How a sperm triggers
the fertilisation calcium wave
in the sea urchin egg

Ian Brian Crossley
The Department of Physiology
University College London
Gower Street,
London WC1E 6BT

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Abstract

Fertilisation is characterised by an increase in cytosolic free calcium (Ca\(_i\)). This crucial event transforms the egg, releasing it from its state of cell cycle arrest and stimulating embryonic development. In the sea urchin egg the Ca\(_i\) increase is the result of increased polyphosphoinositide (PPI) hydrolysis and the production of the calcium mobilising internal messenger inositol 1,4,5–trisphosphate.

I discuss the contribution of inositol polyphosphates to the calcium changes at fertilisation particularly their ability to stimulate calcium entry. I have used cortical granule exocytosis as an indicator of Ca\(_i\) and measured Ca\(_i\) in single eggs with the calcium sensitive fluorescent dye fura2. I show that, contrary to other reports, inositol phosphates activate eggs by a mechanism that is entirely independent of external calcium.

A sperm activates an egg by triggering a regenerative response that involves calcium–stimulated PPI hydrolysis: in this way calcium release leads to further calcium release in an explosive manner. It has been suggested that the sperm triggers this response through a GTP–binding protein. However I show that an analogue of GDP, Guanosine–5’–O–(2–thiodiphosphate), that inhibits GTP–binding protein activation, does not prevent the sperm–induced increase in Ca, or incorporation of the sperm into the egg. Also a GTP analogue, Guanosine–5’–O–thiotriphosphate, which stimulates GTP–binding proteins and causes egg activation, does so by a different mechanism than sperm. This data argues against the idea that a sperm triggers egg activation through a GTP–binding protein mechanism.
The mobilisation of intracellular calcium through PPI hydrolysis and the production of inositol phosphates is a common signal transduction pathway present in many tissues. My data suggests that inositol phosphates act only to mobilise intracellular calcium and not to stimulate calcium influx. They also suggest that fertilisation in the sea urchin may involve a novel transduction pathway because the PPI hydrolysis is not linked to a GTP–binding protein as in other systems.
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Some Abbreviations used in this thesis

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATPyS</td>
<td>Adenosine-5’-O-thiotriphosphate</td>
</tr>
<tr>
<td>ASW</td>
<td>Artificial Sea Water</td>
</tr>
<tr>
<td>BAPTA</td>
<td>bis-(o-aminophenox)-ethane-N,N,N’N’-tetraacetic acid</td>
</tr>
<tr>
<td>Ca_i</td>
<td>Cytosolic free calcium ion concentration</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis-(β-aminoethylether) N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>FE</td>
<td>Fertilisation Envelope</td>
</tr>
<tr>
<td>GDPβS</td>
<td>Guanosine 5’-O-(2-thiodiphosphate)</td>
</tr>
<tr>
<td>GTPyS</td>
<td>Guanosine 5’-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>Ins(1,4,5)P₃</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>Ins(2,4,5)P₃</td>
<td>Inositol 2,4,5-trisphosphate</td>
</tr>
<tr>
<td>Ins(1,3,4)P₃</td>
<td>Inositol 1,3,4-trisphosphate</td>
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<td>Inositol 1,2cyclic 4,5-trisphosphate</td>
</tr>
<tr>
<td>Ins(1,3,4,5)P₄</td>
<td>Inositol 1,3,4,5-tetrakisphosphate</td>
</tr>
<tr>
<td>Ins(1,4,5)-S-P₃</td>
<td>Inositol 1,4,5trisphosphorothioate</td>
</tr>
<tr>
<td>pHᵢ</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIC</td>
<td>Phosphoinositidase C</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1 FERTILISATION AND THE SEA URCHIN EGG

Fertilisation is a fundamental biological process essential for the propagation of animal species. The aims of fertilisation are two fold: first the controlled fusion of the sperm with the egg to establish the diploid genome and secondly the release of the egg from its state of cell cycle arrest to allow embryonic development.

Fertilisation in marine invertebrates, particularly the sea urchin, has been a subject of interest to biologists for over a century. Ease of accessibility to large quantities of gametes and their amenability to experimental manipulation means that the process of fertilisation is perhaps best understood in these animals.

In the sea urchin fertilisation results in one of the most obvious and visible signs of cellular activity in any cell, the cortical reaction. The cortical reaction is the concerted exocytosis of cortical granules which lie immediately beneath the plasma membrane, the contents of which form the fertilisation envelope (FE) (Fig. 1.1) (Moser, 1939; Millonig, 1969). It is a visible manifestation of the many changes that occur in the sea urchin at fertilisation. The interaction of the sperm with the egg transforms the egg from a metabolically quiescent cell arrested in cell cycle interphase into an actively proliferating embryo. Table 1.1 lists the key changes that occur at fertilisation with an indication of their timing. From the table it is clear that fertilisation affects all aspects of the egg's physiology. The earliest changes at the plasma membrane, the action potential and the cortical
The egg was stuck to a coverslip with poly-L-lysine and inseminated as described in Chapter 2 and fixed in 4% formaldehyde 5 minutes post-insemination. FE. The fertilisation envelope visible as a distinct halo-like structure extending around the whole egg perimeter. fc. The fertilisation cone which is the point of entry of the successful activating sperm.
reaction, ensure that only one sperm fuses with the egg (Jaffe & Gould, 1985), while the other changes within the egg, the stimulation of enzymes and protein synthesis, release the egg from its state of cell cycle arrest.

The later events, syngamy and the initiation of DNA synthesis, are the result of these earlier changes and lead to mitosis and the first cell division at 60 to 90 minutes, depending on the species.

Table 1.1

Some changes occurring at fertilisation in the sea urchin egg

<table>
<thead>
<tr>
<th>Conductance changes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca - Na action potential</td>
<td>&lt;3 sec</td>
</tr>
<tr>
<td>Activation potential</td>
<td>3 - 120 sec</td>
</tr>
<tr>
<td>Increase in K⁺ conductance</td>
<td>8 - 50 min</td>
</tr>
<tr>
<td>Increase in Ca⁺</td>
<td>10 - 40 sec</td>
</tr>
<tr>
<td>Cortical reaction</td>
<td>40 - 120 sec</td>
</tr>
<tr>
<td>Activation of NAD kinase</td>
<td>40 - 120 sec</td>
</tr>
<tr>
<td>Acid efflux and increase in pH</td>
<td>1 - 5 min</td>
</tr>
<tr>
<td>Increase in oxygen consumption</td>
<td>1 - 3 min</td>
</tr>
<tr>
<td>Initiation of protein synthesis</td>
<td>&gt; 5 min</td>
</tr>
<tr>
<td>Initiation of DNA synthesis</td>
<td>20 - 40 min</td>
</tr>
<tr>
<td>Mitosis</td>
<td>60 - 80 min</td>
</tr>
<tr>
<td>Cleavage</td>
<td>85 - 95 min</td>
</tr>
</tbody>
</table>

The table lists a number of the important changes that occur at fertilisation along with their timings and the internal messengers thought to regulate them. The table is derived from that of Whitaker & Steinhardt (1985). The values for the timings are taken from the same article and refer to Lytechinus pictus eggs at a temperature of 16 - 18 °C. The changes are referred to in the text.

The experiments presented in this thesis deal with the regulation of the process of fertilisation. In this chapter I shall review what is known about the regulation of fertilisation concentrating mainly on the sea urchin but drawing on other species where appropriate. I shall begin with the later
events of fertilisation about which most is known and develop the theme that fertilisation is regulated by ionic changes under the control of the polyphosphoinositide (PPI) 2nd messenger system. Then, moving to review the earlier events of fertilisation, about which less is known, I shall look at how the sperm signal is transduced in the egg. Finally I shall conclude the chapter by looking to see whether there might be a general mechanism of fertilisation for all species.

1.2 REGULATION OF FERTILISATION BY IONIC SIGNALS

The events at fertilisation are regulated by two ionic changes, an increase in intracellular calcium and intracellular pH (Ca, pH) (Whitaker & Steinhardt, 1985). There is evidence for this idea that satisfies all criteria for establishing the role of an intracellular messenger or mediator:

1. Changing the levels of the mediator must cause the changes that the mediator supposedly regulates.
2. Changes in the cellular activity of the mediator must occur at the appropriate time, and be of appropriate magnitude.
3. Inhibition or antagonism of changes in mediator activity should inhibit fertilisation.

(A) The evidence for calcium as a messenger at fertilisation

The first calcium model of egg activation was proposed in the 1930s and 1940s. It was based on the observation that a number of marine species could be activated by damage, but only when in seawater containing calcium (Heilbrunn & Young, 1930 and Tyler, 1941). More recently it has
been shown that treatment of eggs with the calcium-specific ionophore A23187 elicited all features of egg activation: the cortical reaction; the respiratory burst; the stimulation of protein and DNA synthesis (Steinhardt and Epel, 1974 and also see Table 1.1). Indeed A23187 has been found to be a universal activator of eggs of a number of species (Steinhardt et al., 1974 and see also Section 1.2 (D)). Hamaguchi and Hiramoto (1981) showed that eggs could be activated by the microinjection of calcium/EGTA buffers. So the first criterion, that changing $C_{ai}$ should cause egg activation, has been satisfied. Steinhardt et al. (1977) satisfied the second criterion by measuring $C_{ai}$ using the photoprotein aequorin, showing that the calcium rose transiently to a peak of 5 μM at fertilisation. This has been confirmed using the calcium sensitive fluorescent dye fura2 (Poenie et al., 1985). These levels of calcium are sufficient to stimulate the changes that occur at fertilisation. For example exocytosis can be stimulated in vitro by calcium concentrations in the range 1–3 μM (Baker & Whitaker, 1978 and see also Whitaker & Steinhardt, 1985). The third requirement has been met by microinjecting the calcium chelator EGTA which inhibited fertilisation (Zucker & Steinhardt, 1978).

(B) Evidence for pH as a messenger at fertilisation

The idea that a change in $pH_i$, was important at fertilisation came later than Heilbrunn's proposal for calcium. Steinhardt and Mazia (1973) showed that treatment of sea urchin eggs with the weak base ammonia caused the development of the $K^+$ conductance seen at fertilisation. They proposed that membrane-permeant ammonia entered the egg, combined with a proton to form ammonium ions thus increasing $pH_i$. It was the
increase in pH, that increased the K⁺ conductance. Ammonia treatment also stimulated protein synthesis, DNA synthesis (Epel et al., 1974 and Mazia, 1974) and cycles of chromosome condensation with nuclear envelope breakdown (Mazia & Ruby, 1974). The list is impressive, suggesting that a change in pH is as potent an activator as a change in Ca++. However it was subsequently shown that some of the later events (DNA synthesis, chromosome condensation and nuclear envelope breakdown) were due to the ammonia itself rather than the pH change it caused (Epel et al., 1974 and Shen & Steinhardt, 1978). None the less the increase in pH is essential for certain changes at fertilisation such as the increase in protein synthesis and the development of a K⁺ conductance (Whitaker & Steinhardt 1985).

The pH change was first measured unknowingly as an efflux of acid from eggs at fertilisation (Mehl & Swann, 1961). The first direct measurement of the change was achieved using pH-sensitive microelectrodes and showed that fertilisation resulted in an increase in pH from 6.84 to 7.27 (Shen & Steinhardt, 1978). This observation has been confirmed using a method based upon the distribution of the weak acid dimethyloxazolidinedione (Johnson & Epel, 1981). Changes in pH have been correlated with the acid efflux because both are dependent on extracellular Na⁺ and are inhibited by amiloride, an inhibitor of Na⁺/H⁺ exchangers (Shen & Steinhardt, 1978 and Vigne et al., 1984). This suggests that both phenomena are the result of the activation of a plasma membrane Na⁺/H⁺ exchanger. Normal development requires the presence of extracellular Na⁺ for a few minutes after fertilisation which clearly demonstrates the essential nature of change in pH is (Chambers & Dimich, 1975 and Chambers, 1976).
(C) The ionic regulation of fertilisation in the sea urchin

The two ionic changes can act singly or together to stimulate almost all the events of egg activation (see Table 1.1). For example the cortical reaction requires only an increase in $\text{Ca}_i$ (Vacquier, 1975) but the full stimulation of protein synthesis requires both an increase in $\text{Ca}_i$ and pH. Neither signal on its own is capable of producing the increase that is seen at fertilisation (Winkler et al., 1980).

It should be stressed that, while these two ionic events bring about almost all of the changes associated with fertilisation, parthenogenetic activation (for example by A23187) will not lead to cytokinesis because this requires a sperm centriole. This fact that can be demonstrated by generating cytasters within ionophore-activated eggs using hypertonic seawater. The two treatments together can lead to activation with subsequent cell division and eventual development of an adult sea urchin, albeit with a haploid genome (Brandriff et al., 1975). These experiments clearly demonstrate the importance of ionic changes in regulating egg activation, as the only additional factor required for development is the presence of microtubule organising centres.

(D) Ionic regulation of fertilisation in other species

There is evidence to show that ionic regulation is important in many other species. This is particularly so for the increase in $\text{Ca}_i$. An increase in $\text{Ca}_i$ has been measured in a wide range of species as well as in echinoderms: in amphibians (Busa & Nuccitelli, 1985), in fish (Ridgeway et al., 1977) and in mammals (Igusa & Miyazaki, 1986). A23187, as mentioned briefly in Section 1.2 (B), activates the eggs of a wide range of species
including amphibians and mammals (Steinhardt et al., 1974), surf clams (Schuetz, 1975), medaka (Ridgeway et al., 1977) and tunicates (Bevan et al., 1977). Clearly an increase in Ca\textsubscript{i} is a crucial event in the activation process and it may be that an increase in Ca\textsubscript{i} is a universal event at fertilisation in all species (Jaffe, 1985 and Whitaker & Steinhardt, 1985).

An increase in pH\textsubscript{i} however may not be such a universal signal. An acid efflux that is dependent on external Na\textsuperscript{+} has been observed in Urechis caupo, Spisula solidissima and Barnea candida which is suggestive of a change in pH\textsubscript{i} (Paul, 1975; Ii & Rebhun, 1979 and Dube & Guerrier, 1982). Also, transferring these eggs to low pH seawater soon after fertilisation reverses the activation process, despite the fact that sperm have entered the eggs (Gould & Stephano, 1989). In Xenopus laevis however, while pH\textsubscript{i} increases by 0.3 pH units, the pH change is not dependent on Na\textsuperscript{+}, nor is it required for egg activation (Lee & Steinhardt, 1981 and Webb & Nuccitelli, 1981).

Eggs where a Na\textsuperscript{+}–dependent acid efflux is observed are all arrested in an interphase-like state prior to fertilisation. Perhaps the pH change is peculiar to those species and reflects a type of cell cycle block. Sea urchin eggs for example, which are arrested in interphase, have a low resting pH\textsubscript{i} of 6.8 while Xenopus laevis eggs, which are arrested in 2\textsuperscript{nd} meiotic metaphase, have an pH\textsubscript{i} of 7.2. Experiments in activated Xenopus laevis eggs show that lowering pH\textsubscript{i} with weak acids below 7.2 prevents cell division: this block can be relieved by raising pH\textsubscript{i} again (Lee & Steinhardt, 1981). There exists the possibility then that control of pH\textsubscript{i} can be used as a cell cycle control in those animals, such as sea urchins, whose eggs are arrested in interphase.
1.3 IONIC REGULATION OF CELL FUNCTION

Before moving away from ionic regulation it is worth considering how these ions are regulated in the cell and how they control cell processes. A consideration of the simple bookkeeping of these messengers, their quantities, storage and buffering is useful in understanding how they function as regulators.

(A) Calcium as an intracellular messenger

Looking first at calcium, one of the most striking features about intracellular calcium is its powerful intracellular buffering. The sea urchin for example has a calcium content of 3000 μmoles/litre of eggs (Azarnia & Chambers, 1976) but a resting Ca$_i$ of only 0.1 μM (Poenie et al., 1985). The binding of calcium also occurs during increases in Ca$_i$, which as a consequence, require much greater amounts of calcium than a simple calculation would suggest. The binding places constraints on the mobility of calcium in the cell and allows for local changes of calcium to occur (Baker & Dipolo, 1984 and Rose & Loewenstein, 1975). These constraints are important in the activation of the sea urchin egg where the increase in Ca$_i$ has a distinct spatial organisation as discussed below. It is also a useful phenomenon for biologists as it allows the use of calcium binding dyes to measure Ca$_i$. If there were no significant cellular buffering of calcium the dyes would, at the concentrations they are used experimentally, abolish most calcium changes that occur.

The binding of intracellular calcium has two components: a component that is sensitive to metabolic inhibitors and a component that is not (Baker
The insensitive component reflects binding to intracellular proteins and, while this provides a significant component of the buffering (Baker & Dipolo, 1984), it is of primary importance because this is the way that calcium exerts its effects in a cell. Cell activity is governed by the activities of its constituent proteins. Calcium regulates cell function by binding to and adjusting the activities of certain proteins. This can affect some cell processes directly, for example glycolysis through phosphofructo-kinase, or indirectly as the calcium protein complex affects other cell proteins for example cAMP phosphodiesterase (Campbell, 1983). The enzyme is regulated by one of the best characterised calcium binding proteins calmodulin. Calmodulin is also present in the sea urchin egg (Head et al., 1979) where it has been shown to regulate the activation of the NAD kinase at fertilisation (Epel et al., 1981).

The energy-dependent buffering is important because it sets the resting Ca\textsubscript{i} and, is the source of calcium during cell stimulation. It has three components. First the mitochondria which have a large calcium sequestering capacity but only begin to store calcium when Ca\textsubscript{i} rises above 500nM (Becker et al., 1980 and Carafoli, 1987). This means that they play little, if any, role in determining resting calcium in cells which is of the order of 100nM but may sequester calcium during cell stimulation when Ca\textsubscript{i} increases. It is the other two components, the endoplasmic reticulum and the plasma membrane, that determine the resting Ca\textsubscript{i}. They both contain a Ca\textsuperscript{2+}-ATPase pump that actively pump calcium down to 100nM when these fluxes are balanced by passive fluxes into the cytosol (Baker & Dipolo, 1984 Becker et al., 1980 and Carafoli, 1987). These two components of calcium buffering are also the sources of calcium that are
used during cell stimulation and this will be discussed below.

Experiments in sea urchin eggs have provided some indication of the calcium movements occurring at fertilisation. Sea urchin eggs have the interesting property that their cytoplasmic contents can be stratified by centrifugation, separating the endoplasmic reticulum from the mitochondria while leaving the egg intact (Anderson, 1970 and Eisen & Reynolds, 1985). Using the calcium-sensitive photoprotein aequorin to image the intracellular calcium in single eggs Eisen and Reynolds (1985) showed that the calcium increased first and predominantly in the microsomal region of the egg. Subsequent application of carbonyl cyanide 4-trifluoromethoxyphenyl hydrazone, an inhibitor of oxidative phosphorylation, caused a release of calcium from the mitochondrial region of the cell. They concluded that at fertilisation calcium is released from the endoplasmic reticulum and taken up, at least in part, into the mitochondria. These experiments are interesting because they attempt to study calcium metabolism in vivo and look at the interaction of two of its components in a physiological environment.

(B) pH as an intracellular messenger

Little is known about the regulation of cell function by $\text{pH}_i$. From the pH activity profiles of enzymes it is possible to speculate that changes in $\text{pH}_i$ will affect cell function. However most pH profiles are determined in vitro and it is not clear that the same would be true in vivo (Busa, 1986). There is also much speculation that changes in $\text{pH}_i$ alter the activity of other intracellular messenger systems (Busa, 1986). In the sea urchin egg, while the importance of the pH change is not in question, the
molecular mechanisms of pH regulation are unknown.

Much more is known about the regulation of pH$_i$ than regulation by pH$_d$. As with calcium there are two components to be considered: one active and one passive. pH$_i$ is passively buffered by cytoplasmic constituents. In sea urchin eggs, for example, the buffering is approximately 12 mmol H$^+$/litre$^{-1}$ of eggs pH$^{-1}$, at physiological pH (Swann & Whitaker, 1985). pH$_i$ is also regulated by active processes. Particularly important in the sea urchin egg is a plasma membrane Na$^+$/H$^+$ exchanger described above. Other pH-regulating mechanisms such as a HCO$_3^-$ exchanger have not been identified in the sea urchin and the pH change is not dependent upon bicarbonate. In this respect the sea urchin egg is different from other cells types, where bicarbonate exchange is thought to play an important role in regulating pH$_i$, following growth stimulation (Thomas, 1989).

1.4 THE POLYPHOSPHOINOSITIDE 2$^\text{ND}$ MESSENGER SYSTEM

(A) An historical perspective

Inositol phospholipid hydrolysis has been implicated in many signal transduction processes which involve a mobilisation of calcium (Berridge & Irvine, 1984; 1989 and Berridge, 1987). Fertilisation is no exception (Swann et al., 1987).

Hokin and Hokin (1953) first showed that inositol phospholipids in pancreatic slices treatment with carbamylcholine increased $^{32}\text{P}_i$ incorporation into phosphatidylinositol (PI). They also showed that a similar effect in the cerebral cortex resulted in the increased incorporation of labelled inositol,
but not glycerol, into PI (Hokin & Hokin, 1958). This suggested that the
effect is the result of increased turnover of the inositol group rather than
de novo synthesis of PI. The turnover has been shown to reflect an
increased breakdown of the phospholipid by detecting the production of
water soluble inositol phosphates (Jungawala et al., 1971 and Dawson et al.,
1971). This effect was observed in a number of tissues and its correlation
with responses involving calcium mobilisation but its independence of
calcium led Michell to suggest that PI turnover may gate calcium entry
(Michell, 1975).

Durrell et al. (1968) showed that acetylcholine stimulated the
production of inositol 1,4-bisphosphate, suggesting that the hydrolysis of
polyphosphoinositides (PPIs), in addition to PI hydrolysis, also occurs.
Subsequent studies though looking at PPI levels in smooth muscle cells,
while confirming this earlier result, found that phosphatidylinositol 4,5-
bisphosphate (PIP₂) hydrolysis required calcium (Akhtar & Abdel-Latif,
1980). This suggested that phospholipid breakdown was a consequence of
calcium mobilisation rather than the cause, and so argued against their role
as mobilisers of intracellular calcium. However with the discovery that
(anti-Diuretic Hormone)
ADH stimulated the more rapid breakdown of phosphatidyl inositol
4-monophosphate (PIP) and PIP₂ in hepatocytes in a relatively calcium-
independent manner, it was realised that the breakdown of PI was a
secondary effect reflecting a metabolic flux of PI in the resynthesis of PIP
and PIP₂ (Michell et al., 1981 and Kirk et al., 1981). In a careful kinetic
study looking at the production of the water soluble inositol phosphates
Berridge (Berridge, 1983) showed that serotonin stimulated a production of
inositol trisphosphate and inositol bisphosphate in the blowfly salivary gland
that clearly preceded the production of inositol or inositol monophosphate and the onset of the physiological response. This showed that the stimulated response was the hydrolysis of PIP and PIP$_2$, which could potentially then play a role in the mobilisation of calcium.

