca^{2+}-channel blocker [12]. Here, this finding was confirmed using Bisoxonol (data not shown), a negatively charged fluorescent potential indicator [13].

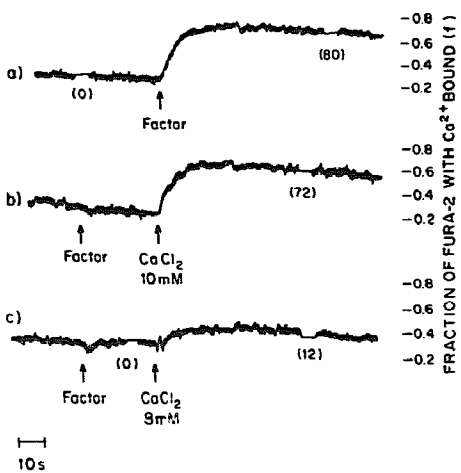

Fig. 4. Effect of the purified-egg jelly factor on $[Ca^{2+}]_i$ at different extracellular $[Ca^{2+}]$. $[Ca^{2+}]_e$ was 10 mM for (a), 0.5 mM for (b) and 2 mM for (c). The conditions are the same as those in Fig. 1, except that the AR was induced by factor (see Materials and Methods).

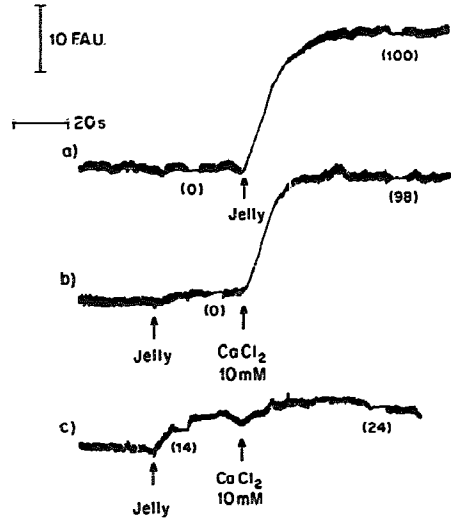

Fig. 5. The role of extracellular Ca^{2+} in the AR-associated depolarization. Plasma membrane potential was monitored with Bisoxonol. The cells were left to equilibrate with the probe 2 min before the recording was started. The traces are from one experiment representative of three. Values in parentheses are the percentages of AR.

These results suggest that, at least in part, the depolarization is caused by the uptake of Ca^{2+}. To test this, Bisoxonol was used to study the effect of $[Ca^{2+}]_e$ on the jelly-induced depolarization.

At 0.5 mM $[Ca^{2+}]_e$, the jelly triggered a significant depolarization, but only after the addition of 10 mM $CaCl_2$ (Fig. 5b). At 2 mM $[Ca^{2+}]_e$, the jelly induced a small depolarization; a subsequent increase in $[Ca^{2+}]_e$ to 10 mM produced a relatively small depolarization and no AR (Fig. 5c). The reduced depolarization induced in the latter case by the addition of 8 mM $CaCl_2$ was in accordance with the small increase in $[Ca^{2+}]_i$ detected under these conditions (see Fig. 1c).

Effect of calmodulin antagonists on both the AR and the increase in $[Ca^{2+}]_i$ induced by egg jelly

As shown in Fig. 3, the inhibition of Ca^{2+} influx is associated with a small increase in $[Ca^{2+}]_i$, which did not trigger the AR. Thus, it was investigated whether calmodulin was involved in the process. The anti-calmodulin agents, TFP and W-7, inhibited both the jelly-induced Ca^{2+} uptake and the AR. W-5 at a concentration equivalent to that of W-7 exhibited only a modest effect (Fig. 6a). W-7 (60 μM) increased $[Ca^{2+}]_i$ at rest from $f = 0.098 \pm 0.005$ ($n = 3$) to 0.292 ± 0.075 ($n = 3$), while W-5 (50 μM) increased $[Ca^{2+}]_i$ only to 0.111 ± 0.026 ($n = 3$). Fig. 6b shows the effect of W-7 and W-5 on $[Ca^{2+}]_i$ after addition of egg jelly. W-7 blocked Ca^{2+} uptake with an IC_{50} (29.2 μM) similar to that required for AR inhibition (32 μM); W-5 had no

