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Calcium mobilization via type III inositol 1,4,5-trisphosphate receptors is not altered by PKA-mediated phosphorylation of serines 916, 934 and 1832

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Abstract

Several studies have shown that PKA-mediated phosphorylation of IP₃R1 at serines S¹⁵⁸⁸ and S¹⁷⁵⁵ enhances the receptor's ability to mobilize Ca²⁺. In contrast, much less is known about whether Ca²⁺ mobilization via IP₃R2 and IP₃R3 is regulated by PKA. We report here that IP₃R2 is only very weakly phosphorylated in response to PKA activation and is probably not a physiological substrate for this kinase. IP₃R3, however, is known to be phosphorylated by PKA at three sites (S⁹¹⁶, S⁹³⁴ and S¹⁸³²) and, thus, we examined how phosphorylation of these sites affects Ca²⁺ mobilization in DT40-3KO cells stably expressing either exogenous wild-type or mutant IP₃R3s; an antibody raised against phospho-serine 934 of IP₃R3 was used to demonstrate that the exogenous IP₃R3s are strongly phosphorylated in response to PKA activation. Surprisingly, our data show that IP₃R3-mediated Ca²⁺ mobilization is unaffected by phosphorylation of S⁹¹⁶, S⁹³⁴ and S¹⁸³². In contrast, phosphorylation of exogenous IP₃R1 (monitored with an antibody against phospho-serine 1755) enhances Ca²⁺ mobilization, indicating that DT40-3KO cells have the capacity to respond to phosphorylation of IP₃Rs. Overall, these data suggest that modification of Ca²⁺ flux may not be the primary effect of IP₃R3 phosphorylation by PKA.

Keywords

Inositol 1,4,5-trisphosphate receptor; cAMP-dependent protein kinase; phosphorylation; calcium mobilization; DT40

1. Introduction

Inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃Rs) are a family of proteins which form Ca²⁺ channels in endoplasmic reticulum (ER) membranes and govern Ca²⁺ release from this organelle by opening in response to IP₃ and Ca²⁺ binding [1-3]. In mammals, type I, II and III IP₃ receptors (IP₃R1, IP₃R2, and IP₃R3) are highly homologous and have the same overall structure; with an N-terminal “ligand-binding domain”, a C-terminal “channel domain”, and

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an intervening “coupling domain” containing putative binding sites for several regulatory factors [1-3]. Despite these similarities, however, expression of IP₃R types varies between tissues [4,5] and subtle differences in their functional properties, such as their regulation by IP₃, Ca²⁺ and ATP, are apparent [3,6-8]. Functional differences between the three receptor types may also arise from their differential phosphorylation by cAMP-dependent protein kinase (PKA) [9-11] but, with the exception of IP₃R1, there is no clear consensus regarding the functional consequences of these phosphorylation events.

PKA phosphorylates IP₃R1 at two sites (S¹⁵⁸⁸ and S¹⁷⁵⁵) [12-14] and studies on IP₃R1 expressed in IP₃R-null DT40-3KO cells (hereafter referred to as 3KO cells) indicate that this phosphorylation enhances IP₃R1-mediated Ca²⁺ release [15,16], a finding consistent with biophysical studies on purified IP₃R1 reconstituted into lipid bilayers [17]. In contrast, PKA-mediated phosphorylation of IP₃R2 has not been examined in detail, and while IP₃R3 is known to be phosphorylated at three sites (S⁹¹⁶, and S⁹³⁴ and S¹⁸³²) [18], the effects of phosphorylation at these sites has not been examined. Attempts to ascribe functional effects to PKA-mediated IP₃R2 and IP₃R3 phosphorylation, however, have been made by activating PKA in cells that express relatively high levels of each receptor type. Enhancement of Ca²⁺ signaling in parotid acinar cells, for example, has been attributed to IP₃R2 phosphorylation [19]. In contrast, inhibition of Ca²⁺ signaling has been attributed to phosphorylation of IP₃R3 in pancreatic acinar cells [20,21]. While these findings suggest that IP₃R2 and IP₃R3 might be differentially affected by PKA, the presence of multiple IP₃R types and other PKA-regulated proteins in these systems makes it impossible to unequivocally attribute effects on Ca²⁺ signaling to the phosphorylation of one particular IP₃R type.

It should be possible to overcome these difficulties using 3KO cells [22], as exogenous wild-type and mutant IP₃Rs can be expressed and analyzed without interference from endogenous receptors [15,16]. Thus, our initial goal was to define the effects of PKA activation on IP₃R2 and IP₃R3-mediated Ca²⁺ mobilization in 3KO cells. However, as our preliminary studies showed that IP₃R2 is phosphorylated only very weakly by PKA, we focused on IP₃R3. We report here that, surprisingly, phosphorylation of S⁹¹⁶, S⁹³⁴ and S¹⁸³² has no effect on IP₃R3-mediated Ca²⁺ mobilization in transfected 3KO cells. In contrast, phosphorylation of IP₃R1 enhances Ca²⁺ signaling, demonstrating that the 3KO cell-based system has the capacity to respond to IP₃R phosphorylation.

2. Materials and methods

2.1 Materials

CT1, CT2 and CT3, affinity purified rabbit antisera specific to the C-termini of IP₃R1, IP₃R2 and IP₃R3, respectively, have been described previously [4]. Anti-IgM (clone M-4), a mouse monoclonal antibody directed against chicken IgM, was from Southern Biotechnology Associates. Trypsin was from Sigma (bovine pancreas; #T4665). TL3, a mouse monoclonal antibody directed against amino acids 22–230 of IP₃R3, was from BD Biosciences. DT40 and 3KO cells [22] were kindly provided by Dr. D. Yule (University of Rochester, NY, U.S.A) with the permission of Dr. T. Kurosaki (Kansai Medical University, Japan).