The link between PPI hydrolysis and calcium mobilisation was provided by Streb et al. (1983) and Burgess et al. (1984), who showed that inositol 1,4,5-trisphosphate [Ins(1,4,5)P$_3$] specifically released calcium from permeabilised pancreatic cells and hepatocytes respectively. This observation has since been extended and Ins(1,4,5)P$_3$ has been shown to release calcium from a large number of cells (Berridge, 1987). Cell fractionation studies (Streb et al., 1983) showed that Ins(1,4,5)P$_3$ released calcium from a microsomal fraction, probably the endoplasmic reticulum (ER). Using radiolabelled Ins(1,4,5)P$_3$, high affinity receptors have been demonstrated in both permeabilised cells (Spat et al., 1986) and microsomal membrane fractions of a number of cell types (Baukal et al., 1985; Spat et al., 1986a and Guillemette et al., 1987). The hierarchy of inositol phosphate affinities for these sites parallels their abilities to release calcium (Spat et al., 1986), suggesting that Ins(1,4,5)P$_3$ releases calcium through a receptor interaction. Supattapone et al. (1988) have purified the high affinity binding site from cerebellum, where it is particularly abundant, to homogeneity, showing that it is a high molecular weight protein (260 kdal). An Ins(1,4,5)P$_3$-receptor has recently been cloned by Mignery et al. (1989) and it has some sequence homology to the skeletal muscle ryanodine receptor. The precise nature of the calcium store has not been determined. Immunochemical methods have been used to localise the receptor to the ER and nuclear membrane but not to the cell plasma membrane (Ross et al., 1989). In
Figure 1.2 Basic scheme of PIP$_2$ hydrolysis and Ins(1,4,5)P$_3$ metabolism

The figure is drawn from Irvine (1989)
contrast Volpe et al. (1987) have suggested that there is a specialised
Ins(1,4,5)P\textsubscript{3} - sensitive calcium store 'the calciosome', distinct from the ER,
which holds calcium pumps and calsequestrin (a calcium binding protein
similar to the sarcoplasmic reticulum of skeletal muscle). How these two
potential calcium stores might be related has yet to be resolved.

More recently a further layer of complexity has been added to this
picture (Irvine et al., 1988) with discovery of a number of metabolites of
Ins(1,4,5)P\textsubscript{3}. Initially it was thought that Ins(1,4,5)P\textsubscript{3} was metabolised by
sequential dephosphorylation to form inositol, which was then re-
incorporated of the phospholipids (Berridge, 1984) (Fig. 1.2). However
studies into inositol phosphate production in parotid cells revealed that some
of the inositol trisphosphate produced was not Ins(1,4,5)P\textsubscript{3}, but Ins(1,3,4)P\textsubscript{3},
(Irvine et al., 1984) and the proportion of this isomer increased with
increasing stimulus duration (Irvine et al., 1985). With the discovery of
inositol 1,3,4,5 - tetrakisphosphate (Ins(1,3,4,5)P\textsubscript{4}) which can be
dephosphorylated to Ins(1,3,4)P\textsubscript{3} (Batty et al., 1985) and an Ins(1,4,5)P\textsubscript{3}
3 - kinase (Irvine et al., 1986), it became clear that Ins(1,3,4)P\textsubscript{3} was in fact
a metabolite of Ins(1,4,5)P\textsubscript{3}, produced via its phosphorylation to
Ins(1,3,4,5)P\textsubscript{4}. These two metabolites have generated a great deal of
interest as possible second messengers (Berridge, 1987; Petersen, 1989;
Putney et al., 1989 and Berridge & Irvine, 1989) which might act with
Ins(1,4,5)P\textsubscript{3} to regulate Ca\textsubscript{4}.

Agonist - induced calcium increases in many cells can be divided into
two phases: an early phase, independent of external calcium, that is due to
release of calcium from internal stores, and a later phase of sustained
calcium influx (Putney, 1979; Putney et al., 1981 and Putney, 1986). This
type of response has been observed in cells as diverse as parotid acinar cells (Putney, 1986a), endothelial cells (Hallam et al., 1988) and neutrophils (Lew et al., 1984) (see also Putney, 1987 and Hallam & Rink, 1989 for reviews). The ability of Ins(1,4,5)P$_3$ to release calcium from internal stores provides a ready explanation for the first phase of the calcium response. It is not clear however, what mechanisms underly the second part of the response. It has been suggested, from work on sea urchin eggs, that Ins(1,3,4,5)P$_4$ regulates calcium entry into cells (Irvine & Moor, 1986; 1987) and so might be responsible for the later part of the calcium increase. This will be dealt with in more detail below.

The other product of PPI hydrolysis, DAG, also has second messenger functions (Nishizuka, 1984,1986). Takai et al. (1979) have shown that DAG is a potent activator of protein kinase C, a calcium-dependent protein kinase. DAG, acting together with phosphatidylserine, greatly enhances the activity of the enzyme and shifts the calcium activation profile of the enzyme so that it is active at resting Ca$_i$ (Kaibuchi et al., 1981). Protein kinase C is a ubiquitous protein kinase that controls cell processes as diverse as secretion and lymphocyte activation (Nishizuka, 1984). The cloning of protein kinase C revealed that it was in fact not a single protein but a family of proteins (Nishizuka, 1988). The various forms of the enzyme show distinct tissue distributions and have different activation characteristics which creates the potential for a complex and highly versatile regulatory system.

It is only recently that attention has been directed at the quantities of Ins(1,4,5)P$_3$ and DAG produced during cell stimulation and relating this to the quantities required to produce physiological effects. Most of the work
has centred on the amounts of Ins(1,4,5)P₃ produced during cell stimulation. A variety of techniques have been used which estimate that the concentration of Ins(1,4,5)P₃ may increase from submicromolar levels to as high as 10 μM (Irvine, 1989) values which straddle the concentration of Ins(1,4,5)P₃ required to release calcium from a number of different cells (Berridge, 1987).

Data is now available for the phosphoinositide system which satisfies most, if not all the criteria for the identification of internal messengers (see Section 1.2 (A)). However the complexity of this system: the large numbers of inositol phosphates (Irvine, 1989), multiple isoenzymes of Protein Kinase C (Nishizuka, 1988) and the potential for complex interactions within the two arms of the PPI system are only just being realised (Berridge, 1987).

(B) Phosphoinositides and fertilisation

Returning to the sea urchin egg and fertilisation, it seems that here the two limbs of the PPI messenger system act to regulate the two ionic changes discussed earlier. Fertilisation results in a large increase in PPI turnover (Turner et al., 1984), and the generation of the two products of PPI hydrolysis, InsP₃ and DAG (Ciapa and Whitaker, 1986). This clearly precedes observable physiological responses such as the cortical reaction (Turner et al., 1984 and Ciapa & Whitaker, 1986). Ins(1,4,5)P₃ is a potent parthenogenetic activator of sea urchin eggs; its microinjection causes the cortical reaction (Whitaker and Irvine, 1984), membrane potential changes typical of those seen at fertilisation (Slack et al., 1986) and an increase in Ca, (Swann and Whitaker, 1986). As in other systems, Ins(1,4,5)P₃ releases
calcium from a microsomal fraction in sea urchin eggs (Clapper and Lee, 1985). The data is all consistent with the idea that \( \text{Ins(1,4,5)P}_3 \) is the intracellular messenger mediating the increase in \( \text{Ca}^{2+} \) at fertilisation.

The other messenger, DAG, is believed to cause the increase in \( \text{pH}_i \) that occurs at fertilisation through the activation of protein kinase C. 12-\(\text{O-}\)tetradecanoyl phorbol acetate, a phorbol ester and known activator of Protein Kinase C (Castagna et al., 1982), and synthetic \( \varepsilon\alpha \) -diacylglycerols cause an acid efflux and increase in \( \text{pH}_i \) in sea urchin eggs (Swann and Whitaker, 1985; Shen and Burgart, 1986). This effect could be blocked by dimethylamiloride (Swann and Whitaker, 1985), an inhibitor of the \( \text{Na}^+/\text{H}^+ \) exchanger (Vigne et al., 1984), and by removal of extracellular \( \text{Na}^+ \) suggesting that it was due to activation of a \( \text{Na}^+/\text{H}^+ \) exchanger as occurs at fertilisation (Swann and Whitaker, 1985; Shen and Burgart, 1986). Recently, it has been shown that the fertilisation \( \text{pH} \) change can be inhibited by calmodulin antagonists suggesting that an increase in \( \text{Ca}^2+ \) might also be important in causing the \( \text{pH} \) change (Shen, 1989).

While there is evidence that meets some of the criteria outlined in Section 1.2 for defining an internal messenger some conditions remain unsatisfied. Although the two products of \( \text{PIP}_2 \) hydrolysis can produce the ionic changes associated with egg activation it is not known whether sufficient quantities of the messengers are produced at fertilisation to produced the observed ionic changes. Also there is a paucity of compounds available which have specific effects on the PPI messenger system. While neomycin, used at concentrations which inhibit PPI hydrolysis in an \textit{in vitro} preparation of sea urchin eggs (Whitaker & Aitchison, 1985) inhibits fertilisation (Swann & Whitaker, 1986) the concentrations required are

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rather high and it is possible that neomycin might be having other additional effects. Perhaps most importantly (see Section 1.4 (D)) neomycin has been shown to inhibit calcium-induced calcium release (Palade, 1987).

Recently several new pharmacological tools have become available that have relatively specific effects on the PPI messenger system. First, the mucopolysaccharide heparin, which has been shown to compete with Ins(1,4,5)P₃ for its specific binding site (Worley et al., 1987), also inhibits Ins(1,4,5)P₃-induced calcium release (Hill et al., 1987; Cullen et al., 1988; Guillemette et al., 1989 and Rice & Joseph, 1989). Heparin is a competitive inhibitor of calcium release and is effective at concentrations in the micromolar range. It is a potentially useful tool then in the study of the actions of Ins(1,4,5)P₃. Experiments are presented in this thesis that look at the effect of heparin on the actions of Ins(1,4,5)P₃ and sperm on the activation of sea urchin eggs.

Potter and his colleagues have synthesised a thio-analogue of Ins(1,4,5)P₃, inositol 1,4,5-trisphosphorothioate (Ins(1,4,5)-S-P₃, (Cooke et al., 1987) which is resistant to intracellular inactivation, either by phosphorylation or dephosphorylation (Taylor et al., 1989). I have tested what effect this compound has on intracellular calcium and the size of the Ins(1,4,5)P₃-sensitive calcium store in sea urchin eggs. With the development of these, and other tools, to manipulate the PPI 2nd messenger system it should be possible to test ideas about intracellular signalling in whole cells. This may be important as it is now realised that the spatial organisation of the signalling apparatus could be crucial in generating calcium signals and controlling cell function (Berridge & Irvine, 1989).
Recently it was reported that Ins(1,4,5)P₃ required the presence of external calcium to activate sea urchin eggs (Slack et al., 1986; Irvine and Moor, 1986). Furthermore Irvine and Moor (1986) found that Ins(1,3,4,5)P₄ was a potent activator of eggs when co-injected with a sub-activating dose of an inositol trisphosphate and this effect too was dependent on extracellular calcium. Ins(2,4,5)P₃, which is not metabolised to Ins(1,3,4,5)P₄, was much less able to activate eggs than Ins(1,4,5)P₃ and so they proposed that it was not Ins(1,4,5)P₃ but Ins(1,3,4,5)P₄ that activated eggs by inducing calcium entry. The action of Ins(1,4,5)P₃, they suggested, was due to its metabolism to Ins(1,3,4,5)P₄.

The idea that Ins(1,3,4,5)P₄ might control plasma membrane calcium fluxes has received some support from patch clamp studies in lacrimal acinar cells (Morris et al., 1987). These cells have a calcium-dependent potassium current which can be used as an indicator of Ca⁺. Ins(1,4,5)P₃ caused only a transient activation of this current either in the presence or absence of external calcium. However a combination of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ produced a sustained activation of this Ca²⁺-dependent K⁺ current. The effect required extracellular calcium and could not be produced by either of these inositol phosphates alone.

These results are surprising in light of the fact that fertilisation can occur in the absence of extracellular calcium (Takahashi & Sugiyama, 1973; Chambers, 1980; Chambers & Angeloni, 1981 and Schmidt et al., 1982). The implication from Irvine and Moor's work is that neither Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄ are the internal messengers that mediate the increase in Ca⁺ at fertilisation. Experiments presented in this thesis address the question of
whether inositol phosphates can cause calcium influx in the sea urchin egg and so can be the messengers at fertilisation.

(D) The increase in calcium at fertilisation takes the form of a wave

Fertilisation is an unusual example of cell stimulation. Outside the nervous system when an agonist stimulates a cell it binds to receptors over a large part of the cell’s surface. At fertilisation the agonist is the sperm and this interacts with the egg at a single point representing less than 0.1% of the egg’s surface. This is reflected in the increase in Ca, that occurs in the egg which is not uniform but takes the form of a wave of calcium release emanating from the point of sperm contact (Eisen et al., 1984; Swann and Whitaker, 1986 and Hafner et al., 1988).

The wave of calcium release is not unique to sea urchins but is a general feature of eggs of deuterostome species (Jaffe, 1985). A wave of cortical granule exocytosis visibly reflects the wave of calcium release and early studies looked at this wave of exocytosis. In medaka eggs Yamamoto (1961) proposed that the exocytosis reflected an invisible wave as it propagated through regions of the egg devoid of cortical granules. He also suggested that this wave was a propagated reaction and could not be explained by simple diffusion of a substance. This also seems to be true of the sea urchin, as the time taken for the wave of exocytosis induced by microinjection of Ins(1,4,5)P₃ to traverse the egg could not be explained by the simple diffusion of Ins(1,4,5)P₃ (Whitaker & Irvine, 1984). It was constant for a five fold range of Ins(1,4,5)P₃ concentrations. Sea urchin eggs contain a PIC that is strongly stimulated by calcium concentrations in the physiological range (Whitaker & Aitchison, 1985) suggesting a possible
mechanism for the propagated response. An increase in Caᵢ stimulates PIC liberating Ins(1,4,5)P₃ which will cause further increases in Caᵢ (Whitaker & Irvine, 1984; Whitaker & Aitchison, 1985; Swann & Whitaker, 1986). Neomycin, an inhibitor of PPI hydrolysis (Whitaker & Aitchison, 1985) inhibits the propagation of the wave induced by the microinjection of Ins(1,4,5)P₃ (Swann & Whitaker, 1986) supporting the idea that the calcium wave is propagated by PPI hydrolysis. However neomycin has a number of other intracellular effects including the inhibition of calcium–induced calcium release (see Section 1.4 (B)) and so this might not be the only interpretation of these results. Busa et al. (1985) have suggested that in frog eggs the calcium wave is the result of calcium–induced calcium release and so the results of Swann and Whitaker (1986) would be consistent with this idea also. However, in contrast to the frog egg (Cross, 1981), the activation wave cannot be triggered by calcium (Chambers & Hinckley, 1979 and Swann & Whitaker, 1986) in sea urchin eggs and so the mechanisms of propagation might be different in these species.

(E) The PPI messenger system at fertilisation in other species

The role of the PPI messenger system at fertilisation like that of Caᵢ seems to be a general one. Ins(1,4,5)P₃ produces activation responses in a wide variety of species: Xenopus eggs (Busa et al., 1985); starfish eggs (Picard et al., 1985); hamster eggs (Miyazaki, 1988) and medaka eggs (Nuccitelli, 1987). The activation of protein kinase C also seems to be important. In many species treatment of eggs with phorbol esters stimulates germinal vesicle breakdown. While strictly speaking this is a maturational event, in species such as Spisula solidissima (Dube et al., 1987) it occurs
post fertilisation. In *Xenopus laevis* eggs phorbol esters stimulate cortical granule exocytosis, cortical contractions and cleavage furrow formation (Bement & Capco, 1989). This provides clear evidence that the protein kinase C arm of the PPI 2nd messenger system has an important role to play at fertilisation.

1.5 MODELS OF FERTILISATION

There is considerable evidence to show that at fertilisation the egg is transformed through two ionic changes linked by a common factor, the PPI 2nd messenger system. Little is known though about how the sperm initiates these changes: what are the molecular mechanisms that link the sperm to PPI hydrolysis? Two different models have been proposed to explain how a sperm fertilises an egg and these are illustrated schematically in Fig. 1.3.

In the first model the sperm is thought to activate the egg in the same way that some hormones stimulate cells, through a receptor/G protein linkage to an effector enzyme (Turner et al.; 1986 and 1987). The sperm would bind to receptors on the egg surface which are linked to PIC through a G−protein, in the same way that the enzyme adenylate cyclase is linked to receptors in other cells (Levitzki, 1986).

The second model proposes that a sperm activates an egg by fusing with it, introducing some activating factor which triggers the events already described (Fig. 1.3).

(A) The receptor/G−protein model of egg activation

If a sperm interacts with a receptor then a reasonable expectation is
Figure 1.3 Models of fertilisation

The figure shows two hypothetical models of how a sperm might activate an egg. These models are described in detail in the text. Factor X is the unknown factor introduced by the sperm. The dashed lines indicate possible sites of action of the factor.
that the interaction of the sperm with the egg should show some specificity. This seems to be the case because cross-species fertilisation of even quite closely related species does not normally occur (Hultin, 1948). Also, prior to egg activation the sperm bind to the egg in large numbers (Tegner & Epel, 1973 and Summers & Hylander, 1975). This binding is species-specific (Summers & Hylander, 1975) and can be abolished by treatment of the eggs with trypsin or antibodies raised against the egg surface (Aketa et al., 1972). Also pretreatment of sperm with extracts of egg membrane inhibits fertilisation (Rossignol et al., 1981). The data strongly suggests the presence of a sperm-specific receptor on the egg surface.

While a sperm-specific receptor is present on the egg surface it is not clear that it is involved in the process of egg activation. The receptor seems to reside not in the egg plasma membrane but in the vitelline membrane, a proteinaceous coat that surrounds the egg. Procedures that disrupt the coat, such as tryptic digestion, while abolishing the species-specific binding do not inhibit fertilisation (Aketa et al., 1972). Rather these treatments allow sperm of other species to fertilise the eggs. So while there seems to be a species-specific barrier to fertilisation that involves a receptor, this barrier does not seem to be involved in the process of egg activation.

This situation is complicated, however, by experiments looking at the sperm ligand that would interact with the egg receptor. The acrosomal granule, which is that part of the sperm that first interacts with and binds to the egg, consists of predominantly one protein, bindin (Vacquier & Moy, 1977). Bindin agglutinates sea urchin eggs in a species-specific manner (Glabe & Vacquier, 1977 and Glabe & Lennarz, 1979). The agglutination
reaction is blocked by pretreatment of the bindin with egg membrane extracts (Vacquier & Moy, 1977 and Glabe and Lennarz, 1979). Bindin, then, seems to interact with the specific sperm receptor described above and might not then be expected to play a role in egg activation. However the bindin protein isolated from Urechis caupo sperm activates Urechis eggs and partially activates sea urchin eggs (Gould et al., 1986). Also the sea urchin bindin causes conductance changes in sea urchin eggs (Longo et al., 1986). The data suggest that bindin might be the sperm ligand but this contradicts the receptor data that suggests that the specific sperm receptor is not involved in the process of activation.

Turning to the other component of the first model, the G-protein, the situation seems more clear. G-Proteins acts as transducers of external signals, linking receptors to their effector enzymes (Gilman, 1987). They are trimeric proteins with α, β and γ subunits, which in their inactive state form a complex to which GDP is bound. Interaction of the G-Protein with an activated agonist/receptor complex stimulates the exchange of GDP for GTP, subunit dissociation and G-Protein activation. The α subunit interacts with and activates the effector enzyme to produce the cellular effect. G-Proteins are inactivated by the hydrolysis of the GTP which allows subunit reassociation, so completing the cycle.

While the role of G-Proteins in signal transduction processes has been most rigorously determined for the cAMP 2nd messenger system (Levitzki, 1986) and for transducin in visual transduction (Stryer, 1986), there is good evidence to suggest that G-Proteins link receptors to PIC (Cockcroft & Stutchfield, 1988). Guanosine 5′-O-(3′-thiotriphosphate) (GTPγS), a non-hydrolysable analogue of GTP which chronically activates
G-Proteins, activates a neutrophil PIC (Cockcroft & Gomperts, 1985) and GTP is required for hormone stimulated PPI hydrolysis in blowfly salivary glands (Litoch, 1985). Additionally AlF₄⁻, a potent G-Protein activator (Bigay et al., 1985) stimulates PPI hydrolysis in hepatocytes (Blackmore et al., 1985).

Cholera and pertussis toxin, which ADP-ribosylate the α-subunits of certain G-Proteins, label proteins of the appropriate molecular weight in sea urchin plasma membranes (Turner et al., 1987). This indicates that there are G-Proteins present in the sea egg and indeed a GTP-binding protein has been partially purified from egg plasma membranes (Oinuma et al., 1986). The microinjection of GTPyS or cholera toxin will activate sea urchin eggs showing that at least one of the G-proteins is linked to PIC. Guanosine-5’-O-(2-thiodiphosphate) (GDPβS), a non hydrolysable analogue of GDP which inhibits G-protein activation (Eckstein et al., 1979), inhibits fertilisation as assayed by scoring cortical reactions (Turner et al., 1986) which suggests that the sperm is somehow linked to a G-protein.

(B) The fusion model of egg activation

The second model proposes that a sperm fertilises an egg by fusing with it and introducing an activating substance. Fusion of the sperm membrane with the egg results in an increase in the surface area of the egg’s membrane and so increases its capacitance. Capacitance measurements show an increase at fertilisation that is coincident with the appearance of a sperm-induced current (McCulloh & Chambers, 1986). The sperm-induced current depolarises the egg’s membrane potential which results in
an action potential (Dale et al., 1978 and Chambers & de Armendi, 1979). This action potential is followed some 10 to 30 seconds later by a sustained period of depolarisation, the activation potential, the result of cation channels opened by an increase in Ca\textsuperscript{2+} (Chambers & de Armendi, 1979 and David et al., 1988). Clearly fusion, measured as a capacitance change, is a very early event at fertilisation, preceding the increase in Ca\textsuperscript{2+} (the appearance of the activation potential) by at least 10 seconds.

Determination of the time of sperm/egg fusion using either the electron microscope (Longo et al., 1986) or a fluorescence assay (Hinckley et al., 1986) show it occurs 5 seconds after the onset of the sperm-induced current. While these techniques suggest that fusion occurs later than the capacitance measurements suggest, the difference probably results from the use of fixatives in the morphological studies which might disrupt any labile fusion state (Whitaker et al., 1989). Regardless of this all studies show that fusion occurs several seconds before the increase in Ca\textsuperscript{2+}, clearly a prerequisite of the fusion model.

The idea that the sperm introduces an activating substance is supported by the work of Dale et al. (1985). They showed that the microinjection of sperm extracts activates sea urchin eggs and that the activating substance is not calcium (Dale et al., 1985). There is no indication as to the identity of the putative sperm messenger, but there are several possible candidates. In contrast to Dale et al., Jaffe (1985) favours calcium as a possible activating substance on the basis that sperm take up large quantities of calcium during the acrosome reaction, just prior to fertilisation (Schackmann et al., 1978). Whitaker and Irvine (1984) have suggested that Ins(1,4,5)P\textsubscript{3} may be the activating substance: Ins(1,4,5)P\textsubscript{3} has recently been shown to
increase in the sperm immediately following the acrosome reaction (Domino & Garbers, 1988). Finally, it has recently been shown that cGMP activates both sea urchin and medaka eggs (Swann et al., 1987 and Iwamatsu et al., 1988) and given the high levels of guanylate cyclase in the sperm (Gray et al., 1976), cGMP must be a possible candidate.

