Inositol trisphosphate and calcium signalling mechanisms

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Studies on control of fluid secretion by an insect salivary gland led to the discovery of inositol trisphosphate (IP₃) and calcium (Ca²⁺) have been my scientific companions over the last 25 years. My relationship with these two messengers has passed through two distinct phases. Firstly, there was the work that led up to the discovery that IP₃ was a Ca²⁺-mobilizing second messenger. The second phase was characterized by the excitement of finding that this IP₃/Ca²⁺ signalling system was a key regulator of many different cellular control mechanisms.

1. Introduction

Inositol 1,4,5-trisphosphate (IP₃) and calcium (Ca²⁺) have been my scientific companions over the last 25 years. My relationship with these two messengers has passed through two distinct phases. Firstly, there was the work that led up to the discovery that IP₃ was a Ca²⁺-mobilizing second messenger. The second phase was characterized by the excitement of finding that this IP₃/Ca²⁺ signalling system was a key regulator of many different cellular control mechanisms.

1.1. Stimulus-secretion coupling in the insect salivary gland

My interest in Ca²⁺ began when I was trying to understand the mechanisms that control fluid secretion using insect salivary glands as a model system. These glands are normally quiescent and are only called into action when stimulated by 5-hydroxytryptamine (5-HT) during feeding [1]. These studies were carried out at Case Western Reserve University where Sutherland and Rall had just discovered cyclic adenosine monophosphate (cyclic AMP) and had developed their novel second messenger concept [2]. I was very excited to find that the stimulatory effect of 5-HT on fluid secretion was exactly duplicated by the addition of cyclic AMP [13]. The next obvious step was to find out how cyclic AMP worked. I approached this problem by characterizing the electrophysiological properties of the secretory response by placing glands in a small Perspex perfusion chamber that enabled the trans-epithelial potential to be monitored while adding and removing 5-HT or cyclic AMP. When the glands were at rest, the lumen had a slightly positive potential relative to the bathing medium and depolarized rapidly in response to 5-HT but hyperpolarized after addition of cyclic AMP [4]. Subsequent experiments revealed that cyclic AMP activated an electrogenic potassium pump, which was the prime mover for fluid secretion. Chloride followed passively and this transport of KCl created the osmotic gradient to drive the flow of water. Subsequent resistance measurements showed that the passive flux of chloride was facilitated by a 5-HT-dependent increase in chloride conductance that occurred independently of cyclic AMP [5]. It seemed that 5-HT was having two actions, one mediated by cyclic AMP to drive potassium transport and a second mechanism that increased the flux of chloride (Fig. 1).

It soon became apparent that Ca²⁺ might regulate this passive chloride flux. A collaborative study with Howard Rasmussen clearly showed that 5-HT was having a profound effect on Ca²⁺ dynamics and suggested that Ca²⁺ was being released from an internal store [6]. We subsequently used both electrophysiological and pharmacological techniques to demonstrate that the blowfly salivary gland had two 5-HT receptors operating through separate second messengers [7-9]. One receptor used cyclic AMP to drive potassium transport whereas the other employed Ca²⁺ to open chloride channels.

1.2. Inositol lipid hydrolysis and Ca²⁺ signalling

The most puzzling aspect of the putative Ca²⁺ messenger system in the insect blowfly was the source of Ca²⁺ much of which was derived from an internal store [7]. As I began to search for the messenger that connected cell surface receptors to the internal store, I became aware of the work by Hokin and Hokin who discovered that external agonists stimulated the turnover of phosphatidylinositol (PI) [10]. Although this PI response had been measured in many different cells in response to many different stimulants, its function was somewhat mysterious. This began to change in 1975 when Bob Michell proposed that this lipid hydrolysis was responsible for Ca²⁺ signalling [11]. In order to test out this hypothesis, I began to use ³H-
In order to investigate the link between PI hydrolysis and Ca\(^{2+}\) signalling, it seemed important to find out more about the biochemical processes underlying the PI response. I began by exploring some interesting work by Allison and his colleagues on Li\(^+\) and inositol phosphate metabolism [16,17]. They found that Li\(^+\) was a potent inhibitor of the inositol monophosphatase that hydrolysed inositol 1-phosphate (IP\(_1\)) to inositol (Fig. 1). This seemed to be an important observation because it was this Li\(^+\)-sensitive step that released the free inositol that I had been measuring to monitor PI hydrolysis. This inositol efflux method offered a simple way of testing the inhibitory effect of Li\(^+\). Since this inositol was derived from the IP\(_1\) produced by hydrolysing PI, Li\(^+\) should reduce this efflux by inhibiting the hydrolysis of IP\(_1\) to inositol, which is exactly what happened [18]. When Li\(^+\) was withdrawn, there was the very large overshoot of inositol efflux that resulted from the inositol monophosphatase suddenly hydrolysing the IP\(_1\) that had built up behind the Li\(^+\) block.

