

Phosphoprotein Inhibition of Calcium-stimulated Exocytosis in Sea Urchin Eggs

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Abstract. We have investigated the role of protein phosphorylation in the control of exocytosis in sea urchin eggs by treating eggs with a thio-analogue of ATP. ATP γ S (adenosine 5'-O-3-thiotriphosphate) is a compound which can be used as a phosphoryl donor by protein kinases, leading to irreversible protein thiophosphorylation (Gratecos, D., and E. H. Fischer. 1974. *Biochem. Biophys. Res. Commun.* 58:960-967). Microinjection of ATP γ S inhibits cortical granule exocytosis, but has no effect on the sperm-egg signal transduction mechanisms which normally cause exocytosis by generating an increase in $[Ca^{2+}]_i$. ATP γ S requires cytosolic factors for its inhibition of cortical granule exocytosis: it does not affect exocytosis when applied directly to the isolated exocytotic apparatus. Our data suggest that ATP γ S irreversibly inhibits exo-

cytosis via thiophosphorylation of proteins associated with the egg cortex. We have identified two thiophosphorylated proteins (33 and 27 kD) that are associated with the isolated exocytotic apparatus. They may mediate the inhibition of exocytosis by ATP γ S. In addition, we show that okadaic acid, an inhibitor of phosphoprotein phosphatases, prevents cortical granule exocytosis at fertilization without affecting calcium mobilization. Like ATP γ S, okadaic acid has no effect on exocytosis in vitro. Our results suggest that an inhibitory phosphoprotein can obstruct calcium-stimulated exocytosis in sea urchin eggs; on the other hand, they do not readily support the idea that a protein phosphatase is an essential component of the mechanism controlling exocytosis.

ONE of the first visible signs of fertilization in sea urchin eggs is the elevation of the fertilization envelope, a structure formed by the exocytosis of cortical secretory granules (Longo, 1973). Exocytosis is due to an elevation in the intracellular free calcium ion concentration, $[Ca^{2+}]_i$, from a resting concentration of ~ 200 nM to a stimulatory concentration of several micromolar (Whitaker and Steinhardt, 1982; Swann and Whitaker, 1986). A similar increase in $[Ca^{2+}]_i$ is a physiological trigger for exocytosis in a number of mammalian secretory cells such as adrenal chromaffin cells (Knight and Kesteven, 1983; Cobbold et al., 1987), pancreatic β -cells (Rorsman et al., 1983), and platelets (Rink and Hallam, 1984). Exocytosis can be conveniently studied in sea urchin eggs since the exocytotic apparatus (the egg cortex) can be straightforwardly isolated (Vacquier, 1975; Baker and Whitaker, 1978). The isolated cortex responds to the same calcium ion concentrations as cause exocytosis in the egg at fertilization (Whitaker and Baker, 1983; Swann and Whitaker, 1986). An increase in

$[Ca^{2+}]_i$ is the sole requirement for exocytosis in sea urchin eggs (Whitaker, 1987); the calcium sensitivity of exocytosis in vitro is not affected by either guanine nucleotides (Swann, K., and M. Whitaker, unpublished observations) or modulators of protein kinase C activity (Whitaker and Aitchison, 1985).

There are indications that calcium may trigger exocytosis by altering protein phosphorylation states at the plasma membrane. However, there is no immediate requirement for ATP during secretion in permeabilized mast cells (Cockcroft et al., 1987; Howell et al., 1989), PC12 cells (Ahnert-Hilger and Gratzl, 1987), chromaffin cells (Holz et al., 1989), *Paramecium* (Vilmart-Seuwen et al., 1986), nor in sea urchin eggs, either in vivo (Baker and Whitaker, 1978) or in vitro (Baker and Whitaker, 1978; Moy et al., 1983; Sasaki and Epel, 1983; Crabb and Jackson, 1985). These observations tend to suggest that no calcium-stimulated phosphorylation step is required. On the other hand, these same cells show a diminished secretory response some minutes after removal of ATP (Howell et al., 1989; Wagner and Vu, 1989; Holz et al., 1989; Vilmart-Seuwen et al., 1986; Baker and Whitaker, 1978; Moy et al., 1983; Sasaki and Epel, 1983), suggesting that ATP (and thus perhaps protein phosphorylation) may protect or prime the exocytotic apparatus in some way. One explanation has been that a phosphoprotein is an essential substrate for a phosphoprotein phosphatase that is part of the

1. *Abbreviations used in this paper:* AppCH₂p, β , γ -methyleneadenosinetriphosphate; AppNHp, β , γ -imidoadenosinetriphosphate; ASW, artificial sea water (435 mM NaCl, 40 mM MgCl₂, 15 mM MgSO₄, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 1 mM EDTA, pH 8.0); ATP γ S, adenosine 5'-O-3-thiotriphosphate; $[Ca^{2+}]_i$, intracellular free calcium concentration; IM, intracellular medium (200 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 5 mM MgCl₂, 2.5 ATP, 10 mM EGTA, pH 6.7).

stimulus-secretion coupling pathway (Momayezi et al., 1987; Tatham and Gomperts, 1989). In *Paramecium*, there is good evidence that dephosphorylation of a 65-kD protein occurs (Gilligan and Satir, 1982; Ziesenis and Plattner, 1985; Stecher et al., 1987; Satir et al., 1989). Artificially stimulating dephosphorylation of the protein with alkaline phosphatase or the calcium-calmodulin regulated phosphoserine protease calcineurin can trigger exocytosis (Momayezi et al., 1987). There is a paradox inherent in linking dephosphorylation to the priming by ATP, however, since it requires that exocytosis be stimulated by dephosphorylation of the inhibitory phosphoprotein substrate by phosphatases, but not by dephosphorylation due to removal of ATP. The paradox can only be resolved by ad hoc arguments that call for complex phosphorylation/dephosphorylation of the inhibitory protein at multiple sites, or by supposing that the ATP requirement for exocytosis reflects an energy-requiring step unrelated to protein phosphorylation.

Nonetheless, another line of evidence supports the idea that the ATP requirement may reflect protein phosphorylation. Secretion in permeabilized chromaffin cells is blocked by adenosine 5'-O-3-thiotriphosphate (ATP γ S), a thio analogue of ATP (Brooks et al., 1984; Brooks and Brooks, 1985). The thio-ATP derivative is a substrate for certain protein kinases, but the resulting thiophosphoproteins are not readily dephosphorylated by phosphoprotein phosphatases (Gratecos and Fisher, 1974). We report here the effects on exocytosis of changing the phosphorylation state in sea urchin eggs using ATP γ S (Crossley et al., 1989). We show that microinjecting ATP γ S blocks exocytosis without affecting normal sperm-egg interactions or the fertilization signal transduction pathway. Exocytosis is inhibited downstream of calcium mobilization, the result of thiophosphorylation by an unidentified cytoplasmic kinase. We have identified two thiophosphorylated protein substrates which may be responsible for the inhibition of exocytosis by ATP γ S.

We also show that exocytosis at fertilization can be inhibited by the compound okadaic acid, which is an inhibitor of protein phosphatases (Bialojan and Takai, 1988; Haystead et al., 1989; Cohen et al., 1990). Yet okadaic acid does not inhibit cortical granule exocytosis in vitro. These data suggest that phosphoproteins can prevent exocytosis, but that activation of a phosphoprotein phosphatase is not an essential component of calcium-stimulated cortical granule exocytosis in vitro.