(C) Conclusions on fertilisation models

While there is more evidence for the receptor/G-protein model than the fusion model the data is by no means conclusive. This is especially true for the sperm receptor where its existence seems clear but the role that it might play in egg activation does not.

The evidence for a G-Protein, while not confused, only fulfils some of the criteria for establishing the role of a G-Protein in a signal transduction process. Only the data showing that GDPβS inhibits fertilisation indicates that a sperm activates an egg through a G-protein mechanism. Experiments with bacterial toxins and GTPyS demonstrate only the existence of a G-protein/PPI signal transduction pathway. They provide no indication that sperm utilise this pathway to activate an egg. This criticism can be made of more recent experiments in eggs of the frog Xenopus laevis. Kline et al. (1988) expressed receptors to acetyl choline (the muscarinic type) and serotonin, which exert their cellular effects through the PPI messenger system via a G-protein linkage, in immature Xenopus eggs by microinjecting brain messenger RNA. These eggs, when matured, could be activated by treatment with the appropriate neurotransmitter.

Other criteria for determining the role of a G-Protein, the requirement of GTP for the initiation of the response and the demonstration
of a reduction of ligand receptor affinity in the presence of GTP, while
difficult to demonstrate for fertilisation must be satisfied to show G–Protein
involvement (Gilman, 1987). I shall describe some experiments that test
predictions of the G–Protein model in the sea urchin egg.

1.6 A GENERAL MODEL FOR FERTILISATION

While the description of fertilisation presented here has dealt mainly
with the sea urchin it should be apparent that there are similarities between
fertilisation in this and other species. Most notable is the general role that
calcium plays in egg activation. An increase in Ca$_i$ at fertilisation has been
found in all species where it has been looked for. Is there a general
mechanism to explain how sperm bring about this increase?

Jaffe (1985) has suggested that there are two mechanisms to explain
how a sperm activates an egg and that deuterostome species use one
mechanism and protostome species the other.

Deuterostome species, which includes sea urchins, are activated by an
explosive wave of calcium release that emanates from the point of sperm
contact. The mechanism of this calcium explosion may be a general one
involving the PPI 2nd messenger system. As mentioned earlier Ins(1,4,5)P$_3$
induces activation responses in a wide variety of deuterostome species. The
mechanism that initiates this wave may also be a general one involving a
GTP–binding Protein. GTPyS produces activation responses in frog,
hamster and fish eggs (Kline & Jaffe, 1987; Miyazaki, 1988 and Iwamatsu,
1989).

The activation processes in eggs of protostome species are not as well
characterised as for deuterostomes. Protostome eggs are certainly not
activated by an explosive wave of calcium release as in the case of deuterostome species. The cortical reaction, which reflects an increased \(\text{Ca}_1\), in those species where it occurs is slow and asynchronous (Jaffe, 1985). This contrasts with deuterostome species where it is rapid and synchronous.

It has been suggested that protostome species are activated by slow increases in \(\text{Ca}_1\) resulting from the influx of calcium through voltage dependent channels in the egg plasma membrane (Jaffe et al., 1985 and Gould & Stephano, 1989). This idea is supported by the fact that these eggs, in contrast to deuterostome eggs, can be activated by exposure to high \(\text{K}^+\) sea water and that the removal of external calcium reverses the fertilisation process (Jaffe, 1985). However it has been reported that higher levels of \(\text{K}^+\) are required to cause egg activation than to cause maximum \(^{45}\text{Ca}^{2+}\) influx (Dube, 1988). This suggests that the \(\text{K}^+\) is doing more than promoting calcium influx when it activates protostome eggs. Recently it has been observed that the microinjection of Ins(1,4,5)P\(_3\) will activate the eggs of the surf clam *Spisula solidissima* (a protostome) and that fertilisation in this species results in an increased turnover of PIP\(_2\) (Bloom et al., 1988). Preliminary data exists then suggesting that the PPI 2\(^{nd}\) messenger system plays a role activating protostome eggs.

In conclusion then fertilisation may have two general features. Firstly it is almost certain that an increase in intracellular calcium is a general activating signal in all species. Secondly, and more speculatively, the PPI 2\(^{nd}\) messenger system might play a general role in generating this increase in \(\text{Ca}_1\). The precise mechanics of how the PPI messenger system causes this change varies depending upon whether it is a protostome or deuterostome species.
1.7 EXPERIMENTS PRESENTED IN THIS THESIS

The experiments presented in this thesis deal with the intracellular signalling processes in the sea urchin egg. Chapter 3 deals with the actions of inositol phosphates particularly their requirement for external calcium. Also in this chapter the activity of an analogue of Ins(1,4,5)P$_3$, Ins(1,4,5)$-S-P_3$, is tested and the nature of the calcium release it causes is compared with that of Ins(1,4,5)P$_3$.

Heparin, a reported in vitro inhibitor of Ins(1,4,5)P$_3$-induced calcium release, is used in Chapter 4. Its ability to inhibit the actions of Ins(1,4,5)P$_3$ in intact eggs and its effect on the process of fertilisation is tested. This compound is used to see what role Ins(1,4,5)P$_3$ might play in triggering the fertilisation calcium wave.

In Chapter 5 guanine nucleotide analogues are used to test the G-protein model of egg activation. GDP$\beta$S is used to try to inhibit the actions of sperm on sea urchin eggs. Also the activation of eggs by GTP$\gamma$S and sperm are compared to determine whether both use the same signal transduction pathway, an essential prediction of the G-protein model.

Finally Chapter 6 looks briefly at the role of phosphorylation in regulating egg activation. Using Adenosine-5'-O-thiotriphosphate (ATP$\gamma$S), an analogue of ATP which can be used by certain protein kinases to irreversibly phosphorylate their target proteins (Gratecos & Fischer, 1974), I look at what role phosphorylation might play both in the signalling processes but also the cellular changes that constitute egg activation.
Chapter 2

Materials and Methods

2.1 Gamete handling

Sea urchins of the species Lytechinus pictus (Pacific Biomarine Laboratories Inc., Venice, CA., USA and Marinus Inc., Long Beach, CA, USA) were held in thermostatted tanks at 15–16 °C in order to keep them continually gravid. They were fed fortnightly on seaweed (Marine Biological Station, Milport, Scotland).

Gametes were obtained by the intracoelomic injection of 0.5 M KCl. Eggs were collected by upending injected females in a beaker of artificial seawater (ASW), stored in a thermostatted bath at the experimental temperature (16 ± 1 °C) and used within 8 hours of shedding.

Eggs are shed from urchins in a jelly-like substance that protects the eggs and which was only removed immediately prior to egg use. The jelly was removed by multiple passage of the eggs through bolting silk (mesh size ~100 μm). The eggs were allowed to settle by gravity and the jelly containing sea water removed by suction. The eggs were resuspended in fresh ASW and the process repeated.

The viability of the eggs from each female was tested at the beginning and the end of experiments on the eggs by fertilising a sample of eggs. For all experiments reported here >90% of the eggs elevated complete fertilisation envelopes, that is a fertilisation envelope was visible around the whole egg circumference.

Sperm were collected in a minimal volume of ASW and stored at 4°C until use. In this form they retained their ability to fertilise eggs for
several days. Fertilisations were performed by diluting this concentrated sperm 10000 times which produced typical sperm concentrations of $0.5 - 1 \times 10^5$ sperm/ml (see Section 2.10).

2.2 Microinjection Techniques

For microinjection dejellied eggs were stuck down onto glass coverslips pretreated with poly(L-lysine) (0.02 mg/ml). Micropipettes were pulled from glass capillary tubes (1.5 mm i.d.; Clarke Electromedical, Pangbourne, U.K.) using a Palmer Bioscience Microelectrode Puller (Sheerness, U.K.). The micropipettes typically had a resistance of $5 - 10 \text{ Mohm}$ when filled with 3 M KCl, with a tip diameter such that a $10 - 15 \text{ ms}$ pulse of 450 kPa pressure ejected a volume of 1 pl. The microinjection pipettes were backfilled with the solution to be injected: a process facilitated by an internal glass filament in the capillaries that draws the solution to the pipette tip by capillarity. The micropipettes were held in a Clarke Electromedical EH-2MS electrode holder, which was in turn held in a micromanipulator (either a P0045, Prior Instruments, Cambridge, U.K. or an MO-103 hydraulic manipulator, Narishige Instruments, Japan).

Eggs were microinjected using a high pressure pulse system. An oxygen cylinder was used to provide a pressure head of between 350 and 1000 kPa. This was connected by means of narrow bore (1.5 mm i.d.) teflon tubing (Omnifit, Cambridge, U.K.) to the micropipette via a self venting electric valve (Radiospares, U.K.). The valve was driven by two 555 stimulators (Radiospares) connected in series, one driving the other at a frequency of 1Hz to produce pressure pulses of variable duration. This prevented contamination of the pipette contents during periods of
immersion in seawater.

The micropipette was manoeuvred to the egg surface by means of the micromanipulator and advanced along its axis indenting into the egg surface a distance of 20 – 30 μm. Entry into the egg was achieved by a light, but sharp tap on the micromanipulator. High pressure pulses continued to be delivered to the pipette until the required amount was injected, the pulses were then switched off and the pipette withdrawn from the egg. All microinjections were into the centre of the egg. Any eggs that appeared to damaged after injection (<5%) were rejected.

Microinjections were performed using standard brightfield microscopy on a Leitz Diavert (Leitz Instruments, Luton, U.K.) inverted microscope using a Leitz 40x, 0.65 NA objective and 10x eyepieces. The injected volume was controlled by varying the pulse duration and was estimated by measuring the cytoplasmic displacement of the injected fluid with an eyepiece graticule, calibrated using a stage micrometer.

2.3 Accuracy of the high pressure microinjection technique

To determine both the qualitative and quantitative accuracy of the microinjection technique used in this thesis I have used the fluorescent dye Lucifer Yellow, comparing the fluorescence intensity of drops of the dye with the fluorescence intensity of eggs injected with Lucifer Yellow. All fluorescence measurements were performed on a Leitz Diavert microscope as described for the measurement of pH, and the 450 nm bandpass filter used to provide the exciting light.

A stock solution of Lucifer Yellow (5mM in 0.5 M KCl, 50 mM PIPES, 50 mM EGTA, pH=6.8) was used for all the experiments. This
stock solution was microinjected directly into eggs as described in section 2.2 and the fluorescence intensity determined.

To prepare the drops the stock solution was diluted to give concentrations of 100 and 500 μM. These were mixed with paraffin oil in a 1:1000 ratio (by volume) and sonicated briefly (Ultrasonic Homogeniser 4710, Cole Palmer, Bishop’s Stortford, U.K.) to give droplets with a size range of 5 to 120 μm. These were observed microscopically by allowing them to settle through the paraffin oil onto coverslips that had been pretreated with dimethyldichlorosilane (2% in 1,1,1-trichloroethane). Dimethyldichlorosilane reduces the surface tension effects of water on glass and so prevents the drops from spreading onto the coverslips. The fluorescence intensity of the drops was determined and their diameter measured with an eyepiece graticule. The drops were assumed to be hemispheres for the purpose of determining volume.

Fig. 2.1 shows the results of these experiments. The graph contains two plots: the calculated quantity of Lucifer Yellow in the drops plotted against their fluorescence intensity and the estimated quantities of Lucifer Yellow injected into eggs plotted against their fluorescence intensity. The good linear relationship between the fluorescence signal and either the calculated quantity of dye in drops or estimated quantity of dye in eggs is illustrated by the correlation coefficients which are 0.96 and 0.98 respectively. For any single volume of Lucifer Yellow injected into eggs the SEM of the fluorescence was, on average, 13% of the mean fluorescence signal, giving a 97.5% confidence interval for a Student’s t-test (two-tailed) of +/- 30%. This suggests that the microinjection is reasonably accurate.

The slopes of the best-fit lines (constrained to pass through zero) for
Figure 2.1  Assessment of the microinjection technique

The graph shows the results from two different experiments. (A) O  The fluorescence intensity of drops of Lucifer Yellow dye are plotted against the calculated quantity of the dye in the drop. Each point is a single observation. (B) ▲ The fluorescence intensity of eggs injected with Lucifer Yellow is plotted against the estimated quantity of dye injected into the egg. Each point is a mean of 10 observations. The SEMs are smaller than the symbol size. The best-fit lines (constrained to pass through zero) for the two sets of data, calculated from a linear plot of the data, are also shown. The solid line is the best-fit for injected eggs and the dotted line the best-fit for the drops of dye.
the drops of dye and eggs are 830 and 320 counts/pmole of dye respectively. This indicates that there is a systematic under estimation of the quantity of dye injected into egg by a factor of 2.6 and provides some indication of the precision of the injection technique. The error is not corrected for in the experiments presented here.

2.4 Measurement of $C_{ai}$

$C_{ai}$ was measured using the calcium sensitive fluorescent dye fura2. The dye has the useful property that on binding calcium its excitation spectra maxima shift to shorter wavelengths (Gryniewicz et al., 1985). This means that at certain excitation wavelengths a change in calcium causes a reduction in fluorescence intensity while at other wavelengths an increase in fluorescence intensity results for the same change in calcium. The ratio of the fluorescence intensities at two such wavelengths provides an indication of the calcium concentration that is independent of factors that affect the fluorescence intensity at a single wavelength eg. dye concentration, cell movement, illuminating intensity. The dye is also relatively insensitive to changes in other biological ions notably $Mg^{2+}$ and $H^+$ (Gryniewicz et al., 1985). This makes it a particularly useful tool for measuring $C_{ai}$ in single cells.

The fura2 (pentapotassium salt; Molecular Probes, Junction City, OR, U.S.A.) was introduced into the eggs by microinjection (see Section 2.2) to give a cytoplasmic concentration of $10-100 \, \mu M$. All fluorescence measurements were performed on a Leitz Diavert inverted microscope with a Ploemopak epifluorescence attachment. Single eggs were alternately excited with 350 nm and 380 nm light from a 50 W high pressure mercury
vapour lamp (Technical Lamps, Slough, U.K.) by means of two interference bandpass filters (bandwidth 10.3 and 11.3 nm respectively; Ealing Optics, Watford, U.K.) fixed into a wheel arrangement driven by a stepper motor (Radiospares). Emitted light was passed through a 510 nm bandpass interference filter (bandwidth 8 nm; Ealing Optics, Watford, U.K.) and its intensity was measured using a photomultiplier tube (Thorn EMI, Middlesex, U.K.). Both the exciting and emitted light passed through the microscope objective (Leitz 40x, 0.65NA): the one was split from the other using a dichroic mirror (Leitz A2 filter block, 400 nm cut off).

Data was collected by and stored on either an Apple microcomputer or an IBM compatible PC. The computer subtracted the preloaded background signal, which was always less than 5% of the signal, from the fluorescence signals and calculated the ratio (350 nm signal/380 nm signal). The intensity data was on occasion either displayed on a chart recorder or stored on magnetic tape. The stepper motor was driven by the computer at a rate sufficient to provide 1 ratio measurement per second.

For some experiments a further dichroic mirror was introduced into the system. This had a cut off of 560 nm and was positioned to reflect 500 nm emitted fluorescent light to the photomultiplier tube and transmit longer wavelength light allowing the egg, which was illuminated with red light, to be observed throughout the experiment.

$Ca_i$ was calculated from the fluorescence ratio using the equation given by Grynkiewicz et al. (1985):

$$Ca_i = \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{F_0}{F_s} \times K_d$$
Where:

\[ R \] is the ratio observed in the egg

\[ R_{m\text{in}} \] is the ratio observed in zero calcium

\[ R_{\text{max}} \] is the ratio observed in saturating calcium

\[ F_0 \] is the fluorescence intensity at 380nm in zero calcium

\[ F_s \] is the fluorescence intensity at 380nm in saturating calcium

\[ K_d \] is the dissociation constant of the dye

The \( K_d \) was taken from Poenie et al. to be 0.774 \( \mu \text{M} \). The other constants in the equation were determined using the methods of Poenie et al. (1985). 100 \( \mu \text{M} \) fura2 was added to a buffer designed to mimic ooplasm (155 mM KCl, 25 mM NaCl, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH = 7.0) which contained either 10 mM Ethyleneglycol-bis-(\( \beta \)-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA) (zero calcium) or 1 mM CaCl\(_2\) (saturating calcium). 5 \( \mu \text{l} \) drops of each solution were placed on a coverslip and the intensities of fluorescence at 350 and 380nm were measured from which it was possible to calculate the remainder of the constants.

Poenie et al. (1986) have reported that the excitation spectra of fura2 in cytosol is not the same as the spectra in standard buffers. The difference takes the form of an enhanced fluorescence at longer wavelengths so the fura2 350:380 excitation ratio for a particular calcium concentration is reduced in cytosol as compared to a buffer. I have observed this effect while measuring \( \text{Ca}_i \) in sea urchin eggs where the excitation ratio observed in an egg was sometimes less than the \( R_{m\text{in}} \) value determined from the \textit{in vitro} calibration. Poenie et al. (1986) have suggested that this effect is due
to viscosity differences between the cytosol and calibration buffer and can be corrected for by applying a factor to reduce both $R_{\text{min}}$ and $R_{\text{max}}$, thus accounting for the enhanced 380nm fluorescence. Mathematically it can be shown that the difference between the fluorescence signals at a single wavelength for two calcium concentrations ratioed against the difference in fluorescence signals at another wavelength produces a constant for those particular wavelengths. This constant can be calculated for the calibration solutions and for the egg. The correction factor used by Poenie et al. (1986) is the fraction of these two constants. In the calcium measurements reported here I have applied a correction factor. This was typically 0.8 – 0.9 and similar in magnitude to the correction factor applied to sea urchins in other studies (Penie et al., 1986 and Alderton and Steinhardt, 1988).

The need to apply a correction factor calls into question how quantitatively accurate is the measurement of $C_{a_i}$ by fura2. The values measured with fura2 are of the same order of magnitude as those made with another indicator aequorin (Swann & Whitaker, 1986). However for the arguments presented here this is not relevant as the comparisons made are qualitative. Additionally where comparisons are made the experiments were made on the same batch of eggs using the same calibration values.

2.5 Measurement of $pH_i$

$pH_i$ was measured using the fluorescent dye BCECF. This dye has a similar property to fura2 except that instead of a change in calcium concentration it is a change in $pH$ causes a shift in the excitation spectra. The techniques used were basically the same as for measuring $C_{a_i}$ except that single eggs were excited with 450 and 490 nm light using bandpass
Figure 2.2  A BCECF calibration curve

The calibration curve is constructed as described in the text. Each point is the mean of 6 fluorescence measurements at each wavelength.
interference filters (bandwidth 6.5 and 7 nm respectively; Ealing Optics, Watford UK), emitted light was passed through a 530nm bandpass filter (bandwidth 8.5 nm) and the exciting and emitted light were split with a dichroic mirror (Leitz F filter block, 510nm cut off).

The calibration however was a little different. A glutamate/glycine medium was used that mimics the properties of egg together with a range of buffers (50 mM) covering a suitable pH range (see Section 2.10). These buffers were mixed with 10 μM BCECF, 5 μl aliquots of these solutions were placed on coverslips and the fluorescence intensities at the two excitation wavelengths measured, ratios were calculated and a calibration curve was drawn (see Fig. 2.2). pH$_i$ can be calculated using the following formula derived from that in Section 2.4:

$$\text{pH}_i = \text{pK}_a - \log_{10} \frac{(R - R_{min})}{(R_{max} - R)}$$

Where pK$_a$ is the logarithm of the apparent dissociation constant K$_a$ which is the real dissociation constant multiplied by F$_o$/F$_s$ (450 nm excitation). pK$_a$, R$_{min}$ and R$_{max}$ can be read from the graph (see Section 2.4). The ratios are F$_{490}$/F$_{450}$.

2.6 Observation of cortical granules

In assaying the extent of cortical reactions in some cases, rather than score fertilisation envelope elevation which is an indirect assay of exocytosis, the cortical granules were observed directly. The granules were observed using differential interference contrast microscopy with a Leitz 100x, 1.2NA oil immersion objective.
2.7 Fluorescence assay for sperm egg fusion

This assay was based directly on that described by Hinckley et al. (1986). Jellied eggs were incubated in 20 μM of the DNA staining vital fluorescent dye Hoechst 33342 for 20 minutes. The eggs were then dejellied as described above and washed 5 times with ASW to remove all traces of external dye. The eggs were then stuck down onto a coverslip with poly(L-lysine) and the appropriate experimental procedure performed and the eggs inseminated with sperm (0.5 – 1x10^5 sperm/ml). Only those sperm nuclei that had fused with the egg could stain with the dye and become fluorescent. The eggs were observed using epifluorescence microscopy on a Nikon Diaphot TMD inverted microscope with a 40x, 0.55 NA objective. A 100W high pressure mercury vapour lamp was used as the light source with a 340–380 nm bandpass excitation filter and 450nm longpass emission filter. The exciting and emitted light were split with a dichroic mirror (cut off 400 nm). Occasionally eggs were photographed using Kodak Recording film.

2.8 Electrophysiological techniques

Microelectrodes were pulled from glass capillary tubing (Clarke Electromedical, Pangbourne, U.K.) (o.d. 1 mm) with an internal glass filament on a David Kopf pipette puller (Tujunga, CA, U.S.A.). They were backfilled with 3 M KCl and had resistances of 30–80 Mohm. The electrodes were held in an electrode holder (Clarke Electromedical, Pangbourne, U.K.) which contained a Ag/AgCl pellet to make the solid/liquid junction. The bath electrode also contained a Ag/AgCl pellet and was filled with ASW.
Single electrode voltage recording was achieved using a preamplifier based on the design of Purves (1981). Voltages were measured with reference to the bath potential and zero was determined at the end of each experiment after removal of the electrode from the cell. Microelectrode resistances were determined by passing current pulses of 1 nA through the electrode and compensating the subsequent p.d. changes with a bridge balance.

Egg penetration was achieved by the momentary application of negative capacitance just sufficient to oscillate the preamplifier. As reported in other studies the membrane potentials of unfertilised eggs fell into two populations, the majority of eggs (>90%) had a resting potential of the order of $-10$ mV while the smaller population had a potential of $-70$ to $-80$ mV (Chambers & De Armendi, 1979 and Hagiwara & Jaffe, 1979). The true resting potential of an unfertilised egg is believed to be $-70$ to $-80$ mV and the low resting potential the result of an electrode leak artefact. The high input resistance of the egg and the negative slope conductance of the egg means that only a small leakage current is required to result in a drastic shift of the membrane potential from $-70$ mV to $-10$ mV (Hagiwara & Jaffe, 1979).

