

REGULATION OF ION CHANNELS BY INOSITOL TRISPHOSPHATE AND DIACYLGLYCEROL

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SUMMARY

Calcium-mobilizing receptors function to regulate ion channels located not only in the plasma membrane but also across the membranes of intracellular organelles, particularly the endoplasmic reticulum. A characteristic feature of such receptors is that they stimulate the hydrolysis of an inositol lipid to generate a pair of second messengers. Diacylglycerol remains within the plasma membrane where it activates protein kinase C leading to the phosphorylation of proteins some of which may regulate specific ionic channels, such as the calcium-dependent potassium channel or the Na^+/H^+ exchanger which regulates intracellular pH. The inositol trisphosphate ($\text{Ins } 1,4,5\text{P}_3$) released to the cytosol functions as a second messenger to release calcium from the endoplasmic reticulum. The $\text{Ins } 1,4,5\text{P}_3$ acts on a specific receptor to enhance the passive efflux of calcium while having no effect on the active calcium pump. There are indications that this $\text{Ins } 1,4,5\text{P}_3$ -induced release of calcium from an internal membrane store might provide an explanation of excitation–contraction coupling in skeletal muscle. Skinned skeletal muscle cells can be induced to contract by adding $\text{Ins } 1,4,5\text{P}_3$. Mobilization of calcium from intracellular reservoirs by $\text{Ins } 1,4,5\text{P}_3$ may thus prove to be a ubiquitous and fundamental mechanism for regulating cellular activity.

INTRODUCTION

Many cellular processes are regulated by calcium-mobilizing receptors which can use the hydrolysis of an inositol lipid as part of a transduction mechanism for generating second messengers. The inositol lipid used by the receptor is phosphatidylinositol 4,5-bisphosphate ($\text{PtdIns } 4,5\text{P}_2$) which is cleaved to diacylglycerol (DG) and inositol 1,4,5-trisphosphate ($\text{Ins } 1,4,5\text{P}_3$) (Berridge, 1984; Berridge & Irvine, 1984; Hokin, 1985; Downes & Michell, 1985). Both products seem to function as second messengers in that DG activates protein kinase C (C-kinase) (Nishizuka, 1984) whereas $\text{Ins } 1,4,5\text{P}_3$ diffuses into the cytosol to release calcium from the endoplasmic reticulum (Berridge, 1984; Berridge & Irvine, 1984). By stimulating the hydrolysis of $\text{PtdIns } 4,5\text{P}_2$, a whole variety of hormones and neurotransmitters initiate a bifurcating signal pathway which functions to control numerous cellular processes including secretion, contraction, metabolism, fertilization, phototransduction and cell proliferation. In this article I shall concentrate on the role of

Key words: inositol trisphosphate, phosphatidylinositol, diacylglycerol.

Ins 1,4,5P₃ in regulating calcium channels within the endoplasmic reticulum. I shall also consider how the DG/C-kinase limb of this bifurcating signal pathway functions to modulate ion channels within the plasma membrane. Before considering how these two signals pathways regulate ion channels it is necessary to describe the biochemistry underlying inositol lipid signal transduction.

AGONIST-DEPENDENT PtdIns 4,5P₂ HYDROLYSIS TO DG AND Ins 1,4,5P₃

The PtdIns 4,5P₂ used by the receptor mechanism to generate intracellular second messengers is a relatively minor membrane component comprising less than 0.5% of the total cellular phospholipids. It is derived from phosphatidylinositol (PtdIns) by two phosphorylation reactions. First, a PtdIns kinase phosphorylates PtdIns to phosphatidylinositol 4-phosphate (PtdIns 4P) which is then converted to PtdIns 4,5P₂ by a second kinase (Fig. 1). These two kinases appear to be extremely active and thus ensure a ready supply of the PtdIns 4,5P₂ required by the receptor as a precursor to generate second messengers. Occupation of an appropriate receptor results in the stimulation of a phosphodiesterase which then cleaves PtdIns 4,5P₂ to DG and Ins 1,4,5P₃. This transduction mechanism thus has much in common with that used to form cyclic AMP. In both cases, a highly phosphorylated precursor is cleaved by an enzyme (which functions as an amplification unit) to release second messengers. Further similarities exist with regard to the way in which receptors are coupled to the underlying amplification unit. In the cyclic AMP system, receptors are coupled to adenylate cyclase by way of specific GTP-binding proteins (G-proteins). A G-protein may also serve to couple receptors to the phosphodiesterase which cleaves PtdIns 4,5P₂ to DG and Ins 1,4,5P₃ (Haslam & Davidson, 1984; Cockcroft & Gomperts, 1985).

