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EFFECT OF CALMODULIN ANTAGONISTS ON CA^{2+} UPTAKE BY BOAR SPERMATOZOA

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Calcium uptake by washed boar sperm suspensions is markedly stimulated by the calmodulin antagonists trifluoperazine and calmidazolium. Both $^{45}Ca^{2+}$ uptake and net Ca^{2+} uptake are increased by these drugs. Drug stimulated Ca^{2+} uptake is blocked by verapamil (1 mM), by ruthenium red (25 μM) and by carbonyl cyanide p-trifluoromethoxyphenyl hydrazone. Calmodulin antagonists do not slow ATP-dependent Ca^{2+} extrusion from plasma membrane vesicles, and they do not inhibit plasma membrane Ca^{2+}-ATPase. It is proposed that calmodulin is involved in the control of Ca^{2+} entry in boar spermatozoa. Most entering Ca^{2+} in uncapacitated spermatozoa is sequestered by mitochondria or rapidly extruded by plasma membrane pumps. In contrast to the uptake mechanism, ATP-dependent Ca^{2+} extrusion does not appear to be regulated by calmodulin.

Calcium transport by spermatozoa is involved in important processes in fertilization including the acrosome reaction and the control of motility (for a review see) (1). The kinetics of transport change during capacitation (2,3), but the basis of these changes is poorly understood. Several processes that may be involved in transport have been identified in sperm plasma membranes and acrosomal membranes including a (Ca^{2+}-Mg^{2+}) ATPase in ram sperm flagellar membranes (4), and a Ca^{2+}-ATPase in rodent sperm acrosomal membranes and boar and bull sperm plasma membranes (5,7,9). Calmodulin has been shown to be present in abundance in spermatozoa (6) and probably plays a key role in organizing events associated with calcium uptake and function. This communication describes the effects of calmodulin antagonists on Ca^{2+} fluxes in intact cells and ^{45}Ca binding to plasma membranes. Results of

Abbreviations: TFP, Trifluoperazine; FCCP, carbonyl cyanide p-trifluoromethoxyphenyl hydrazone; HEPES, ([N-2-hydroxypiperazine]-N^1-ethane sulfonic acid); CMA, calmidazolium (1-[bis(p-chlorophenyl)methyl]-^{3}-[2,4 dichloro-β(2,4 dichlorobenzyloxy) phenethyl] imidazolium chloride).

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this study indicate that calmodulin plays a role in regulating calcium uptake but does not appear to be involved in ATP-driven Ca^{2+} efflux.

METHODS AND MATERIALS

Boar sperm and sperm plasma membranes were prepared as previously described (8), Membranes sedimenting on top of 1.0 M sucrose were used.

Uptake of Ca^{45} by intact cells--Cells were washed several (3-4) times by centrifugation at low speed (∿ 600 xg) in a HEPES based buffer to remove gel particles and then resuspended in buffer at concentrations between 3-10 X 10^7 cells/ml. The buffer contained the following salts (mM): HEPES (20), NaCl (120), KCl (5), sodium pyruvate (5), $MgCl_2$ (0.5) and glucose (5) the pH was 7.4. Ca^{2+} uptake was determined by collecting sperm on fiber glass filters as previously described (10). Specific conditions are described in Table and Figure legends.

Calcium uptake measurements using a calcium electrode--An Orion digital pH meter (model 701) employing a calcium specific electrode was used to measure net calcium uptake and efflux from intact sperm. The electrode was calibrated before each experiment by adding aliquots of a standard solution of $CaCl_2$ to 10 ml of the HEPES buffer used to maintain the washed sperm. Plots of log [Ca^{2+}] apparent vs millivolts were slightly curved at the concentrations of Ca^{2+} used (presumably because of the high Na^+ and the presence of Mg^{2+} in buffers) but indicated that concentration changes of as little as 2 μM could be detected. Calibration curves were considered approximate because they did not include endogenous Ca^{2+} undoubtedly present in buffers, and variable amounts of free calcium that may have been released by the large amounts of sperm used. Titration with EGTA indicated that endogenous Ca^{2+} did not exceed 10 μm. Experiments were carried out in HEPES buffer at room temperature (∿ 22°C) with 10-14 ml of washed sperm containing 3-5 X 10^8 cells/ml. Drugs used in some experiments and the ethanol used to solubize one of them (FCCP), had no effect on the calcium electrode potentials which were recorded on a strip chart recorder. Ruthenium red, at the concentration used, also did not affect electrode potentials.

Ca^{2+} ATPase--in plasma membranes was determined as described previously (9).

Plasma membrane labelling--The incorporation of Ca^{2+} into plasma membrane vesicles, and extrusion from vesicles was determined by Millipore filtration as previously described (9,10).