In agreement with Chambers and de Armendi (1979) it was possible by injecting hyperpolarising current to cause some of the eggs to spontaneously hyperpolarise so that they required the injection of only a small amount of hyperpolarising current to maintain the membrane potential at $-70$ mV. In this thesis only eggs which required $<250$ pA of hyperpolarising current to maintain a potential of $-70$ to $-80$ mV were used.
Once a steady resting potential had been established fura2 was introduced into the egg by microinjection as described above. The insertion of a microinjection pipette caused depolarisation of the egg membrane, however this always recovered on removal of the microinjection pipette without the need to increase the amount of hyperpolarising current.

2.9 Determination of sperm densities

The density of sperm was sometimes determined with a haemocytometer (B.D.H., Dagenham, U.K.). Sperm were diluted 20 fold in distilled water which immobilised the sperm and a drop was applied to the counting chamber. The number of sperm in 40 squares of the chamber was determined. This corresponds to 0.01 µl and the sperm density could then be calculated.

2.10 Solutions

**Artificial Seawater (ASW):** 435 mM NaCl, 40 mM MgCl₂, 15 mM MgSO₄, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 1 mM Ethylenediamine tetracetic acid (EDTA), pH 8.0.

The osmolarity was measured on a vapour pressure osmometer (Wescor Inc., Logan, UT, U.S.A.) and then adjusted to 950 ± 20 mosmoles with either water or NaCl.

**Calcium-free ASW:** 445 mM NaCl, 50 mM MgCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 2 mM EGTA, 1 mM EDTA, pH 8.0.

The osmolarity were adjusted as above. The calcium-free ASW was calculated to have a free calcium ion concentration of less than 100 nM. To make this calculation the manufacturers data on contaminant ions were
used with the stability constants of Martell & Smith (1974) for EGTA. This calculation was confirmed using a calcium-sensitive electrode (World Precision Instruments, New Haven, CT, USA).

**pH calibration buffer:** 220 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 2.5 mM MgCl₂. Several biological buffers were used at a concentration of 50 mM that could cover a range of pH's. Up to pH 5 potassium aspartate; pH 5.5 and 6, 2(Ν-morpholino)ethanesulphonic acid; pH 6.5 and 7.0, piperazine-Ν,Ν-bis(2eth-ane-sulphonic acid); pH 7.5 and 8.0, N-2-hydroxyethylpiperazine-Ν'-2-ethanesulphonic acid; pH 9, N-tris(hydroxymethyl)methyl-3-amino-propanesulphonic acid; pH 10 tris(hydroxymethyl)aminomethane.

**Microinjection buffers.** Two different microinjection buffers were used. The first buffer was used for all microinjection experiments except for the microinjection of GDP-β-S for which the second was used.

Buffer 1: 500 mM KCl, 20 mM PIPES, 100 μM EGTA, pH 6.7.
Buffer 2: 100 mM KAspartate, 50 mM PIPES, 100 μM EGTA, pH 6.7.

### 2.11 Materials

The EGTA, EDTA, biological buffers, Lucifer Yellow dye, heparins were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). The nonhydrolysable nucleotides were from BCL (Lewes, E. Sussex, U.K.). Other reagents were obtained from BDH (Dagenham, Essex, U.K.).

The inositol phosphates were a gift from R.F. Irvine except the nonhydrolysable analogue of Ins(1,4,5)P₃ which was a gift from B.V.L. Potter.
Chapter 3

Actions of inositol phosphates in sea urchin eggs

3.1 Introduction

The question of whether fertilisation in the sea urchin requires external calcium has been controversial (Takahashi & Sugiyama, 1973; Sano & Kanatami, 1980; Chambers, 1980 and Schmidt et al., 1982). Disagreement has centred around the level of calcium in the calcium-free ASW used in the various studies. However the most recent and careful study by Schmidt et al. (1982), in which the calcium chelator EGTA was used in the calcium-free ASW, shows that fertilisation does not require external calcium. Reports then, that Ins(1,4,5)P$_3$ and its phosphorylated metabolite Ins(1,3,4,5)P$_3$ activate eggs by a process dependent upon external calcium (Irvine & Moor, 1986 and 1987) throws doubt on the idea that inositol phosphates are the internal messengers responsible for the increase in $Ca^+$ at fertilisation. This chapter describes experiments that investigate the requirement of fertilisation and inositol phosphate-induced egg activation for external calcium.

In the final part of the chapter I shall describe some experiments using a thio analogue of Ins(1,4,5)P$_3$, inositol 1,4,5-trisphosphorothioate [Ins(1,4,5)S-P$_3$] (Cooke et al., 1987). This synthetic isomer of Ins(1,4,5)P$_3$ is resistant to inactivation by either phosphorylation or dephosphorylation (Taylor et al., 1989). I have used this compound to look at the effects of a raised tonic level of calcium mobilising internal messenger on $Ca_i$. 

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3.2 Calcium changes at fertilisation in the absence of external calcium

To determine whether the presence of external calcium might be important at fertilisation I have measured the calcium changes in single eggs at fertilisation, using the calcium sensitive fluorescent dye fura2, in the presence and absence of external calcium. Mean calcium changes at fertilisation in the presence and absence of external calcium are shown in Fig. 3.1. At fertilisation the Ca$_i$ increases rapidly to a mean value of 2.6µM. Removing external calcium did not significantly affect this increase. Ca$_i$ then declines over a period of 10 minutes and this process is affected by the removal of external calcium. Three minutes after fertilisation the Ca$_i$ of eggs in normal ASW is significantly greater than that of eggs in calcium-free ASW and this difference is maintained for the duration of the transient (p<0.02, one tailed unpaired students t-test). While the calcium changes at fertilisation show some dependence on external calcium it is not clear that this is the result of calcium influx. Removal of external calcium might, by reducing the electrochemical gradient that opposes calcium efflux, facilitate the actions of plasma membrane calcium pumps allowing them to reduce Ca$_i$ more effectively and resulting in the same effect. Whatever the cause of the calcium dependence it does not seem to be functionally important; eggs fertilised in calcium-free ASW reached the stage of 16 cell embryos and may well have developed further. A similar observation has been made by other workers (Chambers, 1980 and Schmidt et al., 1982) who also found that eggs fertilised in the absence of external calcium underwent many cell divisions.
Figure 3.1  The changes in Ca\textsubscript{i} at fertilisation in normal and calcium-free ASW

Ca\textsubscript{i} was measured in single eggs microinjected with fura2 to a final cytoplasmic concentration of 10–30\mu M. The closed symbols represent fertilisations in normal ASW and the open symbols fertilisations in calcium-free ASW (2 mM EGTA). Zero time was taken as the first detectable change in Ca\textsubscript{i}. Each point is the average Ca\textsubscript{i} from 3 experiments and the bars represent S.E.M.s where these are greater than the symbol size. Ca\textsubscript{i} of eggs in normal and calcium-free ASW were compared at each time point with a Students one-tailed unpaired t-test. All points to the right of the * are significantly different p<0.02. The eggs were fertilised with acrosome reacted sperm using a method based on that of Schmidt et al. (1982). They were acrosome reacted with egg jelly, 10 \mu l of sperm were added to 100 \mu l of jelly containing ASW and 5 sec later 0.5 ml of calcium-free ASW (10 mM EGTA, pH=8.9) in order to compensate for the acidification resulting from the chelation of calcium by EGTA) was added. 50 \mu l of this mixture was used immediately to fertilise the eggs.
3.3 Inositol phosphates and cortical granule exocytosis

I microinjected \( \text{Ins}(1,4,5)P_3 \), inositol 2,4,5-trisphosphate, \([\text{Ins}(2,4,5)P_3]\), inositol 1,2-cyclic 4,5-trisphosphate \([\text{Ins}(1,2\text{cyc}4,5)P_3]\), inositol 1,3,4-trisphosphate \([\text{Ins}(1,3,4)P_3]\), and inositol 1,3,4,5-tetrakisphosphate \([\text{Ins}(1,3,4,5)P_4]\) to test their effects on cortical granule exocytosis and fertilisation envelope (FE) elevation. These compounds all stimulated cortical granule exocytosis and FE elevation both in the presence and absence of external calcium. This is illustrated in Fig. 3.2 which shows two eggs injected with \( \text{Ins}(1,4,5)P_3 \), one in ordinary ASW and the other in calcium-free ASW. Both have elevated FEs but, the FE in calcium-free ASW is less refractile and more difficult to see. Using differential interference contrast microscopy it is possible to observe the cortical granules (Fig. 3.3A) directly and show that \( \text{Ins}(1,4,5)P_3 \) stimulates complete cortical granule exocytosis both in the presence (Fig. 3.3B) and absence (Fig. 3.3C) of external calcium. The tenuous FEs, then, are a consequence of impaired FE elaboration which has been shown to require external calcium (Monroy, 1949 and Schon & Decker, 1981) in order for thickening and hardening of the envelope to occur. It is not an effect on the process of exocytosis. So direct observation of the cortical granules was used to assay inositol phosphate activity in this study as it gave a more reliable indication of exocytosis than scoring for the elevation of a fertilisation envelope, which was not always easily seen.

In experiments to determine the sensitivity of eggs to inositol phosphates I injected a constant volume of inositol phosphate (1.5 pl, 0.3% egg volume) and varied the pipette concentration. This approach was adopted for two reasons. Firstly the activation of sea urchin eggs by
Figure 3.2  Ins(1,4,5)P₃ induced FE elevation in normal and calcium-free ASW

Two eggs microinjected with Ins(1,4,5)P₃ (0.3% egg volume, 10 μM pipette concentration). On the left is an egg in normal ASW; on the right an egg in calcium-free ASW. Both have elevated fertilisation envelopes, but the envelope of egg in calcium-free ASW is much less distinct.
Ins(1,4,5)P₃ involves triggering a self-propagating mechanism (Whitaker & Irvine, 1984) that resides within the egg: the sensitivity of the eggs is probably a function of the pipette concentration triggering activation, as well as the total dose injected. Secondly I was concerned about possible damage to eggs caused by microinjecting larger volumes, particularly in calcium-free ASW (see Section 3.5).

The results of microinjecting a number of inositol phosphates into *Lytechinus pictus* eggs are shown in Fig. 3.4. In ASW the most active inositol phosphate was Ins(1,4,5)P₃, half maximal activation occurring at an Ins(1,4,5)P₃ concentration of 2.5μM. Other inositol phosphates also caused cortical granule exocytosis. Ins(1,2cyc4,5)P₃, Ins(2,4,5)P₃ and Ins(1,3,4,5)P₄ activated eggs at half maximal concentrations of 19, 56 and 390μM respectively. I also tested Ins(1,3,4)P₃. It showed little activity: a pipette concentration of 1mM caused only 20% of the eggs injected to undergo complete cortical granule exocytosis. Microinjection of vehicle alone caused no signs of exocytosis (n=28).

Fig. 3.4 also shows that removing external calcium did not affect the sensitivity of cortical granule exocytosis to any of the inositol phosphates tested. These results are very different from those reported previously where an absolute dependence on external calcium was observed. It seems unlikely that a species difference can account for these effects because we have repeated these experiments using eggs from *L. Variegatus* and obtained similar results (Crossley et al., 1988).

3.4 The effect of microinjecting combinations of inositol phosphates

A synergism between inositol trisphosphates and Ins(1,3,4,5)P₄ has
Figure 3.3 Direct observation of the cortical granules in $\text{Ins}(1,4,5)P_3$ injected eggs

Eggs in normal and calcium-free ASW were injected with $\text{Ins}(1,4,5)P_3$ and the cortical granules observed using differential interference contrast microscopy. (A) An egg in normal ASW injected with $\text{Ins}(1,4,5)P_3$ (pipette concentration 0.5 $\mu$M, 0.3% egg volume). This did not stimulate exocytosis. Large numbers of cortical granules, appearing as 1 $\mu$m spherical structures, are visible. (B) An egg in normal ASW injected with $\text{Ins}(1,4,5)P_3$ (pipette concentration 10 $\mu$M, 0.3% egg volume). This stimulated complete exocytosis, only a small number of isolated cortical granules are visible. The egg has elevated an FE. (C) An egg in calcium-free ASW injected with $\text{Ins}(1,4,5)P_3$ (pipette concentration 10$\mu$M, 0.3% egg volume). This caused complete exocytosis, no cortical granules are visible. The egg has elevated an FE. The white semi-circle is a photographic artifact. Scale bar is 10 $\mu$m.
been reported both in sea urchin eggs, and in other cells (Irvine & Moor, 1986, 1987; Morris et al., 1988). I have attempted to reproduce this synergism in *L. pictus* eggs. I co-injected Ins(1,3,4,5)P₄ with either Ins(1,4,5)P₃ or Ins(2,4,5)P₃, choosing concentrations of trisphosphate that are close to the bottom of the dose response curve in Fig. 3.4. The

<table>
<thead>
<tr>
<th>Inositol Phosphates injected</th>
<th>% of eggs elevating</th>
<th>Nº eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No fertilisation</td>
<td>Partial fertilisation</td>
</tr>
<tr>
<td></td>
<td>eggs</td>
<td>envelopes</td>
</tr>
<tr>
<td>Ins(1,4,5)P₃</td>
<td>1μM</td>
<td>62%</td>
</tr>
<tr>
<td>Ins(1,4,5)P₃ with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ins(1,3,4,5)P₄</td>
<td>10μM</td>
<td>58%</td>
</tr>
<tr>
<td>Ins(2,4,5)P₃</td>
<td>10μM</td>
<td>41%</td>
</tr>
<tr>
<td>Ins(2,4,5)P₃ with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ins(1,3,4,5)P₄</td>
<td>10μM</td>
<td>76%</td>
</tr>
</tbody>
</table>

Eggs were microinjected with the inositol phosphates at the pipette concentrations shown (0.3% egg volume) and the degree of fertilisation envelope elevation scored. The data from the experiments using Ins(2,4,5)P₃ is pooled from 3 experiments: no single experiment showed any signs of a synergism.

concentration of Ins(1,3,4,5)P₄ used was 10μM. While this is 20x less than the lowest dose of Ins(1,3,4,5)P₄ that produced activation it is 7 times that used in Irvine and Moor's study (1986). The results are summarised in Table 3.1. The inositol trisphosphates injected alone caused some degree of cortical granule exocytosis and this degree of activation was not substantially increased by the coinjection of 10μM Ins(1,3,4,5)P₄. In no case
Figure 3.4  The ability of a number of inositol phosphates to stimulate cortical granule exocytosis

Eggs were injected with a constant volume of inositol phosphate (0.3% egg volume) and the pipette concentration was varied. The eggs were scored for cortical granule exocytosis by using differential interference contrast microscopy. Ins(1,4,5)P$_3$, Ins(1,2cyc4,5)P$_3$, Ins(2,4,5)P$_3$, Ins(1,3,4,5)P$_4$. Closed symbols refer to injections performed in normal ASW and open symbols refer to injections performed in calcium-free ASW. Each point represents the injection of between 10 and 15 eggs.
was evidence found for the dramatic synergism reported by Irvine & Moor (1986, 1987) who found that 0.1 μM Ins(1,3,4,5)P$_4$ lowered the activation of Ins(2,4,5)P$_3$ 10–50 fold.

3.5 Measurement of Ca$_i$ after the microinjection of Ins(1,3,4,5)P$_4$

Exocytosis is not a strictly quantitative measure of Ca$_i$. So the observation that inositol phosphate–induced cortical granule exocytosis is independent of external calcium ions does not exclude the possibility that inositol phosphates cause a calcium influx that affects the duration and magnitude of the inositol phosphate induced increase in Ca$_i$. I have therefore measured the changes in Ca$_i$ following the microinjection of Ins(1,3,4,5)P$_4$. The results of these experiments are shown in Fig. 3.5. The microinjection of 1.5 pl (0.3% egg vol) of Ins(1,3,4,5)P$_4$ with a pipette concentration of 1mM caused a transient increase in Ca$_i$ to a peak of about 2 μM and duration of 110 seconds. Removal of external calcium did not significantly affect either the magnitude or duration of the calcium transient. This is illustrated in Fig. 3.5. The two calcium transients are almost indistinguishable.

3.6 Damage to eggs microinjected in calcium–free ASW

As these results are so different from those previously reported (Irvine & Moor, 1986 and 1987) attempts were made to reproduce these methods as closely as possible. I attempted to use a low pressure microinjection system and larger injection volumes (2–4%). However this method produced greater visible damage particularly in calcium–free ASW and was not a satisfactory method of injection. Instead I tested the idea
Figure 3.5 Changes in Ca\textsubscript{i} caused by the microinjection of Ins(1,3,4,5)P\textsubscript{4}

Ins(1,3,4,5)P\textsubscript{4} induced changes in Ca\textsubscript{i} were measured with fura2 (20 \( \mu \)M) in single eggs. Ins (1,3,4,5)P\textsubscript{4} was microinjected at \( t = 0 \) (pipette concentration 1 mM, 0.3% egg volume). The two traces each represent averaged calcium measurements from 5 eggs. The dotted line refers to injections performed in normal ASW and the solid line to injections performed in calcium-free ASW (2 mM EGTA).
that the injection of large volumes damaged eggs in calcium-free ASW using the high pressure microinjection system. Eggs were microinjected with either 1.5pl or 20pl (0.3% and 4% egg volume) of 10 µM Ins(1,4,5)P₃ and scored for cortical granule exocytosis. The microinjection of 1.5pl activated 85% of eggs (n=33) while the injection of 20pl activated only 39% (n=38). Thus the microinjection of larger volumes of the same concentration of Ins(1,4,5)P₃ caused significantly less activation (p<0.005). The microinjection of these larger quantities into eggs in ordinary ASW did not result in a reduction in egg activation.

To try and get an estimate of the leakiness of the eggs after microinjection I loaded eggs with a fluorescent dye and monitored the loss of the dye from the eggs after microinjection. Dye leakage was not detectable from eggs microinjected in ordinary ASW with either small (0.3% egg volume) or large volumes (2.4% egg volume). However in calcium free ASW the microinjection of large volumes caused a more rapid loss of dye from eggs so that 2 minutes after injection the fluorescence signal was significantly reduced (p<0.02 one-tailed t-test) in eggs injected with a large volume (14 +/- 1.3% of the dye signal; mean +/- S.D., n=3) as opposed to eggs injected with a small volume (2.7 +/- 2.7% dye signal; mean +/- S.D., n=3).

3.7 Parthenogenetic activation of eggs by Ins(1,4,5)P₃

Ins(1,4,5)P₃ is a potent parthenogenetic activator of sea urchin eggs causing an increase in pHᵢ as well as an increase in Caᵢ (Whitaker & Irvine, 1984). I was interested to see whether Ins(1,4,5)P₃ released eggs from their state of cell cycle arrest. 12 eggs were injected with Ins(1,4,5)P₃
(10 µM pipette concentration, 0.3% egg volume) and scored for nuclear envelope breakdown. All the eggs injected underwent one cycle of nuclear envelope breakdown and 50% of the eggs underwent 2 cycles of nuclear envelope breakdown and reformation before cytolyzing. These cycles of nuclear envelope breakdown were slower than those of fertilised eggs. They took 2–3 hours to undergo one cycle of nuclear envelope breakdown and reformation instead of the usual 60–90 minutes of fertilised eggs.

3.8 The actions of inositol 1,4,5-trisphosphorothioate

The maintenance and adjustment of Ca, is affected by many factors. One important factor is the level of calcium-mobilising second messengers. Recently a phosphorothioate analogue of Ins(1,4,5)P₃, inositol 1,4,5-trisphosphorothioate (Ins 1,4,5-S-P₃), has been synthesised. It is resistant to inactivation by either phosphorylation or dephosphorylation (Taylor et al., 1989). I have used this analogue to investigate the effect of a raised and tonic level of a calcium mobilising second messenger on Ca,.

Firstly, I assayed the activity of this analogue by testing its ability to stimulate fertilisation envelope elevation (see Fig. 3.6). The microinjection of Ins 1,4,5-S-P₃ caused F.E. elevation in 100% of eggs with a pipette concentration of 100µM, thus making it an order of magnitude less effective than the biological isomer Ins(1,4,5)P₃ (see Section 3.2). This is similar activity to that observed in other cells (Taylor et al., 1989).

I used the calcium indicator dye fura2 to investigate the effect of a tonic level of a calcium mobilising second messenger on Ca,. The microinjection of Ins(1,4,5)P₃ at a pipette concentration of 100µM (0.8% egg volume) caused a large increase in Ca, with a peak of 2.42 ± 0.34
Figure 3.6  The ability of Ins(1,4,5)–S–P₃ to cause FE elevation

Eggs were microinjected with a constant volume (0.3% egg volume) of Ins(1,4,5)–S–P₃ and the pipette concentration varied. The eggs were scored for the degree of fertilisation envelope elevation. Cross-hatched bars show that proportion of eggs elevating an envelope around the complete egg circumference, open bars that proportion of eggs elevating an envelope the whole or part of the egg circumference. The filled bar indicates that no envelope elevation was detectable. Numbers above each bar show the number of eggs injected with that pipette concentration.
μM (mean +/- S.D., n = 5) which is similar to values previously reported (Swann and Whitaker, 1986) (Fig 3.7A). A second injection of the same quantity of Ins(1,4,5)P₃ caused an increase in Caᵢ that had a peak of only 1.33 +/- 0.195 μM (mean +/- S.D., n = 5). This was significantly smaller than the change caused by the first injection (p < 0.01; one-tailed, paired Students t-test). A third and fourth injection of Ins(1,4,5)P₃ caused calcium transients that were indistinguishable in both magnitude and duration from the second injection calcium transients.

The microinjection of a quantity of Ins (1,4,5)-S-P₃ (pipette concentration of 500 μM, 0.8% egg volume) with a similar activity as the Ins(1,4,5)P₃ used above caused a transient of similar magnitude and duration (Fig. 3.7B). Subsequent injections of Ins 1,4,5-S-P₃ though, resulted in much smaller changes in Caᵢ than seen with Ins(1,4,5)P₃. The second injection of Ins 1,4,5-S-P₃ caused a transient with a peak of only 0.68 +/- 0.25 μM (mean +/- S.D., n = 5) while the third only a transient with a peak of 0.43 +/- 0.07 μM. These values are significantly smaller than the calcium changes caused by the second and third injections of Ins(1,4,5)P₃ (p < 0.01 and p < 0.001 respectively; one tailed, unpaired students t-test). There are several possible explanations for this attenuation of the calcium response to Ins(1,4,5)-S-P₃. Firstly the continued occupation of the receptor might prevent refilling of the calcium store. Alternatively the presence of a tonic raised level of the messenger might desensitise the Ins(1,4,5)P₃ receptor which would allow the store to refill but prevent further inositol phosphate-induced calcium release. To try and distinguish between these possibilities the eggs were exposed to the calcium ionophore Br-A23187 and the change in Caᵢ measured to provide an indication of
Figure 3.7 A comparison of calcium changes caused by Ins(1,4,5)P₃ and Ins(1,4,5) - S - P₃

Caᵢ was measured in single eggs with fura2 following the microinjection of either Ins(1,4,5)P₃ (pipette concentration 100 μM, 0.8% egg volume) (Panel A) or Ins(1,4,5) - S - P₃ (pipette concentration 500 μM, 0.8% egg volume) (Panel B) at the times indicated by the open arrowheads. Calcium-free ASW replaced the normal ASW for the time indicated by the labelled line. Br-A23187 (50 μM in calcium-free ASW) was perfused onto the egg from a pipette positioned 10 μm away from the egg for the period indicated by the other labelled line. Both panels are representative records from a total of 5 experiments.
the size of the intracellular calcium stores. Br–A23187 is a non-
fluorescent analogue of the better-known ionophore A23187 which releases
calcium from intracellular stores in the sea urchin (Steinhardt & Epel, 1974).
A23187 was unsuitable for use in these experiments, however, because it is
highly fluorescent and interferes with the measurement of Ca$_i$ with fura2.
To eliminate the problem of ionophore–induced calcium entry from
outside the egg this part of the experiment was performed in calcium–free
ASW.