In keeping with the proposed role of DG and Ins 1,4,5P₃ as second messengers, they are rapidly inactivated once the external signal is withdrawn. The neutral DG which operates within the plane of the membrane can either be converted to phosphatidic acid by a DG kinase or it can be fed to a DG lipase which will release arachidonic acid (Fig. 1). There also are two pathways for degrading Ins 1,4,5P₃. First, it can be dephosphorylated to free inositol through a stepwise series of phosphatases (Storey, Shears, Kirk & Mitchell, 1984). Alternatively, it can be phosphorylated through a newly discovered kinase to form inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5P₄) (Irvine, Letcher, Heslop & Berridge, 1986). The latter was first identified in brain cortical slices and appears to be the precursor of Ins 1,3,4P₃ (Batty, Nahorski & Irvine, 1985). When cells are stimulated not only do they produce Ins 1,4,5P₃ but, after a short lag, they also begin to form Ins 1,3,4P₃ (Irvine, Letcher, Lander & Downes, 1984*b*, 1985; Burgess, McKinney, Irvine & Putney, 1985). When Ins 1,4,5P₃ is released from the membrane it can thus flow along two separate routes, a degradative pathway to free inositol or *via* a novel pathway to generate other inositol polyphosphates which could have additional messenger functions (Irvine *et al.* 1984*b*; Batty *et al.* 1985). At present the only

I shall concentrate on the evidence that Ins 1,4,5P₃ is a calcium-mobilizing second messenger.

MOBILIZATION OF INTRACELLULAR CALCIUM BY Ins 1,4,5P₃

The importance of Ins 1,4,5P₃ as an intracellular second messenger is clearly demonstrated by the ability of this molecule to stimulate a number of complex physiological processes when injected into intact cells (Table 1). Many of these processes are regulated by calcium. Using appropriate techniques, increases in intracellular calcium have been recorded following injection of Ins 1,4,5P₃ into either *Limulus* photoreceptors (Brown & Rubin, 1984) or *Xenopus* oocytes (Busa *et al.* 1985). The properties of this Ins 1,4,5P₃-induced release of intracellular calcium have been studied using either permeabilized cells (Table 2) or membrane fractions (Table 3). All the calcium released by Ins 1,4,5P₃ seems to originate solely from the endoplasmic reticulum. Once the plasma membrane has been permeabilized either by incubation in low calcium media, treatment with detergents or by means of high electric discharges, the internal organelles become accessible to molecules supplied in the bathing medium. Such permeabilized cells sequester calcium into both the endoplasmic reticulum and mitochondria but only the former is sensitive to Ins 1,4,5P₃. If the uptake of calcium into the endoplasmic reticulum is inhibited by vanadate, the subsequent release of calcium by Ins 1,4,5P₃ is severely curtailed (Streb, Irvine, Berridge & Schulz, 1983). Blocking the uptake of calcium into the mitochondria using various inhibitors (e.g. oligomycin, antimycin A, ruthenium red) had no effect on uptake into the endoplasmic reticulum, nor did it inhibit the subsequent release of calcium following addition of Ins 1,4,5P₃ (Streb *et al.* 1983; Biden *et al.* 1984; Burgess *et al.* 1984*a,b*; Gershengorn, Geras, Purrello & Rebecchi, 1984; Joseph *et al.* 1984*a,b*). Conversely, if the mitochondria of permeabilized liver cells are intentionally loaded with calcium by incubating them in