2.2 IP₃R cDNAs

Mouse SI+/SII+ IP₃R1 (a kind gift from Dr. K. Mikoshiba, The University of Tokyo, Japan), rat IP₃R2 (a kind gift from Dr. G. Mignery, Loyola University, Chicago, IL, U.S.A.) and rat IP₃R3 (a kind gift from Dr. G. Bell, University of Chicago, Chicago, IL, U.S.A.) cDNAs have been described elsewhere [14,23-25]. To facilitate transfection of 3KO cells, the coding region of wild-type rat IP₃R3 was excised from its original vector backbone [24] with *NotI* and was

ligated into the multiple cloning region of correspondingly cut pcDNA3 (Invitrogen). The mutation of PKA phosphorylation sites was then performed as described [18].

2.3 Generation of phospho-IP₃R antibodies

Anti-pS934 and anti-pS1755 were raised in rabbits as described [4] against the synthetic peptides C-V-L-R-K-Q-*phospho*S-V-F and C-S-G-R-R-E-*phospho*S-L-T-S, which correspond to amino acids 929-936 of rat IP₃R3 [24] and 1750-1758 of mouse IP₃R1 [25], respectively, with an additional cysteine at the N-termini to facilitate coupling reactions. Crude antisera were affinity-purified on peptide-coupled SulfoLink columns (Pierce). Briefly, columns were incubated with antisera overnight at 4 °C and washed with 1 M guanidine HCl, followed by 50 mM Tris, pH 7.4. Specifically bound antibodies were eluted from the columns with 4.5 M MgCl₂ in 50 mM Tris-HCl, pH 7.4, containing 0.1% bovine serum albumin. The eluate was then dialyzed against 155 mM NaCl in 50 mM Tris-HCl, pH 7.4, for 2 hr at room temperature and then overnight at 4 °C against the same buffer supplemented with 10% glycerol and 0.005% NaN₃.

2.4 Cell culture

DT40 and 3KO cell lines were cultured at 40°C, under 5% CO₂ in L-glutamine-containing RPMI 1640 (Mediatech Inc) supplemented with 1% chicken serum, 10% fetal bovine serum and antibiotics (100 units penicillin and 100 µg streptomycin/ml). Human embryonic kidney (HEK) 293 and African green monkey COS kidney cells were cultured as described [4]. The concentration of DMSO, used as a vehicle for forskolin and 3-isobutyl-1-methylxanthine (IBMX), was less than 1% in all experiments.

2.5 Transfection of IP₃R cDNAs

Stable transfection of 3KO cells was achieved by electroporation at 550 V and 25 µF (4-mm gap cuvette) using a BioRad GenePulser XCell. Briefly, 10 million cells were incubated for 10 min on ice in 800 µl of phosphate buffered saline (PBS) containing 25 µg of IP₃R cDNA linearized in the non-coding region. The mixture was then electroporated, incubated for 10 min on ice, and incubated overnight in 25 ml culture medium. Finally, the cells were centrifuged, re-suspended in 80 ml culture medium supplemented with 2 mg/ml G418, seeded into 96-well plates, and grown for 7-10 days prior to selection of G418-resistant clones for expansion. Transient transfection of HEK and COS cells was performed as described [14].

2.6 IP₃R phosphorylation in intact cells

Phosphorylation of IP₃R_s was analyzed using [³²P]P_i-labeled HEK cells, as described [14], or through immunoblotting of electrophoresed cell lysates or immunoprecipitated IP₃R_s (the ~200-300 kDa regions of gels are shown). Cell lysates were prepared via solubilization with ice-cold, phosphatase-inhibitor-supplemented lysis buffer [14] and were either probed directly, or phosphorylated IP₃R_s were immunoprecipitated by overnight incubation at 4°C with Protein A-Sepharose CL-4B beads and anti-pS1755 or anti-pS934. Immune complexes were then electrophoresed and probed with IP₃R1-specific CT1 or IP₃R3-specific TL3. To ensure that the conditions for measurement of IP₃R phosphorylation were the same as those used in Ca²⁺ mobilization experiments (see section 2.8), DT40 or transfected 3KO cells were incubated with 3 µM Fura-2AM, washed, and resuspended in Krebs-HEPES buffer (KHB) [26] prior to stimulation.

2.7 Immunofluorescence microscopy

COS cells were seeded on poly-L-lysine-coated glass coverslips (500,000 cells/9.6 cm²), transfected with 1 µg IP₃R3^{SSS} cDNA, stimulated, fixed with 1% paraformaldehyde in PBS for 1 hr at room temperature, washed with PBS, incubated at -20°C for 10 min with methanol,

rinsed for 10 sec with ice-cold acetone, and re-hydrated in PBS for 30 min at room temperature. Fixed cells were then incubated for 45 min in blocking buffer (10% goat serum, 0.1% BSA in PBS) before overnight incubation at 4°C in blocking buffer supplemented with primary antibody (TL3 or anti-pS934). Following three washes in PBS, the cells were incubated for 45 min at room temperature in blocking buffer supplemented with secondary antibody (FITC- or TRITC-conjugated goat anti-rabbit IgG, respectively). Finally, the fixed cells were washed three times, stained with 100 ng/ml 4'-6-diamidino-2-phenylindole (DAPI) for 5 min, washed three more times, and coverslips were mounted using Vectashield reagent (Vector Labs Inc). Images were acquired using a Zeiss Axioplan 2 microscope equipped with a 63x oil immersion objective (Plan Apochromat from Zeiss).