Materials and Methods

Obtaining Gametes

Gametes of the sea urchin species *Lytechinus pictus* (Pacific Biomarine Laboratories Inc., Venice, CA) were spawned by intracoelomic injection of 0.5 M KCl and were collected in artificial sea water (ASW: 435 mM NaCl, 40 mM MgCl₂, 15 mM MgSO₄, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 1 mM EDTA, pH 8.0). The egg jelly was removed by triple passage through 100 μ m Nitex mesh and the eggs were washed twice with ASW. Sperm were collected in a minimum volume of ASW and were stored at 4°C until use. When eggs were immobilized for microinjection or the preparation of cortices, they were attached to glass coverslips coated with poly(L-lysine) (0.02 mg/ml).

Microinjection Techniques

Micropipettes were pulled from glass capillary tubes (1.5-mm internal diameter; Clark Electromedical, Pangbourne, UK) using a microelectrode

puller (Palmer Bioscience, Sheerness, UK). Pressure pulses were applied to the pipette at a frequency of 1 Hz, so that 1 μ l of solution was ejected with each pulse (Swann and Whitaker, 1986). The micropipette was held in a micromanipulator (Narashige Scientific Instruments, Tokyo, Japan) mounted on the stage of a Leitz Diavert microscope. Microinjection was performed by using brightfield microscopy with a 40 \times , 0.65 NA achromat objective. The injected volume was estimated by measuring the extent of cytoplasmic displacement by the injected fluid with an eyepiece graticule.

Assaying Sperm-Egg Fusion

Sperm-egg fusion was assayed using the dye transfer technique described by Hinckley et al. (1986). In this assay, eggs are loaded with a DNA-staining dye, and, upon fusion, sperm nuclei become brightly fluorescent as the dye gains access and binds to the nucleus. Eggs were loaded with the DNA-staining vital dye Hoechst H333342 (20 μ M in ASW for 30 min) and were washed five times in ASW before being stuck onto coverslips. Excitatory light was provided by using a 380-nm bandpass interference filter (10 nm bandwidth) and emitted light passed through a 490-nm bandpass filter. Eggs were viewed within 5 min of insemination and the number of fluorescent sperm nuclei scored.

Fluorescence Measurement of [Ca²⁺]_i

[Ca²⁺]_i was measured by using the calcium-sensitive fluorescent dye fura2. The fura2 (pentapotassium salt; Molecular Probes, Inc., Junction City, OR; 10 mM in 0.5 M KCl/20 mM Pipes, pH 6.7) was introduced into the eggs by microinjection to give a cytoplasmic concentration of \sim 100 μ M. The fluorescence from single eggs was measured as described by Swann and Whitaker (1986). [Ca²⁺]_i was calculated using the methods and binding constants of Poenie et al. (1985).

Preparation of Cortices

Cortices were prepared as previously described (Whalley and Whitaker, 1988). Eggs were attached to poly(L-lysine)-coated coverslips and were rinsed gently with intracellular medium (IM: 220 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 5 mM MgCl₂, 2.5 mM ATP, 10 mM EGTA, pH 6.7). The eggs were sheared with a jet of medium leaving isolated cortical granule fragments attached to the glass. Exocytosis in response to calcium was assessed using a light-scattering assay described previously (McLaughlin and Whitaker, 1988). Calcium-EGTA buffers were prepared using the constants of Martell and Smith (1974).

Preparation of Samples and Protein Analysis by SDS-PAGE

To identify substrates for ATP γ S-mediated phosphorylation, it was necessary to introduce ³⁵S-labeled ATP γ S into large populations of eggs. This was done using an electrical permeabilization technique based on the method of Swezey and Epel (1988). A suspension of eggs was washed into a buffer consisting of 300 mM glycine, 175 mM potassium gluconate, 185 mM mannitol, 50 mM Pipes, 20 mM NaCl, 5 mM MgCl₂, 2 mM EGTA, 2.5% (wt/vol) dextran T10, pH 6.8 (permeabilization buffer [PB]). Eggs were permeabilized as a 25% suspension in 1 ml PB containing 1 mCi ³⁵S-labeled ATP γ S (Amersham International, Amersham, UK). This was done by placing the suspension in a perspex chamber between two stainless steel plates 1 cm apart. The suspension was pulsed three times by discharging a 1 μ F capacitor charged to 100 V using an electrophoresis power pack. Immediately after permeabilization, the eggs were poured into 35-mm-diam petri dishes coated with 1 mg/ml polylysine. They were left for 30 min after which time cortices were prepared in the usual manner. Cortex protein was extracted into 1 M NaOH, precipitated with 25% TCA (final concentration), and washed with acetone several times. Protein was finally dissolved in sample buffer. Proteins separated by SDS-PAGE, as described by Laemmli (1970). After electrophoresis, gels were dried and autoradiographed using β -max film (Amersham International).

Electron Microscopy

To assess the effects of ATP γ S on the interactions between the plasma membrane and the cortical granules, electron micrographs were made of eggs previously microinjected with ATP γ S. After microinjection, eggs were fixed in 20 mM sodium cacodylate-buffered ASW containing 4% formaldehyde and 2% glutaraldehyde for 1 h at 20°C. Eggs were washed in ASW

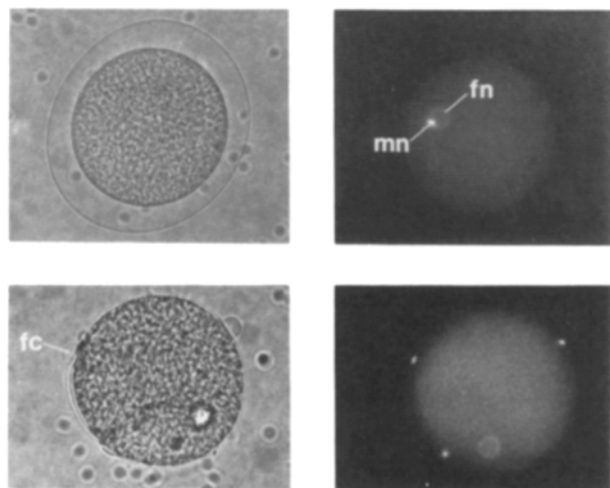
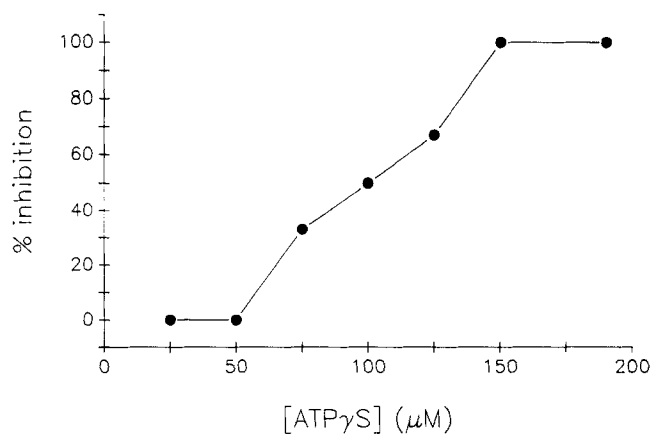


