Regulation of IP$_3$ receptors by cyclic AMP

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**ABSTRACT**

Ca$^{2+}$ and cAMP are ubiquitous intracellular messengers and interactions between them are commonplace. Here the effects of cAMP on inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) are briefly reviewed. All three subtypes of IP$_3$R are phosphorylated by cAMP-dependent protein kinase (PKA). This potentiates IP$_3$-evoked Ca$^{2+}$ release through IP$_3$Rs1 and IP$_3$Rs2, but possibly has little effect on IP$_3$Rs3. In addition, cAMP can directly sensitize all three IP$_3$R subtypes to IP$_3$. The high concentrations of cAMP required for this PKA-independent modulation of IP$_3$Rs is delivered to them within signalling junctions that include type 6 adenyl cyclase and IP$_3$Rs.

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1. Introduction

Cyclic AMP and Ca$^{2+}$ are ubiquitous intracellular messengers used by all eukaryotic cells from plants and animals to coordinate their behaviours in response to both extracellular signals and intracellular activity [1–3]. These messengers create a signalling ‘bottleneck’ through which many extracellular signals funnel to regulate diverse cellular responses. The capacity of a rather limited repertoire of intracellular messengers to selectively regulate cellular activities depends in large part on the spatial organization of the messengers within the cell, the time frames over which they are delivered, and interactions between messengers. The latter often endows signalling pathways with capacities to function as coincidence detectors: conveying signals onward only when several conditions are met [4]. As might be expected of the prototypical intracellular messengers, analyses of the interactions between cAMP and Ca$^{2+}$ have a long history [5,6] that has revealed interactions at many levels. Ca$^{2+}$, for example, regulates formation and degradation of cAMP [2,7], and cAMP can regulate both the channels that allow Ca$^{2+}$ to flow into the cytosol and the Ca$^{2+}$ pumps that extrude it [8,9].

A ubiquitous pathway from extracellular stimuli to cytosolic Ca$^{2+}$ signals is provided by receptors that stimulate phospholipase C (PLC), production of inositol 1,4,5-trisphosphate (IP$_3$) and thereby Ca$^{2+}$ release through IP$_3$ receptors (IP$_3$Rs) [10]. Cyclic AMP also modulates this pathway by, for example, regulating PLC [11] and the coupling of receptors to PLC [12]. However, in this short review, I focus on just one level of interaction, that between cAMP and IP$_3$Rs [13,14]. IP$_3$R subunits are encoded by three genes in vertebrates. The three large, closely related subunits assemble into homo- and hetero-tetrameric structures, which form large-conductance Ca$^{2+}$-permeable channels within

**Abbreviations:** AC, adenylyl cyclase; EPAC, exchange protein activated by cAMP; IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, IP$_3$ receptor; IRAG, IP$_3$R-associated cAMP kinase substrate; IBMT, IP$_3$R-binding protein released by IP$_3$; PKA, protein kinase A (cAMP-dependent protein kinase); PKG, protein kinase G (cGMP-dependent protein kinase); PLC, phospholipase C; P$_o$, single-channel open probability; PTH, parathyroid hormone.

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intracellular membranes, primarily those of the endoplasmic reticulum [10]. Opening of the central pore is initiated by binding of IP₃ to all four IP₃R subunits [15], which evokes conformational changes within the N-terminal domains of the IP₃R [16]. These conformational changes are proposed to facilitate binding of Ca²⁺, which then triggers opening of the pore. Hence, the IP₃R is itself a coincidence detector, responding only when provided with both cytosolic IP₃ and Ca²⁺. High-resolution structures of the N-terminal region of an IP₃R with and without IP₃ bound [16], and cryo-electron microscopy reconstructions of the entire IP₃R in a closed state [17] have begun to reveal the workings of the IP₃R machinery. However, the mechanisms linking IP₃ binding to channel gating are not yet fully resolved. While IP₃ and Ca²⁺ are the essential regulators of IP₃R gating, many additional signals modulate IP₃R behaviour [18]. My focus on cAMP therefore provides only a rather restricted view of the capacity of IP₃Rs to integrate information provided by different signalling pathways.