2.8 Ca²⁺ mobilization

Five million cells were harvested by centrifugation (2000 x g for 10 min), re-suspended in 1 ml culture medium, and incubated with 3 μM Fura-2AM for 20 min at 37°C. Following three washes with KHB, the cells were incubated for 10 min at 37°C in 1 ml KHB to allow for de-esterification of Fura-2AM, were washed once more, resuspended in 2 ml KHB, and excited at 340 and 380 nm and emission intensity at 509 nm was recorded with an LS-50B luminescence spectrometer (Perkin-Elmer). Cytoplasmic Ca²⁺ concentration ([Ca²⁺]_i) was calculated as described [27] utilizing 0.1% Triton X-100 and 7 mM EGTA to obtain R_{max} and R_{min} , respectively. All combined data are presented as mean ± S.E.M from ≥ 3 independent experiments except as noted. Values of "[Ca²⁺]_i increase" were calculated by subtracting basal [Ca²⁺]_i values from [Ca²⁺]_i values recorded ~60 s after stimulation with anti-IgM or ~15 s after trypsin, and are expressed as a percentage of maximum; the average response ($n \geq 3$) to a maximal dose of stimulus in the absence of forskolin plus IBMX. Statistical significance was then assessed using the unpaired Student's *t* test; asterisks indicate when $p < 0.05$.

3 Results

3.1 PKA-mediated phosphorylation of IP₃R_s in intact cells

As our overall goal was to define the effects of PKA-mediated phosphorylation of IP₃R_s on Ca²⁺ signaling in intact cells, we first sought to define which of the receptors are phosphorylated. We, and others, have already shown that IP₃R1 and IP₃R3 are phosphorylated by PKA in intact cells; at Ser¹⁵⁸⁸ and Ser¹⁷⁵⁵ in IP₃R1 [12-14], and Ser⁹¹⁶, Ser⁹³⁴ and Ser¹⁸³² in IP₃R3 [18]. The extent to which IP₃R2 is phosphorylated, however, is not clear. To determine this, we examined exogenous IP₃R2 expressed in [³²P]_i-labeled HEK cells stimulated with 10 μM forskolin, a condition that results in maximal and equivalent phosphorylation of exogenous IP₃R1 and IP₃R3 [14,18]. Fig 1A demonstrates that IP₃R2 (lanes 1-2) was only very weakly phosphorylated under conditions that allowed for substantial phosphorylation of IP₃R3 (lanes 3-4) and IP₃R1 [14,18]. Fig 1B shows that there was ~90% less radioactivity associated with exogenous IP₃R2 than IP₃R3. Thus, IP₃R2 is a very poor substrate for PKA and we did not examine it further. Rather, we focused on elucidating the functional effects of PKA-dependent IP₃R3 phosphorylation.

3.2 Validation of anti-pS934

To facilitate the study of IP₃R3 phosphorylation, we developed an antibody against phospho-S⁹³⁴ of IP₃R3, the predominant site of PKA-mediated phosphorylation [18]. The specificity of this antibody, termed anti-pS934, was demonstrated in immunoblots of cell lysates prepared from HEK cells expressing exogenous wild-type and mutant IP₃R3s (Fig 2A), a system in which we have previously characterized IP₃R3 phosphorylation using [³²P]_i [18]. Anti-pS934 exhibited minor immunoreactivity towards wild-type IP₃R3 (IP₃R3^{SSS}; lane 3) and IP₃R3 mutated at S⁹¹⁶ and S¹⁸³² (IP₃R3^{ASA}; lane 9) in the absence of stimulus, and this was increased substantially under conditions of maximal IP₃R phosphorylation (lanes 4 and 10). Anti-pS934

did not, however, exhibit any immunoreactivity towards IP₃R3 mutated at S⁹³⁴ (IP₃R3^{SAS}; lanes 7-8), IP₃R3 mutated at S⁹¹⁶, S⁹³⁴, and S¹⁸³² (IP₃R3^{AAA}; lanes 5-6), or wild-type IP₃R1 (IP₃R1^{SS}; lanes 11-12) in the absence or presence of forskolin, demonstrating that it is entirely specific towards IP₃R3 phosphorylated at S⁹³⁴. This was confirmed by immunofluorescence microscopy of transfected COS cells. Exogenous IP₃R3^{SSS} was recognized by TL3 in ~50% of the cells, as indicated by punctate staining throughout the cytoplasm (panels v and vi), and anti-pS934 produced a similar signal in IP₃R3^{SSS}-expressing cells after the addition of forskolin (panel viii). Thus, anti-pS934 is specific and is effective in both immunoblots and immunofluorescence (Fig 2), and subsequent studies (Fig 3) show it also to be effective in immunoprecipitations.