Figure 1. The effects of ATP γ S on cortical granule exocytosis at fertilization. (*Top*) Concentration-effect curve for ATP γ S. ATP γ S was microinjected into eggs and these were fertilized \sim 10 min after microinjection. Exocytosis was assessed by a visual inspection of the eggs 5 min after the addition of sperm. At least six eggs were injected at each of the concentrations shown. (*Bottom*) Photomicrographs of eggs after fertilization. The top panels show a control egg. This has a full fertilization envelope visible as a clear ring surrounding the egg. The bottom panels show an egg which was injected with ATP γ S to a final cytoplasmic concentration of 200 μ M. This egg has several fertilization cones but no fertilization envelope. The fluorescence micrographs (*right*) show the control and ATP γ S-injected egg, preloaded with the DNA-staining vital dye H33342, after fertilization. Excitation: 340–380 nm; emission: 450–490 nm. In the control egg, a single bright spot (*mn*) is visible. It indicates the nucleus of the single sperm that has fused with this egg. The female pronucleus (*fn*) is also indicated. The ATP γ S-injected egg is polyspermic: three fluorescent sperm nuclei have fused and are visible. The egg is 100 μ m in diameter. *L. pictus*. 16°C.

and post-fixed with 1% OsO₄ in ASW for 30 min. After washing, eggs were taken through a graded ethanol series and were dehydrated in propylene oxide for 30 min and embedded in Spurr's resin (EMscope Laboratories Ltd., Ashford, UK). Samples were sectioned and stained with lead citrate.

Reagents

The okadaic acid was a gift from Prof. Philip Cohen, University of Dundee (Dundee, Scotland). ATP and H33342 were from Sigma Chemical Co. (Poole, UK), ATP γ S from Boehringer Chemicals (Lewes, UK), and fura2

from Molecular Probes, Inc. All other chemicals were of analytical grade and were obtained from BDH Limited (Poole, UK).

Results

Microinjection of ATP γ S Inhibits Fertilization Envelope Elevation

Eggs were microinjected with ATP γ S (tetralithium salt, 100 mM in 450 mM KCl, 50 mM Pipes, pH 6.7) to give final cytoplasmic concentrations of between 10 μ M and 2 mM in order to construct a concentration-effect curve. Injected eggs were left for 10 min, inseminated, and examined for exocytosis, judged by the elevation of fertilization envelopes. Fig. 1 shows that exocytosis was inhibited in a dose-dependent fashion. In this experiment, ATP γ S fully inhibited exocytosis at a final cytoplasmic concentration of \sim 150 μ M. Lithium chloride, injected at final concentrations as high as 5 mM, had no effect on fertilization envelope elevation (results not shown).

ATP γ S Does Not Inhibit Sperm-Egg Signal Transduction

We thought that ATP γ S might inhibit exocytosis by preventing the interactions between sperm and egg that lead to a stimulatory increase in [Ca²⁺]_i. A visual inspection of eggs treated with ATP γ S before insemination showed that sperm incorporation was not prevented. Fig. 1 also shows a control egg and an egg injected with ATP γ S to give a final cytoplasmic concentration of 200 μ M after insemination. The control egg has a complete fertilization envelope. The egg injected with ATP γ S has no fertilization envelope, but a number of fertilization cones are clearly visible. Fertilization cones are structures formed by a movement of egg cytoplasm into the region surrounding the site of sperm nucleus, mitochondria and axonemal complex, leading to a protrusion at the site of sperm entry (Longo, 1973). These data indicate that multiple sperm-egg interactions had occurred in ATP γ S-injected eggs.

The effects of ATP γ S on sperm-egg interactions was investigated more fully using the gamete fusion assay described by Hinckley et al. (1986). In this assay, eggs are loaded with the DNA staining vital dye Hoechst 33342. When sperm fuse with eggs that have taken up this dye, their nuclei become brightly fluorescent (Hinckley et al., 1986, Swann et al., 1987). Fig. 1 shows a control egg and an egg that had been injected with 200 μ M ATP γ S. Sperm have penetrated both eggs. In the control egg, one fluorescent sperm nucleus is visible. In the egg injected with ATP γ S, there are three fluorescent sperm nuclei. We scored the numbers of sperm incorporated in both control and injected eggs. Table I shows that the injection of ATP γ S leads to multiple

Table I. The Effect of ATP γ S on the Transfer of H33342 between Egg and Sperm

Eggs	Number of fluorescent sperm nuclei per egg
	mean \pm SEM
Control	1.00 (\pm 0.00, <i>n</i> = 12)
ATP γ S (250 μ M–1 mM)	6.73 (\pm 1.60, <i>n</i> = 15)

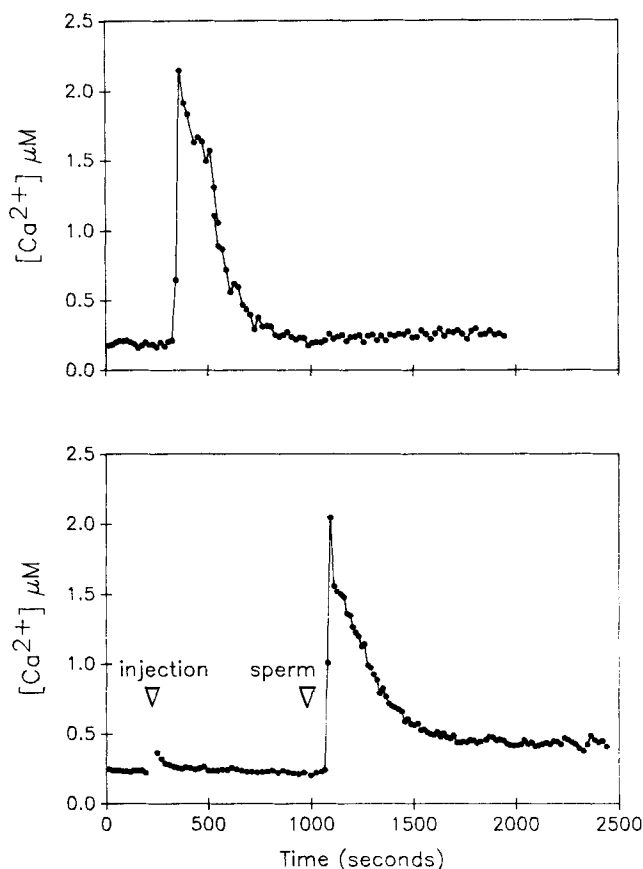


Figure 2. Intracellular free calcium ion concentration measured in single eggs at fertilization. Calcium transients at fertilization in (top) a control egg and (bottom) an egg injected with ATP γ S (final cytoplasmic concentration of 250 μ M) measured with the dye fura2 (final cytoplasmic concentration 25 μ M). In the control egg at fertilization, $[Ca^{2+}]_i$ rapidly increases from \sim 200 nM to over 2 μ M. This then returns to resting levels over the next 10 min. In the ATP γ S-injected egg, the addition of sperm causes a calcium transient very similar in both extent and duration to that at a normal fertilization. These records are typical of six such experiments. Peak $[Ca^{2+}]_i$ (mean and SEM): controls, $1.9 \pm 0.18 \mu$ M; ATP γ S, $1.9 \pm 0.21 \mu$ M. *L. pictus*. 16°C.

sperm fusion events, a consequence of the lack of a fertilization envelope (Whitaker and Steinhardt, 1982). These data indicate that ATP γ S fails to prevent sperm-egg fusion from occurring.