In eggs that had been microinjected with Ins(1,4,5)P$_3$ the application
of Br–A23187 caused an increase in Ca$_i$ that had a mean value of
360 $\pm$ 168 nM (mean $\pm$ S.D., n=4). This is much smaller than the
change in Ca$_i$ caused by the injection of Ins(145)P$_3$. This difference in the
magnitude of the response could be due to the difference in the time
courses of the two responses. On the one hand, the microinjection of
Ins(1,4,5)P$_3$ causes an increase in Ca$_i$ that peaks within 3 seconds, while the
calcium increase caused by ionophore takes about 2 minutes to peak. The
much slower action of ionophore will mean that sequestering mechanisms
that reduce Ca$_i$ can operate and reduce the apparent size of the calcium
increase. In the eggs that had been microinjected with Ins(1,4,5)–S–P$_3$ the
application of Br–A23187 caused an increase in Ca$_i$ that was smaller, only
143 $\pm$ 82 nM (mean $\pm$ S.D., n=5). However this change was even
slower than that caused by Br–A23187 in eggs injected with Ins(1,4,5)P$_3$
and given the reasons outlined above it is difficult to compare these
responses directly. It is however possible to compare directly the initial rate
of rise of Ca$_i$. This is a parameter that will give some indication of the
fullness of the store. The rate of increase in Ins(1,4,5)P$_3$ injected eggs was
482 +/− 237 nM min⁻¹ (mean +/− S.D., n=4) while for eggs injected with Ins(1,4,5)–S–P₃ the rate was only 30 +/− 18 nM min⁻¹. The size of the ionophore releasable calcium store in Ins(1,4,5)–S–P₃ injected eggs is reduced. This suggests that the loss of responsiveness of eggs to Ins(1,4,5)P₃ cannot be explained in terms of a receptor desensitisation model and that the loss of responsiveness is due to prevention of the store refilling by continued receptor occupation.

3.9 Summary

1. Fertilisation does not require external calcium and, although the fertilisation calcium transient has a partial dependence on external calcium this is not functionally important.

2. FE in calcium–free ASW are less refractile and easily seen than those in ordinary ASW. They are not a reliable assay of egg activation in calcium–free ASW.

3. Egg activation stimulated by inositol phosphates, injected either alone or in combination, does not require external calcium.

4. The order of potency of inositol phosphates is Ins(1,4,5)P₃ > Ins(1,2cyc4,5)P₃ > Ins(2,4,5)P₃ > Ins(1,3,4,5)P₄ > Ins(1,3,4)P₃.

5. The calcium changes caused by Ins(1,3,4,5)P₄ have no detectable dependence on external calcium.

6. The microinjection of Ins(1,4,5)P₃ releases egg from their state of cell cycle arrest.

7. Ins(1,4,5)–S–P₃ activates sea urchin eggs, being an order of magnitude less effective than Ins(1,4,5)P₃.

8. Eggs become refractory to stimulation by Ins(1,4,5)–S–P₃ but not Ins(1,4,5)P₃. The refractoriness is probably due to the fullness of the Ins(1,4,5)P₃–sensitive calcium store.
Chapter 4

The actions of heparin in sea urchin eggs

4.1 Introduction

The increase in Ca\textsubscript{i} at fertilisation is thought to be the result of Ins\textsubscript{(1,4,5)}P\textsubscript{3} production (Turner et al., 1984; Whitaker & Irvine, 1984; Ciapa & Whitaker, 1985 and Swann & Whitaker, 1986). A scheme explaining this process (Swann & Whitaker, 1986) suggests that the sperm is a trigger, activating an autocatalytic response that resides within the egg and has as a key component a calcium-sensitive phosphoinositidase C (PIC) (Whitaker & Aitchison, 1985). Stimulation of this enzyme causes the production of Ins\textsubscript{(1,4,5)}P\textsubscript{3}, leading to the release of calcium and resulting in further stimulation of the enzyme (Swann & Whitaker, 1986).

Recently it has been found that the mucopolysaccharide, heparin inhibits the ability of Ins\textsubscript{(1,4,5)}P\textsubscript{3} to release calcium from an intracellular store (Hill et al., 1987; Cullen et al., 1988; Guillemette et al., 1989 and Joseph & Rice, 1989). In this chapter I shall describe some experiments using this compound in intact eggs. The first part of the chapter looks at the ability of heparin to inhibit Ins\textsubscript{(1,4,5)}P\textsubscript{3}–induced calcium release. The second part of the chapter looks at the effects of heparin on fertilisation, particularly the calcium changes that occur and their relationship to other early events of egg activation such as the egg plasma membrane conductance changes. Heparin is also used to test the idea that it is the production of Ins\textsubscript{(1,4,5)}P\textsubscript{3} that is responsible for the wave of calcium release that sweeps across the egg at fertilisation.
Figure 4.1 The actions of heparin on the process of Ins(1,4,5)P₃ induced calcium release

Caᵢ was measured in single eggs with fura2 microinjected to a final concentration of 10–20 μM. Each egg was microinjected at the time indicated by the arrow heads with the same quantity of Ins(1,4,5)P₃, a pipette concentration of 20 μM and 0.3% egg volume. (A) Control experiment, egg elevated a complete FE. (B) An egg in which heparin was co-injected with the fura2 to a final concentration of 300μg/ml; the egg did not elevate an FE. (C) An egg in which De-N-sulphated heparin was co-injected with fura2 to a final concentration of 300 μg/ml; the egg elevated a complete FE. Each panel is representative of 3 experiments.
4.2 Heparin inhibits Ins(1,4,5)P₃ – induced calcium release

I looked at the actions of heparin by measuring Ins(1,4,5)P₃ – induced changes in Caᵢ in eggs pre-injected with heparin. The microinjection of heparin in the standard microinjection vehicle (see section 2.10) caused a large number of eggs to activate (55%, n=11) and the injectate tended to form vesicles within the egg, which suggests a high degree of calcium contamination (Swann & Whitaker, 1986). Including 2 mM EGTA in the injection vehicle reduced activation to less than 10%, but had no effect on the ability of Ins(1,4,5)P₃ or sperm to activate eggs.

Fig. 4.1 illustrates that heparin inhibited the ability of Ins(1,4,5)P₃ to mobilise calcium. Microinjection of twice the quantity of Ins(1,4,5)P₃ normally required to completely activate eggs caused a large increase in Caᵢ in a control egg, to a peak of 1.8 μM, which declined over a period of 2 minutes. The microinjection of this quantity of Ins(1,4,5)P₃ into an egg that had been pre-injected with heparin to a final concentration of 300 μg/ml failed to cause any increase in Caᵢ. It has been suggested that the inhibitory action of heparin is dependent on its sulphation (Worley et al., 1987 and Hill et al., 1987). To test this idea I used the compound De-N-sulphated heparin. This compound when used at a similar concentration as heparin had no effect on the Ins(1,4,5)P₃ response (see Fig. 4.1C).

To quantify the degree of inhibition, dose response curves were constructed, plotting the peak calcium response against the pipette concentration of Ins(1,4,5)P₃. Fig. 4.2 shows that the microinjection of Ins(1,4,5)P₃ into control eggs caused a dose-dependent increase in Caᵢ to a peak value of 2–2.5 μM at an Ins(1,4,5)P₃ pipette concentration of 10μM. 50% response was obtained with a pipette concentration of 3 μM which is
Figure 4.2 The ability of heparin to inhibit Ins(1,4,5)P₃-induced calcium release

Ca₄ was measured in single eggs with fura2 which was co-injected with either heparin to a final concentration of 250–350 µg/ml (△) or a similar volume of injection vehicle (○) following the microinjection of Ins(1,4,5)P₃ (0.3% egg volume). The peak of the change in Ca₄ was plotted against the pipette concentration of Ins(1,4,5)P₃. The bars show S.E.M. and the figures against each symbol the number of eggs injected.
very similar to the dose response curve when cortical granule exocytosis was scored. However, in eggs that had been microinjected with heparin to a final concentration of 250–350 μg/ml, the curve is shifted to the right. In other words, in eggs that had been microinjected with heparin, it was possible to overcome the block by increasing the amount of Ins(1,4,5)P₃ injected. A 50% response now required a pipette concentration of 60 μM. The slope of the dose–response curve and the obtainable is the same in heparin–injected as in control eggs. This indicates that heparin is a competitive inhibitor of calcium release.

4.3 Heparin does not inhibit the fertilisation calcium transient.

I have tested the actions of heparin on the calcium transient at fertilisation. Averaged fertilisation calcium transients of heparin–injected and buffer–injected eggs are shown in Fig. 4.3. Surprisingly, since Ins(1,4,5)P₃ is thought to play a key role in the generation of the fertilisation calcium transient, the transients are essentially indistinguishable. Heparin had no effect on either the magnitude or duration of the calcium transient.

4.4 The actions of heparin on FE elevation.

The inhibition of Ins(1,4,5)P₃–induced calcium release was not the only action of heparin in sea urchin eggs. Despite the fact that fertilisation of heparin injected eggs always resulted in a calcium transient that was similar to that at fertilisation, a normal FE did not always elevate. In many cases the FE only elevated around a part of the egg. This effect was not related to the dose of heparin as illustrated in Table 4.1.
The calcium changes at fertilisation were measured with fura2 co-injected with either heparin (250–350 μg/ml) (solid line) or a similar volume of injection vehicle (dotted line). The traces are averages of 6 and 5 experiments respectively. Individual traces were standardised temporally by reference to the transient peaks. Using a Student’s t-test on the two traces showed that they were not significantly different at any point.
Increasing the concentration of heparin injected did not affect the percentage of eggs elevating envelopes. If anything, the degree of envelope elevation increased with increasing heparin concentration. The mechanism of this inhibition is not clear although it might be related to heparin's ability to inhibit certain phosphatases (Gergely et al., 1984).

Table 4.1

<table>
<thead>
<tr>
<th>Quantity of heparin injected</th>
<th>% eggs elevating fertilisation envelopes</th>
<th>N° eggs injected</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Partial</td>
</tr>
<tr>
<td>50 – 150μg/ml</td>
<td>0%</td>
<td>70%</td>
</tr>
<tr>
<td>200 – 500μg/ml</td>
<td>5%</td>
<td>55%</td>
</tr>
</tbody>
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It has been suggested in some cells that exocytosis involves a dephosphorylation reaction and preliminary evidence is presented in this thesis to suggest that this is the case in sea urchin eggs also (see Chapter 6). Heparin might inhibit exocytosis by partially inhibiting this dephosphorylation reaction.

4.5 The effect of heparin on sperm/egg fusion

In Section 4.4 I showed that heparin partially inhibited fertilisation envelope elevation, the major permanent block to polyspermy in sea urchin eggs. It seemed possible, therefore, that heparin – eggs might be polyspermic. To see if this was the case I have used the fluorescence assay of sperm/egg fusion developed by Hinckley et al. (1986). In the assay eggs are loaded with the fluorescent DNA staining vital dye Hoechst 33342.
Figure 4.4  The Hoechst 33342 sperm/egg fusion assay

A brightfield (Panel A) and fluorescence picture (Panel B) of an egg loaded with Hoechst 33342 and inseminated. The egg was fixed in 4% formaldehyde 5 minutes post-insemination before photography. (mn) the male pronucleus is identifiable as a bright punctate dot overlying the female pronucleus (fn) which is larger but more diffusely fluorescent. The bar represents 50 μm.
and then extensively washed to remove all traces of external dye and inseminated. Only the nuclei of sperm that have fused with the egg will stain with the dye and become fluorescent. This is illustrated in Fig. 4.4 which shows two pictures of an egg treated in this way. Fig. 4.4A is the brightfield picture of the egg showing that it has successfully activated. The nucleus of the fertilising sperm is clearly visible in the fluorescence picture (Fig. 4.4B) as a bright punctate dot. It is the only such fluorescent body associated with the egg. The diffuse fluorescent body adjacent to this is the egg pronucleus which is easily distinguished because of its greater size. Fig.

Table 4.2

<table>
<thead>
<tr>
<th></th>
<th>Number of fluorescent sperm nuclei (mean ± S.D.)</th>
<th>Number of eggs injected</th>
</tr>
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<tbody>
<tr>
<td>Control non-injected eggs</td>
<td>1.0 ± 0.0</td>
<td>20</td>
</tr>
<tr>
<td>Control vehicle-injected eggs</td>
<td>1.1 ± 0.3</td>
<td>8</td>
</tr>
<tr>
<td>Heparin-injected eggs (250–350μg/ml)</td>
<td>7.1 ± 3.5</td>
<td>24</td>
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</table>

Eggs preloaded with Hoechst 33342 were treated as shown in the in the left most column and inseminated. The eggs were fixed 5 minutes post insemination and the number of fluorescent sperm nuclei in each egg scored.

4.5 shows pictures of an egg that had been treated as above except that it had been injected with heparin prior to insemination. This egg had elevated a complete fertilisation envelope (Panel A) but is clearly polyspermic, 3 fluorescent sperm nuclei are visible. The results of injecting a number of eggs with heparin is shown in Table 4.2. Table 4.2 shows heparin-injected eggs to be highly polyspermic, with a significantly greater number
Figure 4.5  The effect of heparin on the process of sperm/egg fusion

Pictures of an egg loaded with Hoechst 33342, injected with heparin (300 µg/ml) and inseminated. The egg was fixed in 4% formaldehyde 5 minutes post-insemination and photographed. Panel A - The brightfield picture showing that the egg has elevated a complete FE. Panel B - A fluorescence picture of the egg shown in A. (n) male pronuclei, (fn) female pronucleus, (on) out of focus male pronucleus.
of fused sperm than vehicle-injected eggs which are themselves slightly polyspermic relative to non-injected eggs. The reason for the slight polyspermy in vehicle-injected eggs may be the damage caused to the vitelline envelope by the process of microinjection. The vitelline envelope provides a containment for the contents of the exocytosed cortical granules and plays an important role in the elaboration of the FE. Damage to this structure may prevent the FE from providing such a complete block to polyspermy.

4.6 The number of sperm required to activate a heparin-injected egg

While heparin is apparently without effect on the fertilisation calcium transient, heparin-injected eggs are highly polyspermic. A possible explanation of these data is that heparin is inhibiting fertilisation and so it requires more than one sperm to fertilise a heparin-injected egg, hence heparin injected eggs are polyspermic. To try and determine whether this is the case I have titrated the fertilising activity of sperm. A concentrated suspension of sperm was diluted until they fertilised less than 100% of the eggs that had been stuck down onto a coverslip, as described for microinjection. The Ca\textsubscript{i} was then measured with fura2 at fertilisation in heparin-injected eggs using this concentration of sperm. Any calcium changes measured in these eggs then, are likely to be due to the actions of only one sperm. The Hoechst 33342 sperm/egg fusion assay provided a further confirmation that only one sperm interacted with a heparin-injected egg. When heparin was microinjected into eggs to a final concentration of 250–350μg/ml insemination of the eggs with the titrated concentration of sperm resulted in the fusion of only 1.05+/−0.68 sperm
Figure 4.6  Calcium changes in a heparin–injected egg fertilised with one sperm

$\text{Ca}_i$ was measured with fura2 which was co–injected with heparin (300 µg/ml). The egg was inseminated at the point shown with the dilution of sperm described in the text. This egg did not elevate an FE. The record is representative of 6 experiments.
per egg (mean +/− S.D., n=20) (data combined from two experiments).

Fig. 4.6 shows the calcium changes at fertilisation in a heparin-injected egg (250–350μg/ml) using the diluted concentration of sperm. The calcium transient is indistinguishable from that of control eggs. In a total of 6 experiments the mean calcium change was 1.6 +/− 0.16 μM (mean +/− SEM) as opposed to 1.8 +/− 0.04 μM (mean +/− SEM, n=4).

4.7 Heparin and the generation of the fertilisation calcium transient

If Ins(1,4,5)P₃ is the messenger responsible for releasing calcium at fertilisation it is surprising that heparin, an inhibitor of Ins(1,4,5)P₃ induced calcium release, has no effect on the fertilisation calcium transient. I have therefore looked more closely at the calcium transient and its relation to other events occurring at the same time. The increase in Caᵢ is not the earliest observable change at fertilisation; earlier events occur at the plasma membrane which are detectable as changes in the egg's membrane potential.

Fig. 4.7 A (i) shows a membrane potential recording from an egg at fertilisation. Following the interaction of the sperm with the egg there are a number of potential changes that are well characterised (Hagiwara & Jaffe, 1979; Chambers & de Armendi, 1979). The membrane potential changes can be divided into 3 stages as shown in Fig. 4.7 A (i). The first stage, (A), occurring before FE elevation has several components: a rapid depolarisation that is the result of a sperm-induced current; an action potential triggered by the sperm-induced current; a depolarisation maintained by the sperm-induced current until the onset of the second stage (Dale et al., 1978 and Chambers & De Armendi, 1979). The second stage (B), during which the FE elevates, is characterised by a further stage
Figure 4.7  The simultaneous measurement of the membrane potential and calcium changes at fertilisation

(A) The membrane potential (i) and Ca_4 changes (ii) in an egg with an initial resting potential of -70mV. 150 pA of hyperpolarising current was injected continuously in this experiment. The details of the three periods A,B and C are described in the text. This is one of three experiments.

(B) The membrane potential (i) and Ca_4 changes (ii) in an egg with an initial resting potential of -20mV. This is one of two experiments.

In both experiments the fluorescence signals have entirely arbitrary units.
of depolarisation that is slower in onset and is believed to be a calcium stimulated non specific cation conductance (Lynn et al., 1984 and David et al., 1988). The final stage, (C), is a slow repolarisation due in large part to a H$^+$-stimulated K$^+$ conductance that develops as a result of the increase in pH$^+_i$ (Steinhardt & Mazia, 1973). The membrane potential changes in stages (A) and (B) and their role in providing a block to polyspermy is well documented (Jaffe & Gould, 1985). However, the temporal relationship between these electrical changes and the increase in Ca$_i$ is unknown. A study by Eisen et al. (1984) simultaneously measured the membrane potential and calcium changes at fertilisation in the sea urchin egg. However, they used the calcium sensitive photoprotein aequorin which has K$_{0.5}$ luminescence (measured as photomultiplier current) for calcium concentration of 3µM (Eisen et al., 1984). So aequorin is not a reliable indicator of resting Ca$_i$ and would not detect subtle increases in Ca$_i$ from a resting level of only 0.1 µM.

Fig. 4.7 A (ii) shows the changes in Ca$_i$ measured in the same egg as the membrane potential changes in Fig. 4.7 A (i). Ca$_i$ was measured with fura2 but using only a single excitation wavelength (380nm), where an increase in calcium results in a decrease in fluorescence intensity. The trace shows that the calcium changes at fertilisation have 2 components. The first increase, which begins coincidently with the onset of phase A of the conductance change, is a small sustained increase in Ca$_i$. The second component of the response is the main activating calcium increase. The period between the interaction of the sperm with the egg and egg activation (the main calcium increase) is termed the latent period (Allen & Griffin, 1958). During the latent period the sperm is generating the signals that
Figure 4.8 The effect of membrane depolarisation on $Ca_i$

The figure shows the effect of membrane depolarisation achieved using two different protocols on $Ca_i$.

(A) Membrane depolarisation was brought about by switching off the hyperpolarising current (150 pA) for two periods of approximately 1 minute. The upper record (i) in the panel shows the membrane potential changes, the lower panel (ii) the calcium changes as a reduction in fluorescence at 380 nm.

(B) The effect of membrane depolarisation by high $K^+$ - ASW on $Ca_i$. $Ca_i$ was measured in a single egg with fura2. At the times indicated with the horizontal lines ASW with 60 mM KCl was washed onto the eggs. In these experiments $Ca_i$ was measured using a ratioing method. This is representative of 3 experiments.
result in egg activation.

The small increase in Ca\textsubscript{i} that occurs during the latent period is a result of the depolarisation of the egg. This can be shown in a number of ways. Firstly, in eggs that are held at a depolarised potential then this step increase in Ca\textsubscript{i} is absent (see Fig. 4.7 B). However, in the same egg which was held at -70mV prior to fertilisation the increase in Ca\textsubscript{i} could be elicited by depolarisation of the egg (see Fig. 4.8 A). A final demonstration that the increase in calcium is a consequence of depolarisation of the egg is given in Fig. 4.8 B. An egg treated with a high K\textsuperscript{+} ASW, which should depolarise the egg to about -20mV, shows a similar small increase in Ca\textsubscript{i}. This suggests that at fertilisation it is the depolarisation of the egg’s membrane potential that causes the small increase in Ca\textsubscript{i} preceding the main calcium transient.

I have looked at the effects of heparin on the latent period using the small step increase in calcium to indicate the beginning of the latent period. This is illustrated in Fig. 4.9 and 4.10 which show typical fertilisation calcium transients from an egg injected with vehicle (Fig. 4.9) and heparin (Fig. 4.10). The upper panel of each figure shows the complete fertilisation transient and in each case a small step-like increase in Ca\textsubscript{i} preceding the main increase is clearly visible. The lower panel shows that portion of the upper record on an expanded time scale with a logarithmic plot of Ca\textsubscript{i}. It was more convenient to take measurements of the latent period from the logarithmic plots and a definition of the various parameters measured is given by the construction lines in Fig. 9.B.

From panel B in Fig. 4.9 and 4.10 it is clear that the latent period is longer in the heparin injected egg than in the control. Table 4.3 shows the
accumulated data from a number of experiments. From this it is clear that the latent period is significantly prolonged in heparin-injected eggs. I also looked at the time taken from the initiation of the main calcium increase to its peak. This parameter must in some way reflect the explosive and regenerative increase in Ca, that occurs at fertilisation and is thought to be mediated by the calcium-stimulated production of Ins(1,4,5)P₃ (see Section 1.4 D).

Table 4.3

<table>
<thead>
<tr>
<th></th>
<th>Latent period duration (sec) (mean +/- S.D.)</th>
<th>Time to transient peak (sec) (mean +/- S.D.)</th>
</tr>
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<tbody>
<tr>
<td>Vehicle injected eggs</td>
<td>20.1 +/- 5.5 (n = 5)</td>
<td>27.3 +/- 4.7 (n = 5)</td>
</tr>
<tr>
<td>Heparin-injected eggs (250 - 350 μg/ml)</td>
<td>84.5 +/- 23.0* (n = 6)</td>
<td>49.6 +/- 20.5* (n = 6)</td>
</tr>
</tbody>
</table>

The table shows the duration of the latent period and the time taken to reach the peak of the calcium response for control and heparin-injected eggs. The timings were measured as described in the text. * significantly greater than control, p < 0.01 (one-tailed unpaired Student's t-test). † significantly greater than control p < 0.05 (one-tailed unpaired Student's t-test). Both groups of experiments used a low dilution of sperm to fertilise the eggs.