2. Regulation of IP₃Rs by PKA

Cyclic AMP-dependent protein kinase (protein kinase A, PKA), exchange proteins activated by cAMP (EPACs), cyclic nucleotide-activated cation channels (CNGs), and some cyclic nucleotide phosphodiesterases (PDEs) are the major targets of cAMP in mammals. At least some of these targets regulate IP₃-evoked Ca²⁺ signalling. PKA, for example, stimulates Ca²⁺ uptake into the sarcoplasmic reticulum, and EPACs through the small G protein rap2B stimulate PLCζ [11]. However, only PKA has been convincingly shown to interact directly with IP₃Rs. The three IP₃R subtypes are closely related, but each has a distinctive distribution of PKA phosphorylation sites. The many effects of cAMP within Ca²⁺ signalling pathways were sources of some confusion in the pioneering studies of IP₃R phosphorylation [19], but the consensus now is that PKA-mediated phosphorylation of IP₃R1 and IP₃R2 enhances their activity, while the functional significance of such phosphorylation for IP₃R3 is less clear [14,20].

Two residues (S¹⁵⁸⁸ and S¹⁷⁵⁵) within the central cytosolic domain of IP₃R1 are phosphorylated by PKA, and their replacement by non-phosphorylatable alanine residues confirms that they are the only sites [21]. Phosphorylation of IP₃R1 by PKA or introduction of phosphomimetic residues (S¹⁵⁸⁸E/S¹⁷⁵⁵E) do not themselves open the channel, but they increase the open probability (Pₒ) of channels activated by IP₃. The increased Pₒ results from shortening of the duration between bursts of channel openings and an increase in the duration of the bursts, with no obvious effect on IP₃ binding or the sensitivity to Ca²⁺ regulation [22]. Hence, phosphorylation of IP₃R1 by PKA improves the coupling of IP₃ and Ca²⁺ binding to channel gating by both stabilizing the bursting state of the IP₃R and destabilizing a prolonged closed state. An alternative splice site (S₂ residues 1693–1732), which encodes 40 residues and is removed from non-neuronal IP₃R1, abuts the second phosphorylation site (S¹⁷⁵⁵). For the neuronal S₂ form of IP₃R1, S¹⁷⁵⁵ entirely mediates the effects of PKA, while in the peripheral S₂ form both residues (S¹⁵⁸⁸ and S¹⁷⁵⁵) must be phosphorylated for PKA to enhance IP₃-evoked Ca²⁺ release [23]. Effective phosphorylation and dephosphorylation of IP₃R1 are facilitated by tethering of PKA to IP₃R1 by AKAP9 (A-kinase-anchor protein 9) [24] and of the protein phosphatase, PP₁C, by IRBIT [25]. AKAP9 or directly to the C-terminal tail of IP₃R1 [26].

The consensus sequences for PKA and cGMP-dependent protein kinase (PKG) are similar, so that some residues (e.g. S¹⁷⁵⁵ in IP₃R1S²) are phosphorylated by either kinase. Yet in native tissues PKG and PKA often exert opposing effects on IP₃-evoked Ca²⁺ release. The difference may, at least in part, be due to expression of IRAG (IP₃R-associated cGMP kinase substrate), which blocks phosphorylation of IP₃R1 by PKA, and IRAG phosphorylated by PKG inhibits IP₃R [27]. Hence, IRAG diverts PKG from the PKA-phosphorylation sites and imposes its own inhibition. PKA also modulates the interaction of IP₃R1 with its endogenous antagonist, IRBIT (IP₃R-binding protein released by IP₃), apparently decreasing the affinity for IRBIT so that IP₃ more effectively competes for occupancy of their shared binding site on the IP₃R [28]. Hence in secretory epithelia, receptors that stimulate formation of cAMP and IP₃ synergistically stimulate release of IRBIT from IP₃Rs, and IRBIT then directly stimulates two of the ion transporters that sustain fluid transport [28].