ATP γ S Does Not Affect the Sperm-Induced Rise in $[Ca^{2+}]_i$ at Fertilization

Sperm-egg fusion precedes the generation of the fertilization calcium transient (Chambers and de Armendi, 1979; Longo et al., 1986; Hinckley et al., 1986), so the uptake of H33342 into sperm nuclei in ATP γ S-injected eggs did not preclude the possibility that the rise in $[Ca^{2+}]_i$ was prevented. We examined whether the microinjection of ATP γ S was preventing the sperm-induced rise in intracellular free calcium by measuring the intracellular free calcium ion concentration using the calcium-sensitive fluorescent dye, fura2 (Swann and Whitaker, 1986). Fig. 2 shows that the microinjection of ATP γ S does not prevent a calcium transient occurring upon insemination. In both cases insemination causes an increase

in $[Ca^{2+}]_i$ from a resting concentration of \sim 200 nM to a peak concentration of \sim 2 μ M, in keeping with previous results (Swann and Whitaker, 1986; Crossley et al., 1988).

The Effect of ATP γ S on the Calcium Sensitivity of Exocytosis In Vitro

ATP γ S microinjection inhibits cortical granule exocytosis downstream of the rise in $[Ca^{2+}]_i$. We treated cortices with ATP γ S in two different ways to investigate whether ATP γ S was inhibitory when applied directly to the isolated exocytotic machinery. In a first set of experiments, we incubated cortices for 10 min with 1 mM ATP γ S before calcium stimulation. The calcium sensitivity of preincubated cortices was determined using the light scattering assay previously described (McLaughlin and Whitaker, 1988). Fig. 3 shows the calcium sensitivity of both control and ATP γ S-treated cortices. Treatment with ATP γ S does not affect the calcium-sensitivity of exocytosis: in both treated and untreated cortices, half-maximal exocytosis occurred at a calcium concentration of \sim 5 μ M.

The second set of experiments was designed to test the possibility that ATP γ S acted as a competitive inhibitor of ATP during exocytosis. We prepared Ca^{2+} /EGTA buffers in

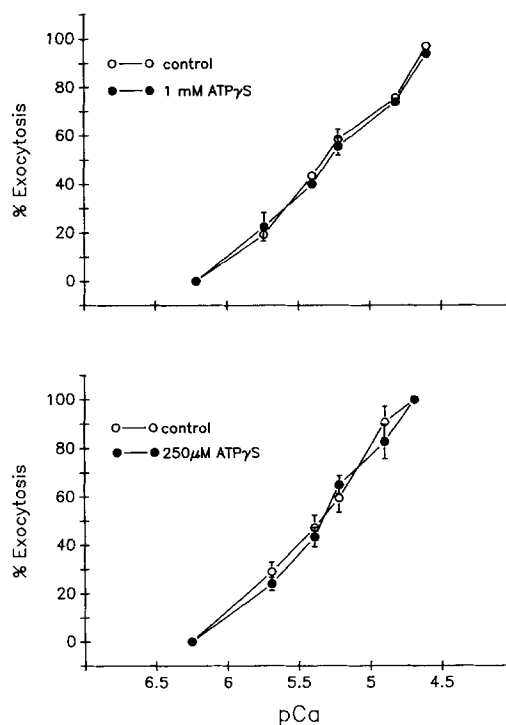


Figure 3. The effect of ATP γ S on the calcium sensitivity of cortices in vitro (Top) The effects of ATP γ S pretreatment on the calcium dependent exocytosis of cortical granules in vitro. Cortices were treated with ATP γ S (1 mM in IM) or IM for 10 min before stimulation with calcium buffers. The extent of exocytosis was measured using a light scattering assay described by McLaughlin and Whitaker (1988). (Bottom) The effects of including ATP γ S (250 μ M) in the calcium buffers used to stimulate cortical granule exocytosis in vitro. Cortices were stimulated with calcium buffers in the presence or absence of 250 μ M ATP γ S. The extent of exocytosis was assessed using the light scattering assay. Mean and SEM are shown ($n = 4$). *L. pictus*. 16°C.

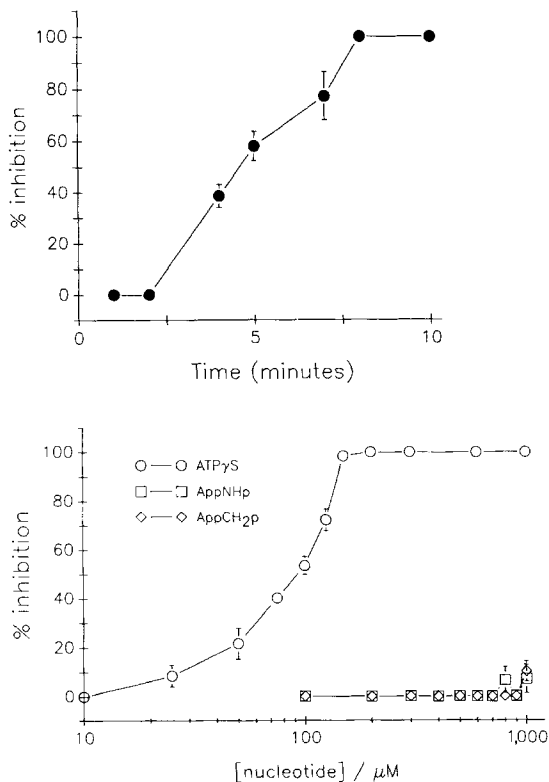


Figure 4. The time course of the inhibition of exocytosis by ATP γ S and the effects of other non hydrolyzable ATP analogues. (*Top*) Eggs were microinjected with ATP γ S to give a final cytoplasmic concentration of 250 μ M and were fertilized at various times after microinjection. Each point represents at least three eggs injected at each time in three separate experiments. Mean and SEM are shown. (*Bottom*) Eggs were microinjected with various concentrations of ATP analogues and fertilized 10 min after microinjection. Results (mean and SEM) are shown from experiments on three egg batches in which 6–10 eggs were injected at each concentration of each analogue. *L. pictus*. 16°C.

IM containing 250 μ M ATP γ S, a concentration which completely inhibits exocytosis in vivo (see Fig. 1). The presence of ATP γ S did not affect the free calcium ion concentration measured with a calcium-sensitive electrode. Fig. 3 also shows that ATP γ S does not significantly affect the calcium sensitivity of exocytosis when present in the triggering calcium buffers. Again, in the presence or absence of ATP γ S, half-maximal exocytosis occurred with a calcium concentration of \sim 5 μ M. These two results show that although ATP γ S inhibits exocytosis in the intact egg, it has no effect on exocytosis in vitro. This suggests that the inhibition in vivo is due either to thiophosphorylation or to an inhibitory compound produced by the metabolism of ATP γ S. The inhibition clearly requires the action of cytosolic factors that are lost when cortices are prepared.

The Effect of β , γ -Imidoadenosinetriphosphate (AppNHp) and β , γ -Methyleneadenosinetriphosphate (AppCH₂p) on Cortical Granule Exocytosis

We microinjected eggs with both AppNHp and AppCH₂p, nonhydrolyzable analogues of ATP that are not kinase substrates (Yount et al., 1971). Eggs were microinjected with

different concentrations of AppNHp and AppCH₂p and sperm were added at least 10 min after microinjection. Neither AppNHp or AppCH₂p had any effect at final concentrations of up to 1.2 mM (Fig. 4). In eggs from the same batches, ATP γ S inhibited exocytosis completely at a concentration of 150 μ M. This confirms the suggestion from our in vitro experiments that ATP γ S is not inhibiting exocytosis by competing with normal, hydrolyzable cellular ATP and supports the idea that the inhibition of exocytosis is due to the production of a metabolite or a thiophosphoprotein.