Table 1. *Effects of injecting Ins 1,4,5P₃ into intact cells*

Tissue	Effect	References
<i>Limulus</i>	Phototransduction and adaptation	Brown <i>et al.</i> (1984) Fein <i>et al.</i> (1984)
<i>Limulus</i>	Calcium mobilization	Brown & Rubin (1984) Wilson, Neufeld & Majerus (1985 <i>b</i>)*
Salamander rods	Modulation of light response	Waloga & Anderson (1985)
Sea urchin oocytes	Fertilization membrane	Whitaker & Irvine (1984) Turner, Jaffe & Fein (1985)
<i>Xenopus</i> oocytes	Depolarizing Cl ⁻ current	Oron, Dascal, Nadler & Lupu (1985) Busa <i>et al.</i> (1985)
<i>Xenopus</i> oocytes	Fertilization membrane	Picard <i>et al.</i> (1985) Busa <i>et al.</i> (1985)

* Used cyclic Ins 1,4,5P₃.

Table 2. *Effects of adding Ins 1,4,5P₃ to permeabilized cells*

Tissue	Effect	References
Pancreas	Calcium mobilization	Streb, Irvine, Berridge & Schulz (1983)
Liver	Calcium mobilization	Burgess <i>et al.</i> (1984a,b) Joseph <i>et al.</i> (1984a)
Insulinoma	Calcium mobilization	Biden <i>et al.</i> (1984) Joseph <i>et al.</i> (1984b)
Neutrophils	Calcium mobilization	Prentki, Wollheim & Lew (1984b)
Pituitary	Calcium mobilization	Gershengorn, Geras, Purrello & Rebecchi (1984)
Adrenal glomerulosa	Calcium mobilization	Kojima, Kojima, Kreutter & Rasmussen (1984)
Macrophages	Calcium mobilization	Hirata <i>et al.</i> (1984)
	Calcium mobilization	Hirata <i>et al.</i> (1985a)
Swiss 3T3	Calcium mobilization	Berridge, Heslop, Irvine & Brown (1984)
Parathyroid	Calcium mobilization	Epstein, Prentki & Attie (1985)
Blood platelet	Calcium mobilization	Wilson, Neufeld & Majerus (1985b)
T-cell line	Calcium mobilization	Imboden & Stobo (1985)
Smooth muscle	Calcium mobilization	Suematsu, Hirata, Hashimoto & Kuriyama (1984)
Smooth muscle	Calcium mobilization	Yamamoto & van Breemen (1985)
Smooth muscle	Calcium mobilization	Hashimoto, Hirata & Ito (1985)
Smooth muscle	Calcium mobilization	Smith, Smith & Higgins (1985)
Smooth muscle	Contraction	Somlyo, Bond, Somlyo & Scarpa (1985)
Skeletal muscle	Contraction	Vergara, Tsien & Delay (1985)
Skeletal muscle	Contraction	Volpe, Salviati, Di Virgilio & Pozzan (1985)
<i>Dictyostelium</i>	Cyclic GMP formation	Europe-Finner & Newell (1985)
Blood platelet	Exocytosis	Brass & Joseph (1985)
Sea urchin egg	Exocytosis	Clapper & Lee (1985)

Table 3. *Summary of tissues where calcium mobilization in response to Ins 1,4,5P₃ has been demonstrated in microsomal preparations*

Tissue	Reference
Pancreas	Streb <i>et al.</i> (1984)
Insulinoma	Prentki <i>et al.</i> (1984a) Joseph <i>et al.</i> (1984b)
Liver	Dawson & Irvine (1984) Joseph <i>et al.</i> (1984b) Dawson (1985)
Platelets	Muallem, Schoeffield, Pandol & Sachs (1985) Adunyah & Dean (1985) O'Rourke, Halenda, Zavoico & Feinstein (1985)
Smooth muscle	Carsten & Miller (1985)
Plant hypocotyl	Drøbak & Ferguson (1985)
Skeletal muscle	Volpe, Salviati, Di Virgilio & Pozzan (1985)
Sea urchin egg	Clapper & Lee (1985)

media containing a high concentration of calcium, there was no release upon addition of Ins 1,4,5P₃ (Burgess *et al.* 1984a). Separation of organelles and membrane fractions by differential centrifugation supports the notion that Ins 1,4,5P₃ acts predominantly to release calcium from the endoplasmic reticulum (Table 3). While release can clearly be seen after addition of Ins 1,4,5P₃ to microsomes, there was no release from either mitochondria or secretion granules (Prentki *et al.* 1984a; Prentki, Wollheim & Lew, 1984b).