In order to complete this Li\(^+\) study, I tried to measure PI metabolites after separating them out on anion-exchange columns [19]. As expected, there was a large accumulation of IP\(_2\) during Li\(^+\) inhibition, but what was unexpected was the appearance of two additional peaks running after the IP\(_2\). On the basis of standards supplied by Rex Dawson and Robin Irvine, these two peaks turned out to be inositol 1,4-bisphosphate (IP\(_2\)) and inositol 1,4,5-trisphosphate (IP\(_3\)). On the basis of what was known previously, the simplest hypothesis was that IP\(_3\) was formed first and was then sequentially hydrolysed to IP\(_2\), IP\(_1\) and then inositol (Fig. 1). In order to test this hypothesis, I developed a rapid perfusion system [20] that enabled me to establish the rate at which these inositol phosphates were produced following 5-HT stimulation. These studies on the kinetics of 5-HT-induced inositol phosphate formation revealed that IP\(_3\) and IP\(_2\) increased first with no apparent latency whereas IP\(_1\), followed by inositol appeared much later. I was very excited by this result because it revealed that IP\(_3\) was being generated very quickly. I already knew that there was a 1–2 s latency before the onset of the electrical change caused by the Ca\(^{2+}\)-dependent activation of the chloride channels and I now found that IP\(_3\) was being elevated before this physiological response. It was this observation that led me to propose that IP\(_3\) might be the diffusible messenger that coupled receptor activation to the mobilization of internal Ca\(^{2+}\) [21].

In order to test whether IP\(_3\) could release Ca\(^{2+}\) from internal stores, I set up a collaboration with Irene Schulz and Robin Irvine. The latter provided large amounts of IP\(_3\) that was then tested on a permeabilized pancreatic cell preparation developed by Streb and Schulz. We were all very excited to find that IP\(_3\) did indeed mobilize Ca\(^{2+}\) from an internal store that we identified as the endoplasmic reticulum and this was reported in Nature in 1983 [22]. In the following year, there were 7 papers (with 4 published in Nature) that confirmed this initial observation in many other cell types including liver cells [23], Swiss 3T3 cells [24], insulin-secreting cells [25,26], Limulus photoreceptors [27,28] and leukocytes [29]. It seemed clear therefore that IP\(_3\) was the long sought after Ca\(^{2+}\)-mobilizing second messenger [30–32].

Fig. 1. Stimulus–secretion coupling in the blowfly salivary gland.
The second and continuing phase of my relationship with this signalling system has been the excitement of seeing the IP3/Ca2+ pathway emerge as a highly versatile signalling system capable of regulating many different cellular processes. We now know that much of this remarkable versatility depends on the way this signal system is organized in both time (Ca2+ oscillations) and space (elementary events and waves) [33–35].

1.4. IP3 and Ca2+ oscillations

My interest in the spatiotemporal aspects of signalling began very early when we discovered that the trans-epithelial potential across the insect salivary gland oscillated in response to 5-HT [4]. An interesting feature of these oscillations is that their frequency varied with agonist concentration [4,36]. Since these changes in frequency occurred over the same range of 5-HT concentrations that caused changes in fluid secretion, we proposed that the signalling system operated through frequency modulation (FM) rather than amplitude modulation (AM) [37]. Since these potential oscillations depended on changes of resistance, which were controlled by Ca2+, we speculated that the spontaneous release of Ca2+ often occurred when the internal store became over loaded with Ca2+. IP3 receptors seem to have a similar sensitivity to store loading. Ludwig Missiaen showed that Ca2+ loading resulted in a spontaneous release of Ca2+ [47].