The Time Course of Inhibition by ATP γ S in Intact Eggs

If ATP γ S were inhibiting exocytosis as a result of some metabolic conversion, or through protein thiophosphorylation, we might expect its inhibitory effects to show a latency. We looked at the time dependency of ATP γ S-mediated inhibition of exocytosis by injecting into eggs a fixed concentration of ATP γ S (250 μ M) and fertilizing after various times. The results of these experiments are also shown in Fig. 4. They show that inhibition of exocytosis by ATP γ S is a time-dependent phenomenon. In these experiments, 100% inhibition did not occur until \sim 7 min after injection, adding weight to the idea that ATP γ S acts as an enzyme substrate in inhibiting exocytosis, rather than as a competitive inhibitor of an ATP-requiring step in the exocytotic pathway.

The Inhibition of Exocytosis by Microinjection of ATP γ S Is Irreversible

We wanted to know whether ATP γ S inhibited exocytosis by being converted into another compound, or if it inhibited exocytosis by thio- (and hence irreversible) phosphorylation. To determine which of these possibilities is the most likely, we prepared cortices from eggs that had been previously microinjected with ATP γ S. If the inhibition were due to the production of an inhibitory metabolite of ATP γ S, then preparing cortices should remove the inhibition by washing the inhibitor away. In contrast, if the inhibition is due to the thiophosphorylation of a cortical protein, the inhibitory effect should not be removed by preparing cortices, so long as the inhibitory protein is tightly associated with the exocytotic apparatus.

We microinjected eggs with either ATP γ S (final concentration: 250 μ M) or a similar volume of injection vehicle (450 mM KCl/50 mM Pipes, pH 6.7). The eggs were left for 10 min and cortices prepared in the usual manner. Cortices were washed several times with IM before the addition of calcium buffers. The cortices were treated with a calcium buffer containing 10 μ M free Ca²⁺. Fig. 5 shows photomicrographs of these experiments. Fig. 5, *top* is a cortex from a normal egg in the presence of 10 mM EGTA. It is covered with cortical granules. Fig. 5, *center* illustrates a cortex prepared from a control egg microinjected with vehicle, then treated in vitro with 10 μ M Ca²⁺. Exocytosis of 70% or more of the cortical granules has occurred. The bottom panel shows the result of adding 10 μ M Ca²⁺ to a cortex prepared from an egg that had been previously microinjected with 250 μ M ATP γ S. A comparison of this panel with the control cortex treated with 10 μ M Ca²⁺ shows that the microinjection of ATP γ S has inhibited exocytosis in the subsequently-isolated cortex. These data show that the inhibition by ATP γ S is irreversible, mak-

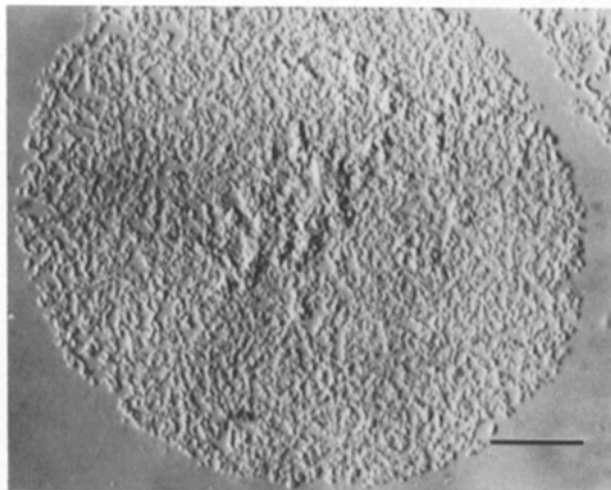
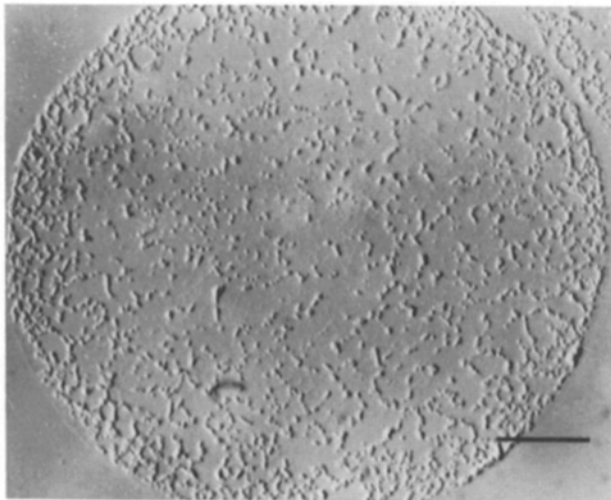
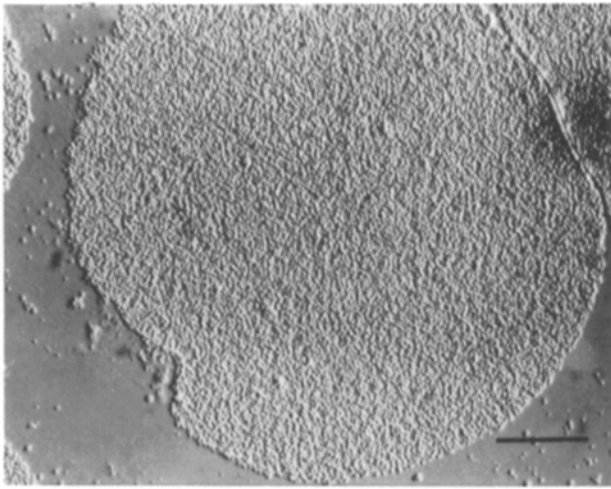


Figure 5. ATP γ S irreversibly inhibits exocytosis. ATP γ S has an irreversible inhibitory effect upon cortical granule exocytosis *in vitro* if the cortex is prepared from an egg microinjected with ATP γ S. Cortices were prepared from eggs microinjected with either KCl/Pipes vehicle or ATP γ S to give a final concentration of 250 μ M. The top panel shows a cortex in the presence of 10 mM EGTA. The center panel is a control cortex after stimulation with 10 μ M Ca $^{2+}$.

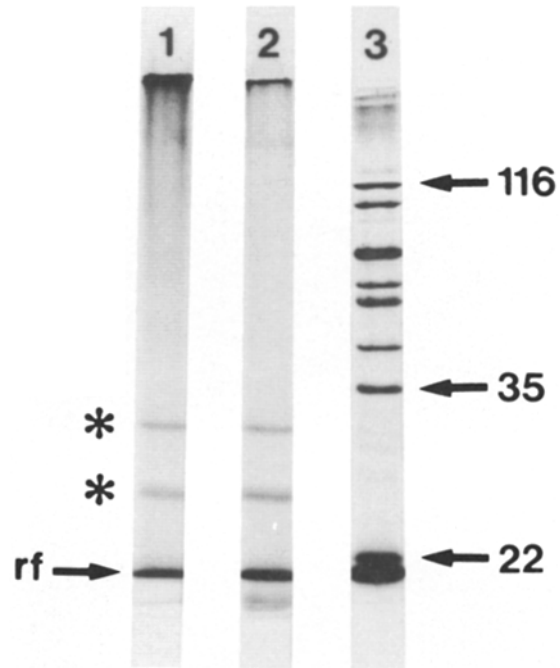


Figure 6. Thiophosphorylation of cortex proteins. An autoradiogram of a polyacrylamide gel of proteins prepared from eggs permeabilized in the presence of 1 mCi [35 S]ATP γ S is shown. Eggs (20%, [vol/vol]) were transiently permeabilized in 1 ml of IM containing 1 mCi [35 S]ATP γ S (>1,000 Ci/mmol). Samples were prepared 30 min later. The sample in lane 1 was prepared from egg cortex. Corresponding whole egg samples were run in lane 2. Approximately 50 times more protein was run in the whole egg lanes. No labeling was detected in either cortex or whole egg samples from eggs incubated with 1 mCi ATP γ S without permeabilization. The two 35 S-labeled bands are marked with an asterisk. No corresponding bands were evident when the gels were stained with Coomassie blue, suggesting that the proteins are not abundant. The arrow indicates the position of the dye front (*rf*) [14 C]-*N*-Ethylmaleimide-labeled protein standards were run in lane 3. The figures to the sides of the autoradiogram are the molecular masses (in kilodaltons) of the appropriate marker proteins. *L. pictus*. 16°C.