3.3 Phosphorylation of IP₃R3s expressed in 3KO cells

To study the effects of phosphorylation on IP₃R3-mediated Ca²⁺ signaling, we chose to use transfected 3KO cells, since they provide a system to examine Ca²⁺ signaling mediated exclusively via exogenous IP₃Rs, free from the interference of endogenous IP₃Rs [15,16]. As expected, endogenous IP₃R3 was present in DT40 cells (Fig 3A, upper panel, lanes 1-2), but was absent from 3KO cells (lanes 3-4). Stable expression of exogenous IP₃R3^{SSS} (3KO-SSS, lanes 5-6), IP₃R3^{SAS} (3KO-SAS, lanes 7-8), IP₃R3^{ASA} (3KO-ASA, lanes 9-10), and IP₃R3^{AAA} (3KO-AAA, lanes 11-12) was achieved at levels comparable to, or above, endogenous IP₃R3. To examine whether these exogenous receptors could be phosphorylated, cell lysates were incubated with anti-pS934 and immunoprecipitated IP₃R3s were visualized with TL3 (Fig 3A, lower panel). This showed that IP₃R3^{SSS} (lane 6) and IP₃R3^{ASA} (lane 10) were phosphorylated at S⁹³⁴ upon PKA activation and that, as expected, IP₃R3^{SAS} (lane 8) or IP₃R3^{AAA} (lane 12) were not. Surprisingly, we did not detect phosphorylation of endogenous IP₃R3 (lanes 1-2), most likely because anti-pS934 does not recognize phosphorylated chicken IP₃R3, due to minor sequence differences in the antibody epitope.

We next set out to define the basic characteristics of IP₃R3 phosphorylation in 3KO-SSS cells. Fig 3B (upper panel) shows, surprisingly, that forskolin was not a potent stimulus and that very little phosphorylation of IP₃R3 was detected at doses of forskolin (10-30 μM) considered supramaximal in many mammalian cell lines; e.g. maximal phosphorylation of exogenous IP₃R3 in HEK cells (Fig 3C) or endogenous IP₃R3 in Rat-1 fibroblasts (data not shown) occurs at 1 μM forskolin. However, inclusion of IBMX (200 μM), an inhibitor of cAMP phosphodiesterases, greatly enhanced IP₃R3 phosphorylation in 3KO-SSS cells (Fig 3B, lower panel) and reduced the concentration of forskolin required for maximal IP₃R3 phosphorylation to ~10 μM.

The use of IBMX to enhance IP₃R3 phosphorylation proved to be essential for the studies on Ca²⁺ signaling in 3KO-derived cells, as we found that forskolin alone caused Ca²⁺ mobilization in 3KO-SSS cells at concentrations of 10 μM and above (Fig 3D). The cause of this mobilization remains unknown, but appears to reflect IP₃R-independent Ca²⁺ release from intracellular stores, as it still occurred in 3KO cells (Fig 3D), and in the absence of extracellular Ca²⁺ (data not shown). Thus, to study the effects of IP₃R3 phosphorylation on Ca²⁺ mobilization, we chose to use 1 μM forskolin plus 200 μM IBMX, as this condition causes near maximal IP₃R3 phosphorylation (Fig 3B), but did not itself cause Ca²⁺ mobilization (Figs 4-5). Further, we could demonstrate that at least 50% of exogenous IP₃R3^{SSS} was phosphorylated by forskolin plus IBMX under these conditions (Fig 3E).

3.4 Effects of IP₃R3 phosphorylation on BCR-mediated Ca²⁺ mobilization

Stimulation of DT40 cells with anti-IgM results in endogenous B cell receptor (BCR) activation, PLCγ-mediated generation of IP₃, and IP₃R-mediated Ca²⁺ mobilization [22,28]. Fig 4A shows typical Ca²⁺ responses to various doses of anti-IgM in DT40 (i) and 3KO-SSS

(*ii*) cells, and dose-response curves (*iii*). Clearly, exogenous IP₃R3^{SSS} (*ii*) couples BCR activation to Ca²⁺ mobilization essentially identically to endogenous IP₃Rs (*i*), with the exception that 3KO-SSS cells are slightly less sensitive to anti-IgM than DT40 cells (EC₅₀ = 0.70 ± 0.06 and 0.50 ± 0.04 μg/ml, respectively). This minor difference may be explained by the fact that, in addition to IP₃R3, DT40 cells express IP₃R1 and IP₃R2 [22], both of which have a higher affinity for IP₃ than IP₃R3 [6,7,22]. 3KO-SSS, 3KO-AAA, 3KO-SAS and 3KO-ASA cells exhibited virtually identical maximum responses to anti-IgM (544 ± 40, 570 ± 52, 533 ± 37 and 522 ± 48 nM Ca²⁺, respectively) and anti-IgM dose-response curves (EC₅₀ = 0.70 ± 0.04, 0.72 ± 0.08, 0.79 ± 0.10, and 0.72 ± 0.06 μg/ml), demonstrating that basal phosphorylation of S⁹¹⁶, S⁹³⁴ and S¹⁸³² does not affect IP₃R3-mediated Ca²⁺ mobilization.

To determine the effects of increased IP₃R3 phosphorylation on anti-IgM - induced Ca²⁺ mobilization, we stimulated cells with a high (5 μg/ml), medium (1 μg/ml) and low (0.05 μg/ml) dose of anti-IgM, 10 min after addition of either vehicle or 1 μM forskolin plus 200 μM IBMX, a condition which phosphorylates at least 50% of IP₃R3s in transfected 3KO cells (Fig 3E) and causes phosphorylation of endogenous IP₃Rs in DT40 cells (Fig 5B). Fig 4B shows that treatment with forskolin plus IBMX had no effect on anti-IgM induced Ca²⁺ mobilization in DT40 cells (*i-iii*), but slightly inhibited Ca²⁺ mobilization in 3KO-SSS cells (*iv-vi*). However, this inhibition was unrelated to IP₃R3 phosphorylation at S⁹¹⁶, S⁹³⁴ or S¹⁸³², as it occurred to a similar extent in all IP₃R3 mutant-expressing cell lines (*vii*). It also occurred in 3KO-SSS and 3KO-AAA cells incubated with extracellular EGTA (Fig 4C), indicating that it is due to reduced IP₃R3-mediated Ca²⁺ release from intracellular stores, rather than reduced Ca²⁺ entry. Surprisingly, therefore, the data in Fig 4 demonstrate that phosphorylation of IP₃R3 at S⁹¹⁶, S⁹³⁴ and S¹⁸³² has no effect on Ca²⁺ mobilization in response to BCR activation, but also reveals a slight inhibitory effect of forskolin plus IBMX that is mediated by an, as yet, unidentified mechanism. This inhibition appears to be a consequence of cAMP elevation and/or PKA activation, as it was not seen when cells were incubated with either 1 μM forskolin or 200 μM IBMX alone (data not shown).

