THE CONTROL OF EXOCYTOSIS IN SEA URCHIN EGGS.

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ABSTRACT.

Fertilisation in the sea urchin egg is characterised by a transient increase in intracellular free calcium. This triggers the exocytosis of the cortical secretory granules which lie immediately beneath the plasma membrane, producing a structure called the fertilisation envelope. The exocytotic apparatus or cortex can be isolated and responds to the physiological trigger – Ca$^{2+}$ – in vitro; it is an ideal system in which to study exocytosis directly. I have developed a method of reconstituting the exocytotic apparatus. This is a very useful technique because it allows manipulation of individual components of the system. I have used this technique to study the effects of calcium ion treatment of each component of the system. I show that this does not affect the responsiveness of subsequent reconstitution.

I have investigated the mechanisms by which various compounds activate eggs and cause the increase in [Ca$^{2+}$]$_i$ which triggers exocytosis. Using a permeabilised egg preparation I show that neither guanosine 3',5'-cyclic monophosphate (cGMP) nor guanosine 5'-O-(3'-thio-triphosphate) (GTPyS – a compound which causes irreversible activation of GTP–binding proteins), directly stimulate the release of calcium from intracellular stores. Using an indirect single cell assay for DAG by measuring pH changes due to activation of a Na$^+$/H$^+$ antiport, I show that GTPyS but not cGMP can stimulate the enzyme phosphoinositidase C in the absence of an increased intracellular free calcium. I also show that, under the conditions of this assay, insemination fails to activate the Na$^+$/H$^+$ antiport. This suggests that sperm do not trigger the array of events which occur at fertilisation by interaction with GTP–binding proteins.
It has been suggested that a change in the phosphorylation state is important in secretory control in many cells. I have investigated whether this also the case in the sea urchin egg by microinjecting into eggs the compound adenosine-5'-O-(3′-thiotriphosphate) (ATPyS) which can be used to irreversibly phosphorylate proteins. ATPyS prevents fertilisation envelope elevation after insemination but does not prevent the sperm induced rise in intracellular free calcium. I present evidence to show that ATPyS requires the presence of cytoplasm in order to elicit its inhibitory effects. I also show that the effect is irreversible and that ATPyS is not itself the inhibitor. The results with this compound strongly suggest that a protein, when phosphorylated inhibits exocytosis in the sea urchin egg.
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Chapter 1. Introduction.

1. Sea Urchin Egg Activation at Fertilisation.

One of the fundamental problems in biology is to understand the mechanism by which interactions between sperm and egg at fertilisation can lead to the plethora of responses required for the onset of cell proliferation and development. One of the best studied systems of fertilisation is that of the sea urchin. The reason for using sea urchins as models of fertilisation is that the gametes are easily obtained in large numbers. This allows the use of biochemical approaches to elucidate activation events at fertilisation. The sea urchin egg has also been much studied because fertilisation is accomplished relatively simply in this animal. All that is required is the mixing of eggs and sperm in sea water.

1.1 WHAT HAPPENS AT FERTILISATION?

When a sperm fertilises an egg, a whole array of events occur which change it from being a quiescent to an actively-dividing cell. Table 1.1, taken from Whitaker and Steinhardt, 1982, shows the events which occur in the egg during the first few minutes following fertilisation.

<table>
<thead>
<tr>
<th>Table 1.1</th>
<th>Events Initiated at Fertilisation.</th>
</tr>
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<tbody>
<tr>
<td>Na dependent action potential</td>
<td></td>
</tr>
<tr>
<td>release of $\text{Ca}^{2+}$ from intracellular stores</td>
<td></td>
</tr>
<tr>
<td>cortical granule exocytosis</td>
<td></td>
</tr>
<tr>
<td>changes in the cytoskeleton</td>
<td></td>
</tr>
<tr>
<td>activation of NAD kinase</td>
<td></td>
</tr>
<tr>
<td>increased levels of reduced nicotinamide nucleotides</td>
<td></td>
</tr>
<tr>
<td>cytoplasmic alkalinisation</td>
<td></td>
</tr>
<tr>
<td>activation of mitochondria and increased oxygen uptake</td>
<td></td>
</tr>
<tr>
<td>lipid peroxidation</td>
<td></td>
</tr>
<tr>
<td>increased tyrosine kinase activity</td>
<td></td>
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1.2 THE IONIC HYPOTHESIS OF EGG ACTIVATION.

We now know that the activation of all of the events triggered by gamete interactions at fertilisation, is caused by changes in the concentrations of two ions, \( \text{Ca}^{2+} \) and \( \text{H}^+ \). That these two signals alone are sufficient to cause all of the other events associated with egg activation constitutes the ionic hypothesis of egg activation (Whitaker and Steinhardt, 1982). Let us consider the evidence that has shown that these two ionic changes, an increase in \([\text{Ca}^{2+}]_i\), and a decrease in \([\text{H}^+]_i\), can initiate the array of events at fertilisation.

(i) The Role of \( \text{Ca}^{2+} \) in Egg Activation.

It was first demonstrated by Steinhardt et al. (1977) that a transient rise in the intracellular free calcium concentration occurred at fertilisation. This was shown by microinjecting the \( \text{Ca}^{2+} \)-sensitive photoprotein aequorin into eggs and measuring the light output on insemination. \( \text{Ca}^{2+} \)-specific ionophores were shown to cause parthenogenetic activation (Chambers et al., 1974; Steinhardt and Epel, 1974). These too, cause activation by an increase in \([\text{Ca}^{2+}]_i\), (Zucker et al. 1978). Ionophores cause egg activation by releasing \( \text{Ca}^{2+} \) from intracellular stores, since there is no requirement for calcium in the extracellular medium (Chambers et al. 1974; Schmidt et al. 1982). It has also been shown that egg activation cannot occur in the absence of an increase in the intracellular free calcium ion concentration. Microinjection of the calcium chelator EGTA prevents the elevation of a fertilisation membrane upon insemination (Zucker and Steinhardt, 1978). These experiments showed that \([\text{Ca}^{2+}]_i\), increased at fertilisation, that activation could be caused by the addition of \( \text{Ca}^{2+} \) ionophore to unfertilised eggs, and that blocking an increase in intracellular free calcium prevented activation, demonstrating that calcium is an essential signal in the activation of sea urchin eggs.
(ii) The Role of pH in Egg Activation.

However, an increase in \([\text{Ca}^{2+}]_i\) is not the only ionic signalling mechanism occurring at fertilisation. There is also an increase in intracellular pH. Using homogenates, the pH was shown to increase from 6.4 to 6.7 at fertilisation (Johnson et al. 1976) but using pH-sensitive microelectrodes in *L. pictus* eggs the pH was found to rise from 6.84 to 7.27 (Shen and Steinhardt, 1978). The latter values were confirmed using a DMO distribution method (Johnson and Epel, 1981) and these latter values are now regarded as fairly accurate in describing the pH of eggs before and after activation. The cytoplasmic alkalinisation is essential to the development of a newly fertilised egg. Indeed, some aspects of activation at fertilisation can be mimicked by the application of bases such as ammonia which cause activation in the absence of a significant increase in intracellular free calcium (Patel et al. 1989), and acts by causing cytoplasmic alkalinisation. Activation with bases such as ammonia stimulates both protein and DNA synthesis (Grainger et al. 1979).

If \(\text{Na}^+\) is removed from the sea water at fertilisation, the alkalinisation is prevented, (Shen and Steinhardt, 1978), and development arrested. This block can be overcome by the addition of NH\(_3\) (Chambers, 1976) illustrating that pH changes are required for successful development. A \(\text{Na}^+/\text{H}^+\) antiport is activated at fertilisation (Johnson et al. 1976, Payan et al. 1983) and this is responsible for the rapid proton efflux that accompanies cytoplasmic alkalinisation (Mehl and Swann, 1961; Johnson et al. 1976). Although \(\text{Ca}^{2+}\) itself can activate the \(\text{Na}^+/\text{H}^+\) antiport, the change in pH is essential for development. The changes initiated by alkalinisation are not themselves calcium dependent. Of all of the events occurring at fertilisation only cortical granule exocytosis and NAD\(^+\) kinase activation occur solely under the control of \([\text{Ca}^{2+}]_i\) (Whitaker and Steinhardt, 1982).

This, very briefly, then, is the evidence in favour of the ionic hypothesis of egg activation in which the increases in the intracellular concentrations of \(\text{Ca}^{2+}\) and pH alone are sufficient to trigger all of the
events associated with the transition from quiescence to proliferation. In summary, 1) the two ionic signals occur at fertilisation, 2) activation of all of the processes at fertilisation can be caused by changing the concentrations of Ca$^{2+}$ and H$^{+}$, and 3) the events at fertilisation can be blocked by preventing these ionic changes from taking place. The two ionic signals synergise in nearly all aspects of activation with the exception of cortical granule exocytosis and NAD$^{+}$ kinase activation.

1.3 THE FERTILISATION CALCIUM WAVE.

The elevation of the fertilisation envelope, a morphological marker for egg activation, proceeds from the point of sperm entry across the egg and is caused by the exocytosis of the cortical secretory granules which lie immediately beneath the plasma membrane. This progressive elevation is due to a progressive increase in Ca$^{2+}$ across the egg. Exocytosis of the cortical secretory granules can be triggered solely by the addition of micromolar calcium ion concentrations. This has been shown most elegantly using an in vitro preparation first described by Vacquier (1975). Sea urchin egg plasma membranes can be isolated by shearing eggs stuck down on polylysine–coated glass in the presence of calcium–free buffers. These plasma membrane fragments or cortices keep their array of cortical granules and these exocytose in response to micromolar calcium ion concentrations (Baker and Whitaker, 1978). An endoplasmic reticulum network exists between the plasma membrane and the cortical granules (Chandler, 1984; Sardet, 1984). However, the calcium wave does not seem to propagate through this network since addition of Ca$^{2+}$ to a small area of cortical lawn does not propagate exocytosis across the whole of the lawn (Whitaker and Steinhardt, 1982). The wave of Ca$^{2+}$ has been illustrated using the Ca$^{2+}$–sensitive protein aequorin in different species (Eisen et al. 1984; Swann and Whitaker, 1986). In L. pictus, the increase in [Ca$^{2+}$], proceeds from the point of sperm egg interaction and sweeps across the egg in 15–25 seconds (Swann and Whitaker, 1986). This time course of calcium progression across the egg is consistent with the hypothesis that it is responsible for the
exocytosis of the cortical granules since the wave of exocytosis has a duration of 22 seconds in *L. pictus* (Whitaker and Irvine, 1984).

2. The PPI Second Messenger System.

It is now known that the activation of a sea urchin egg occurs through the increase in \([Ca^{2+}]_i\), and a decrease in \([H^+]_i\), but the question is, how are these signals generated? It is thought that these signals are linked to changes in the levels of the phosphoinositide second messengers, the water soluble inositol 1,4,5-trisphosphate (InsP₃), and the highly lipophilic molecule sn-1,2-diacylglycerol (DAG), which are generated through the action of the enzyme phosphoinositidase C (PIC) on the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PtdInsP₂).

It was suggested by Michell (1975) that turnover of phosphatidylinositol phospholipids was linked to changes in intracellular Ca²⁺ but the distinction between this being an effect of the rise in calcium rather than the cause could not be made. In 1983, Berridge showed that in blowfly salivary glands, there was a rapid agonist-stimulated breakdown of polyphosphoinositides prior to the calcium increase (Berridge, 1983). InsP₃ formation was stimulated by 5-HT with no apparent lag time but the mobilisation of Ca²⁺ was delayed by at least 1 second. Increases in InsP₃ following agonist stimulation prior to any physiological responses were also seen in liver cells (Drummond *et al.* 1984), confirming that the synthesis of InsP₃ was rapid enough for it to be ascribed a second messenger function. Furthermore, InsP₃ was shown to release Ca²⁺ from non-mitochondrial stores in both pancreatic acinar cells (Streb *et al.* 1983) and in hepatocytes (Burgess *et al.* 1984). This evidence all pointed towards InsP₃ being the second messenger responsible for agonist-stimulated release of Ca²⁺ from intracellular stores, and this is now a widely agreed mechanism by which agonists can cause an increase in intracellular free calcium.

The enzyme phosphoinositidase C also generates another second messenger molecule, diacylglycerol or DAG. This molecule is a potent
activator of protein kinase C, a Ca\(^{2+}\) and phosphatidylserine-dependent cellular kinase (Nishizuka, 1984; Castagna et al. 1982). The generation of InsP\(_3\) and DAG by the hydrolysis of PtdInsP\(_2\) is a cellular process thought to be under the control of GTP-binding proteins (Cockcroft and Gomperts, 1985) which were previously shown to be involved in the signal transduction mechanism of hormones which act through adenylate cyclase (Pfeuffer and Helmreich, 1975). Figure 1.1 illustrates a generalised outline of the polyphosphoinositide messenger system.

2.1 THE ROLE OF InsP\(_3\) IN [Ca\(^{2+}\)]\(_i\) HOMEOSTASIS.

In mammalian cells there are both mitochondrial and non-mitochondrial Ca\(^{2+}\) buffering pools. The mitochondrial pool is characterised by having a high capacity but a relatively low affinity (in the micromolar range) whereas the non-mitochondrial pool is of smaller capacity but has an affinity for calcium in the nanomolar range (Burgess et al. 1983). The role of InsP\(_3\) in the control of intracellular free calcium has now been well characterised in a number of cells. In saponin-permeabilised hepatocytes, InsP\(_3\) released Ca\(^{2+}\) from an ATP-dependent non-mitochondrial store with a half maximal concentration of 200 nM (Burgess et al. 1984) but only a maximum of 30% of the sequestered calcium could be released by InsP\(_3\).

In the rat insulinoma cell line RINm5F, InsP\(_3\) released 90% of the A23187-releasable (and hence non-mitochondrial) pool (Prentki et al. 1985). In amoebae of the slime mold Dictyosteleum discoideum, 5 \(\mu\)M InsP\(_3\) released 70% of \(^{45}\)Ca sequestered by the non-mitochondrial store in response to ATP (Europe-Finner and Newell, 1986). So in cells from both simple and complex eukaryotes, InsP\(_3\) is important in the regulation of intracellular calcium concentrations. It acts upon a non-mitochondrial calcium store. In permeabilised fibroblasts, InsP\(_3\) induced the rapid release
Figure 1.1) The PtdInsP$_2$ second messenger signalling system.

An outline of the cellular mechanisms involved in receptor coupled transmembrane signalling through the phosphoinositide C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate and in the metabolism of the inositol 1,4,5-trisphosphate and 1,2-diacylglycerol signals generated by this signalling system. Agonist-stimulation of PIC through a GTP-binding transducing protein results in the formation of the two second messengers, InsP$_3$ and DAG. InsP$_3$ releases calcium from intracellular stores which can activate calmodulin, while DAG activates protein kinase C. These two second messengers synergise in the initiation of responses to hormones.
of 85% of sequestered $^{45}$Ca taken up in response to ATP. In these cells both the production of InsP$_3$ by GTP and the agonist–induced release of calcium from intracellular stores were inhibited by pre–incubation with the protein kinase C agonist TPA, (Muldoon et al. 1987). These results suggest that the down–regulation of the phosphoinositide second messenger system is accomplished through DAG–stimulated protein kinase C activation.

2.2 THE ROLE OF DAG AS A SECOND MESSENGER.

As well as InsP$_3$, DAG, the other product of PIC hydrolysis of PtdInsP$_2$ has a second messenger role (Nishizuka, 1984, 1986). DAG is a potent activator of a Ca$^{2+}$ and phospholipid protein kinase, protein kinase C, (Takai et al. 1979; Castagna et al. 1982). Together with phosphatidylserine, DAG enhances the affinity of protein kinase C towards calcium so that it is active at resting [Ca$^{2+}$], (Kaibuchi et al. 1981). Protein kinase C can stimulate an increase in intracellular pH in fibroblasts (Moolenaar et al. 1984). DAG, by activating protein kinase C, can, as I shall discuss later, modulate exocytosis in a number of different cells.

2.3 PPI SECOND MESSENGERS AT FERTILISATION.

As outlined above, the phosphoinositide second messengers could be used to generate the two ionic changes responsible for triggering the onset of development at fertilisation. There is now good evidence in support of these two second messengers being produced by the interaction of sperm and eggs. At fertilisation there is a rapid turnover of the phosphoinositide phospholipids (Sheetz, Turner and Jaffe 1984, Kamel et al. 1985) with increased levels of both PtdInsP and PtdInsP$_2$ through the activity of PI and PIP kinase (Oberdorf, Vilar–Rojas and Epel, 1989). Ciapa and Whitaker (1986) have shown that InsP$_3$ and DAG production occurs at fertilisation, coincident with the fertilisation calcium transient, which is consistent with them being important signals in the activation process.
(i) **InsP₃ Causes Calcium Release in Sea Urchin Eggs.**

Work investigating lipid metabolism at fertilisation showed that PtdInsP₂ was hydrolysed at fertilisation. There is also substantial evidence in support of a role for InsP₃ and DAG in causing the ionic signals at fertilisation. Whitaker and Irvine (1984) showed that the microinjection of InsP₃ caused activation as judged by the elevation of fertilisation envelopes. This suggests that InsP₃ is the messenger which causes the increase in [Ca²⁺], essential for activation at fertilisation. It was shown that InsP₃ did indeed release calcium from intracellular stores by Clapper and Lee (1985). Using both calcium sensitive electrodes and the calcium sensitive indicator dye quin2 they showed calcium release on InsP₃ addition with half maximal release at 50–60 nM and maximal release at 1 μM. Density gradient centrifugation showed that this calcium sequestering/releasing component co–purified with the endoplasmic reticulum. Furthermore, 30 nM InsP₃ caused 20% of maximal release, this concentration is close to the 10 nM reported by Whitaker and Irvine (1984) to be the minimum concentration of InsP₃ capable of causing fertilisation envelope elevation. In intact eggs, the microinjection of InsP₃ causes an increase in [Ca²⁺], (Swann and Whitaker, 1986). Thus in sea urchin eggs, InsP₃ releases calcium from endoplasmic reticulum stores.

At fertilisation, the elevation of the fertilisation envelope is initiated from the site of sperm–egg fusion. Whitaker and Irvine (1984) showed that microinjection of InsP₃ caused an exocytotic wave to move across the egg in a manner consistent with a self–propagating wave that started at the point of injection. InsP₃ microinjection also caused cytoplasmic alkalinisation similar to that which occurs at fertilisation. Calcium can stimulate an egg plasma membrane PIC (Whitaker and Aitchison, 1985), and Swann and Whitaker (1986) have shown that neomycin inhibits the propagation of the calcium wave when InsP₃ is microinjected into eggs. These results suggest that at fertilisation, the wave of calcium release from the point of sperm–egg interaction may be due to a self–propagating wave of calcium release and calcium–stimulated PtdInsP₂ hydrolysis.

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DAG Causes Cytoplasmic Alkalinisation in Sea Urchin Eggs.

The other second messenger generated by PIC, DAG, is also important in egg activation at fertilisation. Exogenously added DAG, or the protein kinase C "agonist" TPA (but not a less active methylated TPA) cause cytoplasmic alkalinisation (Shen and Burgart, 1986; Swann and Whitaker, 1985). This effect has been shown to be due to activation of the Na⁺/H⁺ antiport since dimethylamiloride, which is a specific inhibitor of this transporter (Vigne et al. 1984), prevents the pH change caused by TPA (Swann and Whitaker, 1985). The Na⁺/H⁺ exchanger is thought to be responsible for cytoplasmic alkalinisation at fertilisation (Payan et al. 1983). Treatment of eggs with TPA also initiates protein synthesis (Swann and Whitaker, 1985; Lau et al. 1986), one of the consequences of a rise in pH at fertilisation. Finally, it is important to note that these effects are independent of changes in [Ca²⁺]i (Ciapa et al. 1988).

These results provide good evidence to show that the two products of PtdInsP₂ hydrolysis are responsible for the production of the two ionic signals occurring at fertilisation. InsP₃ causes Ca²⁺ release from intracellular stores while DAG can stimulate H⁺ efflux by stimulating protein kinase C and the Na⁺/H⁺ antiport.

2.4 HOW DOES A SPERM ACTIVATE AN EGG?

All of the evidence presented thus far indicates that the activation of an egg by sperm is caused by an increase in intracellular free calcium which in turn causes cytoplasmic alkalinisation, and that this ionic signalling involves the phosphoinositide second messenger system. There are two ideas which have been suggested to describe how a sperm activates an egg. The first is that it acts in a manner analogous to the interaction of a hormone with a G–protein–linked receptor. The second is that the sperm injects some activator compound. These two schemes are illustrated in figure 1.2.
Figure 1.2) A schematic representation of egg activation mechanisms.

The scheme on the left illustrates egg activation through a mechanism analogous to that of a hormone interacting with a cell surface receptor. The sperm binds to a receptor on the egg plasma membrane, and activates PIC through a G-protein. InsP$_3$ then causes the release of Ca$^{2+}$ from intracellular stores. The scheme on the right shows how fusion of gametes might cause egg activation. Upon fusion, the sperm introduces a factor which causes localised calcium release. This calcium stimulates PIC and initiates the autocatalytic self-propagating wave of calcium release across the whole egg.

In the first scheme, the sperm binds to an egg surface receptor and this transduces the message across the plasma membrane, PIC being activated by a regulatory G-protein. The evidence in support of this is that there is a specific sperm binding protein on the egg surface (Vacquier and Moy, 1977) and in addition, eggs can be activated by GTP\(_\text{yS}\), a non-hydrolysable analogue of GTP which irreversibly activates GTP-binding proteins (Turner et al. 1986), and cholera toxin (Turner et al. 1987), both of which are known to interact with receptor/effector coupling-proteins or GTP-binding proteins (Pfeuffer and Helmreich, 1975; Helmreich and Pfeuffer, 1985). Ciapa and Whitaker (1986) have shown that production of InsP\(_3\) and DAG occur some seconds before the onset of an increase in [Ca\(^{2+}\)], suggesting sperm-induced activation of PIC. These results suggested that egg phosphoinositidase C could be activated by a route independent of the previously described calcium activation route (Whitaker and Aitchison, 1985). This evidence has been used as the basis for a scheme in which sperm-egg binding is coupled to the activation of PIC through the mediation of a G-protein in a manner analogous to the mechanism of activation of somatic cells by some, for instance, hormones which stimulate cells by receptor binding coupled to G-protein activation of phosphoinositidase C.

(ii) The Fusion Model of Egg Activation.

The second hypothesis is that upon fusion of sperm and egg the sperm injects some activator compound which triggers the multitude of events which occur at fertilisation. It is known that the fusion of gametes occurs prior to the elevation of [Ca\(^{2+}\)]\(_i\) and cortical granule exocytosis (Hinckley et al, 1986; Longo et al, 1986; Chambers and de Armendi, 1979), and this is consistent with this second possible activation route; the delay between the first electrophysiological signs of fertilisation and the onset of the Ca\(^{2+}\) transient being conceivably due to the diffusion of an activator molecule.
from sperm to egg (Whitaker et al. 1989). One piece of evidence in favour of this hypothesis is that an extract made from sperm causes activation when microinjected (Dale et al. 1985). One possible candidate for an activator molecule is cGMP. This molecule occurs in high concentrations in activated sperm (Kopf et al. 1979) and when microinjected activates eggs by causing a Ca\(^{2+}\) transient similar to that occurring at fertilisation (Swann, Ciapa and Whitaker, 1987). Other possible activator molecules include nicotinamide nucleotides which Clapper et al. (1988) showed could release calcium from non–mitochondrial stores and InsP\(_3\) itself which activates eggs when microinjected (Whitaker and Irvine, 1984).

3. Exocytosis – A Universal Secretory Mechanism.

Exocytosis is a ubiquitous cellular mechanism for the export of secretory products and the insertion of proteins and lipid into the plasma membrane. All cases of secretory exocytosis involves the fusion of pre–packaged secretory granules with the plasma membrane and the release of the secretory granule contents into the extracellular space. Exocytosis may be described as being of two types, constitutive (Kelly, 1985), and triggered. The work in this thesis concentrates on one example of triggered exocytosis, that is where secretory granules are stimulated to fuse with the plasma membrane in response to particular stimuli.

A general feature of all exocytotic events is that fusion and the release of the granule contents occurs without the release of cytoplasmic constituents. It was found that the proportion of catecholamines to other substances was very similar in both chromaffin granules and in the perfusate of stimulated adrenal glands (Douglas, 1968). This suggested that exocytosis involved the expulsion of discretely packaged intracellular compartments. In other words, it is a means of releasing materials from cells in a highly specific fashion whilst conserving the biochemical integrity of the cell. The quantal nature of exocytosis, in which prepackaged granules fuse with the
plasma membrane has been most elegantly demonstrated by measuring capacitance changes in patch clamped secretory cells. Discrete changes of the capacitance (and thus surface area) were demonstrated upon stimulation of single chromaffin cells (Neher and Marty, 1982). These capacitance changes were due to the fusion of individual secretory granules.

Exocytosis in the sea urchin egg is an example of triggered exocytosis, the trigger being, at fertilisation, a sperm induced rise in the intracellular free calcium ion concentration. Sea urchin egg exocytosis results in the formation of the fertilisation envelope which is a physical barrier to further sperm reaching the egg and is thus an effective barrier to polyspermy. The prevention of polyspermy is essential in allowing normal embryonic development to progress.

3.1 THE ROLE OF CALCIUM IN THE CONTROL OF EXOCYTOSIS.

Exocytosis (or more correctly triggered exocytosis since many cells undergo continuous non-stimulated exocytosis) is controlled by a number of second messengers. Until recently it has been viewed as a process universally under the control of Ca\(^{2+}\). A general feature of stimulated exocytosis is a transient increase in the intracellular free calcium concentration, typically from a resting level of approximately 100 nM to a stimulated level approaching micromolar (Llinas et al. 1981; Knight and Kesteven, 1983; Rink and Hallam, 1983; Rorsman et al. 1984; O'Sullivan et al. 1989).

Many of the ideas about how exocytosis may be regulated and its underlying mechanisms have been determined using biophysical approaches; that is, using artificial lipid bilayers to study the changes in membrane properties that occur upon fusion, and the factors that induce it. Using such model systems it is clear that the repulsive forces between the opposing bilayers need to be overcome in order for the necessary juxtaposition of bilayers to occur. There is both electrostatic repulsion and,
more importantly at low separation (20–30 Å), hydrostatic forces (Cowley et al. 1978). Fusion of bilayers has been suggested to involve phase transitions. Changes from lamellar to inverted-hexagonal phase may encourage the fusion of bilayers (Siegel 1987), and the tendency for bilayers to undergo such transitions is dependent on biological lipids such as cholesterol (Cullis et al. 1978), cholesterol esters (Tilcock et al. 1974), diacylglycerol (Dawson et al. 1984, Das and Rand, 1985, 1986) and lysophospholipids (Poole et al. 1970).

In artificial systems, fusion of vesicles to pure lipid bilayers requires Ca$^{2+}$, but at high (mM) concentrations (Cohen et al. 1980), the same factor associated with the stimulation of exocytosis in vivo. When a calcium-binding protein was incorporated into the bilayer, fusion was greatly enhanced in the presence of Ca$^{2+}$ at more physiologically relevant concentrations. This effect was specific for Ca$^{2+}$, which was effective at 10 μM, (Mg$^{2+}$ at 2 mM had no effect) (Zimmerberg et al. 1980). Here is an instance of a biologically-relevant concentration of Ca$^{2+}$ affecting an artificial fusion event. This obviously suggests that biological fusion can not be explained solely in terms of lipid interactions and that protein factors are involved in the control of exocytosis in a catalytic manner. I will now discuss some of the ways in which the membrane fusion may be stimulated in exocytotic secretory events and consider what proteins calcium may be stimulating.

(i) **Intracellular Targets for Calcium in Exocytosis.**

(a) **The Role of Calmodulin in Exocytosis.**

One possible candidate for a Ca$^{2+}$-sensitive regulatory protein is calmodulin (CaM), a ubiquitous Ca$^{2+}$-sensitive protein which is important in the regulation of many different processes such as the activation of cAMP phosphodiesterase (Cheung 1970), membrane protein phosphorylation (Schulman and Greengard, 1978), the regulation of glycogen phosphorylase.
and Cheung, 1979). In many cell types there is some evidence for CaM playing a role in exocytosis. In platelets, the CaM antagonist W-7 prevented thrombin–stimulated serotonin release and simultaneously inhibited Ca$^{2+}$–dependent myosin light chain kinase activity, an enzyme activity known to be under the control of CaM (Nishikawa et al. 1980). In adrenal chromaffin cells high affinity binding sites for calmodulin are present on the chromaffin granules (Trifaró, Bader and Doucet, 1985), and trifluoperazine inhibits Ca$^{2+}$–dependent catecholamine release (Baker and Knight, 1981), without affecting chromaffin granule translocation to the plasma membrane (Burgoyne, Geisow and Barron, 1982). However, these authors argue that this inhibition may be due to the detergent–like behaviour of trifluoroperazine rather than an effect specifically on CaM. More convincing evidence for a role of CaM in adrenal chromaffin cells has been obtained using antibodies directed against CaM. When erythrocyte ghosts containing anti−CaM were fused to cultured chromaffin cells, there was a significant inhibition of catecholamine secretion in response to either high [K$^+$] or acetyl choline; fusion of ghosts containing pre−immune serum had no effect (Kenigsberg and Trifaró, 1985). So in both adrenal chromaffin and other secretory cells, CaM has been implicated as a modulator of exocytosis in studies using both antibodies and so called CaM inhibitors.

(b) Calcium−Activated PIC and The Generation of Fusogens.