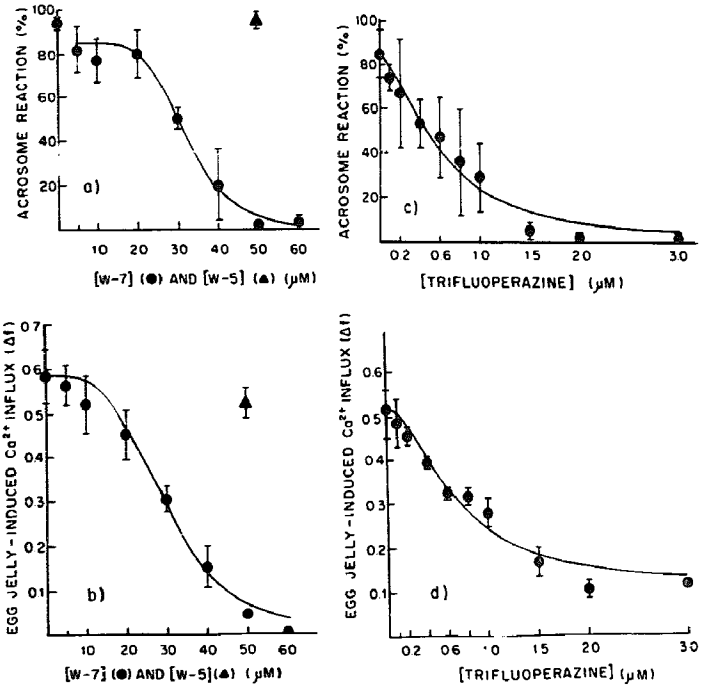

Fig. 6. Inhibition of the egg-jelly-induced Ca^{2+} influx and AR by calmodulin antagonists. $[Ca^{2+}]_i$ was recorded in sperm cells resuspended in ASW with different calmodulin antagonists and Δf, due to the addition of the egg jelly determined. Panels (a) and (b) show the inhibitory effect of the naphthalenesulfonamides on both AR and Ca^{2+} influx. The IC_{50} values were 32 and 29.2 μM, respectively. Panels (c) and (d) show the inhibitory effect of trifluoperazine on both AR and Ca^{2+} influx. The IC_{50} values were 0.57 and 0.67 μM, respectively. All the drugs were dissolved in DMSO and the volume added was never more than 0.3% of the total volume. The points shown are the average of three experiments ± S.D. Only the averages were used for fitting.

effect. The IC_{50} values obtained are in close agreement to the values reported for inhibition of calmodulin action [24]. This observation and the fact that the effect is specific for W-7 were highly suggestive that calmodulin was involved in the egg-jelly-induced Ca^{2+} uptake.

TFP inhibited AR with an IC_{50} of 0.57 μM (Fig. 6c). However, TFP at 3 μM (the higher concentration tested) did not alter $[Ca^{2+}]_i$ significantly at the resting state; $[Ca^{2+}]_i$ changed only -0.021 ± 0.069 ($n = 3$) units of f. As shown in Fig. 6d, TFP inhibited Ca^{2+} uptake with an IC_{50} of 0.67 μM. The main difference between the two dose-response curves is that TFP completely inhibited the AR when there was still 30% of Ca^{2+} uptake. The IC_{50} values obtained are at least one order of magnitude lower than those required to directly affect calmodulin action [25]. The data obtained with TFP seemed to indicate that it modifies a calmodulin-independent mechanism. Since TFP showed a much greater potency than W-7, its effect was further characterized. Fig. 7 shows that TFP inhibited the AR only when it blocked Ca^{2+} uptake. When TFP was added before the jelly, the same inhibition of both Ca^{2+} uptake and the AR was obtained. On the other hand, addition of TFP after the initiation of Ca^{2+} influx showed no inhibition of the subsequent Ca^{2+} uptake and the AR. These experiments indicate that in sea

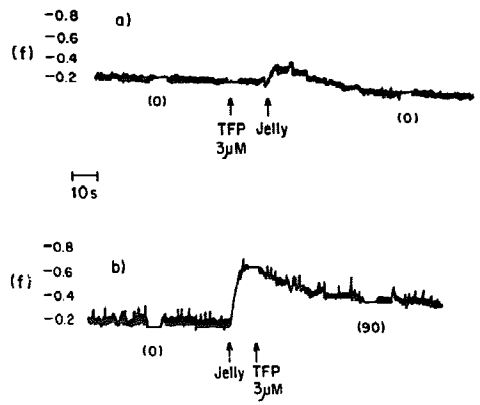

Fig. 7. Addition sequence dependence of the inhibitory effect of trifluoperazine. The artifact due to the intrinsic fluorescence of TFP was subtracted. TFP was added 10 s before (a), or after (b) egg jelly. Values in parentheses are the percentages of AR. The recordings are representative of at least three experiments.