3.5 IP₃R1 phosphorylation enhances BCR-mediated Ca²⁺ mobilization

Given the lack of effect of forskolin plus IBMX on Ca²⁺ signaling in DT40 cells and 3KO-SSS cells (Fig 4), we sought evidence that IP₃R phosphorylation can have measurable effects on Ca²⁺ signaling in our experimental system. In this regard, it has been shown previously that PKA-mediated phosphorylation of IP₃R1 at S¹⁵⁸⁸ and/or S¹⁷⁵⁵ enhances Ca²⁺ release in individual IP₃R1-expressing 3KO cells [15,16]. Thus, PKA activation should also enhance IP₃R1-mediated Ca²⁺ release in our experiments with cell suspensions.

To measure IP₃R1 phosphorylation, we developed an antibody against phospho-S¹⁷⁵⁵. This antibody, termed anti-pS¹⁷⁵⁵, did not recognize phosphorylated IP₃R1 in immunoblots or via immunofluorescence microscopy (not shown), but was effective in immunoprecipitations and was specific towards phospho-S¹⁷⁵⁵ of IP₃R1 (Fig 5A). Thus, we could demonstrate forskolin plus IBMX-induced phosphorylation of endogenous IP₃R1 in DT40 cells (Fig 5B, lane 2, lower panel), and of exogenous IP₃R1^{SS} in 3KO-SS cells (lane 4, lower panel), but not in 3KO-AA cells (lane 6 lower panel). Further, using the same approach utilized for IP₃R3 (Fig 3E), we could show that at least 50% of the receptors in 3KO-SS cells are phosphorylated in response to forskolin plus IBMX (Fig 5C).

Fig 5D shows that treatment with forskolin plus IBMX enhanced the Ca²⁺ response to 5 μg/ml anti-IgM by ~30 % in 3KO-SS cells. This effect was also seen at 1 μg/ml anti-IgM, but was not evident in 3KO-AA cells (Fig 5E), indicating that it is a direct consequence of IP₃R1 phosphorylation at S¹⁷⁵⁵ and/or S¹⁵⁸⁸. Thus, IP₃R phosphorylation at defined sites is capable of modulating Ca²⁺ signals in our experimental system.

3.6 Effects of IP₃R phosphorylation on trypsin-mediated Ca²⁺ mobilization

To determine whether activation of Ca²⁺ mobilization via a different signaling pathway might uncover effects of IP₃R phosphorylation, we examined Ca²⁺ signaling in response to trypsin, which activates the PAR2 G protein-coupled receptor in DT40 cells [29] and which elevates intracellular Ca²⁺ in our experimental system (Fig 6A). However, as with anti-IgM, trypsin-induced Ca²⁺ mobilization was not affected by forskolin plus IBMX treatment in DT40 cells, and was inhibited slightly by this treatment in both 3KO-SSS and 3KO-AAA cells (Fig 6B). Thus, again, this inhibitory effect is not due to phosphorylation of S⁹¹⁶, S⁹³⁴, or S¹⁸³². Enhancement of trypsin-induced responses by forskolin plus IBMX treatment in 3KO-SS cells, but not in 3KO-AA cells, again confirms that Ca²⁺ mobilization can be regulated by IP₃R phosphorylation (Fig 6B).

4. Discussion

The major findings presented herein are (i) that IP₃R2 is a very poor substrate for PKA and (ii) that phosphorylation of S⁹¹⁶, S⁹³⁴ and S¹⁸³² has no effect on the ability of IP₃R3 to mobilize Ca²⁺.

The inability of maximally activated PKA to substantially phosphorylate exogenous IP₃R2 expressed in HEK cells (Fig 1) is consistent with findings that endogenous IP₃R2 in AR42J cells is only very weakly phosphorylated by PKA [9], and that PKA catalytic subunit does not affect IP₃-mediated Ca²⁺ release in permeabilized cells that predominantly express IP₃R2 [11]. Taken together, these data indicate that IP₃R2 is not a physiological substrate for PKA. Therefore, the enhanced Ca²⁺ signaling that has been attributed to PKA-mediated phosphorylation of IP₃R2 in parotid acinar cells [19] is most likely the result of phosphorylation of other proteins, possibly other IP₃R types. Indeed, the evidence for IP₃R2 phosphorylation in parotid acinar cells was that IP₃R2 was immunoprecipitated from forskolin-stimulated cells by an anti-phosphoserine/threonine antibody [19]; however, as parotid cells also express IP₃R1 and IP₃R3 [30], and IP₃Rs heterotetramerize [4,5], it is entirely plausible that non-phosphorylated IP₃R2 was immunoprecipitated because of its interaction with phosphorylated IP₃R1 and/or IP₃R3.

