PROPERTIES OF THE NICOTINIC ACID ADENINE DINUCLEOTIDE PHOSPHATE-BINDING PROTEIN IN SEA URCHIN EGGS

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ABSTRACT

Nicotinic acid adenine dinucleotide phosphate (NAADP) has recently emerged as a novel intracellular calcium mobilising messenger in a variety of cells. Whilst increasing evidence suggests that NAADP acts on a distinct binding protein, little is known regarding the biochemical properties of the putative NAADP "receptor". My thesis investigates properties of the NAADP-binding protein in sea urchin eggs.

Firstly, I show that NAADP binding to its target protein is inhibited by altering the protein:lipid ratio of soluble sea urchin egg homogenates – an effect prevented and reversed specifically by addition of exogenous phospholipids. These data highlight the importance of the lipid environment in maintenance of NAADP binding to its target protein.

In addition, I show that upon binding its ligand, the NAADP-binding protein undergoes an unusual stabilization process that is dependent upon the time the receptor is exposed to its ligand. This property endows the NAADP-binding protein with the extraordinary ability to detect the duration of its activation.

Finally I describe the development of a highly sensitive radioreceptor assay (based upon the sea urchin egg NAADP-binding protein) that is capable of detecting low levels of NAADP from cellular extracts. I apply this technique to determine NAADP levels in a variety of extracts prepared from cells under resting and stimulated conditions.

DECLARATION

I, Dev Churamani, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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"If you are first you are first. If you are second you are nothing."

Bill Shankly

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CHAPTER 1 Introduction

1.1 Calcium signalling - An overview

Numerous cellular events occur as a direct result of changes in the cytosolic calcium concentration (Berridge et al., 2000). Calcium, a ubiquitous signalling ion, is rather extraordinary as it is capable of producing opposing effects often within the same cell. For example, the onset of fertilisation is accompanied by a localised increase in calcium at the site of sperm-egg fusion. This concentrated bolus of calcium then spreads across the egg as a "wave", signalling the start of a variety of cellular events leading to the development of a complex organism (Berridge, 1993). Conversely, changes in intracellular calcium also regulate the rather complicated series of events involved in programmed cell death (Hanson et al., 2004). It is now understood that a simple rise in cytosolic calcium is not sufficient to account for the multitude of cellular responses dependent on calcium. To understand how calcium is able to regulate such a large number of different events, one must consider how calcium signals are generated and presented within a cell.

The calcium signal generated can range from localised calcium transients to repetitive calcium oscillations organized as global calcium waves (Berridge et al., 2000;Bootman et al., 2002;Bootman et al., 2001). These different calcium signals are produced and maintained by an array of different molecules (Berridge et al., 2003). Considering all the possible components that regulate calcium within a cell, one can start to envisage a scenario where recruitment of different cellular factors can generate a multitude of calcium "signatures" resulting in a vast array of cellular responses.

Changes in the intracellular calcium concentration occur either by influx from the extracellular space across the plasma membrane, release of calcium from intracellular calcium stores or more commonly via a combination of both these events (Berridge et al., 2003). The calcium channels involved can be sub-divided into distinct families: ion channels activated by membrane depolarisation (voltage-operated channels), ion channels that open in response to direct binding of ligands (ligand-gated channels) and store-operated channels that open in response to intracellular calcium store depletion (Berridge et al., 2000).

1.2 Calcium Channels

Voltage-operated calcium channels

The voltage-operated calcium channels (VOCC), as the name suggests are modulated by changes in voltage and at resting membrane potential are closed. VOCCs have an intrinsic ability to detect changes in voltage by use of a "voltage sensor" (Catterall, 2000) and depolarisation of excitable cells leads to their opening. The different VOCCs can be classified into two groups depending on their voltage dependence. Broadly speaking VOCCs are divided into low and high threshold activated channels. An example of a low-threshold activated calcium channel is the T-type channel (Perez-Reyes, 2003). Cardiac T-type currents were initially described in dog atrium (Bean, 1985) and have been proposed to be involved in the pacemaker current of diastolic depolarisation (Hagiwara et al., 1988). In contrast, N-type and P/Q-type calcium channels open as a result of stronger depolarisations compared to T-type calcium channels. Influx of calcium through the N-type and P/Q-type sub family of calcium channels contributes to neurotransmitter release (Catterall, 1998).

Ligand-gated calcium channels

Ligand-gated calcium channels (LGCC) upon binding their ligand become permeable to ions. Most LGCCs are non-selective cation ion channels (Krusek et al., 2004). An example of a LGCC is the N-methyl-D-aspartate (NMDA) receptor, a target for glutamate (Dingledine et al., 1999). Upon binding glutamate, NMDA receptors become permeable to calcium ions as well as monovalent cations. These channels play an important role in excitatory neurotransmission (Kemp and McKernan, 2002).

The IP_3 receptor

LGCCs are also present within the cell. A classical example is the inositol trisphosphate receptor (IP_3R), located predominately on the endoplasmic reticulum, an established calcium storing organelle (Berridge, 1993). Solubilization and purification of the IP_3R was first described from rat cerebellar membranes, an abundant source of the protein and its size approximated to be 260 kDa (Supattapone et al., 1988). Shortly after this purification, full cloning was achieved (Furuichi et al., 1989). Electron microscopy studies of the IP_3R isolated from aortic smooth muscle indicated four-fold symmetry consistent with its tetrameric structure and native molecular weight of ~ 1000 kDa (Chadwick et al., 1990). IP_3 is required to sequentially bind all four binding sites of the IP_3R tetramer in order to open the channel (Berridge, 1993). IP_3 binding leads to a large conformational change in the protein resulting in the opening of the channel, allowing movement of calcium from the calcium store into the cytosol of the cell. Binding sites for IP_3 exist on the cytosolic N-terminus of the IP_3R (Mignery and Sudhof, 1990) and as the IP_3R traverses the membrane six times, the C-terminus is also present in the cytosol of the cell (Michikawa et al., 1994). Different isoforms of the IP_3R exist displaying distinct affinities for IP_3 suggesting that each IP_3R may

control specific cellular functions (Newton et al., 1994;Patel et al., 1999). The IP_3R also possesses a "regulatory domain" allowing it to be modulated by calcium, accessory proteins and phosphorylation by several kinases (Patel et al., 1999). Regulation of the IP_3R by calcium is biphasic (Iino, 1990;Bezprozvanny et al., 1991) and particularly important since it allows released calcium to control further release by calcium-induced calcium release (CICR) (Iino and Endo, 1992;Berridge, 1993;Patel et al., 1999).

The ryanodine receptor

Another intracellular LGCC is the ryanodine receptor, a specialized calcium channel that exists on the (sarco)endoplasmic reticulum (SER). Upon ryanodine receptor channel opening, calcium is able to move from the SER into the cytosol of the cell. The ryanodine receptor was initially solubilized and partially characterised from rabbit skeletal muscle (Pessah et al., 1986). Purification and reconstitution of the ryanodine receptor revealed a high molecular weight protein (approximately 400 kDa) which, when incorporated into planar lipid bilayers displayed pharmacological characteristics of the native channel (Lai et al., 1988). Electron microscopy revealed structures similar in shape to the four-leaf clover described for the IP_3R (Lai et al., 1988). Indeed, the native molecular weight of the ryanodine receptor is ~1600 kDa (Lai et al., 1988), almost twice the size of the IP_3R complex. The ryanodine receptor was subsequently cloned from rabbit fast-twitch skeletal muscle (Marks et al., 1989). Ryanodine binding sites have since been located to the C-terminus of the channel (Callaway et al., 1994). Binding of ryanodine leads to a change in diameter of the pore, thus actively modulating the channel (Fill and Copello, 2002).

The ryanodine receptor is modulated by many factors, however its interaction with calcium is likely the most significant (Fabiato, 1985;Fill and Copello, 2002). Ryanodine receptors possess binding domains for calcium, allowing for direct interactions (Fill and Copello, 2002) leading to calcium-induced calcium release (CICR). This process is important, since many cellular events requiring calcium do so some distance away from the origin of the trigger calcium release. Diffusion of calcium within cells is normally impeded by buffers. CICR overcomes this allowing a small trigger release of calcium to be amplified into larger calcium signals. In cardiac muscle cells for example, calcium entry through VOCCs induces further release of calcium from ryanodine receptors located on the SER, allowing the calcium signal to propagate through the cell (Berridge, 1993;Fill and Copello, 2002) leading to muscle contraction.

Sphingosine 1-phosphate activated "calcium channel"

Initial attempts to identify the intracellular molecular target for sphingosine 1-phosphate (an intracellular lipid signalling molecule) were made by Mao and colleagues. They 'discovered' a putative calcium release channel which was sensitive to sphingosyl-phosphocholine (Mao et al., 1996). This channel was termed SCaMPER (sphingolipid Ca^{2+} release-mediating protein of endoplasmic reticulum) and described as a 181 amino acid protein mediating sphingolipid-gated calcium release. The authors portrayed SCaMPER as a protein with two membrane spanning domains, in contrast to the six membrane spanning domains of ryanodine and IP_3 receptors, suggesting that SCaMPER belonged to a unique family of ion channels. However, following the initial description of SCaMPER, further confirmation of this novel ion channel was not forthcoming. Indeed, the next examination of this ion channel, some

years later, led to a different conclusion as to its identity. Using the reverse transcription polymerase chain reaction (PCR), Schnurbus and colleagues re-evaluated the structure of SCaMPER. The conclusions drawn by this group differed greatly from those made by Mao et al. The SCaMPER protein was in fact a 110 amino acid protein spanning the membrane only once (Schnurbus et al., 2002). Furthermore it was unlikely that SCaMPER, once synthesised, remained at the endoplasmic reticulum, since Schnurbus and colleagues showed the migration of SCaMPER away from this organelle. Sphingolipids had no affinity for the SCaMPER protein and finally, over-expression of this protein was toxic. However, controversy still remains regarding the intracellular molecular target for sphingolipids with respect to calcium signalling. Adding to the pool of information surrounding SCaMPER, a recent study expressed and characterised SCaMPER in cardiac myocytes (Cavalli et al., 2003). Though acknowledging the original sequence, determined by Mao and colleagues to be incorrect, Cavalli et al still suggest SCaMPER to be a single-pass membrane protein likely to be either a calcium channel or calcium channel modulator. With the information available at present, it is difficult to confirm or refute the role of SCaMPER in calcium signalling. Much more work is required before the actual intracellular molecular target of sphingolipids is positively identified.

The polycystin-2 calcium channel

Autosomal-Dominant Polycystic Kidney Disease (ADPKD) affects 1 in 400-1000 live births and is the most common genetic cause of kidney failure in man (Arnaout, 2001). ADPKD has been linked to mutations in either of two membrane spanning proteins named polycystin-1 and polycystin-2 (Nauli et al., 2003). Mutations in the genes encoding for these proteins result in growth of fluid-filled cysts in the kidney,

liver and pancreas. Polycystin-1 and Polycystin-2 may interact directly with each other through cytosolic domains in the C-termini of both proteins and possibly form a heteromultimeric complex that functions as a receptor and an ion channel (Emmons and Somlo, 1999;Xu et al., 2003). It has been considered that polycystin-1 which has a large extracellular domain may function as a receptor transducing sensory information. Polycystin-2 however, has sequence similarity with TRP (transient receptor potential, discussed below) channels and studies have indicated that it can form an ion channel when expressed exogenously or when reconstituted into lipid bilayer membranes (Hanaoka et al., 2000;Gonzalez-Perrett et al., 2001). Koulen and colleagues have provided evidence that polycystin-2, is localised to the endoplasmic reticulum (Cai et al., 1999) and can function as an intracellular calcium release channel exhibiting behaviour similar to ryanodine and inositol trisphosphate receptors (Koulen et al., 2002).

Transient receptor potential channels

The TRP family of ion channels, located primarily on the plasma membrane, are permeable to many monovalent cations as well as calcium (Harteneck et al., 2000;Minke and Cook, 2002). They share structural similarity to voltage-gated channels (Padinjat and Andrews, 2004). TRP channels possess a conserved structure made up of six transmembrane domains, a pore forming region as well as possessing a voltage sensor (Minke and Cook, 2002). However, TRP channels are modulated by a variety of stimuli including pH, mechanical force, as well as interactions with ligands and cellular proteins (Clapham et al., 2001), and appear to be gated by the phosphatidylinositol transduction pathway (Montell et al., 2002) allowing calcium to enter the cell. Therefore, TRP channels have varied functions. First discovered in

Drosophila melanogaster (Cosens and Manning, 1969), TRP channels were shown to be required for visual transduction (Montell and Rubin, 1989;Hardie and Minke, 1992). Since then, TRP channels have been found in a multitude of cells from yeast to humans (Clapham et al., 2001;Clapham, 2003;Montell et al., 2002) and play important roles in development (Xu and Sternberg, 2003;Minke and Cook, 2002) as well as potentially being candidates for the store-operated calcium channels (Berridge, 2004;Clapham, 2003), discussed below.

Store-operated calcium entry channels

A major route for calcium to gain access into the cell is via store-operated calcium entry, also known as capacitative calcium entry (Parekh and Putney, Jr., 2005). This is a process whereby a fall in calcium concentration within calcium-storing organelles activates plasma-membrane calcium channels (Putney, Jr., 2001;Berridge, 2004). Capacitative calcium entry is thought to maintain calcium levels within intracellular calcium storing organelles, provide a source of calcium for prolonged, sustained intracellular calcium elevations and adjust calcium levels to maintain amplitude of calcium spikes (Parekh and Putney, Jr., 2005;Putney, Jr., 2001). The mechanism underlying capacitative calcium entry is unknown. It has been suggested that the endoplasmic reticulum releases a calcium influx factor (Randriamampita and Tsien, 1993) which modulates store operated calcium entry. Another suggestion is that IP_3Rs located in the endoplasmic reticulum couple with plasma membrane store-operated calcium channels - a process known as conformation coupling (Berridge, 2004). The exact nature of the store-operated calcium channel, as well the mechanism coupling calcium store depletion with calcium entry is unclear. Possible candidates for the

store-operated calcium channel are the TRP (transient receptor potential) channel family (Liu et al., 2000).

1.3 Calcium buffers and binding-proteins

A large proportion of calcium that enters the cytosol, either through ion channels located on the plasma membrane or through activation of intracellular calcium channels is buffered and it is these buffers (calbindin, parvalbumin) that can play a role in modulating the amplitude and duration of the calcium signal (Schwaller et al., 2002). A variety of calcium sensors including calmodulin, troponin C and synaptotagmin also exist allowing the calcium signal generated to be translated into functional responses (Chin and Means, 2000;Farah and Reinach, 1995). Upon binding calcium, these binding proteins undergo conformational changes allowing them to regulate downstream effectors. By having a multitude of calcium sensors, cells can respond to changes in intracellular calcium in many different ways.

1.4 Calcium pumps and exchangers

The calcium signal, once generated must eventually recede. To achieve this numerous pumps and exchangers exist that return the intracellular calcium concentration to resting levels (Berridge et al., 2003). Removal of calcium to the extracellular space is performed by the Ca^{2+}-ATPase pump (Shull et al., 2003) and the Na^{+}/Ca^{2+} exchanger (Blaustein and Lederer, 1999), both located in the plasma membrane. Alternatively, the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps calcium back into the internal calcium stores (East, 2000). Mitochondria are also capable of removing intracellular calcium, and as a result are important in modulating calcium signals (Duchen, 1999).

1.5 Calcium mobilising messengers

Mobilisation of calcium from intracellular calcium stores requires the production of intracellular calcium-mobilising messengers. The most common of these messengers, inositol 1,4,5 trisphosphate (IP_3), is generated via activation of receptors coupled to phospholipase C (PLC) (Berridge, 1993). IP_3 stimulates calcium release by binding to the inositol trisphosphate receptor (IP_3R) calcium channel which as mentioned is located predominately on the endoplasmic reticulum (ER) (Clapper and Lee, 1985).

The ER also houses ryanodine-sensitive calcium channels which are modulated by cyclic adenosine diphosphate ribose (cADPR), another established intracellular calcium-mobilising messenger (Lee, 2001;Galione and Churchill, 2000). This molecule is discussed in greater detail below.

Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most recently discovered intracellular calcium-mobilising messenger and the subject of this thesis (Lee, 2003). The molecular identity of the NAADP binding protein is as yet unknown, as is that for sphingosine 1-phospate (Section 1.2), another potentially important molecule in the control of calcium dynamics.

Inositol trisphosphate (IP_3)

Without doubt, the most studied intracellular calcium release pathway is that stimulated by the calcium mobilising messenger inositol 1,4,5 trisphosphate (IP_3) (Berridge, 1993). IP_3 is a metabolite of the lipid precursor phosphatidylinositol 4,5 bisphosphate (PIP_2). The calcium mobilising properties of IP_3 were first discovered in

pancreatic acinar cells providing an exciting link between extracellular stimuli and intracellular calcium mobilisation (Streb et al., 1983).

IP_3-induced calcium release

The breakthrough establishing IP_3 as a physiologically relevant calcium-mobilising messenger was made in the laboratories of Berridge and Schulz (Streb et al., 1983). IP_3 was shown to release calcium from a non-mitochondrial calcium pool in permeabilised pancreatic acinar cells. Addition of carbachol also induced calcium release from intracellular stores in this preparation. However, if carbachol was added after IP_3, the carbachol-induced calcium release was abolished suggesting that carbachol was releasing calcium from the same pool as IP_3. These data suggested that carbachol-induced calcium release was mediated by IP_3 (Streb et al., 1983). Calcium release induced by IP_3 was unaffected by mitochondrial inhibitors, suggesting IP_3 was releasing calcium from a separate calcium store. In sea urchin eggs, IP_3-sensitive calcium stores were shown to migrate with ER microsomes (Clapper and Lee, 1985). IP_3 has since been established as a messenger mediating calcium release in numerous systems (Berridge, 1993).

Metabolism of IP_3

IP_3 is synthesised via a well characterised pathway involving phospholipase C (PLC). Activation of PLC by G-protein coupled receptors, or tyrosine kinase coupled receptors results in the hydrolysis of PIP_2 yielding two signalling molecules IP_3 and diacylglyercol (DAG) (Berridge, 1993). DAG activates protein kinase C, whereas IP_3 mobilises calcium from intracellular calcium stores. The existence of various PLC isoforms activated via different transduction pathways (Rhee and Bae, 1997;Berridge

et al., 2003) may allow IP_3 to produce distinct calcium signatures within a cell resulting from the different dynamics of IP_3 production.

Metabolism of IP_3 occurs either via dephosphorylation by IP_3 5-phosphatase to yield inositol 1,4-bisphosphate (Storey et al., 1984) or through phosphorylation by IP_3 3-kinase to produce inositol 1,3,4,5-tetrakisphosphate (IP_4), another potential messenger regulating intracellular calcium (D'Santos et al., 1994).

Cyclic adenosine diphosphate ribose (cADPR)

Like IP_3, cyclic ADP-ribose (cADPR), a metabolite of the pyridine nucleotide NAD, is able to mobilise calcium from intracellular calcium stores. Hon Cheung Lee and co-workers first discovered the ability of cADPR to mobilise calcium in sea urchin egg microsomes (Clapper et al., 1987). Initial experiments showed that the pyridine nucleotides NAD and NADP were as effective as IP_3 in releasing calcium from the sea urchin egg homogenate system. IP_3-induced calcium release was rapid however, calcium release mediated by NAD occurred after a short delay (Clapper et al., 1987). Therefore it was suggested that NAD was first converted to an active form before releasing calcium. "Activated" NAD was purified by HPLC and its structure determined. The molecule was a cyclized form of adenosine diphosphate ribose (ADPR) and was given the name cyclic ADP-ribose (Lee et al., 1989). cADPR-induced calcium release was subsequently shown to proceed via activation of the ryanodine receptor (Galione et al., 1991).

cADPR-induced calcium release

Since the initial discovery, intracellular calcium mobilisation by cADPR has been demonstrated in a variety of systems from plants to humans (Guse, 2002;Lee, 2001;Galione and Churchill, 2000;Guse, 2004). Unlike IP_3 binding to the IP_3R however, no evidence exists to support direct binding of cADPR to the ryanodine receptor, the calcium channel likely modulated by cADPR (Galione et al., 1991). Although inhibitors of the ryanodine receptor such as ruthenium red block the action of cADPR (Fill and Copello, 2002), photoaffinity labelling demonstrates cADPR binding to a distinct protein, smaller than the ryanodine receptor (Walseth et al., 1993). A proposed binding protein is FKBP12.6, an immunophillin binding protein that is known to associate with the ryanodine receptor (Noguchi et al., 1997).

Regardless of its exact molecular target, cADPR is an important calcium mobilising messenger. In sea urchin eggs, cADPR-induced calcium signalling is involved in mediating the fertilisation wave (Lee et al., 1993;Galione et al., 1993). In addition, IP_3-activated calcium transients in activated T-cells are prolonged by cADPR (Guse et al., 1999).

In pancreatic acinar cells, the brain-gut peptide cholecystokinin (CCK) activates repetitive calcium spiking, similar to that seen by infusion of cADPR (Thorn et al., 1994). It appears that cADPR is required for CCK-induced calcium spikes since application of the specific antagonist 8-NH_2-cADPR blocked CCK-induced calcium spiking (Cancela and Petersen, 1998). This derivative of cADPR is the most potent antagonist described, and likely competes with cADPR by acting at the same binding site. However other antagonists of cADPR-induced calcium release such as ruthenium

red and procaine likely act on the ryanodine receptor to block calcium release induced by cADPR (Lee, 2000;da Silva and Guse, 2000;Galione and Churchill, 2002). That cADPR is now established as a physiologically important calcium-mobilising messenger, attention must focus on the exact nature describing how it modulates the ryanodine receptor calcium channel.

Metabolism of cADPR

At present, synthesis of cADPR is believed to be regulated by the enzyme ADP-ribosyl cyclase (Hellmich and Strumwasser, 1991;Lee and Aarhus, 1991). Early studies indicated that an NADase purified from *Aplysia californica* ovotestis synthesised as its primary product cADPR from NAD and not ADPR as expected (Hellmich and Strumwasser, 1991). The purified enzyme was a 29 kDa protein existing as multiple isoforms. This was the first description of a purified enzyme capable of synthesising cADPR. Conversion of NAD to cADPR by ADP-ribosyl cyclase was most effective in the presence of neutral / alkaline conditions (Aarhus et al., 1995). The discovery of ADP-ribosyl cyclase has provided a viable route for cADPR synthesis and hence a role in calcium signalling. ADP-ribosyl cyclase can also use NADP as a substrate to produce 2-phospho-cADPR (Vu et al., 1996) another molecule capable of mobilising calcium as well as NAADP (Aarhus et al., 1995) which will be discussed later.

Two mammalian homologues of ADP-ribosyl cyclase have since been identified, CD38 and BST-1 (Howard et al., 1993;Hirata et al., 1994). These enzymes share 20-30% sequence identity with the aplysia ADP-ribosyl cyclase (Lee, 1999). Most is known about CD38. CD38 was first described as a lymphocyte surface antigen but

has since been found in intracellular organelles (Lee, 2001). In both locations, the active site appears inaccessible to cytosolic NAD. Synthesis of cADPR would occur either extracellularly or within organelles and therefore unable to act upon its molecular target the ryanodine receptor. To overcome the "topological paradox" (DE Flora et al., 2004), evidence has been provided to suggest that CD38 can transport molecules across the membrane during the catalytic process (Franco et al., 1998). Another hypothesis is that upon binding NAD, the cyclase is internalised (Zocchi et al., 1996).

Remarkably, in addition to synthesising cADPR, CD38 and BST-1 are also capable of hydrolysing it to ADP-ribose (Howard et al., 1993;Hirata et al., 1994;Kim et al., 1993;Takasawa et al., 1993) In fact, cADPR has been proposed to be an allosteric modulator of the ADP-ribosyl cyclases (Guse, 2000).

ADP-ribosyl cyclase and its homologues are therefore rather remarkable in that they are able to synthesise four structurally distinct molecules using different substrates. That the products, cADPR, 2-phospho-cADPR, ADPR and NAADP all possess calcium-mobilising activity, suggests a pivotal role for ADP-ribosyl cyclases in calcium signalling.

Sphingolipids

Sphingolipids have recently emerged as important regulatory molecules, mediating a variety of cellular events including cell proliferation, apoptosis and calcium signalling (Young and Nahorski, 2002). The release of intracellular calcium by sphingolipids was first described by Ghosh and colleagues. Using a permeabilised smooth muscle

cell line, sphingosine, after a short delay, induced a large release of calcium from a pool also sensitive to IP_3 (Ghosh et al., 1990). Using enriched rough endoplasmic reticulum sub-fractions containing IP_3Rs, Ghosh and colleagues demonstrated that heparin (an IP_3R antagonist) had no effect on calcium release by sphingosine (Ghosh et al., 1994). A similar release of calcium was also induced by sphingosylphosphorylcholine, however this response was almost instantaneous. Because sphingosine-induced calcium release occurred after a lag, was temperature-dependent and also required the presence of ATP, Ghosh et al suggested that calcium release was in fact due to sphingosine being first converted by sphingosine kinase to sphingosine 1-phosphate which possessed the calcium release activity. Intriguingly, this discovery was similar to that of cADPR in sea urchin egg homogenates where NAD induced calcium release after a short delay due to its metabolic conversion (Clapper et al., 1987).