One of the biophysical explanations of membrane fusion is that phase transitions are necessary to generate intermediary structures likely to favour the formation of intralamellar attachments, one of the topological structures proposed to explain membrane fusion, the likely phase changes being from lamellar phase to hexagonal phase. One of the products of PtdInsP$_2$ hydrolysis, DAG is highly effective at eliciting such phase changes in artificial systems (Siegel, 1987). In some cells the increase in calcium which triggers exocytosis can also activate phosphoinositidase C for example, in neutrophils (Cockcroft et al. 1980, 1981), endocrine pancreas (Laychok,
1983), adrenal chromaffin cells (Whitaker, 1985; Eberhard and Holz, 1987; 1988) and pancreatic beta cells (Wollheim et al. 1987). In these cells, it is conceivable that DAG has a role to play as a fusogen rather than, or as well as, activating protein kinase C. In other cells however, exocytosis does not appear to require the production of fusogenic DAG. In permeabilised mast cells, exocytosis can be stimulated by GTPyS and Ca\(^{2+}\) in the presence of neomycin, an aminoglycoside antibiotic which blocks the production of DAG by phosphoinositidase C (Cockcroft et al. 1987). Here is a clear case in which exocytosis occurs without the production of this putative fusogen.

\begin{enumerate}
\item [(c)] \textit{Phospholipase A\textsubscript{2} and Exocytosis.}
\end{enumerate}

Phospholipase A\textsubscript{2} is a calcium-sensitive phospholipase (Wong and Cheung, 1979) which cleaves phosphoglycerides to produce a free fatty acid and a lysophosphoglyceride. Lysophosphoglycerides are fusogenic (Poole et al. 1970). PLA\textsubscript{2} is implicated in exocytosis in a number of secretory systems such as mast cells (Nakao et al. 1980), anterior pituitary cells (Kurihara et al. 1986) and neutrophils (Smolen and Weissman, 1980). In adrenal chromaffin cells, the PLA\textsubscript{2} inhibitors \(p\)-bromophenacylbromide, compound 1002 and mepacrine block exocytosis in stimulated cells but also block Ca\(^{2+}\) uptake (Frye and Holz, 1983). This shows that results with compounds such as these must be interpreted with caution.

Arachidonic acid, the other product of phospholipase A\textsubscript{2}, was shown to promote granule aggregation and fusion in the presence of a binding protein (synexin) and high concentrations of calcium (Creutz, 1981). However, subsequent work by Drust and Creutz (1988) showed that arachidonic acid stimulated fusion of adrenal chromaffin granules at physiological Ca\(^{2+}\) concentrations in the presence of Mg\(^{2+}\) and calpactin, a protein which is closely related to synexin (Burns et al. 1989). Arachidonate is produced when chromaffin cells are stimulated (Frye and Holz, 1984). In this cell then, the generation of both fusogenic lysophospholipids and
arachidonate may play a role in secretory control possibly together with granule membrane-binding proteins.

(d) The Cytoskeleton and Secretory Granule-Binding Proteins.

In many secretory systems, secretory granule transport is an essential factor in stimulated exocytosis. The secretory granules are embedded in a cytoskeletal network and must reach the plasma membrane in order for fusion to occur. In adrenal chromaffin cells, the granules appear to be trapped within a cytoskeletal mesh (Kondo, Wolosewick and Pappas, 1982), and in pancreatic β-cells, insulin-containing granules are held away from the plasma membrane by a cortical cytoskeletal lattice (Orci, Gabbay and Mallaisse, 1972). In digitonin-permeabilised adrenal chromaffin cells, cytochalasin D (which causes disruption of F-actin networks) promotes secretion whereas phalloidin (an actin filament stabiliser), prevents secretion (Lelkes et al. 1986), although in electrically-permeabilised cells these compounds did not affect exocytosis (Knight and Baker, 1982). Cytochalasin lowers the calcium required for secretion in neutrophils (Lew et al. 1986). In pancreatic β-cells, cytochalasin B enhances glucose-induced insulin secretion (Orci et al. 1972). In these cells, an essential calcium-sensitive step during secretion might be the disassembly of the cytoskeleton. Indeed, in adrenal chromaffin cells, in which the importance of the cytoskeleton in secretion has been widely studied, the transient reorganisation of peripheral actin filaments is required before exocytosis can occur (Cheek and Burgoyne, 1986; Burgoyne and Cheek, 1987).

Actin-binding proteins may be important in regulating secretion in cells where granule transport is a precursor to fusion. One such protein is fodrin. In adrenal chromaffin cells, α-fodrin antibodies inhibit Ca\(^{2+}\) induced catecholamine release (Perrin et al. 1987). Caldesmon is a calcium-sensitive actin-binding protein which is present on adrenal chromaffin granules (Burgoyne, Cheek and Norman, 1986). The cross-linking of actin filaments by this protein is inhibited by micromolar calcium. This
breakdown of the cytoskeletal mesh would increase secretory granule mobility and thus might be expected to enhance secretion.

Another protein which may be important in the control of secretion is calpactin. In adrenal chromaffin cells this heterotetramer has been shown to stimulate granule aggregation at micromolar calcium ion concentrations. Furthermore, the presence of Mg$^{2+}$ and arachidonic acid induces granule fusion (Drust and Creutz, 1988). Of course, this is not the same thing as granule–plasma membrane fusion, but the fusion of granule with granule is an event which occurs during compound exocytosis (Chandler, 1984; Rohlich et al. 1971). More recently, Ali et al. (1989) have shown that antibodies raised against the heavy chain (p36) of calpactin can inhibit exocytosis, and calpactin (and the heavy p36 chain alone) can restore secretory activity in response to elevated [Ca$^{2+}$] to cells rendered refractory by being made leaky. Another observation about calpactin is that the p36 subunit can be phosphorylated on a serine residue in the N-terminal region by protein kinase C (Johnsson et al. 1986). This phosphorylation prevents the reassociation of p36 with the light chain p11. Calpactin is also phosphorylated in adrenal chromaffin cells following nicotinic stimulation and both calpactin and protein kinase C are chromaffin granule–binding proteins (Creutz, Zaks and Hamman, 1987). It is an interesting possibility that this protein may be involved in the regulation of interactions of secretory granules with the cytoskeleton and may play a role in exocytosis by allowing access of the granules to the plasma membrane.

Another possible regulatory protein in adrenal chromaffin cells is a plasma membrane protein of 51 kD which binds strongly to chromaffin granules. Antibodies to this protein, applied by erythrocyte ghost fusion inhibit noradrenaline release. These antibodies also inhibit the capacitance increases associated with membrane fusion when perfused through patch pipettes (Schweitzer et al. 1989).

In view of these findings that the cytoskeleton may be an important regulator of exocytosis, it is likely that there is regulation at the level of the
interactions of secretory granules with the cytoskeleton as well as regulation at the level of membrane fusion itself. In short, the regulation of exocytosis in secretory cells resides at the level of modifications to the cytoskeleton and secretory granule-binding proteins or at the secretory granule–plasma membrane fusion event.

3.2 THE MODULATION OF CALCIUM SENSITIVITY.

Having discussed some of the intracellular receptors for Ca\(^{2+}\) which might play a role in exocytosis, I will now discuss how the calcium-sensitivity of secretion might be modulated. It is clear in some cases, exocytosis can proceed in the absence of elevated \([Ca^{2+}]_i\) (Fernandez et al. 1984; Rink, Sanchez and Hallam, 1983). In particular, I shall look at the modulatory roles of protein kinases and GTP-binding proteins on the control of exocytosis.

(i) Protein Kinase Modulation of Exocytosis.

By measuring \([Ca^{2+}]_i\) in platelets using the indicator dye quin2, Rink, Sanchez and Hallam (1983), showed that 5-HT secretion could be stimulated in the absence of elevated \([Ca^{2+}]_i\). Exogenously added DAG, or TPA, also stimulated exocytosis at basal calcium levels. This led to the proposition that secretion, independent of changes in \([Ca^{2+}]_i\), occurred through the modulatory effects of protein kinase C (Rink et al. 1983; Rink and Hallam, 1984). Subsequent studies of the platelet secretory response using electrically-permeabilised platelets, indicated that DAG, one of the products of thrombin stimulation (Haslam and Davidson, 1984), and a physiological activator of most sub-types of protein kinase C (Kikkawa et al. 1988), caused a shift in the Ca\(^{2+}\)-sensitivity of exocytosis such that it occurred in the absence of elevated calcium concentrations (Knight and Scrutton, 1984ab).
Protein kinase C causes increased Ca\textsuperscript{2+} - sensitivity of secretory systems (Knight and Scrutton, 1984ab; Baker and Knight, 1983) such that exocytosis can appear to be Ca\textsuperscript{2+} - independent (Rink et al. 1983). It can also affect the extent of secretion. For instance, in platelets, TPA affects the calcium - sensitivity of 5 - HT secretion but the extent of secretion of the lysosomal enzyme β-N-acetylglucosaminidase without affecting the calcium - sensitivity (Knight, Niggli and Scrutton, 1984). In isolated pancreatic islets, TPA causes both a shift in the Ca\textsuperscript{2+} - sensitivity of insulin release (Jones et al. 1985), but also affects the extent of secretion (Tamagawa et al. 1985; Jones et al. 1985).

There is also some evidence that other protein kinases may have a modulatory role to play in the control of exocytosis. In both pancreatic islets (Tamagawa et al. 1985), and in RINm5F cells (Wollheim et al. 1984), forskolin, an activator of adenylate cyclase and thus, indirectly, cAMP - dependent protein kinase, stimulates insulin release. Again, at least in RINm5F cells, this effect seems to be through the modulation of Ca\textsuperscript{2+} - sensitivity since no change in [Ca\textsuperscript{2+}], was measured when cells secreted insulin in response to forskolin (Wollheim et al. 1984).

In platelets both cAMP and cGMP - dependent protein kinases may modulate secretion. cGMP has been shown to increase, and cAMP decrease, the calcium - sensitivity of thrombin - stimulated 5 - HT release from permeabilised platelets (Knight and Scrutton, 1984b). In intact platelets cAMP reduces the affinity of ATP secretion for calcium, suggesting that the intracellular target for cAMP - dependent protein kinase is an intracellular Ca\textsuperscript{2+} receptor (Collazos and Sanchez, 1987).

These data from a variety of different secretory cells indicate that the calcium - sensitivity of secretion can be modulated by the actions of protein kinases. These phosphorylations can affect either the Ca\textsuperscript{2+} - sensitivity, or the extent of exocytosis, depending upon the cell type and the experimental conditions used.
(ii) G-Protein Control of Exocytosis.

As mentioned above, the provision of DAG and the activation of protein kinase C is one potential way in which exocytosis at resting Ca\(^{2+}\) levels can occur. DAG is produced by the hydrolysis of PtdInsP\(_2\). The enzyme responsible for this, phosphoinositidase C, is under the control of G-proteins in both secretory cells; for instance, platelets (Haslam and Davidson, 1984), mast cells (Cockcroft and Gomperts, 1985) and an insulinoma cell line (Vallar, Biden and Wollheim, 1987; Wollheim et al. 1987); and non-secretory cells (Uhing et al. 1986; Muldoon et al. 1987). Thus it could be argued that the stimulatory effects of non-hydrolysable GTP analogues (Fernandez et al. 1984; Haslam and Davidson, 1984; Knight and Scrutton, 1985) are due solely to the production of DAG and changing the Ca\(^{2+}\)-affinity of the secretory apparatus. There is however, good evidence to support a role for G-proteins different from that of producing DAG and activating protein kinase C.

Firstly, in electrically permeabilised bovine adrenal chromaffin cells, non-hydrolysable analogues of GTP inhibited exocytosis, whilst under the same conditions the protein kinase C activator, TPA, stimulated exocytosis (Knight and Baker, 1985). This shows that the two compounds must have different sites of action. An involvement of G-proteins in catecholamine secretion was further illustrated in these cells by Bittner, Holz and Neubig (1986). These authors found Ca\(^{2+}\)-independent secretion with GTPyS or GppNHp without phosphoinositide hydrolysis. In this case, though, the effect was stimulatory at very low [Ca\(^{2+}\)] rather than inhibitory as described by Knight and Baker (1985).

G-proteins also modulate exocytosis in neutrophils. Barrowman et al. (1986) showed that GTPyS stimulated exocytosis in the absence of Ca\(^{2+}\) (less than 2x10\(^{-11}\) M free Ca\(^{2+}\)). TPA inhibited Ca\(^{2+}\)-induced exocytosis whereas GTPyS stimulated exocytosis in these cells. This showed that GTPyS was not stimulating exocytosis through the production of DAG, since, if this were the case, it would be expected that the modulatory effects of
both TPA and GTPyS on exocytosis would be the same. Indeed, it has been shown that under the same conditions, GTPyS fails to stimulate phosphoinositidase C (Cockcroft, 1986), thus DAG is not produced through this route. In mast cells, too, a G–protein different from Gp seems to stimulate exocytosis. Cockcroft et al. (1987) differentiated between exocytosis and the production of DAG using the aminoglycoside neomycin. They found that GTPyS and Ca\(^{2+}\) still stimulated exocytosis when PPI hydrolysis was inhibited. GTPyS could still be generating DAG under these conditions (Bocckino et al. 1985; Irving and Exton, 1987). However, exocytosis occurs in the absence of ATP (Cockcroft et al. 1987), showing that protein kinase C–mediated phosphorylation is not required to support exocytosis. The G protein which stimulated exocytosis was termed Ge (Cockcroft et al. 1987).

In many cells, then, there seems to be a G–protein acting on exocytosis downstream of Ca\(^{2+}\)–mobilisation and the production of DAG. The nature of this putative G–protein Ge is unknown but low molecular weight G–proteins have been localised to the cytoplasmic face of the secretory granules of adrenal cells (Burgoyne and Morgan, 1989). G–proteins have also been shown to be involved in the constitutive secretion of yeast. Here, an important gene product in the secretory pathway, SEC4, is a ras–like G–protein and here too, the G–protein is localised to the cytoplasmic faces of the secretory granules and plasma membrane (Goud et al. 1988). This localisation to the secretory granule may be a common feature in all secretory cells in which G–proteins play a modulatory role.

Thus it now appears that although Ca\(^{2+}\) is often a trigger for exocytosis, other regulatory processes such as protein phosphorylation, and the actions of G–proteins, can control the sensitivities of exocytotic systems to Ca\(^{2+}\).
3.3 THE REQUIREMENT FOR ATP IN EXOCYTOSIS.

There is a general consensus that phosphorylation or dephosphorylation reactions are important in exocytosis since in most systems there is a requirement for Mg$^{2+}$–ATP. In adrenal chromaffin cells, there is an absolute requirement for ATP. ATP affects the extent of exocytosis but not the Ca$^{2+}$–sensitivity (Knight and Baker, 1982). The kinetics of secretion suggested that the rate of phosphorylation or dephosphorylation was the controlling factor in secretion rather than the extent of protein phosphorylation. This finding suggested that Ca$^{2+}$ may stimulate exocytosis by the activation of either a kinase or phosphoprotein phosphatase. Lee and Holz (1986) demonstrated that Ca$^{2+}$ caused the phosphorylation of at least 17 proteins during secretion and this phosphorylation was enhanced by both TPA and DiC$_8$ which suggested that secretion was modulated by the activation of protein kinase C.

It has also been shown in adrenal chromaffin cells that the ATP analogue, ATPyS, blocks secretion (Brooks et al. 1984; Brooks and Brooks, 1985). ATPyS is a substrate for cell kinases but the resulting thiophosphoproteins are relatively resistant to the action of phosphatases (Gratecos and Fischer, 1974). Thus blocking a dephosphorylation seems to prevent exocytosis in adrenal chromaffin cells. In neurotransmitter release, activation of protein kinases occurs in response to an array of secretagogues, from nerve impulses to neurotransmitters to hormones (Nestler et al. 1984), and in exocrine pancreas, both amylase secretion and protein phosphorylation followed the same biphasic dose–response behaviour (Roberts and Butcher, 1983). On CCK stimulation, at least 9 proteins change in their phosphorylation state, both protein kinase C–dependent and independent (Burnham et al. 1986).
(i) The Effects of ATP on Exocytosis in Mast Cells.

In mast cells, the situation is rather different in that in the permeabilised cell ATP shifts the Ca$^{2+}$ sensitivity but not the extent of exocytosis (Howell et al. 1987). These authors argued that, in this system, ATP acts by changing the affinity of exocytosis for Ca$^{2+}$ through protein kinase C, but is not essential for exocytosis. This can be stimulated solely by the addition of calcium and GTPyS. More recent studies have suggested that exocytosis in mast cells may require a dephosphorylation reaction since, ATP retards the kinetics of exocytosis in permeabilised cells in response to GTPyS and Ca$^{2+}$, whereas non-hydrolysable analogues of ATP such as AppNHp do not (Tatham and Gomperts, 1989). After permeabilisation, mast cells will become refractory to GTPyS and Ca$^{2+}$ if maintained in ATP-free media. The subsequent addition of ATP to refractory cells restores full responsiveness (Howell, Kramer and Gomperts, 1989), suggesting a certain state of phosphorylation is necessary to support secretion. The phosphorylation which causes this restoration of secretory efficacy is probably through protein kinase C (Howell, Kramer and Gomperts, 1989). These results suggest that in mast cells, a dephosphorylation may be obligatory to support secretion.

The quest for a significant phosphorylation or dephosphorylation has, however, proved elusive. In both permeabilised platelets (Knight et al. 1984) and adrenal chromaffin cells (Niggli et al. 1984; Lee and Holz. 1986), there is no single phosphorylation event which is uniquely associated with exocytosis though there are a number of possible candidates.

(ii) Exocytosis and Protein Dephosphorylation in Paramecium.

The search for a phosphorylation event uniquely associated with exocytosis has proved unrewarding in mammalian cells. However, in one system there is a well-documented change in the phosphorylation state of a single protein which correlates well with secretory activity. In
Paramecium, exocytosis involves the dephosphorylation of a 65 kD membrane-bound phosphoprotein (Gilligan and Satir, 1982; Zieseniss and Plattner, 1985), which has been called parafusin (Satir et al. 1989). The beauty of this system is that non-discharge strains of Paramecium are available. In the nd9 strain there is no dephosphorylation of a 65 kD protein when they are challenged with secretagogues. This finding suggests a link between dephosphorylation and exocytosis of trichocysts. Further evidence for this involvement is provided by Zieseniss and Plattner, who showed that the extent of exocytosis and the extent of dephosphorylation go hand in hand, and by Stecher et al. (1987), who showed that both exocytosis and parafusin dephosphorylation were inhibited by antisera to this protein. Calmodulin was found at the preformed exocytotic sites (Momayezi et al. 1986) and a calcineurin-like protein has also been found. Calcineurin is a Ca$^{2+}$- and calmodulin-dependent protein phosphatase (Stewart et al. 1982).

As is the case with the sea urchin egg exocytotic apparatus, the secretory granules (trychocysts) and plasma membrane of the Paramecium can be isolated and remain active in vitro and studies of exocytosis in these cells are also not complicated by the necessity of secretory granule transport as a prelude to exocytosis. These fragments have also been termed cortices. Momayezi et al. (1987) investigated the effects of adding exogenous phosphatases and antibodies to CaM and calcineurin (CaN) on exocytosis. They found that injection into intact cells or application on cortices, of either alkaline phosphatase or a Ca$^{2+}$-CaM-CaN complex induced exocytosis and a parallel dephosphorylation of the 65 kD protein. They also found that inhibitors of phosphatase activity and anti-CaN antibodies blocked these events. The microinjection of Ca$^{2+}$-CaM-CaN into non-discharge strains did not cause exocytosis. Previously, microinjection of wild type cytoplasm had been shown to overcome the block to exocytosis (Lefort-Tran et al. 1981) in these strains. These results suggested that other factors had to be involved in the stimulation of exocytosis. These experiments suggest that in Paramecium, exocytosis may be mediated by a
Ca\(^{2+}\)–CaM–CaN complex acting perhaps on parafusin, together with some other as yet unidentified factors.

4. Exocytosis In The Sea Urchin Egg.

The work in this thesis is concerned with one example of triggered exocytosis, cortical granule exocytosis in sea urchin eggs. The first physical sign of successful fertilisation is the elevation of the fertilisation envelope, brought about when the egg’s cortical granules fuse with the plasma membrane, starting from the point of sperm fusion and progressing from this point to the opposite pole of the egg. The fertilisation envelope surrounds the fertilised egg as a spherical shell with the fertilisation membrane separated from the newly formed embryo by the perivitelline space. The fertilisation envelope serves to exclude further sperm from interacting with the egg and so acts as a complete physical barrier to polyspermy (Epel, 1978). Figure 1.3 shows a sea urchin egg before and after fertilisation.

4.1 SEA URCHIN EGG EXOCYTOSIS – A MODEL SYSTEM.

The cortical granules are already primed for exocytosis. Studies of exocytosis in this cell are free of the complication of secretory granule transport and cytoskeletal disassembly which are required in other cells. The sea urchin egg is therefore ideally and, with the exception of perhaps, Paramecium, uniquely suited for the study of triggered exocytosis. This is made easier because the secretory apparatus can be studied in vitro: egg plasma membranes can be isolated with the cortical granules still attached. Using a polybase, eggs can be stuck to glass or plastic slides and most of the egg can be sheared away with a jet of appropriate medium leaving behind fragments of plasma membrane termed cortices (Vacquier, 1975). Using this isolated system it was found that in suitable media, of similar ionic composition to that of ooplasm, exocytosis can be triggered by adding
micromolar Ca\(^{2+}\) (Baker and Whitaker, 1978). This is the same [Ca\(^{2+}\)] as occurs during cortical granule exocytosis at fertilisation (Steinhardt et al. 1977; Swann and Whitaker, 1986). This exocytosis is also very easy to see. The cortical granules have a diameter of about 1 \(\mu\)m and when they fuse with the plasma membrane they lose their integrity and seem to disappear, leaving a flattened dome in their place. Cortical granule exocytosis is shown in figure 1.4.

Thus the sea urchin egg represents an exocytotic system which can be easily isolated, responds to the same stimulus in vitro as in vivo and is easy to visualise. Furthermore, the cortical granules can be separated from the plasma membrane and attach when reapplied. These reconstituted cortical lawns undergo exocytosis when calcium is added (Crabb and Jackson, 1985). Here, then, is a system which should allow an investigation of the individual contributions made by these components to exocytosis.

### 4.2 CALCIUM IS THE SOLE REQUIREMENT FOR EXOCYTOSIS.

(i) The Effect of Protein Kinases and Inhibitors of Cytoskeletal Function.

In the sea urchin egg, Ca\(^{2+}\) seems to be the only requirement for exocytosis. Neither protein kinase C activators such as PMA or DiC\(_8\) nor cyclic nucleotides, affect exocytosis directly nor do they modulate the sensitivity towards Ca\(^{2+}\) (Whitaker, Aitchison and Swann, unpublished observations). Inhibitors of cytoskeletal function do not affect exocytosis, either (Byrd and Perry, 1980; Whitaker and Baker, 1983). This may be due to the fact that in the sea urchin egg the secretory cortical granules are already firmly attached to the plasma membrane and thus no granule transport is necessary, unlike the situation in many other cells where an important aspect of exocytosis is the transport of secretory granules towards the plasma membrane. Thus, in this cell, our observations about exocytosis
Figure 1.3) Fertilisation envelope elevation.

The top panel shows an unfertilised sea urchin egg. The bottom panel shows an egg, 5 minutes after insemination. The transparent halo surrounding the egg is the fertilisation envelope which is formed by the calcium-stimulated exocytosis of the cortical secretory granules which lie immediately beneath the plasma membrane (Moser, 1939; Schuel et al., 1972).
Figure 1.4) Cortical granule exocytosis in vitro.

This figure shows the effect of elevated calcium ion concentrations on isolated cortices. The panel on the left shows a cortical fragment isolated in a medium containing 10 mM EGTA. On the right is the same fragment after the addition of 10 μM Ca²⁺. The addition of calcium has caused the disappearance of many of the cortical granules. This is due to exocytosis.
relate to the fusion event itself rather than other essential events which are precursors to membrane fusion.

(ii) **G - Proteins and Exocytosis in Sea Urchin Eggs.**

An involvement of G - proteins in exocytosis has now been established in a number of cells. In the sea urchin egg there is some evidence for the involvement of a regulatory G - protein (G_e) in exocytosis *in vivo*. Although GTPyS and cholera toxin activate eggs (Turner *et al.* 1986; 1987), they do this by raising [Ca^{2+}]_i (Swann *et al.* 1987). Neither have any effect when applied directly to cortices before or during the application of calcium buffers (Swann and Whitaker, unpublished communication). However, GDPβS prevents cortical granule exocytosis at fertilisation. Turner *et al.* (1986) reported that the "block" by this compound was overcome by the microinjection of InsP_3 which they suggested meant that the inhibitory effect was due to an inhibition of the sperm induced rise in intracellular free calcium. However, more exhaustive studies have subsequently shown that with high concentrations of GDPβS, exocytosis is completely inhibited (Crossley and Whitaker, 1989; Whitaker, Swann and Crossley, 1989). Furthermore, microinjection of GDPβS causes an increase in [Ca^{2+}]_i and does not block a sperm induced calcium transient. These results suggest that there may be a role for G_e or other G - proteins in exocytosis in intact sea urchin eggs. However the only requirement for exocytosis *in vitro* is Ca^{2+}. I will now discuss some of the factors which may play a role in controlling calcium - stimulated exocytosis in sea urchin eggs.

4.3 **CALCIUM TARGETS IN SEA URCHIN EGG EXOCYTOSIS.**

(i) **The Role of Calmodulin in Cortical Granule Exocytosis.**

There is evidence to suggest that calmodulin is important in the control of exocytosis in the sea urchin egg. In this cell, the CaM antagonist
trifluoperazine inhibited exocytosis (Baker and Whitaker, 1979; Whitaker and Baker, 1983). A more direct relationship between CaM and exocytosis was shown using antibodies directed against CaM (Steinhardt and Alderton, 1982). In this study a complete block of exocytosis with calcium concentrations up to 1 mM was found in vitro with long incubations of the antibody. The block was rapidly reversible by the addition of exogenous CaM. Using immunofluorescence, the CaM was seen to lie on the plasma membrane beneath the cortical granules and occurred at the site of plasma membrane/cortical granule attachment. This study provides strong evidence for CaM regulating exocytosis in the sea urchin egg.

It is important to bear in mind the difficulty in interpreting experiments using calmodulin inhibitors because of the chemical properties of these compounds. Thus, experiments using antibodies provide much stronger evidence for the involvement of CaM than those studies using "specific" inhibitors TFP and W-7 which may have effects other than those pharmacologically ascribed to them (Whitaker and McLaughlin, 1988). Indeed, Whitaker and de Mey (unpublished observation) have shown that calmidazolium, another potent CaM inhibitor, has no effect on the calcium sensitivity of cortical granule exocytosis in the sea urchin egg. The evidence for the involvement of CaM in the control of exocytosis is inconclusive. On the one hand calmodulin inhibitors such as TFP can inhibit exocytosis whereas calmidazolium does not; on the other, antibodies raised against CaM can inhibit cortical granule exocytosis.

(ii) Calcium-Stimulated Phospholipases and Exocytosis.

(a) Ca\(^{2+}\) - Activated PIC And The Generation Of DAG.

As was previously mentioned, DAG is highly effective at inducing lipid bilayer phase transitions (L-H\(_{17}\)) which are likely intermediaries in fusion events (Siegel, 1987). In the sea urchin egg at fertilisation InsP\(_3\) and DAG are generated through the hydrolysis of PtdInsP\(_2\) (Ciapa and Whitaker,
1986), and phosphoinositidase C is activated at identical calcium concentrations as those required to stimulate exocytosis (Whitaker and Aitchison, 1985). These data provide a possible link between the production of DAG at fertilisation and cortical granule exocytosis as well as in the activation of the Na⁺/H⁺ antiport through the activation of protein kinase C. It has also been found that inhibiting polyphosphoinositide hydrolysis with the aminoglycoside antibiotic neomycin, inhibits exocytosis in response to calcium (Whitaker and Aitchison, 1985) which is consistent with DAG acting as a fusogen but Whitaker and McLaughlin (1988) have shown that this may be due to neomycin's effects on the surface potential of the membranes. What is clear is that since neither TPA nor oleylactetylgllycerol (an analogue of DAG) stimulate exocytosis in vitro, it is unlikely that DAG stimulates exocytosis or modulates the calcium sensitivity of exocytosis through the activation of protein kinase C.

(b) Ca²⁺—Activated PLA₂ And The Generation Of Fusogens.

There is some evidence to suggest that phospholipase A₂ may play a role in cortical granule exocytosis (Schuel, 1978). Phospholipase A₂ cleaves phospholipids to produce arachidonate and a lysophosphoglyceride. Both arachidonate (Creutz, 1981; Drust and Creutz, 1988) and lysophospholipids (Poole et al. 1970) are fusogenic. Melittin, a PLA₂ activator triggers cortical granule exocytosis (Shimada et al. 1982). Quinacrine, an inhibitor of PLA₂ function blocks exocytosis but not sperm incorporation, and the PLA₂ in sea urchin egg homogenates is Ca²⁺—sensitive and maximally active at the resting pH of the unfertilised egg (Ferguson and Shen, 1984). This finding suggested that the calcium transient at fertilisation activated phospholipase A₂ which produced fusogenic lysophosphoglycerides. Since PLA₂ is an enzyme under the control of calmodulin (Wong and Cheung, 1979), one might argue that the effect of calmodulin antibodies on cortical granule exocytosis (Steinhardt and Alderton, 1982) is by inhibiting PLA₂.
It is possible, though, that the inhibition of exocytosis seen in the sea urchin egg when quinacrine is applied, is due to the prevention Ca\(^{2+}\) mobilisation rather than inhibiting exocytosis downstream of the calcium signal. It has been shown in adrenal chromaffin cells, that inhibitors of PLA\(_2\) block calcium uptake (Frye and Holz, 1983). Without more thorough studies, it is unclear whether quinacrine inhibits exocytosis or Ca\(^{2+}\)–mobilisation.