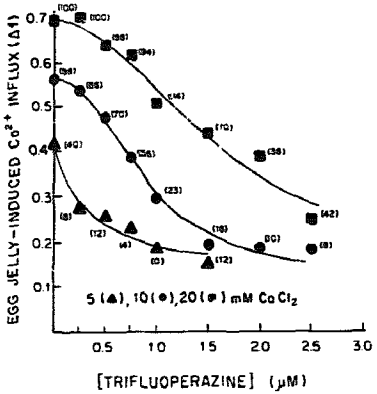

Fig. 8. The role of extracellular Ca^{2+} as antagonist of trifluoperazine. Sperm cells were resuspended in ASW with 5 (▲), 10 (●) and 20 (■) mM of Ca^{2+} and Δf, due to the addition of egg jelly, was determined at different TFP concentrations. The IC_{50} values were 0.37, 0.9 and 1.66 μM of TFP for 5, 10 and 20 mM Ca^{2+}, respectively. The values in parentheses are the percentages of AR for each point. The data are from one experiment representative of three.

urchin sperm, the main target of TFP, and probably of W-7, is the entry pathway of Ca^{2+} and not calmodulin.

Antagonism between calmodulin inhibitors and $[Ca^{2+}]_e$

It is possible to distinguish a calmodulin antagonist from a Ca^{2+}-channel antagonist by measuring their effect at increased $[Ca^{2+}]_e$. Increments of $[Ca^{2+}]_e$ would not alter or enhance the effect of a calmodulin antagonist [26], but it would decrease the potency of a Ca^{2+}-channel antagonist [27]. Fig. 8 shows that the potency of TFP to inhibit the jelly-induced AR and the uptake of Ca^{2+} decreased as $[Ca^{2+}]_e$ increased. Although less evident, this was also true for W-7 (data not shown). In the presence of W-7 and at high $[Ca^{2+}]_e$, sperm were unable to keep their $[Ca^{2+}]_i$ at the resting level. This might be due to the inhibition by W-7 of the Ca^{2+}-ATPase present in the sea urchin sperm plasma membrane [28]. TFP did not produce this effect since its concentration was not high enough to inhibit calmodulin.

Discussion

In sea urchin sperm, Ca^{2+} plays a key role in the response of the cell to egg factors. Fluorescent Ca^{2+} chelators are now being used to measure the influx of Ca^{2+} through the plasma membrane of cells [19,20]. Their use has the advantage of allowing a continuous recording of $[Ca^{2+}]_i$ with a time resolution of seconds or less. This is particularly important in cells as small as sperm, where standard electrophysiological measurements are quite difficult [29]. In the sea urchin sperm, the AR occurs within about 15 s [30]; therefore, these indicators allow the recording of the changes in Ca^{2+} influx that take place before the reaction is over.

An interesting observation is that sperm in 0.5 mM $[Ca^{2+}]_e$ did not change their $[Ca^{2+}]_i$ when jelly was added, even though the Ca^{2+} gradient was around 5000. Two possibilities could explain this finding. One is that the Ca^{2+} influx pathway opened by jelly was unable to move Ca^{2+} at this low $[Ca^{2+}]_e$, as has been shown for the dihydropyridine-sensitive Ca^{2+} channel [31]. A second possibility is that the jelly did not bind to its receptor, since it has been reported that the binding of factor depends on $[Ca^{2+}]_e$ [32]. Reported data from Ref. 33 and the results presented here (Fig. 3) do not support this latter possibility.

In contrast, when $[Ca^{2+}]_e$ was 2 mM, the jelly induced a small increase in $[Ca^{2+}]_i$ without inducing the AR. Adding 8 mM $CaCl_2$ to sea water, 30 s later, did not induce the AR, nor an increase in $[Ca^{2+}]_i$ to the level found when the AR was triggered. These results would be consistent with a mechanism in which the Ca^{2+} pathway opened by jelly is regulated by the levels of $[Ca^{2+}]_i$. At present, the inhibition mechanism of the increase in $[Ca^{2+}]_i$ is not known, it could be an inactivation of Ca^{2+} channels due to voltage and/or $[Ca^{2+}]_i$ [34], or through another mechanism. As mentioned before, speract can induce a transient increase in $[Ca^{2+}]_i$ and could be somehow related to the inhibition. Since the effects of both egg jelly and factor (free of speract) were similarly modified by $[Ca^{2+}]_e$, it is very likely that the inhibition of the increase in $[Ca^{2+}]_i$ was not affected by speract.

