Modulation of Ca^{2+} oscillations by phosphorylation of Ins(1,4,5)P_{3} receptors

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Abstract
Activation of InsP_{3}Rs (InsP_{3} receptors) represents the major mechanism underlying intracellular calcium release in non-excitable cells such as hepatocytes and exocrine cells from the pancreas and salivary glands. Modulation of calcium release through InsP_{3}Rs is therefore a major route whereby the temporal and spatial characteristics of calcium waves and oscillations can potentially be ‘shaped’. In this study, the functional consequences of phosphoregulation of InsP_{3}Rs were investigated. Pancreatic and parotid acinar cells express all three types of InsP_{3}R in differing abundance, and all are potential substrates for phosphoregulation. PKA (protein kinase A)-mediated phosphorylation of InsP_{3}Rs in pancreatic acinar cells resulted in slowed kinetics of calcium release following photo-release of InsP_{3}. In contrast, activation of PKA in parotid cells resulted in a marked potentiation of calcium release. In pancreatic acinar cells the predominant InsP_{3}R isomorph phosphorylated was the type 3 receptor, while the type 2 receptor was markedly phosphorylated in parotid acinar cells. In order to further decipher the effects of phosphorylation on individual InsP_{3}R subtypes, DT-40 cell lines expressing homotetramers of a single isoform of InsP_{3}R were utilized. These data demonstrate that phosphoregulation of InsP_{3}Rs results in subtype-specific effects and may play a role in the specificity of calcium signals by ‘shaping’ the spatio-temporal profile of the response.

The spatio-temporal properties of Ca^{2+} signals define effector specificity
Stimulus-induced changes in the [Ca^{2+}](cytosolic Ca^{2+} concentration) represent the most versatile and ubiquitous signalling system in biology. Elevations in [Ca^{2+}], regulate a host of cellular events, including gene transcription [1,2] and regulated exocytosis [3], and under defined circumstances can control such seemingly diametrically opposite outcomes as smooth muscle contraction and relaxation [4], as well as mitochondrial energy production and mitochondrially induced cell death through apoptosis [5]. A critical question which remains is how such a varied array of cellular outcomes can be encoded by this one signal. Experiments by Cobbold and colleagues [6–9] provided the initial clues, by demonstrating that Ca^{2+} signals themselves can be as diverse as the effectors they regulate and therefore potentially carry enough information to code a multitude of cellular endpoints. Groundbreaking experiments measuring [Ca^{2+}], in single hepatocytes and mouse oocytes with the photoprotein aequorin showed that physiologically relevant stimulation elicited repetitive Ca^{2+} transients, not simply sustained Ca^{2+} elevations [6–8]. Furthermore, in hepatocytes, the frequency of the transients was dependent on the concentration of hormone or neurotransmitter applied and intriguingly the shape or profile of the transients was characteristic of a particular agonist [9]. These observations provided the initial evidence to support the now widely held contention that the precise spatio-temporal patterns of Ca^{2+} oscillations and Ca^{2+} waves supply specific information underpinning the downstream specificity of Ca^{2+} signals. Thus understanding the mechanisms which are responsible for shaping these signals is of obvious importance. In hepatocytes and other electrically non-excitable cells such as exocrine cells of the pancreas and salivary glands, Ca^{2+} release through InsP_{3}Rs [Ins(1,4,5)P_{3} receptors] plays a central role in the initiation and propagation of Ca^{2+} oscillations and Ca^{2+} waves. Therefore, acute regulation of Ca^{2+} release at the level of InsP_{3}Rs potentially plays a major role in shaping Ca^{2+} signals.

Regulation of Ca^{2+} release by InsP_{3}R phosphorylation
At least three genes for InsP_{3}Rs, termed types 1, 2 and 3, are expressed in mammals [10–14]. The functional protein forms a tetramer, with each monomer expressing a binding site for InsP_{3} within the N-terminus and a putative pore-forming region in the C-terminus. In the approx. 2000-amino-acid stretch between the InsP_{3}-recognition site and the pore is the ‘regulatory and coupling domain’ where motifs are located which predict modulation by a host of potential regulatory factors [12]. Whereas it is clear that the most important co-regulators of the InsP_{3}R are InsP_{3} and Ca^{2+} itself [15], each subtype of the receptor has the potential to undergo phosphorylation by numerous kinases, including PKA (cAMP-dependent protein kinase) [16–23], cGMP-dependent protein kinase [24,25], calmodulin-dependent...
protein kinase [26], protein kinase C [26] and various tyrosine kinases of the Src family [27]. Phosphoregulation of InsP3Rs therefore provides a potentially important nexus for cross-talk between Ca2+ and other signalling networks. Thus altering the kinetics of Ca2+ release through InsP3Rs could be a major contributing factor to the profile of the Ca2+ signal. InsP3Rs can be phosphorylated by numerous kinases and at least the type 1 InsP3R appears to be a particularly good substrate for phosphorylation by PKA, although there is currently little consensus as to the physiological consequence of its phosphorylation, by PKA or any other kinase.