This sensitivity of IP3 receptors to the level of Ca2+ within the lumen of the endoplasmic reticulum has led to a store loading model of Ca2+ oscillations [32,48,49]. The basis of this model is that the oscillations are so sensitive to variations in the external Ca2+ concentration that the rate of Ca2+ entry is controlled by agonist concentration.

This store loading Ca2+ oscillator model provides an explanation of the rhythmical contractions of vascular, lymphatic, airway and corpus cavernosum smooth muscle cells [50]. Neurotransmitters and hormones modulate the frequency of this cytosolic oscillator by increasing either the level of IP3 or the influx of Ca2+. A similar oscillatory mechanism may also operate in pacemaker cells such as the interstitial cells of Cajal (ICCs) that drive the rhythmical contractions found in gastrointestinal and urethral smooth muscle cells.

1.5. Spatial aspects of Ca2+ signalling

As imaging techniques have improved, it has been possible to observe spatial organization of the IP3/Ca2+ signalling system. It is now possible to visualize the activity of the individual building blocks of calcium signals as described in cardiac cells [51,52], which...
revealed the existence of elementary events that were called sparks. These sparks are small bursts of calcium released from a localized group of ryanodine receptors. The IP$_3$/Ca$^{2+}$ signalling system might be similarly organized into elementary events [53,54]. The elementary events produced by IP$_3$ receptors have been called puffs [54]. The puffs were then characterized by Martin Bootman and Peter Lipp who showed how they are recruited to produce global calcium signals in HeLa cells [55,56]. Such studies on the elementary events have greatly increased our understanding of how calcium signals are constructed [33,34].

1.6. The role of IP$_3$/Ca$^{2+}$ signalling pathways in cellular control mechanisms

A remarkable feature of the IP$_3$/Ca$^{2+}$ signalling pathway is that it participates in the control of a large number of cellular processes (Fig. 2). In some cases, it plays a direct role in generating Ca$^{2+}$ signals whereas in other cases it functions to modulate the Ca$^{2+}$ signals produced by other signalling pathways. The following examples will illustrate the versatility of this Ca$^{2+}$ signalling mechanism. Control of salivary secretion by the insect gland described earlier is an example where IP$_3$/Ca$^{2+}$ signalling has a direct role by activating the trans-epithelial flux of Cl$^-$ (Fig. 1). In the case of these insect salivary glands and in many other cell types, the Ca$^{2+}$ signal appears as regular oscillations as has been described for liver cells [39], pancreatic cells [57], smooth muscle cells [50,58–60], interstitial cells of Cajal [61] and mammalian oocytes [40–42]. In the last case, there is an unusual activation mechanism that depends upon the sperm fusing with the oocyte to introduce the sperm-specific phospholipase C$\zeta$ (PLC$\zeta$), which then begins to hydrolyse PtdIns4,5P$_2$ to generate the IP$_3$ that initiates the Ca$^{2+}$ oscillations [62] (Fig. 3). These oscillations then control a number of egg responses such as cortical granule release, the...
block to polyspermy, the completion of meiosis and the initiation of the cell cycle programme leading to embryonic development. The Ca^{2+} pulses thus activate the oocyte to begin the multiple rounds of cell proliferation that occur at the beginning of development.