4.4 THE EFFECT OF ATP ON EXOCYTOSIS IN SEA URCHIN EGGS.

In the sea urchin egg, as in other cells, exocytosis is dependent on ATP. When eggs were poisoned with cyanide, there was a decrease in intracellular ATP and a concomitant decrease in the elevation of fertilisation envelopes at fertilisation (Baker and Whitaker, 1978). Of course, this could reflect a decrease in the level of Ca\(^{2+}\) in the eggs because ATP is required to maintain an intact intracellular pool of Ca\(^{2+}\). It could equally have arisen because of a decrease in the level of PtdInsP\(_2\), since there was no ATP with which PtdInsP kinase could replenish the store of this lipid, essential in the generation of InsP\(_3\). However, using the cortical preparation, Baker and Whitaker found that when incubated in ATP free medium, cortices had the same affinity for Ca\(^{2+}\) but a proportion of the granules remained refractory to the stimulus. The addition of ATP to these cortices did not allow the refractory granules to become responsive. This suggests that in this system it is not ATP *per se* that is important but rather something derived from it – perhaps a phosphoprotein, a finding similar to that of Knight and Baker (1982).

5. IS A CALMODULIN–ACTIVATED PHOSPHATASE IMPORTANT IN CORTICAL GRANULE EXOCYTOSIS?

As mentioned previously, CaM antagonists (Baker and Whitaker, 1983), and anti–CaM antibodies (Steinhardt and Alderton, 1982), both inhibit
exocytosis in the sea urchin egg and, in the in vitro cortical preparation, the
time dependent decrease in Ca\textsuperscript{2+} -sensitivity can be substantially slowed by
the inclusion of Mg -ATP in the incubation medium (Whitaker and Baker,
1978; Sasaki and Epel, 1983; Moy et al. 1983). CaM is present at the sites
of cortical granule attachment and the anti-CaM block can be overcome
by the addition of exogenous CaM (Steinhardt and Alderton, 1982). In
Paramecium, the Ca\textsuperscript{2+} -CaM -CaN complex is not sufficient to trigger
exocytosis in incompetent strains suggesting the involvement of other factors.
In sea urchin eggs, the sensitivity of exocytosis towards Ca\textsuperscript{2+} is reduced if
cortices are prepared in media containing chaotropic agents (Sasaki, 1984),
suggesting that here too factors other than those directly involved in the
exocytotic mechanism play a modulatory role in the control of exocytosis
probably by affecting the Ca\textsuperscript{2+} -affinity of the exocytotic mechanism. It has
been reported that unfertilised eggs contain a Ca\textsuperscript{2+} -CaM -dependent
phosphoprotein phosphatase (Iwasa and Ishiguro, 1986). This protein
consists of two subunits. Protease peptide mapping showed that the 17K
subunit showed homology to the 19K subunit of calcineurin but the 55K
subunit was not homologous. It is an interesting possibility that this
phosphatase may play a role in exocytosis and thus exhibit similarities to
exocytosis in Paramecium. However, it has not been satisfactorily
determined as to whether this protein occurs in isolated cortices. There is
little substantial evidence to suggest the involvement of a dephosphorylation
in cortical granule exocytosis, except for the data of Whitaker and Baker
(1978) showing the requirement of ATP to keep the cortex responsive to
Ca\textsuperscript{2+} and the finding of Sasaki and Epel (1983) that in the absence of ATP,
calcium sensitivity is reduced. However, by analogy with both mast cells
and Paramecium, where there is also a decrease in responsiveness following
the removal of ATP, it is a possible mechanism of exocytotic control and
certainly one which is worth pursuing.
5. Experimental Approach.

The work in this thesis consists of three parts. In the first part, I have developed a reconstituted system to study the contributions made by the plasma membrane and cortical granules in exocytosis. This involves a simple separation of the secretory components and subsequently bringing them back together to form an exocytotically competent system. I have used this technique to examine some factors which may be important in mediating interaction between the secretory components. I have also tried to define the role of calcium ions in the control of exocytosis.

The second part of the thesis is concerned with the possible role of phosphatases in controlling exocytosis. I used the compound ATPyS to irreversibly phosphorylate proteins and show that this can inhibit exocytosis without affecting sperm egg interactions and a rise in \([\text{Ca}^{2+}]_i\). I also show that this inhibition is time dependent and requires the presence of cytoplasm although the inhibition, once established, is permanent. I have also identified proteins which are thiophosphorylated.

The final part of the thesis concentrates on mechanisms of egg activation. I have studied some of the ways in which compounds activate eggs and cause cortical granule exocytosis. I show that sea urchin egg phosphoinositidase C can be activated by a GTP-binding protein. I also show that this is not the mechanism used by the egg to generate the increase in \([\text{Ca}^{2+}]_i\) at fertilisation. This investigation has used techniques of measuring calcium-fluxes and phosphoinositide hydrolysis in permeabilised eggs, and I have developed a single cell assay for PIC activation using intracellular pH as a sign of protein kinase C activation.

1. HANDLING OF SEA URCHIN GAMETES.

All experiments were carried out on the eggs of either *Lytechinus pictus* or *Lytechinus variegatus*. *L. pictus* were obtained off the coast of California by Pacific Biomarine Laboratories Incorporated (Venice, Ca). *L. variegatus* were obtained off the coast of Florida. Both species were despatched to London by air. *L. pictus* were kept in tanks containing natural sea water at 16°C and remained gravid throughout the year. *L. variegatus* were kept in similar tanks at 20°C and were used from January to April during which time they were producing fully mature gametes. Eggs were obtained by injecting female urchins with 0.5M KCl (0.1 ml for *L. pictus*, and 1.0 ml for *L. variegatus*) and were collected by placing the urchins upside down on top of a 50 ml beaker full of artificial sea water (ASW, 435 mM NaCl, 40 mM MgCl\(_2\), 15 mM MgSO\(_4\), 11 mM CaCl\(_2\), 10 mM KCl, 2.5 mM NaHCO\(_3\), 1 mM ethylenediaminetetra-acetic acid (EDTA), pH 8.0). Male urchins were shed in a similar manner but were placed right side up in a small volume of ASW. Sperm was collected "dry" by pipetting directly from the gonadopores. Sperm was kept undiluted at 4°C and used within 2 days of collection. Eggs were scored for the elevation of fertilisation envelopes and only batches of eggs in which fertilisation was greater than 95% were used. Eggs were used within four hours of shedding.

All of the experiments described used eggs which had their jelly coats removed. This was done by passing the jellied eggs several times through a Nitex mesh. The mesh consists of fibres approximately 100 μm apart. This allows eggs to pass through but removes the jelly coat which surrounds them. The eggs were allowed to settle and were washed several times before use in order to remove the egg jelly from the ASW. Occasionally eggs were washed into solutions other than ASW. This was done by spinning egg suspensions in a hand centrifuge and resuspending in the
appropriate medium. Eggs were routinely used as soon after dejellying as possible in order to retain their capability of fertilisation.

2. MICROINJECTION TECHNIQUES.

The injection of solutions into eggs was performed on glass slides or coverslips mounted on the stage of an inverted microscope (Leitz Diavert). Eggs were stuck to the glass using poly-D-lysine. The glass was coated with a solution of polylysine at 0.01 mg/ml for 30 sec and this was then rinsed with distilled water and allowed to dry. Using this concentration of polylysine and this time ensured that the eggs stuck down firmly but did not become flattened which makes microinjection difficult. A perspex rectangle with a slot in it was used to construct a bath by sticking it to the coverslip using silicone grease. A slurry of eggs was applied to the completed bath and, after settling, the eggs were washed. Eggs were viewed using bright field optics with 10x or 40x objectives. Microinjections were always performed with the eggs viewed at 40x. Glass micropipettes made from capillary glass with an internal diameter of 1.5 mm (type 150F, Clark Electromedical, Pangbourne, U.K.) using a Bioscience puller (Bioscience, Shearness, U.K.) were filled using fine plastic tubing attached to a syringe and typically 0.1 to 1 μl of solution was used. All injections were performed using high pressure pulses applied to the back of the solution. This was done by holding the pipettes in an electrode holder (Clark Electromedical, Pangbourne, U.K.), to which a length of teflon tubing was attached. This was attached to a gas cylinder via a solenoid valve (British Fluidics, Bishops Stortford, U.K.) which allowed pulses of pressure to force solutions from the tips of the micropipettes. The whole assembly was held in a hydraulic micromanipulator (Narishige, Japan) which was attached to the microscope stage. While the pipette assembly was outside the egg, continuous pulsing at 1 Hz was used to prevent contamination of the tip contents by sea water.
To inject an egg the micropipette was moved so that it just touched the egg and was moved into the egg such that it caused a large deformation. In order to penetrate the egg the top of the pipette holder was tapped very gently. Once the desired volume of solution had been injected the pulsing was stopped and the micropipette withdrawn carefully. The volume of injection was estimated by observing the cytoplasmic displacement following each pulse, and its size was estimated with reference to an eyepiece graticule. Injections expressed as a percentage of total egg volume can then be converted to a final cytoplasmic concentration given the pipette concentration. Eggs which showed any cytolysis following microinjection were rejected from the experiment.

3. MEASUREMENT OF INTRACELLULAR ION CONCENTRATIONS IN SINGLE EGGS.

(i) The Measurement of \([\text{Ca}^{2+}]_i\) using Fura2.

The intracellular free calcium ion concentration in single eggs was measured using the calcium–sensitive fluorescent dye fura2. This is a compound with an effective dissociation constant in physiological situations for calcium of less than 1 \(\mu\text{M}\). A useful property of this dye is that it changes its fluorescent properties on binding calcium: the characteristics of the excitation spectrum alter. I have used similar techniques for the measurement of intracellular free calcium as have been previously described (Poenie et al. 1985; Swann and Whitaker, 1986); in these experiments, fura2 (pentapotassium salt; Molecular Probes, Junction City, Oregon, U.S.A.; 10 mM in 0.5 M KCl/20 mM PIPES (Piperazine–N,N’-bis[2-ethane sulphonic acid]), pH 6.7), was injected at final concentrations of 20–100 \(\mu\text{M}\). These concentrations of fura2 did not prevent fertilisation as judged by the elevation of a fertilisation envelope.

The measurements were performed on a Leitz Diavert microscope fitted with a Leitz Ploemopak epifluorescent attachment. A mercury vapour
lamp provided the excitation light and this beam passed through bandpass interference filters (Ealing Optics, Watford, U.K.) of 350 nm or 380 nm (with half max. bandwidths of 11.2 and 10 nm respectively). This excitatory light was reflected onto the object by a 400 nm dichroic mirror. The subsequent emitted light passed back through this mirror and onto a photomultiplier via a 490 nm bandpass interference filter (7.2 nm half max. bandwidth). The photomultiplier was a type 9824B (EMI Electron Tubes Ltd, Hayes, U.K.), and it was run with a photocathode voltage of between 600 and 900 V. The current signal was converted to an output voltage by a current to voltage converter and this output voltage was recorded on a chart recorder and also to an Apple microcomputer where it was stored on magnetic disc. A stepper motor (Radio Spares, Corby, U.K.) was used to alternate the excitation interference filters, and the output voltage from the photomultiplier was stored on magnetic disc and converted to a calcium concentration (Swann and Whitaker, 1986). The calcium concentration was calculated using the equation described by Poenie et al. (1985). $Ca^{2+}$ was calculated from the 350/380 ratio of fluorescent signals ($R$) according to the equation,

$$Ca^{2+} = K_0 (F_o/F_s)(R - R_{min})/(R_{max} - R)$$

where $K_0$ is the effective dissociation constant for $Ca^{2+}$ and fura2 in an intracellular medium,

$F_o$ is the fluorescence of fura2 in the $Ca^{2+}$ free form

$F_s$ is the fluorescence of fura2 in the $Ca^{2+}$ saturated form,

The calibration constants were determined by placing drops of fura2 (100 µM) on the microscope stage in either 10mM ethyleneglycol- $bis$-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) or 10mM CaCl$_2$ in a medium containing 155mM KCl, 25mM NaCl, 100mM MOPS (3-(N-morpholino) propanesulphonic acid), pH 7.0. In this solution the $K_0$ for fura2 is 774 nM (Poenie et al. 1985). For each experiment, the signal at each excitation wavelength from an uninjected egg was used as a background and values were subtracted from the signals from fluorescent eggs.
Typically, the signal from non-injected eggs was about 0.5−2 % of the signal from a fura2−injected egg.

(ii) The Measurement of pH\textsubscript{i} using BCECF.

The intracellular pH of cells can be conveniently measured using the fluorescent dye fluorescein and its derivatives (Thomas \textit{et al.} 1979, Rink \textit{et al.} 1982). This technique uses the fact that the fluorescence of these dyes is dependent upon pH in the physiological range. In these experiments I used the dye BCECF (Molecular Probes, Junction City, Oregon, U.S.A.), which, has the advantage that it is more highly charged and thus less leaky than fluorescein. BCECF (10 mM in 0.5 M KCl, 20 mM PIPES, pH 6.7) was introduced into eggs by microinjection to give a final concentration of 20−50 \(\mu\text{M}\). The techniques of fluorescence measurements were essentially the same as for fura2 except that a 5% neutral density filter (Ealing Optics, Watford, UK) was placed between the light source and the excitation filters, the excitation wavelengths were 450 nm and 490 nm (12 nm bandwidth) and the emission intensity was measured using a 530 nm (15 nm bandwidth) filter.

Calibration was performed using a drop method similar to that used for fura2. The medium used for drop calibration was similar to that of ooplasm, 220 mM K gluconate, 500 mM N−methyl glucamine, 10 mM KCl, 5 mM MgCl\textsubscript{2}, 5 mM EGTA and 2.5 mM ATP. This was adjusted to the required pH using a number of biological buffers at a concentration of 50 mM. These were: pH 4.0−5.5, aspartic acid; pH 5.5−6.3, MES (2[N]−Morpholinoljethanesulphonic acid); pH 6.3−7.1, PIPES; pH 7.1−8.0, HEPES (N−2−Hydroxyethyl piperazine N’−2−ethanesulphonic acid; pH 8.0−9.0, TAPS (tris−[hydroxymethyl]−methylamino propane sulphonic acid). BCECF was added to these at a final concentration of 20 \(\mu\text{M}\) and the fluorescence ratios were measured on the microscope. An effective pK was calculated for the dye. pH was calculated using the equation:
\[
pH = - \log \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right) + pK_a
\]
where \( R \) is the measured ratio of fluorescence for a given pH
\( R_{\text{min}} \) is the ratio of the unbound form of the dye
\( R_{\text{max}} \) is the ratio of the bound form of the dye

Using these values the pH of unfertilised eggs was between 6.60 and 6.80, in keeping with previously measured values using microelectrodes (Shen and Steinhardt, 1979), and dimethyloxazolidine distribution (Johnson and Epel, 1981), and using the calculated calibration values the pH change at fertilisation was approximately 0.4, similar to the value previously reported (Whitaker and Steinhardt, 1982).

4. TECHNIQUES USED TO INVESTIGATE CORTICAL GRANULE EXOCYTOSIS IN VITRO.

(i) Preparation of Cortices.

The cortical preparation was described first by Vacquier (1975). It has since proved to be a very useful preparation in the study of exocytosis in sea urchin eggs. The cortical preparation is a means by which plasma membrane fragments with the cortical granules attached are isolated and remain active to their physiological trigger. The beauty of this system is that it is an easily isolable exocytotic apparatus which responds to the same signal \textit{in vitro} as that which causes exocytosis in the intact cell, that is a rise in the intracellular free \( \text{Ca}^{2+} \) concentration. It is also useful because exocytosis is easily seen using conventional light microscopy. Cortices respond to \( \text{Ca}^{2+} \) at 1–3 \( \mu \text{M} \) when kept in a medium similar in composition to that of ooplasm (Baker and Whitaker, 1978) and this [\( \text{Ca}^{2+} \)] is similar to the peak concentration occurring at fertilisation (Steinhardt \textit{et al.} 1977; Swann and Whitaker, 1986).

Eggs were attached to glass slides pretreated for 1 minute with 0.05–0.1 mg/ml poly-L-lysine and were rinsed gently with intracellular
medium (IM: 220 mM K glutamate, 500 mM glycine, 10 mM, 2.5 mM MgCl₂, 2.5 mM ATP, 10 mM EGTA, pH 6.7) or a medium based on KCl (PKME: 50 mM PIPES, 425 mM KCl, 2.5 mM MgCl₂, 2.5 mM ATP, 10 mM EGTA, pH 6.7). The eggs were sheared with a jet of the appropriate medium leaving the isolated cortices behind. Cortices were generally used as soon as they had been prepared unless incubations with particular chemicals were required. In order to achieve rapid perfusion of Ca²⁺ containing solutions across a cortical field a cell was constructed from a block of perspex with a slot milled in it. Over this slot was attached a No.1 coverslip. A piece of black rubber was glued on the slide side of the cell to give a clearance of approximately 1 mm when firmly pressed down. A hole at one end of the cell allowed solutions to be added to the cell and a needle attached to a vacuum line at the other end allowed for rapid change of solutions.

(ii) Measuring the Extent of Exocytosis in Cortices.

The extent of exocytosis was determined using the method of Zimmerberg (1984). The cortical lawn was illuminated using dark-field optics so that the light passing through the specimen is related to the degree of light scattering. As cortical granules fuse with the plasma membrane so they lose their refractility and light scattering ability and thus the extent of exocytosis is inversely proportional to light scattering. This was determined quantitatively by attaching a photodiode to the camera port of the microscope and sending the output directly to a potentiometric chart recorder. For each experiment, maximum exocytosis was determined by perfusing with 10 mM Ca²⁺ solution. The effects of other solutions were expressed in terms of the decrease in light scattering compared to that when a solution containing 10 mM calcium was added.
(iii) Preparation of Calcium-containing Solutions.

Calcium-EGTA buffers were used in experiments on exocytosis in isolated cortical fragments. IM contained 10 mM EGTA and various total calcium concentrations. The free calcium concentrations were calculated using the constants of Martell and Smith (1974). The calcium-EGTA ratios and free calcium were 0.528, 1.78 µM; 0.715, 3.98 µM; 0.797, 5.9 µM; 0.877, 11.2 µM; 0.914, 16.6 µM; 0.943, 25.1 µM. For exocytosis in reconstituted lawns sufficient 100 mM CaCl$_2$ in IM or PKME was added to give the desired [Ca$^{2+}$]$_{\text{free}}$. The pH of calcium-EGTA buffers was routinely checked before and immediately after each experiment and a calcium-sensitive electrode was used to measure the free calcium ion concentration.

Free calcium was measured using a calcium-sensitive electrode. The electrode was purchased from World Precision Instruments Inc. (New Haven, CT, USA). This electrode and a reference electrode (Pye Instruments, Cambridge, UK), were attached to a pH meter set in the voltage recording mode. I calibrated the electrode using serial dilutions of CaCl$_2$ for pCa 2 to pCa 4.6 and calcium/EGTA buffers (10 mM EGTA, pH 6.7) for pCa values higher than this. Figure 2.1 shows a calibration curve for the calcium electrode I used.

5. RECONSTITUTION OF EXOCYTOSIS.

The reconstitution of an exocytotically-competent system from the sea urchin egg was first achieved by Crabb and Jackson (Crabb and Jackson, 1985). In this method, cortical granules were purified by homogenisation of a cortical suspension at pH 9.1 in a solution containing ammonia. This caused the dissociation of a proportion of the cortical granules which were purified by differential centrifugation and were used for reconstitution onto denuded plasma membrane lawns. I used substantially different methods to
Figure 2.1. Calcium sensitive electrode calibration curve.

This figure shows a calibration curve for the calcium electrode I used for assessing the [Ca$^{2+}$] in the solutions used to stimulate exocytosis. Solutions containing pCa of 2 to 4.6 were prepared by serial dilution of a 1 M CaCl$_2$ stock. Solutions with a pCa higher than this were prepared using Ca/EGTA buffers. Each point represents the mean of four separate readings. The bars show sem n = 4.
achieve reconstitution since, homogenisation of *L. pictus* cortices succeeded only in breaking them up into smaller fragments and produced only a few monomeric cortical granules which could not be removed from these plasma membrane fragments by differential centrifugation.

(i) Preparation of Cortical Granules.

All glassware used for the preparation of cortical granules was washed with ethanolic KOH for one week and thoroughly rinsed in deionised water before use. Eggs were dejellied and washed into either IM or PKME and were attached to glass petri-dishes which had been pretreated with 1 mg/ml poly-L-lysine and rinsed several times in IM or PKME. Cortical lawns were prepared as described in section 2.6 and were exhaustively washed until completely free of cytoplasmic organelles. Washed cortices were examined using dark field optics and were not used for the preparation of cortical granules until the washing solution was free of cytoplasmic particles. The cortical granules were removed from the washed cortical lawns by shearing with either IM or KEA (450 mM KCl, 5 mM EGTA, 50 mM NH₄Cl, pH 9.1). This shearing was achieved by forcing 1 ml of the appropriate solution from a disposable syringe. Removal of the granules was accompanied by a clearing of the translucent cortical lawn. This proved to be a convenient measure of the extent of granule detachment. The petri-dish was periodically examined using dark field optics during the procedure as a further guide to the progress of the preparation and also to ensure that plasma membrane was not detaching from the petri dish. When isolation was complete, the cortical granule suspension was examined by differential interference contrast microscopy in order to estimate the purity of the preparation and the degree of aggregation. When granules were prepared in KEA, they were neutralised to pH 6.7 by the addition of 1 M PIPES immediately before use.
(ii) Preparation of Plasma Membrane Lawns.

Cortical lawns were prepared in either IM or PKME on glass slides as previously described in section 2.7. These cortical lawns were then further sheared with a more powerful jet of medium which removed more than 95% of the cortical granules but left the plasma membrane fragments adhering to the slides. This process did not appear to tear away fragments of plasma membrane together with cortical granules, since isolated cortical granules did not lyse when treated with high concentrations of calcium as they do if still attached to small plasma membrane fragments (Crabb and Jackson, 1985).

(iii) Preparation of Reconstituted Cortical Lawns.

Reconstituted cortical lawns were prepared in a cell constructed on a glass slide. Strips of PTFE tape were pressed onto the edges of the slide and the centre of the slide was coated with 0.1–1 mg/ml poly-L-lysine. The centre was rinsed and a slurry of eggs was applied and allowed to settle for 2 minutes and from these eggs plasma membrane lawns were prepared. A #1 coverslip was pressed onto the PTFE supports to complete the perfusion cell. These perfusion cells typically held a volume of 20 μl. A suspension of cortical granules was applied to the edge of the cell and perfused over the lawns by capillary action. The granules were allowed to settle for 10 to 20 minutes after which time unbound cortical granules were removed with several washes of the perfusion cell with the appropriate buffer. All perfusions were aided by the placing of strips of filter paper at one end of the cell.

(iv) Assessment of the Extent of Exocytosis in Reconstituted Lawns.

The extent of exocytosis in reconstituted lawns was determined by examining a field of at least 5 plasma membrane fragments at 40x
magnification using differential interference contrast (DIC) microscopy before and after the addition of a particular calcium-containing solution. For each field, the number of fused and unfused granules was determined.

(v) Calcium Pretreatment of Cortical Granules.

Cortical granules used for pretreatment experiments were prepared as described previously except that the medium contained 2 mM EGTA. The pH was adjusted to pH 6.7 and a quantity of 100 mM CaCl₂ in PKM (PKME lacking EGTA) was added to give a final concentration of 1 mM. The granules were incubated for 5 minutes in 1 mM calcium followed by the addition of sufficient 100 mM EGTA in PKM to give a final EGTA concentration of approximately 10 mM. The pH was adjusted by the addition of 1 M KOH and the cortical granules were used as soon as this treatment was completed.

(vi) Calcium-pretreatment of Plasma Membranes.

Plasma membranes were prepared as described previously. They were then perfused 5 times with 100 μl of a solution containing 1 mM Ca²⁺ to ensure the removal of any EGTA from the chamber. A further 100 μl of 1 mM Ca²⁺ was added and left for 5 minutes. After this time the plasma membrane fragments were washed with 5 100 μl aliquots of PKME.

6. PREPARATION OF AN EGG EXTRACT.

Extracts were prepared from eggs using a homogenisation technique with a minimum volume of extraction buffer in order to attempt to purify factors which would convey increased Ca²⁺ sensitivity on reconstituted lawns. This was done in the following way. Packed dejellied eggs (1 ml) were resuspended in 10 ml ice cold calcium-free sea water (445 mM NaCl, 50
mM MgCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 2 mM EGTA, 1 mM EDTA, pH 8.0) and gently centrifuged for 1 minute in a hand centrifuge. This was repeated 5 times so that the external environment was essentially calcium-free. The eggs were washed once in PKME, and resuspended in 0.5 ml PKME containing 2.5 mM ATP, 2.5 mM PMSF (phenylmethylsulphonyl fluoride), 50 µg/ml leupeptin, 50 µg/ml pepstatin A and 25 µg/ml aprotinin and were transferred to a teflon centrifuge tube. The suspension was gently homogenised by passage through a 1 ml Gilson pipette tip several times. The homogenate was examined periodically and homogenisation ceased when free of intact eggs. The homogenate was left on ice for 10 minutes. It was then centrifuged at 100,000x g for 1 hour at 4°C in a Sorvall ultracentrifuge. After this the supernatant was carefully removed and stored on ice until use. Examination of the supernatant by differential interference contrast microscopy showed it to be free of particulate material. The KCl egg extract was dialysed overnight at 4°C against IM, containing ATP and protease inhibitors as for the extract preparation solution, and 1% dextran (molecular weight 200–250 kdal, BDH, Poole, UK). The final volume after dialysis was approximately 40% of the starting volume.

7. THE USE OF PERMEABILISED EGGS TO INVESTIGATE ⁴⁵Ca RELEASE FROM INTRACELLULAR STORES.

Permeabilisation techniques have been used in many cell types in order to study the release of Ca²⁺ from intracellular stores, using either Ca²⁺ sensitive microelectrodes (Biden et al. 1986), Ca²⁺-sensitive dyes (Gershengorn et al. 1984) or ⁴⁵Ca release (Muldoon et al. 1986) to investigate release. I have used ⁴⁵Ca release to investigate Ca²⁺ fluxes in permeabilised sea urchin eggs.

Permeabilisation experiments were performed on eggs which were stuck on 2.5 cm diameter plastic petri dishes coated with 0.05 mg/ml poly-L-lysine for 3 minutes. Sea urchin eggs had their jelly coats removed and were washed and gently centrifuged 3 times in calcium free
sea water (445 mM NaCl, 50 mM MgCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 2 mM EGTA, 1 mM EDTA, pH 8.0), and 2 ml of a 4% suspension was applied to each dish. The eggs were allowed to settle for 3 minutes after which time they were rinsed and aspirated 3 times with 4.0 ml IM. Eggs were permeabilised in 4 ml IM containing 0.005% (0.4 mM) digitonin for 3 minutes after which the solution was aspirated and the eggs rinsed twice in 4 ml IM. The permeabilised eggs were rinsed for 10 minutes in 4 ml IM. They were aspirated and rinsed once, rapidly, in 4 ml IM and then 2 ml of IM containing 0.5 μCi/ml ⁴⁵Ca was added. After 5 minutes the compound under investigation was added. This was done by removing 100 μl of the ⁴⁵Ca bathing solution and mixing this with a concentrated solution of the test compound. This mixture was immediately added back to the permeabilised eggs and was circulated throughout the dish by gentle agitation. Each experiment was stopped by the aspiration of the solution and three rounds of rinsing and aspiration with 4 ml IM. Eggs were extracted into 1 ml 1% Triton-X100 and this was counted for radioactivity and the total egg protein determined by the method of Bradford (1974). The release of ⁴⁵Ca was expressed in terms of the amount of ⁴⁵Ca remaining associated with the eggs as a proportion of that in eggs treated with ATP alone. In each series of experiments controls of 0 and 2.5 mM ATP alone were used. The experimental conditions used were designed to maximise the difference between the uptake in the presence and absence of ATP.

8. THE USE OF ISOTOPES TO INVESTIGATE PPI METABOLISM IN PERMEABILISED EGGS.

I investigated stimulated polyphosphoinositide hydrolysis in permeabilised eggs using 2-[³H]-D-myoinositol which is taken up into the eggs and is incorporated into the phosphoinositide phospholipids. The advantage of using labelled inositol rather than ³²P is that the label is incorporated solely into the metabolic pool under consideration. The use of ³²P presents a problem in that it is incorporated into the ATP pool and
ATP co-elutes with Ins(1,4,5)P₃ in ion exchange analyses. (Irvine et al. 1985).

(i) Treatment of Eggs and Sample Preparation.

Jellied eggs were incubated for 18 hours at 16°C with 100 μCi of 2-[^3]H-D-myoinositol (Amersham International plc, Amersham U.K.) as a 50% suspension in 10 ml ASW. The eggs were dejellied and washed several times in 100 ml ASW until the external solution was essentially free of labelled inositol. The eggs were washed and resuspended 3 times in IM containing 10 mM EGTA followed by a further 3 washes in IM containing 10 mM EGTA or 10 μM EGTA when calcium buffers were to be used. 50 μl of eggs were used in each experiment and were used as a 5% suspension in IM. The eggs were allowed to settle in Eppendorf tubes, the supernatant was removed and the eggs were resuspended in 1 ml of IM containing the drug or calcium buffer and a digitonin concentration of 0.0025% (250 μM). The IM used for the permeabilisation/stimulation contained 20 mM LiCl which has been shown to inhibit Ins−1 P phosphatase (Hallcher and Sherman, 1981). After 5 minutes experiments were stopped by the addition of 100 μl 50% perchloric acid and were left on ice for 30 minutes. The solutions were centrifuged at 10,000×g for 3 minutes and the supernatants were removed and retained. The pellets were resuspended and vortexed vigourously in 400 μl IM and centrifuged at 10,000×g for 3 minutes. The supernatant was removed and pooled with the previous supernatants. The pooled supernatants were stored at −20°C before analysis.

(ii) Analysis of Inositol Phosphates.