Sphingosine kinase conversion of sphingosine to sphingosine 1-phosphate was later confirmed by the same group where they demonstrated the release of intracellular calcium by sphingosine 1-phosphate without any delay (Ghosh et al., 1994).

Increasing evidence now suggests that sphingosine 1-phosphate is an intracellular calcium mobilising messenger, but the intracellular target for sphingosine 1-phosphate (as discussed in Section 1.2) still remains unclear. That IP_3 antagonists are without effect on calcium release mediated by sphingolipids (described above) suggests the existence of a distinct binding site.

Sphingosine 1-phosphate has the rather interesting property that it is able to signal at both the extra- and intracellular level. Extracellular receptors for sphingosine 1-phosphate have been identified as members of the seven transmembrane domain superfamily of G-protein coupled receptors (Lee et al., 1998). These receptors have been termed EDG (endothelial differentiation gene) 1, 3, 5, 6 and 8. Activation of these cell surface receptors can stimulate intracellular calcium release indirectly through the production of IP_3 (Spiegel and Milstien, 2003). However, as described previously, sphingosine 1-phosphate is able to mobilise intracellular calcium without prior activation of extracellular receptors. Discovery of these intracellular effects has led to the re-evaluation of some data obtained by external application of sphingolipids.

1.6 Nicotinic acid adenine dinucleotide phosphate (NAADP)

More recently, nicotinic acid adenine dinucleotide phosphate (NAADP) has emerged as a novel intracellular calcium mobilising messenger (Patel, 2004). It appears that this signalling molecule has properties distinct from the established calcium-mobilising messengers thus adding to the pool of components capable of modulating intracellular calcium. I will now discuss in detail properties of this rather *extraordinary* signalling molecule.

NAADP – A new player in intracellular calcium signalling

As mentioned earlier, observations from the laboratory of Hon Cheung Lee identified NAD and NADP as potential intracellular calcium mobilising agents. NAD-induced calcium release in sea urchin egg homogenates was attributed to its enzymatic conversion to cADPR (Clapper et al., 1987).

In contrast, calcium release induced by NADP was immediate (Clapper et al., 1987). NADP-induced calcium release was subsequently shown to be the result of a contaminant present in the commercially available preparations. This contaminant was later identified as nicotinic acid adenine dinucleotide phosphate (NAADP) (Aarhus et al., 1995;Chini et al., 1995), and thus spawned a decade of research into the calcium mobilising properties of this novel intracellular calcium-mobilising messenger (Churchill et al., 2003;Patel, 2004;Yamasaki et al., 2005a;Lee, 2005).

Initial work in establishing the properties of NAADP was conducted in the sea urchin egg, a model favoured by many researchers in investigating calcium signalling mechanisms (Clapper et al., 1987;Lee and Aarhus, 1995). Cross desensitization studies revealed that NAADP-induced calcium release was distinct from calcium release in response to IP_3 and cADPR (Lee and Aarhus, 1995;Chini et al., 1995;Genazzani et al., 1996). High concentrations of NAADP induced rapid calcium release such that upon re-uptake of calcium, a second application of NAADP was unable to induce a further release of calcium. Calcium release by cADPR and IP_3 however was unaffected by this treatment. In this model system therefore, desensitization was homologous to each of the calcium mobilising messengers (Aarhus et al., 1996;Genazzani et al., 1996), suggesting NAADP mobilised calcium via a separate channel. Furthermore, heparin and 8-NH_2-cADPR, inhibitors of IP_3 and cADPR-induced calcium release did not block calcium release by NAADP (Lee and Aarhus, 1995). Now a wide variety of cells responsive to NAADP exist, ranging from plants (Navazio et al., 2000) to humans (Berg et al., 2000;Johnson and Misler, 2002;Langhorst et al., 2004). This has provided further impetus in investigating the significance of NAADP as a signalling molecule.

Structure-activity relationships

NAADP is a metabolite of NADP, the only difference between the two molecules being the replacement of a nicotinamide group in NADP with nicotinic acid (Figure 1.1). In the sea urchin egg homogenate, alkaline treatment of NADP generated a compound capable of inducing calcium release (Clapper et al., 1987). Lee and Aarhus were rather surprised to discover no differences between the NMR spectra of NADP and the "active factor" (Lee and Aarhus, 1995). This suggested that all the non-exchangeable protons in NADP were also present in this active derivative. Lee and Aarhus therefore concluded that the most likely difference would occur in the exchangeable protons, with the amide group of nicotinamide being the ideal candidate. Consistent with this hypothesis, mass spectroscopy revealed that the active compound was one atomic mass unit heavier than NADP. Conversion of the amide group of the nicotinamide to a carboxyl group would exactly account for this difference in atomic mass (Figure 1.1). This relatively simple change in structure endowed NAADP with potent calcium release activity, making it one of the most effective calcium-mobilising agents described to date.

By synthesising a variety of NAADP analogues, Lee and Aarhus concluded that certain structural requirements were necessary for NAADP to retain its ability to release calcium. Removal of the negative charge from the 3-position of the pyridine ring completely abolished activity. Moving the negative charge to the 4-position also rendered the molecule inactive, suggesting that the negative charge at the 3-position was crucial for NAADP to function (Lee and Aarhus, 1997). Consistent with this observation was the lack of negative charge in NADP at the same 3-position (existence of an amide group which has no charge), and that NADP was also

ineffective in releasing calcium. The exact location of the 2-phosphate group on the ribose was far less critical. Whether the phosphate was positioned on the 2' or 3' carbon or attached to both cyclically, the resulting molecule was able to effectively release calcium (Lee and Aarhus, 1997). Removal of the phosphate group however generates NAAD, which is unable to invoke calcium release. By synthesising a caged derivative of NAADP, Lee and colleagues further emphasised the importance of the 2-phosphate group. Attachment of the caging group to the 2-phosphate produced caged NAADP with no calcium release activity (Lee et al., 1997). Upon photolysis with ultraviolet light however, the resulting molecule exhibited identical properties to authentic NAADP.

Lee and Aarhus also discovered the importance of the amino group on the 6-position of the adenine ring (Lee and Aarhus, 1997). Changing the amino group to –OH group resulted in the compound being approximately 1000-fold less effective in releasing calcium. More recently Billington and colleagues re-affirmed the importance of the negative charge at the 3-position of the pyridine group (Billington et al., 2005). A variety of analogues were synthesised and the study concluded that though the negative charge at the 3-position was important for NAADP binding to its target protein with increased affinity, the size of the attached group was less important. Taken together these studies have provided substantial evidence to suggest that NAADP-induced calcium release was highly sensitive to the molecules structure and that this response was likely to be mediated by a specific NAADP receptor.

Recent attempts to purify the putative NAADP receptor have led to the discovery of triazine dyes as potential agonists of the NAADP receptor (Billington et al., 2004a).

These compounds which are structurally unrelated to NAADP have been shown to release calcium in the sea urchin egg homogenate via the NAADP receptor. These dyes may help elucidate the exact nature of the NAADP receptor.

Specific binding sites for NAADP within cells

Although the molecular identity of the NAADP receptor remains to be established, specific binding sites for NAADP have now been described in a number of systems (Aarhus et al., 1996;Genazzani et al., 1997a;Billington and Genazzani, 2000;Patel et al., 2000b;Patel et al., 2000a;Bak et al., 2001;Berridge et al., 2002b;Masgrau et al., 2003). Of the systems studied, NAADP binding has best been characterised using the sea urchin egg. Specific binding of NAADP was first demonstrated in purified egg microsomes (Aarhus et al., 1996). Binding of radiolabelled NAADP was inhibited by nanomolar concentrations of unlabeled NAADP however, micromolar concentrations of structurally related nucleotides (NAD, NAAD, cADPR or cyclic-ADP-ribose 2'-phosphate) did not affect NAADP binding. High micromolar concentrations of NADP modestly inhibited binding (Aarhus et al., 1996;Billington and Genazzani, 2000), however this inhibition is likely due to contamination of the commercially available NADP with NAADP (Aarhus et al., 1996).

Binding of NAADP in the sea urchin egg was further characterised in two independent studies. Both Patel and colleagues and Billington and Genazzani demonstrated specific high affinity (IC_{50} in the picomolar range) binding of NAADP to sea urchin egg homogenates (Patel et al., 2000a;Billington and Genazzani, 2000). A rather interesting feature of NAADP binding in the sea urchin egg homogenate is that binding is irreversible. This is discussed further in Chapter 3.

The first report describing specific binding of NAADP in mammalian tissues was by Patel and colleagues (Patel et al., 2000b). Similar to the sea urchin egg homogenate, binding of NAADP to mouse brain homogenates was unaffected by the structurally related nucleotides (Patel et al., 2000b). However, binding of NAADP in the mouse brain was reversible (determined by addition of a molar excess of unlabeled NAADP), a noticeable difference compared to sea urchin egg homogenates (Patel et al., 2000b;Aarhus et al., 1996;Billington and Genazzani, 2000) (see Chapter 3). The authors hypothesised that the difference in reversibility of the two systems maybe due to the existence of differentially regulated receptor subtypes (Patel et al., 2000b). The difference in reversibility of NAADP binding in the mouse brain and sea urchin egg homogenates could also explain the difference in the relative affinities displayed in the two systems. Autoradiographic localisation revealed widespread distribution of NAADP binding throughout the brain. Interestingly, NAADP binding sites were abundant in the medulla, midbrain and thalamus (Patel et al., 2000b). That the localisation of these binding sites was different from other intracellular calcium-release channels suggested that different calcium mobilising messenger molecules may mediate different neuronal functions (Patel et al., 2000b). NAADP binding has since been reported in rabbit heart (Bak et al., 2001) and MIN-6 (mouse insulinoma β-cell line) cells (Masgrau et al., 2003). Like in sea urchin egg homogenates and mouse brain, NAADP binding is unaffected by related analogues.

More recently triazine dyes, capable of acting as NAADP mimetics, were also shown to inhibit binding of radiolabelled NAADP to the NAADP binding protein (Billington et al., 2004a).

Inhibition of NAADP-induced calcium responses

A unique attribute of NAADP-induced calcium release in the sea urchin egg homogenate is the ability of low concentrations of NAADP that do not induce calcium release to inhibit further calcium release by maximal concentrations of NAADP (Aarhus et al., 1996;Genazzani et al., 1996). This inactivation phenomenon is explored further in Chapter 3.

In pancreatic acinar cells, application of low (nanomolar) concentrations of NAADP induced a mixture of calcium responses (Cancela et al., 1999). These were categorised as short-lasting calcium spikes, a mixture of short and long lasting calcium transients and sustained calcium release. However, in the presence of high (micromolar) NAADP concentrations, Cancela and colleagues failed to elicit a calcium response in these cells (Cancela et al., 1999). Similarly, using an isolated nuclei preparation from pancreatic acinar cells, low concentrations of NAADP induced a calcium response (Gerasimenko et al., 2003), however no response was evoked at high concentrations of NAADP (Gerasimenko et al., 2003).

Detailed studies in other mammalian systems have clearly demonstrated a "bell-shaped" activation curve for NAADP-induced calcium release. Application of low concentrations of NAADP in T-lymphocytes stimulated long lasting, small amplitude calcium spikes. In contrast, high concentrations of NAADP failed to elicit calcium responses (Berg et al., 2000). In human pancreatic β-cells too, nanomolar concentrations of NAADP evoked complex calcium signals whereas micromolar NAADP concentrations were less effective. Further increasing the NAADP concentration resulted in complete desensitisation of the NAADP receptor (Johnson

and Misler, 2002). Similarly, in MIN-6 cells, Masgrau and colleagues also reported the inactivation of an NAADP sensitive calcium release mechanism at high ligand concentrations (Masgrau et al., 2003). Low NAADP concentrations were also more effective than higher ligand concentrations in evoking a calcium response in arterial smooth muscle (Boittin et al., 2002). Inactivation in all systems occurs without the prior release of calcium. This suggests that rather than depletion of calcium stores by NAADP, inactivation is likely an intrinsic property of the NAADP receptor.

In contrast to studies in intact cells, microsomal preparations from mammalian cells (with the exception of isolated nuclei from pancreatic acinar cells; (Gerasimenko et al., 2003) did not inactivate at high NAADP concentrations (Yusufi et al., 2001;Bak et al., 2001;Bak et al., 1999;Mojzisova et al., 2001). Thus possible cytosolic factors required for NAADP-induced receptor inactivation may be lost during cell fractionation.

In an attempt to find selective antagonists of NAADP-induced calcium release, Chini and colleagues described one such compound; thio-NADP. This compound selectively inhibited NAADP-induced calcium release without affecting release by cADPR or IP_3 (Chini et al., 1995). The antagonistic effect of thio-NADP, derived from NADP, was later attributed to a contaminant (Dickey et al., 1998). HPLC purification of thio-NADP suggested that the contaminant was NAADP itself and that thio-NADP was not an antagonist of NAADP (Dickey et al., 1998).

The L-type calcium channel blockers, diltiazem, nifedipine and verapamil have also been reported to block calcium release by NAADP (Genazzani et al., 1996;Genazzani

et al., 1997a;Yusufi et al., 2001). However, the concentrations at which these antagonists were effective (high micromolar range), was higher than usually required for their action on L-type calcium channels. Bay K 8664, an agonist of the L-type calcium channel also inhibited the NAADP-sensitive calcium release (Genazzani et al., 1997a). In addition, micromolar concentrations of K^+ channel blockers inhibited calcium release mediated by NAADP without significantly affecting calcium mobilisation by IP_3 and cADPR (Genazzani et al., 1997a).

Cross-talk between calcium release channels

As discussed, substantial evidence exists to suggest that NAADP mediates its effects through a molecular target distinct to IP_3 and cADPR (Patel, 2004;Galione et al., 2004). Current available data however, indicates that NAADP-induced calcium release in intact cells requires the recruitment of IP_3 and cADPR-activated calcium channels. An important example of this interaction was described by Cancela and colleagues in pancreatic acinar cells (Cancela et al., 1999). As previously mentioned, intracellular application of NAADP in pancreatic acinar cells produced localised short and long lasting calcium oscillations and more global calcium waves (Cancela et al., 1999). These responses mimicked those produced by CCK (discussed below). Interestingly, NAADP-mediated calcium responses were blocked by heparin or 8-NH_2-cADPR, antagonists of IP_3 and cADPR, respectively. This was in stark contrast to sea urchin egg homogenates, where NAADP-induced calcium release was unaffected by these antagonists (Lee and Aarhus, 1995). Cancela and colleagues hypothesised that the effects of NAADP were propagated by CICR mediated by IP_3 and ryanodine receptors (Cancela et al., 1999). To support this theory, inactivation of NAADP receptors with high concentrations of NAADP had little affect on calcium

responses induced by IP_3 and cADPR (Cancela et al., 1999). This suggested that NAADP was acting upstream of IP_3 and ryanodine receptors.

NAADP responses in starfish oocytes and sea urchin eggs also involves ER calcium channels (Santella et al., 2000;Churchill and Galione, 2000). However in these cells, simultaneous block of IP_3 and ryanodine receptors is required to inhibit the calcium signals induced by NAADP. This suggests that NAADP responses are amplified by either IP_3 or cADPR receptor activation. Inhibition of one calcium channel is compensated by the other.

Another variation in channel "chatter" (Patel et al., 2001) is observed in arterial smooth muscle cells (Boittin et al., 2002). Globalisation of calcium waves generated by NAADP in this system requires ryanodine and not IP_3-acitvated calcium channels.

In ascidian oocytes and T-lymphocytes, both Albrieux and colleagues and Berg et al have demonstrated a functional relationship between IP_3 and NAADP-mediated calcium release (Albrieux et al., 1998;Berg et al., 2000). In ascidian oocytes, NAADP mobilisation of calcium results in the complete abolition of the subsequent IP_3 response (Albrieux et al., 1998). In T-lymphocyte too, high concentrations of NAADP that inactivate the NAADP-calcium release channels also inhibit the calcium response to IP_3 (Berg et al., 2000). In both systems, it is unlikely that IP_3 and NAADP calcium channels interact via CICR as NAADP-induced calcium release is unaffected by cytosolic calcium (Chini and Dousa, 1996). Nevertheless it is evident that some interaction does occur between different calcium release channels.

Agonist-specific recruitment of calcium mobilising messengers

As mentioned above, calcium signals induced by CCK stimulation of pancreatic acinar cells are mimicked by NAADP. Intriguingly, inactivating NAADP receptors with high concentrations of NAADP greatly attenuates calcium responses to CCK (Cancela et al., 1999), suggesting the requirement of a functional NAADP pathway to mediate the effects of CCK. In contrast, high desensitising concentrations of NAADP are unable to block responses to acetylcholine (ACh) and bombesin (Cancela et al., 2000;Burdakov and Galione, 2000). Bombesin-induced calcium spiking in pancreatic acinar cells was abolished by antagonists of IP_3 and cADPR (Burdakov et al., 2001;Burdakov and Galione, 2000), whereas antagonists of cADPR had no effect on calcium spikes induced by ACh (Cancela et al., 2000). These observations suggest agonist-specific recruitment of calcium-mobilising messengers to generate characteristic calcium signals. In support, recent studies have demonstrated increases in cellular NAADP levels in response to CCK but not ACh (Yamasaki et al., 2005b).

Recruitment of NAADP-mediated calcium release pathways by specific agonists has since been reported in MIN-6 and smooth muscle cells. Evidence in support of this will be discussed later.

NAADP "activation" of the ryanodine receptor

A far simpler explanation for the paradoxical block of NAADP responses by antagonists of IP_3 and ryanodine receptors, is the controversial hypothesis favoured by some authors that NAADP directly modulates the ryanodine receptor rather than a dedicated NAADP calcium release channel (Galione and Petersen, 2005). Hohenegger and colleagues described the direct activation of type 1 ryanodine receptors by

NAADP (Hohenegger et al., 2002). Using a sarcoplasmic reticulum preparation from skeletal muscle, NAADP-induced rapid calcium release was blocked by ruthenium red and ryanodine. By reconstituting purified ryanodine receptors, NAADP was demonstrated to activate single channel currents and the effects of NAADP again found to be sensitive to ryanodine receptor blockers.

NAADP was also shown to activate type 2 ryanodine receptors from cardiac microsomes (Mojzisova et al., 2001), however experimental findings were not confirmed in purified preparations and similar studies by Copello and colleagues failed to demonstrate NAADP modulation of ryanodine receptors (Copello et al., 2001). Furthermore, Bak and colleagues demonstrated that in cardiac microsomes, NAADP-induced calcium release was *not* blocked by specific antagonists of IP_3 or ryanodine receptors (Bak et al., 2001).

More recently however, Gerasimenko and co-workers have shown that NAADP is able to induce calcium release from the nuclear envelope of isolated nuclei from pancreatic acinar cells, and that this effect is blocked by ruthenium red and ryanodine (Gerasimenko et al., 2003). The authors argued that the effect was due to NAADP activation of the ryanodine receptor. Additionally, NAADP-induced calcium release could be blocked by ruthenium red in T-lymphocytes (Langhorst et al., 2004) but not heparin (Dammermann and Guse, 2005). Knockdown of ryanodine receptor expression also significantly reduced the NAADP-induced calcium response (Langhorst et al., 2004). Furthermore by combining calcium imaging with microinjection, subcellular calcium signals induced by NAADP were blocked in the presence of ruthenium red and more importantly in T-cell clones lacking ryanodine

receptors. The authors therefore concluded that NAADP likely activated the ryanodine receptor. However, as previously suggested, NAADP-induced calcium responses may require functional ryanodine receptors to amplify the response by CICR. Molecular identification of the NAADP-binding protein seems to be the likely solution to resolve these arguments.

NAADP-sensitive calcium stores

Mobilisation of intracellular calcium stores by second messengers has generally been accepted important in generating cytosolic calcium signals in a variety of cells (Berridge et al., 2000). The endoplasmic reticulum is established as a key calcium storing organelle (Meldolesi and Pozzan, 1998), possessing receptors for the second messengers IP_3 and cADPR (Bootman et al., 2002). The endoplasmic reticulum however is not the only organelle to store and release calcium. Other putative calcium-containing organelles include the mitochondria (Duchen, 1999), golgi (Hu et al., 2000), secretory granules (Petersen, 1996) and endosomes (Berridge et al., 2003). Increasing evidence now suggests that NAADP mobilises calcium from an acidic compartment (Churchill et al., 2002).

Fractionation of sea urchin egg homogenates by percoll density centrifugation revealed calcium release induced by IP_3, cADPR and NAADP was differentially distributed (Lee and Aarhus, 1995). Calcium release assayed from fractionated sea urchin egg homogenates indicated that the IP_3- and cADPR-sensitive calcium stores comigrated and that these fractions were enriched in glucose-6-phosphatase activity, a marker for the endoplasmic reticulum (Lee and Aarhus, 1995). However, unlike IP_3 and cADPR, NAADP-sensitive stores were more widely distributed. This simple

fractionation revealed that NAADP-sensitive stores were distinct from those activated by IP_3 and cADPR. The large size of sea urchin eggs allowed Lee and Aarhus to stratify the organelles into distinct layers and further characterise the NAADP-responsive calcium store. The eggs were loaded with fluo-3 (fluorescent calcium indicator) and caged calcium mobilising messengers. The uncaging of NAADP, cADPR or IP_3 resulted in localised calcium release. Calcium release by NAADP was mainly in a pole distal to the nucleus (Lee and Aarhus, 2000). This however was strikingly different from IP_3- and cADPR-induced calcium release which was localised at the nuclear pole. Stratified eggs treated with Bodipy-thapsigargin, a fluorescent analogue used to visualise stores containing Ca^{2+}-ATPase, resulted in staining close to the nuclear pole (Lee and Aarhus, 2000). This was in agreement with Genazzani and Galione who had earlier shown that NAADP mobilised calcium from a thapsigargin-insensitive pool (Genazzani and Galione, 1996). Calcium release by IP_3 and cADPR in sea urchin egg homogenates treated with thapsigargin was significantly reduced compared to that in the absence of thapsigargin. However, NAADP-induced calcium release was unaffected. Taken together, these studies suggested the existence of two distinct calcium pools one mobilised by IP_3 and cADPR, and the other by NAADP.

Recent data has positively identified the calcium store mobilised by NAADP to be an acidic organelle. Using glycylphenyl alanine 2-napthylamide (GPN), (a substrate for the lysosomal exopeptidase, cathepsin C leading to osmotic lysis), Churchill and colleagues demonstrated significant decrease of calcium release in response to photorelease of NAADP (Churchill et al., 2002). However calcium release in response to IP_3 and cADPR was unaffected.

Calcium uptake into these acidic organelles was investigated using an enriched lysosomal preparation, thus overcoming the effects of multiple calcium stores. Uptake of calcium was inhibited by apyrase, which suggested the requirement of ATP, but not by pretreatment with thapsigargin (Churchill et al., 2002). The inhibition of calcium uptake in the presence of bafilomycin (an inhibitor of vacuolar H^+ pumps powered by ATP), and other proton collapsing agents (FCCP, nigericin and NH_3) suggested that the NAADP-sensitive calcium store was acidic.

Mounting evidence now suggests that NAADP mobilises calcium from a thapsigargin insensitive pool in a variety of systems (Boittin et al., 2002;Brailoiu et al., 2003;Brailoiu et al., 2005). NAADP was shown to significantly decrease the calcium content of secretory vesicles in MIN-6 cells, without affecting calcium within the endoplasmic reticulum (Mitchell et al., 2003). This was further demonstrated in intact MIN-6 cells using caged NAADP. Depletion of the endoplasmic reticulum store with thapsigargin was without effect on the magnitude of calcium release induced by the uncaging of NAADP (Mitchell et al., 2003). Yamasaki and colleagues also suggested that NAADP may release calcium from an acidic organelle in pancreatic acinar and β-cells. It was hypothesised that specific agonists recruit lysosomal-related organelles via an NAADP messenger pathway, whilst other agonists recruit IP_3 and cADPR as second messengers to mobilise calcium from the endoplasmic reticulum. Pretreatment of acinar cells with GPN, or β-cells with bafilomycin significantly attenuated NAADP-induced calcium responses but was without effect on IP_3- and cADPR-induced calcium release (Yamasaki et al., 2004). Furthermore, disrupting the acidic organelles, selectively inhibited calcium responses in response to CCK (in pancreatic acinar cells) and glucose (in pancreatic β-cells) but was without effect on calcium

responses induced by acetylcholine. Similarly, pretreatment of pulmonary smooth muscle cells with bafilomycin also blocked calcium signals induced by NAADP and the potent vasoconstrictor, endothelin-1 (Kinnear et al., 2004). However calcium signals in response to IP_3, ryanodine and prostaglandin-$F_{2\alpha}$ were unaffected (Kinnear et al., 2004). These studies further suggest that NAADP can mobilise calcium from stores insensitive to IP_3 and cADPR, and that the recruitment of NAADP-mediated calcium release pathway is agonist-specific as previously discussed.

NAADP-induced calcium release requires two calcium pools

The ability of NAADP to induce long-term calcium oscillations in sea urchin eggs, even though NAADP self-inactivates, further suggests the requirement of more than one calcium store and release pathway. Churchill and Galione investigated the mechanism underlying NAADP-induced calcium oscillations. It was demonstrated that NAADP, but not IP_3 or cADPR induced calcium oscillations (Churchill and Galione, 2001a). The fact that NAADP caused more calcium release than cADPR and IP_3 was indicative of calcium being released possibly from a common pool as well as from a store responsive to NAADP alone. Calcium oscillations produced by NAADP were not due to re-sensitisation of NAADP-induced calcium release, although desensitisation of calcium release was shown to be reversible (Churchill and Galione, 2001b). This was because cells were shown to be insensitive to NAADP for longer then the onset of calcium oscillations (Churchill and Galione, 2001a).