We have also found that phosphorylation of IP₃R3 at S⁹¹⁶, S⁹³⁴ and S¹⁸³², the only sites in IP₃R3 known to be modified by PKA [18], does not affect the receptor's ability to mobilize Ca²⁺. This conclusion was validated by the positive control that Ca²⁺ mobilization via IP₃R1^{SS}, but not IP₃R1^{AA}, was enhanced by PKA activation; thus, 3KO cells contain the requisite proteins to allow for Ca²⁺ signaling via exogenous IP₃Rs to be modified by their phosphorylation. Phosphorylation of IP₃R3 at S⁹¹⁶, S⁹³⁴ and S¹⁸³², therefore, may play a role other than regulation of Ca²⁺ channel activity, such as providing docking sites for ancillary proteins. Interestingly, similar findings have recently been reported for Akt kinase-mediated phosphorylation of IP₃R1; this kinase phosphorylates IP₃R1 at S²⁶⁸¹, but this modification does not affect the receptor's ability to mobilize Ca²⁺ [31].

Surprisingly, we also found that forskolin plus IBMX treatment inhibited Ca²⁺ release by ~10% for each of the exogenous IP₃R3s, irrespective of whether S⁹¹⁶, S⁹³⁴ or S¹⁸³² were mutated or not. Conceivably, this inhibition is a result of phosphorylation of a previously uncharacterized site in IP₃R3 that is revealed when the receptor is expressed in 3KO cells, although this possibility is unlikely, as S⁹¹⁶, S⁹³⁴ and S¹⁸³² comprise the full complement of sites phosphorylated by PKA in intact HEK cells and *in vitro* [18]. Alternatively, it could be the effect of an, as yet, unidentified PKA substrate that specifically associates with and inhibits IP₃R3. Either event may go some way to explaining the PKA-mediated inhibition of Ca²⁺ signaling that has been attributed to IP₃R3 phosphorylation in pancreatic acinar cells [20,21].

The development of anti-pS934 and anti-pS1755 allowed us, for the first time, to characterize the phosphorylation of exogenous IP₃Rs expressed in 3KO cells and endogenous IP₃Rs in DT40 cells. Interestingly, our data revealed that forskolin is a very weak stimulus in these cells and that co-incubation with IBMX is required for efficient IP₃R phosphorylation (Fig 3B), perhaps indicating that DT40-derived cell lines contain high levels of cAMP phosphodiesterase activity. Clearly, this should be taken into account when examining PKA-mediated events in DT40-derived cells. Further, and somewhat surprisingly, while endogenous IP₃Rs are phosphorylated by PKA in DT40 cells (Fig 5B), this does not affect Ca²⁺ signaling (Fig 4). As IP₃R1, IP₃R2 and IP₃R3 are all present in DT40 cells [22], this could reflect the combined effects of enhanced IP₃R1 activity and inhibited IP₃R3 activity. Alternatively, as DT40 cell IP₃R complexes are likely to be heterotetrameric [5], and as nothing is currently known about how phosphorylation affects IP₃R heterotetramers, it is possible that the IP₃R complexes in DT40 cells are unaffected by phosphorylation, even though they contain IP₃R1 and/or IP₃R3.

In summary, we demonstrate the differential phosphorylation of IP₃R types by PKA, with IP₃R2 being only very weakly phosphorylated compared to IP₃R1 and IP₃R3. Further, phosphorylation of IP₃R3 at the sites known to be targeted by PKA (S⁹¹⁶, S⁹³⁴ and S¹⁸³²) does not affect its ability to mobilize Ca²⁺, suggesting that modification of Ca²⁺ flux may not be the primary effect of IP₃R3 phosphorylation.

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5. References

1. Taylor CW, da Fonseca PCA, Morris EP. IP₃ receptors: the search for structure. *Trends in Biochemical Sciences* 2004;29:210–219. [PubMed: 15082315]
2. Patterson RL, Boehning D, Snyder SH. Inositol 1,4,5-trisphosphate receptors as signal integrators. *Annual Review of Biochemistry* 2004;73:437–465.
3. Bezprozvanny I. The inositol 1,4,5-trisphosphate receptor's. *Cell Calcium* 2005;38:261–272. [PubMed: 16102823]
4. Wojcikiewicz RJH. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J. Biol. Chem* 1995;270:11678–11683. [PubMed: 7744807]
5. Taylor CW, Genazzani AA, Morris SA. Expression of inositol trisphosphate receptors. *Cell Calcium* 1999;26:237–251. [PubMed: 10668562]
6. Wojcikiewicz RJH, Luo SG. Differences Among Type I, II, and III Inositol-1,4,5-Trisphosphate Receptors in Ligand-Binding Affinity Influence the Sensitivity of Calcium Stores to Inositol-1,4,5-Trisphosphate. *Mol Pharmacol* 1998;53:656–662. [PubMed: 9547355]
7. Tu H, Wang Z, Nosyreva E, et al. Functional Characterization of Mammalian Inositol 1,4,5-Trisphosphate Receptor Isoforms. *Biophys. J* 2005;88:1046–1055. [PubMed: 15533917]
8. Maes K, Missiaen L, Parys JB, et al. Mapping of the ATP-binding Sites on Inositol 1,4,5-Trisphosphate Receptor Type 1 and Type 3 Homotetramers by Controlled Proteolysis and Photoaffinity Labeling. *J. Biol. Chem* 2001;276:3492–3497. [PubMed: 11035010]
9. Wojcikiewicz RJH, Luo SG. Phosphorylation of Inositol 1,4,5-Trisphosphate Receptors by cAMP-dependent Protein Kinase. Type I, II, and III receptors are differentially susceptible to phosphorylation and are phosphorylated in intact cells. *J. Biol. Chem* 1998;273:5670–5677. [PubMed: 9488697]
10. Tu H, Tang T-S, Wang Z, et al. Association of Type 1 Inositol 1,4,5-Trisphosphate Receptor with AKAP9 (Yotiao) and Protein Kinase A. *J. Biol. Chem* 2004;279:19375–19382. [PubMed: 14982933]