The supernatants from the permeabilisation experiments were analysed using a high performance liquid chromatography (HPLC) method (Irvine et al. 1985). I used a partisil SAX anion exchange column or a partisphere
SAX cartridge (Whatman Lab. Sales, Maidstone U.K.). Both of these columns separate inositol phosphates on the basis of charge. HPLC was performed on an LKB HPLC system (LKB Instruments Ltd. Croydon U.K.). Samples were prepared by neutralising with 1 M K$_2$CO$_3$ and filtering through Gelman LC13 2 micron Acrodiscs (Gelman Sciences Ltd. Northampton) and to them was added 50 μl of a mixture of AMP, ADP and ATP, all at concentrations of 1 mg/ml. I also added 50 μl of $^{32}$P–labelled inositol phosphates prepared from red blood cells (Downes, Mussat and Michell, 1982) to give approximately 500 dpm of Ins(1,4,5)P$_3$. 0.95 ml of each sample was applied to the column for analysis.

A gradient method was used to elute the inositol phosphates. The elution solutions were a) distilled H$_2$O and b) 1.7 M ammonium formate taken to pH 3.7 by the addition of o–phosphoric acid. These solutions were filtered through 0.22 μm filters and were degassed before use. A flow rate of 1.0 ml/minute was used and 1 ml fractions were collected using a Gilson model 203 fraction collector. Radioactivity of the fractions was determined by scintillation counting using Hi–salt scintillant (Raytest Instruments, Sheffield; 4 ml per ml of eluate) and a Hewlett–Packard Tri–Carb 1500 Scintillation counter. Figure 2.2 shows an ammonium formate gradient together with an inositol phosphate elution profile using a Partisil SAX HPLC column.

9. AN ASSAY OF β–1,3–GLUCANASE ACTIVITY.

β–1,3–Glucanase is a marker enzyme for the cortical secretory granules (Moy et al. 1983). Assays of glucanase were performed using the following procedure. To a particular egg extract in IM containing 0.1% Triton–X100 was added 20 μl 2.5 M Na$_2$PO$_4$ pH 6.0 and 5 μl of 200 μg/ml laminarin. The sample was incubated at 40°C for 3 hours and was then boiled for 3 minutes. To this was added 100 μl of a solution of 100 ml 0.25 M Na$_2$PO$_4$ pH 6.0 containing 0.1 ml glucose oxidase, 1 mg horseradish peroxidase and 10 mg dianisidine hydrochloride (3,3'–
Figure 2.2) An HPLC analysis of $^{32}\text{P}$-labelled inositol phosphates.

This figure shows an HPLC analysis of $^{32}\text{P}$-labelled inositol phosphates prepared from erythrocyte plasma membranes. The dashed line shows the % of 1.7 M ammonium formate in the buffer used to elute inositol phosphates on a Partisil SAX HPLC column. The remainder of the eluate consisted of distilled water. The points joined by the solid line are the counts per sample of 1 minute fractions eluted using this gradient.
dimethoxybenzidine hydrochloride). The reaction was performed by incubating at 40°C for 10 minutes and the reaction was stopped by the addition of 200 μl of 50% H₂SO₄. Absorbance was read at 530 nm.

10. AN ASSAY FOR SPERM-EGG FUSION.

One of the earliest events at fertilisation is the fusion of the sperm membrane with the plasma membrane of the egg (Hinckley et al. 1986; Longo et al. 1986). This gives cytoplasmic continuity between the two gametes and because of this a simple assay has been developed to study fusion using the DNA staining vital dye H33342 (Hinckley et al. 1986). Eggs were loaded with H33342 (20 μM in ASW for 30 minutes) and were washed 5 times in ASW before being stuck onto coverslips in the usual manner. This procedure means that only the egg cytoplasm contains the dye and the extracellular medium is free of the dye. H33342 shows enhanced fluorescence upon binding to DNA and so upon insemination, when cytoplasmic continuity occurs the dye binds to the sperm nuclei and these become brightly fluorescent. Excitatory light was provided using a 380 nm bandpass interference filter (10 nm bandwidth), and a 490 nm emission side filter was used. Eggs were viewed within 5 minutes of insemination and the number of fluorescent sperm nuclei scored.

11. ELECTRICAL PERMEABILISATION OF EGGS.

For some experiments using ATPyS I used the technique of electro-permeabilisation in order to gain access to the egg interior without causing structural perturbation. To do this I employed a method modified from that of Swezey and Epel (1988). Before permeabilisation eggs were washed twice in calcium-free sea water and once in permeabilisation buffer (PB: 300 mM glycine, 175 mM K 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). Eggs were resuspended to a final concentration of 25 % in PB
and were transferred to a perspex chamber with 2 stainless steel plates separated by 2 cm. The suspension was pulsed 3 times by discharging a 1 μF capacitor charged to 100 V using an electrophoresis power pack. The suspension was immediately transferred to petri–dishes or glass slides pre–coated with 1 mg/ml polylysine.

12. ANALYSIS OF PROTEINS USING SDS–PAGE.

(i) Preparation of Samples.

Samples from eggs or plasma membrane fragments were dissolved in 0.5 ml 1 M NaOH and were cooled on ice for 15 minutes. To this was added 1 ml ice cold 50 % TCA (trichloroacetic acid). The samples were left on ice for a further 10 minutes. The TCA solution was centrifuged in a micro–centrifuge model 320 a (Mechanika Precyzyjna, Warsaw, Poland), for 30 seconds at 10,000 x g. The supernatant was discarded and the pellet resuspended in 0.05 ml distilled water containing 1 mM PMSF. The samples were centrifuged for 30 seconds at 10,000 x g and the pellet retained. The pellet was washed and centrifuged as above in 0.5 ml ice–cold acetone to remove any TCA from the pellet. This step was repeated. The acetone was removed and the washed protein pellet dried at 37°C for 90 minutes. The dried pellet was dissolved in 0.1 ml sample buffer and was boiled for 10 minutes. Samples were briefly sonicated to ensure solubilisation using a 4710 series ultrasonic homogeniser (Cole Parmer Instrument Co. Chicago. USA.). Samples were briefly centrifuged to remove air bubbles and were allowed to cool before application to gels.

(ii) Polyacrylamide Gel Electrophoresis.

Egg proteins and proteins from isolated plasma membrane fragments were analysed using SDS polyacrylamide gel electrophoresis as described by Laemmlli (1970). Briefly, slab gels of 3 mm thickness were used with a
proportion of 0.8 NN'-methylenebisacrylamide to 30 acrylamide. The final acrylamide concentration in the running gel was 10 % and 3 % in the stacking gel. Samples were dissolved in a sample buffer consisting of 2 % SDS (sodium dodecyl sulphate), 10 % glycerol, 80 mM Tris/HCl, pH 6.8 and 5 % 2-mercaptoethanol. Sample buffer also contained bromophenol blue (1 % of a 0.2 % stock in ethanol). This allowed the running of the gel to be followed. The running buffer consisted of 0.38 M glycine, 0.05 M Tris and 0.1 % SDS. Samples were run into the stacking gel using 100 V and this voltage was increased to 200 V (approx. 30 mA), as soon as the sample had entered the running gel. Electrophoresis was stopped when the running front was within 0.5 cm of the end of the gel. Proteins were visualised by staining in a solution of coomassie blue R250 in 45 % methanol, 10 % acetic acid for 30 minutes with agitation. Gels were destained in 25 % methanol, 7 % acetic acid for 20 hours. Gels were dried at 80°C using a Bio-Rad 543 gel dryer. In order to estimate the molecular weights of the separated polypeptides, I also ran lanes of 14C-labelled proteins. This allowed estimates of molecular weight to be made both in coomassie blue stained gels and in autoradiographs.

(iii) Autoradiography of SDS-PAGE Gels.

In order to determine radioactive incorporation into proteins, autoradiography of gels was performed. For autoradiography of 35S-labelled proteins I used Hyperfilm Betamax film (Amersham International plc. UK.). Gel and film were placed in Kodak X-Omatic cassettes. Films were developed using Kodak Dental X-ray reagents (Eastman Kodak Company, Rochester, USA).
Chapter 3. Reconstitution of Exocytosis.

1. INTRODUCTION.

One of the major problems in beginning to understand the mechanistic events underlying exocytosis is that exocytotic systems are very difficult to study in isolation. This is due mainly to the fact that interaction between the plasma membrane and a subcellular organelle, the secretory granule, has to occur in an exocytotic event. In most secretory cells, the secretory granules are only loosely associated with the plasma membrane and so any attempt to isolate the secretory apparatus is dogged by this fundamental problem. The sea urchin egg is one example of a cell in which studies of the secretory apparatus are possible in vitro. This means that we are able to test the effects of compounds on exocytosis directly. For instance, one can test the effect of a putative inhibitor compound by applying it directly to sea urchin cortices. It is now possible to reconstitute the secretory apparatus of the sea urchin egg. This has the virtue that one can treat individual components and examine the effect of such treatments on subsequent exocytotic efficacy.

The reconstitution of exocytosis in the sea urchin egg was first achieved by Crabb and Jackson (1985). In this chapter, I have achieved an in vitro reconstitution using different methods and have used this preparation to examine some of the factors involved in cortical granule/plasma membrane binding and to define the role of calcium in the secretory process by treating the isolated components (cortical secretory granules and plasma membranes) with calcium and reconstituting cortical lawns with these treated components.
2. PREPARATION OF CORTICAL GRANULES.

In the reconstituted system described by Crabb and Jackson, cortical granules were prepared by the preparation of cortices (plasma membrane fragments with the cortical secretory granules still attached) in suspension (Detering et al. 1977) followed by treatment with a KCl-based solution containing 50 mM ammonium chloride at pH 9.1 (KEA), and rounds of homogenisation and differential centrifugation to purify the released cortical granules (CG's). Treatment of eggs with local anaesthetics or ammonia-containing solutions causes a dissociation of the cortical granules from the plasma membrane (Hylander and Summers, 1981). Crabb and Jackson made use of this observation to develop a procedure for the isolation of pure cortical granules from plasma membrane fragments of *Strongylocentrotus purpuratus*. Application of these purified CG's to plasma membrane lawns from which the cortical granules had been removed allowed cortical granule—plasma membrane reassociation to occur and these reconstituted lawns subsequently underwent calcium-stimulated fusion. Studies with antibodies raised against the hyalin, one of the protein components of the granule interior, showed that the calcium-induced morphological change was indeed fusion rather than calcium induced lysis of the attached cortical granules. Using this method with eggs of *Lytechinus pictus*, I found that homogenisation of cortices in KEA succeeded in releasing some cortical granules. However, the most noticeable effect was the breaking down of the cortices into small fragments with the CG's still attached. These fragments settled onto the plasma membrane lawns and fused in response to calcium but most of the polymeric particles also fused in the absence of interaction with plasma membrane fragments attached to the glass which implied that the granules were still attached to their own plasma membrane fragments. This method of isolation of cortical granules was clearly inappropriate for *L. pictus* eggs. High speed centrifugation (10,000xg for 1 minute) removed the majority of these larger fragments from the suspension and left a suspension of monomeric granules and aggregates of up to 10 cortical granules. The protocol shown below was used to obtain mainly monomeric
CG's free of large aggregates using a different homogenisation/centrifugation method.

A 10 % suspension of eggs was homogenised in PKME (i) and this was centrifuged at 300x g centrifugation for 3 minutes to give a cortex pellet (ii) and cytoplasmic supernatant (iii). The cortex pellet was resuspended, incubated in KEA for 10 minutes and gently homogenised in a loose-fitting glass homogeniser. The homogenate was centrifuged at 300x g for 3 minutes to give a pellet (iv) containing large sheets of plasma membranes and a supernatant (v) containing small fragments of plasma membrane and free cortical granules. The CG-containing supernatant (v) was centrifuged at 10,000x g for 1 minute to give a pellet (vi) and a CG containing supernatant (vii). I assayed the activity of the enzyme β-1,3-glucanase, a marker for the cortical granule contents, in order to determine the fate of the CG's in this procedure. The results of this are illustrated in table 3.1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucanase activity as a % of starting homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>homogenate (i)</td>
<td>100</td>
</tr>
<tr>
<td>cortex pellet (ii)</td>
<td>1.75</td>
</tr>
<tr>
<td>cytoplasmic supernatant (iii)</td>
<td>95.5</td>
</tr>
<tr>
<td>pellet (iv)</td>
<td>0.19</td>
</tr>
<tr>
<td>supernatant (v)</td>
<td>1.15</td>
</tr>
<tr>
<td>pellet (vi)</td>
<td>0.675</td>
</tr>
<tr>
<td>CG supernatant (vii)*</td>
<td>0.255</td>
</tr>
</tbody>
</table>

* Cortical Granule containing suspension.
From this table it is apparent that most of the cortical granule glucanase activity remains in the cytoplasmic supernatant, either because of imperfect fractionation of the egg cortex, the lysis of the cortical granules or by the release of CG's by shearing forces applied to the cortex during homogenisation. Only a maximum of about 0.25 % of the cortical granules are present in the purified CG supernatant. In order to increase the yield of cortical granules substantially for reconstitution experiments I devised the protocol described in chapter 2; cortical granules were removed by shearing from cortices attached to polylysine coated glass. The recovery of CG’s using this method was much higher as judged by the recovery of glucanase activity in the purified cortical granule suspension. I assayed the glucanase activity during the preparation of CG’s using this shearing method to determine the efficiency of this method. The results are shown in table 3.2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucanase activity as a % of starting homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg lysate*</td>
<td>86.4</td>
</tr>
<tr>
<td>CG suspension</td>
<td>10.7</td>
</tr>
<tr>
<td>Denuded PM</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*: This includes the egg cytoplasm together with plasma membrane fragments washed away during the preparation of the cortical lawns.

This data shows that using this method over 10 % of the glucanase activity in the starting egg suspension was associated with the cortical granule suspension. This is 40x more efficient than cortical granule isolation using the homogenisation and centrifugation method described in section 3.1. For all of the subsequently described experiments, this simpler and more
efficient way of isolating cortical granules which avoids the problem of contaminating plasma membrane, was used.

3. AGGREGATION OF CORTICAL GRANULES.

The preparation of cortical granules by the shearing method produced mainly monomeric CG's but aggregates (2 or more granules) were also present. These made up approximately 10 % of the total number of particles in suspension. Following preparation there was a time-dependent increase in the extent of aggregation and this was found to be pH dependent. Table 3.3 shows the time dependency of aggregation at pH 6.7 (PKME) and at pH 9.1 (KEA). I counted the number of monomeric and aggregated particles at the times indicated in order to assess this aggregation. CG's prepared in IM exhibited similar aggregation properties as those prepared in PKME at pH 6.7 and so CG's were prepared in KEA for most of the experiments below except when otherwise indicated.

<p>| Table 3.3  Aggregation of cortical granules. |
| % of particles as aggregates * |</p>
<table>
<thead>
<tr>
<th>time/minutes</th>
<th>pH6.7 (PKME)</th>
<th>pH9.1 (KEA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.5</td>
<td>7.0</td>
</tr>
<tr>
<td>30</td>
<td>19.5</td>
<td>10.0</td>
</tr>
<tr>
<td>60</td>
<td>31.0</td>
<td>12.5</td>
</tr>
<tr>
<td>120</td>
<td>45.0</td>
<td>20.0</td>
</tr>
<tr>
<td>180</td>
<td>70.5</td>
<td>30.3</td>
</tr>
</tbody>
</table>

* These percentages were calculated by counting the numbers of aggregates and monomers in solution. An aggregate was defined as any particle consisting of two or more cortical granules. Each value was obtained by counting at least 100 particles.
For each of the experiments described below, similar results were obtained in both PKME and IM. When prepared in media other than KEA, they were used as soon after preparation as possible so as to minimise the extent of aggregation.

4. RECONSTITUTION OF AN EXOCYTOTICALLY–COMPETENT SYSTEM.

I used cortical granules isolated using the plasma membrane shearing technique to reconstitute a competent exocytotic system. Figure 3.1 shows a typical reconstitution experiment performed in IM. Isolated cortical granules were added back to the plasma membrane lawns and were allowed to settle for 10 minutes, after which time the chamber was washed free of unbound cortical granules. Figure 3.1a shows a plasma membrane fragment. The plasma membrane fragments are completely depleted of cortical granules following shearing. Figure 3.1b illustrates the same fragment after CG settling and washing of the chamber. In this experiment the granules were allowed to settle for 10 minutes before the chamber was cleared of unbound granules by the perfusion of 100 µl of IM. Monomers, dimers, and oligomers are attached to the plasma membrane fragment. Figure 3.1c shows the result of adding 100 µM Ca²⁺ in IM. All of the cortical granules which were attached to the plasma membrane fragment have changed morphology. This morphological change represents membrane fusion. Figure 3.1d shows an area of plasma membrane fragments in the same experiment. In the centre is a region free of plasma membrane. Granules are present in this area. They have not undergone fusion although treated with 1 mM Ca²⁺. In other words fusion only occurs when cortical granule–plasma membrane interactions occur.

Occasionally I have observed that a string of cortical granules attach to the plasma membrane through only one of their number. When calcium is added, all undergo exocytosis. This result is not without precedent as
Figure 3.1) Reconstitution of exocytosis \textit{in vitro} in sea urchin eggs.

Fig. 3.1a) Plasma membrane fragments attached to a glass coverslip with polylysine. The fragments are completely free of cortical granules. Fig. 3.1b) The same fragments after the addition of 20 µl of a suspension of cortical granules allowed to settle for 10 minutes. Monomers (▼), dimers (▲), and oligomers (●) are all present. Fig. 3.1c) After the addition of 100 µM calcium in IM. All of the granules attached to the plasma membrane have undergone exocytosis. Figure 3.1d) This shows a region between plasma membrane fragments. None of the granules which are present in this area have undergone the morphological change associated with fusion. Scale bar is 10 µm.
Figure 3.2) Isolated cortical granules.

Fig. 3.2a) Cortical granules settled onto a glass slide. Both monomers (▼) and aggregates (♦) are present. Fig. 3.2b) After the addition of 10 mM calcium. All of the granules, monomers or aggregates remain unaffected by this treatment. Scale bar is 10 μm.
compound exocytosis in which granules fuse with each other does occur in both sea urchin eggs (Chandler 1984) and mast cells (Rohlich et al. 1971). In contrast, aggregates of cortical granules remain morphologically unaffected by calcium concentrations as high as 10 mM in the absence of interactions with the plasma membrane. This is illustrated in figure 3.2. Here, cortical granules were isolated and allowed to settle onto glass in a perfusion chamber similar to that used in the reconstitution experiments. 10 mM Ca\textsuperscript{2+} was added and the granules left in this medium for 2 minutes. This result shows that calcium affects neither monomers nor oligomers in the absence of interactions with the plasma membrane. It also strongly suggests that the purification of cortical granules by shearing from cortices causes the release of pure cortical granules; they are not attached to tiny plasma membrane fragments. In contrast, Crabb and Jackson reported substantial lysis (30%) of granule aggregates and attributed this to small fragments of plasma membrane being present. In view of the homogenisation technique required to isolate cortical granules using the protocol described by these authors this discrepancy is not surprising. Another feature of the reconstituted system is that specificity is involved in the cortical granule–plasma membrane interaction. In other words, more granules bind to the plasma membrane fragments than to the glass between these fragments. This is illustrated in table 3.6 on page 84.

5. CALCIUM SENSITIVITY OF EXOCYTOSIS.

(i) The Effects of Different Media on Calcium Sensitivity.

I investigated the differences in the calcium sensitivities of cortical lawns and preparations reconstituted in different systems. Figure 3.3 shows the effect of different calcium concentrations on exocytosis in native cortical fragments and from reconstituted lawns prepared in IM or PKME. Both of the systems have lost some sensitivity towards calcium. Reconstituted exocytosis in IM is 10 fold less sensitive than native cortical lawns and 50
Figure. 3.3) A comparison of in vitro exocytosis in native and reconstituted preparations.

Reconstituted exocytosis in IM (■) is 10 fold less sensitive to calcium than native in vitro exocytosis (○) and 100 fold less sensitive in PKME medium (▲). Each point represents the mean of 8 experiments. Error bars are the sem.
times less sensitive in PKME. Half maximal exocytosis occurs at 6.3 μM in cortical fragments, 26 μM in RL's prepared in IM and 250 μM in PKME. This decrease in the calcium sensitivity of reconstituted lawns has been previously reported (Crabb and Jackson, 1985). However, I found that the decrease in calcium sensitivity was dependent on the media in which the experiment was performed.

(ii) The Effects of Cytoplasmic Extracts on Calcium Sensitivity.

Anions such as Cl⁻ are capable of solubilising proteins (Hatefi et al. 1974), and the cortical lawns of the sea urchin species *Hemicentrotus pulcherrimus* are an order of magnitude less sensitive to calcium if treated with KCl (Sasaki, 1984). Furthermore, sensitivity could be restored in KCl treated cortices by adding back particular protein fractions. I attempted to restore the sensitivity of PKME reconstitutions by the application of cytoplasmic extracts. Cortical granules were prepared using 0.5 ml of KEA and were diluted 1 in 4 with cytoplasmic extract prior to application to the reconstitution cell. The plasma membrane lawns were prepared in PKME and were treated with 20 μl of cytoplasmic extract for 10 minutes prior to reconstitution. Control lawns were treated with IM. The results of these experiments are shown in figure 3.4. In the presence and absence of the cytoplasmic extract, there was no difference between the calcium sensitivities of reconstituted lawns. In both cases, [Ca^{2+}]_{50\%} was approximately 270 μM. These data indicate that the application of a cytoplasmic KCl extract does not increase the calcium sensitivity of reconstituted lawns.

6. CORTICAL GRANULE – PLASMA MEMBRANE INTERACTIONS.

(i) The Effect of Proteases on CG – PM Interactions.

That granules preferentially bind to the plasma membrane rather than to glass is strongly suggestive of specific protein-mediated interactions.
Figure 3.4) The effect of a cytoplasmic extract on the calcium sensitivity of reconstitution.

The effects of a cytoplasmic extract on the calcium-sensitivity of reconstituted lawns was investigated. Both PM and CG's were incubated with the cytoplasmic extract dialysed against IM. After incubation for 10 minutes reconstitutions were performed. The extract did not alter the calcium sensitivity of exocytosis.
mediating binding. I looked for evidence that this was the case by treating plasma membrane fragments and cortical granules with the protease, trypsin and seeing if this decreased cortical granule binding. Protease treatment has previously been shown to decrease the calcium sensitivity of isolated cortices in suspension (Jackson et al., 1985), and attached to glass (Whitaker and Aitchison, unpublished), and trypsin – treatment of cortices causes a gradual decrease in plasma membrane – cortical granule attachment (Vacquier, 1975). These experiments were performed in PKME buffers, reagents were purchased from Boehringer. I treated isolated plasma membranes with 50 \( \mu \)g/ml trypsin for 20 minutes followed by washing with 500 \( \mu \)g/ml soybean trypsin inhibitor (SBTI). Cortical granules from non – treated cortices were then added and granule density assessed after 10 minutes. For cortical granule proteolysis, granules were treated with 100 \( \mu \)g/ml trypsin for 20 minutes followed by the addition of SBTI to give a final concentration of 500 \( \mu \)g/ml. Control granules were diluted to the same final concentration with SBTI alone. The results of this are shown in table 3.4.

<table>
<thead>
<tr>
<th>Protease treatment</th>
<th>Number of CG per 1000 ( \mu )m(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neither</td>
<td>11.5 +/- 3.1</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>1.1 +/- 0.9</td>
</tr>
<tr>
<td>Cortical granules</td>
<td>1.4 +/- 1.2</td>
</tr>
</tbody>
</table>

Mean +/- sem are shown; n = 5.

Table 3.4 shows that tryptic digestion of either component inhibits the interactions between cortical granules and plasma membranes which are

81
needed for the formation of reconstituted lawns. These data show that proteins on both the cortical granules and the plasma membrane are required to interact in order to allow reconstitution to occur. Binding between the two secretory components is not mediated solely by lipidic interactions between the two.

(ii) The Effect of ATP, DTT and Protease Inhibitors on the Calcium Sensitivity of Reconstituted Exocytosis.

A general feature of exocytosis is a requirement for ATP in order to retain calcium sensitivity. In isolated sea urchin egg cortices, the removal of ATP gradually causes the cortical granules to become refractory to calcium (Baker and Whitaker, 1978; Sasaki and Epel, 1983). I investigated whether the inclusion of ATP in the isolation media and calcium containing solutions increased the calcium sensitivity of the reconstituted system. I also investigated whether the calcium sensitivity was increased by the inclusion of dithiothreitol (DTT), which reduces disulphide bonds, and a cocktail of protease inhibitors. Proteolysis by trypsin, and sulphydryl moiety modification by N-ethylmaleimide have both been reported to increase the calcium ion concentration required to stimulate exocytosis in vitro (Jackson et al. 1985) and I have already demonstrated that trypsin treatment inhibits the association of cortical granules and plasma membrane. I included protease inhibitors and DTT to see if this increased cortical granule binding or decreased the calcium required for fusion. All solutions used in both the preparation of cortical granules and plasma membrane lawns and the calcium buffers used to challenge the reconstituted lawns were IM-based and contained 2.5 mM ATP, 10 mM DTT, 1 mM PMSF and 200 μg/ml pepstatin, leupeptin and aprotinin. Table 3.5 shows the results of these experiments. Each value represents the mean and sem of five reconstitution experiments.

This table shows that the reconstituted system is unaffected by these additions. The loss of calcium sensitivity is probably due to factors other
than protease activity or reduction of disulphide-linked proteins. The additions did not significantly affect the extent of cortical granule binding to the plasma membrane fragments. The mean number of cortical granules per 1000 μm² of plasma membrane and the SEM values were 10.05 +/- 3.2 for control lawns and 9.89 +/- 2.9 for lawns in which additions of the

---

**Table 3.5** The effect of ATP, DTT and protease inhibitors on the calcium sensitivity of reconstituted exocytosis.

<table>
<thead>
<tr>
<th>[Ca²⁺] / μM</th>
<th>% Exocytosis</th>
<th>Control</th>
<th>With Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.2</td>
<td>7.8 +/- 1.5</td>
<td>6.7 +/- 2.1</td>
<td></td>
</tr>
<tr>
<td>17.8</td>
<td>24.6 +/- 2.2</td>
<td>23.1 +/- 2.3</td>
<td></td>
</tr>
<tr>
<td>28.4</td>
<td>53.3 +/- 4.3</td>
<td>50.0 +/- 4.1</td>
<td></td>
</tr>
<tr>
<td>40.0</td>
<td>79.3 +/- 3.7</td>
<td>81.3 +/- 2.4</td>
<td></td>
</tr>
<tr>
<td>64.5</td>
<td>96.8 +/- 3.8</td>
<td>94.1 +/- 2.6</td>
<td></td>
</tr>
</tbody>
</table>

various compounds were made. In isolated cortical fragments, ATP retards the system from becoming refractory to calcium (Whitaker and Baker, 1978; Sasaki and Epel, 1983), one of the reasons for this might be by keeping a protein in a phosphorylated state. The inclusion of ATP in these experiments did not increase the calcium sensitivity of reconstituted lawns which suggests that the loss of Ca²⁺-sensitivity is not due to a gradual dephosphorylation of a control protein.

7. **THE EFFECT OF CALCIUM PRETREATMENTS.**

I investigated the effects of calcium pretreatments on the efficacy of reconstitution. In these experiments, either cortical granules, plasma membrane or both of these were pretreated with 1 mM calcium for 10
minutes. Calcium was removed by the addition of EGTA to a final concentration of 10 mM, and the pretreated granules and plasma membranes were reconstituted in the usual manner. Calcium pretreatment of the cortical granules did not cause any fusion or aggregation.

Table 3.6 shows that the number of cortical granules bound to plasma membrane fragments was unaffected by calcium pretreatments of either the plasma membrane or the cortical granules or both of the components of the secretory system.

Table 3.6  The Effect of Calcium Pretreatments on Cortical Granule Binding.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Number of CG per* 1000 μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) PM lawns.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.5 +/- 0.82 a</td>
</tr>
<tr>
<td>PM pretreated with 10 mM Ca²⁺</td>
<td>8.1 +/- 0.51</td>
</tr>
<tr>
<td>CG pretreated with 10 mM Ca²⁺</td>
<td>8.2 +/- 1.3</td>
</tr>
<tr>
<td>PM &amp; CG pretreated with 10 mM Ca²⁺</td>
<td>7.4 +/- 0.49</td>
</tr>
<tr>
<td>b) Polylysine coated coverslip.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.0 +/- 0.31</td>
</tr>
</tbody>
</table>

* Mean & sem are shown (n = 6, except a: n = 12).

* Each mean was calculated from the values obtained in 2 experiments, each of which counted granules on at least ten 1000μm² areas.

Figure 3.5 illustrates that none of the calcium pretreatment procedures affected the secretory response of the reconstituted system. Clearly neither the Ca₅₀%, nor the calcium required for 100% exocytosis is affected in
Figure. 3.5) The effect of calcium pretreatments on reconstitution.

Treatning either plasma membrane, cortical granules or both with 1 mM calcium prior to reconstitution does not affect the subsequent sensitivity to calcium. This experiment was performed in PKME medium. Control (♦); calcium pretreated plasma membrane (●); calcium pretreated cortical granules (■); both calcium pretreated (▲). All show half maximal exocytosis at approximately 250 µM calcium and maximal exocytosis at 1 mM calcium.
these reconstitutions performed in PKME and KEA buffers. Identical results were obtained in the presence or absence of 2.5 mM ATP. I also found that if cortical granules were added back to plasma membrane lawns in the presence of 1 mM calcium, they spontaneously fused immediately they contacted the plasma membrane. These results suggest that if calcium sensitive enzyme–substrate interactions are an important step during exocytosis then obviously the enzyme and substrate must reside in different sub–cellular compartments. For instance, one could envisage an enzyme on the plasma membrane acting upon a substrate whose localisation was the cortical granule. It seems unlikely the substrate could otherwise have escaped depletion during the calcium pretreatments if this were not the case.

These results show that calcium pretreatments are without effect on either the calcium–sensitivity of reconstituted lawns or on the mechanism involved in plasma membrane–cortical granule association.