The type 1 receptor possesses two PKA consensus sequences at Ser-1589 and Ser-1755, which become phosphorylated upon elevating cAMP [16]. Interestingly, a 40-amino-acid region between these residues is spliced out in the peripheral form of the type 1 receptor (S2 variant) and evidence suggests that, as a consequence of splicing, phosphorylation preferentially switches from Ser-1755 in the brain receptor (S2 variant) to Ser-1589 in the peripheral form of the receptor [28]. Preferential phosphorylation of individual sites may, in some part, explain discrepancies reported in the effects of phosphorylation. For example, reports suggest that phosphorylating the peripheral form of the type 1 receptor in megakaryocytes appears to attenuate Ca2+ release [22]. In contrast the majority (but not all) of the reports using neuronally derived tissue, where the long form of the receptor is abundant, suggest that PKA phosphorylation potentiates Ca2+ release [19–21,23].

**Phosphorylation of InsP3R in exocrine acinar cells**

While there are relatively plentiful data regarding PKA phosphorylation of the type 1 InsP3R, there are only a handful of studies examining phosphoregulation of type 2 and type 3 receptors [17,18,23,29–31]. Indeed, although it has been demonstrated directly that both type 2 and type 3 receptors can be phosphorylated by PKA, the specific sites have not been elucidated, since the canonical motifs expressed in the type 1 receptor are not conserved in the type 2 and type 3 InsP3Rs. However, additional potential PKA-phosphorylation motifs are present in the regulatory domain of type 2 and type 3 receptors [13,14]. In contrast to functional studies on the type 1 InsP3R, the limited data on phosphorylation of type 2 InsP3Rs are fairly consistent. Data from hepatocytes, which predominately express type 2 as well as type 1 InsP3Rs, show enhanced Ca2+ release under conditions consistent with PKA-mediated phosphorylation of InsP3Rs [18,32].

Similarly, parotid acinar cells express a majority of type 2 InsP3Rs, but also express a significant complement of type 3 InsP3R, together with approx. 5% type 1 InsP3R [33,34]. Stimulation of these cells with muscarinic or purinergic agonists to raise InsP3 levels, concurrently with manoeuvres to increase levels of cAMP, resulted in a larger initial Ca2+ spike than is observed with Ca2+-mobilizing agonists alone [29]. Raising cAMP during sub-threshold agonist stimulation resulted in an oscillatory Ca2+ signal, while raising cAMP during an oscillatory response converted the oscillating signal into a ‘peak and plateau’-type signal consistent with a leftward-shift in the concentration–response relationship for InsP3-induced Ca2+ release [29]. Reinforcing the contention that the effects of raising cAMP were primarily on InsP3-induced Ca2+ release, no changes in the formation of InsP3, in Ca2+ influx, Ca2+ release through ryanodine receptors [29] or sarcoplasmic/endoplasmic-reticulum Ca2+–ATPase pump activity [35], were observed under similar conditions. Importantly, in a permeabilized cell system a marked potentiation of InsP3-induced Ca2+ release was evident (Figure 1A).

Moreover, using an immunoprecipitation assay, robust phosphorylation of type 2 InsP3Rs was demonstrated upon forskolin treatment (Figure 1B) [29]. All these effects were inhibited significantly by treatment with PKA inhibitors, indicating that the effects on InsP3-induced release were likely to result from a PKA-dependent action of cAMP [29]. Collectively, these data demonstrate that a primary mechanism underlying potentiated Ca2+ signalling in salivary acinar cells when InsP3 and cAMP are elevated is through phosphorylation of type 2 InsP3Rs.

This effect may have important physiological relevance to fluid secretion from parotid acinar cells. Peak saliva secretion occurs when salivary glands are stimulated through sympathetic and parasympathetic neural pathways simultaneously. This stimulation will result in both an increase in cAMP and InsP3, as a consequence of concurrent β-adrenergic and muscarinic receptor stimulation. Fluid secretion is initiated by the transcellular flux of Cl− into the acinar cell lumen resulting from activation of a Ca2+-activated Cl− channel. The transcellular potential established by the movement of Cl− subsequently promotes the paracellular movement of Na+, and water follows osmotically. An enhanced Ca2+ signal mediated by PKA phosphorylation of type 2 InsP3Rs appears to play a major role in enhancing the activity of Ca2+-activated Cl− channels and thus Cl− flux, ultimately leading to peak fluid secretion.