11. Dyer JL, Mobasher H, Lea EJA, et al. Differential effect of PKA on the Ca²⁺ release kinetics of the type I and III InsP₃ receptors. *Biochemical and Biophysical Research Communications* 2003;302:121–126. [PubMed: 12593857]
12. Ferris CD, Cameron AM, Bredt DS, et al. Inositol 1,4,5-trisphosphate receptor is phosphorylated by cyclic AMP-dependent protein kinase at serines 1755 and 1589. *Biochemical and Biophysical Research Communications* 1991;175:192–198. [PubMed: 1847804]
13. Haug LS, Jensen V, Hvalby O, et al. Phosphorylation of the Inositol 1,4,5-Trisphosphate Receptor by Cyclic Nucleotide-dependent Kinases in Vitro and in Rat Cerebellar Slices in Situ. *J. Biol. Chem* 1999;274:7467–7473. [PubMed: 10066812]
14. Soulsby MD, Alzayady K, Xu Q, et al. The contribution of serine residues 1588 and 1755 to phosphorylation of the type I inositol 1,4,5-trisphosphate receptor by PKA and PKG. *FEBS Letters* 2004;557:181–184. [PubMed: 14741364]
15. Wagner LE II, Li W-H, Joseph SK, et al. Functional Consequences of Phosphomimetic Mutations at Key cAMP-dependent Protein Kinase Phosphorylation Sites in the Type 1 Inositol 1,4,5-Trisphosphate Receptor. *J. Biol. Chem* 2004;279:46242–46252. [PubMed: 15308649]
16. Wagner LE II, Li W-H, Yule DI. Phosphorylation of Type-1 Inositol 1,4,5-Trisphosphate Receptors by Cyclic Nucleotide-dependent Protein Kinases: A mutational analysis of the functionally important sites in the S2+ and S2- splice variants. *J. Biol. Chem* 2003;278:45811–45817. [PubMed: 12939273]
17. Tang T, Tu H, Wang Z, et al. Modulation of Type I inositol (1,4,5)-trisphosphate Receptor Function by Protein Kinase A and Protein Phosphatase 1 alpha. *J. Neurosci* 2003;23:403–415. [PubMed: 12533600]
18. Soulsby MD, Wojcikiewicz RJH. The type III inositol 1,4,5-trisphosphate receptor is phosphorylated by cAMP-dependent protein kinase at three sites. *Biochemical Journal* 2005;392:493–497. [PubMed: 16107208]
19. Bruce JIE, Shuttleworth TJ, Giovannucci DR, et al. Phosphorylation of Inositol 1,4,5-Trisphosphate Receptors in Parotid Acinar Cells. A mechanism for the synergistic effects of cAMP on Ca²⁺ signaling. *J. Biol. Chem* 2002;277:1340–1348. [PubMed: 11694504]
20. Giovannucci DR, Groblewski GE, Sneyd J, et al. Targeted Phosphorylation of Inositol 1,4,5-Trisphosphate Receptors Selectively Inhibits Localized Ca²⁺ Release and Shapes Oscillatory Ca²⁺ Signals. *J. Biol. Chem* 2000;275:33704–33711. [PubMed: 10887192]
21. Straub SV, Giovannucci DR, Bruce JIE, et al. A Role for Phosphorylation of Inositol 1,4,5-Trisphosphate Receptors in Defining Calcium Signals Induced by Peptide Agonists in Pancreatic Acinar Cells. *J. Biol. Chem* 2002;277:31949–31956. [PubMed: 12065595]
22. Sugawara H, Kurosaki M, Takata M, et al. Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. *The EMBO Journal* 1997;16:3078–3088. [PubMed: 9214625]
23. Sudhof TC, Newton CL, Archer BT III, et al. Structure of a novel InsP₃ receptor. *The EMBO Journal* 1991;10:3199–3206. [PubMed: 1655411]
24. Blondel O, Takeda J, Janssen H, et al. Sequence and functional characterization of a third inositol trisphosphate receptor subtype, IP3R-3, expressed in pancreatic islets, kidney, gastrointestinal tract, and other tissues. *J. Biol. Chem* 1993;268:11356–11363. [PubMed: 8388391]
25. Furuichi T, Yoshikawa S, Miyawaki A, et al. Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400. *Nature* 1989;342:32–38. [PubMed: 2554142]
26. Soulsby MD, Wojcikiewicz RJH. 2-Aminoethoxydiphenyl borate inhibits inositol 1,4,5-trisphosphate receptor function, ubiquitination and downregulation, but acts with variable characteristics in different cell types. *Cell Calcium* 2002;32:175–181. [PubMed: 12379177]
27. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem* 1985;260:3440–3450. [PubMed: 3838314]
28. Kubista H, Hawkins T, Moss SE. Characterisation of calcium signalling in DT40 chicken B-cells. *Biochimica et Biophysica Acta - Molecular Cell Research* 1998;1448:299–310.
29. Morita T, Tanimura A, Nezu A, Kurosaki T, Tojyo Y. Functional analysis of the green fluorescent protein-tagged inositol 1,4,5-trisphosphate receptor type 3 in Ca²⁺ release and entry in DT40 B lymphocytes. *Biochemical Journal* 2004;382:793–801. [PubMed: 15175012]