Long before the discovery of IP₃Rs, synergistic stimulation of a Ca²⁺-sensitive K⁺ channel by cyclic AMP and β-adrenoceptors (which stimulate PLC and β-adrenoceptors which stimulate formation of cAMP) in hepatocytes suggested that cAMP might enhance receptor-mediated Ca²⁺ release from intracellular stores [29]. Subsequent studies confirmed that PKA stimulates phosphorylation of hepatic IP₃Rs [30] and potentiates IP₃-evoked Ca²⁺ release [31,32]. IP₃R2, the major IP₃R subtype in hepatocytes, is phosphorylated by PKA at a single residue (Ser⁹³⁷), although others suggest that IP₃R2 is a rather poor substrate for PKA [20]. Ser⁹³⁷ is unique to IP₃R2, but the functional consequences of the phosphorylation appear similar to those seen with IP₃R1, namely enhanced bursts of IP₃R gating [33]. Additional effects of PKA, including an increase in IP₃ binding affinity [30] and recruitment of IP₃Rs into functional Ca²⁺ stores [32], may also contribute to the effects of PKA on IP₃R2 in intact cells.

The effects of PKA on IP₃R3 have been least explored. In intact cells, IP₃R3 is phosphorylated by PKA at three sites (S¹⁵⁹⁸, S¹⁷⁵⁴, C¹⁸³²) that are unique to IP₃R3, with S¹⁷⁵⁴ being the most extensively phosphorylated [34]. But, at least in cells expressing only IP₃R3, PKA has no effect on IP₃-evoked Ca²⁺ release triggered by cell-surface receptors [34]. Whether the phosphorylation affects other aspects of IP₃R3 behaviour remain to be determined.

3. Direct regulation of IP₃Rs by cAMP

In HEK-293 cells stably expressing human type 1 receptors for parathyroid hormone (PTH), PTH stimulates formation of cAMP, but it does not alone evoke an increase in cytosolic Ca²⁺ concentration ([Ca²⁺]ₑ). However, PTH potentiates the increase in [Ca²⁺]ₑ evoked by receptors that stimulate PLC, the endogenous muscarinic M₂ receptors of HEK-293 cells, for example, which can be activated by carbachol (Fig. 1A). This effect of PTH is mimicked by stimulation of endogenous prostaglandin receptors or β-adrenoceptors, by direct activation of adenylyl cyclase with forskolin or by addition of a membrane-permeant analog of cAMP, 8-Br-cAMP. The non-additive effects of maximally effective concentrations of PTH and 8-Br-cAMP confirm that the effect of PTH on carbachol-evoked Ca²⁺ signals is entirely mediated by cAMP (Fig. 1B) [35,36]. Responses to other PLC-coupled receptors are also potentiated by PTH, and the enhanced responses are not associated with increased production of IP₃ [35,37]. Furthermore, cAMP also potentiates the Ca²⁺ signals evoked by a membrane-permeant form of IP₃ (IP₃-BM) [38]. These results, demonstrating that cAMP acts downstream of IP₃, are important because cAMP can, through EPACs, stimulate PLCs [11]. However, the effects of PTH are neither mimicked by EPAC-selective analogs of cAMP [36] nor blocked by an EPAC antagonist [39]. The enhanced IP₃-evoked increases in [Ca²⁺]ₑ are not due to inhibition of Ca²⁺ extrusion from the cytosol by cAMP [38]. Furthermore, cAMP potentiates IP₃-evoked Ca²⁺ release in permeabilized cells [40], and it enhances IP₃-gated channel activity in nuclear patch-clamp recordings of IP₃R [40]. These results, where cAMP potentiates the activation of IP₃R by IP₃, seem consistent with the many reports suggesting that phosphorylation of IP₃R1 and IP₃R2 by PKA enhances responses to IP₃ (see preceding section). However,
The concentrations of 8-Br-cAMP (in intact cells) and of cAMP (in permeabilized cells) needed to sensitize IP3Rs to IP3 are much higher than those required to activate PKA [36].

4. In DT40 cells expressing single IP3R subtypes, high concentrations of cAMP potentiate IP3-evoked Ca2+ release through IP3R1, IP3R2 and IP3R3 [40]. This does not align with the consensus that PKA increases the sensitivity of only IP3R1 and IP3R2 [14].