There is a constant cycling of calcium across the endoplasmic reticulum which under steady-state conditions reflects a balance between passive efflux and ATP-dependent influx. The calcium pump is electrogenic and requires a compensating flow of potassium in the opposite direction to maintain electroneutrality (Muallem, Schoeffield, Pandol & Sachs, 1985). A constant supply of potassium counter ions within the endoplasmic reticulum is provided by a furosemide-sensitive potassium/chloride cotransporter. Release of calcium induced by Ins 1,4,5P₃ also requires an opposite flow of potassium as a counter ion (Muallem *et al.* 1985).

A net release of calcium from the endoplasmic reticulum could occur either by promoting the efflux pathway or by inhibiting the active pump. All the evidence points to an action of Ins 1,4,5P₃ on the passive efflux pathway. Once the endoplasmic reticulum has sequestered calcium, blocking the pump by the addition of vanadate or the removal of ATP does not mimic the effect of Ins 1,4,5P₃, nor does it prevent this agent from releasing calcium (Prentki *et al.* 1984b; Clapper & Lee, 1985; Hirata *et al.* 1985a; Muallem *et al.* 1985). Release of calcium from the endoplasmic reticulum by Ins 1,4,5P₃ is independent of temperature, again suggesting a passive phenomenon (Brass & Joseph, 1985; Hirata *et al.* 1985a; Smith, Smith & Higgins, 1985). Smith *et al.* (1985) have argued that this temperature-independence suggests an action of Ins 1,4,5P₃ on a channel rather than a carrier.

In response to the sudden addition of Ins 1,4,5P₃ more than 50% of the stored calcium is released within seconds but, on continued incubation, the calcium is taken up again, so restoring the initial steady-state condition (Joseph *et al.* 1984a; Biden *et al.* 1984). The re-uptake of calcium occurs because the pulse of Ins 1,4,5P₃ is degraded by an inositol trisphosphatase (Joseph *et al.* 1984a). In these permeabilized cells, the release of calcium from the endoplasmic reticulum by Ins 1,4,5P₃ occurs fast enough for it to be able to stimulate various physiological processes such as the cortical reaction in sea urchin eggs (Clapper & Lee, 1985), exocytosis in blood platelets (Brass & Joseph, 1985) or contraction of smooth (Somylo, Bond, Somylo & Scarpa, 1985) and skeletal muscle (Vergara, Tsien & Delay, 1985; Volpe, Salviati, Di Virgilio & Pozzan, 1985). It has been suggested that Ins 1,4,5P₃ could regulate the intracellular level of calcium, not only during cell stimulation but also when the cell is at rest, by controlling the rate of calcium cycling across the endoplasmic reticulum (Prentki, Corkey & Matschinsky, 1985). By varying the rate of infusions of Ins 1,4,5P₃ to counteract losses *via* the degradation enzyme, Prentki *et al.* (1985) have achieved a series of steady-state calcium levels.

Release of calcium from the endoplasmic reticulum by enhancing the passive efflux component seems to occur through a specific receptor for Ins 1,4,5P₃. No