The effect of heparin on this parameter was not as clear. The difference was only just significant at p < 0.05. So heparin may also have an effect on the propagation of the activation calcium wave.
Figure 4.9  The latent period determined from the calcium record in a control egg.

$C_{a_i}$ was measured with fura2 which was co-injected with the heparin-injection vehicle. Panel A is the complete calcium record. A small step-like increase in $C_{a_i}$ clearly precedes the main increase. Panel B is an expanded record of that part of A indicated by the time scale in B. $C_{a_i}$ is shown on a logarithmic scale to accentuate the small step-like change in calcium and to provide a clear indication of its beginning and end.
4.8 Summary

1 Heparin causes a 20-fold inhibition in the ability of Ins(1,4,5)P₃ to cause an increase in Ca²⁺ in intact eggs.

2 The actions of heparin are specific and related to its degree of sulphation.

3 Heparin did not significantly inhibit either the magnitude or duration of the fertilisation calcium transient.

4 Heparin partially inhibited FE elevation.

5 Heparin-injected eggs tended to be polyspermic.

6 A single sperm is capable of activating a heparin-injected egg.

7 It is possible to determine the duration of the latent period at fertilisation from a small increase in Ca²⁺ that precedes the main activating increase in Ca²⁺, and is the result of a depolarisation of the egg's membrane.

8 Heparin significantly prolongs the duration of the latent period.
Figure 4.10  The latent period determined from the calcium record in a heparin-injected egg

Ca_{i} was measured with fura2 which was co-injected with heparin (300 µg/ml). See figure legend to Fig. 4.9 for further details.
Chapter 5

Effects of some guanine nucleotides on sea urchin eggs

5.1 Introduction

Little is known about how the interaction of the sperm with the egg leads to the generation of those messengers causing the increase in Ca\textsubscript{i} and pH\textsubscript{i} that constitute egg activation. This chapter describes experiments using two guanine nucleotides, Guanosine 5'–O–(3–thiotriphosphate) (GTPyS) and Guanosine 5'–O–(2–thiodiphosphate) (GDP\beta S), to investigate these early events of egg activation.

Turner et al. (1986,1987) have proposed that at fertilisation the sperm activates phosphoinositidase C (PIC) in the same way that some hormones activate the enzyme in other cells: through a receptor linked to PIC by a G–Protein (Turner et al., 1986; Turner et al., 1987). They have shown that cholera toxin and GTPyS, agents which activate G–Proteins, cause fertilisation envelope elevation (FE) and GDP\beta S, which inhibits G–Protein activation, inhibits the ability of sperm to cause FE elevation. However as explained in Chapter 1 this only fulfils some of the conditions for establishing that a G–protein is involved (see Section 1.5 C). Furthermore in some cells the process of exocytosis may involve a G–Protein (Howell et al., 1987), so exocytosis and FE elevation may not be a good criteria for establishing a role for a G–Protein during the early stages of egg activation. The chapter describes some experiments using guanine nucleotides to investigate whether a G–Protein is involved in egg activation. I have used GDP\beta S and GTPyS and looked at their effects on the earlier events of fertilisation, the two ionic changes and the fusion of
the sperm with the egg. These events constitute the process of egg activation and are not, unlike the process of exocytosis, a consequence of it.

5.2 GDPβS inhibits fertilisation envelope elevation

I have investigated some of the actions of GDPβS in sea urchin eggs. In agreement with an earlier study (Turner et al., 1986) GDPβS inhibited fertilisation as assayed by scoring FE elevation (see Fig. 5.1A). The concentrations required to inhibit were similar to those reported: concentrations greater than 4 mM inhibiting fertilisation in 85% of eggs (n = 17), 50% inhibition occurring at a concentration of 2 mM.

5.3 GDPβS and sperm/egg fusion

I have extended these observations to look at the effects of GDPβS on the earlier events at fertilisation. Fusion of the sperm with the egg precedes the cortical reaction (Longo et al., 1986 and Hinckley et al., 1986). I used the fluorescence assay of sperm/egg fusion developed by Hinckley et al. (1986) to look at the effect of GDPβS on this process. GDPβS was microinjected into eggs at concentrations sufficient to inhibit FE elevation completely. The eggs were fertilised and scored for fluorescent sperm nuclei. Fig. 5.1 shows two pictures of such an egg. Fig. 5.1A, the brightfield picture, shows that GDPβS has inhibited FE elevation, despite the large numbers of sperm associated with the egg. However, the fluorescence picture (Fig. 5.1B) shows that GDPβS has not inhibited the fusion of the sperm with the egg. In this case, a total of 3 sperm nuclei can be seen. The results of injecting a number of eggs is shown in Table 5.1.
Figure 5.1  The effect of GDPβS on FE elevation and sperm/egg fusion

Pictures of an egg loaded with Hoechst 33342 dye, microinjected with GDPβS (final concentration 3 mM) and fertilised. The egg was fixed 10 minutes post-insemination in 4% formaldehyde and photographed. (A) Brightfield picture showing that GDPβS inhibited FE elevation. (B) Fluorescence picture of the same egg showing that GDPβS failed to inhibit sperm/egg fusion. The scale bar represents 50 μm.
Table 5.1

<table>
<thead>
<tr>
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<th>Number of fluorescent sperm nuclei per egg (mean +/− S.D.)</th>
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<tbody>
<tr>
<td>Control non-injected eggs</td>
<td>1.0 +/− 0.0 (n = 12)</td>
</tr>
<tr>
<td>GDPβS-injected eggs</td>
<td>1.2 +/− 1.6 (n = 26)</td>
</tr>
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Eggs were loaded with Hoechst 33342 dye for 30 minutes and treated as shown in the left hand column of the table. The eggs were inseminated and fixed 5 minutes post insemination in 4% formaldehyde before the number of fluorescent sperm nuclei per egg was scored. The numbers of eggs scored are shown in parentheses.

5.4 Calcium changes in GDPβS injected eggs

As GDPβS failed to inhibit the interaction of the sperm with the egg as determined by the fluorescence assay I tested whether it was able to block the sperm-induced increase in Ca\textsuperscript{i}. Ca\textsuperscript{i} was measured in single eggs with fura2 following the microinjection of GDPβS and insemination.

The microinjection of GDPβS invariably caused an increase in Ca\textsuperscript{i} to greater than micromolar levels, similar to those seen at fertilisation (Poenie et al., 1985 and Swann & Whitaker, 1986). In most cases (78%, n = 22) Ca\textsuperscript{i} remained elevated above micromolar levels but in those cases where Ca\textsuperscript{i} recovered to below 500nM the subsequent addition of sperm caused an increase in Ca\textsuperscript{i} to above 1 μM (n = 4). However, neither the microinjection of GDPβS nor the subsequent addition of sperm caused the elevation of a FE; this is despite the fact that Ca\textsuperscript{i} increased to levels that would normally stimulate exocytosis. Even in eggs where Ca\textsuperscript{i} did not recover the addition
Figure 5.2 Changes in $Ca_i$ in an egg injected with GDP$\beta$S

$Ca_i$ was measured in a single egg using fura2. GDP$\beta$S was microinjected (final concentration 5 mM) and sperm added to the bath at the time indicated. The egg did not elevate an FE.
of sperm caused further increases in \( \text{Ca}_i \) but due to the initial high resting level these increases were only 100–300 nM in magnitude.

It seemed possible that the observations made with GDP\( \beta \)S, particularly those relating to the increase in \( \text{Ca}_i \), might be due to a contaminant of the GDP\( \beta \)S. However the microinjection of the main contaminants (GMP, GDP and Li\( + \)) failed to either inhibit FE elevation or cause an increase in \( \text{Ca}_i \). Furthermore the calcium concentration in the GDP\( \beta \)S was measured, using fura2, and was less than 450 nM. The GDP\( \beta \)S–induced calcium increase then, cannot be explained by calcium contamination. A similar effect may occur in the hamster egg where an increase in \( \text{Ca}_i \) can be measured indirectly through a calcium–activated potassium conductance and a membrane hyperpolarisation. The microinjection of GDP\( \beta \)S into hamster eggs frequently causes a transient hyperpolarisation indicating an increase in \( \text{Ca}_i \) (Miyazaki, 1988).

5.5 The actions of GTP\( \gamma \)S in sea urchin eggs

Turner et al. (1986) have shown that the microinjection of GTP\( \gamma \)S, a G–protein activator, activates sea urchin eggs. However, showing that GTP\( \gamma \)S activates sea urchin eggs demonstrates only the presence of a G–protein transduction mechanism. It does not show that this is the transduction mechanism used by a sperm when it fertilises an egg. In the Introduction I suggested that several of the conditions for determining the involvement of a G–Protein at fertilisation remained to be satisfied. Particularly important are the criteria that link the actions of the agonist directly to GTP: the requirement of GTP for the initiation of the response and the demonstration of a reduction of receptor ligand affinity in the
Figure 5.3  The ability of GTPyS to stimulate FE elevation

(A) The sensitivity of eggs to GTPyS. Eggs were microinjected with GTPyS to the cytoplasmic concentrations indicated and the eggs were scored for FE elevation 5-10 minutes afterward. ○ percentage of eggs elevating partial envelope. ● percentage of eggs elevating a complete envelope. At least 10 eggs were injected at each concentration.

(B) The time required after microinjection of GTPyS for FE elevation to occur. The time interval between the time of injection to the start of FE elevation is plotted against the concentration of nucleotide. Only eggs elevating complete envelopes are included.
presence of GTP. However, these other conditions are difficult to study in the sea urchin egg at fertilisation because there is no in vitro system, such as isolated egg plasma membranes, for investigating sperm stimulated PPI hydrolysis or receptor binding affinity. I have adopted another approach and investigated the relation of a G-Protein to egg activation by comparing the actions of sperm with those of GTPyS.

As a first step in this study I investigated the activity of GTPyS in sea urchin eggs. In agreement with other studies (Turner et al., 1986 and Swann et al., 1988) the microinjection of GTPyS activated sea urchin eggs, causing them to elevate complete envelopes, with 50% activation occurring at a final cytoplasmic concentration of 80 μM (Fig. 5.3). This is a quantitatively different result from the study of Turner et al. (1986) where 50% activation occurred with a cytoplasmic concentration of 2 μM. The reason for this difference is not clear. It may be due to a species variation or perhaps a difference in the microinjection techniques. There was a delay between the microinjection of the GTPyS and elevation of a FE. The delay was highly variable (Fig. 5.3B) but showed some dose-dependency so that eggs microinjected with GTPyS to a concentration of 50 μM had a delay of 100+/−32 seconds (mean+/−S.D., n=19) while the delay with a concentration of 150 μM to 450 μM was 35+/−22 seconds (mean+/−S.D., n=14). These two sets of data are significantly different (p<0.001, one-tailed unpaired Students t-test).

The latency in FE elevation reflects a delay before an increase in Ca,

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Figure 5.4 Calcium changes caused by the microinjection of GTPyS

(A) A representative trace of the calcium changes caused by the GTPyS. Ca\textsubscript{i} was measured in a single egg with fura2 following the microinjection of GTPyS (150 \textmu M) at the time indicated. The egg raised a complete FE.

(B) The effect of removing external calcium on the GTPyS induced calcium changes. Each trace shows averaged calcium records from 4 eggs injected with GTPyS (150 - 200 \textmu M). The dotted line represents averaged records of eggs in normal ASW and the solid line eggs microinjected in calcium-free ASW (2 mM EGTA). Traces from individual eggs were standardised with reference to the peak of the calcium transient. The calcium concentrations at each time point were compared using a one-tailed Students t-test (unpaired) and the two records are significantly different (p < 0.01) for the period shown by the horizontal line.
Ca	extsuperscript{2+}. This is a feature of the microinjection however as a similar increase is seen when injecting the vehicle buffer. There is a clear delay however, between the injection and the main increase in Ca	extsuperscript{2+}. This suggests that the actions of GTPyS involve the generation of an intermediate that leads to the release of calcium.

G-proteins, as well as stimulating enzymes that generate intracellular messengers, also control some ion channels (Neer & Clapham, 1988) and so can alter the plasma membrane permeability to certain ions. Given that fertilisation can occur in the absence of extracellular calcium an important prediction, if egg activation involves a G-protein, is that GTPyS can activate eggs independently of external calcium. This is demonstrated in the experiments of Fig. 5.4B which shows averaged calcium records from 4 eggs microinjected with GTPyS in normal and calcium-free ASW. It shows that GTPyS microinjected into eggs in calcium-free ASW causes an increase in Ca	extsubscript{i} that is indistinguishable in magnitude from that caused by the nucleotide in normal ASW. Although, as in the case of the fertilisation calcium transient, there is a later part of the response that is dependent upon external calcium: by about 3–4 minutes the Ca	extsubscript{i} is significantly smaller in calcium-free ASW than in eggs in normal ASW (p<0.01, one-tailed unpaired students t-test). (see Fig. 5.4B).

5.6 The actions of fluoride in sea urchin eggs

Cholera toxin and GTPyS are not the only compounds that stimulate G-Proteins. Fluoride ions are well known G-protein activators. The activation process has been shown to require Al	extsuperscript{3+} (Sternweis & Gilman, 1982) and the two are thought to act by forming a quaternary complex
Figure 5.5  Fluoride induced calcium changes

Calcium was measured with fura2. NaF was microinjected (final concentration 150 μM) at the time indicated. The egg raised a complete FE during the large increase in Ca\textsubscript{i}. This record is representative of 3 experiments.
AlF₄⁻. Bigay et al. (1985) suggest that the AlF₄⁻ complex activates the G-Protein with GDP, the quaternary complex resembling the gamma phosphate that the GDP lacks. The GDP and AlF₄⁻ together imitate GTP and so activate the G-Protein. I have tested whether fluoride too is capable of activating sea urchin eggs. Fig. 5.5 shows that the microinjection of fluoride activates sea urchin eggs in a similar way to GTPyS, by causing an increase in Caᵢ resembling in magnitude and duration that seen at fertilisation. Similar to GTPyS there is also a delay between the microinjection of the fluoride and the activating calcium transient.

5.7 Sperm cannot cause a change in pHᵢ without a change in Caᵢ

Whalley and Whitaker (1988) have previously shown that GTPyS is capable of causing an increase in pHᵢ that is not dependent upon an increase in Caᵢ. The mechanism thought to underly this observation is shown in Fig. 5.6. GTPyS, by stimulating a G-Protein, activates PIC which results in the generation of the two intracellular messengers, Ins(1,4,5)P₃ and DAG. The DAG activates a Na⁺/H⁺ antiporter through Protein Kinase C and causes an increase in pHᵢ. So the production of DAG can be measured indirectly as a change in pHᵢ. However the Ins(1,4,5)P₃ releases calcium from an intracellular store which would set off the regenerative response already described (see Section 1.4 D), whereby the released calcium would cause further stimulation of PIC. In fact, as calcium ionophores cause a change in pHᵢ (Shen & Steinhardt, 1979), if GTPyS released calcium by any other mechanism an increase in pHᵢ might also occur. To avoid this, Whalley and Whitaker used the calcium chelator, bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA) to prevent any
Figure 5.6  The mechanism of calcium–independent GTPyS induced pH change

The diagram shows how GTPyS is thought to act in sea urchin eggs. The feedback pathway whereby calcium stimulates PIC is also shown to demonstrate why it is necessary to use BAPTA in these experiments. The site of action of R59 022 is also shown.
increase in Ca\textsubscript{i}. They also showed that the DAG--kinase inhibitor R59022 (de Courcelles et al., 1985), which should potentiate the actions of DAG by preventing its metabolism, potentiated the increase in pH\textsubscript{i} caused by GTPyS.

If sperm activate an egg through a G--Protein mechanism then they too should cause an increase in pH\textsubscript{i} as GTPyS does. I have tested this prediction and the results are shown in Fig. 5.7. An egg was microinjected with BCECF and BAPTA prior to insemination. pH\textsubscript{i} was measured for a period of 15 minutes following insemination and no increase in pH\textsubscript{i} was detected. Observation of the egg showed that there were several fertilisation cones suggesting that several sperm had interacted with the egg. This was confirmed using the fluorescence assay of sperm/egg fusion which showed that on average 9.1 ± 2.4 (mean ± S.D., n=10) sperm fused with BAPTA injected eggs. At the end of this period the microinjection of GTPyS caused an increase in pH\textsubscript{i}.

These data suggest that the actions of sperm are different from those of GTPyS. GTPyS stimulates DAG production and causes an increase in pH\textsubscript{i}. Sperm cause no increase in pH\textsubscript{i} suggesting that a sperm activates an egg, not by directly stimulating PPI hydrolysis, but by bringing about an increase in Ca\textsubscript{i} by some other means.

5.8 Heparin inhibits GTPyS activation of sea urchin eggs

An alternative explanation of the above data might be that the actions of GTPyS are not different from those of sperm but that GTPyS is a much more potent stimulator of G--Proteins than sperm. To test this idea I have investigated the actions of heparin on GTPyS--induced egg activation. As discussed in the previous chapter heparin inhibits the
Figure 5.7 Changes in $pH_i$ in a BAPTA-injected egg following insemination and injection of GTP$_y$S

$pH_i$ was measured with BCECF (10 μM) which was co-injected into the egg with BAPTA (final concentration 2 mM). The sperm were added to the bath at the time indicated. Surrounding eggs elevated FEs within 60 seconds. GTP$_y$S was microinjected (final concentration 250 μM) at the time indicated. This record is representative of 4 experiments.
ability of Ins(1,4,5)P$_3$ to release calcium from an intracellular store. Heparin inhibited the ability of GTPyS to increase Ca$^+$, as shown in Fig. 5.8. In this experiment the microinjection of 200 $\mu$M GTPyS, a quantity of nucleotide that activates 100% of eggs injected, caused only a small increase in Ca$^+$. In contrast the subsequent addition of sperm caused a calcium transient that is indistinguishable from that at fertilisation. In a series of experiments the microinjection of GTPyS caused an average increase in Ca$^+$ to 470 $\pm$ 180 nM (mean $\pm$/S.D., n = 6), which is probably an injection artefact, whereas subsequent fertilisation of heparin–injected eggs always resulted in a large increase in Ca$^+$ with an average peak of 1.71 $\pm$ 0.42 $\mu$M (mean $\pm$/S.D., n = 4).

There is a possibility however that heparin might have other actions as well. Heparin might for example inhibit the ability of GTPyS to stimulate G-proteins. It has been shown that heparin disrupts the link between receptors and G-proteins (Salomon et al., 1978 and Willuweit & Aktoris, 1988). To control for these possibility I tested the ability of GTPyS to cause an increase in pH$_i$ in an egg injected with heparin Fig. 5.9. Clearly heparin does not prevent the ability of GTPyS to stimulate an increase in pH$_i$.

5.9 Summary
1. GDP$\beta$S inhibits FE elevation.
2. GDP$\beta$S does not inhibit the fusion of the sperm and egg.
3. GDP$\beta$S does not prevent the sperm–induced increase in Ca$^+$.
4. GTPyS activates sea urchin eggs with a dose–dependent delay causing an increase in Ca$^+$, a calcium increase that is similar to that seen at fertilisation both in its magnitude and duration, and in its dependence on external calcium.
Figure 5.8 Changes in $\text{Ca}_i$ in an egg microinjected with heparin and GTPyS

$\text{Ca}_i$ was measured in a single egg with fura2 which was co-injected with heparin (final concentration 300 $\mu$g/ml). GTPyS was microinjected (final concentration 150 $\mu$M) and sperm added to the bath at the times indicated. This record is representative of 6 experiments.
Figure 5.9 Changes in pHᵢ in a heparin-injected egg following the microinjection of GTPγS

pHᵢ was measured using BCECF which was co-injected with heparin (final concentration 300 μg/ml). GTPγS was microinjected at the time indicated. The egg showed no signs of cortical granule exocytosis. This record is representative of 5 experiments.
5. Another G-protein activator, fluoride, activates eggs in a similar way to GTPyS.

6. GTPyS causes an increase in pH, in BAPTA-injected eggs while sperm do not.

7. Heparin inhibits egg activation by GTPyS but not sperm.
Chapter 6

The actions of Adenosine – 5’ – O – (thiotriphosphate)

6.1 Introduction

Many cell processes are regulated by adjustments in the level of protein phosphorylation. To investigate whether phosphorylation might have a direct role to play in the processes occurring at fertilisation I have used the compound Adenosine – 5’ – O – (thiotriphosphate) (ATPyS). This substance, a thio analogue of ATP, is a substrate for some kinases but in contrast to ATP the resultant phosphorylation is irreversible (Gratecos & Fischer, 1974 and Lokha et al., 1987). The irreversible phosphorylation will cause the processes regulated by these kinases to become chronically activated or inhibited: ATPyS is therefore a useful tool for determining which processes might be controlled by phosphorylation.

6.2 ATPyS inhibits FE elevation

The microinjection of ATPyS up to a final cytoplasmic concentration of 500 µM failed to cause egg activation. However when these eggs were subsequently inseminated they failed to elevate fertilisation envelopes (see Fig. 6.2A). ATPyS was found to be a potent inhibitor of fertilisation envelope elevation as shown in Fig. 6.1 with 100% inhibition occurring at a final cytoplasmic concentration of 150 µM.

6.3 ATPyS does not inhibit sperm egg fusion

Clearly ATPyS could be inhibiting a number of mechanisms: it could be inhibiting the process of exocytosis itself or the generation of the signals
Figure 6.1  The ability of ATPγS to inhibit FE elevation

Eggs were microinjected with ATPγS to the concentrations indicated and fertilised 10–15 minutes post-injection. The eggs were scored for FE elevation 5 minutes later when all the surrounding eggs had raised complete FEs. Cross-hatched bars indicate the percentage of eggs elevating complete envelopes, open bars the percentage of eggs elevating complete or partial envelopes and the filled bar at 150 μM ATPγS indicates that no eggs raised any sort of envelope. The numbers above each bar represents the number of eggs injected with that concentration.
that stimulate exocytosis.

Inspection of eggs that had been microinjected with ATPyS and subsequently inseminated showed the presence of a number of structures that resembled enlarged fertilisation cones.

Table 6.1

<table>
<thead>
<tr>
<th>Number of fluorescent sperm nuclei</th>
<th>(mean +/− S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control non-injected eggs</td>
<td>1.0 +/− 0.0 (n = 20)</td>
</tr>
<tr>
<td>Control vehicle injected eggs</td>
<td>1.3 +/− 0.7 (n = 9)</td>
</tr>
<tr>
<td>ATPyS injected eggs (150−200 μM)</td>
<td>14.6 +/− 6.7 (n = 10)</td>
</tr>
</tbody>
</table>

Eggs loaded with Hoechst 33342 for 30 minutes and extensively washed to remove external dye were treated as described in the left hand column of the table. They were subsequently inseminated and, after 5 minutes, fixed in 4% formaldehyde before the number of fluorescent sperm nuclei in each egg was determined.