8. HETEROLOGOUS RECONSTITUTED LAWNS.

The trigger for exocytosis, an increase in [Ca$^{2+}$],, is conserved from species to species. Therefore it is a possibility that the mechanism controlling exocytosis is conserved. As a first step towards looking at this, I attempted to form heterologous lawns between the components of eggs from two species of sea urchin, *L. pictus* and *L. variegatus*. I found that the cortical granules derived from *L. variegatus* would recombine with plasma membranes from their own eggs or those of *L. pictus*. Both kinds of reconstituted lawns underwent exocytosis on the addition of calcium. Figure 3.6 shows that the calcium sensitivity of both types of reconstituted lawn was very similar. This experiment indicates that at least between these two species the mechanism controlling exocytosis is conserved.

Crabb *et al.* (1987) reported that there was very little binding of cortical granules when applied to plasma membrane fragments from red
Figure 3.6) Cross-reactivity of reconstitution.

Both homologous and heterologous reconstituted lawns can be stimulated to undergo exocytosis by similar concentrations of calcium. Homologous lawns were prepared using both cortical granules and plasma membrane from *L. variegatus*. Heterologous lawns were prepared from cortical granules from *L. variegatus* and plasma membrane derived from *L. pictus*. In both lawns granules were settled for 10 minutes, the reconstituted lawns were washed and calcium containing solutions added sequentially. In both preparations, half-maximal calcium concentration was approximately 300 μM.
blood cells. This suggests that some degree of specificity of binding between granule and plasma membrane exists. Of course, it is more likely that interactions would occur between the secretory granules and plasma membranes of different kinds of secretory cell. In an attempt to examine this possibility, I prepared secretory granules from adrenal chromaffin cells. I prepared the granules in a medium consisting of 140 mM K glutamate, 5 mM MgCl₂, 2.5 mM ATP, 1 mM EGTA, 20 mM PIPES pH 6.7. All procedures were performed at 4°C. The medulla was finely sliced and homogenised in a dounce tissue homogeniser. The suspension was centrifuged at 1,200 x g for 10 minutes, the supernatant retained and spun at 24,200 x g for 10 minutes. The fluffy mitochondrial layer was discarded and the remaining pellet resuspended and centrifuged again. This second pellet was resuspended and retained on ice until use. Calcium containing solutions were made in the above medium or IM, in both cases in the absence of EGTA, by the addition of 0.1 M CaCl₂. These granules, when applied to L. pictus plasma membranes, bound to the fragments and did so at a greater extent than to areas free of sea urchin egg plasma membrane. However, the application of calcium up to concentrations of 10 mM in either the chromaffin granule preparation medium, or in IM did not cause fusion. This result shows that we cannot form a functional heterologous reconstituted lawn between the secretory components of sea urchin eggs and one type of mammalian secretory cell.

9. CONCLUSIONS.

In this chapter I have devised a method of reconstituting exocytosis from the secretory components from the eggs of the sea urchin Lytechinus pictus. I have shown that when brought together, the components form a competent secretory system. This has a reduced calcium-sensitivity compared to native in vitro cortices and exhibits different sensitivity in different isolation media, as has been shown to be the case in native cortices (Sasaki, 1984). I have shown that the interactions between the plasma membranes and cortical granules can be prevented by protease
treatment which suggests specific protein interactions are responsible for the docking of granules to the plasma membrane.

I have also shown that pretreating the components with calcium affects neither the binding of the components or the calcium–sensitivity of the reconstituted lawns. This suggests that if exocytosis involves the actions of a Ca$^{2+}$–sensitive enzyme on a substrate, then these two components must reside on different sub–cellular compartments. An alternative explanation for the lack of effect of calcium pretreatments is that a Ca$^{2+}$–stimulated allosteric mechanism is important in stimulating exocytosis. The finding that strings of granules will fuse when only one is contacting the plasma membrane suggests that exocytosis may involve the generation of fusogens.

Finally, I have shown that reconstitution is possible between the secretory components derived from different species of sea urchin, suggesting that at least here, there is conservation of the exocytotic mechanism.

Some of the work described in this chapter has been published (Whalley and Whitaker, 1988).
Chapter 4. Irreversible Phosphorylation Inhibits Exocytosis In Sea Urchin Eggs.

1. INTRODUCTION.

In this chapter, I will describe some experiments in which I have used nucleotide analogues as tools to try and identify some of the proteins and mechanisms which may be involved in the control of exocytosis in sea urchin eggs. I will describe some experiments in which I have used a thio analogue of ATP, adenosine 5'-O-(3-thiotriphosphate) (ATPyS) in order to try and determine the role of phosphorylation or dephosphorylation in exocytosis in the sea urchin egg. ATPyS is a substrate for some cellular kinases but the resulting thiophosphoproteins are resistant to the action of phosphatases (Gratecos and Fischer, 1975) which effectively means that ATPyS can be used to phosphorylate some proteins irreversibly. I have used this compound to investigate the possible involvement of phosphorylation or dephosphorylation during exocytosis in the sea urchin egg.

In most cells which have been investigated, Mg$^{2+}$-ATP is required to support a competent secretory system. In adrenal chromaffin cells, ATP affects the extent of secretion but not the calcium sensitivity and the removal of ATP prevents exocytosis from occurring (Knight and Baker, 1982). In Paramecium the dephosphorylation of a 65 kD protein has been shown to be an important step in the exocytosis of trichocysts (Zieseness and Plattner, 1985). In mast cells, a kinetic analysis of secretion has shown that ATP retards the onset of agonist induced exocytosis which suggests the involvement of a dephosphorylation in stimulating exocytosis in this cell (Tatham and Gomperts, 1989). In sea urchin eggs, poisoning with cyanide to deplete them of intracellular ATP results in an inhibition of fertilisation envelope elevation, both in vivo and in vitro (Baker and Whitaker, 1978). Furthermore, adding ATP back to these poisoned cortices does not restore the calcium sensitivity to an unpoisoned state. This result suggests that ATP
is necessary to "prime" the secretory apparatus rather than to participate in the exocytotic reaction itself. One possibility for this being that ATP is required as a phosphate donor for a phosphoprotein essential for exocytosis. In this chapter I have tried to define a role for phosphorylation state in the control of exocytosis in the sea urchin egg.

2. THE EFFECTS OF ATPyS ON EGG ACTIVATION.

(i) ATPyS Inhibits Fertilisation Envelope Elevation.

I microinjected eggs with ATPyS (100 mM in 450 mM KCl, 50 mM PIPES, pH6.7) to give final concentrations of between 10 μM to 2 mM. The eggs were left for 10 minutes after injection and were examined for exocytosis, judged by the elevation of fertilisation envelopes. These concentrations of ATPyS did not cause exocytosis in any of 30 eggs. When these eggs were subsequently inseminated, I found that there was a dose dependent inhibition of fertilisation envelope elevation. Figure 4.1 shows the dose dependency of this inhibition. In these experiments, eggs were inseminated at least 10 minutes after microinjection and eggs were examined for fertilisation envelopes 5 minutes after the application of sperm. Eggs which had partial envelopes were scored as non-inhibited. This figure shows that ATPyS causes 100 % inhibition at final concentration of about 100 μM. The ATPyS used was microinjected as a tetra-lithium salt. Lithium chloride, injected at final concentrations as high as 5 mM had no effect upon fertilisation envelope elevation (results not shown).

Figure 4.2 shows a control egg and an egg injected with ATPyS to give a final cytoplasmic concentration of 200 μM after insemination. The control egg has a complete fertilisation envelope. In contrast, the egg injected with ATPyS does not have a fertilisation envelope but a number of fertilisation cones are clearly visible. This suggests that multiple sperm-egg interactions had occurred. Fertilisation cones are structures formed by
Figure 4.1) The effects of ATPγS on cortical granule exocytosis.

ATPγS was microinjected into eggs and these were fertilised approximately 10 minutes after microinjection. Exocytosis was assessed by a visual inspection of the eggs five minutes after the addition of sperm. At least 6 eggs were injected for each of the concentrations shown in this figure.
Figure 4.2) Photomicrographs of eggs after fertilisation.
The top panel shows a control egg. This has a full fertilisation envelope visible as a clear ring surrounding the egg. The bottom panel shows an egg which was injected with ATPyS to a final cytoplasmic concentration of 200 μM. This egg has several fertilisation cones but no fertilisation envelope.
a movement of egg cytoplasm into the region surrounding the site of sperm nucleus, mitochondria and axonemal complex that leads to a protrusion at the site of sperm entry (Longo, 1973). The localised polymerisation of actin is probably important in the formation of these structures. The presence of these suggests that ATPyS was not preventing sperm–egg interactions.

(ii) ATPyS does not Inhibit Sperm–Egg Interactions.

The presence of fertilisation cones in inseminated ATPyS injected eggs suggested that ATPyS was not affecting sperm–egg interactions. This was investigated more fully using a gamete fusion assay as 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 which have taken up this dye, their nuclei become brightly fluorescent. This is an easy way to judge the success of early sperm–egg interactions. Figure 4.3 shows a control egg and an egg which had been injected with 150 μM ATPyS. Both eggs have interacted with sperm. In the control egg, one fluorescent sperm nucleus is visible. In the egg injected with ATPyS, three fluorescent sperm nuclei are visible. I scored the numbers of fusing sperm in both control and injected eggs. Table 4.1 shows that the injection of ATPyS leads to multiple sperm–fusion events.

<table>
<thead>
<tr>
<th>Table 4.1</th>
<th>The effect of ATPyS on the transfer of H33342 between egg and sperm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs</td>
<td>Number of fluorescent sperm nuclei per egg. Mean (+/- sem)</td>
</tr>
<tr>
<td>Control</td>
<td>1.00 (+/- 0.00, n = 12)</td>
</tr>
<tr>
<td>ATPyS</td>
<td>6.73 (+/- 1.60, n = 15)</td>
</tr>
<tr>
<td>250μM – 1mM</td>
<td></td>
</tr>
</tbody>
</table>

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Figure 4.3) Fluorescence micrographs of eggs loaded with H33342, after fertilisation.

Figure 4.3a. A control egg stained with the DNA-staining vital dye H33342, after fertilisation. This egg had a full fertilisation envelope. This shows a fluorescence micrograph of the egg illuminated with 340–380 nm light. Emission was at 450–490 nm. A single bright spot is visible which shows the single sperm which has fused with this egg.

Figure 4.3b. An egg stained with H33342 after the microinjection of ATPyS (final cytoplasmic concentration of 200 µM) and insemination. The egg had no fertilisation envelope. The egg was illuminated as in figure 4.3a. 3 fluorescent sperm nuclei have fused with this egg.
This table shows that ATPyS does not prevent gamete fusion and allows many sperm to fuse with eggs. This result is entirely expected, since the absence of a fertilisation envelope means that there is no physical block to prevent polyspermy.

(iii) ATPyS does not Affect the Sperm—Induced Rise in $[\text{Ca}^{2+}]$, at Fertilisation.

Another obvious explanation for this inhibition of exocytosis is that ATPyS is preventing the sperm induced rise in intracellular free calcium which we know is a requirement of cortical granule exocytosis. I used the calcium sensitive fluorescent dye fura2 to measure the changes in intracellular free calcium at fertilisation in both untreated eggs and eggs microinjected with ATPyS.

Figure 4.4 shows changes in intracellular free calcium ion concentrations in a) a control non— injected egg and b) an egg microinjected to give a cytoplasmic final concentration of approximately 250 $\mu$M. Figure 4.4a shows that upon insemination $[\text{Ca}^{2+}]$, increases from a resting level of approximately 200 nM to over 2 $\mu$M. The calcium ion concentration then decreases over the next 10 minutes to levels approaching those seen prior to fertilisation. Figure 4.4b shows the calcium changes that occur following microinjection of ATPyS and subsequent insemination. The record shows firstly that a small calcium increase of approximately 200 nM occurs on injection. Secondly, it shows that when the egg was inseminated at about 12 minutes after injection, a calcium transient occurred that was very similar in both extent and duration to that which occurs in control eggs. No cortical granule exocytosis was seen. This record is typical of 6 records of calcium changes at fertilisation in eggs microinjected with ATPyS. These data indicate that ATPyS does not prevent the sperm—induced rise in intracellular free calcium. This indicates that the effect of ATPyS on cortical granule exocytosis resides downstream of calcium mobilisation and is thus a direct effect on exocytosis.
Figure 4.4) Intracellular free calcium ion concentration measured in single eggs at fertilisation.

Calcium transients at fertilisation in a) a control egg and b) an egg injected with ATPyS (final cytoplasmic concentration of 250 μM) measured with the dye fura2 (final cytoplasmic concentration 25 μM). In the control egg at fertilisation, \([\text{Ca}^{2+}]_i\) rapidly increases from approximately 200 nM to over 2 μM. This then returns to resting levels over the following 10 minutes. In the ATPyS injected egg, the addition of sperm causes a calcium transient very similar in both extent and duration to that at a normal fertilisation. These records are typical of 6 such experiments.
ATPyS can Cause Spontaneous Calcium Transients.

Occasionally, the microinjection of ATPyS, particularly at high concentrations gave rise to a calcium transient several minutes after injection. Figure 4.5 illustrates an example of this in an egg microinjected with 400 μM ATPyS. Approximately 5 minutes after microinjection [Ca$^{2+}$]$_i$ increases to approximately 1.8 μM and gradually decreases to 0.7 μM in the following 5 minutes. The application of sperm causes a very similar increase in [Ca$^{2+}$]$_i$. Neither of these calcium increases caused exocytosis. It is apparent, though, that this calcium transient is not necessary for inhibition of exocytosis to occur, since in figure 4.4 I showed an egg which did not have a spontaneous transient. This egg failed to undergo exocytosis.

The reason for spontaneous calcium transients is unknown, but a possible explanation is that the thiophosphate moiety of ATPyS is used to phosphorylate the 5' position of inositol on PIP. The turnover of the resultant thiophosphorylated – PIP$_2$ would lead to the production of non-hydrolysable InsP$_3$, which is resistant to hydrolysis (Cooke, Nahorski and Potter, 1989). When a critical concentration of this is reached the autocatalytic wave thought to be responsible for the calcium transient at fertilisation would be set off with the result of a "fertilisation-like" rise in intracellular free calcium.

Another possibility is that InsP$_3$ could be produced through another route. There is evidence that the enzyme nucleoside diphosphokinase can transfer the thiophosphate group from ATPyS to GDP leading to the formation of GTPyS (Wieland and Jakobs, 1989). It is a possibility that the ATPyS injected into eggs is being used to produce GTPyS. This would then cause an increase in [Ca$^{2+}$]$_i$. 

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Figure 4.5) ATPyS caused spontaneous calcium transients.
A calcium transient in an egg injected with 400 μM ATPyS measured with the dye fura2 (cytoplasmic concentration 25 μM). Approximately 5 minutes after microinjection $[\text{Ca}^{2+}]_i$ increases to approximately 1.7 μM and then falls to about 0.7 μM. The application of sperm causes a further calcium transient to about 1.7 μM. Fertilisation envelope elevation does not occur.
3. MECHANISMS OF INHIBITION OF EXOCYTOSIS BY ATPyS.

(i) The Effect of ATPyS on the Calcium-Sensitivity of Cortices.

ATPyS clearly does not affect the interactions between sperm and egg, nor the sperm-induced rise in intracellular free calcium which normally leads to cortical granule exocytosis. It is inhibiting cortical granule exocytosis more directly. The two obvious ways in which this inhibition might occur are either by causing irreversible phosphorylation as described earlier, or by acting as a competitive inhibitor of some process essential for cortical granule exocytosis to occur. In order to assess which of these is the most likely explanation for this inhibition I treated cortices with ATPyS in two different ways and examined the calcium sensitivity. Firstly I incubated cortices for 10 minutes with 1 mM ATPyS prior to calcium stimulation. If ATPyS is used to thiophosphorylate a protein then this procedure should lead to inhibition providing the kinase responsible for this is present in the egg cortex. The calcium sensitivity of pre-incubated cortices was determined using the light scattering assay described in chapter 2. Figure 4.6a shows the calcium sensitivity of both control and ATPyS-treated cortices. The experiment shows that treatment with ATPyS does not affect the calcium-sensitivity of exocytosis. In both treated and untreated cortices, half maximal exocytosis occurred with a calcium concentration of about 5 μM.

It is possible that the inhibitory effects of ATPyS requires it to be present at the same time as calcium. In other words, ATPyS may inhibit exocytosis as a competitive inhibitor of some essential process. To look at this second possibility, I prepared Ca^{2+}/EGTA buffers in IM with a fixed EGTA concentration of 10 mM, containing 250 μM ATPyS. This is a concentration of ATPyS which completely inhibits exocytosis in vivo. The presence of ATPyS did not affect the free calcium ion concentration. Figure 4.6b shows that ATPyS does not significantly affect the calcium sensitivity of exocytosis when present in the calcium buffers used to stimulate exocytosis. Again, in the presence or absence of ATPyS, half maximal
Figure 4.6) The effect of ATPyS on the calcium sensitivity of cortices \textit{in vitro}.

Figure 4.6a. The effects of ATPyS pretreatment on the calcium dependent exocytosis of cortical granules \textit{in vitro}. Cortices were treated with ATPyS (1 mM in IM) or IM for 10 minutes prior to stimulation with calcium buffers. The extent of exocytosis was measured using the light scattering assay described in chapter 2.

Figure 4.6b. The effects of including ATPyS (250 μM) in the calcium buffers used to stimulate cortical granule exocytosis \textit{in vitro}. Cortices were stimulated with calcium buffers in the presence or absence of 250 μM ATPyS. The extent of exocytosis was assessed using the light scattering assay described in chapter 2. Mean and sem are shown, n = 4.
exocytosis occurred with a calcium concentration of about 5 μM. These two results using ATPyS in vitro lead us to the conclusion that ATPyS inhibits exocytosis in vivo but has no effect in vitro, either in pre-incubation experiments or when present in the calcium containing solutions used to stimulate exocytosis. This suggests that the inhibition is probably due to thiophosphorylation or metabolism of ATPyS to produce an inhibitory compound and that ATPyS inhibits exocytosis only when present in whole eggs. The inhibition clearly requires the presence of cytosolic factors.

(ii) The Effect of AppNHp on Cortical Granule Exocytosis.

From the data above we have two possible ways of describing the mode of action of ATPyS; it or one of its metabolites inhibits some process involved in controlling exocytosis in the intact egg, or it is used as a kinase substrate. I microinjected eggs with another non-hydrolysable analogue of ATP, but one which is not a kinase substrate. The compound I used was AppNHp. This compound is an ATP analogue but cannot be used by kinases (Yount et al. 1971). Eggs were microinjected with different concentrations of AppNHp and sperm were added at least 10 minutes after microinjection. AppNHp had no effect at concentrations up to 1.2 mM in 18 eggs. This suggests that ATPyS is not inhibiting exocytosis by competing with normal, hydrolysable cellular ATP. It also suggests that the inhibition is due to the production of a metabolite or a thiophosphoprotein.

(iii) The Time Course of Inhibition by ATPyS.

In order to find out more about the mode of inhibition by ATPyS, I looked at the timing of the inhibition for a given concentration of ATPyS. The concentration of ATPyS which I used was 250 μM, a concentration at which 100% inhibition occurred. I looked at the time dependency by microinjecting eggs at various times prior to insemination and then scoring for fertilisation envelope elevation. The results of this are shown in figure
4.7. This shows that inhibition of exocytosis by ATPyS is a time-dependent phenomenon. In this time course experiment, 100% inhibition does not occur until approximately 7 minutes after injection. This result indicates that ATPyS is not the inhibitor compound. The time-dependency of this inhibition again suggests that the inhibition is due to either the production of a metabolite of ATPyS, or that ATPyS has been used as a substrate by cellular kinases and that the resultant thiophosphorylation of protein(s) inhibits exocytosis. What is clear from the studies of the action of ATPyS in vitro is that the enzyme or substrate responsible for the modification of ATPyS which causes inhibition must be cytosolic since ATPyS had no effect on exocytosis in vitro whether used for pre-incubation or in the calcium buffers.

(iv) The Inhibition of Exocytosis by ATPyS is Irreversible.

The results from the timing experiments suggest that ATPyS is inhibiting exocytosis through the production of some metabolite or by being used to irreversibly phosphorylate important proteins. In order to determine which of these possibilities is the most likely, I prepared cortices from eggs which were microinjected with ATPyS prior to preparation. Using this approach, it is possible to distinguish between these two possibilities since if the inhibition is due to metabolite production, 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. In this experiment I microinjected eggs with either ATPyS to a final concentration of 250 μM, or a similar volume of injection vehicle (450 mM KCl/50 mM PIPES, pH 6.7). The eggs were left for 10 minutes and then cortices were prepared in the usual manner. Cortices were washed with IM prior to the addition of calcium buffers. The cortices were treated with a calcium buffer containing 10 μM free Ca²⁺ and photographs taken. Figure 4.8 shows the
Figure 4.7) The timing of the inhibition of exocytosis by ATPyS.

Eggs were microinjected with ATPyS to give a final cytoplasmic concentration of 250 μM and were fertilised at various times after microinjection. Each point represents at least 3 eggs injected at each concentration in 3 separate experiments. Mean and sem are shown.
results of this experiment. The top panel shows a cortex from a normal egg in the presence of 10 mM EGTA. It is covered with cortical granules. The panel in the centre illustrates a cortex from an egg microinjected with vehicle after the addition of 10 μM Ca$^{2+}$. Exocytosis of at least 70% of the cortical granules is seen as a clearance of the lawn. This value of about 70% is typical of the extent of exocytosis seen in normally prepared cortices at this concentration of calcium. The bottom panel shows a cortex from an egg which had been microinjected with ATPyS to a final concentration of approximately 250 μM after treatment with 10 μM Ca$^{2+}$. A comparison of this panel with the control cortex in the presence of 10 μM calcium shows that the microinjection of ATPyS into this egg has inhibited exocytosis in the isolated cortex. This result shows us that the effect of ATPyS is irreversible; the lower panel shows a cortex from an egg injected with ATPyS but none of this compound is present at the time of calcium treatment. This result and the results obtained using the in vitro preparation in section 4.5 also suggests that cytosolic factors are required for inhibition to occur. This result strongly suggests that ATPyS elicits its inhibitory effect through the irreversible phosphorylation of some protein(s) rather than through the production of a metabolite and that the kinase responsible for this, or the protein which is phosphorylated, is cytosolic.

4. INTRACELLULAR TARGETS FOR THIOPHOSPHORYLATION.

(i) ATPyS Inhibits Exocytosis in Permeabilised Eggs.

All of the results so far indicate that the probable mode of action of ATPyS is through the irreversible phosphorylation of proteins. A useful approach in identifying important exocytotic control proteins is to look at which proteins are phosphorylated by ATPyS. This can be done by using $^{35}$S-labelled ATPyS. However, I have shown that this inhibitory effect will only occur in the presence of egg cytoplasm, in other words the kinase responsible for the inhibitory thiophosphorylation is cytosolic. In order to bring about this inhibition I have had to microinject eggs with ATPyS but
Figure 4.8) ATPyS irreversibly inhibits exocytosis.

ATPyS has an irreversible inhibitory effect upon cortical granule exocytosis \textit{in vitro} if the cortex is prepared from an egg microinjected with ATPyS. Cortices were prepared from eggs microinjected with either KCl/PIPES vehicle or ATPyS to give a final concentration of 250 μM. The top panel shows a cortex in the presence of 10 mM EGTA. The centre panel is a control cortex after stimulation with 10 μM Ca$^{2+}$. The bottom panel shows a cortex prepared from an ATPyS injected egg after treatment with 10 μM Ca$^{2+}$ for 10 minutes. Clearly, exocytosis has been inhibited in this cortex, although ATPyS is no longer present. This result suggests that ATPyS elicits its inhibitory effects through thiophosphorylation of proteins. Scale bar is 20 μm.
it is not a realistic proposition to use a microinjection technique with \(^{35}\text{S}\) labelled ATPyS in sea urchin eggs in order to try to detect thiophosphorylated proteins. It is obviously desirable to be able to use high activities of labelled ATPyS in large populations of eggs in order to have a good chance of identifying thiophosphorylated proteins. I have used the technique of electropermeabilisation to allow ATPyS to gain access to large populations of eggs. I used an electrical permeabilisation technique based on the method of Swezey and Epel (1988), in order to determine whether this allows access of ATPyS to the egg interior without the loss of the cytosolic protein which I suggested is responsible for the inhibition of exocytosis. A 25% suspension of eggs in permeabilisation buffer (PB) was permeabilised as described in chapter 2 in the presence (1 mM) or absence of ATPyS. As soon as permeabilisation was accomplished the suspension was applied to glass slides treated with 0.1 mg/ml polylysine. The eggs were left for 10 minutes after which time cortices were prepared in IM in the normal way. Exocytosis in response to calcium was assessed using the usual light scattering assay.

Figure 4.9 shows the calcium-sensitivity of cortices prepared from eggs permeabilised in the presence or absence of ATPyS. This figure shows that the process of permeabilisation itself decreases the calcium sensitivity of the cortices. In the control permeabilised cortices the calcium required for half maximal exocytosis is about 20 \(\mu\text{M}\) whereas in cortices prepared from non-permeabilised eggs (see figure 4.5) this is about 5 \(\mu\text{M}\). The figure also demonstrates that when the eggs were permeabilised in the presence of 1 mM ATPyS, the cortices from these eggs were significantly less sensitive to calcium. The calcium required for half maximal exocytosis in this case is about 50 \(\mu\text{M}\). The figure also shows that at a calcium concentration of 100 \(\mu\text{M}\) only 75% exocytosis occurs in cortices prepared from eggs treated with ATPyS. Indeed increasing the calcium concentration to 1 mM did not stimulate any further exocytosis in these cortices. This shows that ATPyS will enter these transiently permeable eggs and elicit its inhibitory effects although not fully. This is not surprising. This relatively
Figure 4.9) The calcium sensitivity of cortices prepared from eggs permeabilised in the presence of ATPγS.

Cortices were prepared from eggs which were transiently permeabilised in a perspex cell by 3 discharges with a 1 μF capacitor charged to 100 V. Eggs were permeabilised in PB in the presence or absence of 1 mM ATPγS. Cortices were prepared in the normal manner and the extent of exocytosis determined using the light scattering assay.
gentle permeabilisation procedure does not permeabilise all of the eggs in a suspension and so the calcium sensitivity shown in figure 4.9 is for a mixture of cortices prepared from permeabilised and unpermeabilised eggs. It was apparent in these experiments that the response of the cortices to calcium was variable. Some did not seem to be significantly less sensitive to calcium than control cortices. On the other hand, others were much less sensitive, such that there appeared to be no secretory response at all. This reflects the gently permeabilisation technique which was employed in this study in order to maximise the likelihood of ATPyS-inhibition occurring by reducing the rate at which cytosolic proteins leak out of the eggs.

(ii) Identification of Substrates for ATPyS-Mediated Thiophosphorylation.

Using the technique of electropermeabilisation I allowed $^{35}$S-ATPyS access to the interior of a large population of eggs in an attempt to identify cortical proteins which are targets for phosphorylation by this substrate. I permeabilised a 25% suspension of eggs in PB containing 1 mCi $^{35}$S-ATPyS and immediately poured the suspension into plastic petri dishes coated with 0.1 mg/ml polylysine. The eggs were left at 16°C for 30 minutes after which time cortices were prepared. Proteins were extracted with 1 M NaOH and prepared for PAGE as described in chapter 2. A sample of whole eggs was also prepared for PAGE. To illustrate that thiophosphorylation was a process which required access to the cytoplasm I prepared cortex and whole egg samples from eggs which were incubated with $^{35}$S-ATPyS without permeabilisation.

Figure 4.10 shows that $^{35}$S incorporation into cortices from permeabilised eggs is up to 40 x greater than in non-permeabilised eggs illustrating that the uptake requires access to the cytoplasmic side of the plasma membrane, either through thiophosphorylation or uptake into cortical organelles, for instance the cortical granules.
Figure 4.10) Uptake of $^{35}$S into egg cortices.

Cortices were made from eggs made permeable in the presence of 1 mCi $^{35}$S–ATPyS. They were dissolved in 1 M NaOH and a fraction counted in a scintillation counter. As a control, cortices were also prepared and counted from non-permeabilised eggs incubated with 1 mCi $^{35}$S–ATPyS.
Figure 4.11 shows an autoradiogram of a polyacrylamide gel of these samples. Lanes 1–3 and 5–7 are samples prepared from eggs permeabilised in the presence of 1 mCi $^{35}$S-ATP with lanes 1,2,5 and 6 cortical proteins and 3 and 7, proteins prepared from whole eggs. Lanes 8–10 are cortical proteins (8 and 9) and whole eggs (10) from eggs which were incubated with 1 mCi $^{35}$S-ATP without being permeabilised. Lanes 4 and 11 are $^{14}$C-labelled protein markers. The autoradiogram clearly shows that two polypeptides are strongly labelled in both cortices and whole eggs permeabilised with $^{35}$S-ATP. Eggs which were not permeabilised do not have these proteins labelled which shows that their localisation is intracellular. The lanes containing samples of egg cortex show quite clearly that both of these labelled bands are associated with the exocytotic apparatus. This shows that the proteins thiophosphorylated by ATP in eggs are localised to the plasma membrane and supports the suggestion made previously that ATP inhibits exocytosis through protein-thiophosphorylation. It is interesting that in the lanes containing whole egg samples there are no other obvious labelled polypeptides. The two protein bands have approximate molecular weights of 33 and 27 kD. In several other experiments only the higher molecular weight band is present which suggests that the 27 kD protein may be a proteolytic fragment of the 33 kD protein.

This experiment clearly shows that ATP is used by an egg kinase to thiophosphorylate protein(s) which are associated with the egg cortex. This suggests that ATP does indeed inhibit exocytosis through the thiophosphorylation of proteins. These proteins when thiophosphorylated, are associated with the egg cortex and can inhibit exocytosis. Of course, this data does not necessarily show that the dephosphorylation of this protein is a requirement for exocytosis to occur. It could equally suggest that this thiophosphorylation confers on the substrates an inhibitory function which is not of physiological importance. However, this data provides us with a possible mechanism by which exocytosis might be regulated, and the finding that $^{35}$S-ATP labels only 2 polypeptides provides us with a useful first
step towards identifying the biochemical nature and function of these proteins.