release occurs upon addition of inositol, inositol 1-phosphate, inositol 2-phosphate, inositol 1,2-cyclic phosphate, inositol 1,4-bisphosphate, 2,3-diphosphoglyceric acid, fructose 2,6-bisphosphate, fructose 1,6-bisphosphate (Streb *et al.* 1983; Joseph *et al.* 1984*a,b*; Berridge, Heslop, Irvine & Brown, 1984; Biden *et al.* 1984; Burgess *et al.* 1984*a,b*; Irvine *et al.* 1984*b*). The specificity of the response is highlighted by the fact that there is no release following stimulation with inositol 1,4-bisphosphate which is the natural breakdown product resulting from the dephosphorylation of Ins 1,4,5P₃ by the inositol trisphosphatase. Preliminary measurements suggest that Ins 1,3,4,5P₄ is incapable of releasing calcium (M. J. Berridge & R. F. Irvine, unpublished observation). Preliminary structure-activity studies have begun to identify those aspects of the molecule which are crucial for release to occur (Irvine *et al.* 1984*b*; Burgess *et al.* 1984*b*). All analogues capable of stimulating release have phosphates in the 4- and 5-positions of the molecules. The phosphate on the 1-position is not necessary because inositol 4,5-bisphosphate can release calcium, albeit at much higher concentrations, suggesting that this phosphate plays a role in enhancing the affinity of the molecule for its receptor. First attempts to isolate and purify the Ins 1,4,5P₃ receptor have begun with the identification of a specific binding site in permeabilized cells and microsomes using [³²P]Ins 1,4,5P₃ (Baukal *et al.* 1985; Spät *et al.* 1986*a*; Spät, Fabiato & Rubin, 1986*b*). This binding site has a similar pharmacological profile to that detected in the calcium release experiments. Another approach has been to develop a photoaffinity label which could be used specifically to tag the receptor and thus to facilitate its identification during purification procedures. Hirata *et al.* (1985*b*) have described an arylazide derivative of Ins 1,4,5P₃ which can irreversibly inhibit the release of calcium induced by Ins 1,4,5P₃ in macrophages. Since the inhibitory effect of this derivative can be prevented by adding a 10-fold excess of Ins 1,4,5P₃, it would seem that the normal receptor is being labelled. The aim of all these studies will be the identification of the Ins 1,4,5P₃ receptor as a first step in working out how it functions to trigger the explosive release of calcium from the endoplasmic reticulum.

Ins 1,4,5P₃ AND EXCITATION-CONTRACTION COUPLING IN MUSCLE

A major unsolved problem in physiology concerns the nature of the mechanism whereby excitation of the sarcolemma of skeletal muscle results in a release of calcium from the sarcoplasmic reticulum (Caille, Ildefonse & Rougier, 1985). The two membranes are separated by cytosol so it is necessary to explain how information is transferred from the surface membranes to the sarcoplasmic reticulum. There is growing evidence that Ins 1,4,5P₃ may function to relay information between the two membranes in smooth muscle and perhaps also in skeletal muscle. Somlyo & Somlyo (1968) coined the term 'pharmacomechanical coupling' to describe the phenomenon whereby smooth muscle cells contract in response to transmitters such as acetylcholine, norepinephrine, vasopressin and angiotensin II. It has been known for some time that such transmitters act on smooth muscle to stimulate the hydrolysis of PtdIns 4,5P₂ to give Ins 1,4,5P₃ (Akhtar & Abdel-Latif, 1980, 1984; Smith *et al.*

1984; Nabika, Velletri, Lovenberg & Beaven, 1985). That Ins 1,4,5P₃ might function as a second messenger in smooth muscle is supported by the finding that it will stimulate the release of calcium when applied to skinned muscle cells (Suematsu *et al.* 1984; Somylo *et al.* 1985; Yamamoto & van Breemen, 1985) or from microsomes derived from uterine sarcoplasmic reticulum (Carsten & Miller, 1985). What is even more interesting is that Ins 1,4,5P₃ can trigger vascular smooth muscle to contract (Somylo *et al.* 1985). Similar evidence has been advanced to support the proposal that Ins 1,4,5P₃ may function in excitation-contraction coupling in skeletal muscle (Vergara *et al.* 1985; Volpe *et al.* 1985). In addition, Ins 1,4,5P₃ can release calcium from the sarcoplasmic reticulum, and when the latter was separated into two parts much more calcium was released from the terminal cisternae than from the longitudinal tubular region (Volpe *et al.* 1985). The current hypothesis is that depolarization of the T-tubule membrane somehow stimulates the hydrolysis of PtdIns 4,5P₂ to release Ins 1,4,5P₃; which then diffuses across the 20-nm gap to trigger the release of calcium from the sarcoplasmic reticulum. Preliminary measurements of Ins 1,4,5P₃ in skeletal muscle indicate that the level does increase in response to electrical stimulation (Vergara *et al.* 1985). While many questions remain unanswered, the possibility that Ins 1,4,5P₃ might function in skeletal muscle is certainly worth pursuing further as it may provide an elegant solution to a problem that has puzzled physiologists for decades.