Addition of both heparin and 8-NH_2-cADPR however, eliminated calcium oscillations generated by NAADP (Churchill and Galione, 2001a). Similarly, thapsigargin abolished NAADP-induced calcium oscillations. However, neither antagonists for IP_3

and cADPR nor thapsigargin prevented the release of calcium by NAADP. Therefore, a possible mechanism to explain these calcium oscillations is calcium released by NAADP from thapsigargin-insensitive stores may be taken up in to thapsigargin-sensitive calcium stores, causing the latter to overload and spontaneously release calcium. These stores were shown to contain more calcium after NAADP-mediated calcium release, providing strong evidence for the proposed calcium oscillation model (Churchill and Galione, 2001a). This calcium oscillation model has since been promoted in arterial smooth muscle (Boittin et al., 2002). Thapsigargin was able to inhibit calcium release by NAADP in smooth muscle cells however, localised calcium bursts produced by NAADP were unaffected. Therefore the requirement of a secondary pool of calcium to modulate calcium release in the arterial smooth muscle (Boittin et al., 2002) was similar to the model proposed by Churchill and Galione for NAADP-induced calcium oscillations in the sea urchin egg (Churchill and Galione, 2001a). Cross-talk between these calcium-release channels combined with the interaction of different calcium pools, may partially explain how different calcium signalling molecules produce distinct signature calcium responses.

NAADP-induced calcium release requires calcium entry

As discussed, NAADP has been established as an intracellular calcium mobilising messenger, inducing calcium release from internal stores. However, recent reports suggest that calcium entry may also play a role in NAADP-induced calcium responses. For example, in T-lymphocytes, NAADP-mediated calcium responses were abolished by the calcium channel blocker SK&F 96365 (Langhorst et al., 2004). However, a small sustained release of calcium was still evident under these conditions. Furthermore, pre-incubating T-lymphocytes with gadolinium (a specific

calcium channel blocker, inhibiting calcium entry), prior to addition of NAADP resulted in the delayed onset of the calcium transient (Langhorst et al., 2004). That the amplitude of the calcium transient was also reduced suggested NAADP-induced calcium responses in T-lymphocytes required calcium entry.

Fertilisation of the sea urchin egg also involves calcium entry (Shen and Buck, 1993). Upon sperm-egg fusion, one of the first events to occur is the cortical flash, resulting in the increase of calcium around the cortex of the egg. This depolarisation induced by sperm, is amplified by an action potential meditated by the L-type calcium channels (McDougall et al., 1993) .

Churchill and colleagues showed that photorelease of NAADP (but not IP_3 or cADPR) within the sea urchin egg resulted in the cortical flash. The removal of calcium from artificial sea water, or the addition of cadmium was sufficient to abolish the NAADP-induced cortical flash (Churchill et al., 2003). Thus it appears that NAADP is able to regulate both calcium mobilisation from internal stores as well as calcium influx.

In mature starfish oocytes, NAADP-induced calcium signals were greatly attenuated when extracellular calcium was removed (Santella et al., 2000). Furthermore, NAADP-induced calcium release was shown to initiate in the cortex of the matured oocyte (Lim et al., 2001). This would suggest that calcium entry was a critical component of NAADP signalling at fertilisation. Close localisation of NAADP receptors to the plasma membrane has also been recently hypothesised (Moccia et al., 2003). That NAADP was shown to activate an inwardly rectifying calcium current

dependent on the F-actin cytoskeleton (Moccia et al., 2003), suggest that the calcium store maybe coupled to the calcium channel in the plasma membrane. Thus the calcium current described by Moccia and colleagues has been proposed to explain changes in calcium witnessed during the cortical flash and calcium entry in T-lymphocytes.

Intracellular NAADP levels change in response to extracellular stimuli

To achieve full second messenger status, a change in endogenous levels of NAADP must occur in response to a physiological stimulus. This change has now been reported. The first study demonstrated changes in NAADP levels in two cell types: sea urchin sperm and egg (Churchill et al., 2003). Using a radio-receptor assay, Churchill and colleagues were able to demonstrate an approximate four-fold increase in the endogenous levels of NAADP within the sea urchin egg upon fertilisation. This increase was likely due to the delivery of preformed NAADP from the sperm. It was revealed that upon contact with egg jelly, the level of NAADP in the sperm increased, entirely consistent with the increase seen at fertilisation (Churchill et al., 2003). However, a second rise in NAADP observed post-fertilisation maybe a result of synthesis within the egg itself.

Sea urchin egg homogenates prepared shortly after fertilisation, were able to release calcium when challenged with IP_3 and cADPR (Churchill et al., 2003). However, when fertilised homogenates were challenged with NAADP, calcium release was attenuated (Churchill et al., 2003). These results were entirely consistent with an increase in cellular NAADP levels at fertilisation and subsequent desensitization of

the NAADP receptor. Thus Churchill and colleagues successfully recorded the first rise in NAADP levels within a cell.

The first report demonstrating an increase of NAADP within a mammalian system was in MIN-6 cells (Masgrau et al., 2003). As discussed earlier, high inactivating concentrations of NAADP were effective in attenuating calcium release in response to glucose, suggesting a possible messenger role for NAADP. Subsequently, Masgrau and colleagues, using a radioreceptor assay, described a two-fold increase in NAADP levels in MIN-6 cells stimulated with glucose (Masgrau et al., 2003). Changes in intracellular NAADP levels in response to extracellular stimuli have since been reported in rat arterial smooth muscle cells (Kinnear et al., 2004) and mouse pancreatic acinar cells (Yamasaki et al., 2005b) and will be discussed in Section 4.1.

Synthesis of NAADP

As discussed, ADP-ribosyl cyclase from *Aplysia Californica*, can catalyse the cyclization of NAD to cADPR. This enzyme can also catalyse the exchange of nicotinamide for nicotinic acid in NADP resulting in the production of NAADP (Figure 1.1). This base-exchange reaction at present is the only known enzymatic route of NAADP synthesis. The discovery of two mammalian homologues CD38 and BST-1 (as described in Section 1.5) further highlight the importance of ADP-ribosyl cyclase in producing intracellular calcium-mobilising messengers (Lee, 1999;Chini et al., 2002). Chini and colleagues had initially described using crude extracts from rat tissues that NAADP could be synthesised by exchange of nicotinamide for nicotinc acid (Chini and Dousa, 1995). Importantly synthesis of NAADP and cADPR was impaired in tissues prepared from CD38 knock-out mice (Chini et al., 2002). Aarhus

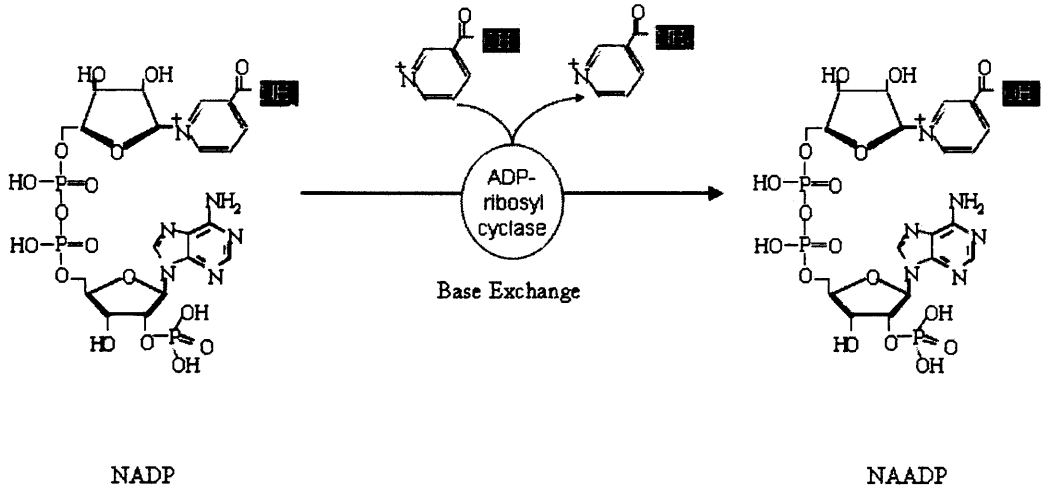

Figure 1.1: Synthesis of NAADP by ADP-ribosyl cyclase Shown here are the schematic structures of NADP (left) and NAADP (right). ADP-ribosyl cyclase in the presence of nicotinic acid and NADP can catalyse the replacement of nicotinamide with nicotinic acid. The net result is the replacement of the $-NH_2$ group with an –OH group (shown in the shaded regions).

and colleagues investigated how one enzyme was able to catalyse the synthesis of two different molecules. Their study found that the products formed by ADP-ribosyl cyclase were dependent upon pH. Base-exchange reactions were favoured in acidic conditions whereas, at neutral or alkaline pH, ADP-ribosyl cyclase favoured the cyclization reaction (Aarhus et al., 1995). Another critical requirement for the production of NAADP by ADP-ribosyl cyclase was the need for nicotinic acid. In the absence of nicotinic acid, even under acidic conditions, this enzyme would preferentially favour the cyclization reaction (Aarhus et al., 1995). This property was evident for both *Aplysia* ADP-ribosyl cyclase and recombinant human CD38 (Aarhus et al., 1995). Recent evidence suggests that nicotinic acid acts as an allosteric regulator of ADP-ribosyl cyclase, increasing the affinity of the enzyme for NADP under acidic conditions and reducing the affinity under alkaline conditions (Bacher et

al., 2004). Further characterisation of preferential NAADP synthesis was provided by two independent studies. Graeff and colleagues and Wilson and Galione suggested the existence of two forms of ADP-ribosyl cyclase. A membrane-bound cyclase, which was insensitive to cGMP but required cAMP and a soluble cyclase dependent on cGMP (Graeff et al., 1998;Wilson and Galione, 1998). That the membrane fractions were required to synthesise NAADP and were potentiated by cAMP suggested a role for the membrane-bound cyclase in producing NAADP (Wilson and Galione, 1998). In both cases, acidic pH was required to drive NAADP synthesis. It is unlikely that within the cytosol of a cell such an acidic pH would exist. Furthermore, the half-maximal concentration of nicotinic acid required to synthesise NAADP is far greater than that expected within the cytosol (Aarhus et al., 1995). That intracellular organelles possess ADP-ribosyl cyclase (Liang et al., 1999) suggest that membrane-bound cyclase may exist within an acidic compartment and provide suitable conditions for the synthesis of NAADP. However it must be noted that though the synthesis of NAADP is optimal under acidic conditions, synthesis at neutral pH is still significantly higher than cADPR synthesis under neutral conditions (Bak et al., 1999). Given that NAADP is active in the low nanomolar concentrations, it is suggested that cytosolic ADP-ribosyl cyclase below optimal conditions may synthesise physiological amounts of NAADP (Aarhus et al., 1995).

In recent developments, a new member of the ADP-ribosyl cyclase family has been identified from *Schistosoma mansoni*. $NAD(P)^+$ catabolising enzyme (NACE), catalyses the synthesis of ADPR and NAADP (Goodrich et al., 2005). However, NACE rather surprisingly, is unable to catalyse cADPR efficiently. Further

characterisation of this novel ADP-ribosyl cyclase may shed light on how one enzyme can specifically catalyse the synthesis of a variety of calcium-mobilising messengers.

Metabolism of NAADP

As discussed, evidence now exists describing the synthesis of NAADP in a variety of systems. However, little remains known regarding the metabolism of NAADP. Chini and colleagues initially showed that incubating NAADP with various rat tissue extracts resulted in the degradation of NAADP determined using a calcium-release bioassay (Chini and Dousa, 1995). Berridge and colleagues further addressed this issue and characterised the metabolic route of NAADP inactivation. Incubating NAADP with crude brain membranes from mice resulted in a significant decrease of the nucleotide quantified by HPLC. The decrease in NAADP was directly associated with the appearance of another product having a similar retention time to that of NAAD. Various enzymatic treatments on this purified product yielded compounds with similar retention times to that of enzymatic treatment of authentic NAAD but not NADP. Degradation therefore was likely to occur via dephosphorylation of NAADP at the 2' position to yield NAAD (Berridge et al., 2002a). Metabolism of NAADP by brain membranes was critically dependent upon calcium. In the absence of calcium, the rate of NAADP metabolism was greatly attenuated. The half-maximal calcium concentration required to stimulate NAADP metabolism was well within the resting physiological range of intact cells (Berridge et al., 2002a). Thus, the phosphatase involved in the breakdown of NAADP would clearly possess the ability to detect changes in cytosolic calcium. This then would provide a feedback mechanism to regulate the NAADP response. More recently Billington et al described another possible method of inactivating NAADP. Cellular enzymes such as glucose-6-

phosphate dehydrogenase were able to reduce NAADP to NAADPH (Billington et al., 2004b). That NAADPH was inert towards the receptor led the authors to the conclusion that NAADPH could act possibly as a pool of inactivated NAADP or as a metabolic intermediate (Billington et al., 2004b).

1.7 Aims

As discussed in this chapter, evidence suggests that NAADP is a novel intracellular calcium mobilising messenger involved in generating calcium signals in a number of systems (Patel, 2004). However, information regarding the molecular target remains scarce.

The major focus of my thesis was to investigate the properties of the NAADP receptor in sea urchin eggs. The objectives were:

i To explore the modulation of the NAADP-binding protein by its lipid environment.

ii To examine in detail the effect of ligand binding on receptor function.

iii To exploit the binding properties of the target protein to measure levels of NAADP in cell extracts.

CHAPTER 2

NAADP binding to its target protein in sea urchin eggs requires phospholipids

2.1 Introduction

In sea urchin egg homogenates, NAADP can modulate the release of intracellular calcium independently of IP_3 or ryanodine receptor activation (Galione et al., 2000). However the molecular target of NAADP has yet to be isolated. Patel and co-workers have recently described the successful solubilization of the NAADP target protein from sea urchin egg homogenates (Berridge et al., 2002b), and the evidence presented suggests that this protein is the same as that involved in the calcium release in response to NAADP.

Washed sea urchin egg homogenates were treated with the non ionic detergent Triton X-100, and the supernatant, recovered after centrifugation was tested for [^{32}P]NAADP binding. [^{32}P]NAADP binding to the supernatant was readily detectable. Binding of [^{32}P]NAADP to solubilized homogenates was inhibited by NAADP and its analogues with the same rank order of potency as that reported for binding to membranes (Patel et al., 2000a) and calcium release (Lee and Aarhus, 1997). Furthermore, the recovered solubilized protein bound NAADP irreversibly, a property consistent with NAADP binding to its receptor in the sea urchin egg homogenate. Therefore initial evidence suggested that the membrane bound and solubilized protein was the same. That the size of NAADP receptors determined from gel filtration (440 kDa) and sucrose gradient (120 kDa) centrifugation were much smaller than the IP_3 (Supattapone et al., 1988) and ryanodine receptors (Lai et al., 1988), also suggested that NAADP induced

calcium release through a novel channel. However, it remains to be established whether NAADP directly modulates a calcium channel. This requires the isolation and functional expression of the target protein. Thus relatively little is currently known about the biochemical properties of the NAADP receptor.

The lipid environment has the capacity to modulate ion channels through direct and indirect interactions. Lipids play an important role in maintaining the tertiary structure of receptors and ion channels and can also act as second messengers allowing them to directly modulate ion channels.

Sphingolipids are structural lipids that can be metabolized to synthesize ceramide and sphingosine 1-phosphate, two molecules involved in cell signaling. Sphingosine 1-phosphate (as described in Section 1.5) is an important lipid mediator, possessing the ability to modulate G-protein coupled receptors (An et al., 1999) as well as directly mobilizing intracellular calcium from the endoplasmic reticulum via an unidentified calcium channel (Ghosh et al., 1990;Ghosh et al., 1994;Young and Nahorski, 2002).

K^+ channels, found in virtually all cells allow rapid movement of K^+ ions across cell membranes. This family of ion channels, present in an array of excitable and non-excitable cell types, has been demonstrated to be modulated by unsaturated free fatty acids (Kim, 2003). Arachidonic acid (AA), an unsaturated free fatty acid directly activated inwardly rectifying K^+ channels (Liu et al., 2001). AA is also believed to modulate members of the tandem-pore K^+ channel family (Patel et al., 1998;Fink et al., 1998;Bang et al., 2000;Kim, 2003). When expressed in COS cells, TRAAK (TWIK-related arachidonic acid-stimulated K^+ channel) channels produced

instantaneous and non-inactivating currents that were not gated by voltage (Fink et al., 1998). These currents were stimulated by perfusion of AA in a reversible, concentration-dependent and direct manner (Fink et al., 1998). Saturated fatty acids were without effect, suggesting these ion channels were sensitive specifically to unsaturated free fatty acids.

Phosphatidylinositol-4,5-bisphosphate (PIP_2), a lipid and precursor of many signaling molecules is also involved in modulating a variety of ion channels (Hilgemann et al., 2001). PIP_2 has been shown to bind and activate inward rectifier K^+ channels (Huang et al., 1998). Conversely, PIP_2 modulation of vanilloid receptor (VR1) ion channels is inhibitory (Chuang et al., 2001). Phospholipase C-mediated hydrolysis, or sequestration of PIP_2 by antibodies enhanced basal and capsaicin-induced currents. Thus, changes in endogenous PIP_2 levels can exert opposing regulatory effects (Huang et al., 1998;Chuang et al., 2001).

Stable association of lipids with ion channels can also regulate their function. The nicotinic acetylcholine receptor requires acidic and neutral lipids to maintain ion flux through the channel (Fong and McNamee, 1986). This requirement of lipids has been attributed to stabilization of the secondary structure via distinct binding sites (Tillman and Cascio, 2003). Furthermore, the amount of lipid associated with the nicotinic acetylcholine receptor is also crucial for functional properties (Jones et al., 1988). Negatively charged lipid molecules are also associated with K^+ channels and required for ion conductance (Valiyaveetil et al., 2002).

Modulation of ion channels by lipids can occur indirectly, by altering biophysical properties of the membrane in which they are found (Tillman and Cascio, 2003). It is therefore, important to fully understand the interactions between lipids and ion channels in order to appreciate how these channels function.

Much effort in characterizing the effects of lipids on cell-surface ion channels has been conducted however in contrast very little work has been performed on intracellular calcium channels. In this chapter I will highlight the importance of phospholipids in maintaining NAADP binding to its target protein in sea urchin eggs.

2.2 Materials and Methods

Preparation of sea urchin egg homogenates

Sea urchins (*Lytechinus pictus*) were obtained from Marinus (Long beach, CA, USA). Eggs were harvested in Instant Ocean® aquarium salt solution (specific gravity, 1.02-1.023; Sigma) after intracoelomic injection of 500 mM KCl. Egg jelly was removed by passing through a nylon mesh (85 μM) and washed sequentially with Ca^{2+}-free artificial sea water (470 mM NaCl, 27 mM $MgCl_2$, 28 mM $MgSO_4$, 10 mM KCl, 2.5 mM $NaHCO_3$, 1 mM EGTA, pH 8; two washes) and nominally Ca^{2+}-free artificial sea water (Ca^{2+}-free artificial sea water without EGTA; two washes) at 4°C. The eggs were then resuspended (50 %, v/v) in KGluIM (intracellular-like medium) composed of 250 mM potassium gluconate, 250 mM N-methyl-D-glucamine, 1 mM $MgCl_2$ and 20 mM NaHepes (pH 7.2, with acetic acid) supplemented with an ATP-regenerating system (1 mM ATP, 10 mM phosphocreatine and 10 unit/ml creatine phosphokinase) and a protease inhibitor cocktail (EDTA-free; Roche, Lewes, UK). Homogenisation was performed at 4°C using a Dounce Homogeniser (clearance 0.026mm – 0.076mm,

six strokes). Homogenates were immediately centrifuged at 11,600 g for 8 seconds to remove cortical granules and the supernatant stored at –80°C before use.

Receptor solubilization

Sea urchin egg homogenates (50 %, v/v) were washed twice by centrifugation at 100,000 g for 5 minutes at 4°C in KGluIM. Washed homogenates (8 %, v/v) were incubated with either Triton X-100 or CHAPS (1 %, w/v) for 60 minutes at 4°C and then centrifuged at 100,000 g for 60 minutes at 4°C. Supernatant fractions containing soluble NAADP-binding proteins were recovered and stored at –80°C until required.

Synthesis of radiolabelled NAADP

[^{32}P]NAADP was synthesised from [^{32}P]NAD (specific activity 1000Ci/mmol; Amersham Biosciences) by incubation with 75 units/ml NAD kinase (Sigma) and 10 mM MgATP for 3 hours at 37°C in a buffer containing 20 mM NaHepes (pH 7.2). The remaining ATP and ADP was converted into AMP by incubation with apyrase (5 units/ml; Sigma) for 30 minutes at 37°C. The reaction was then diluted into a medium containing 100 mM nicotinic acid and 1 μg/ml ADP-ribosyl cyclase (Sigma) and incubated for a further 1 hour in order to convert [^{32}P]NADP into [^{32}P]NAADP by base-exchange. The final mixture was separated by anion-exchange high performance liquid chromatography (HPLC) on a 3 mm × 150 mm column packed with AG® MP1 (Bio-Rad). Elution was performed at a flow rate of 1 ml/min using a gradient of trifluoroacetic acid (TFA) (with water) that increased linearly from 0-2 % over the first 6 minutes, to 4 % at 11 minutes, to 8 % at 16 minutes, to 16 % at 21 minutes, to 32 % at 26 minutes and to 100 % (150 mM TFA) at 26.1 minutes. Fractions were

collected every minute, neutralized by the addition of NaHepes (final concentration 190 mM), and their radioactivity was determined by Cerenkov counting.

Radioligand binding to solubilized sea urchin egg homogenates

Solubilized sea urchin egg homogenates were diluted 10-fold into KGluIM supplemented with [^{32}P]NAADP (0.5-3 nM) and γ-globulin (4 mg/ml) in either the absence or presence of the indicated concentration of detergent and lipid. Stock solutions (50-100 mg/ml) of phosphatidylcholine (PC; from egg yolk), lysophosphatidylcholine (LPC; from egg yolk), phosphatidylethanolamine (PE; from bovine brain), phosphatidylserine (PS; from bovine brain), sphingomyelin (SPM; from egg yolk), diacylglycerol (1-stearoyl-2-arachidonoyl-sn-glycerol), arachidonic acid (AA) and palmitoleic acid (PA) (all from Sigma) were prepared in the appropriate detergent (10-20 %, w/v) and stored at –20°C. All binding reactions were performed at room temperature for 30 minutes and terminated by precipitation of protein with polyethylene glycol (15 %, w/v; average molecular weight, 8000) for 30 minutes at 4°C. Precipitated samples were centrifuged at 100,000 g for 5 minutes at 4°C. The recovered pellets were washed with ice-cold polyethylene glycol (15 %, w/v) and dissolved in water. Radioactivity associated with the pellets was determined by Cerenkov counting.

In some experiments, Triton X-100 solubilized preparations were serially diluted in binding medium supplemented with 1 % (w/v) Triton X-100 with or without phospholipids before radioligand binding. The final Triton X-100 concentration in the binding assay was 0.1 % (w/v). For reversibility experiments (Figure 2.1C), solubilized sea urchin egg homogenates were first diluted 4-fold with Triton X-100 (1

%, w/v), and then samples were adjusted to 1.5 % (w/v) Triton X-100 or 1.5 % (w/v) Triton X-100 + 2.5 mg/ml PC after a 5 minute incubation using concentrated stock solutions such that total incubation volume was increased <5 %. Radioligand binding was compared with samples that had not been diluted, but where the appropriate amount of detergent had been added. The final Triton X-100 concentration in the binding assay in this set of experiments was therefore 0.15 % (w/v).

Gel-filtration analysis of prelabelled NAADP receptors

Sea urchin egg homogenates (2.5 %, v/v) were incubated for 30 minutes at room temperature in KGluIM supplemented with 100 pM [^{32}P]NAADP. Samples were subsequently washed and solubilized with Triton X-100 (1 %, w/v) as described above for receptor solubilization. [^{32}P]NAADP labelled soluble extracts (50 µl) were injected on to a Superdex 200 HR 10/30 size-exclusion chromatography column (Amersham Biosciences) linked to a HPLC system (Waters). Fractionation was performed at room temperature (flow rate, 0.5 ml/min) in either KGluIM or KClIM (KCl-based intracellular-like medium) composed of 250 mM KCl and 20 mM NaHepes, pH 7.2, in either the absence or presence of PC (1 mg/ml). Both media were supplemented with Triton X-100 (1 %, w/v). In some experiments, unlabelled soluble extracts were incubated with [^{3}H]phosphatidylcholine (0.02 nM, 66 Ci/ml) (Amersham Biosciences) for 5 minutes prior to separation. Collected fractions were analysed directly for radioactivity by Cerenkov (for [^{32}P]NAADP) or scintillation (for [^{3}H]phosphatidylcholine) counting. NAADP receptor migration was compared with that of apoferritin (molecular mass of 443 kDa) determined by measuring absorbance of the column eluate at 280 nm.

Data analysis

The curve in Figure 2.4 was fitted to the following equation:

$B = (B_{max} * [L]) / (K_d + [L])$

Where L is the amount of ligand bound and K_d is the concentration of [^{32}P]NAADP (L) producing half maximal binding (B_{max}). All data are presented as mean ± standard error of the mean (S.E.M.)