5. CONCLUSION.

In this chapter, I have shown that ATPyS inhibits cortical granule exocytosis. It does not prevent gamete fusion or the sperm−induced rise in intracellular free calcium which normally stimulates cortical granule exocytosis. Indeed, ATPyS can cause a rise in $[\text{Ca}^{2+}]_i$, possibly due to the production of InsP$_3$. ATPyS has no effect on exocytosis in vitro, unless present in the intact egg before the preparation of cortices. This result strongly suggests that ATPyS inhibits exocytosis by thiophosphorylating proteins and strongly suggests that either the kinase or the phosphorylated protein are cytosolic. Using a permeabilised egg preparation, I have shown that only one or two proteins are both thiophosphorylated, and associated with the egg cortex. This means we may have a way of identifying control proteins involved in cortical granule exocytosis.

Some of this work has already been presented in abstract form (Crossley, Whalley and Whitaker, 1989).
Figure 4.11) An autoradiogram of a polyacrylamide gel of proteins prepared from eggs permeabilised in the presence of 1 mCi $^{35}$S-ATPyS.

Eggs were permeabilised and after 30 minutes samples were prepared. Lanes 1,2 and 5,6 are cortex proteins. Corresponding whole egg samples for these experiments are lanes 3 and 7. Approximately 50 x more protein was run in the whole egg lanes. Lanes 8,9 are cortices and lane 10 whole eggs incubated with 1 mCi ATPyS without permeabilisation. Lanes 4 and 11 are $^{14}$C-N-ethylmaleimide labelled protein standards. The figures to the sides of the autoradiogram are the molecular weights in kDa of the appropriate marker proteins.
Chapter 5. The Role Of G–proteins In Exocytosis.

1. INTRODUCTION.

For several years there have been suggestions that G–proteins might have a direct role in stimulating exocytosis. The first suggestion of this came from patch–clamp experiments in mast cells where GTPγS was shown to stimulate exocytosis (Fernandez, Neher and Gomperts, 1984). A definitive role was shown in mast cells by Cockcroft et al. (1987), who showed that GTPγS directly stimulated exocytosis by using neomycin to uncouple the secretory response from activation of PIC. This study showed that the effect of GTPγS was not merely to change the affinity of exocytosis to Ca^{2+} through DAG–activated PKC. There is now evidence from a number of other cells which suggests this role for G–proteins. In RINm5F cells GTPγS stimulates secretion at low levels of Ca^{2+} but in this cell PIC–activation is calcium–dependent (Vallar, Biden and Wollheim, 1987; Wollheim et al. 1987). GTPγS can also stimulate exocytosis in adrenal chromaffin cells (Bittner, Holz and Neubig, 1986) and in neutrophils (Barrowman, Cockcroft and Gomperts, 1986). Low molecular weight G–proteins have been found to be associated with adrenal chromaffin granules (Burgoyne and Morgan, 1989). Yeast, too, possess G–proteins which regulate secretion. The SEC4 gene product is essential for the yeast secretory pathway. It has been cloned (Salminen and Novick, 1987) and characterised (Goud et al. 1988), and it is a ras–like G–protein.

From many cells then, there is accumulating evidence to implicate G–proteins in controlling exocytosis. In sea urchin eggs, there is some evidence to support the idea of a regulatory G–protein being involved in exocytosis. GDPβS inhibits cortical granule exocytosis when injected into eggs without affecting the increase in intracellular calcium which normally triggers exocytosis (Crossley and Whitaker, 1989). However, in vitro, the only stimulus for exocytosis is an increase in the free calcium ion concentration. A more important role for G–proteins in sea urchin eggs
might be in stimulating the calcium transient at fertilisation (Turner et al. 1986; Swann, Ciapa and Whitaker, 1987).

The cytoplasmic signal stimulating exocytosis at fertilisation is an increase in $[\text{Ca}^{2+}]_i$, which is caused by the interaction of sperm and egg. What is not known is how the interaction can trigger this ionic signal. In the experiments described in this chapter, I have investigated the ways in which various compounds can cause the release of calcium from intracellular stores. I have tried to draw analogies between these mechanisms and the actions of sperm in an attempt to understand more fully the molecular events occurring at fertilisation which lead to an increase in $[\text{Ca}^{2+}]_i$.

The polyphosphoinositide second messenger system has a central role to play in the control of the ionic changes in the egg which are known to occur at fertilisation (Whitaker, 1989). Both limbs of the second messenger system are implicated in causing the dual ionic changes which initiate development. $\text{InsP}_3$ is responsible for the release of $\text{Ca}^{2+}$ from intracellular stores (Whitaker and Irvine, 1984; Swann and Whitaker, 1986; Crossley et al. 1988; Clapper and Lee, 1985), and DAG, through the activation of protein kinase C (Nishizuka, 1984), can activate the $\text{Na}^+ / \text{H}^+$ antiport, which leads to cytoplasmic alkalinisation, (Swann and Whitaker, 1985; Shen and Burgart, 1986). What is not clear is how interactions of sperm and egg can lead to the generation of these second messengers.

In many instances of stimulus–response coupling there is now good evidence for the regulatory involvement of a family of proteins called GTP–binding proteins or G–proteins (Gilman, 1987). The PPI second messenger system is also capable of being activated through interactions with regulatory G–proteins (Cockcroft and Gomperts, 1985). In the sea urchin egg, Turner et al. (1986, 1987) have suggested that sperm trigger the rise in intracellular free calcium by activating phosphoinositidase C through a pathway involving a G–protein. The evidence for this is that a non–hydrolysable analogue of GTP (GTPyS: guanosine 5’–O–thiotriphosphate), causes the elevation of a fertilisation envelope, (Turner et al. 1986), presumably by increasing the
intracellular free calcium ion concentration. They also found (Turner et al. 1987) that cholera and pertussis toxins, which ADP-ribosylate the alpha-subunits of particular G-proteins (Gill, 1982; Ui, 1984), have substrates in sea urchin eggs which are of similar molecular weights to the alpha-subunits of known G-proteins (Gilman, 1984; Joseph, 1985) and that cholera toxin also caused cortical granule exocytosis and fertilisation envelope elevation.

Other nucleotides have been tested and one other guanine nucleotide has been shown to cause effects similar to those of GTPyS. Swann et al. (1987) reported that the cyclic nucleotide cGMP caused egg activation and a transient increase in intracellular free calcium similar to that occurring at fertilisation. In this chapter I describe some of the approaches I have used to determine how GTPyS and cGMP cause an increase in [Ca^{2+}]_i leading to activation and cortical granule exocytosis.

2. GUANINE NUCLEOTIDE ACTIVATION OF SEA URCHIN EGGS.

I microinjected various nucleotides into eggs and scored for egg activation five minutes after microinjection. Figure 5.1 shows the results of this study. As has been previously reported both GTPyS and 3',5'cGMP caused activation in approximately 90% of eggs at a final cytoplasmic concentration of 100 µM (Swann et al. 1987). In contrast neither ATPyS nor GDPβS caused significant egg activation judged by fertilisation envelope elevation. This result suggests that the activation by GTPyS is through the activation of a G-protein rather than through non-specific effects of injecting either guanine nucleotides or compounds with thiophosphate moieties. Swann et al. (1987) previously reported that a non-hydrolysable analogue of cGMP, 8-Br-cGMP was ineffective. I have confirmed this finding and also found that this compound did not prevent sperm from activating eggs when injected to concentrations as high as 250 µM. I investigated the modes of action of GTPyS and cGMP, the two compounds which caused egg activation.
Figure 5.1) The effects of some nucleotides on egg activation.

Eggs were microinjected with nucleotides to the concentration indicated. Egg activation was assessed by scoring eggs for fertilisation envelope elevation 5 minutes after microinjection. Eggs were fertilised after injection experiments. These results were obtained using batches of eggs in which at least 95% of non-injected eggs activated following insemination. The results for each nucleotide represent at least 15 individual eggs.
3. CALCIUM RELEASE FROM INTRACELLULAR STORES.

Both GTPyS and cGMP cause a transient increase in intracellular free calcium (Swann et al. 1987). Both of these compounds are equally efficacious in eliciting this response in the presence or absence of extracellular calcium (Crossley, I. unpublished observation). This shows that mobilisation from intracellular stores is an important event in this parthenogenetic activation. I wanted to distinguish between the two possible ways in which this calcium release might be occurring, that is through direct effects on the eggs' reticular calcium store or through the generation of calcium releasing second messengers, the most likely candidate being InsP$_3$.

I examined the first of these two possibilities by studying the ability of these two compounds to release $^{45}$Ca, sequestered in response to ATP, from permeabilised eggs which were attached to petri−dishes coated with polylysine (see chapter 2). The protocol used was developed such that the difference between sequestration in the presence and absence of ATP was maximised. That is, non−specific binding of $^{45}$Ca was minimised and uptake into an ATP−sensitive store maximised.

Figure 5.2 illustrates the results of experiments designed to find the best concentration of digitonin for permeabilisation. In this experiment, I permeabilised eggs with increasing concentrations of digitonin and measured $^{45}$Ca uptake at 10 minutes in the presence and absence of ATP. The graph shows mean values from 2 experiments of $^{45}$Ca uptake (closed circles), and ATP−independent uptake as a percentage of ATP−dependent uptake (open circles). The figure shows that $^{45}$Ca uptake was maximum, and ATP−independent uptake minimum using a digitonin concentration of 0.22 mM (0.0025%). This concentration of digitonin was used for all subsequent experiments.
Figure 5.2) The effect of varying the concentration of digitonin on $^{45}$Ca uptake in permeabilised eggs.

The concentration of digitonin was varied and $^{45}$Ca uptake (closed circles), and ATP-independent $^{45}$Ca uptake as a % of ATP-dependent uptake (open circles), determined 10 minutes after the addition of $^{45}$Ca.
(i) $^{45}\text{Ca}$ Uptake is an ATP-Dependent Process.

Figure 5.3 shows that the sequestration of $^{45}\text{Ca}$ in the permeabilised egg preparation was dependent on the presence of ATP. The graph shows uptake of $^{45}\text{Ca}$ into permeabilised eggs in the presence and absence of 2 mM ATP. The free calcium ion concentration was calculated to be about 200 nM (using the constants of Martell and Smith, 1975). Each point represents the mean and sem of three experiments. Clearly, at all time points uptake in the presence of ATP was greater than in the absence of ATP. At the 1 and 3 minute time points uptake in the absence of ATP was approximately 25% of ATP dependent uptake. By 5 minutes this ATP independent uptake had decreased to 18% and remained at this level for subsequent time points. Because of this, test compounds were always introduced to eggs after they had been allowed to sequester $^{45}\text{Ca}$ for 5 minutes. Calcium uptake in sea urchin egg fractions has previously been shown to be dependent on ATP in both isolated cortical fragments (Payan et al. 1986) and in homogenates (Clapper and Lee, 1985).

(ii) $^{45}\text{Ca}$ Uptake is into a Non-Mitochondrial Store.

To show that calcium sequestration was into non-mitochondrial stores, I measured ATP-dependent $^{45}\text{Ca}$ uptake in the presence and absence of 10 μM FCCP (carbonyl cyanide 4-trifluoromethoxyphenyl-hydrazone) which is a mitochondrial uncoupler. I permeabilised eggs and added $^{45}\text{Ca}$ in the presence or absence of 2 mM ATP. A further ATP sample also contained 10 μM FCCP (taken from a 10 mM stock in DMSO). I measured the $^{45}\text{Ca}$ uptake at 6 and 10 minutes. Figure 5.4 shows that 10 μM FCCP does not significantly affect $^{45}\text{Ca}$ sequestration. This result indicates that the ATP-dependent uptake of calcium in permeabilised eggs is into non-mitochondrial calcium stores when the calcium is buffered to sub-micromolar levels. This is in keeping with findings in mammalian cells, where non-mitochondrial pools are characterised by having an affinity for calcium in the nanomolar range, whereas mitochondrial calcium pools

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Figure 5.3) $^{45}$Ca uptake into permeabilised eggs is dependent upon ATP. $^{45}$Ca uptake was determined at various times after the addition of permeabilisation buffer containing 0.5 μCi/ml $^{45}$Ca, in the presence (open circles), or absence (closed circles), of 2 mM ATP. Each value represents the mean and sem of at least 3 experiments.
Figure 5.4) $^{45}\text{Ca}$ uptake is into a non-mitochondrial calcium store.

$^{45}\text{Ca}$ uptake was determined 6 and 10 minutes after the addition of $^{45}\text{Ca}$ in the presence and absence of 2 mM ATP. The presence of the mitochondrial uncoupler FCCP (10 µM) did not significantly affect uptake. Each value represents the mean and sem of 3 experiments.
sequester calcium at higher concentrations (Burgess et al. 1983; Biden et al. 1986).

(iii) Permeabilised Eggs are Sensitive to InsP₃.

In order to determine the calcium releasing activity of the nucleotides under examination, it was important to ensure that the system was sensitive to known calcium releasing agents. I examined the ability of InsP₃ to release $^{45}$Ca from permeabilised eggs. Figure 5.5 shows a typical experiment in which I challenged permeabilised eggs with either InsP₃ or the calcium ionophore A23187. These compounds were added 5 minutes after the addition of $^{45}$Ca, experiments were stopped after a further 1 or 5 minutes and the radioactivity associated with the eggs determined. In this experiment, 2 μM InsP₃ caused a 40 % reduction in the $^{45}$Ca associated with the eggs and 10 μM InsP₃ a 90 % reduction. When A23187 was used to a final concentration of 10 μM a very similar result was obtained. These decreases in $^{45}$Ca associated with the eggs are due to calcium release. This experiment shows that calcium release in this preparation is sensitive to the dose of InsP₃ and also that the $^{45}$Ca is sequestered almost exclusively into an InsP₃ sensitive calcium store. A high dose of InsP₃ released the same calcium as 10 μM A23187 which suggests that the non-released calcium is not sequestered into an InsP₃-insensitive membrane Ca²⁺ store. Similar results to this were found by Oberdorf et al. (1986); in both isolated cortices and microsomes, A23187 released approximately 85 % of the $^{45}$Ca sequestered in the presence of ATP.

(iv) Neither GTPyS nor cGMP Cause $^{45}$Ca Release.

I tested the ability of GTPyS and cGMP to release calcium from permeabilised eggs. I permeabilised eggs, allowed them to sequester $^{45}$Ca for 5 minutes and added nucleotides or InsP₃ as a control. The $^{45}$Ca
Figure 5.5) InsP$_3$ causes the release of $^{45}$Ca from permeabilised eggs.

The effects of adding InsP$_3$ at 2 and 10 $\mu$M, and 10 $\mu$M A23187 were investigated. These agents were added at 5 minutes and the $^{45}$Ca associated with the eggs was determined a further 1 or 5 minutes later. 2 $\mu$M InsP$_3$ caused the release of approximately 40 % of the sequestered calcium. Both 10 $\mu$M InsP$_3$ and 10 $\mu$M A23187 caused the release of approximately 90 % of sequestered calcium. This record is typical of 3 such experiments.
associated with the eggs was measured 1 or 5 minutes after these additions. Figure 5.6 shows the results of these studies. In this figure mean and sem values are shown for 5 experiments in which the effects of these nucleotides was investigated. It also shows values obtained using InsP$_3$ in 5 experiments. These experiments clearly show that neither of these nucleotides cause any calcium release from permeabilised eggs at concentrations of 100 µM. Indeed, in single experiments using 500 µM GTPyS or cGMP no calcium release was detected.

In contrast InsP$_3$ causes a dose–dependent release of $^{45}$Ca with half–maximal release occurring at about 2 µM. This value is considerably higher than the 50–60 nM reported using sea urchin egg homogenates (Clapper and Lee 1985). However, this value is similar to values reported for other permeabilised cells (Biden et al. 1986; Stoehr et al. 1986). These studies in permeabilised eggs show that neither GTPyS nor cGMP can release $^{45}$Ca from permeabilised eggs.

Of course one might argue that the lack of effect of cGMP and GTPyS on calcium efflux from the endoplasmic reticulum is that $^{45}$Ca is preferentially taken up into an InsP$_3$–sensitive store and that cGMP and GTPyS release calcium from a different store which has a reduced rate of calcium sequestration. There is a precedent for this. Biden et al. (1986), showed that in permeabilised GH$_3$ cells the InsP$_3$–sensitive calcium store preferentially sequestered Ca$^{2+}$ when compared to InsP$_3$–insensitive endoplasmic reticulum calcium stores. In sea urchin eggs, Clapper et al. (1987) have shown that a number of different calcium stores exist in homogenates, which are sensitive to different calcium mobilising agents.

In order to investigate if these nucleotides released calcium from a different calcium store, one which did not sequester $^{45}$Ca under these conditions, I used the calcium sensitive fluorescent dye fura2 to monitor calcium changes in permeabilised egg suspensions. In these experiments I
Figure 5.6) GTPyS and cGMP cannot release $^{45}$Ca from permeabilised eggs.

$^{45}$Ca release was measured 1 or 5 minutes after the addition of the compound under test. In$\text{P}_3$ causes a dose dependent release of calcium with half-maximal release occurring at approximately 2 $\mu$M. In contrast, neither GTPyS nor cGMP caused any $^{45}$Ca release. Each data point is the mean of 6 experiments. The error bars are sem values.
added calcium releasing agents at the same time as the digitonin used for permeabilisation.

\[
[\text{Ca}^{2+}] = \frac{K_d (F - F_{\text{m in}})}{(F_{\text{max}} - F)}
\]

where \( F \) is the fluorescence value measured at 510 nm
\( F_{\text{m in}} \) is the fluorescence in nominally calcium free medium
\( F_{\text{max}} \) is the fluorescence in high calcium medium

Table 5.1. shows that only InsP\(_3\) released calcium from permeabilised eggs in these studies.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Change in [Ca(^{2+})]/(\mu)M</th>
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<tbody>
<tr>
<td>control</td>
<td>0.375 +/- 0.03 (n=5)</td>
</tr>
<tr>
<td>10 (\mu)M InsP(_3)</td>
<td>1.73 +/- 0.21 (n=4)</td>
</tr>
<tr>
<td>100 (\mu)M GTP(_y)S</td>
<td>0.40 +/- 0.08 (n=3)</td>
</tr>
<tr>
<td>500 (\mu)M GTP(_y)S</td>
<td>0.35</td>
</tr>
<tr>
<td>100 (\mu)M cGMP</td>
<td>0.37 +/- 0.05 (n=3)</td>
</tr>
<tr>
<td>500 (\mu)M cGMP</td>
<td>0.40</td>
</tr>
</tbody>
</table>

These results conclusively show that neither GTP\(_y\)S nor cGMP cause the direct release of calcium from internal stores. This suggests that they must mobilise calcium indirectly. Swann, Ciapa and Whitaker (1987) reported a latency in the activation by these compounds. This suggests that they activate eggs by producing calcium-releasing second messengers. They also indicate that the indirect effects of cGMP and GTP\(_y\)S are prevented in permeabilised eggs, presumably because of a loss of components essential to
the mechanism. The obvious first possibility is that these compounds can stimulate the production of InsP$_3$.

4. PPI METABOLISM IN PERMEABILISED EGGS.

I used permeabilised eggs to determine whether either of these activator compounds caused the hydrolysis the plasma membrane phospholipid, PtdInsP$_2$, the lipid precursor of the calcium releasing second messenger InsP$_3$. I did this by preloading eggs with 2-$[^3]$H-$D$-myo-inositol and permeabilising with digitonin in the presence of agents which may cause PtdInsP$_2$ hydrolysis. Metabolism in the permeabilised eggs was stopped by the addition of perchloric acid. Water soluble inositol phosphates were analysed by HPLC. Figure 5.7 illustrates one such experiment. This figure shows the production of inositol phosphates 5 minutes after permeabilisation. Counts for all of the different inositol phosphates were pooled and expressed as a percentage of the total aqueous counts. This single experiment shows that significant production of inositol phosphates occurs in the presence of both 10 $\mu$M Ca$^{2+}$ and 100 $\mu$M GTPyS. However cGMP does not cause any inositol phosphate production. 10 $\mu$M Ca$^{2+}$ has previously been shown to cause PtdInsP$_2$ hydrolysis in isolated sea urchin egg cortices (Whitaker and Aitchison, 1985). This result suggests tentatively that GTPyS can also stimulate PtdInsP$_2$ hydrolysis but that cGMP is not capable of activating phosphoinositidase C.

However, the use of permeabilised eggs to measure inositol phosphate production proved not to be a very reliable method for introducing compounds into eggs without causing egg activation. In most cases permeabilisation itself led to substantial accumulation of inositol phosphates. One such experiment is illustrated in figure 5.8. This Figure shows mean values from experiments performed in duplicate. Substantial accumulation of inositol phosphates, similar to the levels produced by 10 $\mu$M Ca$^{2+}$ and 100 $\mu$M GTPyS shown in figure 5.7, occurred in all cases where eggs were permeabilised.

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GTPyS and calcium can stimulate phosphoinositidase C in permeabilised eggs.

In this single experiment I measured the production of inositol phosphates in response to various stimuli. Stimuli were added at the time of permeabilisation and inositol phosphates analysed by HPLC. Both 10 \( \mu \text{M} \) Ca\(^{2+} \) and 100 \( \mu \text{M} \) GTPyS caused an increase in inositol phosphates compared to a control of 10 mM EGTA. Neither 2 \( \mu \text{M} \) Ca\(^{2+} \) nor 100 \( \mu \text{M} \) cGMP stimulated inositol phosphate production.
Figure 5.8) Permeabilising eggs with digitonin causes accumulation of inositol phosphates.

In this experiment inositol phosphates were analysed by HPLC from non-permeabilised eggs or eggs permeabilised with 0.22 mM digitonin. The x-axis describes the inclusions in the permeabilisation media. The top line is the free calcium concentration in pM. The bottom line indicates the absence (−), or presence (+), of 100 pM GTPyS. Clearly, in these experiments, permeabilisation itself, led to the production of inositol phosphates to similar levels as seen in stimulated eggs in figure 5.7. The data shown are mean determinations from duplicate samples.
5. MEASURING PPI METABOLISM IN SINGLE EGGS.

(i) A Single Cell Assay for DAG Production.

Because of the problems measuring PIC activity in permeabilised eggs, I used different methods to look at the ability of GTPyS and cGMP to activate PIC. This involved the use of an assay for diacylglycerol (the other second messenger produced through the hydrolysis of PtdInsP$_2$) in single cells. I made use of the fact that diacylglycerol-activated protein kinase C stimulates the Na$^+$/H$^+$ antiport (Swann and Whitaker, 1985; Shen and Burgart, 1986) and in other cells (e.g. Moolenaar et al. 1984). We know that Ca$^{2+}$ can stimulate the Na$^+$/H$^+$ antiport because it stimulates PIC and since GTPyS and cGMP cause an increase in intracellular free calcium (Swann, Ciapa and Whitaker, 1987), I devised an assay which would circumvent this complicating problem and measure Ca$^{2+}$-independent pH changes. The way in which I performed this assay was to measure the intracellular pH using the fluorescent dye BCECF in single eggs previously microinjected with the calcium chelator BAPTA to prevent changes in intracellular Ca$^{2+}$ from occurring. Figure 7.9 shows a schematic representation of the method.

It shows that although Ca$^{2+}$ cannot stimulate phosphoinositidase C, GTPyS and cGMP should be able to if they work through a route independent of [Ca$^{2+}$]. In order to show that any effects are due to the activation of PKC by DAG I have used the compound R59022 which has been reported to be an inhibitor of diacylglycerol kinase (De Chaffoy de Courcelles et al. 1985). This compound causes the persistence of DAG because it can block the phosphorylation of DAG to phosphatidic acid by the enzyme DAG kinase.

In order to show that any pH changes occurred through the activation of the antiport, I used the compound dimethylamiloride (DMA) which specifically inhibits the Na$^+$/H$^+$ antiport (Vigne et al. 1984), and has been
Figure 7.9) A schematic diagram of the method used to measure \( \text{Ca}^{2+} \)-independent activation of the \( \text{Na}^+ / \text{H}^+ \) antiport.

To prevent any changes in \([\text{Ca}^{2+}]_i\) from occurring and activating PIC, I injected BAPTA into the eggs. Production of DAG was enhanced by the presence of an inhibitor of DAG kinase, (R59022), in the sea water. To show that any pH changes were due to \( \text{Na}^+ / \text{H}^+ \) exchanger activation, DMA was sometimes included in the sea water.
shown to be effective in inhibiting Na⁺/H⁺ stimulated alkalinisation in sea urchin eggs (Swann and Whitaker, 1985). Using this system I looked for evidence that GTPyS or cGMP could activate phosphoinositidase C directly.

In order to ensure that the fluorescent dye BCECF was appropriate for measuring pH changes in sea urchin eggs, I measured pH changes in fertilising, Ca²⁺-ionophore and TPA treated eggs. Figure 5.10 shows that at fertilisation there is a pH change of about 0.4 units. This is mimicked by the application of the calcium ionophore Br−A23187. Cytoplasmic alkalinisation also occurs when eggs are treated with 250 nM TPA although this is only about two thirds of the pH change at fertilisation or when eggs are stimulated with Br−A23187. This suggests that there is a calcium component in the stimulation of the Na⁺/H⁺ antiport to its new steady-state at fertilisation. These results are similar to those previously reported (Swann and Whitaker, 1985). This shows that this technique can be used to measure changes in intracellular pH in single sea urchin eggs. Microinjection of both GTPyS and cGMP causes pH changes very similar to those which occur at fertilisation or with calcium ionophore (I. Crossley, unpublished results). This is because these two compounds cause an increase in intracellular free calcium (Swann et al. 1987).

(ii) GTPyS Causes a pH Change Independent of Increased \([\text{Ca}^{2+}]_i\). 

I used this single cell assay of intracellular pH to see if GTPyS or cGMP could stimulate PIC. I used the calcium sensitive dye fura2 to check that the concentration of BAPTA used would block calcium changes in eggs. Figure 5.11 shows the intracellular calcium ion concentration changes when 150 pM GTPyS was injected into eggs. In the top panel, microinjection of GTPyS causes \([\text{Ca}^{2+}]_i\), to increase from about 200 nM to over 2 μM after a delay of 2 minutes. In contrast, the lower panel shows that when the egg was previously injected with BAPTA to 2 mM, the injection of GTPyS does not cause any change in \([\text{Ca}^{2+}]_i\). These records are typical of 3 such
Figure 5.10) pH changes measured in single eggs using the pH-sensitive fluorescent dye BCECF.

The pH change at fertilisation measured using BCECF was 0.35 (top panel). TPA (centre panel) stimulated a smaller pH change of approximately 0.25 pH units. These values are similar to those reported using different techniques (Whitaker and Steinhardt, 1982; Swann and Whitaker, 1985). The calcium ionophore A23187 also stimulated a pH change of approximately 0.35 pH units (bottom panel), indicating that the pH change which occurs at fertilisation can be mimicked by artificially raising [Ca^{2+}]_i. Each record is typical of at least 3 separate experiments.
experiments. These records show that any pH changes which are observed are independent of the calcium ion concentration.

I investigated whether GTPyS could cause pH changes independent of an increase in [Ca\(^{2+}\)]. Figure 5.12 illustrates the results of one such experiment. In figure 5.12a it can be seen that the microinjection of 100 \(\mu\)M GTPyS caused an alkalinisation of 0.13 pH units. The time between microinjection and the maximum increase in pH was 350 seconds. In contrast panel b shows a similar experiment in which the egg was preincubated with the diacylglycerol kinase inhibitor R59022 (10 \(\mu\)M) which should enhance the effect of GTPyS if the alkalinisation is due to the production of DAG. R59022 was present throughout the experiment. This experiment demonstrates that R59022 slightly increases the extent of the alkalinisation to 0.21 pH units. More striking, however, is the more rapid onset of this effect. Almost immediately after recording commenced after microinjection, the pH began to increase and the whole increase had occurred after 140 seconds compared to 350 seconds in the absence of R59022.

Figure 5.12c shows an experiment in which the egg was incubated in 10 \(\mu\)M R59022 and 100 \(\mu\)M dimethylamiloride (DMA). This inhibits the Na\(^+\)/H\(^+\) antiport. This figure shows that after microinjection, GTPyS does not cause a pH change. After about 400 seconds there is a slight alkalinisation but on the removal of DMA there is a rapid increase in pH of 0.19 units indicating that the effect of GTPyS is through the activation of the Na\(^+\)/H\(^+\) antiport.

Table 5.2 shows the mean and sem values from 4 experiments in the presence and absence of R59022 when I measured both the increase in pH and the time lapse between microinjection and the maximum pH change.

This table shows that the presence of the diacylglycerol kinase inhibitor R59022 increased the pH change caused by GTPyS and also greatly decreased the time required to bring about a pH change.
Figure 5.11) Ca\(^{2+}\) changes in eggs microinjected with GTPyS.

\([\text{Ca}^{2+}]_i\) was measured in single eggs using the calcium-sensitive fluorescent dye fura2. The top panel shows a calcium transient in an egg microinjected with 100 nM GTPyS. After a delay of about 2 minutes the \([\text{Ca}^{2+}]_i\) increases to about 2 μM and then decreases to resting levels over the following 15 minutes. The bottom panel shows a record of \([\text{Ca}^{2+}]_i\) in an egg microinjected with the same concentration of GTPyS. This egg had been previously injected with BAPTA to a final concentration of 2 mM. Clearly, 2 mM BAPTA is sufficient to prevent increases in \([\text{Ca}^{2+}]_i\) from occurring.
Figure 5.12) pH changes in single eggs microinjected with GTPyS measured with the pH-sensitive dye BCECF in the presence of 2 mM BAPTA.