ing it improbable that ATP γ S is converted to an inhibitory metabolite within the egg. The most likely explanation of these results is that protein thiophosphorylation via a cytosolic kinase is responsible for the inhibition of exocytosis.

Identification of Substrates of ATP γ S-mediated Thiophosphorylation

We looked for thiophosphorylated proteins by using the technique of electropermeabilization to introduce [35 S]ATP γ S into the cytoplasm of a large number of eggs. 35 S incorporation into permeabilized eggs was \sim 40 times greater than in nonpermeabilized eggs (data not shown). Fig. 6 shows an autoradiogram of a polyacrylamide gel of these samples. The

The bottom panel shows a cortex prepared from an ATP γ S-injected egg after treatment of the cortex with 10 μ M Ca $^{2+}$ for 10 min. Exocytosis has been inhibited in this cortex, although ATP γ S is no longer present. This result suggests that ATP γ S elicits its inhibitory effects through protein thiophosphorylation. *L. pictus*. 16°C. Bar, 10 μ m.

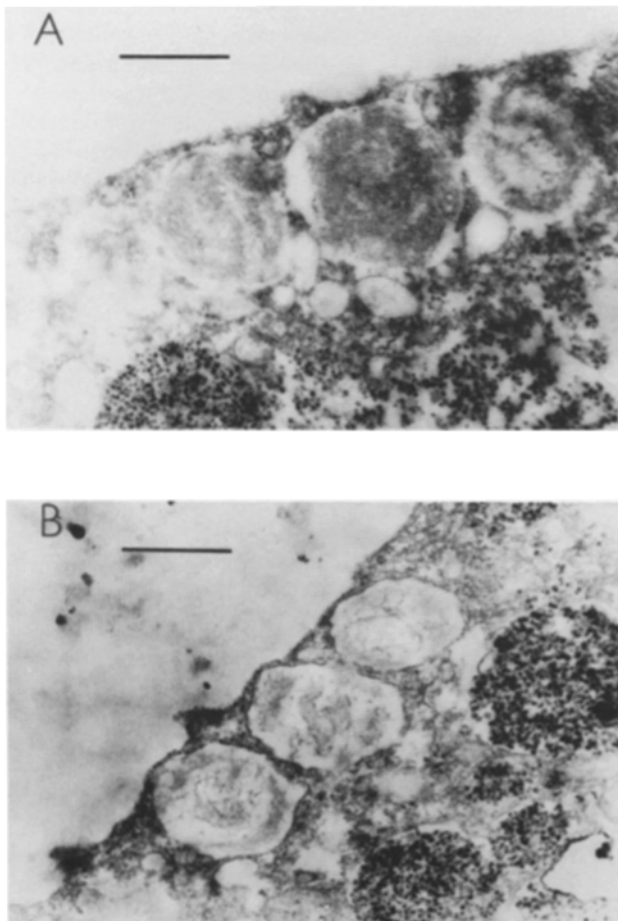


Figure 7. Transmission electron micrographs of egg cortex in whole eggs. (A) An unfertilized egg. (B) An egg that had been microinjected with 200 μM $\text{ATP}\gamma\text{S}$ 20 min before fixation. *L. pictus*. 16°C. Bar, 0.5 μm .

autoradiogram shows that two polypeptides of apparent molecular masses of ~ 33 and 27 kD are strongly labeled both in whole eggs permeabilized with [^{35}S]ATP γS and in cortices prepared from [^{35}S]ATP γS -labeled eggs. The two proteins are relatively enriched in the egg cortex, indicating that the proteins thiophosphorylated by ATP γS in eggs are localized to the plasma membrane.

ATP γS Does Not Affect Plasma Membrane–Cortical Granule Apposition

In the unfertilized egg, the cortical granules are closely apposed to the plasma membrane (Chandler, 1984) and this close contact allows for the isolation of the entire secretory apparatus or cortex (Vacquier, 1975). One explanation for the inhibition of exocytosis by ATP γS is that this close contact might be abolished (Chandler et al., 1989). We investigated this possibility by examining the ultrastructure of ATP γS -injected eggs using electron microscopy. We prepared samples of control eggs and eggs which had been injected with ATP γS to give a final concentration of between 300 and 500 μM . Fig. 7 shows electron micrographs obtained from these eggs. There is no apparent difference between the plasma membrane–cortical granule interactions in eggs injected with ATP γS and control eggs, indicating that

the inhibition of exocytosis is not due to translocation of the cortical granules away from the plasma membrane.

The Effects of Okadaic Acid on Cortical Granule Exocytosis

The above data suggest that protein phosphorylation can antagonize calcium-stimulated exocytosis. We used okadaic acid, a potent inhibitor of several types of protein phosphatase (Bialojan and Takai, 1988; Haystead et al., 1989; Cohen et al., 1990) to test the idea that calcium-stimulated exocytosis may involve activation of a protein phosphatase. Okadaic acid (0.5 mM in ethanol) was microinjected into eggs which were then inseminated. It inhibited exocytosis in a dose-dependent fashion (Fig. 8). Half-maximal inhibition occurred at ~ 400 nM final cytoplasmic concentration, with maximal inhibition at 1 μM . In contrast to the delay seen with ATP γS , microinjection of okadaic acid led to rapid inhibition of exocytosis: eggs fertilized within 2 min of microinjection failed to raise fertilization envelopes. Okadaic acid-injected eggs in which exocytosis was inhibited had a very similar appearance to eggs treated with ATP γS , with pronounced fertilization cones at the sites of gamete fusion (not shown). Inhibition of exocytosis by ATP γS and okadaic acid is additive (Fig. 8).

We tested whether okadaic acid prevented normal sperm-induced calcium transients using the calcium indicator dye fura2. Fig. 9 shows fertilization calcium transients in control and okadaic acid-injected eggs. There is no apparent difference in magnitude between these calcium transients. The transient in okadaic acid-injected eggs declined more rapidly than in control eggs, but this difference showed itself only 60 s after fertilization, while exocytosis in control eggs was complete by 40 s after the onset of the Ca_i transient.

Okadaic Acid Does Not Prevent Calcium-induced Exocytosis In Vitro. Okadaic acid (1 μM) did not affect the calcium sensitivity of exocytosis in cortices isolated from unfertilized eggs (Fig. 9).