2.3 Results

Binding of [^{32}P]NAADP to diluted solubilized sea urchin egg homogenates in the presence of a fixed detergent concentration

Patel and co-workers have previously described the solubilization of the NAADP-binding protein from the sea urchin egg homogenate (Berridge et al., 2002b). I examined binding of [^{32}P]NAADP to sea urchin egg homogenates solubilized in the presence of Triton X-100. Initially, binding was performed to solubilized sea urchin egg homogenates that had been serially diluted in the presence of Triton X-100 (1 %, w/v). Surprisingly, [^{32}P]NAADP binding to diluted solubilized sea urchin egg homogenates deviated from the linear relationship expected between maximum binding and protein concentration (Broken line; Figure 2.1A example of a typical experiment). The amount of [^{32}P]NAADP bound at arbitrary receptor concentrations of 1 and 0.125 was 12 ± 1 (n = 6) and 0.38 ± 0.26 (n = 3) fmol/incubation respectively; the latter value only 27 ± 12 % of that expected following an 8-fold dilution [(12 ± 1)/8 = 1.5 ± 0.1 fmol/incubation; Figure 2.1B). Since in these experiments the Triton X-100 concentrations remained constant, I hypothesised that the inhibitory effect of dilution on [^{32}P]NAADP binding was due to a change in the

endogenous protein/lipid ratio of the soluble extracts. To test this binding of [^{32}P]NAADP to diluted solubilized sea urchin egg homogenates was performed in the presence of exogenous phospholipid. In the presence of PC [^{32}P]NAADP binding was, as expected linear with respect to dilution (Figure 2.1A and B). Thus binding of [^{32}P]NAADP to soluble samples diluted 8-fold in the presence of PC was 1.6 ± 0.3 fmol/incubation and therefore close (109 ± 7 %) to the theoretical (1.5 ± 0.1 fmol/incubation) values assuming a linear relationship between ligand binding and protein concentration. Binding of [^{32}P]NAADP to soluble preparations that had been diluted 4-fold with detergent was similar whether PC was added during or 5 minutes after dilution (Figure 2.1C); average values were 24 ± 1 % and 26 ± 1 % of control (undiluted) incubations (n = 3). These data thus reveal a possible requirement for phospholipids in order for NAADP binding to its target protein, and that the inhibitory effects of lipid removal on [^{32}P]NAADP binding are fully reversible.

Binding of [^{32}P]NAADP to solubilized sea urchin egg homogenates in the presence of increasing detergent concentration

I next examined radioligand binding at a fixed concentration of soluble extract in the presence of increasing detergent concentration. Increasing the Triton X-100 concentration inhibited [^{32}P]NAADP binding (Figure 2.2A) and 50 % inhibition (IC_{50}) of [^{32}P]NAADP binding was achieved at 0.17 % (w/v) Triton X-100. PC prevented the inhibitory effects of Triton X-100 on [^{32}P]NAADP binding (Figure 2.2A). Thus, decreasing the endogenous protein/lipid ratio by this independent method inhibited [^{32}P]NAADP binding, an effect prevented by the presence of exogenous phospholipid. [^{32}P]NAADP binding to soluble preparations was also examined in the presence of the zwitterionic detergent CHAPS. Similar to Triton X-

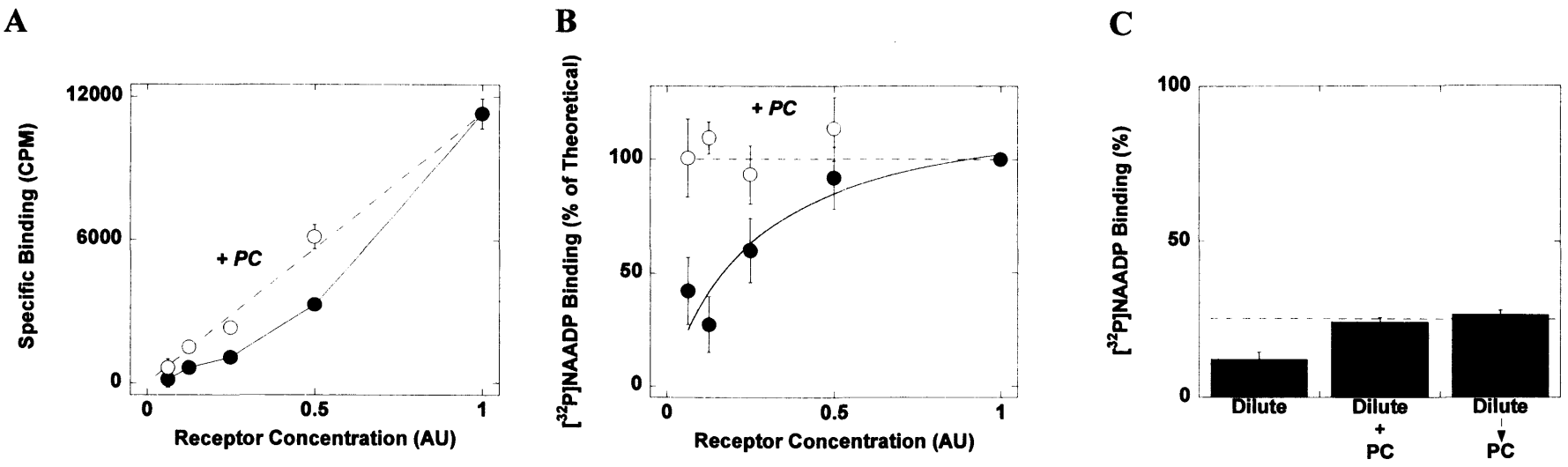

Figure 2.1: Binding of [^{32}P]NAADP to solubilized sea urchin egg homogenates is inhibited when receptors are diluted at a fixed detergent concentration

(A) Specific binding of [^{32}P]NAADP (0.5 nM) to varying concentrations of egg homogenates solubilized with Triton X-100 from a typical experiment (performed in triplicate; mean ± S.D.). Binding was performed to solubilized preparations diluted 10-fold into detergent-free medium (final Triton X-100 concentration = 0.1 %, w/v). Solubilized sea urchin egg homogenates were directly used for binding or first serially diluted (2-16-fold) in binding medium + 1 % (w/v) Triton X-100 with (open circles) or without (closed circles) 2.5 mg/ml PC, to yield arbitrary concentrations (AU) of 1 and 0.5-0.125 respectively. (B) Results from at least 3 experiments (described in A) are shown. Data are expressed as a percentage of that expected, assuming a linear relationship between receptor concentration and [^{32}P]NAADP binding (broken line). (C) Binding of [^{32}P]NAADP to soluble preparations that had been diluted 4-fold (receptor concentration = 0.25) with Triton X-100 (1 %, w/v) either in the absence (Dilute) or presence of PC added simultaneously (Dilute + PC) or 5 minutes after dilution (Dilute → PC). See Material and Methods section for further details. Data are normalised to binding to undiluted samples (receptor concentration = 1).

100, binding was inhibited in the presence of CHAPS, and less so in the presence of exogenous PC (Figure 2.2B). However, the inhibitory effects of CHAPS were less marked than Triton X-100 (IC_{50} = 1.4 %, w/v), likely reflecting the differences in critical micelle concentration (CMC; lowest concentration above which monomers cluster to form micelles) between the two detergents. Furthermore, since the CMC for CHAPS was greater than Triton X-100, the lipid to detergent ratio was adjusted accordingly.

As described in Figure 2.2A, 0.4 % (w/v) Triton X-100 inhibited [^{32}P]NAADP binding by 40 ± 5 % (Figure 2.2C). Following further addition of Triton X-100 (to a final concentration of 1.6 %), 5 minutes after the first addition, [^{32}P]NAADP binding was reduced by 75 ± 11 % (Figure 2.2C). In parallel incubations, binding of [^{32}P]NAADP to soluble preparations following subsequent addition of detergent in the presence of PC was similar to control incubations where protein/lipid ratio was unchanged (final detergent concentration, 0.1 %, w/v) (Figure 2.2C). Taken together, these data indicate that the effects of delipidation on radioligand binding to solubilized sea urchin egg homogenates by high detergent concentrations is reversible.

Binding properties of the delipidated NAADP target protein

Having established a possible role for lipids in maintaining binding of [^{32}P]NAADP, I investigated the interaction between lipids and related molecules on [^{32}P]NAADP binding to its target protein. Triton X-100 (1.6 %, w/v) inhibition of [^{32}P]NAADP binding was prevented by PC in a concentration dependent manner (Figure 2.3A). The half-maximal effect occurred at approximately 2 mg/ml (Figure 2.3A). Similar effects were seen in the presence of LPC (lysophosphatidylcholine), however this lipid was

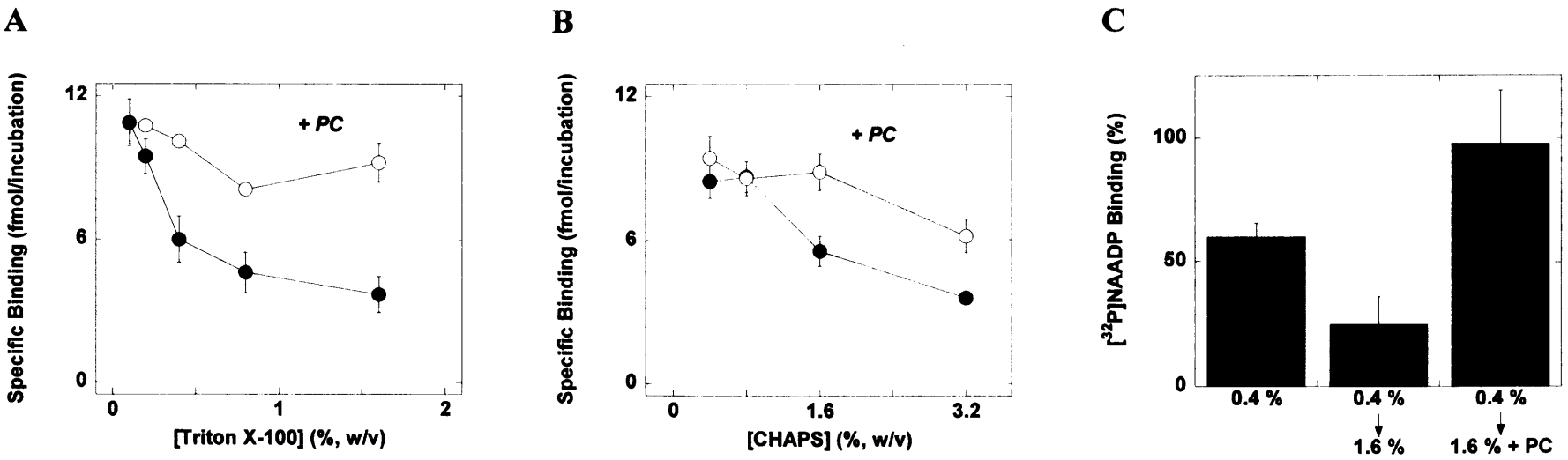

Figure 2.2: Binding of [^{32}P]NAADP to solubilized sea urchin egg homogenates is inhibited by increasing detergent concentrations

Binding of [^{32}P]NAADP to Triton X-100 solubilized extracts (arbitrary receptor concentration = 1) was determined in the presence of Triton X-100 (A) or CHAPS (B) at the indicated concentrations in the absence (closed circles) or presence (open circles) of PC (5:2 ratio, PC:Triton X-100 or 1:1 ratio, PC:CHAPS). Results are from at least four independent experiments. (C) Binding of [^{32}P]NAADP was determined to Triton X-100-solubilized extracts adjusted to a submaximal inhibitory concentration of Triton X-100 (0.4 %, w/v) and compared with samples to which further detergent was added (after 5 minutes incubation) to a final concentration of 1.6 % (w/v) either in the absence (0.4 % → 1.6 %) or presence (0.4 % → 1.6 % + PC) of PC. Data are normalised to [^{32}P]NAADP binding at 0.1 % (w/v) Triton X-100.

less effective than PC (Figure 2.3A). The sphingosine-based phospholipid, sphingomyelin afforded little protection against the inhibitory effects of high detergent (Figure 2.3A) on [^{32}P]NAADP binding. Diacylglycerol, and non-esterified fatty acids also failed to protect against Triton X-100 inhibition (Figure 2.3B). In fact AA and PA (palmitoleic acid) further inhibited [^{32}P]NAADP binding to delipidated proteins. Inhibitory effects of Triton X-100 on ligand binding were partially prevented by the polar head group phosphocholine (Figure 2.3C). However, high concentrations of phosphocholine were required relative to PC when considering the molecular mass of the two molecules. Choline alone had little effect (Figure 2.3C). Finally, I investigated the effects of PE and PS, a neutral and negative phospholipid, respectively. Like PC, both PE and PS were effective in preventing the inhibitory effects of Triton X-100 (Figure 2.3D) on [^{32}P]NAADP binding to solubilized sea urchin egg homogenates. Taken together, these data illustrate the specific importance of phospholipids in maintaining binding of NAADP to its target protein.

Saturation analysis of [^{32}P]NAADP binding to soluble NAADP receptors in low and high detergent

In an attempt to uncover a mechanism describing inhibition of [^{32}P]NAADP binding to its target protein upon lipid removal, saturation binding analysis was performed in the presence of low and high detergent. A decrease in the apparent affinity for [^{32}P]NAADP to its target protein was observed upon delipidation (Figure 2.4A and B). At a Triton X-100 concentration of 0.1 % (w/v), the apparent affinity was approximately 100 ± 3 pM (n = 5). In the presence of a high Triton X-100 concentration (1.6 %, w/v) this value clearly increased, however no curve fit was applied as saturation was not observed (Figure 2.4A and B). Use of higher ligand

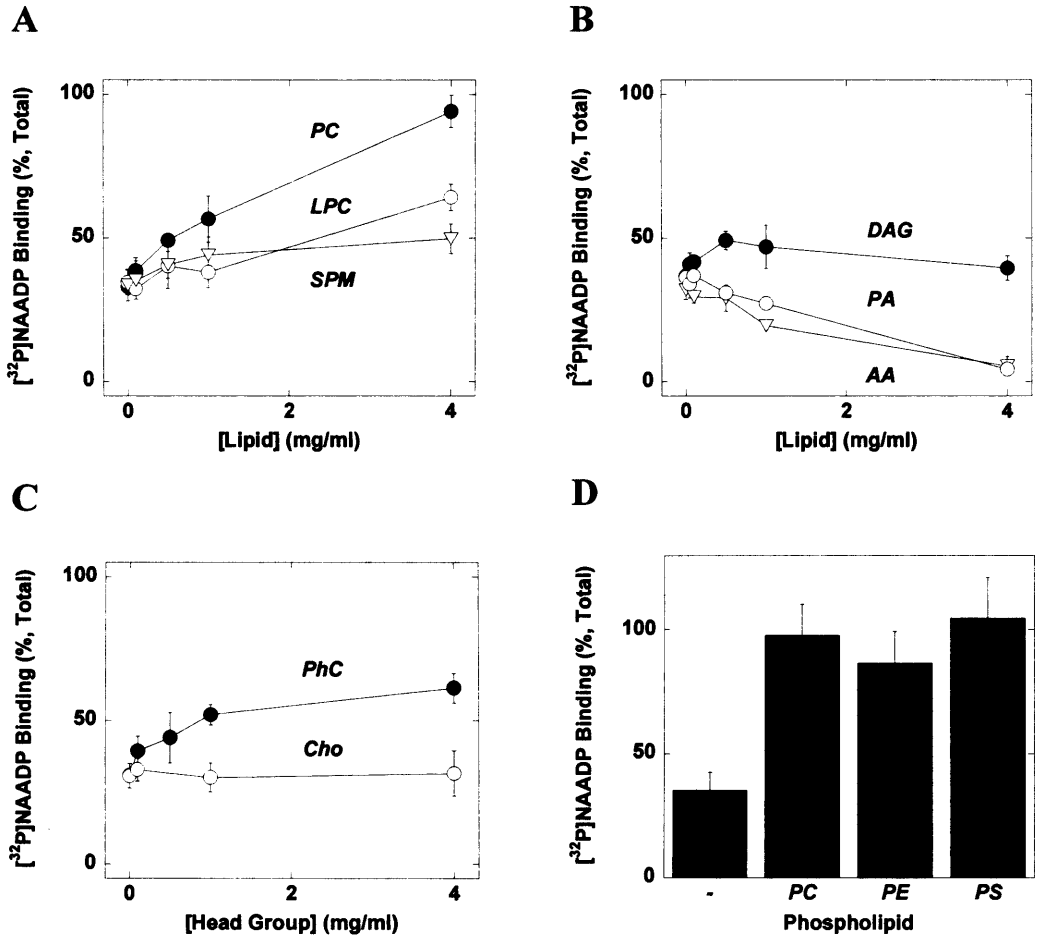

Figure 2.3: Phospholipids specifically prevent the inhibitory effects of delipidation on [^{32}P]NAADP binding

Binding of [^{32}P]NAADP to solubilized samples was determined in the presence of Triton X-100 (1.6 %, w/v) and the indicated concentration of (A) PC (closed circles), LPC (open circles) or SPM (inverted triangles), (B) DAG (closed circles), PA (open circles) or AA (inverted triangles) or (C) head group constituents phosphocholine (PhC, closed circles) and choline (Cho, open circles). (D) Binding of [^{32}P]NAADP to solubilized samples was also determined in the presence of Triton X-100 (1.6 %, w/v) and either PC, PS or PE (4 mg/ml), or in the absence of phospholipid (-). All data (from at least three experiments) are expressed relative to binding of [^{32}P]NAADP determined at 0.1 % (w/v) Triton X-100. Data expressed as mean ± S.E.M.

concentration was precluded since ratio of non-specific to total binding approached unity (inset Figure 2.4A). The total level of [^{32}P]NAADP binding at highest concentration of ligand tested (3 nM) was similar at Triton X-100 concentrations of 0.1 % (w/v) (6.3 ± 1 fmol/incubation) and 1.6 % (w/v) (6 ± 1 fmol/incubation; n = 3).

Delipidation of solubilized sea urchin egg homogenates by gel filtration

To further examine the effect of delipidation on NAADP binding, solubilized sea urchin egg homogenates were separated by gel filtration. Gel filtration is a technique commonly applied to delipidate detergent extracts. By exploiting the irreversible nature of NAADP binding to its target protein (Aarhus et al., 1996;Billington and Genazzani, 2000;Patel et al., 2000a), sea urchin egg homogenates were labelled with [^{32}P]NAADP, unbound ligand removed by centrifugation, and soluble protein-ligand complexes recovered following detergent treatment. As previously reported (Berridge et al., 2002b), when "tagged" NAADP-binding protein was fractionated by gel filtration in KGluIM (intracellular-like medium) supplemented with Triton X-100 (1 %, w/v), NAADP receptor-ligand complexes were recovered as a single peak of radioactivity (Figure 2.5A) co-eluting with the molecular weight marker apoferritin (440 kDa, Figure 2.5, inverted triangle). However, gel filtration performed in KClIM (K^+ based intracellular-like media) using soluble preparations otherwise prepared in an identical manner, resulted in the recovery of two radioactive peaks (Figure 2.5B). The first peak corresponded to [^{32}P]NAADP bound to its target protein. The second peak was not readily recoverable by polyethylene glycol precipitation, had a broad distribution and was indicative of ligand dissociation from its target protein during gel filtration fractionation (inset Figure 2.5B). Having previously described the

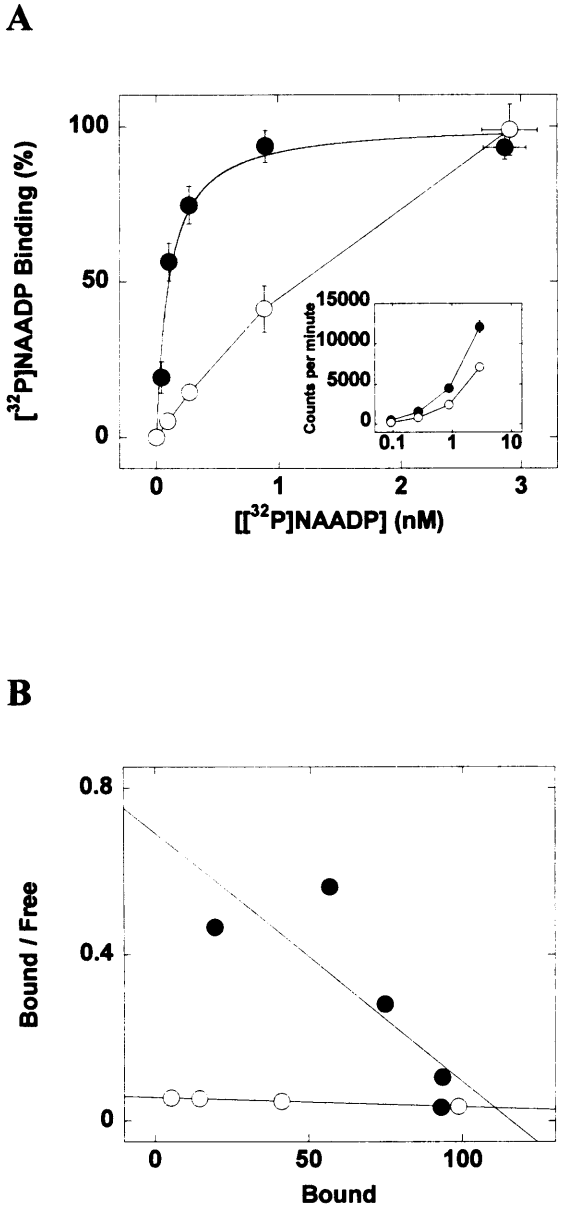

Figure 2.4: Saturation analysis of [^{32}P]NAADP binding to solubilized sea urchin egg homogenates in the presence of low and high concentrations of detergent

Binding of increasing concentrations of [^{32}P]NAADP to Triton X-100 solubilized extracts at a final detergent concentration of 0.1% (closed circles) or 1.6% (open circles) (w/v). Data are from at least three experiments (A) and analysed using a Scatchard plot (B). The inset of A shows total (closed circles) and non-specific (open circles) binding of [^{32}P]NAADP to solubilized extracts at a final detergent concentration of 1.6% (w/v) from a typical experiment.

requirement of phospholipids for NAADP binding to its target protein (Figure 2.1 – 2.3), I examined the possibility that fractionation in the two different media resulted in differential delipidation of soluble sea urchin egg homogenates and thus accounting for the observed difference in recovery of radioactivity bound to its target protein. Therefore, I analysed the phospholipid content of collected fractions. The majority of phospholipid was recovered in late fractions in both media, consistent with separation of protein and lipid in the presence of detergent (Figure 2.5C and D). In addition to this fraction, a second much smaller phospholipid fraction was detected eluting in earlier fractions. When phospholipid elution was determined in the presence of KGluIM, the early phospholipid pool co-eluted with NAADP binding sites (Figure 2.5C). However, when phospholipid elution was performed in KClIM, co-elution of phospholipid with NAADP binding sites was not observed (Figure 2.5D). Therefore it seemed that fractions enriched in [^{32}P]NAADP bound to its target protein, also contained significant levels of phospholipids when fractionated in KGluIM, but not KClIM. These observations thus undoubtedly suggest that binding of [^{32}P]NAADP to its target protein during gel filtration correlates with associated phospholipid.

Finally, to provide further evidence that dissociation of protein-ligand complexes was due to delipidation in KClIM, I examined the effect of fractionating soluble complexes in the presence of PC (Figure 2.6). When samples were separated in KClIM, in the presence of PC, ligand dissociation was prevented. PC however had little effect when included in KGluIM fractionation. These data provide further confirmation that NAADP binding to its target protein is highly dependent upon the presence of phospholipids.