The top panel shows a pH change caused by the microinjection of GTPyS. After microinjection, the pH increased slowly by 0.13 pH units. The centre panel shows a similar experiment except that the egg was incubated with 10 μM R59022, an inhibitor of diacylglycerol kinase. Clearly, the rate of the pH increase in more rapid and the pH increased by 0.21 pH units. The bottom panel shows an experiment in which the sea water contained 100 μM dimethylamiloride. Cytoplasmic alkalinisation did not occur until after the removal of DMA. Each trace is typical of at least 3 experiments.
These data show that GTPyS is capable of activating the \( \text{Na}^+/\text{H}^+ \) antiport through the production of diacylglycerol and the activation of protein kinase C. This suggests that GTPyS is capable of activating the protein PIC in a \( \text{Ca}^{2+} \)–independent manner and that GTPyS normally causes egg activation by producing \( \text{InsP}_3 \).

### Table 5.2
The effect of GTPyS and R59022 on intracellular pH in single eggs.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pH change (mean +/- sem)</th>
<th>time to maximum pH change (secs: mean +/- sem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 ( \mu \text{M} ) GTPyS + 10 ( \mu \text{M} ) R59022</td>
<td>0.20 +/- 0.014</td>
<td>170 +/- 34</td>
</tr>
<tr>
<td>100 ( \mu \text{M} ) GTPyS</td>
<td>0.105 +/- 0.012</td>
<td>422 +/- 30</td>
</tr>
</tbody>
</table>

(iii) cGMP Does not Cause \( \text{Ca}^{2+} \)–Independent Alkalinisation.

I wanted to see if cGMP could also cause this intracellular pH change. I did this by using the same method as for GTPyS. cGMP was injected to give a final concentration of approximately 100 \( \mu \text{M} \), a concentration which normally activates 100 \% of eggs. However, figure 5.13a shows clearly that cGMP does not cause a pH change in the presence of BAPTA. Injection of cGMP in the absence of BAPTA into the same batch of eggs at concentrations of 50 \( \mu \text{M} \) to 100 \( \mu \text{M} \) caused all to activate. In a separate experiment I injected cGMP at a concentration of 100 \( \mu \text{M} \) followed some 10 minutes later by a second injection of 100 \( \mu \text{M} \) GTPyS. This is shown in figure 5.13b. It is clear from this experiment that GTPyS causes cytoplasmic alkalinisation in an egg in which cGMP has no effect. This clearly shows that the mechanism of actions in causing egg activation must be different.
Figure 5.13) pH changes in single eggs microinjected with cGMP measured with BCECF in the presence of 2 mM BAPTA.

The top panel shows that the microinjection of 100 µM cGMP failed to cause any pH change in a BAPTA-injected egg. The bottom panel shows that in an egg which failed to change pH in response to cGMP, the microinjection of GTPyS caused cytoplasmic alkalination.
These results using a single cell assay for the production of DAG have shown that GTPyS can stimulate phosphoinositidase C and cause a calcium transient by producing InsP\textsubscript{3}. This suggests that sea urchin egg PIC can be activated by a G- protein. On the other hand cGMP does not activate PIC under these conditions, nor does it directly release calcium from intracellular stores. Its mode of action in egg activation remains unclear.

(iv) Do Sperm Activate Phosphoinositidase C Through a G- Protein?

I have so far shown in this chapter that there is a G- protein activated phosphoinositidase C and activation by the compound GTPyS occurs through this route. I wanted to see if sperm also activate eggs by using this route. To address this question I used the assay for single cell alkalinisation. I microinjected eggs with BAPTA to a final concentration of 2 mM and then fertilised. Figure 5.14 shows that the addition of sperm did not cause any cytoplasmic alkalinisation. In four such experiments sperm caused a pH change of $-0.0125 \pm 0.007$ (sem). In a separate experiment I microinjected BAPTA into eggs which had been preloaded with the DNA staining vital dye H33342 in order to assess the number of sperm interacting with eggs under these conditions. Table 5.3 shows that the microinjection of BAPTA allows many sperm to fuse with eggs.

<table>
<thead>
<tr>
<th>Eggs</th>
<th>Number of fluorescent sperm nuclei per egg. Mean (+/- sem.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 (+/- 0.00, n=9)</td>
</tr>
<tr>
<td>BAPTA 2.0-3.0 mM</td>
<td>9.10 (+/- 2.40, n=10)</td>
</tr>
</tbody>
</table>

Table 5.3. The effect of BAPTA on the transfer of H33342 between egg and sperm.
These data show that although many sperm fuse with eggs microinjected with 2 mM BAPTA, they are not capable of stimulating cytoplasmic alkalinisation. This result strongly suggests that the way in which sperm activate eggs does not involve the activation of PIC by coupling through a G-protein. In this respect at least, it seems that the actions of cGMP resemble the way in which sperm activate eggs better than GTPyS. The results presented in this chapter indicate that eggs do contain a G-protein activated PIC but that this is not the route used for activation at fertilisation.

6. CONCLUSION.

In this chapter, I have shown that non-mitochondrial Ca\(^{2+}\)-stores in sea urchin eggs are insensitive to both GTPyS and cGMP. Non-mitochondrial Ca\(^{2+}\)-stores release Ca\(^{2+}\) in response to InsP\(_3\). I have investigated PPI metabolism in eggs using both \(^3\)H-labelled inositol and by measuring the activation of the Na\(^+\)/H\(^+\) antiport, an indirect measure of DAG production. Using the isotopic method I have shown that GTPyS but not cGMP might activate PIC but the use of permeabilised eggs is not reliable in assessing PIC activation. Using a single cell assay of cytoplasmic alkalinisation, I have shown that GTPyS can produce DAG independent of changes in [Ca\(^{2+}\)]\(_i\). This suggests that GTPyS can activate PIC and that this enzyme can be activated by G-proteins. In contrast, neither, cGMP nor sperm cause cytoplasmic alkalinisation under conditions in which [Ca\(^{2+}\)]\(_i\) is buffered at resting levels. The data in this chapter provide evidence that sea urchin egg activation at fertilisation does not proceed through a mechanism involving receptor/G-protein activation of PIC.

Some of the work described in this chapter has been previously reported in abstract form (Whalley and Whitaker, 1988).
Figure 5.14) pH measured in an inseminated egg using the pH-sensitive dye BCECF in the presence of 2 mM BAPTA.

The pH was measured in an egg microinjected with 2 mM BAPTA. After insemination there was no detectable change in pH for 16 minutes. Visual inspection showed that many sperm were attached to the egg. In separate experiments, using the dye H33342, BAPTA did not prevent sperm–egg fusion but rather increased the numbers of sperm fusing with eggs. The mean and sem values for 10 eggs was 9.1 +/- 2.4.
Chapter 6. Discussion.

1. Mechanisms of egg activation.

1.1 TWO MODELS OF EGG ACTIVATION.

The generation of an increase in \([\text{Ca}^{2+}]_i\), at fertilisation is a prerequisite for cortical granule exocytosis. The mechanism underlying the \(\text{Ca}^{2+}\) increase is not fully characterised although the involvement of the phosphoinositide second messenger system has been suggested to play an important role (Whitaker and Irvine, 1984; Ciapa and Whitaker, 1986; Swann and Whitaker, 1985, 1986; Shen and Burgart, 1986). Two models have been proposed to explain how a sperm activates an egg at fertilisation. The first model of egg activation postulates that sperm interact with eggs and activate phosphoinositidase C (PIC) to produce \(\text{InsP}_3\) and DAG, through a G-protein (Turner et al. 1986). The basis for this suggestion is that GTPyS causes cortical granule exocytosis when microinjected into eggs (Turner et al. 1986), due to an increase in \([\text{Ca}^{2+}]_i\), (Swann et al. 1987).

The second model is that upon gamete fusion, the sperm introduces some activator compound. The basis for this model is that it is known that gamete fusion occurs prior to the elevation of \([\text{Ca}^{2+}]_i\) (Hinckley et al. 1986; Longo et al. 1986; Chambers and de Armendi, 1979). It has been suggested that during the period between gamete fusion and the onset of the calcium transient, an activator molecule diffuses from sperm to egg (Whitaker et al. 1989). Several putative activators have been identified. Microinjection of \(\text{InsP}_3\) (Whitaker and Irvine, 1984) or cGMP (Swann et al. 1987) activates eggs, and Clapper et al. (1987) have found that a number of nicotinamide nucleotides cause intracellular calcium release. A high molecular weight extract from sperm has also been shown to cause egg activation (Dale et al. 1985; G. Ehrenstein unpublished).
The effects of the two guanine nucleotides which activate eggs are specific. ATPyS is without effect and of the cyclic nucleotides tested, only 3',5'-cGMP (cGMP) is effective in eliciting activation responses. A non-hydrolysable analogue of cGMP, 8-BrcGMP, does not activate eggs when microinjected which suggests that metabolism of cGMP might be a prerequisite for activation (Swann et al. 1987).

Neither GTPyS nor cGMP activates eggs immediately. There is a latency of up to 1 minute between microinjection and the onset of the calcium transient (Swann et al. 1987). This strongly suggests that neither compound releases calcium directly but, rather, that they initiate some other process which triggers an increase in $[\text{Ca}^{2+}]_i$, perhaps by stimulating PIC. I used various techniques to elucidate the ways in which these calcium signals might be generated in eggs and to see if any analogies could be drawn between the modes of action of these compounds and the events occurring at fertilisation. In particular, I wanted to see whether a G-protein model is appropriate in explaining egg activation at fertilisation.

1.2 CALCIUM STORES IN PERMEABILISED EGGS.

(i) Characterisation of the Store and its InsP$_3$-Sensitivity.

I first looked to see if calcium could be released by these compounds directly. I used $^{45}$Ca-release measurements in a digitonin-permeabilised egg preparation in order to investigate this possibility. Previous work on sea urchin egg reticular calcium stores has used microsomal fractions rather than whole eggs (Clapper and Lee, 1985), and showed ATP-dependent uptake and InsP$_3$-sensitive calcium release. Uptake into permeabilised eggs, with $[\text{Ca}^{2+}]$ buffered to sub-micromolar concentrations, was also dependent on ATP and was into non-mitochondrial stores. These stores released $^{45}$Ca in response to InsP$_3$ in a dose-dependent manner. Half-maximal calcium release occurred with 2.5 $\mu$M InsP$_3$ and maximal release at 10 $\mu$M. This InsP$_3$ sensitivity is considerably less than that reported by
Clapper and Lee (1985). This may be due to different microsomal fractions being present. Clapper and Lee used homogenates which do not contain the reticular system which lies immediately beneath the plasma membrane (Chandler, 1984; Sardet, 1984), and has been shown to sequester calcium and release it in response to InsP$_3$ (Payan et al. 1986; Oberdorf, Head and Kaminer, 1986). This cortical store has been reported to be less sensitive to InsP$_3$ than microsomal fractions (Oberdorf, Head and Kaminer, 1986). The sensitivity towards InsP$_3$ which I found in permeabilised eggs was similar to that reported for calcium release in other permeabilised cells, for instance rat insulinoma cells (Prentki et al. 1985), GH$_3$ pituitary cells (Biden et al. 1986; Gershengorn et al. 1984) and pancreatic islets (Wolf et al. 1985).

(ii) Calcium Stores are Insensitive to GTPyS and cGMP.

In contrast to InsP$_3$, the non–mitochondrial calcium stores in permeabilised eggs were insensitive to both GTPyS and cGMP. Neither compound stimulated any $^{45}$Ca release. This lack of effect of GTPyS or cGMP could feasibly be explained by envisaging them acting on an InsP$_3$–insensitive store, and supposing that the InsP$_3$–sensitive store was preferentially filled under the conditions of this assay. It appears that the InsP$_3$–sensitive store of GH$_3$ cells sequesters calcium preferentially to InsP$_3$–insensitive stores (Biden et al. 1986). Preferential filling of an InsP$_3$–sensitive calcium store has also been demonstrated in parotid gland (Aub et al. 1982).

Non–mitochondrial membrane calcium stores in sea urchin egg homogenates, are also sensitive to a number of nicotinamide nucleotide derivatives (Clapper et al. 1987). These agents seem to release calcium from functionally distinct stores rather than acting on different receptors in the same store, since partial separation of microsomes sensitive to these different compounds has been achieved using density gradient centrifugation (Clapper et al. 1987). However, neither GTPyS nor cGMP acts on InsP$_3$–insensitive calcium stores. Using fura2, I showed that neither GTPyS nor
cGMP released any calcium from permeabilised eggs when added at the time of permeabilisation. All of these data indicate that neither have any direct calcium-releasing activity in permeabilised eggs. In view of this, these compounds must activate eggs through another route. One obvious route is through the production of InsP₃.

1.3 MEASURING PPI HYDROLYSIS IN SEA URCHIN EGGS.

(i) Inositol Phosphate Production in Permeabilised Eggs.

I examined the effects of GTPyS, cGMP and Ca²⁺ on the production of inositol phosphates using eggs loaded with 2⁻³H⁻myo-inositol and measuring the production of ³H⁻labelled inositol phosphates. In one experiment I saw production of inositol phosphates when either GTPyS or 10 μM Ca²⁺ was added. A PIC in sea urchin cortices has been shown to be calcium-sensitive (Whitaker and Aitchison, 1985). However, assessing the activation of PIC in digitonin-permeabilised eggs proved unreliable since digitonin itself was capable of stimulating inositol phosphate accumulation in some experiments.

(ii) DAG Production in Single Eggs.

Because of this, I used an indirect assay for the activation of PIC. This was to monitor the production of diacylglycerol through the activation of protein kinase C, in the absence of intracellular changes in [Ca²⁺]. DAG, in the presence of phosphatidyserine greatly enhances the calcium-affinity of PKC, so that it is active at resting calcium levels (Kaibuchi, Takai and Nishizuka, 1981; Nishizuka, 1984). I measured activation of the Na⁺/H⁺ antiport, the activity of which is known to be dependent on the activation of protein kinase C in both sea urchin eggs (Swann and Whitaker, 1985; Shen and Burgart, 1986) and in other cells (Moolenaar et al. 1984),
using the fluorescent dye BCECF, as an indication of DAG production and hence PIC activity.

(iii) GTPyS Stimulates Cytoplasmic Alkalinisation through the Na\(^+\)/H\(^+\) antiport.

GTPyS caused cytoplasmic alkalinisation in eggs in which changes in [Ca\(^{2+}\)], were prevented. The effect was sensitive to R59022 which is an inhibitor of diacylglycerol kinase (de Chaffoy de Courcelles et al. 1985), and has been shown to potentiate DAG-dependent processes (Nunn and Watson, 1987; Dale and Penfield, 1987). This compound increased both the extent and the rapidity of onset of the response, suggesting that the effect of GTPyS was through the production of DAG. In contrast, neither cGMP nor sperm were capable of stimulating the Na\(^+\)/H\(^+\) antiport which suggested that neither could stimulate DAG production. The pH changes caused by GTPyS could be prevented by the presence in the extracellular solution of dimethylamiloride (DMA) which is a specific inhibitor of the Na\(^+\)/H\(^+\) antiport (Vigne et al. 1984) and inhibits pH changes at fertilisation (Swann and Whitaker, 1985).

(iv) Ca\(^{2+}\)-Dependent and Independent Activation of the Na\(^+\)/H\(^+\) Antiport.

Under the conditions of this assay, GTPyS caused a pH change that is only about 50% of that which occurs at fertilisation. TPA also causes an alkalinisation which is lower than that elicited by sperm, whereas the calcium ionophore A23187 mimics the fertilisation pH response. These differences can be attributed to the differences in calcium ion concentrations in these eggs. In both the GTPyS and TPA treated eggs, alkalinisation is independent of [Ca\(^{2+}\)]\(_i\). TPA has been shown to activate eggs without producing a calcium transient (Ciapa et al. 1988), and the studies with GTPyS were in eggs in which BAPTA was preventing changes in [Ca\(^{2+}\)]\(_i\).
In contrast, at fertilisation and in eggs treated with ionophore, $[\text{Ca}^{2+}]_i$ increases do occur. Thus it seems that there is a contribution of calcium towards cytoplasmic alkalinisation. A synergism between TPA and Ca$^{2+}$ has been reported in the activation of protein kinase C in a human monoblast cell line (Alvarez et al. 1989) and it has been suggested that Ca$^{2+}$ synergises with TPA by increasing the recruitment of PKC to the PM (May et al. 1985). It has recently been shown that the sea urchin egg Na$^+$/H$^+$ antiport can be activated either by PKC or by a Ca$^{2+}$-CaM-dependent protein kinase (Shen, 1989), and that this Ca$^{2+}$-CaM-dependent route is used at fertilisation. Certainly, the data presented here also supports a Ca$^{2+}$-dependent component in Na$^+$/H$^+$ antiport activation.

1.4 THE SOURCE OF THE DAG PRODUCED BY GTPyS- ACTIVATION.

The data in chapter 5 show that GTPyS causes cytoplasmic alkalinisation through the Na$^+$/H$^+$ antiport by DAG-stimulated protein kinase C. This finding shows that GTPyS can stimulate DAG production in a calcium-independent manner and suggests that its effect is through the activation of a G-protein. The production of DAG may be due to activation of PIC which has been shown to be sensitive to GTPyS and AlF$_4^-$ (Cockcroft and Gomperts, 1985; Blackmore et al., 1985; UHING et al. 1986; Cockcroft and Taylor, 1987), which suggests the involvement of G-proteins in agonist-stimulated PIC activation (Joseph, 1985). Both GTPyS and AlF$_4^-$ are thought to activate G-proteins by causing the dissociation the $\alpha$- from the $\beta$- and $\gamma$-subunits (Gilman, 1984), GTPyS by virtue of its non-hydrolysable nature and ability to replace GDP, and AlF$_4^-$ by mimicking the $\gamma$-phosphate of GTP (Bigay et al. 1987).
(i) PtdInsP₂ is the Major Substrate for G-Protein Activated PIC.

The PI phospholipids, PtdInsₓ and PtdInsP₂, are potential source of diacylglycerol. In instances of either receptor (Berridge, 1983; Drummond et al. 1984), or GTPyS/AlF₄⁻–stimulated PIC activation (Straub and Gershengorn, 1986; Uhing et al. 1986; Cockcroft et al. 1987; Muldoon et al. 1987), the initial inositol phosphate products are the bis and trisphosphates, showing that PI is not subject to G-protein–activated PIC hydrolysis. PIC–hydrolysis of PI is calcium–dependent (Majerus et al. 1985).

Studies of the kinetics of formation of inositol phosphates suggest that PtdInsP₂ is the major lipid substrate for PIC in G–protein activation in intact cells or in isolated membranes. In GH₃ cells Drummond et al. (1984) showed that TRH rapidly stimulated the production of mono, bis and tris phosphates at 37°C. However, when incubated at 25°C, the production of InsP₁ and InsP₂ was slower and less pronounced. The production of InsP₃ was enhanced and was formed at the same rate as at 37°C. The implication of this is that the production of InsP₁ and InsP₂ is through the catabolism of InsP₃ and that the enzymes responsible for this are temperature dependent. In isolated hepatocyte membranes, the formation of InsP₃ occurs more rapidly than InsP₁ or InsP₂ when stimulated with AlF₄⁻ (Cockcroft and Taylor, 1987), and InsP₃ accounted for 90% of the inositol phosphates produced. These results suggest that the major phospholipid of G–protein–activated PIC is PtdInsP₂. There is no example of PIC hydrolysis of PtdInsP in the absence of PtdInsP₂ hydrolysis. It is therefore correct to assume that the DAG produced in GTPyS activated sea urchin eggs is derived from PtdInsP₂.

(ii) Other Phospholipases and the Production of DAG.

The production of diacylglycerol is not an exclusive property of the PPI–phospholipases. There are other routes for the production of this
compound. The first is through the activation of phospholipase D (PLD) and subsequent dephosphorylation of phosphatidic acid through the enzyme, phosphatidate phosphohydrolase (PAP). PLD has also been shown to be activated by GTPγS or AlF₄⁻ (Bocckino et al. 1987; Anthes et al. 1989), both of which can activate G-proteins. However, a number of observations suggest that GTPγS does not produce DAG by activating this type of phospholipase. Firstly, PLD appears to require Ca²⁺ for activation (Anthes et al. 1989). Secondly, the enzyme which converts phosphatidic acid to diacylglycerol, PAP, is calcium-dependent (Pollard and Brindley, 1984), and finally, when hepatocytes are stimulated with vasopressin, phosphatidic acid production occurs several minutes before any accumulation of diacylglycerol (Bocckino et al. 1987). In the experiments in sea urchin eggs described in chapter 5, no Ca²⁺-stimulated enzyme should be activated since I have shown with fura2 that changes in [Ca²⁺]ᵢ are prevented by BAPTA. Furthermore, alkalinisation begins immediately after microinjection of GTPγS, which shows that DAG production occurs without the delay we would expect if the pathway for DAG production was through the actions of the enzymes PLD and PAP.

Diacylglycerol can also be produced through the activation of non-specific phospholipase C (PLC) and phosphodiester hydrolysis of, for instance, phosphatidylcholine. This has also been shown to be activated by GTPγS and AlF₄⁻ in some cells (Bocckino, Blackmore and Exton, 1985; Irving and Exton, 1987; Diaz-Meco et al. 1989). Again, this seems to be an unlikely source of the DAG which activates protein kinase C in sea urchin eggs.

In all cases thus far reported, phosphatidylcholine hydrolysis by PLC seems to be activated by the same agonists which activate the PI second messenger system (Loffelholz, 1989). It seems likely that the activation of PLC occurs after the activation PIC and may be a consequence of the production of InsP₃ and/or DAG from PtdInsP₂. Besterman et al. (1986), have shown that protein kinase C can directly stimulate PLC, suggesting that this mechanism may be one way of having sustained activation of protein
kinase C in stimulated cells and that the initial agonist–induced hydrolysis of PtdInsP$_2$ leads to subsequent activation of PC–PLC. In one study in intact cells PC–PLC requires an increase in [Ca$^{2+}$], for activation (Martinson, Goldstein and Brown, 1989), and a cytosolic PLC from myocardium is inhibited in the presence of EGTA (Wolf and Gross, 1985).

Kinetic studies in pancreatic acini have shown biphasic production of DAG with the first phase (15 seconds after agonist addition) rising in parallel with an increase in InsP$_3$. The second phase of DAG production (peaking at 5 minutes after agonist addition) coincided with the production of phosphorylcholine, indicating that the activation of a PC–specific PLC occurs after a pronounced delay (Matozaki and Williams, 1989). These results tend to rule out non–specific PLC hydrolysis of PC as a likely source of DAG in my experiments although it is possible that the production of DAG, initially through PIC, may later stimulate other phospholipases by activating protein kinase C. All of these data point towards the initial production of diacylglycerol occurring through PIC action on PtdInsP$_2$. My results suggest that in sea urchin eggs there is a G–protein–stimulated phosphoinositidase C.

(iii) Egg Activation by GTPyS is Inhibited by Heparin.

To further substantiate the idea that GTPyS activates eggs by stimulating PIC and producing InsP$_3$, Crossley and Whitaker (unpublished results) have shown that activation is inhibited if eggs are injected with heparin prior to GTPyS. Heparin is a competitive inhibitor of the InsP$_3$ receptor (Worley et al. 1987) and acts by reducing the number of available receptors for InsP$_3$ rather than reducing the affinity of the receptor (Guillette et al. 1989; Joseph and Rice, 1989). This inhibition of a calcium transient by heparin is further evidence that GTPyS activates eggs through phosphoinositidase C.
The latency of GTPyS activation can also be explained if PIC activation is responsible for the calcium transient. On this hypothesis, the delay is due to the time required to produce a threshold concentration of InsP₃, enough to set off the self–propagating wave of calcium release and cortical granule exocytosis (Swann and Whitaker, 1986). All of these data provide strong evidence that one way of activating eggs is through G–protein stimulated PIC.

1.5 HOW DOES A SPERM ACTIVATE AN EGG?

(i) Neither Sperm nor cGMP Stimulate PIC through a G–Protein.

If we consider the data described in chapter 5, an inevitable conclusion is that sperm cannot activate PIC through a G–protein. Neither sperm nor cGMP cause cytoplasmic alkalinisation in the same way as GTPyS. This shows that they do not activate PIC in a Ca²⁺–independent manner, and strongly suggests that sperm do not stimulate an egg PIC through a receptor coupling to a G–protein. Indeed, sperm seem to more closely resemble cGMP than GTPyS in their mode of action. These findings must throw doubt on the widely espoused hypothesis that sperm act in a manner analogous to hormones (Turner et al. 1986, 1987; Kline and Jaffe, 1987; Miyazaki, 1988), and initiate fertilisation events through a G–protein. Kline et al. (1988) further suggested this role for a G–protein at fertilisation by expressing serotonin or muscarinic M1 receptors in Xenopus oocytes and showing that serotonin or acetylcholine elicited fertilisation–like responses in these eggs. However, this data does no more than indicate that G–proteins which can stimulate the polyphosphoinositide second messenger system, are present in eggs, confirming the suggestion made on the basis of the data obtained using GTPyS in sea urchin eggs (Turner et al. 1986). My data are in agreement with this finding, but my data also shows that sperm do not mimic the actions of GTPyS in activating PIC. The egg G–proteins might be required during egg maturation or during events later in
the cell cycle, but they do not appear to participate in the transduction pathway at fertilisation.

(ii) GDPβS Inhibits Cortical Granule Exocytosis, not the Calcium Transient.

Another finding that rather argues against a G-protein being part of the transduction mechanism is that GDPβS does not inhibit the sperm-induced rise in [Ca\(^{2+}\)]. It was originally shown that GDPβS prevented egg activation but did not prevent InsP\(_3\) from causing calcium-mobilisation as judged by the elevation of a fertilisation envelope (Turner et al. 1986). It was argued from this result that GDPβS was having an inhibitory effect between sperm-egg interaction and the production of InsP\(_3\) by inhibiting the interactions of a sperm receptor and PIC by inhibiting a G-protein. However, it has now been shown very clearly that GDPβS has no effect on fertilisation other than preventing cortical granule exocytosis (Crossley and Whitaker, 1989; Whitaker et al. 1989). GDPβS has no effect on the sperm-induced calcium transient. Indeed, the microinjection of GDPβS often caused a large increase in [Ca\(^{2+}\)] (Crossley and Whitaker, 1989). These results provide very strong evidence against the G-protein model of egg activation being appropriate in describing fertilisation.

(iii) Possible Egg Activators at Fertilisation.

In the introduction, I proposed two models to explain how an egg might be activated at fertilisation. The first model proposed that a sperm was analogous to a hormone and activated PIC through a G-protein. The second model suggested that the sperm "injected" an activator molecule into the egg at fertilisation. The data in this thesis suggest that the first of these models is inappropriate in describing fertilisation in sea urchin eggs. Sperm do not activate eggs in a manner analogous to hormones; that is, they do not cause activation through a G-protein-stimulated PIC. It seems that the second model is better at describing fertilisation. On fusion,
the sperm introduces an activator compound into the egg. When a critical concentration of this is reached, or when it has activated some other process, the calcium wave is triggered.

The identity of such a factor is unknown, but clearly cGMP is a candidate since it reaches a high concentration in sperm (Kopf et al. 1979), and activates eggs when microinjected (Swann et al. 1987). How cGMP activates eggs is as yet unknown but I have shown that it does not have the ability to directly release calcium from intracellular stores, nor does it stimulate phosphoinositidase C. Its latency of activation (Swann et al. 1987) suggests that it activates eggs indirectly; one possibility is through a cGMP-dependent protein kinase. Activation of eggs by cGMP is not confined to sea urchins. cGMP can mimic muscarinic responses in *Xenopus* oocytes (Dascal and Landau, 1982). Having said that, it is unlikely that cGMP alone, is the activator at fertilisation since at concentrations of up to 100 μM, its delay in activating eggs (1–2 minutes [Swann et al. 1987]), is considerably longer than the 12 second latent period at fertilisation (Allen and Griffin, 1958).

Another possible activator compound which a sperm might introduce into the egg upon fusion is InsP$_3$. In a recent report measuring InsP$_3$ levels in sperm (Iwasa et al. 1989), it is suggested that a single sperm contains more InsP$_3$ than is required to activate eggs by microinjection (Whitaker and Irvine, 1984). However, Swann et al. (1987) argued that for a sperm to activate an egg by "injecting" InsP$_3$ would require an InsP$_3$ concentration in the sperm cytoplasm in the order of 10–100 mM. The InsP$_3$ levels reported for acrosome-reacted sperm were several orders of magnitude less than this (Iwasa et al. 1989).

The ability to reconstitute exocytosis is potentially a very useful approach in elucidating mechanisms which may be important in controlling this event. In order to be an appropriate experimental approach, it must be shown that it mimics normal in vitro exocytosis. This has been shown to be the case. The application of calcium-containing solutions to reconstituted lawns results in morphological changes which are qualitatively identical to the changes which occur in cortices (Crabb and Jackson, 1985). Furthermore, immunofluorescence studies using antibodies to hyalin, one of the proteins whose localisation is the interior of the cortical granules, have shown that calcium stimulates vectorial transport of this protein across the plasma membrane (Crabb and Jackson, 1985; Crabb, Modern and Jackson, 1987). These results show that reconstituted lawns are an appropriate experimental model for testing ideas about exocytosis.

I found that isolated cortical granules were insensitive to high calcium concentrations. I did not observe any lysis of either monomeric or aggregated cortical granules in contrast to Crabb and Jackson (1985), who found that up to 30% of aggregated granules fused. This difference reflects the differences in the techniques used to isolate granules. I did not use a homogenisation technique but sheared the cortical granules from isolated cortices. Homogenisation produced aggregates of granules which lysed on the addition of calcium which shows that these aggregates were attached to small fragments of plasma membrane in contrast to granule aggregates prepared by shearing which did not lyse under the same conditions. In contrast, when granules were settled onto plasma membrane fragments and were challenged with calcium, monomeric and aggregated granules all underwent fusion. Granules not associated with the plasma membrane did not. Granules from other secretory cells are also stable to high concentrations of calcium. Drust and Creutz (1988) have shown that isolated chromaffin granules neither aggregate nor fuse when suspended in
a solution containing 1 mM calcium. This lack of calcium sensitivity in isolated secretory granules indicates that the mechanism of exocytosis involves factors other than merely the secretory granules, for instance, plasma membrane proteins.