The IP_{3}/Ca^{2+} signalling pathway also has a role to play in the control of cell proliferation in many other cell types (Fig. 2). This role is well-established in the lymphocyte activation [63], which is initiated by antigen binding to the T cell receptor (TCR) that then recruits phospholipase C_\gamma_1 (PLC_\gamma_1) to produce IP_3 (Fig. 4). This IP_3 acts through the IP_3 receptor (IP_3R) to release Ca^{2+} from the internal store. As the ER empties, it sends a signal to the Ca^{2+} release-activated Ca^{2+} (CRAC) channel responsible for maintaining the Ca^{2+} entry current \( I_{\text{CRAC}} \) for the 2 h period required to induce lymphocytes to grow. The ORAI1 protein has been identified as the channel responsible for carrying \( I_{\text{CRAC}} \) [64]. The coupling between store emptying and activation of the CRAC channel is still unclear. A stromal interaction molecule 1 (STIM1) in the ER may function as the sensor of store emptying [64,65]. Both the release and entry processes create the Ca^{2+} messengers that contribute to the dendritic Ca^{2+} waves that underlie learning and memory [66]. In the case of Purkinje neurons, the IP_3 receptors are located on the endoplasmic reticulum that extends into the multitude of spines that decorate the dendritic tree [67]. The metabotropic receptors, which are also located on the spines, are responsible for generating IP_3 that contributes to the process of long-term depression (LTD) that is responsible for motor learning. Like many learning mechanisms, LTD occurs when the Purkinje neuron detects the near simultaneous arrival of two synaptic inputs, one coming from the climbing fibres that innervate the base of the dendritic tree and the other arriving through the parallel fibres that innervate the spines. In 1993, I proposed that the IP_3 receptor might be that coincident detector that enables the Purkinje cell to associate the information arriving from these two separate inputs [68]. As noted earlier, opening of the IP_3 receptor depends upon the simultaneous presence of both IP_3 and calcium [44–46] and these are exactly the two messengers being supplied by the two inputs to the Purkinje neurons. The climbing fibre results in depolarization of the dendrites to generate a calcium signal whereas the parallel fibres activate the metabotropic receptors to produce IP_3. When these two messengers appear at approximately the same time, there is a large release of calcium within the spine and this may then be responsible for inducing LTD. Although there are other contenders for coincident detectors in these neurons, there has been considerable experimental evidence to support the notion that the IP_3/Ca^{2+} signalling pathway may be responsible for LTD [69,70].

A number of neuronal cells display dendritic Ca^{2+} waves that initiate at synaptic regions and then progress down to the soma through a process of Ca^{2+}-induced Ca^{2+} release (CICR) driven by InsP_3 receptors [71–74]. The function of such waves is largely unknown. I have speculated that they may be responsible for carrying Ca^{2+} signals into the nucleus to activate gene transcription [66], but they may also regulate other processes. For example, such waves located in the pyramidal neurons in the prefrontal cortex may regulate the firing patterns that occur during working memory [75]. Another example is found in the respiratory neurons in the pre-Bötzinger complex (pre-BötzC) where such an InsP_3-dependent dendritic Ca^{2+} wave (Fig. 5) appears to be a central feature of the pacemaker mechanisms that drives breathing [76]. A feature of this rhythm generator is that the pacemaker neurons communicate with each other through glutamatergic synapses, which have a strong positive feedback component that operates periodically to ensure that all the participating neurons fire together to produce a synchronous and robust output signal. The feedback mechanism that serves to amplify the normally weak synaptic signalling process seems to depend on the generation of a dendritic Ca^{2+} wave that is triggered by the formation of InsP_3 by mGluR5 receptors (Fig. 5). The Ca^{2+} wave that travels to the soma is responsible for activating TRPM4 channels that provide the inward \( I_{\text{CAN}} \) current responsible for the membrane depolarization (\( \Delta V \)) that triggers the burst of action potentials. The increase in the level of Ca^{2+} within the soma precedes the pacemaker depolarization that triggers the action potentials that activate the motor neurons that control breathing [76].

Cardiac cells provide another example of how the IP_3/Ca^{2+} signalling pathway can modify the ongoing Ca^{2+} signalling system that drive contraction [77]. In both atrial and ventricular cells, microdomains of Ca^{2+} create the global signal that drives contraction,
but the mode of recruitment is different. In the case of atrial cells, $Ca^{2+}$ sparks are generated at special zones where the junctional sarcoplasmic reticulum (jSR) lies close to the sarcolemma [78]. This atrial jSR has both type 2 RYRs (RYR2s) and type 2 IP3 receptors (IP3R2s). Atrial cells use two distinct mechanisms of $Ca^{2+}$ release. The junctional zones generate $Ca^{2+}$ sparks using a mechanism similar to that of SERCA pumps. In effect, this microdomain of $Ca^{2+}$ is contained near the cell from the plasma membrane.

Under normal conditions, this atrial spark at the surface fails to breach a mitochondrial firewall because the $Ca^{2+}$ is rapidly removed by uptake into the mitochondria and by uptake into the SR by the SERCA pumps. In effect, this microdomain of $Ca^{2+}$ is contained near the cell surface because not enough $Ca^{2+}$ gets across the firewall to activate the internal RYR2s [78]. However, stronger contractions are induced following stimulation of the atrial cells by either $β$-adrenergic agonist or by endothelin. The large positive inotropic response induced by endothelin may depend on the ability of IP3 to enhance the cell surface sparks so that they breach the mitochondrial firewall to stimulate the non-junctional RYR2s to ignite the $Ca^{2+}$ wave that produces the larger global signals. Excessive activation of the IP3/Ca2+ signalling pathway might be the cause of atrial arrhythmias [77,79].