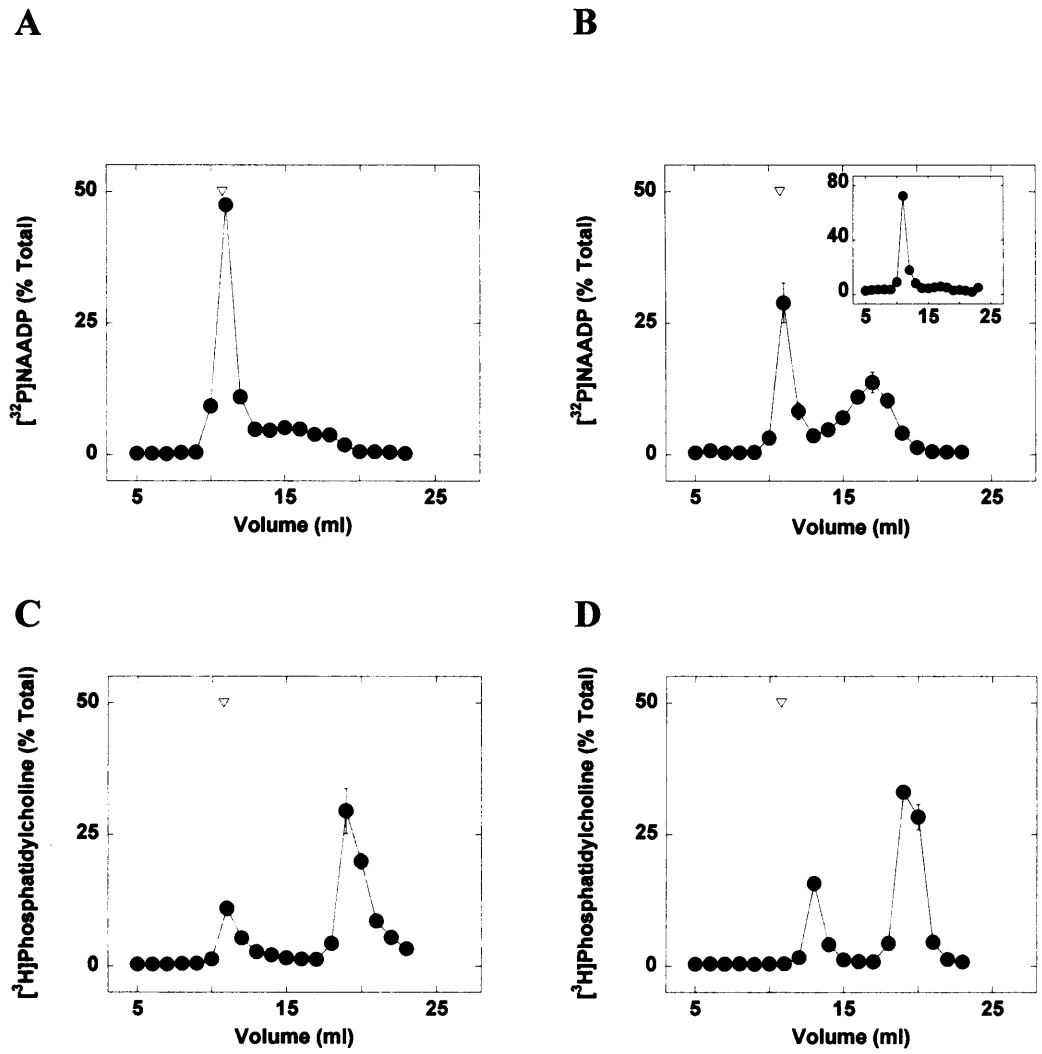

Figure 2.5: Gel filtration analysis of solubilized "tagged" NAADP receptors

Triton X-100 solubilized sea urchin egg homogenates were labelled with [^{32}P]NAADP (A and B) or [^{3}H]phosphatidylcholine (C and D) prior to solubilization and separated by gel filtration in either KGluIM (A and C) or KClIM (B and D) supplemented with 1 % (w/v) Triton X-100. Radioactivity of the collected fractions was determined by Cerenkov ([^{32}P]NAADP), or scintillation ([^{3}H]phosphatidylcholine) counting. Inverted triangle is the migration of the molecular weight marker apoferritin (440 kDa). The inset of B shows the distribution of recovered radioactivity following precipitation of the collected fractions with polyethylene glycol. Data are mean ± S.E.M from at least three experiments. In most cases error bars are smaller than symbols.

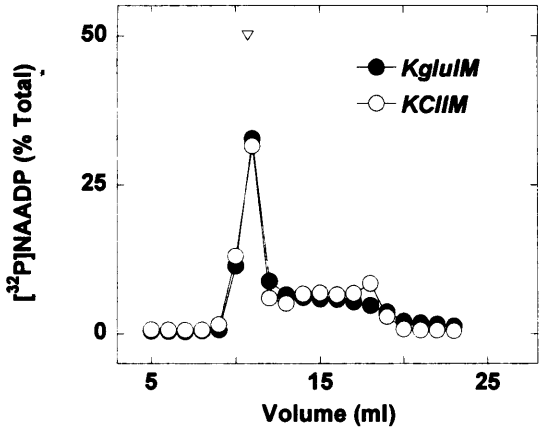

Figure 2.6: Gel filtration analysis of solubilized sea urchin egg homogenates in the presence of PC

[^{32}P]NAADP-labelled sea urchin egg homogenates solubilized with Triton X-100 were separated by gel filtration in KGluIM (closed circles) or KClIM (open circles), in the presence of 1 % Triton X-100 (w/v) and PC (1 mg/ml). Data are mean ± S.E.M. from three experiments.

2.4 Discussion

In this chapter, I have shown that NAADP binding to its target protein is inhibited as a result of decreasing the amount of lipid associated with the soluble sea urchin egg protein. To induce this change, I employed three independent methods: (i) by decreasing the amount of protein (by dilution) in the presence of a fixed detergent concentration, (ii) by increasing the detergent concentration at a fixed protein concentration, and (iii) fractionating soluble extracts by gel filtration in the presence of detergent. Inhibition of NAADP binding to its target protein by either of these methods was prevented by addition of phospholipids. The results described reveal a critical requirement of phospholipids for NAADP binding to its target protein.

That [^{32}P]NAADP binding to solubilized sea urchin egg homogenates was strikingly inhibited in the presence of high detergent (Figure 2.2A and B), allowed me to use a considerably delipidated preparation to investigate the specificity of the observed protective effects of PC compared to other lipids and related molecules (Figure 2.3). Cleavage of one fatty acid side chain from PC produces LPC, a molecule similar to PC. However, LPC was shown to be less effective than PC in preventing the inhibitory effects of delipidation on [^{32}P]NAADP binding to its target protein (Figure 2.3A). These results suggest that hydrophobic interactions between lipids and target proteins are crucial for maintaining NAADP binding. SPM, though not a glycerol-based lipid, contains a phosphorylcholine moiety and takes on a similar conformation to PC. However, SPM provided little protection against the inhibitory effects of detergent (Figure 2.3A) thus providing evidence to suggest that the protective effects of PC were indeed specific. The inhibitory effects of delipidation by high detergent were unaffected by diacylglycerol (which lacks the polar head group of PC), non-esterified fatty acids (Figure 2.3B), or head groups alone (at least at equivalent molar concentrations) (Figure 2.3C). These data provide evidence to suggest that both head groups and lipids tails of PC are required to stabilize NAADP binding to its target protein. That PE and PS also prevented the effects of delipidation (Figure 2.3D) suggest that the head group requirement may be non-specific. However, a balance between the hydrophobic and hydrophilic interactions of the lipid molecule may be critical. Interestingly, that AA further inhibited [^{32}P]NAADP binding to solubilized sea urchin egg homogenates was an effect consistent with previous reports describing inhibition of NAADP-induced calcium release by AA (Clapper et al., 1987).

Significant dissociation of NAADP occurred from protein-ligand complexes fractionated by gel filtration in the presence of detergent using KClIM (Figure 2.5B), when fractionated in the presence of KGluIM (Figure 2.5A), NAADP dissociation was not observed. That the two media contain the same concentration of K^+ (250 mM) and that this has previously been shown to maximally stabilize binding of NAADP to its target protein (Dickinson and Patel, 2003) cannot thus explain this difference. However, analysis of the phospholipid content in both conditions revealed noticeable variations. Phospholipids clearly co-elute with the NAADP-binding protein in the presence of KGluIM. In contrast, phospholipids do not co-elute with the NAADP-binding protein when fractionated in KClIM. A clear correlation between ligand dissociation and phospholipid content is therefore evident, however at present no explanation can be given explaining such differences in phospholipid migration. This correlation between phospholipid content and ligand dissociation is entirely consistent with a role for lipids in maintaining the recognition of NAADP by its target protein.

Little information is available describing the regulation of calcium-release channels by surrounding membrane lipids. Binding of IP_3 to partially purified liver (predominately type II) IP_3R was specifically inhibited by PIP_2 (Kamata et al., 1992). Furthermore, PIP_2 was also shown to inhibit binding of IP_3 to recombinant type I membrane-bound receptors IP_3Rs (Lupu et al., 1998). Thus it was suggested that the inhibitory effects of PIP_2 were likely due to interaction with the IP_3 binding site. PC, PE and PS were shown to have little effect on IP_3 binding to its receptor (Kamata et al., 1992).

Digestion of cardiac sarcoplasmic reticulum vesicles with phospholipase A2 (which cleaves single fatty acid residues from phospholipids), inhibited [^{3}H]ryanodine binding, an effect reversed by PC, PE and PS (Dong et al., 2001). These data thus provide evidence in support of a requirement of phospholipids in maintaining binding of ryanodine to its receptor. That ryanodine binding to soluble type I ryanodine receptors is inhibited by CHAPS in the absence, but not in the presence of PC (Pessah et al., 1986;Du et al., 1998), suggests that NAADP binding to its target protein described in this chapter (Figure 2.2B) shares similar characteristics to the ryanodine receptor.

At present, the mechanism to explain inhibition of NAADP binding to its target protein by removal of phospholipids is not known. Disruption of the quaternary structure of the protein complex that is essential for ligand binding by delipidation is one possible explanation. However to substantiate this claim, isolation and purification of the NAADP target protein is required. In the case of high affinity ryanodine binding to ryanodine receptors, a contribution from each subunit within the tetrameric assembly is required (Lai et al., 1988). In contrast however, each individual subunit of the IP_3R binds IP_3 independently (Mignery and Sudhof, 1990), a property consistent with the reported insensitivity of ligand binding to phospholipids for this family of intracellular calcium release channel (Kamata et al., 1992).

To conclude, in the present chapter, I provide evidence to show that phospholipids are required for NAADP to bind to its target protein. By maintaining the lipid environment of the NAADP receptor, thus preserving ligand binding, new strategies

can be devised to aid purification and functional characterisation of this novel calcium release channel.

CHAPTER 3 Time-sensing by NAADP receptors

3.1 Introduction

Specific binding sites for NAADP have been identified in a variety of biological preparations (reviewed in Section 1.6) including sea urchin eggs (Aarhus et al., 1996;Genazzani et al., 1996;Billington and Genazzani, 2000;Patel et al., 2000a;Berridge et al., 2002b), mouse brain (Patel et al., 2000b), rabbit heart (Bak et al., 2001) and MIN6 cells (Masgrau et al., 2003). Intriguingly, competitive binding studies reveal that addition of an excess of unlabelled NAADP to sea urchin egg homogenates previously exposed to radiolabelled NAADP is ineffective in displacing bound [^{32}P]NAADP (Aarhus et al., 1996;Billington and Genazzani, 2000;Patel et al., 2000a). These data show that binding is irreversible. Analysis of binding at various times after the addition of [^{32}P]NAADP indicate that unlabelled NAADP is able to prevent the further association of [^{32}P]NAADP, however its inability to displace bound [^{32}P]NAADP suggests that the ligand binding site is occluded (Aarhus et al., 1996;Billington and Genazzani, 2000;Patel et al., 2000a). A recent study has unveiled a reversible component of NAADP binding in sea urchin egg homogenates (Dickinson and Patel, 2003). In low K^+-containing media, [^{32}P]NAADP bound to its receptor in sea urchin egg homogenates can be displaced by an excess of unlabelled NAADP (Dickinson and Patel, 2003). These studies suggest that the NAADP-binding site may exist in more than one conformation.

A further extraordinary property of NAADP-induced calcium release in sea urchin eggs is the ability of low concentrations of NAADP that do not induce calcium release to block subsequent release of calcium by otherwise maximal concentrations of NAADP (Aarhus et al., 1996;Genazzani et al., 1996). Inactivation of NAADP-

induced calcium release by prior exposure to sub-threshold concentrations of NAADP appears similar to the irreversible nature of NAADP binding to its receptor. Inactivation of NAADP-induced calcium release is both time- and concentration-dependent. Pretreatment of homogenates with low NAADP concentrations for a short duration greatly reduces calcium release in response to high NAADP concentrations. However, homogenates exposed to sub-threshold concentrations of NAADP for longer durations are totally refractory to maximal NAADP concentrations (Aarhus et al., 1996;Genazzani et al., 1996). Clearly then the duration of prior exposure to sub-threshold concentrations of NAADP influences receptor inactivation. Furthermore, inactivation was more complete the higher the concentration of NAADP used in the pretreatment (Aarhus et al., 1996;Genazzani et al., 1996).

In this chapter, I describe a novel form of molecular "memory" displayed by the NAADP receptor, which manifests as a stable receptor-ligand interaction. Intriguingly, the stabilization of receptor-ligand complexes displays some similarities to NAADP-induced inactivation of calcium release with respect to its time and concentration dependence. By preventing NAADP receptor-ligand stabilization, I investigate the role of this molecular memory in NAADP-induced receptor inactivation and desensitization.

3.2 Materials and Methods

Synthesis of radiolabelled NAADP

[^{32}P]NAADP was prepared as previously described (Section 2.2) with modifications. Briefly, [^{32}P]NAD (specific activity 1000 Ci/mmol; Amersham Biosciences) was incubated for 1 hour at 37°C with 50 mU/ml human NAD kinase (Lerner et al., 2001)

(kindly provided by Dr Grant C Churchill), 5 mM MgATP, 0.5 mM $MnCl_2$ and 60 mM NaHepes. The reaction was then diluted into a medium containing 125 mM nicotinic acid and 1.25 μg/ml ADP-ribosyl cyclase and incubated for 1 hour 30 minutes at room temperature. NAADP was purified by separation of the final mixture by anion-exchange chromatography as described in Materials and Methods, Section 2.2.

Gel-filtration analysis of labelled NAADP receptors

Sea urchin egg homogenates were labelled with [^{32}P]NAADP prior to solubilization with Triton X-100 (Figure 3.1A) similar to that described in Section 2.2. Briefly, sea urchin egg homogenates (2.5 %, v/v) were incubated for 5 – 120 minutes either at room temperature or 4°C (Figure 3.4) in KGluIM supplemented with the indicated concentration of [^{32}P]NAADP. In some experiments, sea urchin egg homogenates were pretreated with staurosporine (100 nM, Sigma) or a phosphatase inhibitor cocktail (1:100 dilution as directed, Calbiochem, 524635) for 15 minutes prior to addition of [^{32}P]NAADP. In other experiments, sea urchin egg homogenates were first washed (100,000 g, 5 minutes × 3), resuspended in KGluIM to a final homogenate concentration of 2.5 % (v/v) before the addition of [^{32}P]NAADP (100 pM) for 5 and 60 minutes.

All labelled homogenates were solubilized and fractionated by gel filtration in KClIM supplemented with Triton X-100 (1 %, w/v) and analysed directly for radioactivity as described in Chapter 2.

In several experiments, sea urchin egg homogenates were first solubilized prior to incubation with their ligand. Sea urchin egg homogenates were washed (100,000 g, 3 × 5 minutes), resuspended (8 %, v/v) in KGluIM, solubilized with Triton X-100 and recovered as otherwise described (Materials and Methods, Section 2.2). Solubilized extracts were incubated for 5 – 60 minutes at room temperature in the presence of 100 pM [^{32}P]NAADP, fractionated by gel filtration and analysed directly for radioactivity by Cerenkov counting. Binding of [^{32}P]NAADP to extracts before gel filtration was determined by precipitating soluble extracts with polyethylene glycol (15 %, w/v) and γ-globulin (4 mg/ml) for 30 minutes at 4°C. Recovered pellets were washed with ice-cold polyethylene glycol (15 %, w/v), dissolved in water and radioactivity determined by Cerenkov counting. NAADP receptor migration was compared with that of apoferritin (as described in Chapter 2) by measuring absorbance of the column eluate at 280 nm.

^{45}Ca flux assay

Sea urchin egg homogenates (50 %, v/v) were serially diluted (2-fold every 30 minutes) at room temperature to a final concentration of 2.5 % (v/v) with KGluIM supplemented with 3 μg/ml oligomycin, 1 mM potassium cyanide (to inhibit mitochondrial uptake), 1.5 μM $^{45}CaCl_2$ (1Ci/mmol) and an ATP-regenerating system. The ATP-regenerating system (to promote uptake of ^{45}Ca into intravesicular Ca^{2+} stores) consisted of 10 U/ml creatine phosphokinase, 10 mM phosphocreatine and 1 mM ATP. Intravesicular calcium stores were allowed to load for 3 hours at room temperature to achieve steady-state levels characterised previously (Chini and Dousa, 1996).

In inactivation experiments (Figure 3.7), ^{45}Ca-loaded sea urchin egg homogenates were first chilled to 4°C or kept at room temperature for 10 minutes and then pretreated with the indicated (sub-threshold) concentrations of NAADP for a further 10 minutes. Release of ^{45}Ca was initiated by the addition of 10 μM NAADP for 2 minutes.

For calcium release experiments (Figure 3.8), sea urchin egg homogenates loaded with ^{45}Ca were either chilled to 4°C or maintained at room temperature prior to stimulation with the indicated concentration of NAADP for 2 minutes.

The ^{45}Ca content of the homogenate was determined by rapid filtration through Whatman GF-B filters under vacuum followed by washing three times with ice-cold KGluIM supplemented with 3 mM $LaCl_3$. Non-specific binding of ^{45}Ca to sea urchin egg homogenates was determined by the addition of the calcium ionophore ionomycin (10 μM, Calbiochem).

Data analysis

The curves in Figure 3.1 were fitted to the following equation:

$$S = S_{max} * (1 - e^{-k*t})$$

Where the observed rate constant (k) is the time (t) taken for specific binding (S) to reach its maximum (S_{max}).

NAADP-induced inactivation and release curves (Figures 3.7 and 3.8) were fitted to the following equation:

$$C = (C_1 / [1 + (IC_{50} / [L])^h]) + C_3$$

Where the IC_{50} is the concentration of ligand (L) causing half-maximal inactivation (Figure 3.7) / or half-maximal depletion (Figure 3.8) of vesicular ^{45}Ca (C), C_1 is inactivation (Figure 3.7) / total NAADP releasable ^{45}Ca pool (Figure 3.8), C_3 is the amount of ^{45}Ca bound non-specifically and h is the Hill coefficient.

3.3 Results

Time and concentration-dependent stabilisation of NAADP receptors by NAADP

As described in Chapter 2 delipidation of "tagged" solubilized sea urchin egg homogenates by gel filtration in KClIM supplemented with Triton X-100 (1 %, w/v) led to the recovery of two distinct radioactive peaks. The first peak, recoverable as a high molecular weight complex, corresponded to the receptor-ligand complex and the second peak was indicative of ligand dissociated from its receptor. Strikingly, reducing the duration of receptor labelling from 60 minutes (Figure 3.1B) to 5 minutes (Figure 3.1C) resulted in greater ligand dissociation from NAADP receptors following gel filtration. This clear time-dependence was remarkable given that exposure of ligand to its receptor had occurred some several hours prior to fractionation.

Intriguingly, though receptor-ligand complexes were more prone to dissociation, and as a result less "stable" when labelled for shorter durations, the extent of labelling was

effectively complete by 5 minutes (Figure 3.1D). Thus, radioactivity associated with membranes was similar following incubation with radioligand for 5 (2.2 ± 0.1 fmol/packed µl, n = 21) or 60 (2.7 ± 0.1 fmol/packed µl n = 23) minutes. These data demonstrate that the rate at which receptors become stabilized is substantially slower then the rate at which receptors bind their ligand.

Stabilization of receptor-ligand complexes was further investigated in the presence of various concentrations of [^{32}P]NAADP. Receptors exposed to 1000 pM [^{32}P]NAADP for 5 minutes (Figure 3.1E) stabilized rapidly compared to receptors labelled for 5 minutes with 50 pM [^{32}P]NAADP.

NAADP receptor-ligand complexes are intact prior to gel filtration

Since I had already shown in Chapter 2 that dissociation of NAADP receptor-ligand complexes under the present gel filtration conditions was due to separation of protein and lipids (Figure 2.5), I hypothesised that NAADP receptors exposed to their ligand for shorter periods were more prone to delipidation than receptors labelled for longer. To test this, labelled receptors were fractionated in gel filtration medium supplemented with phospholipid. The time-dependent effects on NAADP receptor stabilization were not apparent when soluble extracts were fractionated in the presence of phosphatidylcholine (PC). Little dissociation of ligand was observed from receptors labelled for either 5 or 60 minutes following gel filtration (Figure 3.2). These data show that delipidation of labelled sea urchin egg homogenates reveal the duration of receptor activation by NAADP.

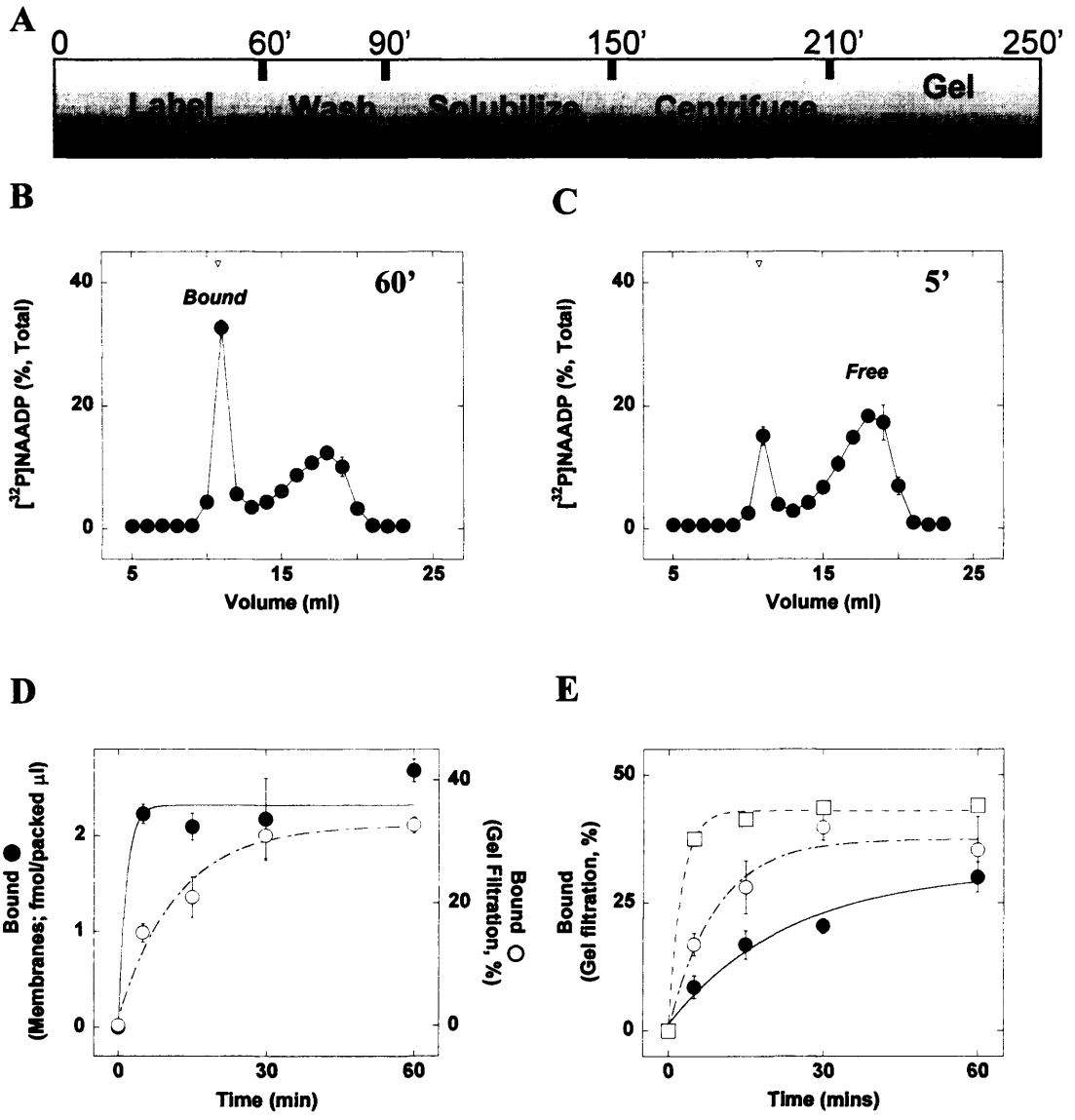

Figure 3.1: Time and concentration-dependent stabilization of NAADP receptors by their ligand

(A) Schematic protocol outlining the preparation and analysis of receptor-ligand complexes. Sea urchin egg homogenates (2.5 %, v/v) were incubated with [^{32}P]NAADP at room temperature in KGluIM. Unbound radioactivity was removed by centrifugation and labelled membranes solubilized in the presence of Triton X-100 (1 %, w/v). Solubilized extracts were fractionated by gel filtration in KClIM supplemented with Triton X-100 (1 %, w/v). Elution profiles of receptors "tagged" for 60 (B) and 5 (C) minutes. The inverted triangle represents the elution of aproferritin (molecular mass 443kDa). (D) Time-course comparing extent of receptor labelling (closed circles) with ligand remaining bound (open circles) to its receptor following gel filtration. (E) Time-course comparing the rate of NAADP receptor stabilization following labelling with 50 pM (closed circles), 200 pM (open circles) and 1000 pM (open squares) [^{32}P]NAADP. Data are means ± S.E.M from 3 – 23 experiments. In some experiments, error bars are smaller than symbols.

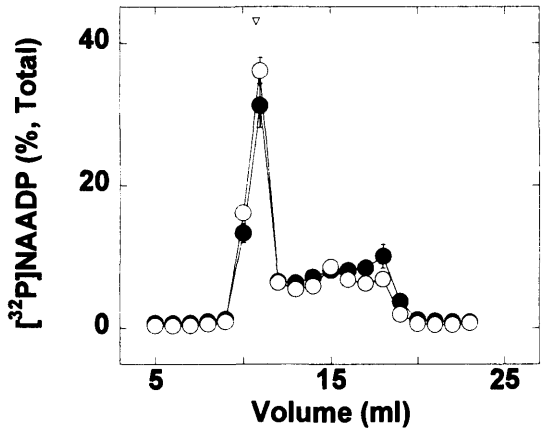

Figure 3.2: Time-dependent effects of NAADP on receptor stabilization are prevented by PC

Triton X-100-solubilized sea urchin egg homogenates labelled with 100 pM [^{32}P]NAADP for 5 (closed circles) or 60 minutes (open circles) were separated by gel filtration in KClIM supplemented with Triton X-100 (1 %, w/v) and PC (1 mg/ml). Data are mean ± S.E.M from 3 experiments.