Other procedures--Sperm motility was rated subjectively at room temperature as percentage motile cells; sperm were counted in a hemocytometer. Protein was determined by the method of Markwell et al. (11).

Trifluoperazine (TFP) was the gift of the Smith Kline and French Company. Calmidazolium (1-[bis(p-chlorophenyl)methyl]-3-[2,4 dichloro-β-(2,4 dichlorobenzyloxy) phenethyl] imidazolium chloride) was purchased from Janssen Pharmaceuticals. Calmodulin was purchased from the CAABCO Co., Houston, Texas. The protein was found to be electrophoretically pure.

RESULTS

^{45}Ca uptake--When washed boar sperm were incubated in buffer containing either trifluoperazine (TFP) or calmidazolium (CMA), ^{45}Ca uptake was increased as shown in Table 1. ^{45}Ca uptake was enhanced markedly at a concentration of 20 μm TFP and a similar effect was produced by CMA at 4 μM. The magnitude of this increase varied between 50% and 150% among preparations collected on different days. Percentage motility and the forward progression of sperm, rated subjectively, were only slightly decreased from controls at

TABLE 1
EFFECT OF CALMODULIN ANTAGONISTS
ON ^{45}Ca UPTAKE BY WASHED BOAR SPERMATOZOA

Exp. No	Pretreatment	Addition (0 time)	CPM/5min/10^8 Sperm
1	None	None	12,872 ± 1,548
	None	CMA (4 μM)	19,409 ± 581
	None	TFP (20 μM)	24,845 ± 2,688
2	None	None	12,364 ± 650
	None	TFP (20 μm)	33,664 ± 1,319
	None	CMA (4 μM)	23,248 ± 1,429
	TFP	CMA	30,862 ± 1,076
3	None	None	15,910 ± 150
	TFP	None	29,970 ± 1,955
	Verapamil(1mM)	None	19,353 ± 150
	Verapamil	TFP	16,297 ± 217
4	None	None	14,728 ± 142
	CMA	None	23,969 ± 2,668
	Verapamil	None	13,761 ± 382
	Verapamil	CMA	14,799 ± 593

a) Effect of calmodulin antagonists on ^{45}Ca uptake by washed boar spermatozoa. Tubes containing sperm (0.5-1.8 X 10^8 cells) in HEPES buffer were incubated at 37° for 15 min before adding 0.5 ml ^{45}Ca (200 μM, 8 μC_1). Sperm were then collected on glass fiber filters and washed rapidly with buffer containing 5mM EGTA (3 ml, 5 times). Counts bound to the filter in the absence of sperm were approximately 10% of the control count rate. Percentage motility averaged about 70% but declined to about 50% in sperm suspensions containing trifluoperazine and calmidazolium. Results are averages of assays carried out in triplicate (± S.E.M.).

these concentrations. Higher concentrations of these drugs gradually decreased motility but even at concentrations as high as 100 μM TFP approximately 30% of sperm were still motile; calmidazolium, however, abolished motility at concentrations higher than 10 μM. The concentration of these drugs was used, therefore, at levels which provided a maximum response and which sustained motility near the control rate. The second experiment in Table 1 shows that the response to CMA and TFP were not additive which suggests that their mechanism of action are similar. The Ca^{2+} channel blocking agent verapamil blocked the effects of TFP and CMA on ^{45}Ca uptake (experiments 3 and 4).

Net Ca^{2+} uptake and efflux--Fig. 1a shows that TFP produced a substantial increase in net calcium uptake by sperm. While these rates varied somewhat among preparations, very motile sperm suspensions accumulated Ca^{2+}

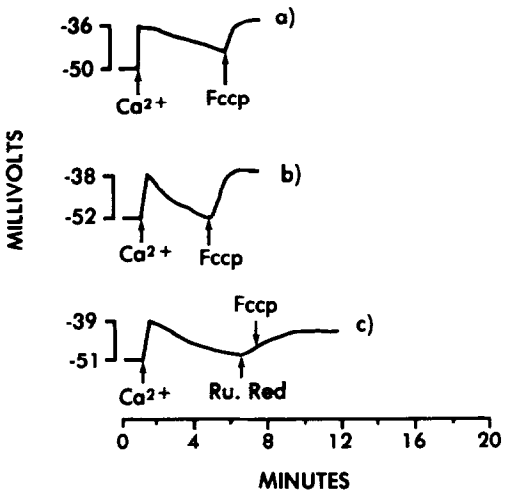

Figure 1: Ca^{2+} fluxes by washed sperm as determined by a Ca^{2+}-specific electrode. Sperm (5 X 10^9 cells, 10.2 ml) were washed and resuspended with stirring in HEPES buffer at room temperature (21°C-23°C) as indicated in Methods. Drugs were added to give the following final concentrations (μM): TFP (traces a-c) (20), FCCP (2), ruthenium red (25). Other conditions were Exp. (a) Ca^{2+} 40 μM, initial motility 70%, final motility 50%. Exp. (b) Ca^{2+} 50 μM, initial motility 70%, final motility 60%. Exp. (c) Ca^{2+} 50 μM, initial motility 70%, final motility 50%.