Fertilisation cones are structures formed normally at fertilisation at the site of sperm entry as the egg incorporates the sperm (Tilney & Jaffe, 1980). Their presence then provides an indication that ATPyS is not inhibiting the process of sperm egg interaction and incorporation. To provide a more direct indication of this I used the bisbenzamide fluorescent sperm/egg fusion assay described previously (see Section 4.5). Fig 6.2 shows pictures of an egg labelled with Hoechst 33342, microinjected with ATPyS and fertilised. Fig. 6.2A, the brightfield picture, shows that ATPyS has inhibited
Figure 6.2  The effect of ATPyS on the process of sperm/egg fusion

Pictures of an egg loaded with Hoechst 33342, microinjected with ATPyS (final concentration 150 µM) and fertilised 10 minutes post-injection. Ten minutes after insemination the egg was fixed in 4% formaldehyde and photographed. (A) Brightfield picture showing that ATPyS has inhibited FE elevation, (fc) – fertilisation cone. (B) Fluorescence picture of the same egg showing 3 fluorescent sperm nuclei.
FE elevation despite the fact that 2 fertilisation cones are visible which suggests that at least 2 sperm have interacted with the egg. Fig. 6.2B shows the fluorescence picture where 3 fluorescent sperm nuclei, 2 of whose positions correlates with those of the fertilisation cones in Fig. 6.2A, are clearly visible. In fact eggs microinjected with ATPyS and inseminated tend to be highly polyspermic as shown in Table 6.1. A significantly greater number of sperm fused with eggs that had been injected with ATPyS than eggs injected with vehicle (p<0.01, two tailed unpaired Students t-test).

6.4 ATPyS does not inhibit the fertilisation calcium transient

Since ATPyS was not inhibiting the interaction of the sperm with the egg I tested whether ATPyS was inhibiting the generation of the main signal for stimulating exocytosis in the sea urchin egg, the increase in Ca\textsuperscript{i}. Fig. 6.3 shows the measurement of Ca\textsuperscript{i} in a single egg with fura2 following the microinjection of ATPyS and subsequent insemination. Clearly the microinjection of ATPyS at a concentration sufficient to inhibit fertilisation envelope elevation does not prevent sperm from causing an increase in Ca\textsuperscript{i} that was indistinguishable in both magnitude and duration from that seen at fertilisation in a control egg.

These experiments indicate that the ATPyS induced inhibition of cortical granule exocytosis is due to an effect on the process of exocytosis itself and not on the generation of the signals that stimulate exocytosis.
Figure 6.3 Calcium changes in an egg microinjected with ATPyS and fertilised

$\text{Ca}_i$ was measured with fura2. The egg was microinjected with ATPyS (final concentration 200 µM) and sperm added to the bath at the times indicated. The egg failed to elevate an FE and resembled the egg in Fig. 6.2A in having a number of fertilisation cones. This record is representative of 4 experiments.
6.5 Summary

1. ATPyS does not activate sea urchin eggs.

2. ATPyS inhibits FE elevation with 100% inhibition occurring with a concentration of 150 μM.

3. ATPyS does not prevent sperm/egg fusion. Indeed ATPyS–injected eggs are highly polyspermic.

4. ATPyS does not inhibit the sperm–induced calcium changes.
Chapter 7

Discussion and Conclusions

7.1 Egg activation, inositol phosphates and calcium influx

The key event that causes sea urchin egg activation and triggers embryonic development is an increase in \( \text{Ca}_i \) (Whitaker & Steinhardt, 1985). A sperm can activate an egg in the absence of external calcium (Takahashi & Sugiyama, 1973; Chambers, 1980 and Schmidt et al., 1982) indicating that the increase results from the release of calcium from intracellular stores. The work of Irvine and Moor (1986,1987) showing that inositol phosphates, the internal messengers thought to cause this increase in \( \text{Ca}_i \), absolutely require external calcium to activate eggs creates a paradox. On the one hand a sperm can activate an egg in the absence of external calcium, while on the other the messenger thought to generate the key increase in \( \text{Ca}_i \) requires external calcium to activate an egg. The experiments in Chapter 3 attempt to resolve this paradox. I investigated the role of external calcium in the calcium response at fertilisation and the possibility that inositol phosphates gate calcium influx.

(A) The fertilisation calcium transient depends partially on external calcium

The data show that the fertilisation calcium transient is similar to calcium responses in other cells. It has two components (Putney 1979, 1986 and Putney et al., 1981). There is an early component that is entirely independent of external calcium and a later component that is dependent upon external calcium. As stated in Chapter 3 (see Section 3.2) the removal of external calcium could either inhibit a calcium influx or, by
reducing the electrochemical gradient which opposes calcium efflux that occurs post fertilisation (Azarnia & Chambers, 1976), enhance the action of plasma membrane calcium pumps allowing a more rapid reduction in \( \text{Ca}_1 \). Either of these possibilities could produce this effect and the experiments do not discriminate between these two possibilities. However in contrast to other cells (Putney, 1979) (see also Section 1.4 C) this later component appears to have little physiological significance as eggs fertilised in calcium-free ASW will undergo several cell divisions (Chambers, 1980 and Schmidt et al., 1982).

(B) Inositol phosphates do not require external calcium to activate eggs

I tested whether inositol phosphates gate external calcium and so could be responsible for the later phase of the calcium response. Using two criteria of egg activation, cortical granule exocytosis and the increase in \( \text{Ca}_1 \), I have compared the actions of range of inositol phosphates in stimulating cortical granule exocytosis in the presence and absence of external calcium. The rank order of potency was similar to that observed for the release of calcium assayed in other cells, that is \( \text{Ins}(1,4,5)P_3 > \text{Ins}(1,2\text{cyc}4,5)P_3 > \text{Ins}(2,4,5)P_3 > \text{Ins}(1,3,4,5)P_4 > \text{Ins}(1,3,4)P_3 \) (Irvine et al., 1984; 1986 and Berridge 1987). It is possible that the activities of some of the inositol phosphates may be due, in part, to contamination by \( \text{Ins}(1,4,5)P_3 \). The \( \text{Ins}(1,3,4,5)P_4 \) used in this study may have had a 1% contamination with \( \text{Ins}(1,4,5)P_3 \) (Irvine, personal communication), accounting for its activity. The \( \text{Ins}(1,2\text{cyc}4,5)P_3 \) may also have been contaminated with \( \text{Ins}(1,4,5)P_3 \) by as much as 10% (Willcocks et al., 1989) which would account for most of its activity. More importantly though, \( \text{Ins}(2,4,5)P_3 \) had two orders of magnitude
greater activity than that reported by Irvine and Moor (1986,1987). The activity reported here is much closer to the activity observed in other cells (Irvine et al., 1984).

The microinjection of inositol phosphates not only produced the early events of egg activation, such as cortical granule exocytosis, but also released eggs from their state of cell cycle arrest. Ins(1,4,5)P$_3$ - injected eggs underwent cycles of nuclear envelope breakdown and reformation. The time course of these cell cycles was somewhat slower than those in ordinary zygotes or A23187 activated eggs (Whitaker & Patel, 1990). The reasons for this are not known but may be related to the different calcium changes that the various agents cause.

All the inositol phosphates stimulated cortical granule exocytosis equally well in either the presence or absence of external calcium. Moreover the Ins(1,3,4,5)P$_4$ - induced calcium transient was not measurably affected by the removal of external calcium. The experiments show that inositol phosphates do not act to cause calcium influx in a way that significantly affects Ca$_{i}$.

These results are clearly very different from those of other studies (Slack et al., 1986, Irvine & Moor, 1986 and Irvine and Moor, 1987) which found that inositol phosphate - stimulated FE elevation had an absolute requirement for external calcium. There could be several explanations for this difference. Firstly a possible source of error arises when assaying activity in calcium - free ASW by scoring FE elevation. FEs in calcium - free ASW are much less refractile and are more difficult to see than FEs in normal ASW. They are not then a reliable assay of activation in calcium - free ASW. Secondly the microinjection of large volumes (>2% egg volume) causes damage to eggs in calcium - free ASW as indicated by
the leakage of fluorescent dye from the egg. The leakage might allow the EGTA present in the calcium-free ASW to enter the egg and chelate calcium released by the inositol phosphate. This idea is supported by the observation that increasing the volume of inositol phosphate microinjected into eggs in calcium-free ASW reduced the percentage eggs undergoing complete cortical reactions. Either of these possibilities could account for the dependence on external calcium observed in other studies (Slack et al., 1986 and Irvine & Moor, 1986). A similar effect has been observed in eggs of *Xenopus laevis*, where continuous impalement of eggs with a microelectrode in a calcium-free media caused visible degeneration and reduced the responses of some eggs to Ins(1,4,5)P₃ (Busa et al., 1985).

The experiments presented here show that neither sperm nor inositol phosphate-induced egg activation require external calcium. These results then are entirely consistent with the idea that Ins(1,4,5)P₃ is the intracellular messenger responsible for the increase in Caᵢ at fertilisation.

I was also unable to reproduce the synergism between inositol trisphosphates and Ins(1,3,4,5)P₄ described by Irvine and Moor (1986, 1987). The reasons for this are not clear because the large differences in the sensitivity of eggs to Ins(2,4,5)P₃ between the two studies make exact reproduction of the experiments impossible.

The actions of inositol phosphates have also been investigated in *Xenopus laevis* oocytes with essentially identical results. Snyder et al. (1988) using a calcium-activated Cl⁻ current in the eggs to monitor calcium mobilisation showed that Ins(1,3,4,5)P₄ was not able to gate external calcium in any detectable way.
The actions of Ins(1,3,4,5)P₄ in other cells

While Ins(1,3,4,5)P₄ might not have a role to play as an internal messenger in sea urchin eggs it may gate calcium in other cells where a calcium influx of functional importance exists (Putney, 1979). The acinar cell may be just such a cell. As described in Chapter 1 (Section 1.4 C) Morris et al., using a calcium-activated K⁺ conductance as an indirect indicator of Ca²⁺, have reported that Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ together gate calcium influx in mouse lacrimal acinar cells. They suggest that Ins(1,3,4,5)P₄ is an important internal messenger controlling calcium influx. Changya et al. (1989) have extended their observations to show that the effect is not the result of Ins(1,3,4,5)P₄'s metabolism to Ins(1,3,4)P₃ or due to its potentiating the effect of Ins(1,4,5)P₃ by acting as an alternative substrate for the 5 phosphatase which metabolises both compounds.

A specific binding site for Ins(1,3,4,5)P₄ has been detected in a number of tissues including HL60 cells and adrenal cortex (Bradford & Irvine, 1987 and Enyedi & Williams, 1988). More recently in rat cerebellum (Thiebert et al., 1990) a binding site with high affinity for Ins(1,3,4,5)P₄ has been separated from Ins(1,4,5)P₃-binding proteins and metabolic enzymes. It has been suggested that this is an Ins(1,3,4,5)P₄-receptor and it provides further support for the idea that Ins(1,3,4,5)P₄ has 2nd messenger functions.

However recent evidence from parotid acinar cells suggests that calcium entry is controlled by another mechanism. Takemura and Putney (1989) measured calcium responses in populations of parotid cells in the absence of external calcium. Readdition of calcium to the cells caused a small increase in Ca²⁺. The same procedure performed on cells that had been stimulated with carbachol (a process terminated by the addition of atropine
and known to discharge the agonist sensitive calcium store (Aub et al., 1982)) also caused an increase in Ca, but this had an additional overshoot. The increase in calcium must represent influx and the additional overshoot shows that the influx is greater in the case of prestimulation. The overshoot was still present even if the calcium was replaced 20 minutes after the addition of atropine. This means that the overshoot cannot be regulated by inositol phosphates which have a half life of only seconds after the termination of stimulation (Hughes et al, 1988). Takemura and Putney (1989) suggest that it is the degree of filling of the agonist-sensitive calcium store that controls influx: this is the capacitative model of calcium entry. They propose that an empty calcium store somehow increases the plasma membrane permeability to calcium and that this phenomenon may account for calcium entry normally seen during cell stimulation. Removal of the stimulus allows the store to refill and this prevents further calcium influx. This mechanism of control does not involve Ins(1,3,4,5)P₄.

Another approach to look at calcium entry has been to use the tumour promoter, thapsigargin, which empties the Ins(1,4,5)P₃-sensitive calcium store without causing PPI turnover or inositol phosphate production. In some cell types such as parotid gland cells this compound causes calcium influx (Takemura et al., 1989). This provides a further indication that inositol phosphates are not required to gate calcium entry in parotid gland cells.

Further support for the capacitative model has been found by Hallam et al. (1988, 1989) in endothelial cells. They used manganese ions to monitor calcium influx. These ions utilise the same entry pathway into endothelial cells as calcium but their entry is more easily monitored because
they strongly quench fura2 fluorescence (Gryniewicz et al., 1985 and Hallam et al., 1988). They showed that stimulated calcium entry did not depend upon either an elevated \(\text{Ca}_i\) (Hallam et al., 1988) or on receptor occupation (Hallam et al., 1989).

It may be however that the mechanisms regulating calcium influx differ from cell to cell. The influx of manganese ions can only be demonstrated in platelets (Sage et al., 1989) and endothelial cells (Hallam et al., 1988) but not in parotid acinar cells (Merritt & Hallam, 1988) suggesting that there are different entry mechanisms.

(D) Multiple injections of \(\text{Ins}(1,4,5)^{\text{P}}_3\)

In chapter 3 I compared the actions of \(\text{Ins}(1,4,5)^{\text{P}}_3\) and \(\text{Ins}(1,4,5)^{\text{S}}_3\). The microinjection of \(\text{Ins}(1,4,5)^{\text{P}}_3\) caused a calcium transient which was similar in magnitude to that seen at fertilisation. Subsequent injections of the same quantity of \(\text{Ins}(1,4,5)^{\text{P}}_3\) were unable to cause the same calcium change. The peak of the response was reduced by 45%. This is despite the fact that the final concentration of \(\text{Ins}(1,4,5)^{\text{P}}_3\) was 0.8 \(\mu\text{M}\) which is close to that required to release 100% of calcium from a reticular preparation of sea urchin eggs (Clapper and Lee, 1985). This indicates that the unfertilised sea urchin egg is in some way primed so that the first calcium response is larger. This priming could take place at several different levels. Firstly the regenerative response that resides within the egg may only be able to be triggered once. Or, again, the size of the calcium store may be adjusted after triggering the first response so that subsequent responses are smaller. There is some evidence for this second idea as after fertilisation the total calcium content of sea urchin egg is reduced by 30%.
(Azarnia & Chambers, 1976). In addition, the membrane structures lying immediately beneath the plasma membrane, which are thought to store calcium, undergo extensive reorganisation after fertilisation (Sardet, 1984).

I have shown that Ins(1,4,5)–S–P₃, a non-hydrolysable analogue of Ins(1,4,5)P₃, activates sea urchin eggs with 10 fold less activity than Ins(1,4,5)P₃. This is similar to permeabilised Swiss 3T3 cells where Ins(1,4,5)–S–P₃ was 7 fold less able to release calcium than Ins(1,4,5)P₃ (Taylor et al., 1988). Both Ins(1,4,5)–S–P₃ and Ins(1,4,5)P₃ caused an increase in Caᵢ to values similar to fertilisation. However in contrast to Ins(1,4,5)P₃, the response to subsequent injections of Ins(1,4,5)–S–P₃ declined continuously until the eggs were refractory to stimulation. As I mentioned in chapter 3, the thio analogue is resistant to metabolism (Taylor et al., 1989), so the effect must be due directly to its action. The experiments with ionophore suggest that the internal store is much less full in eggs that had been microinjected with Ins(1,4,5)–S–P₃. This shows that the continued presence of the calcium–mobilising messenger prevents refilling of the store and so explains why further injections fail to cause an increase in Caᵢ. A similar observation has been reported in an in vitro system (Taylor et al., 1989). These experiments provide a further indication that the Ins(1,4,5)P₃ receptor does not desensitise.

7.2 SOME ACTIONS OF HEPARIN IN SEA URCHIN EGGS

(A) Heparin and Ins(1,4,5)P₃–induced egg activation

I have shown that heparin inhibits the Ins(1,4,5)P₃–induced increase in Caᵢ in intact sea urchin eggs. The inhibition can be overcome
completely by increasing the quantity of Ins(1,4,5)P$_3$ injected by a factor of 20 without changing the slope of the dose response curve. The actions of heparin are therefore competitive. This is similar to an in vitro study on bovine adrenal cortex which showed that heparin could produce a 12-fold shift in the quantity of Ins(1,4,5)P$_3$ required to produce calcium release (Guillemette et al., 1989). The molecular weight of heparin has a range of 4000 – 6000 which means that a concentration of heparin in the range 30 – 60 μM was used to obtain the effects described here.

It has been suggested (Worley et al., 1987) that the actions of heparin are dependent upon its high degree of sulphation. Worley et al. (1987) showed that heparin, but not unsulphated polysaccharides, displaced bound tritiated Ins(1,4,5)P$_3$ with great potency. I have shown that this is also true of calcium release as well. De-N-sulphated heparin (that is heparin treated with nitric acid to remove sulphate groups) failed to inhibit Ins(1,4,5)P$_3$-induced calcium release in any detectable way. The actions of heparin then, are related to its negatively charged sulphates. Perhaps the sulphates resemble the phosphates on the Ins(1,4,5)P$_3$, allowing heparin to inhibit calcium release by competing for the same binding site.

(B) The effect of heparin on fertilisation

Heparin was apparently without effect on the fertilisation calcium transient. Although heparin-injected eggs tended to be polyspermic this did not provide an explanation of this observation. Use of a lower concentration of sperm that allowed only one sperm to fuse with a heparin-injected egg did not significantly reduce the size of the fertilisation calcium transient. The failure of heparin then, to inhibit the calcium
changes cannot be explained by the actions of an increased number of sperm overcoming the inhibitory action of heparin.

It is surprising that heparin does not prevent the sperm-induced calcium increase when it so clearly antagonises the actions of Ins(1,4,5)P$_3$. To investigate this phenomenon further I looked more closely at the calcium increase considering it as two separate processes. Firstly a triggering process that is stimulated by the sperm and which triggers the second, regenerative process that is the wave of calcium release that sweeps across the egg and is a property of the egg (Whitaker & Irvine, 1984 and Swann & Whitaker, 1986).

(C) Triggering the wave of calcium release

During the latent period events are set in motion that lead to the triggering of the regenerative wave of calcium release. The latent period was first defined by Allen and Griffin (1958) as the time that fertilisation becomes insensitive to inhibition by lauryl sulphate to the initiation of the cortical reaction. Lauryl sulphate is believed to act by interfering with sperm motility and preventing the binding of sperm to egg (Presley & Baker, 1970). In the experiments presented here the latent period is considered as beginning with the sperm-induced depolarisation of the eggs membrane potential and ending with the start of the calcium wave. These are roughly equivalent definitions of the latent period as the first electrical change occurs within 3 seconds of sperm binding to the egg (Aarnia & Chambers, 1979).

I have looked at the relationship between the membrane potential changes at fertilisation and the increase in Ca$_i$ measured with fura2. The
fluorescent dye has the advantage that it is sensitive to changes in \( \text{Ca}_i \) at resting concentrations (Gryniewicz et al., 1985), unlike aequorin which was used in an earlier study addressing the same question (Eisen et al., 1984). I showed that the main increase in \( \text{Ca}_i \) was preceded by a much smaller increase that was coincident with the onset of the depolarisation of the egg's membrane potential. I showed that this smaller increase in \( \text{Ca}_i \) was a consequence of the depolarisation. The increase in \( \text{Ca}_i \) is probably the result of calcium influx from the voltage-dependent calcium current activated by the depolarisation induced by the sperm (David et al., 1988) and analogous to the calcium influx observed by Paul & Johnston (1978). It has subsequently been shown that this small calcium increase has the same voltage dependence as the calcium current (Takemoto, Crossley & Whitaker unpublished observation).

I used the small increase in \( \text{Ca}_i \) to measure the duration of the latent period and showed that heparin prolonged the latent period by a factor of 2.5.

The prolongation of the latent period by heparin suggests that the sperm triggers the calcium wave by stimulating the production of \( \text{Ins}(1,4,5)\text{P}_3 \). The production of \( \text{Ins}(1,4,5)\text{P}_3 \) need not be the primary event. It could be that the sperm stimulates some other event such as an increase in \( \text{Ca}_i \) which in turn causes the production of \( \text{Ins}(1,4,5)\text{P}_3 \). The data support the idea that \( \text{Ins}(1,4,5)\text{P}_3 \) is produced during the latent period.

(D) The regenerative wave of calcium release

To look at the effects of heparin on the second, regenerative process I looked at the time taken from beginning to the peak of the main calcium
increase. This interval has been shown to be similar in magnitude to the
time taken for the wave of calcium release to traverse the egg (Swann &
Whitaker, 1986). Here the results are not as clear-cut as the time taken
for the activation calcium wave to reach a peak was only just significant at
\( p < 0.05 \). The data tend to suggest that heparin delays the propagation of
the activation wave indicating that \( \text{Ins}(1,4,5)P_3 \) is involved in process. This
is consistent with the model of the calcium wave proposed by Swann &
Whitaker (1986) which has as its central feature the calcium stimulated
production of \( \text{Ins}(1,4,5)P_3 \). However, the wave of calcium that sweeps
across the egg at fertilisation is decremental in nature, as is the calcium
wave in other species (Allen & Rowe, 1958; Chamber & Hinkley, 1979 and
Swann & Whitaker, 1986). Under certain experimental conditions the
activation wave will fail to propagate across the egg. It is surprising then
that heparin has such a small effect on the fertilisation wave if calcium
generated \( \text{Ins}(1,4,5)P_3 \) release is the only propagative mechanism.

Busa et al. (1985) have suggested that calcium-induced calcium release
is responsible for the propagation of the calcium wave in \textit{Xenopus laevis}
eggs. Perhaps a similar mechanism is present in sea urchin eggs, although
unlike frog eggs (Cross, 1981), the calcium wave in sea urchin eggs cannot
be triggered by the microinjection of calcium buffers (Swann & Whitaker,
1986) or by the local application of calcium ionophore (Chambers &
Hinckley, 1979).

\( \text{(E)} \) The role of \( \text{Ins}(1,4,5)P_3 \) as a second messenger at fertilisation

These results show that heparin does have an effect on the fertilisation
calcium transient but the effect is more subtle than might have been
expected. As heparin inhibits the ability of Ins(1,4,5)P₃ to release calcium from internal stores it is tempting to suggest that this is the reason for the observed effects. However heparin has also been shown to inhibit the interaction of receptors with G-proteins (Salomon et al., 1978 and Willuweit & Aktoris, 1988) and this might provide an alternative explanation for the prolongation of the latent period. Experiments presented here though suggest that the sperm does not utilise a G-protein, so making this explanation less likely.