Okadaic Acid Irreversibly Inhibits Exocytosis. We microinjected eggs with 1 μM okadaic acid and prepared cortices from the injected eggs 10 min after microinjection. The cortices from okadaic acid-injected eggs failed to respond to 10 μM Ca^{2+} , while cortices from injection controls responded normally (not shown).

Okadaic acid-injected eggs, then, were indistinguishable from ATP γS -injected eggs in our experiments, save that the inhibition was more rapid in onset.

Discussion

The Effects of ATP γS on Exocytosis in Sea Urchin Eggs

We have used both intact, unfertilized sea urchin eggs and the isolated egg cortex to study phosphoprotein control of exocytosis. The intact egg offers information about factors that affect exocytosis in situ, while the isolated egg cortex provides information about factors that are essential to bring about calcium-stimulated exocytosis.

Our experiments in the intact egg indicate that ATP γS -induced inhibition of exocytosis occurs distal to the signal transduction pathways that are responsible for triggering the fertilization [Ca^{2+}]_i transient: sperm-egg fusion and the [Ca^{2+}]_i

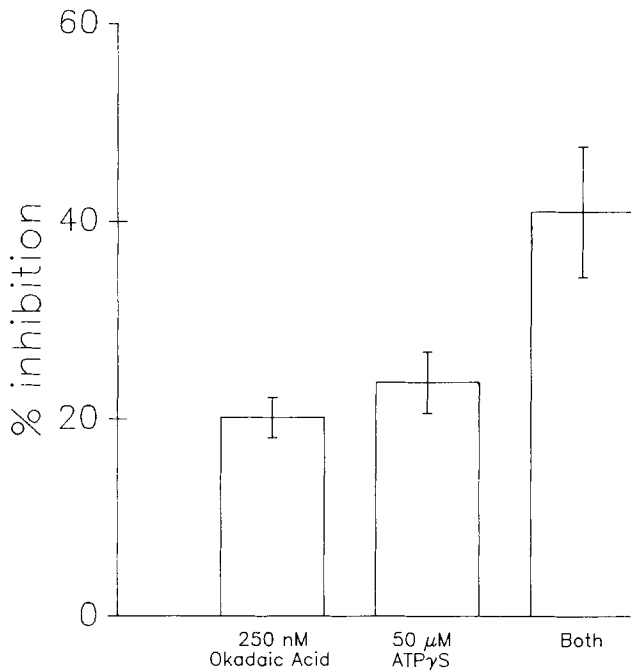
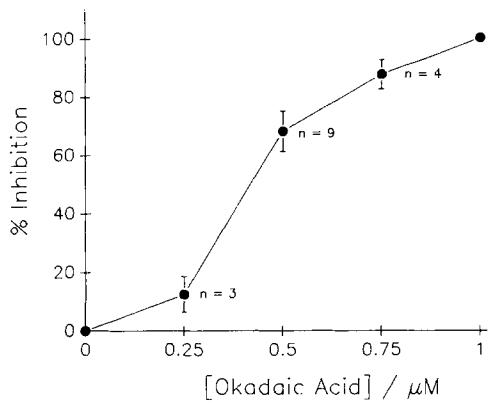


Figure 8. The effects of the phosphatase inhibitor okadaic acid on exocytosis in intact eggs. (Top) Concentration-effect curve for the inhibition of exocytosis at fertilization (fertilization envelope elevation) obtained by microinjecting okadaic acid into unfertilized eggs and then inseminating them. The mean, SEM, and number of experiments at each concentration is indicated. 10–20 eggs were microinjected at each concentration in each experiment. No inhibition of exocytosis was seen in ethanol-injected controls (not shown). (Bottom) Additive effects of okadaic acid and ATP γ S. The data (mean and SEM) are from experiments on six egg batches in which 15–20 eggs were injected for each condition and fertilized 10 min later. *L. pictus*. 16°C.

transient itself are unaffected. In intact eggs, ATP γ S exerts its inhibitory effects on the mechanism that links the rise in $[\text{Ca}_i]$ and exocytosis.

We used the *in vitro* cortical preparation to test whether ATP γ S inhibited exocytosis in the isolated cortical apparatus. We found that it was without effect, either when used to pretreat the cortices, or when present in the Ca^{2+} buffers used to trigger exocytosis. Two pieces of information suggest why calcium-induced exocytosis is blocked in the intact egg, but not in the isolated egg cortex. AppNHp is a nonhydrolyzable analogue of ATP but, unlike ATP γ S, it cannot serve as a phosphate donor for kinases (Yount et al., 1971). It inhibits

processes that require ATP hydrolysis but is incapable of inhibiting exocytosis when injected into eggs. The second pertinent observation is that the inhibition is a time-dependent event. Inhibition is not apparent until several minutes after microinjection, indicating that ATP γ S itself is not immediately inhibitory. These two results suggest that what is important for inhibition of exocytosis is a reaction involving the metabolism of ATP γ S.

Cortices prepared from ATP γ S-injected eggs are unresponsive to calcium, indicating that the effects of ATP γ S are irreversible. Thiophosphorylation has been shown to be

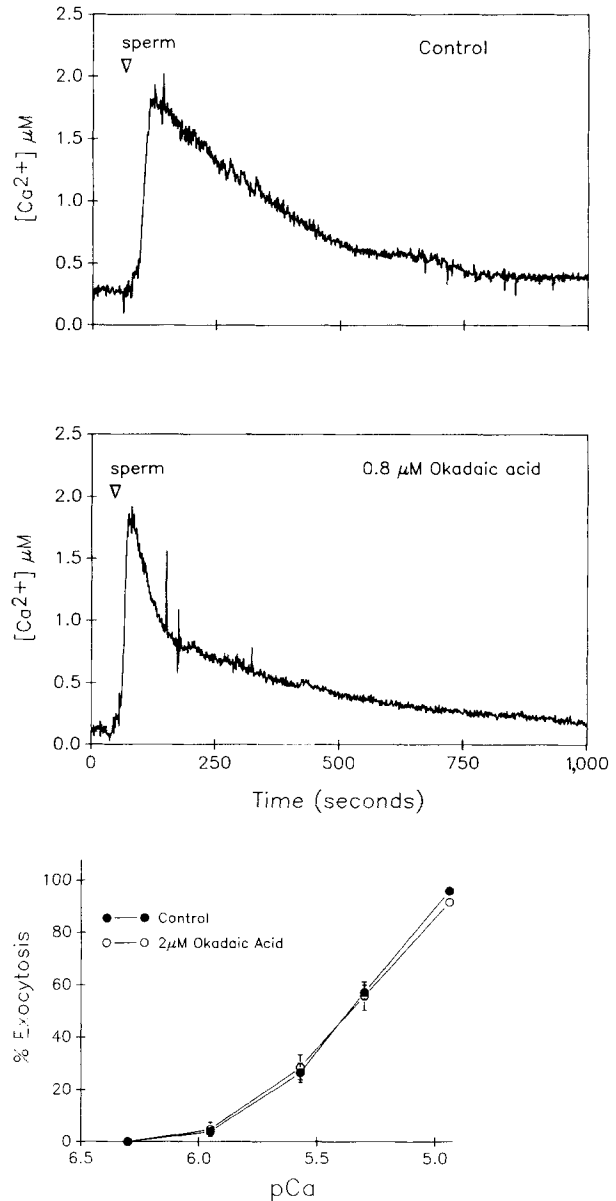


Figure 9. Exocytosis and the fertilization calcium transient in okadaic acid-injected eggs. (Top) The fertilization $[\text{Ca}^{2+}]_i$ transient in okadaic acid-injected eggs. The record is representative of three such experiments. (Bottom) The response of the isolated exocytotic apparatus to calcium under both conditions. Mean and SEM are shown. Each point represents at least four separate determinations. *L. pictus*. 16°C.

irreversible (Gratecos and Fischer, 1974). We have shown that ATP γ S can be used as a thiophosphoryl donor for a kinase in sea urchin eggs. Our data indicate that it is the resulting thiophosphorylation of proteins that is the most likely explanation for the inhibition of exocytosis. The lack of effect of ATP γ S on isolated cortices indicates that the inhibition of exocytosis is due to protein thiophosphorylation by a cytosolic protein kinase.