at about 0.3 n moles/min/10^8 cells. These rates are not too different from the maximum rate calculated from ^{45}Ca uptake (∿ 0.1 n moles/min/10^8 cells from Table 1). At the concentrations of Ca^{2+} used (∿ 40 μM) there was no clear evidence of increased acrosome reactions as viewed by phase contrast microscopy; hence it was surmised that most entering calcium was being sequestered by mitochondria. This was supported by the results also shown in Fig. 1 in which FCCP was given after sperm were allowed to accumulate Ca^{2+}. Calcium efflux was very rapid (traces a,b), and virtually all the calcium taken up was extruded into the medium. Sperm were still motile after this treatment. Comparing the rapid efflux to the slower accumulation of Ca^{2+} along a gradient undoubtedly directed inward, it is doubtful that efflux was simple diffusion but is more likely explained as the result of the action of plasma membrane pumps, still active in the presence of high TFP. Ruthenium red (25 μM) inhibited Ca^{2+} uptake and caused a slow release of calcium from cells which was not markedly accelerated by FCCP (Fig. 1c). Ruthenium red is thought to block Ca^{2+} uptake but not efflux from mitochondria (13), the slow efflux therefore suggests an action of this dye at the plasma membrane.

TABLE 2
EFFECT OF CALMODULIN (CM) ANTAGONISTS ON PLASMA MEMBRANE Ca^{2+}-ATPase

Addition	Pi formed (n moles/mg/hr)	% of control rate
NONE	294.6 ± 26.0	--
Calmodulin (0.2 μg)	304.1 ± 30.8	103.2
CM + CMA (4 μM)	304.1 ± 30.8	103.2
CM + TFP (70 μM)	361.0 ± 23.3	122.5

Effect of Calmodulin (CM) antagonists on plasma membrane Ca^{2+} ATPase. Assays were carried out at 37°C (100 μg protein) as described in (9).

Effects of drugs on Ca^{2+}-ATPase and ^{45}Ca binding to plasma membranes--While the rapid efflux of Ca^{2+}, induced by FCCP, from calcium loaded sperm suggested the presence of plasma membrane pumps, the Ca^{2+}-ATPase present in these membranes (9) was unaffected by trifluoperazine (Table 2). Plasma membranes prepared in a manner to remove endogenous calmodulin (17) show Ca^{2+}-ATPase activity that is not activated by calmodulin, and, therefore, as might be expected the ATPase was not inhibited by TFP. Indeed, enzyme activity was slightly increased by the drug. In other experiments (not shown) in which Mg ATP was added in excess, ATPase activity was not inhibited by TFP and $^{45}Ca^{2+}$ extrusion from Ca^{2+} loaded plasma membrane vesicles, (9) was unaffected by TFP whether or not ATP was present. Moreover, when intact sperm were loaded with ^{45}Ca (as in Fig. 1) and diluted in medium containing FCCP to induce rapid extrusion of the isotope, TFP failed to slow this efflux.

DISCUSSION

The preceding experiments have suggested an hypothesis for calcium transport by uncapacitated boar sperm: 1) calmodulin plays a role in the control of calcium uptake by spermatozoa; 2) plasma membrane pumps restrict this calcium entry, but 3) ATP-dependent pumps are not controlled by calmodulin. The last aspect of the hypothesis is based on the inability of TFP to inhibit plasma membrane Ca^{2+}-ATPase, ATP-dependent extrusion Ca^{2+} extrusion from plasma membrane vesicles, and ^{45}Ca from intact sperm.

Calmodulin may control a Ca^{2+} gating mechanism at the plasma membrane. We note that Skinner et al. (13) suggest that calmodulin plays a role in calcium transport in dog spermatozoa but suggest that the action is at a Ca^{2+} pump.

Ca^{2+} extrusion in boar sperm may depend on regulators other than calmodulin. Such calmodulin independent regulation has been suggested for guinea pig sperm adenylate cyclase by Garbers et al. (14). Moreover, calmodulin regulation of Ca^{2+} extrusion is not a general phenomenon; a Ca^{2+}-ATPase which is not inhibited by calmodulin is thought to be involved in Ca^{2+} transport in rat liver membranes (15) and Thorens (16) has identified a Ca^{2+}-ATPase independent of Mg^{2+}, thought to be involved in calcium transport in vascular smooth muscle.

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