5. In permeabilized DT40 cells expressing IP3R2, cAMP potentiates the Ca2+ release evoked by IP3, and the effect of cAMP is unaffected by addition of either H89 (to inhibit PKA) or the catalytic subunit of PKA.

6. In nuclear patch-clamp recordings from IP3R2 expressed in DT40 cells and stimulated with IP3, cAMP increases channel activity in the absence of ATP [40], confirming that protein phosphorylation is not required.

These observations suggest that cAMP can regulate IP3R activity via both PKA and by mechanisms that do not require activation of either PKA or EPACs. The observations are intriguing because they suggest an effect of cAMP that is not mediated by any of its conventional targets. Our results indicate that while cAMP alone cannot activate IP3Rs and nor does cAMP affect IP3 binding to IP3R2 [40], it does enhance the effectiveness with which the essential co-agonists, IP3 and Ca2+, stimulate channel opening. The mechanisms underlying these non-canonical actions of cAMP are not yet resolved. In light of evidence that the effects of cAMP are preserved in isolated nuclei and permeabilized cells [36,40], it seems likely that binding of cAMP to a low-affinity site within either the IP3R itself or a tightly associated protein mediates this allosteric regulation of IP3Rs by cAMP.

4. Signalling to IP3Rs at cAMP junctions

Despite compelling evidence that cAMP entirely mediates the potentiating effects of PTH on IP3-evoked Ca2+ signals [36], there are some puzzling features of the signalling pathway that initially led us to a different conclusion. Firstly, the direct effects of cAMP on IP3Rs require much higher concentrations of cAMP than are needed for activation of PKA or EPACs, and probably much higher than the average concentrations achieved in stimulated cells [36]. Secondly, although many different stimuli evoke cAMP formation and potentiation of carbachol-evoked Ca2+ signals, the relationship between their effects on cAMP and Ca2+ are entirely different for different stimuli. For example, for concentrations of PTH and isoproterenol (which activates β-adrenoceptors) that cause similar submaximal potentiation of Ca2+ signals, PTH evokes a more than 10-fold greater increase in intracellular cAMP concentration than does isoproterenol [36]. This immediately suggests that the cAMP that regulates IP3R activity cannot be uniformly distributed in the cytosol. Thirdly, and more troublesome, are the many manipulations of cAMP formation that fail to affect the carbachol-evoked Ca2+ signals. Hence substantial inhibition of cAMP formation by either low-affinity inhibitors of adenyl cyclase (AC, ~90% inhibition in Ref. [38] and ~70% in Ref. [36]) or siRNA-mediated knockdown of AC3 (the major subtype in HEK-293 cells), or an enhancement of cAMP accumulation after inhibition of cyclic nucleotide phosphodiesterase [36,38,39] had no effect on the ability of any concentration of PTH to potentiate carbachol-evoked Ca2+ signals. This initially led us to conclude that the effects of PTH were not mediated by cAMP [38], but we had then to revise that conclusion in light of evidence that cAMP does mediate the effects of PTH.

It is easy to envisage how the effects of a maximal concentration of PTH might be unaffected by even very substantial inhibition of AC if there are ‘spare receptors’, such that maximal activation of the receptors can provide more cAMP than needed to cause

several lines of evidence demonstrate that this is not a sufficient explanation:

1. When PTH-evoked protein phosphorylation is blocked by inhibition of either PKA activity (using H89) or the association of PKA with A-kinase-anchoring proteins (AKAPs, using a membrane-permeant form of an uncoupling protein, ht-89), there is no effect on the ability of any concentration of PTH to potentiate the Ca2+ signals evoked by carbachol [36,38,39]. Others have also suggested that potentiation of carbachol-evoked Ca2+ signals by β2-adrenoceptors is insensitive to inhibition of PKA in HEK-293 cells [41]. Similar results were reported for rat osteoblasts, where potentiation of ATP-evoked Ca2+ signals by PTH was unaffected by inhibition of PKA [42].