We identified two proteins which are thiophosphorylated by ATP γ S using ³⁵S-labeled ATP γ S. Only two bands, at 33 and 27 kD, were strongly labeled. They were associated with the egg cortex. These data show that ATP γ S can be used by an egg kinase to thiophosphorylate proteins which are associated with the egg cortex. A 33-kD phosphoprotein has been found in cortical granule membranes (Decker and Kinsey, 1983).

How Might Thiophosphorylation Inhibit Exocytosis?

Thiophosphorylation may alter the interactions between the plasma membrane and cortical granules, perhaps by modifying the cytoskeleton. There is a precedent for this. When intact sea urchin eggs are treated with the tetrasaccharide stachyose and other compounds at high osmotic strengths, exocytosis is inhibited (Zimmerberg and Whitaker, 1985). Studies on the ultrastructure of eggs treated with osmoticants such as stachyose showed that rather than inhibiting exocytosis at the level of membrane fusion, the inhibition was due to the displacement of cortical granules from immediately beneath the plasma membrane, perhaps as a result of actin polymerization (Chandler et al., 1989). ATP γ S might act in a similar way. However, a gross cytoskeletal rearrangement of this sort does not occur in ATP γ S-injected eggs: cortices can be prepared from ATP γ S-treated eggs with the cortical granules still firmly attached. Moreover, our electron micrographs showed no apparent differences in the association of cortical granules and the plasma membrane in control or ATP γ S-injected eggs. ATP γ S may have more subtle effects on the cytoskeleton, but exocytosis in sea urchin eggs is insensitive to cytoskeletal inhibitors, at least in vitro (Whitaker and Baker, 1983).

The processes of membrane fusion during exocytosis have no immediate requirement for ATP (Cockcroft et al., 1987; Howell et al., 1989; Ahnert-Hilger and Gratzl, 1987; Holz et al., 1989; Vilmart-Seuwen et al., 1986; Baker and Whitaker, 1978; Moy et al., 1983; Sasaki and Epel, 1983; Crabb and Jackson, 1985). These observations accord with our finding that exocytosis was unaffected by the nonhydrolyzable ATP analogue AppNHp and suggest that protein phosphorylation per se is not one of the steps in the path to membrane fusion. On the other hand, there is some evidence that a phosphatase activity may trigger exocytosis (Momayezi et al., 1987; Tatham and Gomperts, 1989). The irreversible thiophosphorylation induced by ATP γ S might be expected to frustrate an exocytotic phosphatase; this provides an adequate explanation of the inhibitory effects of ATP γ S. However, this hypothesis is not easily reconciled with our observations of the effects of the phosphatase inhibitor, okadaic acid.

Okadaic Acid Inhibits Cortical Granule Exocytosis Only in Intact Eggs

Okadaic acid is a potent inhibitor of protein phosphatases

PP1 and PP2A and a much less potent inhibitor of the Ca²⁺/calmodulin-dependent protein phosphatase 2B (PP2B) (Cohen et al., 1990). The latter phosphatase (Stewart et al., 1982) is perhaps the best candidate as an exocytotic control in sea urchin eggs, since there is evidence for calmodulin regulation of in vitro exocytosis (Steinhardt and Alderton, 1982). We find that okadaic acid has no effect on calcium-induced exocytosis in isolated cortices. In itself, this observation need not disconcert an advocate of the phosphatase hypothesis, since the only identified phosphatase associated with exocytosis is calcineurin (PP2B)-like (Momayezi et al., 1987) and thus not susceptible to marked inhibition by okadaic acid at concentrations <5 μ M (Cohen et al., 1990). It is our experiments with okadaic acid in intact eggs that are difficult to reconcile completely with the idea that exocytosis in sea urchin eggs is triggered by a calcium-activated phosphatase. The argument is as follows.

Inhibiting exocytosis in intact eggs with okadaic acid in every particular resembles inhibition by ATP γ S, though onset is more rapid. Inhibition by ATP γ S and okadaic acid is additive. Cortices prepared from okadaic acid-injected eggs are refractory to calcium, even though okadaic acid is no longer present, in marked contrast to cortices treated with okadaic acid in vitro. The only plausible explanation of these results is that phosphatase inhibition in the intact egg leads to hyperphosphorylation (Haystead et al., 1989); this cannot occur in vitro: our experiments with ATP γ S indicate that kinase activity is lost when the cortex is separated from the cytoplasm. The corollary is that isolated cortices do not contain active phosphatases either, since any hyperphosphorylation induced by okadaic acid is not intrinsically irreversible and will be susceptible to phosphatases, in contrast to the thiophosphorylation induced by ATP γ S. This argument seems to rule out even a calcineurin-like phosphatase.

Phosphoproteins and the Control of Exocytosis

Tipping the balance of protein phosphorylation/dephosphorylation in unfertilized sea urchin eggs in favor of phosphorylation using ATP γ S or okadaic acid blocks exocytosis. This is evidence that an inhibitory phosphoprotein can intervene in the pathway that links the increase in [Ca_i] to membrane fusion. Phosphatase activity may therefore facilitate exocytosis by relieving this inhibition, as has been suggested by experiments in mast cells, where ATP retards the onset of exocytosis (Tatham and Gomperts, 1989), and in adrenal chromaffin cells, where ATP γ S blocks exocytosis (Brooks et al., 1984) and thiophosphorylates a 43-kD protein (Brooks and Brooks, 1985). However, these data cannot be taken to mean that a phosphatase activity is an essential component of calcium-stimulated membrane fusion. We have shown here using okadaic acid that a thiophosphorylation block does not inevitably imply the participation of a phosphatase in the final steps of exocytosis, as defined by exocytosis in vitro. Our data do not exclude the possibility that activation of a phosphatase forms part of the mechanism that controls exocytosis in the intact egg, only that this aspect of control is absent when exocytosis is studied in vitro.

Only in *Paramecium* is there a well-documented causal relationship between phosphatase activity and secretory granule discharge, both in the intact cell and in vitro (Gilligan and Satir, 1982; Zieseniss and Plattner, 1985; Momayezi et al., 1987; Stecher et al., 1987). In sea urchin eggs, the bal-

ance of evidence argues against a strict causal relationship. Nevertheless, identifying and isolating the inhibitory thiophosphoproteins will enable us to study their interactions with the unknown proteins that mediate membrane fusion and perhaps provide a way of isolating the essential components of the exocytotic mechanism.

We thank Dr. Rajnikant Patel and Dr. Keith Caddy for their help and advice and Professor Philip Cohen for the gift of okadaic acid.

The work was supported by grants from the Science and Engineering Research Council and the Wellcome Trust. T. Whalley was an SERC Scholar and I. Crossley received a Studentship from the Wellcome Trust.

Received for publication 9 August 1990 and in revised form 17 January 1991.

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