2.1 CALCIUM SENSITIVITY OF RECONSTITUTED EXOCYTOSIS.

In agreement with Crabb and Jackson (1985), I found that reconstituted exocytosis was less sensitive than native in vitro exocytosis. However, I found that reconstitutions in PKME were much less sensitive to calcium than reported by these authors. I found half-maximal stimulation with approximately 250 μM Ca^{2+} whereas Crabb and Jackson reported this value to be 18.5 μM. This is obviously a large difference between the calcium sensitivities of the two systems.

(i) The Use of Ca^{2+}–EGTA Buffers.

There are two potential explanations for this discrepancy. The first is that Crabb and Jackson used calcium–EGTA buffers to test the secretory response whereas I used an EGTA–free medium with the calcium concentrations made by serial dilution. EGTA is useful because it can buffer calcium in the micromolar range at near neutral pH values. However, to obtain free Ca^{2+} concentrations of 18 μM requires a high ratio of calcium to EGTA (0.949). Thus, if the EGTA was assumed to be pure whereas often its' purity is only about 95% (Miller and Smith, 1984), the actual free Ca^{2+} concentration would be much higher than anticipated. By way of an illustration, if the purity of the EGTA is 100%, a calcium–EGTA ratio of 0.949 at pH 6.8 would give a free calcium concentration of 18.2 μM. If however the purity was 96%, the free calcium value would be 97 μM. It seems that this was a problem not originally taken into account by these Crabb and Jackson who later reported reproducibility problems.
possibly due to variation in purity levels in different batches of EGTA (Crabb, Modern and Jackson, 1987).

(ii) The Extraction of Calcium—Sensitising Factors.

The second explanation is that differential extraction of potential Ca\(^{2+}\)—sensitising proteins can occur in KCl—based media. It was reported by Sasaki (1984), that in the eggs of *Hemicentrotus pulcherrimus*, cortices were an order of magnitude less sensitive to Ca\(^{2+}\) when prepared in a KCl—based medium, rather than in a medium whose major anion was glutamate and which more closely resembles the cytoplasmic composition of the egg (Rothschild and Barnes, 1953). This decrease in sensitivity could be restored by the application of a KCl cortical extract, dialysed against a glutamate medium. The factor responsible for sensitising cortices to calcium was determined to be a high molecular weight heat and protease—labile protein. Eggs of the sea urchin species *Lytechinus pictus*, that I used for these experiments are also susceptible to KCl desensitisation. In a glutamate—based medium the \([\text{Ca}^{2+}]_{50}\%\) is approximately 3 \(\mu\)M (Steinhardt and Alderton, 1982; Whitaker and Aitchison, 1985), but in KCl—based media \([\text{Ca}^{2+}]_{50}\%\) lies between 9 and 18 \(\mu\)M (Steinhardt, Zucker and Schatten, 1977). However, it was found that eggs of *Strongylocentrotus purpuratus*, from which Crabb and Jackson prepared their reconstituted lawns, do not exhibit this difference in calcium sensitivity in different media (Sasaki and Epel, 1983). This seems a likely source of the discrepancy between my results and those previously obtained (Crabb and Jackson, 1985). Indeed, this view is given further credence by my results that reconstitution in IM, (a medium based on glutamate as the major anion), is an order of magnitude more sensitive to Ca\(^{2+}\) than in PKME. In IM, \([\text{Ca}^{2+}]_{50}\%\) in reconstituted lawns is approximately 26 \(\mu\)M, a 4—fold decrease compared to native cortices whose \([\text{Ca}^{2+}]_{50}\%\) was 3.5 \(\mu\)M. In *Strongylocentrotus purpuratus*, the difference between the \([\text{Ca}^{2+}]_{50}\%\) in cortices and reconstituted lawns was approximately 3—fold. These results show that reconstituted exocytosis from *Lytechinus pictus* is a useful approach in studying exocytotic mechanisms.
2.2 CORTICAL GRANULE – PLASMA MEMBRANE BINDING IS DEPENDENT ON PROTEIN INTERACTIONS.

Isolated cortical granules settle onto denuded plasma membrane fragments in a time-dependent manner. This settling is preferentially on to the plasma membrane. Granules will also settle onto polylysine-coated glass but at a density reduced to approximately 20% of that of granules on plasma membrane. This indicates that specific interactions are responsible for mediating these interactions, either mediated through binding proteins or through lipid interactions. Binding clearly involves the interaction of proteins on both the plasma membrane and the cortical granules since protease treatment of either component inhibits binding. This result is consistent with that of Vacquier (1975) who reported that tryptic digestion of cortices resulted in the liberation of the cortical granules implying protein-mediated interactions exist between the secretory granules and the plasma membrane. Crabb, Ward and Haggerty (1985) reported that mild tryptic digestion reduced the calcium sensitivity of cortices. One of the contents of the cortical granules is a trypsin-like protease which contributes to the prevention of polyspermy (Schuel et al. 1973; Sawada et al. 1984). One might envisage that one reason for reduced calcium sensitivity of reconstituted lawns is the release of this protein during preparation, and proteolysis of proteins important in the exocytotic mechanism. However, the calcium sensitivity of reconstituted lawns was not increased by the inclusion of protease inhibitors to all of the media used to isolate PM and CG's which shows that proteolysis is not a factor responsible for the reduction of calcium sensitivity compared to native in vitro exocytosis.

2.3 FUSOGENS AND EXOCYTOSIS.

Exocytosis faces the topological problem of rearranging two lipid bilayers to form the fusion pore through which continuity of the granule interior and the extracellular space is established. Phase transitions may be important in generating intermediaries which have been proposed to explain
membrane fusion (Siegel, 1987). The presence of DAG in biological lipids increases the likelihood of phase transitions occurring (Dawson et al. 1984; Das and Rand, 1985, 1986). It has been suggested that DAG, generated from the hydrolysis of phosphoinositides may induce fusion (Hawthorne and Pickard, 1979).

A number of observations have led to the hypothesis that the generation of fusogenic DAG may stimulate exocytosis in sea urchin eggs (Whitaker and Aitchison, 1985; Ciapa and Whitaker, 1986; Whitaker, 1987). Calcium causes PtdInsP\(_2\) hydrolysis at the same concentration as stimulates exocytosis (Whitaker and Aitchison, 1985), and at fertilisation the increase in DAG levels follows the same time course as the calcium transient and exocytosis (Ciapa and Whitaker, 1986). Furthermore, neomycin inhibits both exocytosis and PtdInsP\(_2\) hydrolysis with the same concentration dependence (Whitaker, 1987; McLaughlin and Whitaker, 1988).

(i) DAG is not Required for Exocytosis.

The results using calcium-pretreated components of the secretory system showed that these pretreatments had no effect on the subsequent calcium sensitivity. The calcium-sensitivity when either or both components were pretreated with calcium was not significantly different from controls. Calcium stimulates the hydrolysis of both PtdInsP\(_2\) and PtdInsP in isolated cortices (Whitaker and Aitchison, 1985). Thus it seems likely that 10 minute calcium-pretreatments should deplete the stores of poly-phosphoinositides such that on the addition of calcium to reconstituted lawns, no DAG could be produced. This result suggests that DAG production is not required for exocytosis.

One proviso for this is that the enzyme and substrate are able to interact. For instance, one could envisage PtdInsP\(_2\) in the plasma membrane with the enzyme PIC residing on the cortical granules such that the phosphoinositide substrates could escape depletion during pretreatment.
This is not the case. Hylander and Summers (1981) showed that when the cortical granules are detached from the plasma membrane \textit{in vivo} using the anaesthetic procaine, fertilisation still proceeds and these eggs divide. This shows that PIC and the phosphoinositide phospholipids needed to generate the \( \text{InsP}_3 \) and DAG required for egg activation must both reside in the plasma membrane. When the plasma membrane is treated with calcium this should deplete the stores of phosphoinositide phospholipids and so exocytosis would appear to proceed without the production of DAG. However, it has not been determined as to whether calcium pretreatments completely deplete the plasma membrane of all sources of DAG although it is, of course, possible that fusogenic DAG might be derived from other phospholipid precursors.

(ii) The Role of Fusogens in Exocytosis.

The fact that strings of cortical granules will fuse when only one of the string is in contact with the plasma membrane suggests that the generation of a diffusible fusogen might be a prerequisite for exocytosis. This fusogen as I have said, is probably not DAG. It could be that calcium-stimulated PLA\(_2\) produces a fusogen (Wong and Cheung, 1979; Ferguson and Shen, 1984), either arachidonate (Creutz, 1981; Drust and Creutz, 1988), or a lysophospholipid (Poole \textit{et al}. 1970). It could equally be a fusogenic protein which is activated only in the presence of calcium (Pollard, Burns and Rojas, 1988). Whatever the situation, it is abundantly clear that fusion can only occur when interactions between the plasma membrane and the secretory granules exist, and then, only in the presence of calcium. Aggregates of granules do not fuse, even in very high (mM) concentrations of calcium. Exocytosis in mast cells, too, requires the presence of calcium at the time of fusion. In permeabilised mast cells, secretion in response to \( \text{Ca}^{2+} \) and GTP\(_y\)S is immediately abolished if the cells are returned to calcium-free media (B.D. Gomperts, unpublished communication).
2.4 RECONSTITUTION USING COMPONENTS FROM DIFFERENT SECRETORY CELLS.

I showed that reconstitution of exocytosis between the secretory components derived from different sea urchin species was possible with no loss of calcium-sensitivity. This was done using two closely related species, *L. pictus* and *L. variegatus*. This result shows conservation of the secretory mechanism at least between different sea urchin species. That CG binding requires specific plasma membrane proteins has been shown by Crabb *et al.* (1987). They showed that CG's do not bind to the cytoplasmic face of human erythrocyte plasma membranes. This result is not surprising since erythrocytes are terminally differentiated and do not undergo triggered exocytosis. There would be more chance of a positive result if exocytotic components from other secretory cells was used. As a first step towards this, I applied chromaffin granules to isolated *L. pictus* PM fragments. Adrenal chromaffin granules settled on to sea urchin egg plasma membrane fragments but did not fuse when Ca$^{2+}$ from 10 μM to 10 mM was added. This suggests that although the proteins responsible for the binding of secretory granules and plasma membranes might be conserved from species to species, the fusion mechanism is not.

3. **Exocytosis and Protein Dephosphorylation.**

3.1 EXOCYTOSIS AND A REQUIREMENT FOR ATP.

(i) ATP and Second Messenger Signalling Systems.

It is widely accepted that in order to secrete, cells need to be metabolically competent – that is they require ATP. However, it is incorrect to state that exocytosis requires ATP at the level of membrane fusion since, of course, ATP might be required for the competence of other physiological responses associated with exocytosis. For instance, *in vivo*, the
calcium mobilisation thought necessary for exocytotic secretion requires a metabolically intact PPI second messenger system. The PPI second messenger system may be inactivated in the absence of ATP. PtdInsP$_2$ is turned over rapidly and cannot be synthesised by PI-kinase in the absence of ATP (Tou et al. 1970). Another important consideration is the maintenance of cellular GTP levels. If exocytosis involves G-proteins, as it may do in a number of cells, (Barrowman et al. 1986; Cockcroft et al. 1986; Vallar, Biden and Wollheim, 1987; Bittner, Holz and Neubig, 1986) then depletion of ATP will have an effect on G-protein activation. This is because GTP is normally made by the transphosphorylating enzyme, nucleoside diphosphokinase (Kirkland and Taylor, 1959), which uses ATP as the phosphoryl group donor. Clearly, decreased intracellular ATP will result in decreased cytosolic GTP. This means that the GTP-GDP exchange necessary for G-protein activation (Helmreich and Pfeuffer, 1985) would be prevented. Metabolically depressed cells may therefore, be unable to secrete for a number of reasons not necessarily directly associated with the exocytotic event, but rather with cell signalling mechanisms.

(ii) The use of Broken Cell Preparations to Study ATP Requirements.

In studies on exocytotic secretion in permeabilised cell preparations and on isolated secretory systems such as the sea urchin egg cortex and Paramecium, the requirement for ATP in maintaining the Ca$^{2+}$-mobilising second messenger system and G-protein signalling systems intact is obviously by-passed by the exogenous addition of Ca$^{2+}$ and GTP, usually in non-hydrolysable form. Thus, in permeabilised cell preparations, any role for ATP different from that of maintaining second messenger signalling systems should be apparent.
3.2 PHOSPHORYLATION AS A PRELUDE TO EXOCYTOSIS.

(i) Priming of Secretory Systems with ATP.

In sea urchin eggs, *in vivo*, there is a requirement for metabolic competence to support exocytosis at fertilisation. Cyanide treatment of eggs leads to an inhibition of exocytosis and, furthermore, cortices prepared from ATP-depleted eggs are less sensitive to calcium than non-treated eggs. Exocytotic sensitivity is not restored by incubating cortices with ATP (Baker and Whitaker, 1978). When cortices are isolated, there is a gradual time-dependent decrease in the calcium sensitivity which can be prevented by the inclusion of ATP in the incubation medium (Sasaki and Epel, 1983; Moy *et al.* 1983), although ATP is not required at the time of Ca$^{2+}$ application. These results suggest that, in sea urchin eggs it is not the provision of ATP which is required to support secretion but rather some product of ATP, perhaps a phosphoprotein. A similar result has been found as a requirement for trichocyst exocytosis in *Paramecium* (Vilmart–Seuwen *et al.* 1986). Mast cells too, exhibit this dependence on ATP prior to exocytosis rather than at the time of stimulation (Howell, Kramer and Gomperts, 1989). This suggests that ATP is required to maintain the exocytotic apparatus in a state which is receptive to the exocytotic trigger.

(ii) Inhibitory Effects of ATP in Exocytosis.

Here, however, the analogy to *Paramecium* and mast cells ends since in both of these cells, the presence of ATP at the time of exocytosis inhibits exocytosis. In *Paramecium*, 2 mM ATP completely inhibits exocytosis (Vilmart–Seuwen *et al.* 1986). In mast cells the effect of ATP is somewhat different in that rather than inhibiting exocytosis, it retards the onset of secretion, by altering the kinetics of secretion (Tatham and Gomperts, 1989). In sea urchin eggs, ATP is not inhibitory (Vacquier, 1975; Whitaker and Baker, 1978). So although there are parallels between these three secretory cells, in that all three require ATP to keep exocytosis
primed rather than at the time of stimulation, there are also distinct differences in the effects of ATP.

(iii) The Importance of ATP in Adrenal Chromaffin Cells.

Adrenal chromaffin cells are rather different in that there is an absolute requirement for ATP at the time of secretion rather than before exocytosis (Knight and Baker, 1982). Here, then, ATP does not seem to be acting in a "priming" role, similar to that seen in mast cells (Howell et al. 1989). In some ways though, the ATP dependence of adrenal chromaffin cells is similar to that of mast cells. In mast cells, exocytosis is gradually inhibited over a period of 5 minutes in the absence of ATP and secretion is restored by the addition of ATP (Howell, Kramer and Gomperts, 1989). In adrenal chromaffin cells, the addition of ATP to cells permeabilised in the absence of ATP, allows cells to undergo exocytosis in response to Ca\(^{2+}\) (Knight and Baker, 1981, 1982). One could argue that the effect is the same. ATP is used to phosphorylate a regulatory protein essential for exocytosis, the difference between the requirements for ATP at the time of exocytosis reflecting different rates of the loss of phosphorylation of this protein or that in chromaffin cells, phosphorylation and dephosphorylation of regulatory proteins are required.

Finally, with regard to the importance of ATP in supporting exocytosis in adrenal chromaffin cells, it is important to note that endocytosis occurs along with exocytosis (Baker and Knight, 1981). This process occurs after exocytosis has ceased and so the requirements for endocytosis as opposed to exocytosis can be defined (von Grafenstein et al. 1986). It has been shown that endocytosis requires the presence of ATP and involves ATP hydrolysis, since the non-hydrolysable analogue AppNHp inhibits endocytosis (von Grafenstein, 1988). The implication of this is that, in these cells, it is difficult to precisely define the conditions required for exocytosis since endocytosis occurs at the same time. Thus, the absolute requirement for ATP may be for the endocytic part of the cycle rather than for exocytosis,
and thus exocytosis at the level of membrane fusion may have no absolute
dependence on ATP as is the case in sea urchin eggs, Paramecia, and mast
cells. This difficulty in interpreting the requirement for ATP has been
noted previously (Baker and Knight, 1984; Knight, 1987).

3.3 ATPyS INHIBITS CORTICAL GRANULE EXOCYTOSIS.

(i) The Effects of ATPyS in Sea Urchin Eggs.

In chapter 4, I have shown that the compound ATPyS, when injected
into eggs, inhibits cortical granule exocytosis. This inhibition is not at the
level of sperm–egg interactions or in the generation of an increase in
[Ca^{2+}], since ATPyS affects neither sperm–egg fusion as judged by the
transfer of the DNA–staining vital dye H33342 between gametes, nor the
sperm–induced rise in intracellular free calcium, measured with the calcium
sensitive fluorescent dye fura2. Indeed, ATPyS itself, causes a calcium
transient after microinjection in some eggs at high concentrations. This
could be due to the production of thiophosphorylated InsP₃ via PIP–
kinase. This InsP₃ would be resistant to the normal degradative processes
(Cooke, Nahorski and Potter, 1989). Alternatively, the calcium transient
might result from the production of GTPyS. The enzyme, nucleoside
diphosphokinase can produce GTPyS from ATPyS (Wieland and Jakobs,
1989). The GTPyS could then produce InsP₃ by activating PIC, which, as
I have shown, can be activated by GTPyS in sea urchin eggs.

I used the in vitro cortical preparation to test whether ATPyS inhibited
exocytosis in the isolated cortical apparatus. I found that it was without
effect, either when used to pretreat the cortices, or when present in the
Ca^{2+}–buffers used to stimulate exocytosis. Two pieces of information show
why this is. Firstly, the compound AppNHp is incapable of inhibiting
exocytosis when injected into eggs. This is another non–hydrolysable
analogue of ATP. Unlike ATPyS it can not be used as a kinase substrate,
(Yount et al. 1971), but inhibits processes which require ATP hydrolysis.
The second observation is that the inhibition is a time-dependent event. These two results suggest that what is important in this inhibition is the metabolism of ATPyS. This may be through the production of an inhibitory metabolite or the thiophosphorylation of a protein. Thiophosphoproteins have been shown to be resistant to phosphatases (Gratecos and Fischer, 1974), so it seems likely that an irreversible phosphorylation of a protein can inhibit exocytosis.

(ii) Inhibition is Due to Irreversible Protein Phosphorylation.

In order to determine which of these possibilities was correct, I prepared cortices from eggs which had been microinjected with ATPyS. Clearly, this approach distinguishes between the production of an inhibitory compound and a thiophosphoprotein. If an inhibitory compound is produced, it should be washed away during the preparation of the cortices. In contrast, if a thiophosphorylation occurs, and the thiophosphorylated protein is associated with the egg cortex, preparation of cortices should leave the inhibitory effect intact. Cortices prepared from eggs microinjected with ATPyS were resistant to calcium stimulation although at the time of the application of calcium no ATPyS and presumably no low molecular weight metabolite of ATPyS was present. This result, and the finding that ATPyS was without effect on isolated cortices shows that the inhibition of exocytosis is due to protein thiophosphorylation by a cytosolic protein kinase.

(iii) Intracellular Targets for ATPyS-Thiophosphorylation.

I identified proteins which are thiophosphorylated by ATPyS using 35S-labelled ATPyS. Incubating permeabilised eggs with this resulted in only 2 major bands, of 33 and 27 kD, which were labelled and associated with the cortex. Of these, the 27 kD protein was not always present which suggests that it may be a proteolytic fragment of the 33 kD protein. However, the fact that only one or two proteins are substrates for ATPyS
makes the characterisation of these somewhat easier. If these proteins are required to be dephosphorylated during exocytosis then clearly there is a difference in exocytotic control between sea urchin eggs and Paramecium, where the essential phosphoprotein required for exocytosis has a molecular weight of approximately 65 kD (Momayezi et al. 1987; Satir et al. 1989). However, the mechanism behind exocytosis in both cells, involving a dephosphorylation, might be the same.

It is also a possibility that using $^{35}$S–labelled ATPyS to find the inhibitory proteins is not efficient enough to detect the 65 kD protein (parafusin) which is a very minor component of Paramecium (Murtaugh et al. 1987), and that the proteins which are labelled are high abundance cortical proteins with no role in exocytosis. I have not localised the 33 kD protein, but it seems that it is probably localised to the cortical granule membrane since Kinsey et al. (1980) reported a 33 kD protein associated with the cortical granule membranes but much reduced in concentration in purified plasma membranes (Decker and Kinsey, 1983).

3.4 HOW MIGHT THIOPHOSPHORYLATION INHIBIT EXOCYTOSIS?

The inhibition of exocytosis by thiophosphorylation does not imply that part of the mechanism of exocytosis involves a dephosphorylation. As reasonable an interpretation of this result is that a protein can inhibit exocytosis if it is irreversibly phosphorylated. It is possible that thiophosphorylation inhibits the interactions between PM and CG’s in some way. This does not, however, seem to be the case since cortices can be prepared from ATPyS–treated eggs with the cortical granules still firmly attached. Indeed, I observed that CG’s from ATPyS–treated eggs seem to be more firmly anchored to the PM than CG’s prepared from untreated eggs. This was noticeable when trying to remove ATPyS–CG’s by shearing. These CG’s required considerably more force to be detached from the PM. This is not, in itself, enough to show that ATPyS has had no effect on the interactions between PM and CG.
When intact sea urchin eggs are inhibited with the tetrasaccharide, stachyose, and other compounds at high osmotic strengths, exocytosis, as judged by both the elevation of the fertilisation envelope and capacitance changes, is inhibited (Zimmerberg and Whitaker, 1986). This was thought to be due to the inhibition of secretory granule swelling. However, 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 establishment of a granule-free zone (Chandler et al. 1989). It was suggested that this might have been due to actin polymerisation. By analogy it is possible that ATPyS inhibits exocytosis by causing actin polymerisation and the establishment of a granule-free zone. This becomes more likely since I have shown that inhibition is only established in intact eggs. I have argued that this implies that the kinase responsible for inhibitory thiophosphorylation must be cytosolic. It is equally possible that the substrate is cytosolic, and, on the hypothesis outlined above, this substrate might be a protein which promotes and stabilises actin polymerisation.

(i) Phosphatase Inhibitors Inhibit Exocytosis.

However, several other pieces of evidence also point towards a dephosphorylation reaction having a role in exocytotic control. The first is that heparin can inhibit exocytosis without affecting changes in [Ca^{2+}], (I. Crossley and M.J. Whitaker, unpublished observation). Heparin has been shown to inhibit protein phosphatases (Gergely et al. 1984), and also inhibits exocytosis in Paramecium (Momazeyi et al. 1987).

Secondly, cortical granule exocytosis is inhibited in intact eggs by α-naphthyl phosphate (Sheppard and Whitaker, unpublished), which is an inhibitor of calcineurin (Li, 1984). Another protein phosphatase inhibitor, okadaic acid (Bialojan and Takai, 1988; Haystead et al. 1989), also inhibits cortical granule exocytosis when injected into eggs (Sheppard and Whitaker, unpublished). Neither of these phosphatase inhibitors seems to affect
calcium mobilisation since injected eggs went on to develop and divide after fertilisation.

Thirdly, ATP removal is eventually inhibitory, (Whitaker and Baker, 1978; Sasaki and Epel, 1983; Moy et al. 1983) which suggests that it is required to keep the exocytotic system primed. This is like mast cells (Howell, Kramer and Gomperts, 1989) except that the time required for the secretory granules to become refractory to the exocytotic trigger is far greater. The inhibition occurs over periods of hours (Sasaki and Epel, 1983; Moy et al. 1983) rather than minutes as is the case in mast cells (Howell, Kramer and Gomperts, 1989). These data all suggest that cortical granule exocytosis may involve the dephosphorylation of particular proteins. The identity of these is unknown, but there are two obvious candidates for this, a 33 kD and a 27 kD protein. The 27 kD protein may be a proteolytic fragment of the 33 kD protein which is probably localised to the cortical granule membrane (Decker and Kinsey, 1983).

(ii) The Effect of ATPyS in Other Secretory Cells.

The results in chapter 4 show that in sea urchin eggs, thiophosphorylation of cortical proteins can inhibit exocytosis. Thiophosphorylation has previously been shown to inhibit exocytosis from adrenal chromaffin cells (Brooks, Treml and Brooks, 1985; Brooks and Brooks, 1987), implicating a dephosphorylation in catecholamine secretion, but in mast cells ATPyS is unable to inhibit exocytosis (B.D Gomperts, unpublished communication). This discrepancy may be due to the fact that ATPyS is not a substrate for all cellular kinases. For instance, in *Xenopus* eggs, only a subset of those proteins phosphorylated by ATP are also phosphorylated when ATPyS is the substrate (Lohka et al. 1987). Another possible explanation for this discrepancy is that when ATPyS as opposed to ATP is used as the phosphate donor, the rate of phosphorylation is substantially slower (Ranu, 1986).
3.5 A ROLE FOR DEPHOSPHORYLATION IN EXOCYTOSIS.

The work of Whitaker and Baker (1978) and others (Sasaki and Epel, 1983; Moy et al. 1983), showed that there was no requirement for ATP during calcium-stimulated exocytosis in sea urchin eggs. However, they did show a requirement for ATP prior to exocytosis, in other words ATP was required to prime the system. One way in which it may do so is if it were keeping an essential phosphoprotein in a phosphorylated state. Exocytosis would then involve the actions of a calcium-stimulated phosphatase on this protein. In this thesis I have further suggested a role for a dephosphorylation being important in the control of exocytosis since exocytosis is inhibited by irreversibly phosphorylation. However, dephosphorylation of proteins is not a sufficient stimulus for exocytosis in sea urchin eggs because the removal of ATP (and presumably dephosphorylation of proteins essential for exocytosis) does not lead to spontaneous fusion. The secretory granules merely become refractory to calcium. Similarly, in *Paramecium* (Vilmart-Seuwen et al. 1986), and in mast cells (Howell et al. 1989), exocytosis is inhibited in a time-dependent fashion in the absence of ATP which again suggests that dephosphorylation alone is insufficient to explain exocytosis.

In *Paramecium*, the evidence for an involvement of a phosphatase in exocytosis is convincing. The dephosphorylation of a 65 kdal protein is correlated with exocytosis (Gilligan and Satir, 1982; Zieseniss and Plattner, 1985). In the nd9 strain, both exocytosis and the dephosphorylation of the 65 kdal protein are inhibited (Zieseniss and Plattner, 1985). Furthermore, the calcium- and calmodulin-activated phosphatase calcineurin (Stewart et al. 1982), and other phosphatases stimulate exocytosis and a protein with homology to calcineurin has been found at the exocytotic sites (Momayezi et al. 1987). In sea urchin eggs such a protein may also exist (Iwasa and Ishiguro, 1986).

In at least three secretory cells (sea urchin eggs, *Paramecium*, and mast cells), then, there is now good evidence that a dephosphorylation may be
important in exocytosis. In adrenal chromaffin cells, ATP is required for exocytosis (Knight and Baker, 1982) which might suggest that a dephosphorylation is not essential. It is however, important to bear in mind that ATP may be required for cytoskeletal disassembly or some other process, for instance, endocytosis (von Grafenstein, 1988) rather than exocytosis itself; that might be why in adrenal chromaffin cells exocytosis is inhibited very rapidly after the removal of ATP (Knight and Baker, 1982). Another possibility is that ATP is required for binding of regulatory proteins to the secretory granules. Of the many chromaffin granule binding proteins which have been discovered, several require ATP in order to associate with these secretory granules (Creutz et al. 1983). A further possibility is that the secretory granule transport to the plasma membrane required before fusion (Kondo et al. 1982; Burgoyne et al. 1982; Orci et al. 1972) is ATP dependent. Adams (1982) has shown that, in axons, membrane-bound organelle transport requires ATP. Indeed, Baker and Knight (1984) and Knight (1987) have pointed out the difficulty in interpreting the dependency of exocytosis for ATP.

Thus, a dephosphorylation might have a role to play in this cell but other ATP-requiring events are also necessary for secretion to occur. In view of this, it is of interest that the protein strongly implicated in exocytosis in *Paramecium*, PP65 or parafusin, has recently been shown to be present in pituitary, thyroid, brain, adrenal gland and other cells (Satir et al. 1989). It is a possibility that this protein, and its dephosphorylation are essential elements in exocytosis in many cells.
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Some Abbreviations used in the text.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AppNHp</td>
<td>adenylyl-imidodiphosphate</td>
</tr>
<tr>
<td>ASW</td>
<td>artificial sea water</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>ATPγS</td>
<td>adenosine-5'-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>BCECF</td>
<td>2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>intracellular free calcium ion concentration</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CG</td>
<td>cortical granule</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine-3',5'-cyclic monophosphate</td>
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<tr>
<td>DAG</td>
<td>1,2-diacetylglycerol</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>GDPβS</td>
<td>guanosine-5'-O-(2-thiodiphosphate)</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine-5'-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>[H^+]_i</td>
<td>intracellular free hydrogen ion concentration</td>
</tr>
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<td>IM</td>
<td>intracellular medium with glutamate as the major anion</td>
</tr>
<tr>
<td>InsP_3</td>
<td>D-myo-inositol 1,4,5-trisphosphate</td>
</tr>
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<td>kilodalton</td>
</tr>
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<td>phosphatidylinositol</td>
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<td>intracellular medium with chloride as the major anion</td>
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<td>plasma membrane</td>
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<td>polyphosphoinositide</td>
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<td>phosphatidylinositol-4-phosphate</td>
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<td>PtdInsP_2</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
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<tr>
<td>PIC</td>
<td>phosphoinositidase C</td>
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<td>protein kinase C</td>
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<tr>
<td>scm</td>
<td>standard error of the mean</td>
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<td>TPA</td>
<td>tetradecanoyl phorbol acetate</td>
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<td>DMO</td>
<td>dimethylthiazolidine</td>
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