Heparin produces only a small effect on the main increase in Ca⁺, which suggests that Ins(1,4,5)P₃ may not be the only or even the major intracellular messenger responsible for the calcium release at fertilisation. Alternatively it is possible that much larger quantities of Ins(1,4,5)P₃ are generated than are required to cause the calcium increase. The subtle effects observed with heparin then may reflect the greater time required to generate the larger amounts of Ins(1,4,5)P₃ needed to overcome the block to calcium release. Unfortunately there have been no experiments to determine the quantities of Ins(1,4,5)P₃ produced at fertilisation which would resolve this issue. Ciapa and Whitaker (1986) have suggested that the concentration of Ins(1,4,5)P₃ may reach 0.5–5 μM a figure that agrees closely with the 1 μM concentration of Ins(1,4,5)P₃ required to cause maximal release of calcium from sea urchin egg preparations (Clapper & Lee, 1985 and Whalley & Whitaker, unpublished observations). It is also similar to the concentrations of Ins(1,4,5)P₃ measured in other cells (Irvine, 1989). Also, studies of sea urchin lipids indicate that the instantaneous hydrolysis of all the PIP₂ in a sea urchin egg to produce Ins(1,4,5)P₃ would result in a concentration of 6 μM (Turner et al., 1984). All these figures
are entirely consistent with Ins(1,4,5)P₃ being the messenger causing calcium release at fertilisation. Heparin however shifts the dose–response curve for the Ins(1,4,5)P₃–induced calcium transient to 20 fold higher concentrations of Ins(1,4,5)P₃. While the dose–response curve is not a strict measure of the absolute concentration of Ins(1,4,5)P₃ required to release calcium, it is reasonable to suppose that 20 times more Ins(1,4,5)P₃ is required to maximally release calcium. So from Clapper and Lee’s data (1985) a concentration of 20 μM would now be required to cause maximal calcium release. The increase in Caᵢ can not now be explained solely by the production of Ins(1,4,5)P₃ which would only reach a concentration of 0.5–5 μM. This argument is not entirely conclusive as the thumbnail calculation does not take into account the possibility of turnover of PPI, which would allow greater quantities of Ins(1,4,5)P₃ to be produced than a calculation of the total amount of PIP₂ present in the egg suggests.

The evidence presented here is not wholly consistent with Ins(1,4,5)P₃ being the only messenger responsible for the calcium increase at fertilisation. Clearly mass assays of inositol phosphate production at fertilisation are required to resolve this issue.

7.3 G–PROTEINS AND EGG ACTIVATION

(A) The actions of GDPβS in sea urchin eggs

In chapter 5 I investigated the possibility that a G–protein might be involved in the process of egg activation. Perhaps the most convincing evidence that a G–protein plays a role in egg activation are experiments
showing that GDP\(\beta\)S inhibits fertilisation (Turner et al., 1986). Unlike GTP\(\gamma\)S and cholera toxin which cause egg activation and thus demonstrate the presence of a G-protein, the use of GDP\(\beta\)S addresses the question of whether the sperm utilise this signal transduction pathway to activate an egg. I confirmed the observation of Turner et al. (1986) that GDP\(\beta\)S inhibited the ability of sperm to stimulate FE elevation. However GDP\(\beta\)S failed to inhibit either sperm/egg fusion or the sperm-induced increase in Ca\(_i\). Additionally the microinjection of GDP\(\beta\)S caused an increase in Ca\(_i\) similar to the levels observed at fertilisation while failing to stimulate FE elevation. It was not clear how GDP\(\beta\)S caused this calcium increase. It was not a result of calcium contamination or the actions of the major contaminants of GDP\(\beta\)S. A similar effect of GDP\(\beta\)S has been observed by Miyazaki (1988) in hamster eggs where Ca\(_i\) can be measured indirectly as a hyperpolarisation of the eggs membrane potential. In hamster eggs the microinjection of GDP\(\beta\)S frequently caused a hyperpolarisation.

These results are very different from those of Turner et al. (1986). However more recently this group has been unable to overcome the effect of GDP\(\beta\)S on FE elevation by microinjecting Ins(1,4,5)P\(_3\) (Jaffe, unpublished observations). This too suggests GDP\(\beta\)S inhibits FE elevation rather than the generation of Ins(1,4,5)P\(_3\).

Clearly the actions of GDP\(\beta\)S are complex being both stimulatory and inhibitory, stimulating an increase in Ca\(_i\) while inhibiting FE elevation. These experiments suggest that GDP\(\beta\)S inhibits the process of exocytosis, itself rather than the process by which the sperm generates the signals that are responsible for egg activation. The data do not support the idea that the sperm activates an egg through a simple G-protein mechanism.
(B) A comparison of the actions of GTPyS and sperm

Chapter 5 also dealt with experiments using the guanine nucleotide GTPyS. GTPyS activated eggs with 100% activation occurring with a concentration of 150 μM. While this concentration is greater than that previously published (Turner et al., 1986), some variability in the sensitivity of eggs to GTPyS has recently been reported and in some cases concentrations as high as 100 μM were required (Jaffe, 1989).

There was a delay between the microinjection of GTPyS and egg activation that was dose dependent. Ca, remained low during this delay which suggests that GTPyS activates eggs through some intermediate molecule.

The calcium changes caused by GTPyS closely resemble those that occur at fertilisation both in magnitude and duration. This is an important consideration for if egg activation by a sperm involves a G-protein then the calcium changes caused by activating the G-protein mechanism should resemble those of fertilisation. This is illustrated by considering activation of eggs by another parthenogenetic agent, Ins(1,4,5)P₃. The microinjection of Ins(1,4,5)P₃ causes a calcium transient similar in magnitude to that seen at fertilisation but considerably shorter (Swann & Whitaker, 1986), even when the quantity of Ins(1,4,5)P₃ injected is much greater than that required to cause activation (Figure 3.7). It is unlikely then, even though a sperm contains sufficient Ins(1,4,5)P₃ to activate an egg (Iwasa, et al., 1989), that a sperm activates an egg simply by introducing Ins(1,4,5)P₃. Unlike the calcium changes induced by other parthenogenetic agents, notably Ins(1,4,5)P₃, GTPyS-induced calcium changes also show a similar dependence upon external calcium to the fertilisation calcium changes. The early phases of both
responses are independent of external calcium while the later phases, at times greater than 3 minutes, show some dependence.

Fluoride ions, also potent G–protein activators, activate sea urchin eggs, causing Ca\textsubscript{i} changes very similar to those seen with GTP\textsubscript{yS}. This provides another indication of the presence of a potent G–protein mechanism in the egg.

However, here the similarities between the actions of GTP\textsubscript{yS} and sperm ends. While these two agents cause very similar changes in Ca\textsubscript{i}, they do so by different mechanisms. In the sea urchin egg, as described in Chapter 1, DAG, one of the products of PPI hydrolysis, activates a Na\textsuperscript{+}/H\textsuperscript{+} exchanger through Protein Kinase C to cause an increase in pH\textsubscript{i}. Whalley and Whitaker (1988) showed that GTP\textsubscript{yS} is able to stimulate an increase in pH\textsubscript{i} in eggs which had been pre–injected with the calcium chelator BAPTA to prevent any increase in Ca\textsubscript{i}. This effect was enhanced by the DAG–kinase inhibitor R59022 suggesting that it is the result of DAG production and so presumably PPI hydrolysis. I have shown that sperm, on the other hand, are not able to cause an increase in pH\textsubscript{i} under similar experimental conditions. These experiments indicate that GTP\textsubscript{yS} is able to stimulate PPI hydrolysis and an increase in pH\textsubscript{i}, independent of an increase in Ca\textsubscript{i}, while sperm cannot stimulate an increase in pH\textsubscript{i} without an increase in Ca\textsubscript{i}.

There are two possible explanations for these observations. Firstly, that the actions of sperm and GTP\textsubscript{yS} are different: this explanation is not consistent with a G–protein model of egg activation. Secondly, it is possible that GTP\textsubscript{yS} is a much more potent G–protein activator than sperm and so GTP\textsubscript{yS} is able to cause an increase in pH\textsubscript{i} while sperm cannot, despite the fact that they both stimulate the same pathway. The second
explanation seems unlikely though because of the experiments done with heparin, an inhibitor of Ins(1,4,5)P$_3$-induced calcium release. Heparin inhibited the ability of GTPyS to cause an increase in Ca$_i$, but did not prevent sperm from causing a calcium transient that was indistinguishable in magnitude from the control fertilisation transient.

(C) G-proteins and fertilisation

To summarise the results of experiments with guanine nucleotides do not support a G-protein model of egg activation. Firstly I showed that GDPβS, an inhibitor of G-protein activation, did not prevent the sperm-induced increase in Ca$_i$. The actions of this nucleotide are however complex because GDPβS has other effects most notably the inhibition of FE elevation. It is interesting to speculate that the reason the calcium increase caused by GDPβS fails to stimulate FE elevation is that the process of exocytosis itself involves a G-protein. There is a precedent for this in neutrophils and mast cells where Barrowman et al. (1986) and Howell et al. (1987) have suggested that there is a G-protein associated with the exocytotic process, although clearly the evidence in sea urchins is very preliminary.

Secondly GTPyS, while causing egg activation and calcium responses very similar to those seen at fertilisation, differs in its actions from sperm in two respects: the nucleotide, unlike sperm, causes an increase in pH$_i$ independently of an increase in Ca$_i$, but the nucleotide, again unlike sperm, is unable to activate a heparin-injected egg. These two observations together exclude the possibility that GTPyS and sperm stimulate the same signal transduction pathways but with different potencies. If one is a better
stimulus of the signal transduction pathway than the other, then that stimulus should be a more potent in both experiments. This is clearly not the case. The experiments suggest that GTPyS and sperm stimulate different pathways and so argue against a simple G−protein model to explain egg activation.

An assumption that is implicit in these conclusions, and indeed one that is often made in other areas of PPI research, is that PPI hydrolysis is the only source of DAG. In order to compare the experiments measuring pH, with those using heparin and measuring Ca_i it is essential to the argument that the Ins(1,4,5)P_3 and DAG arise from the same source in both cases. Recent work suggests that this is not always so. DAG may be produced independently of Ins(1,4,5)P_3 by the hydrolysis of choline phospholipids, specifically phosphatidyl choline (PC) (Loffelholz, 1989). Coradetti et al. (1982, 1985) first showed that acetylcholine stimulated choline production from a phospholipid pool, indicating that they may be utilised in signal transduction. DAG can be produced from PC either by the direct action of a PC−specific phospholipase C, or by a two step pathway where phospholipase D (PLD) cleaves PC to produce choline and PA, which is dephosphorylated by a PA phosphatase to produce DAG (Loffelholz, 1989). In liver cells, vasopressin and GTPyS both stimulate the production of PA which clearly preceded accumulation of DAG (Bocckino et al., 1987) suggesting the presence of a receptor−linked PLD. A receptor linked PC−specific phospholipase C might also be present in hepatocytes as ATP, presumably acting through a purinergic receptor, enhanced GTPyS stimulated production of phosphocholine (Besterman et al., 1986).

Analysis of the DAG produced in some stimulated cells indicates that
perhaps 80% of it arises from PC (Bocckino et al., 1985 and Martinson et al., 1989). If a similar effect were present in sea urchin eggs then this might explain the observations here. GTPyS would stimulate a greater production of DAG than sperm, and so cause a measurable pH change, while it might cause less Ins(1,4,5)P$_3$ production than sperm and thus fail to activate a heparin-injected egg.

In some cells where DAG production has been compared with PPI hydrolysis a biphasic production of DAG has been observed, the early phase of which correlates well with PPI hydrolysis and the later phase of which does not (Griendling et al., 1986 and Matozaki & Williams, 1989), but correlates with the production of choline and its metabolites instead (Matozaki & Williams, 1989). In sea urchin eggs however, where inositol phospholipids make up a much greater proportion of the total phospholipids than in most other cell types (Kinsey et al., 1980), the time course of InsP$_3$ production closely mirrors that of DAG (Ciapa & Whitaker, 1986). This suggests that the PC pathway does not make a significant contribution to DAG production in sea urchin eggs.

Also a single sperm, in contrast to GTPyS, is able to activate a heparin-injected egg. Were these two agents to stimulate PPI hydrolysis using the same pathway then one would have to conclude that sperm were a more potent stimulus than GTPyS. It would be surprising then if 10 sperm, the average number of sperm that fuse with a BAPTA-injected egg, were unable to stimulate sufficient PPI hydrolysis and DAG production to cause a detectable change in pH. This is especially so since any DAG resulting from PPI hydrolysis might be expected to activate Protein Kinase C and stimulate PC hydrolysis in this way (Besterman et al., 1986 and
In conclusion it can be said that sperm do not utilise the same signal transduction pathways as GTP\(_\text{yS}\). Together with the evidence that GDP\(_\beta\)S does not inhibit fertilisation this suggests that sperm do not activate sea urchin eggs using G–protein mechanism.

### 7.4 THE ACTIONS OF ATP\(_\text{yS}\)

To investigate the role that phosphorylation might play in the fertilisation process I used the analogue of ATP, ATP\(_\text{yS}\). This compound is a substrate for certain kinases but, unlike ATP, the thio–phosphate group is resistant to the action of phosphatases. ATP\(_\text{yS}\) then, can be a useful tool to probe processes regulated by phosphorylation. The irreversible phosphorylation caused by ATP\(_\text{yS}\) will cause an irreversible activation or inhibition of the appropriate process providing a clear indication that phosphorylation plays a regulatory role (Gratecos & Fischer, 1974).

ATP\(_\text{yS}\) failed to inhibit the fertilisation calcium changes suggesting that if a kinase is involved in the activating process it cannot utilise ATP\(_\text{yS}\) as a substrate. Interestingly however despite the fact that Ca\(_i\) increases to levels sufficient to stimulate exocytosis in eggs injected with ATP\(_\text{yS}\), the eggs fail to elevate FE\(_s\). The simplest explanation of these observations is that ATP\(_\text{yS}\) is inhibiting the process of exocytosis. More direct evidence that this is the case has come from experiments on isolated sea urchin egg plasma membranes or cortices. It is possible to isolate the sea urchin egg secretory apparatus by sticking eggs to glass coverslips with poly-L-lysine and shearing away the bulk of the eggs with hydrostatic pressure. The fragments of plasma membrane and egg cortex with associated cortical granules that
remain are secretion competent, being sensitive to calcium concentrations in
the physiological range (Vacquier, 1975 and Baker & Whitaker, 1978). The
so called cortical preparation provides access to the secretory apparatus
allowing direct and complete control of what is effectively the intracellular
space. It is thus a useful tool for the in vitro study of exocytosis. Crossley
et al. (1989) have shown that ATPyS inhibits exocytosis in this system.

Changes in the state of protein phosphorylation are thought to regulate
exocytosis in a number of cells, including mast cells, Paramecium and
adrenal chromaffin cells. In mast cells ATP, but not its non hydrolysable
analogue AppNHp, inhibited the onset of exocytosis (Tatham and Gomperts,
1989). Exocytosis in Paramecium is closely associated with a
dephosphorylation reaction (Gilligan & Satir, 1982 and Zieseness & Plattner,
1985) and can be triggered both in vivo and in vitro by exogenous
phosphatases (Momayezi et al., 1987). Finally in adrenal chromaffin cells
it is possible to inhibit secretion with ATPyS (Brooks et al., 1984). These
observations contradict the general finding that metabolic inhibition or the
removal of ATP inhibits secretion (Knight, 1988). However this seems to
be a another distinct action of ATP which is permissive for secretion and
involves maintenance of the exocytotic apparatus in a secretion competent
state. In mast cells for example secretion can occur in vitro in the absence
of ATP but is inhibited in vivo by metabolic inhibitors because ATP is
required to maintain the phosphoinositide pools that are involved in the
signal transduction processes that trigger secretion (Cockcroft et al., 1987).

To this list can now be added sea urchin eggs, where ATPyS inhibits
the cortical reaction both in vivo and in vitro. Recently we have shown
that $^{35}$S–ATPyS labels two proteins in sea urchin egg cortices (Whalley,
Crossley & Whitaker unpublished obervation) and it is tempting to suggest that one or both of these proteins is involved physiologically in controlling exocytosis. These proteins are of a different molecular weight than the 65 kd protein in *Paramecium tetraurelia* (Zieseness & Plattner, 1985) and the 43 kD protein in chromaffin cells (Brooks & Brooks, 1985) that are phosphorylated and thought to be involved in exocytosis. It will be interesting to determine what role these proteins play in controlling exocytosis and what their relationship is to the proteins in other cells.

### 7.5 ALTERNATIVE MODELS OF EGG ACTIVATION

Experiments discussed in this thesis do not support a simple G-protein model of egg activation and so alternative models must be considered.

The results presented here do not exclude some sort of receptor model to explain egg activation. While many hormones stimulate PPI hydrolysis by a mechanism that probably involves a G-protein (Cockcroft & Stutchfield, 1988 and Casey & Gilman, 1988) others, notably the growth factors Epidermal Growth Factor (EGF) and Platelet Derived Growth Factor (PDGF), stimulate PPI hydrolysis by different pathway (Boyer et al., 1989 and Pandiella et al., 1989).

Studies of these growth factor receptors has shown that they differ from other second messenger receptors that are linked to PPI hydrolysis. Growth factor receptors contain an intrinsic tyrosine kinase activity and have little amino acid homology between other receptors that stimulate PPI hydrolysis (Boyer et al., 1989). Nonhydrolysable analogues of GTP do not synergise with EGF to stimulate PPI hydrolysis in contrast to hormones
which stimulate PPI hydrolysis through a G-protein pathway (Pandiella et al., 1989). Also there is considerable evidence to indicate that EGF receptors directly phosphorylate an isozyme of PIC (Meisenhelder et al., 1989; Boyer et al., 1989 and Pandiella et al., 1989). While it has not been rigorously demonstrated that this tyrosine phosphorylation controls the activity of the enzyme this seems a likely mechanism. It is possible then that sperm, like the growth factors EGF and PDGF, could stimulate PIC through a receptor with intrinsic kinase activity.

The other proposed mechanism to explain egg activation involves the fusion of the sperm with egg and the introduction of an activating factor into the egg. This idea has recently received support from cross-fertilisation studies between Strongylocentrotus nudus sea urchin eggs and Asterina pectinefera starfish sperm. When the eggs have their vitelline layers removed by trypsin treatment and are inseminated by acrosome-reacted starfish sperm they fail to activate. However EM studies show that the egg surface engulfs the sperm without fusion occurring (Kyozuka & Osani, 1988). Treatment of this sperm/egg mixture with polyethylene glycol however allowed sperm/egg fusion and resulted in egg activation with cell division almost to levels seen with sea urchin sperm (Kyozuka & Osani, 1989). Polyethylene glycol is a fusogenic molecule and has been used to promote cell/cell fusion in a number of different cell types. A possible interpretation of these experiments is that the polyethylene glycol promotes the fusion of the sperm with the eggs and it is this that results in egg activation.

In considering the fusion model of egg activation it is interesting to consider potential candidates for an activating substance. For reasons
discussed earlier it is unlikely that Ins(1,4,5)P_3 is the activating substance because the calcium changes caused by the injection of Ins(1,4,5)P_3 do not resemble those of fertilisation: they are too short. It is possible though, that some protein such as PIC introduced by the sperm which generates Ins(1,4,5)P_3 could be responsible for egg activation. Two other possible candidates however are smaller molecules with potential 2^nd messenger functions. These are cGMP and cyclic ADP-ribose, which is a metabolite of pyridine nucleotides that has been shown to have calcium mobilising activity in a sea urchin egg system (Clapper et al., 1987).

Firstly cGMP has been shown to activate not only sea urchin eggs (Swann et al., 1984) but medaka eggs also (Iwamatsu et al., 1988). The calcium changes caused by the injection of cGMP into sea urchin eggs resemble the calcium changes seen at fertilisation both in magnitude and duration. Sea urchin sperm contain large quantities of guanylate cyclase (Gray et al., 1976) and this enzyme is transiently activated by egg peptides (Bentley et al., 1986). This is therefore a potential mechanism of egg activation which warrants further investigated particularly since cGMP does not cause an increase in pH, in eggs preinjected with BAPTA: in this respect the actions of cGMP more closely resemble those of sperm than GTPyS which does cause an increase in pH, under the same experimental conditions (Whalley & Whitaker, 1988). However the quantities of cGMP produced within the sperm (Kopf, et al., 1979) are probably not sufficient to activate an egg.

Finally it is worth considering a metabolite of NAD, cyclic ADP-ribose. Clapper and Lee (1987) showed that a microsomal preparation of sea urchin eggs which responded to Ins(1,4,5)P_3 by releasing calcium also
responded to the addition of NAD and NADP in the same way. They demonstrated that the pyridine nucleotides released calcium from stores functionally distinct from the \( \text{Ins}(1,4,5)\text{P}_3 \)–sensitive store. The action of NAD was not direct but due to its metabolism by a high molecular weight factor in the microsomal preparation to form cyclic ADP–ribose. This compound is as active as \( \text{Ins}(1,4,5)\text{P}_3 \) in releasing calcium and many tissues are capable of metabolising NAD to cyclic ADP–ribose (Ruskindo & Lee, 1989). It is not clear yet whether cyclic ADP–ribose plays a physiological role as an internal messenger but clearly it has the potential to be a messenger at fertilisation. None the less the calcium changes caused by its microinjection (Clapper & Lee, 1987) into sea urchin eggs resemble those of \( \text{Ins}(1,4,5)\text{P}_3 \) and it seems unlikely then that the sperm simply activates an egg by introducing cyclic ADP–ribose.

7.6 Concluding Remarks

The title of this thesis is "How the sperm triggers the fertilisation calcium wave". In Chapter 3 I showed that the sperm does not trigger the calcium wave by gating external calcium and it was unlikely that any other inositol phosphate other than \( \text{Ins}(1,4,5)\text{P}_3 \) alone could be responsible for egg activation. The experiments of Chapter 4 called into question the precise role of \( \text{Ins}(1,4,5)\text{P}_3 \) as a messenger at fertilisation. Although the prolongation of the latent period by heparin indicate that \( \text{Ins}(1,4,5)\text{P}_3 \) is produced during the latent period. The experiments of Chapter 5 suggest that the sperm does not utilise a \( G \)–protein to trigger egg activation. Indeed the data suggests that PPI hydrolysis is not the primary event and that some other event, perhaps an increase in \( \text{Ca}^{2+} \), is required to cause PPI
hydrolysis and egg activation.

The data in this thesis can be used to construct a model of how a sperm triggers the fertilisation calcium wave. The fusion of the sperm with the egg results in the introduction of an activating substance. With present data the best of the possible candidates is cGMP, or a cGMP generator such as guanylate cyclase. cGMP acts to bring about an increase in $Ca_i$ by an unknown mechanism, which must be independent of PPI hydrolysis because BAPTA blocks both the ability of sperm, and cGMP, to cause an increase in $pH_i$ (an indirect measure of PPI hydrolysis). The same experiments with BAPTA indicate that the increase in $Ca_i$ is required for the stimulation of PPI hydrolysis, perhaps through the calcium–sensitive PIC. The finding that heparin prolongs the latent period shows that PPI hydrolysis, with the attainment of a critical level of $\text{Ins}(1,4,5)P_3$, is required to trigger the fertilisation calcium wave. Propagation of the wave, and therefore egg activation, is ensured by this model as the production of $\text{Ins}(1,4,5)P_3$ will release calcium and stimulate further $\text{Ins}(1,4,5)P_3$ production. However it is also possible that a calcium–induced calcium release mechanism, such as the two–store model proposed by Berridge and Irvine (1989), may be involved in wave propagation.

This model of egg activation is distinct from another model (Turner et al., 1986), involving receptor/G–protein interaction. The data presented here is not consistent with this model. While this model incorporates the available data, more work needs to be done on the earlier events in the activation sequence, the identity of the activating substance and its mechanism of action need to be defined.
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