In the case of ventricular cells, the IP3/Ca2+ signalling pathway may also contribute to positive inotropic responses to agonists such as endothelin by enhancing the sparks that are responsible for excitation–contraction coupling [80]. The spatial organization of the enhanced $Ca^{2+}$ signal responsible for this positive inotropic response may be particularly important for understanding the processes responsible for cardiac hypertrophy and heart disease.

A number of signalling pathways have been implicated in the activation of cardiac hypertrophy. One of the characteristics of this hypertrophy is the re-appearance of foetal cardiac genes suggesting that hypertrophic stimuli induce a process of de-differentiation. Many of the extrinsic factors that drive this hypertrophic response (e.g. mechanical load, loss of myocytes and endocrine factors such as endothelin) seem to act through the $Ca^{2+}$ signalling system, which appears to be particularly important for inducing some of the key transcriptional events responsible for activating the foetal genes (Fig. 5). However, the heart receives continuous pulses of $Ca^{2+}$ to drive contraction under normal conditions without initiating a change in transcription. So what is it about the hypertrophic $Ca^{2+}$ signals that initiate the remodelling of cardiac gene transcription? It has been proposed that hypertrophic stimuli induce subtle changes in the characteristics of the individual $Ca^{2+}$ transients (e.g. increases in frequency, amplitude or width) that are sufficient to activate the foetal transcriptional events that induce the phenotypic remodelling that leads to hypertrophy [81–83]. An important feature of this proposal is that the changes in the temporal properties of each transient may reflect an IP3-dependent alteration in the spatial organization of each transient [83] (Fig. 6). During the normal transients, each $Ca^{2+}$ spark is located primarily in the cytoplasm where it functions to drive contraction. However, in the presence of agonists that increase the level of IP3, this cytosolic signal may be amplified by the release of $Ca^{2+}$ by IP3 receptors located near the nucleus to give a nuclear-cytosolic signal capable of stimulating the transcriptional events responsible for hypertrophy. This explanation is based on the fact that the IP3R is a coincident detector in that its activation requires the presence of both IP3 and $Ca^{2+}$ [44–46]. In the absence of IP3, the IP3Rs will fail to respond to the depolarization-induced $Ca^{2+}$ sparks that occur mainly in the cytoplasm. Likewise, the endothelin-induced elevation in IP3 will also fail to stimulate the release of $Ca^{2+}$ during diastole when the $Ca^{2+}$ concentration is low. However, during the course of the brief $Ca^{2+}$ transient, the simultaneous presence of both $Ca^{2+}$ and IP3 will result in the activation of the perinuclear IP3 receptors (Fig. 6). In effect, the IP3Rs will create a nuclear $Ca^{2+}$ microdomain responsible for driving the nuclear transcriptional processes such as HDAC5 phosphorylation [84]. The normal $Ca^{2+}$ signals responsible for excitation–contraction coupling were not able to activate the CaMKII responsible for stimulating the phosphorylation of HDAC5. However, this phosphorylation, which was activated by the endothelin-dependent formation of InsP3, seemed to depend on the local release of $Ca^{2+}$ from perinuclear InsP3 receptors [84]. There is now experimental evidence to show that IP3 receptors can function to enhance nuclear $Ca^{2+}$ transients [77].
is an excellent example of how IP₃ can alter the properties of Ca²⁺ signalling within discrete cellular microdomains [83].

2. Conclusion

I have been privileged to live through a period when many of the major signalling pathways used for cell communication were being discovered. I was caught up in the excitement of the discovery of cyclic AMP, which introduced the concept of second messengers and this prepared me for my work on the role of IP₃ as a second messenger linking inositol lipid hydrolysis to Ca²⁺ signalling. There has been much excitement along the way. In addition to the real buzz of making new discoveries in the laboratory, I have also enjoyed immensely the much excitement along the way. In addition to the real buzz of making new discoveries in the laboratory, I have also enjoyed immensely the much excitement along the way.


[70] S.H. Wang, W. Denk, M. Häusser, Coincident detection in single dendritic spines


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