When Bisoxonol, a potential-sensitive dye, was used under these conditions, a correlation was found between the magnitude of depolarization and the amount of Ca^{2+} uptake. These observations support previous findings [12], which pointed out that Ca^{2+} uptake is responsible for an important fraction of the jelly-induced depolarization observed during the AR.

Electrophysiological recordings [35,36] and $^{45}Ca^{2+}$-uptake measurements [37] indicate that voltage-dependent Ca^{2+} channels can be blocked by calmodulin antagonists. Furthermore, these Ca^{2+} channels are inactivated by an increase in $[Ca^{2+}]_i$ [34,35]. In the sea urchin sperm there is only pharmacological evidence for the participation of Ca^{2+} channels in the jelly-induced Ca^{2+} uptake [6,7]. Sea urchin sperm contain high amounts of calmodulin [14], and it was found that TFP [15] and W-7 [16] inhibited the AR. Since the inhibition occurred at concentration at which calmodulin action is inhibited by these compounds, the overall findings indicated that this protein could be involved in the AR.

In the light of this information it seemed important to determine whether, in fact, 'calmodulin antagonists' block the sperm AR by inhibiting calmodulin or by modifying other processes. This report shows that both TFP and W-7 block the egg-jelly-induced Ca^{2+} uptake

in *S. purpuratus* sea urchin sperm. The antagonists probably act directly upon the jelly-triggered Ca^{2+} uptake pathway, since their inhibition could be overcome by increasing extracellular Ca^{2+}.

TFP and W-7 did not affect the AR after Ca^{2+} uptake had been started. In addition, TFP failed to block Ca^{2+} influx after it was activated in contrast with the effect of Ca^{2+} antagonists in other systems [20,38]. This finding could be explained considering that the sea urchin sperm has two pathways for Ca^{2+} uptake that are activated sequentially. On this assumption, the initial opening of the TFP-sensitive Ca^{2+} influx pathway is followed by its rapid inactivation after jelly addition. The second Ca^{2+} pathway is insensitive to TFP and remains open for minutes. The possibility that there are two Ca^{2+} uptake pathways could explain why verapamil and D-600, which block the AR and $^{45}Ca^{2+}$ uptake induced by jelly, fail to inhibit these processes a few seconds after the addition of jelly [2]. This suggests that there is a link between the two Ca^{2+} influx mechanisms.

A possible mechanism for the secondary Ca^{2+} influx that occurs after the addition of jelly could be through the reversal of Na^+/Ca^{2+} exchanger [39]. Recently, it has been suggested that this takes place in speract-induced Ca^{2+} uptake in sea urchin sperm [11]. Nevertheless, it is possible that the secondary Ca^{2+} influx pathway could involve a Ca^{2+} channel that is activated by an increase in $[Ca^{2+}]_i$ as suggested in neutrophils [40] (but see Ref. 41), or by pH_i. Another, less likely, possibility is that there is only one Ca^{2+} channel, which is no longer sensitive to the blockers after its activation [42].

The egg-jelly-induced Ca^{2+} pathway of sea urchin sperm does not increase $[Ca^{2+}]_i$ when $[Ca^{2+}]_e$ is 0.5 mM, but it inactivates by a mechanism that appears to be sensitive to $[Ca^{2+}]_i$ and is blocked by TFP, W-7 and dihydropyridines. These characteristics correspond to those described for some Ca^{2+} channels [31,34]. Nevertheless, to establish whether this pathway is indeed a Ca^{2+} channel, it will be necessary to demonstrate electrophysiologically the presence of these channels in the plasma membrane of this cell. Recently, it has been possible to incorporate isolated plasma membranes from sperm of this species into planar bilayers and to record single-channel activity. These channels can conduct Ca^{2+} and are blocked by Co^{2+}, Cd^{2+} and La^{3+} [43]. It remains to be seen if this channel corresponds to an egg-jelly-regulated channel.

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