Receptor-ligand stabilization occurs in the presence of kinase and phosphatase inhibitors and is not dependent on cytosolic factors

In an attempt to unravel a mechanism for receptor stabilization, the possible requirement of cytosolic factors, in particular, kinases and phosphatases was investigated. This approach was considered as the stabilization of NAADP receptor-ligand complexes occurred over a period of minutes suggesting a slow developing process.

When membranes were first washed to remove all cytosolic components and otherwise labelled and solubilized in an identical manner (to receptors in Figure 3.1), time-dependent stabilization of receptor-ligand complexes was comparable between control (2.4 ± 0.4-fold increase, n = 4) and washed (2.1 ± 0.1-fold increase, n = 3;

WM) membranes (Figure 3.3A). The extent of labelling of NAADP receptors was unaffected by washing (Figure 3.3B).

Additionally, receptors in their native membrane environment were either pretreated with 100 nM staurosporine (a non-specific kinase inhibitor) or a phosphatase inhibitor cocktail before labelling with [^{32}P]NAADP for 5 and 60 minutes.

Interestingly, prior treatment of receptors with staurosporine (100 nM) or a phosphatase inhibitor cocktail (1:100 dilution) did not affect NAADP receptor stabilization. In control experiments, a 2.4 ± 0.4–fold (n = 4) increase in the amount of ligand remaining bound to its receptor was evident between receptors labelled for 5 and 60 minutes (Figure 3.3A). Similar results were obtained from receptors preteated with staurosporine (2.5 ± 0.1-fold increase, n = 3; KI) and phosphatase inhibitor cocktail (2.7 ± 0.4-fold increase, n = 3; PI). Moreover, no difference was observed in the extent of receptor labelling following pretreatment with kinase or phosphatase inhibitors (Figure 3.3B).

Importantly however, the lack of a positive control cannot exclude the possibility of membrane bound kinases and phosphatases mediating time-dependent receptor stabilization by NAADP or NAADP binding.

Receptor stabilization is temperature-dependent

To further probe the possible involvement of an enzymatic process mediating stabilization, receptors were labelled in their native membrane for 5 and 120 minutes

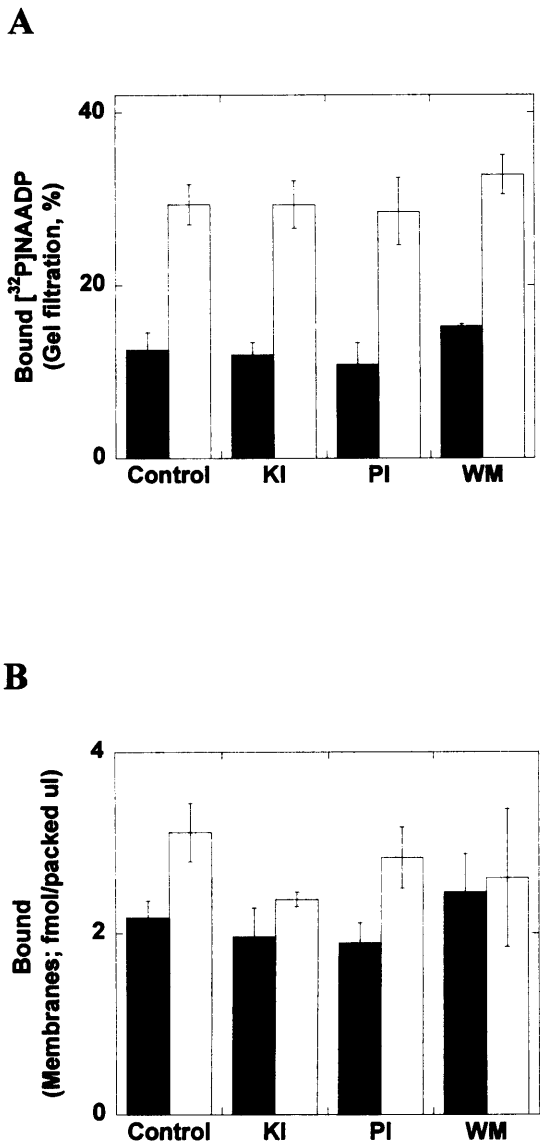

Figure 3.3: NAADP receptor stabilization does not require kinases, phosphatases or cytosolic factors

Sea urchin egg homogenates were pretreated with staurosporine (non-specific kinase inhibitor, KI), a phosphatase inhibitor cocktail (PI) or first washed twice in KGluIM prior to labelling with 100 pM [^{32}P]NAADP (WM) for 5 (closed bars) and 60 minutes (open bars). Results from a minimum of 3 experiments showing (A) the amount of ligand recovered bound to its receptor following gel filtration and (B) the extent of membrane labelling prior to solubilization.

at 4°C. Rather surprisingly, under these conditions, receptor stabilization was prevented (Figure 3.4A), since receptors labelled for longer durations were equally susceptible to the effects of delipidation (Figure 3.4A, B and C). Significantly however, temperature had no effect on the extent of receptor labelling (Figure 3.4D). Thus, labelling receptors at low temperatures did not affect binding but prevented subsequent stabilization of NAADP receptor-ligand complexes.

NAADP receptor stabilization occurs in the absence of free ligand

As observed in Figure 3.1, a clear lack of correlation exists between the extent of receptor labelling and subsequent receptor stabilization. One possible explanation is that stabilization occurs as a result of ligand binding to a reversible binding site. This site would not be observed when determining receptor labelling as ligand bound to reversible binding sites would dissociate upon washing of membranes prior to solubilization.

To investigate the possibility of reversible binding sites contributing to receptor stabilization, sea urchin egg homogenates were labelled for 5 minutes with 100 pM [^{32}P]NAADP, washed to remove free ligand and the homogenate subsequently incubated at room temperature for a further 55 minutes. In parallel incubations, receptors were labelled for 60 minutes without the removal of free ligand. Recovery of receptor-ligand complexes following fractionation (Figure 3.5) showed no difference in the amount of ligand bound to its receptor had receptors been labelled either for 5 minutes prior to the removal of free ligand or labelled with ligand for 60 minutes. Thus reversible binding sites are unlikely to contribute to stabilization of the receptor-ligand complex.

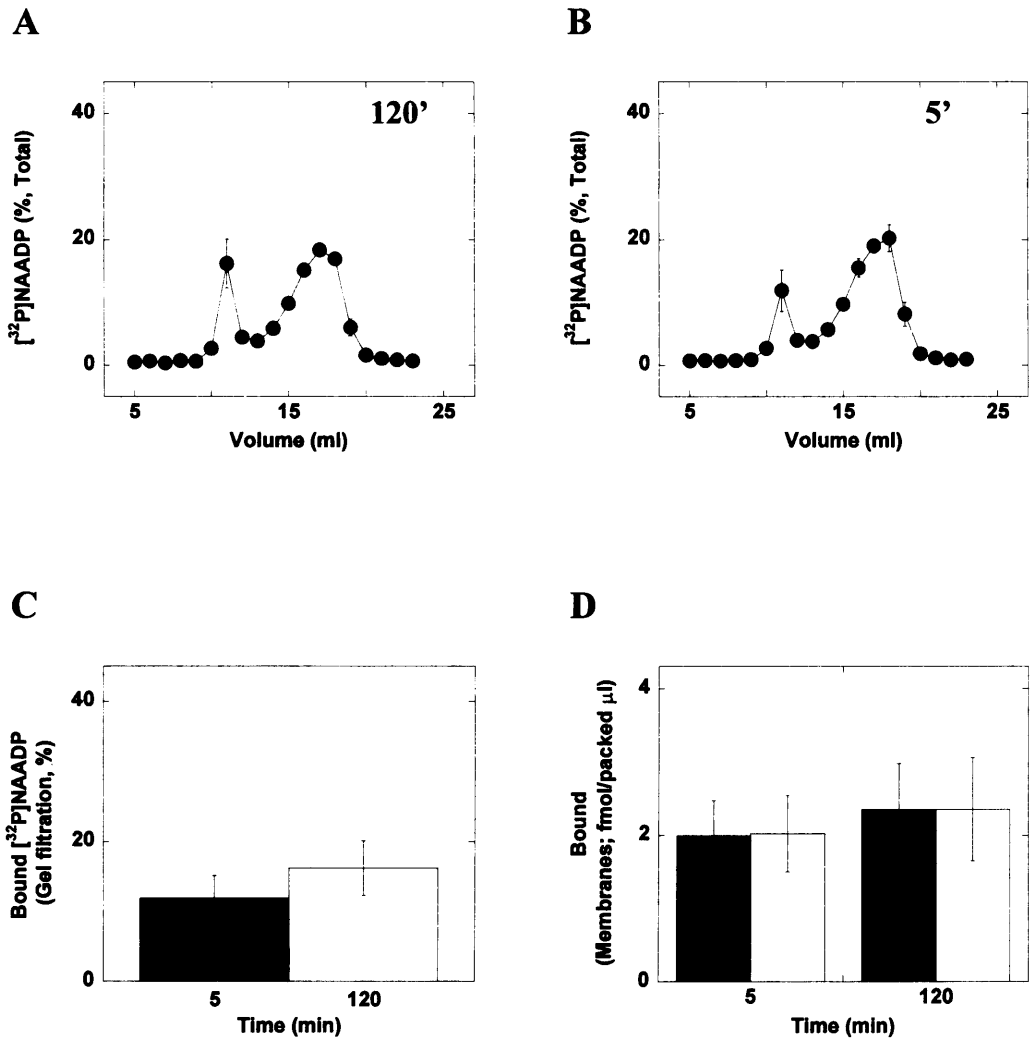

Figure 3.4: NAADP receptor stabilization is prevented when receptors are labelled at 4°C

Sea urchin egg homogenates were labelled with 100 pM [^{32}P]NAADP for 120 (A) and 5 (B) minutes at 4°C prior to solubilization and fractionation by gel filtration. Pooled data from 3 experiments (C) showing the amount of ligand remaining bound to its receptor following gel filtration and (D) extent of labelling at 4°C (closed bars) and room temperature (open bars).

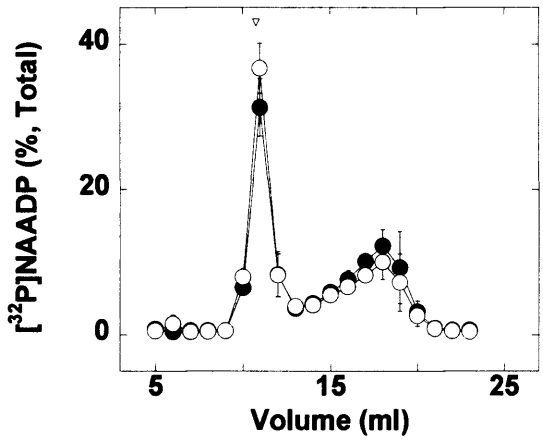

Figure 3.5: NAADP receptors stabilize in the absence of free ligand

Sea urchin egg homogenates were labelled for 5 minutes with 100 pM [^{32}P]NAADP. The remaining free ligand was washed by centrifugation, and labelled homogenates incubated at room temperature for a further 55 minutes (closed circles). In parallel, sea urchin egg homogenates were labelled for 60 minutes (open circles) with 100 pM [^{32}P]NAADP. Labelled homogenates were solubilized and fractioned by gel filtration as otherwise described. Data are mean ± S.E.M from 3 experiments.

Stabilization of solubilized sea urchin egg homogenates

In all experiments thus far NAADP receptors were exposed to their ligand whilst present in their native membrane environment. To investigate the possibility that NAADP receptors could stabilise outside of this setting, sea urchin egg homogenates were first solubilized prior to exposure of ligand. Soluble extracts were labelled with 100 pM [^{32}P]NAADP then separated by gel filtration. Receptors labelled for 5 minutes were more prone to dissociation compared to receptors labelled for 60 minutes (Figure 3.6A). However, radioactivity associated with soluble extracts (determined by protein precipitation with polyethylene glycol) prior to fractionation was no different following short or long incubations (Figure 3.6B). That soluble

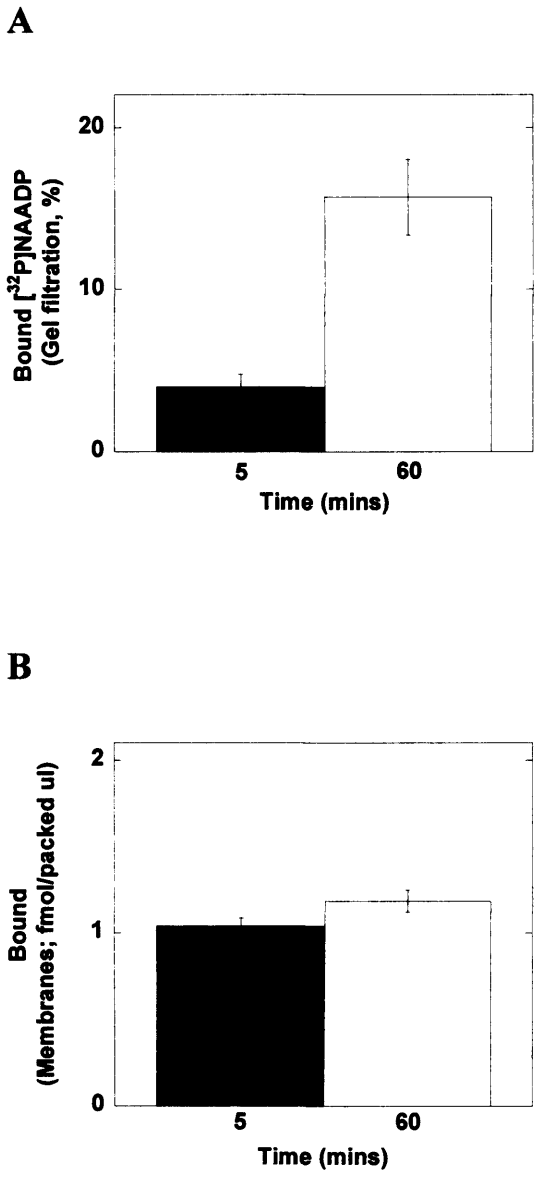

Figure 3.6: Stabilization of solubilized NAADP receptors

(A) Sea urchin egg homogenates were solubilized prior to exposure of [^{32}P]NAADP for 5 (closed bars) and 60 minutes (open bars) and subsequently fractionated in KClIM supplemented with Triton X-100 (1 %, w/v). (B) Extent of receptor labelling of NAADP receptors was determined following precipitation of soluble extracts with polyethylene glycol. Data are mean ± S.E.M from 3 experiments.

extracts remember their duration of activation suggests that this molecular "memory" may be intrinsic to the NAADP receptor.

Stability of receptor-ligand complexes is not related to NAADP receptor inactivation

An unusual property of the release mechanism activated by NAADP is the observed inactivation induced by sub-threshold concentrations of NAADP (see Section 3.1). Given that inactivation and stabilization are slowly developing processes, I considered the possibility that the stabilized conformation of the receptor-ligand complex was representative of an inactivated NAADP receptor. Since the effects of stabilization are clearly temperature dependent (Section 3.4), I investigated the effects of temperature on NAADP-induced receptor inactivation.

Sea urchin egg homogenates were loaded with ^{45}Ca at room temperature prior to investigating the effects of temperature on NAADP-induced inactivation of calcium release. Addition of ionomycin (10 μM) to loaded sea urchin egg homogenates reduced the ^{45}Ca content by 91 ± 1 % (n = 3). NAADP (10 μM) also reduced the intravesicular calcium content (by 43 ± 5 % n = 3), but was without effect when sea urchin egg homogenates were first challenged with 1 nM NAADP which alone did not affect calcium content (Figure 3.7). These data confirm that sub-threshold concentrations of NAADP can inactivate NAADP receptors. Similar results were obtained from experiments performed in ^{45}Ca loaded sea urchin egg homogenates that were first chilled to 4°C prior to NAADP challenge (Figure 3.7B).

To further investigate the effects of NAADP-induced inactivation at different temperatures, full dose-response curves were produced to uncover subtle changes (Figure 3.7C). Homogenates were subsequently incubated with various sub-threshold concentrations of NAADP for 10 minutes to inactivate receptors, prior to the addition

of a maximal stimulatory concentration of NAADP (10 μM). The IC_{50} for NAADP-induced inactivation at room temperature was 595 ± 250 pM (n = 3). Similarly, when inactivation was determined at 4°C, the IC_{50} was 339 ± 30 pM (n = 3). These data suggest that temperature was without effect on NAADP-induced inactivation.

Temperature affects NAADP-induced calcium release

Finally, I investigated the effect of a reduced temperature on calcium release induced by NAADP. NAADP-induced calcium release was elicited by addition of indicated concentration of NAADP for 2 minutes to ^{45}Ca loaded sea urchin egg homogenates at room temperature or first cooled at 4°C. Much to my surprise, a clear shift in the apparent affinity of NAADP to induce calcium release was observed at low temperatures (Figure 3.8). The EC_{50} of NAADP-induced calcium release at room temperature was 89 ± 9 nM (n = 3) and the Hill coefficient was 1.25 ± 0.35. However, when determined at 4°C, the EC_{50} of NAADP-induced calcium release was 21 ± 6 nM (n = 3), a clear 4-fold shift. Furthermore, the Hill coefficient was 0.59 ± 0.05. These data suggest that the NAADP receptor is more sensitive to its ligand at low temperatures.

3.4 Discussion

In this chapter, I have uncovered an unusual property of the NAADP receptor. The NAADP receptor, upon binding its ligand, undergoes a slow stabilization process, dependent on the time receptors are exposed to their ligand. The ability of NAADP receptors to distinguish between the duration of activation indicates that the NAADP receptor possesses a form of molecular memory. Stabilization of the receptor-ligand

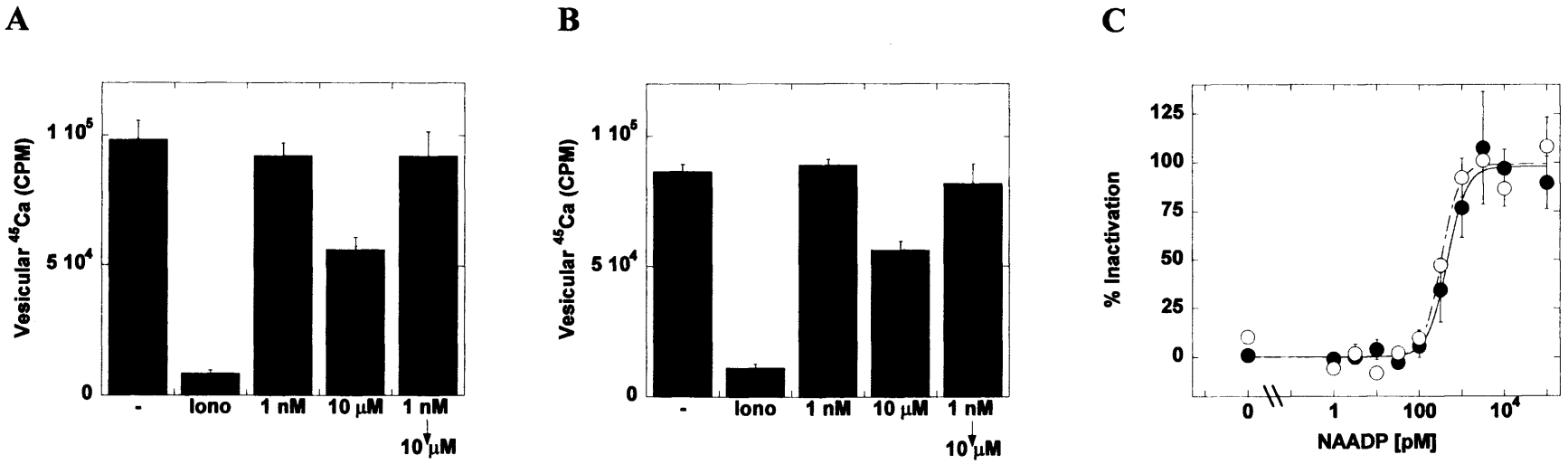

Figure 3.7: Inactivation of NAADP-induced calcium release by NAADP

Intravesicular calcium content in sea urchin egg homogenates (1.25 %, v/v) was determined following addition of NAADP for 2 minutes at the indicated concentrations, or by first adding a low sub-threshold concentration of NAADP for 10 minutes prior to addition of a maximal concentration of NAADP (1 nM → 10 µM) either at room temperature (A) or to homogenates first chilled to 4°C (B). Ionomycin (Iono, 10 µM) was added to determine non-specific binding of ^{45}Ca. (C) Sea urchin egg homogenates (1.25% v/v) were incubated with various sub-threshold concentrations of NAADP for 10 minutes prior to addition of 10 µM NAADP for 2 minutes. NAADP-induced inactivation was determined at room temperature (closed circles) and 4°C (open circles) and expressed relative to the amount of ^{45}Ca remaining in intravesicular stores following pretreatment with a maximally inactivating NAADP concentration. Data are pooled from 3 experiments expressed as mean ± S.E.M.

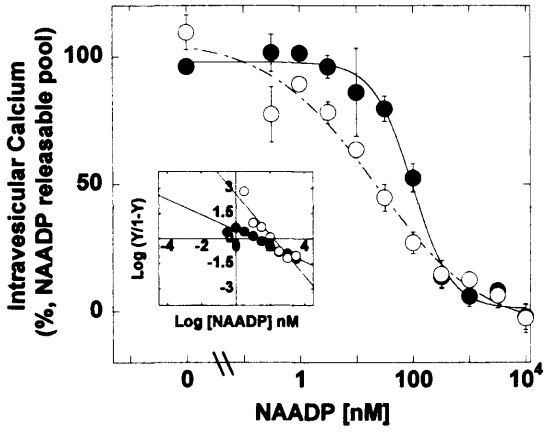

Figure 3.8: NAADP-induced depletion of intravesicular calcium stores in sea urchin egg homogenates

Sea urchin egg homogenates (1.25 %, v/v) were loaded with ^{45}Ca prior to the addition of indicated concentrations of NAADP for 2 minutes. NAADP-induced depletion of intravesicular calcium stores was determined at room temperature (closed circles) and 4°C (open circles). Data are from 3 experiments and expressed relative to the amount of ^{45}Ca remaining in intravesicular calcium stores following treatment with a maximally stimulating concentration of NAADP. The inset shows a Hill plot used to analyse the calcium release data.

complex does not require cytosolic factors or the continued presence of ligand.Stabilization persists upon solubilization but is inhibited at low temperature.

Initial observations revealed that NAADP receptor-ligand complexes were more prone to dissociation by gel filtration when receptors were labelled for shorter durations (Figure 3.1B and C). Rather more surprising was the discovery that when [^{32}P]NAADP binding was determined to membranes the extent of labelling was effectively complete by 5 minutes (Figure 3.1D). Intriguingly, when fractionation was performed in the presence of PC, no difference was apparent between receptors

labelled for 5 or 60 minutes (Figure 3.2). This data suggested that prior to fractionation, labelled NAADP receptors were intact and that delipidation revealed the duration of receptor exposure to its ligand. Thus by exploiting conditions that induce ligand dissociation, I have uncovered a highly unusual stabilization process that develops slowly (relative to receptor-ligand interaction) and can be thought of as an index of the time receptors were previously exposed to their ligand.

Stabilization of receptor-ligand complexes developed slowly over a period of minutes suggesting the possible involvement of an enzymatic-mediated process. In experiments where membranes were first washed (effectively removing cytosolic components) prior to labelling with [^{32}P]NAADP, receptors proceeded to stabilize (Figure 3.3). Furthermore, the ability of NAADP receptors to detect and remember their duration of activation was also apparent in receptors that had first been solubilized prior to exposure of ligand (Figure 3.6). In addition, prior treatment of NAADP receptors with staurosporine (a non-specific kinase inhibitor) or a phosphatase inhibitor cocktail did not affect stabilization of NAADP receptor-ligand complexes (Figure 3.3). Therefore, washing of membranes, or first solubilising receptors prior to exposure of ligand, and that staurosporine has no affect on NAADP receptor-ligand stabilization, suggests that kinases may not be involved since ATP (present during the preparation of homogenates) is effectively removed. However, although phosphatase inhibitors appear not to affect stabilization (Figure 3.3), one cannot rule out the possibility of membrane bound phosphatases playing a role in NAADP receptor-stabilization, since positive controls for phosphatase inhibitors are lacking. To comprehensively exclude both kinases and phosphatases, the activity of both enzyme inhibitors should be determined. For example, dephosphorylation of p-

Nitrophenyl phosphate (pNPP) by phosphatases turns pNPP yellow, and this can be detected at an absorbance of 405nm (Montalibet et al., 2005). By inhibiting phosphatase activity, dephosphorylation of pNPP should be prevented.

Importantly, temperature was able to prevent stabilization of receptor-ligand complexes (Figure 3.4). However, the extent of binding of NAADP to its receptor at different temperatures was unaffected and, was in agreement with earlier work (Patel et al., 2000a). This suggests a possible involvement of enzymatic activity contributing to receptor-ligand stabilization. Alternatively, low temperatures may simply alter the properties of associated lipids and affect membrane fluidity. However, as mentioned above, that NAADP receptor-ligand complexes can stabilise when removed from their lipid environment and still display this form of molecular memory suggests that this property is likely intrinsic to the NAADP receptor. Furthermore, receptor-ligand complexes stabilize in the absence of free ligand (Figure 3.5). These data suggest that binding of [^{32}P]NAADP to a reversible binding site does not contribute to receptor-ligand stabilization and supports the notion of a property built-in to the NAADP receptor.