2. In permeabilized HEK-293 cells, the catalytic subunit of PKA causes minimal phosphorylation of IP3R and a barely detectable increase in the sensitivity of the Ca2+ stores to IP3, while cAMP and 8-Br-cAMP cause substantial increases in IP3 sensitivity [36].
maximal activation of IP_{3}R. However, that argument cannot be employed to explain the lack of effect of AC inhibitors on the Ca^{2+} responses evoked by submaximal concentrations of PTH. We therefore proposed that the ‘spare’ signalling capacity might exist within subcellular compartments or ‘signalling junctions’. We suggest that CaMP is delivered to IP_{3}Rs locally at concentrations substantially greater than required to cause maximal sensitization of the associated IP_{3}Rs (Fig. 1C). The concentration-dependent effects of PTH, we suggest, come from recruitment of these hyperactive signalling junctions, rather than from graded activity within individual junctions. Each signalling junction is, in effect, an ‘on-off’ switch with a considerable safety margin because once activated it delivers more CaMP than needed to fully sensitize the associated IP_{3}Rs. Our scheme neatly accounts for both the inconsistent relationship between CaMP and response for different stimuli (because different stimuli operate with different safety margins) and it provides a mechanism that would allow IP_{3}Rs to be exposed to high concentrations of CaMP. It also accommodates the results showing that even manipulations of CaMP concentration fail to effect the Ca^{2+} signals evoked by PTH (because the large safety margin protects the signalling pathway from even substantial perturbations of CaMP).

The involvement of signalling junctions is supported by additional evidence [36]. Notably, there is a selective association between AC6 (which accounts for only 5% of AC in HEK-293 cells) and IP_{3}R2 in HEK-293 cells, consistent with targeted delivery of CaMP from AC to IP_{3}R. Loss of IP_{3}R2 (using siRNA) selectively attenuates the potentiation of carbachol-evoked Ca^{2+} signals by PTH. Global inhibition of AC activity by low-affinity inhibitors reduces PTH-evoked CaMP formation without affecting Ca^{2+} signals, whereas the converse occurs when expression of AC6 is reduced. Loss of AC6 has no perceptible effect on CaMP levels, but it attenuates the potentiation of carbachol-evoked Ca^{2+} signals by PTH. The rationale, we suggest, is that all signalling junctions feel the effect of the low-affinity inhibitors, which thereby reduces the CaMP delivered within junctions but not sufficiently to obliterate the safety margin, whereas removing AC6 from individual junctions (each perhaps containing only a single AC) will incapacitate that junction. Finally, in cells with diminished expression of γs, which couples receptors to AC, the safety margin is reduced such that further inhibition of AC (using the low-affinity AC inhibitors) does reduce the ability of PTH to potentiate carbachol-evoked Ca^{2+} signals [36].

In conclusion, there are at least two routes through which CaMP can directly modulate IP_{3}R gating. Phosphorylation of IP_{3}R1 and IP_{3}R2 by PKA increases the effectiveness with which IP_{3} and Ca^{2+} evoke bursts of channel openings. In addition, binding of CaMP to a low-affinity site that seems to be closely associated with the IP_{3}R can also increase the apparent efficacy of IP_{3} and Ca^{2+} in gating each IP_{3}R subtype. The need for high concentrations of CaMP for this direct action demands local delivery of CaMP to IP_{3}Rs, and that has so far been shown to occur for only IP_{3}R2 [36]. The low-affinity of IP_{3}Rs for CaMP effectively insulates them from global changes in cytosolic CaMP concentration and allows them to respond only to CaMP delivered to them within signalling junctions (Fig. 1C). Because each active junction delivers CaMP at a super-saturating concentration to associated IP_{3}Rs, the junction behaves as a robust digital switch that can rapidly respond to changes in extracellular stimulus intensity. The CaMP is delivered rapidly and at a high concentration driving rapid association of CaMP with IP_{3}Rs, and as soon as the AC stimulus is removed the focal concentration of CaMP dissipates by diffusion, rapidly terminating the effects of CaMP on IP_{3}Rs.

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