While type 2 InsP3R phosphorylation is primarily responsible for enhanced Ca2+ release in parotid acinar cells, consistent with the original data from hepatocytes the question remains as to how individual InsP3R types are preferentially phosphorylated when multiple subtypes are expressed. An emerging theme in signal transduction is the idea that molecules may be targeted to sites of action, or in present scaffolds to enhance the efficiency and specificity of signalling [36,37]. This idea also appears to be the case for PKA modulation of InsP3R-induced Ca2+ signalling. Primary evidence for this comes from pancreatic acinar cells, where it appears that PKA is targeted to the type 3 InsP3R through an AKAP (kinase anchoring protein) [17]. Pancreatic acinar cells also express all InsP3R types [38]; however, in contrast to parotid cells, Ca2+ release stimulated by photolyis of caged InsP3 is attenuated in magnitude and the kinetics are slowed by raising cAMP (Figure 2A) [17,31]. Interestingly, the type 3 InsP3R is markedly phosphorylated under these conditions (Figure 2B) [30] and furthermore
the phosphorylation appears to be targeted since regulatory subunits of PKA specifically co-immunoprecipitate with type 3 InsP₃Rs, and not other subtypes [17]. The primary evidence that an AKAP is involved in this targeting comes from the use of Ht-31, a peptide which mimics the conserved amphipathic helix present in AKAPs and thought to be involved in binding PKA. Ht-31 completely abrogates the inhibitory effects of raising cAMP [17].

AKAPs often serve as multifunctional scaffolds, targeting signalling molecules, other than PKA to substrates [36]. This appears to be a general phenomenon which also relates to InsP₃R, since it has been recently reported using a two-hybrid screen with the InsP₃R as bait, that both PKA and protein phosphatase-1α form a complex with the type 1 InsP₃R [21,39]. Further work will be necessary to identify the particular AKAP responsible for PKA targeting in pancreatic acinar cells, but an attractive proposition given the co-immunoprecipitation of type 3 InsP₃R with actin and the co-localization with the actin terminal web [17] would be an AKAP that is capable of interacting with microfilaments.

Of particular interest, stimulation with the gut hormone CCK (cholecystokinin) also results in PKA-dependent type 3 InsP₃R phosphorylation at concentrations of hormone which generate Ca²⁺ oscillations (Figure 2B; >10 pM) [30]. In contrast, stimulation with muscarinic agonists does not induce phosphorylation at physiologically relevant concentrations. This phosphorylation event appears to contribute to the distinctive slow baseline-spiking pattern of Ca²⁺ oscillations stimulated by CCK. In support of this idea, stimulation of PKA during sinusoidal oscillations initiated by muscarinic agonists converts the pattern to one reminiscent of the low-frequency baseline oscillations stimulated by CCK [17,31]. Conversely, inhibiting the targeting of PKA to the InsP₃R with Ht-31 peptide markedly effects the baseline pattern of CCK oscillation but has no effect on muscarinic stimulated oscillations. Stimulation with another agonist, bombesin, which acts at a neuromedin receptor, also results in the phosphorylation of type 3 InsP₃R and qualitatively very similar Ca²⁺ oscillations to those stimulated by CCK [31]. Phosphorylation of InsP₃R, therefore, appears to be a general mechanism which contributes to this particular slow baseline spiking pattern of Ca²⁺ oscillations. An intriguing issue is the physiological consequences of the markedly different patterns of Ca²⁺ oscillations stimulated by CCK/bombesin.
and acetylcholine. It is obviously tempting to make the prediction that different effectors preferentially recognize the distinct \( \text{Ca}^{2+} \) signals; however, this hypothesis has yet to be tested experimentally.

Regulation of InsP₃Rs, in particular by phosphorylation, is obviously an important locus for the regulation of \( \text{Ca}^{2+} \) release and thus \( \text{Ca}^{2+} \) oscillations. Clearly, further studies are necessary to precisely define the functional effects of phosphorylation on individual InsP₃R types and to identify the location of phosphorylated sites in the type 2 and type 3 receptors. These studies will be facilitated by the recent generation of DT-40 cell lines with defined InsP₃R populations, including an InsP₃R-null background [40].

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