The slow development of receptor stabilization described here is similar in some respects to the inactivation of calcium release by prior exposure of NAADP receptors to sub-threshold concentrations of NAADP (Aarhus et al., 1996;Genazzani et al., 1996). Thus, I hypothesised that the stabilized form of the NAADP receptor-ligand complex may be representative of an inactivated form of the receptor. However, although reducing the temperature prevented receptor stabilization (Figure 3.5), inactivation of calcium release was unaffected (Figure 3.7). The lack of affect of low

temperature on NAADP-induced inactivation of calcium release (described here) is in total agreement with earlier work (Genazzani et al., 1997b). These data suggest that NAADP receptor stabilization is unrelated to the inactivation phenomenon displayed by the NAADP receptors.

In contrast, a clear 4-fold increase in the apparent affinity of NAADP-induced calcium release was observed at low temperatures (Figure 3.8). It is possible then that the stabilized form of the NAADP receptor-ligand complex may represent a desensitized form of the NAADP receptor. Thus at 4°C NAADP-activated calcium channels are more likely to open since desensitization of NAADP receptors at this lower temperature appears not to occur. Furthermore, the inability to desensitize may explain the apparent shift in affinity of calcium release induced by NAADP. Examination of the Hill coefficients suggest that at room temperature, receptor desensitization may occur by activation of a single site, whereas at the reduced temperature, some form of negative cooperativity exists preventing receptor desensitization.

The change in the apparent affinity for NAADP-induced depletion of calcium stores described above, contradicts an earlier study where low temperature has no obvious effect on calcium release induced by NAADP (Genazzani et al., 1997b). It is worth noting however, that the temperatures in the present study and those in earlier differ by 3°C. Another noticeable difference between the two studies is that in the current work, release is determined by examining the ^{45}Ca content of intravesicular stores whereas in earlier work release is established by fluorimetry. One possible explanation for these differences is that the fluormetic dye used to measure calcium

may exhibit different properties at low and high temperatures. However, whether the change in temperature, or different methodology employed is sufficient to account for the differences in calcium release induced by NAADP remains to be established.

Given that NAADP binding to its receptor and NAADP-induced calcium release are demonstrable at low temperatures suggest that NAADP directly activates an ion channel to induce calcium release. Furthermore, these results exclude the possibility that NAADP activates the NAADP receptor by a metabolic reaction.

A wealth of information exists describing the modulation of ligand binding to intracellular calcium release channels by cytosolic proteins as well as numerous physiological ions (Fill and Copello, 2002;Patel et al., 1999). cADPR, as previously discussed (Section 1.5), is known to modulate the ryanodine receptor and induce calcium release (Galione et al., 1991). However, studies suggest that cADPR is unlikely to bind directly to ryanodine receptors (Walseth et al., 1993;Fill and Copello, 2002). It has been reported that cADPR in fact binds to an accessory protein (Walseth et al., 1993), which has been proposed as FKBP12.6 (Noguchi et al., 1997). Furthermore, purified sea urchin egg microsomes lose sensitivity to cADPR that is restorable by addition of calmodulin (Lee et al., 1994). In contrast IP_3 is thought to bind directly to the IP_3R (Guillemette et al., 1988). Removal of the cytosol does not inhibit NAADP binding and is without effect on receptor-ligand stabilization. These data suggest that NAADP binding to its target protein is similar to IP_3 binding to its receptor and does not require a cytosolic accessory protein. This is in contrast to cADPR binding to the ryanodine receptor. Taken together, these data suggest that soluble cytosolic proteins are unlikely to play a significant role in the stabilization of

NAADP receptor-ligand complexes however the role of closely associated proteins cannot be ruled out.

In summary, I have described an unusual stabilization process displayed by the NAADP receptor characteristic of a novel form of molecular memory. That temperature effectively prevents receptor-ligand stabilization suggests that an enzyme-mediated process, likely intrinsic to the NAADP receptor drives receptor-ligand stabilization. Identification and purification of this NAADP binding protein may help to explain the significance of this rather extraordinary "time-sensing" property.

CHAPTER 4 Determination of cellular NAADP levels

4.1 Introduction

Until recently, little information existed regarding cellular NAADP levels. Using a radioreceptor assay based on the sea urchin egg homogenate, levels of NAADP were quantified within sea urchin sperm (Billington et al., 2002;Churchill et al., 2003) and estimated to be present in the micromolar range. Significantly, these levels were shown to increase when sea urchin sperm were stimulated with sea urchin egg jelly, the first such report of changes in intracellular NAADP levels in response to a physiological stimulus (Churchill et al., 2003). These peculiarly high concentrations of NAADP suggested that upon fertilisation, sperm delivered NAADP into the cytosol of the egg. However, the exact components of egg jelly required to stimulate NAADP synthesis in sea urchin sperm were not investigated and remain unknown.

When sperm are in close proximity to the egg, diffusible carbohydrate molecules from egg jelly bind to targets on the sperm and induce the acrosome reaction (Neill and Vacquier, 2004). The acrosome reaction is a critical step required to fertilize the egg and is a calcium-mediated process. The acrosome reaction can be identified by two physiological events: exocytosis of the acrosomal vesicle and the extension of the acrosomal process (Neill and Vacquier, 2004). These events are required for sperm-egg fusion and successful egg activation.

A number of macromolecules isolated form sea urchin egg jelly have been demonstrated to induce the acrosome reaction (Darszon et al., 2001). The acrosome reaction induced by polysaccharides (carbohydrate signalling molecules) occurs in a species specific manor. For example, polysaccharides present in *L. pictus* egg jelly

can only induce the acrosome reaction in *L. pictus* sperm and not sperm from other species. In *Strongylocentrotus purpuratus*, three molecules in particular have been identified capable of regulating the acrosome reaction, the most important being fucose sulfate polymer (FSP) (Vacquier and Moy, 1997). Binding of FSP to sea urchin sperm leads to numerous ionic changes as well as increases in endogenous IP_3 levels (Domino and Garbers, 1988). In *S. purpurartus*, FSP binds to the sea urchin receptor for egg jelly (suREJ), which leads to two distinct increases in intracellular calcium (Vacquier and Moy, 1997). The first increase in intracellular calcium can be blocked by the calcium selective channel blockers verapamil and dihydropyridines (Darszon et al., 1999). The second increase in calcium has been suggested to occur via store-operated calcium entry channels, and although sperm lack endoplasmic reticulum, the acrosome vesicle has been identified in mice as an intracellular calcium store (Herrick et al., 2005). Other components of egg jelly involved in the acrosome reaction are sialoglycans. Sialoglycans alone do not induce the acrosome reaction, however, they greatly potentiate the effects of FSP (Hirohashi and Vacquier, 2002a). Speract, a sperm-activating peptide and chemoattractant in *S. purpurartus*, has also been implicated in inducing changes in intracellular calcium (Darszon et al., 1999), however, its role in the acrosome reaction remains to be resolved.

Little is known concerning NAADP levels in mammalian cells. NAADP-induced calcium release has been reported in a variety of cell types and demonstrated to occur within the nanomolar range (Patel et al., 2001), therefore, basal NAADP levels are likely to be lower than this value. If NAADP levels in cells are indeed lower, an accurate sensitive method is required to detect cellular levels, especially under non-stimulated conditions.

In this chapter, I have measured NAADP levels within sea urchin sperm using a radioreceptor assay and examined the effects of stimulating sperm with purified egg jelly and its components. Then, by utilizing the non-dissociating nature of NAADP binding to its target protein in sea urchin egg homogenates, I describe a simple method to increase the sensitivity of the sea urchin egg homogenate preparation to NAADP. I use this method to determine low concentrations of NAADP in a variety of cells from bacteria to humans.

4.2 Materials and Methods

Preparation of sea urchin sperm extracts

Sea urchin (*S. purpuratus*) sperm were obtained by intracoelomic injection of 0.5 M KCl and maintained "dry" at 4°C until use. Sperm were activated for 2 minutes by dilution to a final concentration of 4×10^7 /ml into either sea water or sea water containing purified egg jelly, FSP, sialoglycan or speract (kindly provided by the Vacquier Lab, Scripps Institution of Oceanography, San Diego; (Hirohashi and Vacquier, 2002a)). Sperm were pelleted by centrifugation (12,000 g, 30 seconds), the supernatant removed by aspiration and pellets extracted with 5 packed cell volumes of trichloroacetic acid (10 % w/v) for 5 minutes at 4°C. Precipitated protein was removed by centrifugation (12,000 g, 1 minute) and trichloroacetic acid was back-extracted from the supernatant with water-saturated diethyl ether (BDH, 5 × 1 ml). Extracts were neutralised with 100 mM NaHepes, allowed to air dry for 30 minutes to remove small traces of ether and frozen at -20°C until analysed.

Preparation of human red blood cell extracts

Red blood cells were isolated from human blood obtained from volunteers as described previously (Carrey et al., 2003). Cells pelleted by centrifugation (12,000 g, 13 seconds) were extracted with 2 packed cell volumes of trichloroacetic acid (Sigma, 10 %, w/v) for 1 – 240 minutes (as indicated) at room temperature. Precipitated protein was removed and trichloroacetic acid removed as described for sea urchin sperm extracts. Nucleotide recovery was determined by including [^{32}P]NAADP (approximately 20000 c.p.m.) with cells, followed by Cerenkov counting of the recovered sample. Extracts were prepared from 1.1×10^8 cells, 25 mg protein.

Red blood cells were also extracted with $HClO_4$ (perchloric acid, Sigma) with one cell volume of perchloric acid, 1.5 M for 60 minutes on ice. Samples were centrifuged (13,000 g) for 1 minute and recovered supernatant diluted with an equal volume of $KHCO_3$ (2M). Samples were incubated for a further 60 minutes on ice, after which insoluble potassium perchlorate was removed by centrifugation. The final pH of the supernatant was 8-8.5.

Preparation of other cell extracts

Hepatocytes from male Sprague-Dawley rats were isolated by EDTA perfusion of the liver, followed by centrifugation on a Percoll gradient as described in (Meredith, 1988) by Professor Jack Judah.

Competent *Escherichia coli* (JM 109; Promega) were transformed with a vector conferring ampicillin resistence (pGEM® - 3Z) and cultured for 16 hours at 37°C in

Terrific Broth, supplemented with 0.4 % (v/v) glycerol and 100 μg/ml ampicillin (kindly grown by Mr Jez Fabes).

J774 murine macrophages were cultured in Dulbecco's modified Eagle's medium, supplemented with 10 % (v/v) foetal bovine serum and antibiotics, and were maintained as suspensions at 37°C in an atmosphere of 5 % CO_2 (kindly provided by Dr Mathew Frost).

All the cells were harvested by centrifugation and washed twice with phosphate-buffered saline. Cells were pelleted by centrifugation (12,000 g, 13 seconds) and extractions (100 μl packed cell volume + 200 μl of trichloroacetic acid) were performed for 5 minutes at 4°C. The numbers of cells (and total protein content) used were 1.3×10^7 (10 mg), 7×10^7 (5 mg) and 5×10^7 (12 mg) for hepatocytes, *E. coli* and J774 cells respectively.

Determination of protein content

Protein content of cells was determined by a colourmetric assay based on the reduction of Cu^{2+} to Cu^{1+} (Smith et al., 1985) using the Bicinchoninic acid (BCA; Pierce) reagent and bovine serum albumin standards.

Treatment of extracts with alkaline phosphatase

Alkaline phosphatase conjugated to agarose (Sigma) was washed twice by centrifugation and resuspended in a buffer containing 100 mM Tris (pH 9; approximately 700 units/ml). Cell extracts (24 μl) were treated with the alkaline phosphatase or the corresponding vehicle (6 μl) and incubated at room temperature

for 1 hour. The final concentration of alkaline phosphatase-agarose in the incubations was approximately 140 units/ml. Alkaline phosphatase was removed by centrifugation at 12,000 g for 5 minutes before radioligand binding.

Radioligand binding

[^{32}P]NAADP was prepared as described in Section 2.2. [^{32}P]NAADP (0.5 nM) was allowed to bind to sea urchin egg homogenates (0.25 %, v/v) at room temperature in KGluIM (intracellular-like medium). Homogenates were incubated with cell extracts (at the dilutions indicated) or increasing concentrations of NAADP added either before or together with the radioligand. The bound and free radioligand were separated by rapid filtration (with a Tris buffer 100 mM Tris, pH 7.2) through Whatman GF-B filters using a cell harvester.

Determination of NADP levels

NADP levels in the cell extracts were determined (by comparison with known concentrations of NADP) either by high performance liquid chromatography (HPLC) or using a spectrophotometric enzyme-cycling assay.

Extracts were separated by anion-exchange chromatography on a Hypersil® NH_2 column (250 mm × 3.2 mm; Phenomenex, Macclesfield, Cheshire, U.K.). Samples were injected on to the column after equilibration with 5 mM KH_2PO_4 (pH 2.65), and the bound material was eluted with a buffer composed of 0.5 M KH_2PO_4 and 1 M KCl (pH 3.8) using a linear gradient (30 minutes separation; flow rate, 0.5 ml/min). Absorbance of NADP was monitored at 254 nm and area measurements determined using Breeze software (Waters).

The enzyme-cycling assay was based on the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl-2H-tetrazolium bromide; 500 μM) by NADPH, which was formed from NADP present in the extract after enzymatic oxidation of glucose-6-phosphate (5 mM) by glucose-6-phosphate dehydrogenase (2.5 units/ml). Reactions were performed at room temperature in the presence of 2 mM phenazine ethosulphate in a buffer composed of 100 mM Tris (pH 8). Absorbance of the formed product was monitored at 570 nm using a plate reader. NADP levels were similar when determined by HPLC or the cycling assay.

Data analysis

Curves in Figure 4.2 and 4.3 were fitted to the following equation:

$$B = (B_1 / [1 + (IC_{50} / [L])^h]) + B_3$$

Where in Figure 4.2, the IC_{50} is the concentration of egg jelly [L] causing the half-maximal increase in NAADP levels (B), B_1 is the specific change of NAADP levels and B_3 is the resting level of NAADP.

Data in Figure 4.3 shows the concentration of NAADP [L] capable of inhibiting specific binding (B) by 50 % (IC_{50}). In this instance, B_1 is specific binding and B_3 the amount of ligand bound non-specifically.

In both sets of experiments h is the Hill coefficient. All data are expressed as mean ± S.E.M.

4.3 Results

Changes in sea urchin sperm NAADP levels

Churchill and colleagues have previously shown that stimulating sea urchin sperm with sea water containing egg jelly leads to an increase in sperm NAADP levels (Churchill et al., 2003). In collaboration with the Vacquier Lab (Scripps Institution of Oceanography, University of California, San Diego), I stimulated sea urchin sperm with purified egg jelly (Vacquier and Moy, 1997); Figure 4.1A). NAADP levels (determined, using the radioreceptor assay described in (Churchill et al., 2003)) clearly increased in a concentration-dependent manner (Figure 4.1A). NAADP levels increased approximately 2.5-fold from a resting level of 0.29 pmol/mg protein (n = 2) to 0.77 pmol/mg (n =2) when stimulated with a maximal concentration of purified egg jelly. The half maximal concentration of egg jelly required to increase NAADP levels was estimated to be 0.11 μg fucose/ml based on the phenol-sulfuric assay using fucose as a standard (Hirohashi and Vacquier, 2002b) .

I next examined the effect of three purified components of egg jelly on their ability to stimulate increases in sperm NAADP content. When sea urchin sperm were stimulated with a maximal concentration of FSP (0.05 μg/μl), the major component of sea urchin egg jelly involved in the acrosome reaction, sperm NAADP levels remained unchanged (Figure 4.1B). Similarly, stimulation of sea urchin sperm with sialoglycan (0.038 μg/μl) or speract (10 nM) also did not affect cellular NAADP levels (Figure 4.1B, n = 2). These data suggest the major components of sea urchin egg jelly individually are not capable of inducing increases in NAADP levels within sea urchin sperm.

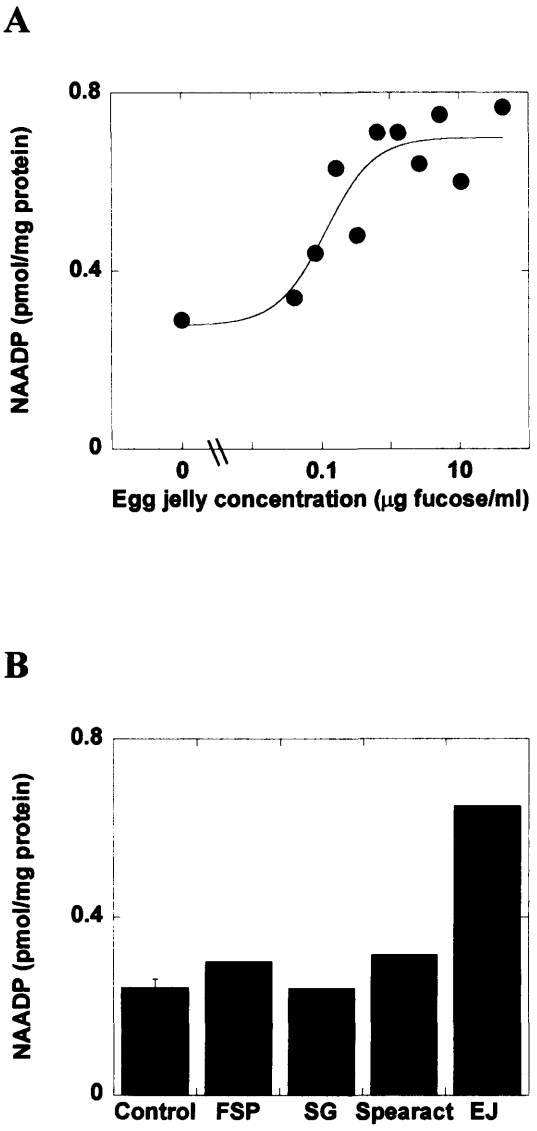

Figure 4.1: Changes in sperm NAADP levels

(A) NAADP levels were estimated in sea urchin sperm stimulated with the indicated concentration of purified egg jelly for 2 minutes prior to extraction with trichloroacetic acid. Results are from two independent extractions of one batch of sea urchin sperm. (B) Sea urchin sperm were stimulated with vehicle (control), fucose sulfate polymer (FSP, 0.05 μg/μl), sialoglycan (SG, 0.038 μg/ul), spearact (10 nM) or purified egg jelly (EJ, 42.5 μg fucose/ml) for 2 minutes prior to extraction with trichloroacetic acid. Results shown are from two independent stimulations of a single batch of sperm.

Increased sensitivity of the radioreceptor assay for NAADP

NAADP in sea urchin sperm is present at micromolar concentrations (Billington et al., 2002;Churchill et al., 2003). However, as discussed in Section 4.1, NAADP levels are likely to be lower in other systems. Therefore in order to determine cellular NAADP levels in cells where NAADP is probably maintained in the low nanomolar rage, I attempted to increase the sensitivity of the radioreceptor assay to NAADP from that described in (Churchill et al., 2003). Previous work has shown that low concentrations of unlabelled NAADP are more effective in inhibiting binding of radiolabelled NAADP to homogenates when added before the radioligand than when homogenates are exposed to the two ligands simultaneously (Patel et al., 2000a). This is likely due to irreversible binding of NAADP to its receptor in sea urchin egg homogenates. I exploited this increase in sensitivity to detect low concentrations of NAADP. Thus from conventional isotope dilution experiments (i.e. binding of radioligand determined in the simultaneous presence of various concentrations of unlabelled NAADP), the concentration of unlabelled NAADP that inhibited [^{32}P]NAADP binding by 50 % (IC_{50}) was 880 ± 220 pM (Figure 4.2). However, treatment of homogenates with the same concentration of NAADP before the addition of radioligand resulted in a marked leftward shift in the competition curves. When assayed under these conditions, sea urchin egg NAADP receptors appeared to be more sensitive (IC_{50} = 40 ± 5 pM) to their ligand (Figure 4.2). Clearly then by pre-incubating sea urchin egg homogenates with NAADP prior to addition of [^{32}P]NAADP, the sensitivity of the assay is significantly increased.

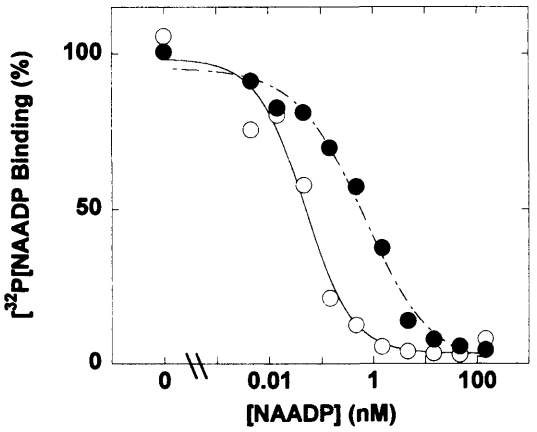

Figure 4.2: Increased sensitivity of sea urchin egg homogenates to NAADP

Binding of [^{32}P]NAADP to sea urchin egg homogenates (0.25 %, v/v) was determined in the presence of indicated concentration of unlabelled NAADP incubated 1 hour before (open circles) or simultaneously (closed circles) with radioligand. Data shown is from a typical experiment repeated at least 3 times.

Quantification of basal NAADP and NADP levels in a variety of cells

Having established conditions for the detection of low concentrations of NAADP, I initially analysed extracts from a readily available human cell type, the red blood cell for NAADP. Binding of [^{32}P]NAADP to sea urchin egg homogenates was inhibited by red blood cell extracts. Inhibition of radioligand binding by red blood cell extracts was more pronounced when incubated with sea urchin egg homogenates prior to the addition of [^{32}P]NAADP (Figure 4.3). By comparison with known concentrations of NAADP, the concentration of NAADP within the extract was estimated to be 16 ± 2 nM (n = 7) when the extract was added to the radioreceptor assay before [^{32}P]NAADP (Table 4.3) and 13 ± 1 nM, (n = 10) when extracts were added simultaneously with radioligand. After normalisation for protein, the cellular NAADP content was

determined to be 142 ± 6 fmol/mg. The data suggest that NAADP is likely present in red blood cell extracts.

In previous reports quantifying cellular NAADP levels, nucleotides were extracted with perchloric acid ($HClO_4$) (Churchill et al., 2003;Masgrau et al., 2003). I therefore compared levels of NAADP in red blood cells extracted with $HClO_4$ (Masgrau et al., 2003) and trichloroacetic acid (present study). After correction for differential nucleotide recovery ($HClO_4$ = 60 ± 3 %, n = 3 and trichloroacetic acid = 80 ± 5 %, n = 3), the NAADP content of red blood cells estimated by $HClO_4$ (345 ± 27 fmol/mg, n = 3) extraction was approximately 2-fold higher than trichloroacetic acid (156 ± 18 fmol/mg, n = 3) extraction (Figure 4.4A). In contrast, NADP levels were similar when determined by either $HClO_4$ (76 ± 14 pmol/mg, n = 3) or trichloroacetic acid (60 ± 6 pmol/mg, n = 5) extraction (Figure 4.4B).

I analysed three other cell types for NAADP content. NAADP was detected in trichloroacetic acid extracts from hepatocytes and *E. coli*, but only after prior incubation of the homogenate with the extract (Figure 4.3). The calculated NAADP concentration was 4.5 ± 0.5 and 2.5 ± 0.8 nM for rat hepatocytes and *E. coli* respectively (Table 4.1). However, NAADP levels were not detected in J774 murine macrophage extracts when determined by either method (Table 4.1).

Prior treatment of red blood cell extracts with alkaline phosphatase abolished the ability of the extracts to inhibit [^{32}P]NAADP binding (Figure 4.5). This was consistent with the removal of free phosphate groups and the inhibitory factor being NAADP.

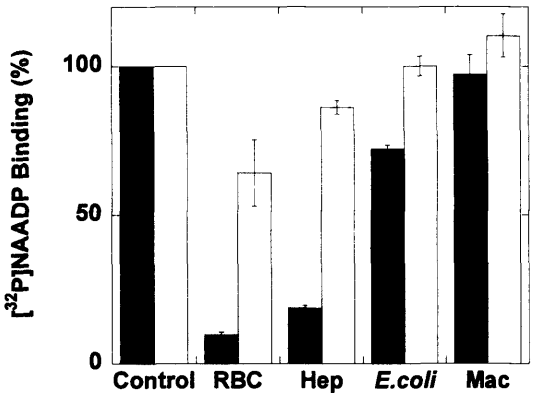

Figure 4.3: Inhibition of NAADP binding by cellular extracts

Binding of [^{32}P]NAADP to sea urchin egg homogenates was determined in the presence of cellular extracts prepared from red blood cells (RBC), rat hepatocytes (Hep), *Escherichia coli* (*E.coli*), J744 murine macrophages (Mac) or vehicle (Control) incubated before (closed bars) or simultaneously (open bars) with [^{32}]NAADP.

As shown in Figure 4.5, dephosphorylation of hepatocyte and *E.Coli* extracts also eliminated their ability to inhibit [^{32}P]NAADP binding.