ROLE OF Ins 1,4,5P₃ AND DG IN REGULATING ION TRANSPORT

Release of calcium from the endoplasmic reticulum by Ins 1,4,5P₃ is but one limb of a bifurcating signal pathway. The other component is DG, which operates within the plane of the membrane to activate protein kinase C (Fig. 1) (Nishizuka, 1984). The importance of this bifurcating signal pathway lies in the fact that both limbs cooperate with each other to control a whole host of cellular processes. I have already described the role of Ins 1,4,5P₃ in releasing intracellular calcium and now shall concentrate on the role of the DG/C-kinase pathway in regulating ion transport. In addition to acting independently of each other, an important aspect of this bifurcating pathway is that the two limbs often act synergistically with each other (Kaibuchi *et al.* 1983; Nishizuka, 1984).

This synergism was uncovered by devising methods for stimulating each signal pathway independently of the other. Calcium ionophores could be used to bypass the action of Ins 1,4,5P₃ in elevating the intracellular level of calcium, whereas phorbol esters were found to duplicate the ability of DG to activate protein kinase C (Castagna *et al.* 1982). When these two agents were administered together they could stimulate cells maximally at concentrations which had no effect when given alone. In the case of blood platelets, such synergism can be explained on the basis of the DG/C-kinase pathway enhancing the sensitivity of the exocytotic process to the stimulatory action of calcium (Knight & Scrutton, 1984). Another example of the interaction between the DG/C-kinase and calcium pathways has been uncovered in *Aplysia* neurones, where the voltage-sensitive calcium current in bag cell neurones is

enhanced following stimulation with a phorbol ester or after injection of purified protein kinase C (De Riemer *et al.* 1985).

Modulation of calcium-sensitive processes may be a particularly important function of the DG/C-kinase pathway. An interesting example has been uncovered in pyramidal hippocampal neurones, where the administration of a phorbol ester blocked accommodation which normally sets in due to the opening of a calcium-dependent potassium channel (Baraban, Snyder & Alger, 1985). The idea is that the DG/C-kinase pathway may act by reducing the sensitivity of potassium channels to the stimulatory action of calcium. Such a mechanism may account for the normal inhibition of accommodation brought about by acetylcholine because the latter is known to stimulate inositol lipid hydrolysis in the hippocampus (Gonzales & Crews, 1984; Fisher & Bartus, 1985). The DG/C-kinase pathway may also control two distinct potassium currents and a voltage-dependent calcium current in photo-receptors of *Hermisenda* (Farley & Auerbach, 1986). Regulation of neuronal excitability by adjusting potassium channels may turn out to be a general mode of action of receptors which operate through the inositol lipids. For example, substance P excites magnocellular cholinergic neurones (Stanfield, Nakajima & Yamaguchi, 1985), whereas a transient outward current in dorsal raphe serotonergic neurones is stimulated by α_1 -adrenoreceptors (Aghajanian, 1985). Both these transmitters are known to stimulate inositol lipid hydrolysis in the brain, but it is not clear yet whether either Ins 1,4,5P₃ or DG has any role to play in regulating potassium channels in either of these tissues.

Another important ionic change regulated by this receptor mechanism is the Na⁺/H⁺ exchanger which has an important role in regulating intracellular pH, especially during the action of mitogenic stimuli. There are large increases in intracellular pH following fertilization (Swann & Whitaker, 1985) or during the action of growth factors on fibroblasts (Moolenaar, Tsien, van der Saag & de Laat, 1983; L'Allemain, Paris & Pouyssegur, 1984). Since these changes in both oocytes and fibroblasts can be duplicated by the addition of a phorbol ester the idea has developed that the DG/C-kinase pathway may act to stimulate the Na⁺/H⁺ exchanger. The two major ionic events which occur when cells are induced to grow may thus be under the independent control of the two separate limbs of the inositol lipid signalling system. DG acts to stimulate the Na⁺/H⁺ exchanger to increase intracellular pH whereas the Ins 1,4,5P₃ released to the cytosol stimulates the release of calcium to account for the large increase in intracellular calcium recorded in cells responding to growth factors (Hesketh *et al.* 1985; Moolenaar, Tertoolen & de Laat, 1984).

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