To exclude the possibility of artificial generation of NAADP by acid breakdown of NADP, I performed a full time-course for extraction of red blood cells and determined NAADP and NADP content. No significant change in NAADP content was observed if red blood cells were extracted with trichloroacetic acid for 1 minute (12 ± 1 nM; n = 3) or 15 minutes (12 ± 1 nM; n = 3; Figure 4.6A). However, increasing the extraction period over 15 minutes increased NAADP levels significantly. For example, NAADP content in extracts was 26 ± 3 nM (n = 3) when extracted for 240 minutes (Figure 4.6A). The increase in NAADP levels following extractions over longer periods was

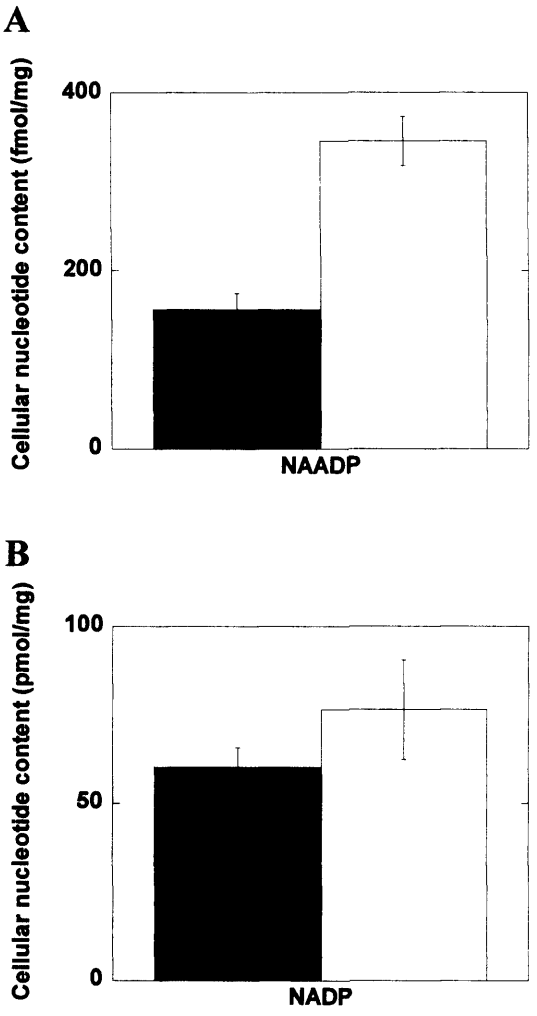

Figure 4.4: Effect of different extraction methods on measured NAADP and NADP levels in human red blood cells

Red blood cells were extracted with either trichloroacetic acid (closed bars) or perchloric acid (open bars) as described in Materials and Methods. NAADP (A) and NADP (B) levels were determined by use of the radioreceptor and cycling assays respectively. Data are corrected for differences in efficiency of nucleotide recovery.

Cell Type	[NAADP]		[NADP]
	Extract (nM)	Cell (fmol/mg)	Extract (μM)
Human red blood cell	16 ± 2	142 ± 6	7.8 ± 1
Rat hepatocytes	4.5 ± 0.5	109 ± 12	20 ± 11
E. coli	2.5 ± 0.8	81 ± 21	4.1 ± 0.6
J774 macrophages	n.d	n.d	3.1 ± 1.2

Table 4.1: Estimated cellular NAADP and NADP levels in a variety of cells. NAADP and NADP levels were determined from acid extracts prepared from a variety of cells. Results are means ± S.E.M for 3 - 10 independent preparations. NAADP was not detectable (n.d) in J7444 macrophages.

not due to increased extraction efficiency as NADP levels remained unchanged over the same time-course. In fact, NADP levels were identical when cells were extracted for 1 (5 ± 1 μM, n = 3) and 240 (5 ± 1 μM, n = 3) minutes (Figure 4.6A).

Finally, I compared the levels of NAADP in the extracts with the levels of NADP. Though the measured levels of NAADP in hepatocytes determined from different extracts was similar (3.2 – 5.2 nM), the NADP content was much more variable (7.7 – 49 μM) (Figure 4.6B). These data indicate little correlation between NAADP and NADP content further suggesting that the NAADP levels detected are unlikely due to acid breakdown of NADP.

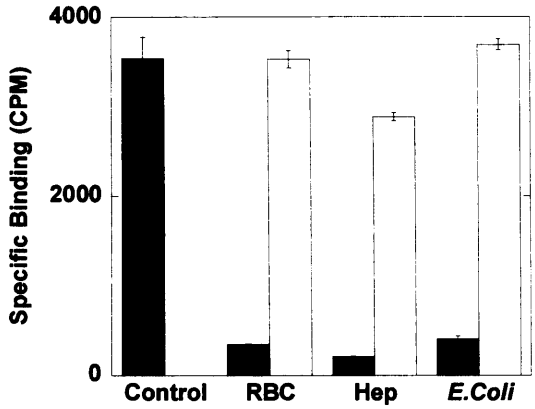

Figure 4.5: Inhibition of NAADP binding by cell extracts is prevented by alkaline phosphatase

Concentrated extracts were treated with either alkaline phosphatase (open bars) or vehicle (closed bars) for 60 minutes prior to incubation with sea urchin egg homogenates. [^{32}P]NAADP was subsequently added and [^{32}P]NAADP binding determined.

4.4 Discussion

Using a radioreceptor assay based upon the ability of sea urchin egg homogenates to bind NAADP, I have estimated NAADP levels in sea urchin sperm stimulated with purified egg jelly and constituent components. My data suggests that purified egg jelly increases NAADP levels within sperm in a concentration-dependent manner (Figure 4.1A). However, egg jelly components in isolation have little effect on NAADP levels (Figure 4.1B). Furthermore, by increasing the sensitivity of the radioreceptor assay for NAADP I have detected NAADP levels in primary cells ranging from bacteria to human.

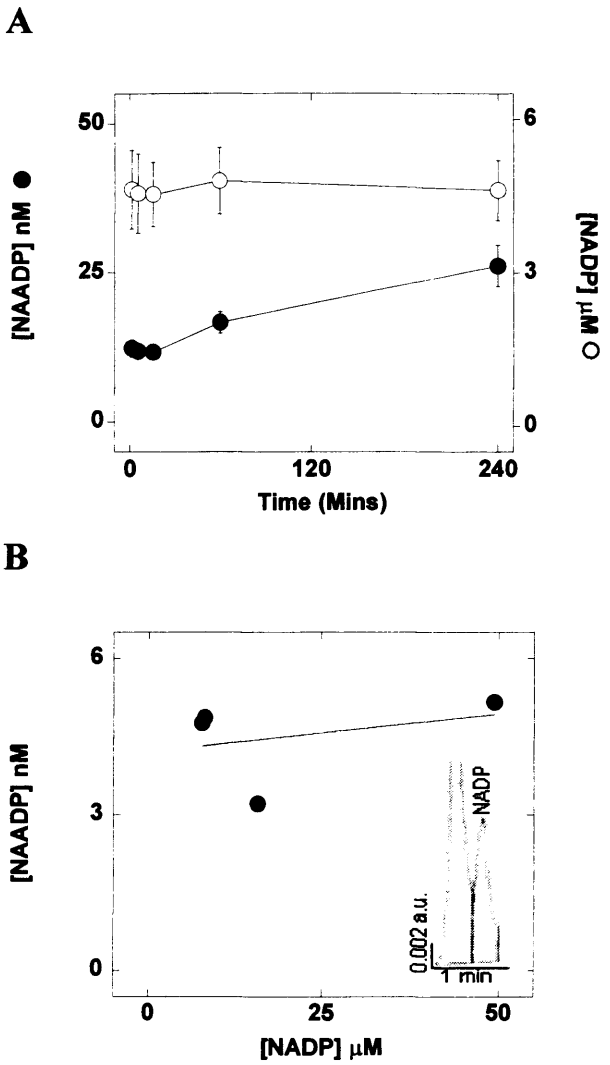

Figure 4.6: NAADP detected in cell extracts is not derived from NADP

(A) NAADP (closed circles) and NADP (open circles) levels were determined in red blood cells extracted with TCA for the indicated times. (B) Lack of correlation between NAADP and NADP levels in rat hepatocytes. Inset of B shows detection of NADP by HPLC.

In agreement with an earlier study (Churchill et al., 2003), NAADP levels within sea urchin sperm increased when stimulated with egg jelly (Figure 4.1). Sperm were stimulated for two minutes with increasing concentrations of purified egg jelly a time point shown previously at which NAADP levels within sperm peak. NAADP levels clearly increased approximately 2.5-fold, an order of magnitude comparable with previous work (Churchill et al., 2003). The effects of purified egg jelly were

concentration-dependent and clearly saturable (Figure 4.1A), suggesting the effects of egg jelly are likely involve receptors located on the sperm. However, the amount of NAADP estimated in the current study was significantly lower than that reported by Churchill and colleagues (Churchill et al., 2003). Interestingly, Billington and colleagues also report a large variation in estimated NAADP levels between sperm batches (Billington et al., 2002). One possibility to explain the discrepancy in NAADP levels is the different methods of nucleotide extraction employed (discussed below). Another possible explanation is that different species of sea urchin have different intracellular levels of NAADP. In the present study, NAADP levels were determined in *S. purpurartus* whereas published work estimated levels within *L. pictus*. Nevertheless, stimulation of sea urchin sperm with egg jelly leads to an increase in NAADP levels thus raising the possibility that NAADP is required at fertilisation.

In contrast, when sperm were stimulated with individual components of egg jelly that are involved in the acrosome reaction, no change in NAADP levels was detected (Figure 4.1B). These data would suggest that NAADP does not play a role in the acrosome reaction. However, the effects of FSP, a known inducer of the acrosome reaction can be potentiated by sialoglycans (Hirohashi and Vacquier, 2002a). Therefore it is important to first examine the effect of stimulating sperm with a combination of purified egg jelly components before completely ruling out the role of NAADP in the acrosome reaction. Nevertheless, other unidentified components of egg jelly (such as proteins) may be important in stimulating changes in sperm NAADP levels. Indeed, agonist-evoked changes in cellular NAADP levels appear to be agonist-specific. In smooth muscle cells for example, both endothelin-1 and

prostaglandin-$F_{2\alpha}$ stimulate increases in cytosolic calcium concentration but only the former elevates NAADP. Similarly, stimulation of pancreatic acinar cells with CCK, but not acetylcholine results in the rise of NAADP levels within this cell (Yamasaki et al., 2005b), results consistent with the effects of NAADP receptor desensitisation and acidic store disrupters on agonist-mediated calcium signalling (as discussed in Section 1.6). Identification of the egg jelly component(s) that mediate NAADP increases in sperm may well provide vital clues as to the mechanism of NAADP synthesis in this cell type.

If, as appears likely, NAADP is not involved in the acrosome reaction, a possible role for NAADP synthesised in sea urchin sperm, as proposed in previous studies is to mediate the cortical flash (Billington et al., 2002;Churchill et al., 2003). The cortical flash as described in Section 1.6 is one of the first events occurring upon sperm-egg fusion. Thus, NAADP produced in sea urchin sperm as a result of stimulation with sea urchin egg jelly maybe delivered in to the egg.

By simple manipulation of the radioreceptor assay used to detect sperm NAADP levels, the sensitivity of sea urchin egg homogenates to NAADP was substantially enhanced (Figure 4.2), thereby providing an ideal method for detecting low concentrations of NAADP. This is of importance as NAADP is active over the low nanomolar range, thus basal NAADP levels are likely to be maintained well below these concentrations.

Graeff and Lee have recently developed a cycling assay for determining NAADP levels (Graeff and Lee, 2002). This assay uses less specialized material than the

radioreceptor assay and is linear with respect to NAADP concentration. However, the specificity of this assay is critically dependent upon prior removal of certain nucleotide substrates (NAD and NAAD), which would be present in cell extracts. Estimation of cellular NAADP levels by this method would be grossly overestimated if NAD and NAAD were not first removed, since these nucleotides act as substrates of the cycling assay. More importantly, the assay described in this chapter is approximately 1000-fold more sensitive than that described by Graeff and Lee (Graeff and Lee, 2002). The ability of the assay developed in this chapter to detect concentrations of NAADP as low as 10 picomolar is significant for the reasons described above.

Having established conditions to detect low levels of NAADP, binding of [^{32}P]NAADP to sea urchin egg homogenates was inhibited by red blood cell extracts (Figure 4.3). Given the highly specific nature of the NAADP receptor for its ligand relative to other nucleotides (Aarhus et al., 1996;Patel et al., 2000a), these results are consistent with the presence of NAADP in the extracts. Furthermore, the extent of inhibition is substantially greater when homogenates are incubated with the same concentration of extract before the addition of radioligand compared to simultaneous incubation with [^{32}P]NAADP. This suggests that the inhibitory factor present in the extract probably binds sea urchin egg NAADP receptors in an irreversible manner providing further evidence that the factor is NAADP.

Low levels of NAADP were detected in extracts from hepatocytes and *E. coli* (Table 4.1). That detection of NAADP was only possible by prior incubation of the

homogenate with the extracts highlights the increased sensitivity and usefulness of the pre-incubation method for determining NAADP.

Treatment of cellular extracts with alkaline phosphatase, which abolished the ability of all extracts to inhibit [^{32}P]NAADP binding, is also consistent with the inhibitory factor being NAADP (Figure 4.5). ATP, ADP and NADP, the other major constituents of cellular extracts determined by anion-exchange chromatography, were unlikely to contribute to inhibition of [^{32}P]NAADP binding, as treatment of cellular extracts with apyrase, or NAD(P)ase, enzymes capable of hydrolysing ATP/ADP (to AMP) and NAD(P) to ADP-ribose(-phosphate) had no effect on the extracts ability to inhibit [^{32}P]NAADP binding (Churamani et al., 2004).

A key concern is that NAADP detected in cellular extracts is simply an artefact of the extraction procedure resulting from the artificial breakdown of NADP. Indeed, the estimated concentration of NADP (7.8 ± 1 μM, n = 10) in the extract was approximately 500 times that of NAADP (16 ± 2 nM, n = 10), such that even moderate conversion of NADP to NAADP (0.19 %) could account for the levels of NAADP detected in cells. Given that incubation of NADP in alkaline media is sufficient to produce NAADP (Chini et al., 1995), it is vitally important that extracts are neutralized. Previous work has indicated that extracts prepared using $HClO_4$ remain slightly basic after neutralization (Masgrau et al., 2003). I compared red blood cell NAADP content after extraction by the previously reported procedure with that determined using my method. After correction for differential nucleotide recovery (as described in Materials and Methods, Section 4.2), NAADP content determined by trichloroacetic acid and $HClO_4$ was in reasonable agreement (Figure 4.4A). The slight

difference in NAADP content determined by these two methods may in part be explained by alkaline hydrolysis of NADP to NAADP (Chini et al., 1995). Alternatively, extraction with $HClO_4$ may be more efficient, however that NADP levels are similar (Figure 4.4B) would argue against this.

Another concern is that acid breakdown of NADP is also capable of artificially generating NAADP. In fact, work by Dr Sandip Patel shows that prolonged incubation of NADP with trichloroacetic acid results in the production of NAADP (Churamani et al., 2004). I found that NAADP content was similar in red blood cells extracts prepared by extraction with trichloroacetic acid for up to 15 minutes (Figure 4.6A). However, NAADP levels did increase significantly following prolonged incubations with trichloroacetic acid. This is likely due to acid breakdown of NADP to NAADP. Since the levels of NAADP in red blood cell extracts were determined by extraction over 1 minute, these levels are likely to be present in red blood cells.

Comparison of NAADP and NADP concentrations in cellular extracts revealed a lack of correlation between the two nucleotides (Figure 4.6B). This further suggests that NAADP is unlikely to be generated simply by NADP breakdown. Indeed, the NADP concentrations in *E. coli* and macrophages were similar, but NAADP was detected only in the former preparation (Table 4.1).

In summary, I have reported increases in sperm NAADP levels upon stimulation with egg jelly. Purified egg jelly components, involved in the acrosome reaction seem unlikely to contribute to these changes in NAADP levels. This suggests a possible role for other unidentified constituents of egg jelly to stimulate increases in sperm

NAADP. By exploiting the unusual binding characteristics of the target protein for NAADP in sea urchin egg homogenates, I also described experimental conditions for determining very low concentrations of NAADP. I have used this improved assay to determine low concentrations of NAADP in cells that are unrelated. I anticipate that this methodology will aid future studies in defining the mechanism of synthesis of NAADP within cells, of which little is known compared with its calcium-mobilizing activity.

CHAPTER 5 Conclusions

Since its initial discovery some 10 years ago, nicotinic acid adenine dinucleotide phosphate (NAADP) has rapidly established itself as an intracellular calcium mobilising messenger (Patel, 2004;Yamasaki et al., 2005a;Lee, 2003). However, unlike inositol trisphosphate and to a lesser extent cADPR, little is known about the molecular target for NAADP (Galione and Petersen, 2005). The widely held view is that NAADP-induced calcium release occurs via a calcium channel distinct from the IP_3 and ryanodine receptors, targets for IP_3 and cADPR, respectively.

Thus, the major aims of this thesis were to unravel the properties of the NAADP receptor in sea urchin eggs, providing further information for this most intriguing of intracellular calcium release pathways. For example, in Chapter 2 I show that binding of NAADP to its target protein in sea urchin eggs is critically dependent on its surrounding lipid environment, in particular phospholipids. Since the NAADP receptor has yet to be purified and cloned, the data presented here suggest that inclusion of lipids in size-exclusion chromatography (a strategy used to separate proteins on basis of size) or other fractionation procedures may help to stabilize the NAADP receptor-ligand complex, thus aiding purification. Furthermore, if on purification the molecular target for NAADP is indeed deemed to be a novel calcium ion channel, reconstitution into lipid bilayers will be required to determine its intrinsic properties. The information provided in Chapter 2 will thus help to ensure efficient composition of the lipid bilayers for functional expression of the NAADP target protein.

The successful purification, sequencing and cloning of this intriguing protein is likely to help explain many of the rather unusual features displayed by the NAADP-binding protein such as the newly described "molecular memory" (Chapter 3). In addition, the physiological relevance of irreversible binding, inactivation and time-sensing may be clarified. Identification of the NAADP receptor protein may also help to resolve many of the controversies and debates that rage namely:

i Does NAADP activate a novel intracellular calcium channel or simply directly modulate the ryanodine receptor (Galione and Petersen, 2005)?

ii Does NAADP induce calcium release from an acidic calcium store (Churchill et al., 2002;Mitchell et al., 2003;Yamasaki et al., 2004;Kinnear et al., 2004;Brailoiu et al., 2005) or the endoplasmic reticulum (Gerasimenko and Gerasimenko, 2004)?

Recently, changes in cellular NAADP levels in response to physiological stimulation have been reported (Churchill et al., 2003;Masgrau et al., 2003;Yamasaki et al., 2005b;Kinnear et al., 2004;Lee, 2003;Rutter, 2003). This substantiates the claim that NAADP is a bona fide intracellular calcium mobilising messenger. Indeed, data presented in Chapter 4 suggest that changes in sperm NAADP levels arise in response to specific, yet unidentified components within sea urchin egg jelly. To identify these compounds for example, egg jelly can first be treated with trypsin (a proteolytic enzyme), or boiled prior to stimulating sperm. If, as a consequence of these treatments, sperm NAADP levels do not increase upon stimulation with egg jelly, one can infer that a protein factor may be involved.

As mentioned, changes in intracellular NAADP levels occur in response to specific agonists, however, the exact mechanism leading to changes in NAADP levels is as yet unknown. At present the proposed route of synthesis is likely via base-exchange and involves ADP-ribosyl cyclase but whether this occurs in vivo is unclear. To test for the role of ADP-ribosyl cyclase, prior treatment of sea urchin sperm with nicotinamide (an inhibitor of ADP-ribosyl cyclase enzyme activity (Inageda et al., 1995;Graeff et al., 1996;Sethi et al., 1996)), should prevent changes in sperm NAADP levels when stimulated with sea urchin egg jelly. This approach could also be extended to other systems where NAADP levels have been demonstrated to increase in response to physiological agonists. If nicotinamide has no affect on sperm NAADP levels upon stimulation, one might suggest the involvement of other synthetic pathways such as phosphorylation of NAAD.

NAADP has also been demonstrated to increase in sea urchin eggs upon fertilisation (Churchill et al., 2003). The first increase has been attributed to delivery from sperm, however, the second increase is likely a result of synthesis within the egg. This suggests that NAADP-induced calcium signalling may be important in other stages of early embryonic development. To investigate this, cellular extracts could be obtained from embryos at various stages of development and NAADP levels measured using the radioreceptor assay described in Chapter 4. Since the sensitivity of the assay for NAADP is greatly increased, this should allow us to detect even the smallest of changes in NAADP levels. Furthermore, incubation of fertilized eggs with nicotinamide may shed light upon the role of ADP-ribosyl cyclase in sea urchin egg development. Using simple light microscopy combined with the biochemical

approach described above, a simple pattern linking ADP-ribosyl cyclase activity, NAADP and sea urchin development may emerge.

Although ADP-ribosyl cyclase and its homologues have been proposed as candidate enzymes involved in the production of NAADP (Lee, 2005), the conditions required to synthesize NAADP necessitates some form of compartmentalization of the enzymatic machinery. Indeed, the catalytic domains of these enzymes appear located extracellularly or within intracellular vesicles. However, the proposed localization of these enzymes would make them inaccessible to their substrates. Furthermore, it appears rather strange then, that the possible location for the synthesis of this intracellular calcium mobilising messenger (i.e. within an acidic organelle) would occur at a site inaccessible to its target protein. Therefore this "topological paradox" (DE Flora et al., 2004) requires further investigation. On the other hand many characteristics of the NAADP signalling pathway are less than straightforward.

NAADP is the most potent calcium mobilising messenger described to date. This indicates that basal levels of this messenger are likely to be extremely low. The current study (Chapter 4) has provided a reliable and highly sensitive method to quantify cellular NAADP levels within the low nanomolar range (Churamani et al., 2004). My method should aid in defining the route of NAADP synthesis which at present is unidentified. For example, incubating various homogenate preparations with nicotinic acid and NADP, if base-exchange activity is present, will generate NAADP. However, current methods of detection such as HPLC and various assays (Graeff and Lee, 2002;Graeff and Lee, 2003;Churchill et al., 2003) may not be sensitive enough to detect small increases in NAADP, if enzyme activity is low.

Therefore, the new method described in Chapter 4 will greatly aid detection of small increases in NAADP levels, thus providing the calcium signalling community with a significant tool to quantify the amount of NAADP within a cell.

Identification of the NAADP-binding protein and cellular apparatus governing NAADP synthesis and metabolism will ensure that NAADP and its signalling pathway remain in the spotlight for decades to come.

APPENDIX

List of publications resulting from this thesis:

Churamani, D., G.D.Dickinson, and S.Patel. 2005. NAADP binding to its target protein in sea urchin eggs requires phospholipids. *Biochem. J.* 386:497-504.

Churamani, D., E.A.Carrey, G.D.Dickinson, and S.Patel. 2004. Determination of cellular nicotinic acid-adenine dinucleotide phosphate (NAADP) levels. *Biochem. J.* 380:449-454.

Churamani, D., G.D.Dickinson, and S.Patel 2003. Effect of protein:lipid ratio on binding of nicotinic acid adenine dinucleotide phosphate (NAADP) to solubilized sea urchin egg homogenates. *Proceedings of the British Pharmacological Society* at http://www.pa2online.org/vol1issue3abst015P.html

ABBREVIATIONS

AA	Arachidonic acid
ACh	Acetylcholine
ADP	Adenosine diphosphate
ADPKD	Autosomal dominant polycystic kidney disease
ADP-ribose	Adenosine diphosphate-ribose
AMP	Adenosine monophosphate
ATP	Adenosine trisphosphate
cADPR	cyclic adenosine diphosphate ribose
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
cGMP	Cyclic guanosine monophosphate
CICR	Calcium-induced calcium release
CMC	Critical micelle concentration
CPM	Counts per minute
DAG	Diacylglycerol
EDG	Endothelial differentiation gene
ER	Endoplasmic reticulum
FCCP	Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
FSP	Fucose sulfate polymer
GPN	Glycylphenyl alanine 2-napthylamide
HPLC	High performance liquid chromatography
IP_3	Inositol 1,4,5-trisphosphate
IP_4	Inositol 1,3,4,5 tetrakisphosphate
IP_3R	Inositol trisphosphate receptor

kDa	Kilodalton
LGCC	Ligand-gated calcium channel
MIN6	Mouse insulinoma β-cell line
mM	Millimolar
μM	Micromolar
NAAD	Nicotinic acid adenine dinucleotide
NAADP	Nicotinic acid adenine dinucleotide phosphate
NACE	$NAD(P)^+$ catabolising enzyme
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
nAChR	Nicotinic acetylcholine receptor
nM	Nanomolar
NMDA	N-methyl-D-aspartate
PA	Palmitoleic acid
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
pM	Picomolar
PS	Phosphatidylserine
SCaMPER	Sphingolipid calcium release-mediating protein of endoplasmic reticulum
SER	(Sarco)endoplasmic reticulum
SERCA	Sarco-endoplasmic reticulum calcium ATPase

SG	Sialoglycan
SPM	Sphingomyelin
suREJ	Sea urchin receptor for egg jelly
TFA	Trifluoroacetic acid
TRAAK	Twik-related arachidonic acid-stimulated K^+ channel
TRP	Transient receptor potential
TWIK	Tandom of P domains in weak inward rectifier K^+ channel
VOCC	Voltage-operated calcium channel
VR1	Vanilloid receptor

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