30. Zhang X, Wen J, Bidasee KR, et al. Ryanodine and inositol trisphosphate receptors are differentially distributed and expressed in rat parotid gland. *Biochem. J* 1999;340:519–527. [PubMed: 10333498]
31. Khan MT, Wagner L II, Yule DI, Bhanumathy C, Joseph SK. Akt kinase phosphorylation of inositol 1,4,5-trisphosphate receptors. *J. Biol. Chem* 2006;281:3731–3737. [PubMed: 16332683]

Figure 1. Phosphorylation of IP₃Rs in intact cells

(A) [³²P]P_i-labeled HEK cells transfected with cDNAs encoding wild-type IP₃R2 (lanes 1-2), or IP₃R3 (lanes 3-4) were exposed to 10 μM forskolin for 5 min as indicated, and IP₃Rs were immunoprecipitated with CT2 or CT3, electrophoresed and assessed for Coomassie blue staining capacity (upper panel) and associated radioactivity (lower panel). Negligible staining and radioactivity was obtained from control (vector-transfected) cells (not shown) [14,18].

(B) Histogram of the data presented in A. Exogenous IP₃R phosphorylation was calculated by subtracting the negligible radioactivity obtained from control cells from that in lanes 1-4 and is expressed as a percentage of the radioactivity associated with IP₃R3 in the presence of forskolin (lane 4). Data shown are representative of three independent experiments.

Figure 2. Validation of anti-pS934

(A) HEK cells transfected with empty vector (lanes 1-2), or cDNA encoding wild-type IP₃R3 (IP₃R3^{SSS}; lanes 3-4), IP₃R3 mutated at S⁹¹⁶, S⁹³⁴, S⁹¹⁶, and S¹⁸³² (IP₃R3^{AAA}; lanes 5-6), mutated at S⁹³⁴ (IP₃R3^{SAS}; lanes 7-8), mutated at S⁹¹⁶ and S¹⁸³² (IP₃R3^{ASA}; lanes 9-10) or wild-type IP₃R1 (IP₃R1^{SS}; lanes 11-12) were incubated with or without 10 μM forskolin for 5 min as indicated, and lysates were prepared and probed with anti-pS934 (*upper panel*), TL3 (*lower panel*; lanes 1-10), or CT1 (*lower panel*; lanes 11-12). (B) COS cells transfected with cDNA encoding IP₃R3^{SSS} were incubated without (*upper panels*) or with (*lower panels*) 10 μM forskolin for 10 min, fixed, and stained with DAPI to visualize nuclei (*panels iii and iv*), TL3 to visualize IP₃R3 (*panels v and vi*), and anti-pS934 to visualize IP₃R3 phosphorylated at S⁹³⁴ (*panels vii and viii*).

Figure 3. Phosphorylation of IP₃R3s expressed in 3KO cells

(A) DT40 cells (lanes 1-2), 3KO cells (lanes 3-4), or IP₃R3-expressing 3KO cells (lanes 5-12) were exposed to 1 μM forskolin plus 200 μM IBMX for 10 min as indicated, lysates were prepared, and IP₃R3s phosphorylated at S⁹³⁴ were immunoprecipitated with anti-pS934. Total IP₃R3 in lysates prior to immunoprecipitation (*upper panel*) or phosphorylated IP₃R3 in immunoprecipitates (*lower panel*) were assessed in immunoblots with TL3. (B) 3KO-SSS cells were exposed to 0-100 μM forskolin in the absence (*upper panel*) or presence (*lower panel*) of 200 μM IBMX for 10 min, and IP₃R3 phosphorylation was assessed as in A. (C) IP₃R3^{SSS}-expressing HEK cells were exposed to 0.01-3.0 μM forskolin for 10 min and IP₃R3 phosphorylation was assessed using anti-pS934 in immunoblots of cell lysates. (D) Traces of typical Ca²⁺ responses to forskolin (added at *t* = 0.5 min) in 3KO cells (dashed line) and 3KO-SSS cells (solid lines). (E) 3KO-SSS cells were incubated with or without 1 μM forskolin plus 200 μM IBMX for 10 min as indicated and levels of IP₃R3 in cell lysates prior to (pre) and following (post) immunoprecipitation of phosphorylated IP₃R3 with anti-pS934 were assessed in immunoblots with TL3. The lack of difference in signal intensity in control lysates pre and post immunoprecipitation (lanes 1 and 2) shows that IP₃R3^{SSS} is not significantly phosphorylated, whereas the difference in signal intensity in stimulated cells (lanes 3 and 4; a reduction of ~50%) shows that at least 50% of IP₃R3^{SSS} is phosphorylated.

Figure 4. Effect of PKA activation on IP₃R3-mediated Ca²⁺ mobilization in response to BCR stimulation

(A) Traces of typical Ca²⁺ responses to 5, 1, 0.05 and 0.005 $\mu\text{g/ml}$ anti-IgM (added at $t = 0.5$ min) in DT40 (i) or 3KO-SSS (ii) cells. Dose-response curves (iii) for DT40 and 3KO-SSS cells are expressed as a percentage of the $[Ca^{2+}]_i$ increase in response to 5 $\mu\text{g/ml}$ anti-IgM. Error bars may be occluded by symbols. (B) Traces of typical Ca²⁺ responses in DT40 (i-iii) or 3KO-SSS (iv-vi) cells exposed to 5 (i and iv), 1 (ii and v), or 0.05 (iii and vi) $\mu\text{g/ml}$ anti-IgM (added at $t = 10.5$ min) in the absence (solid line) or presence (broken line) of 1 μM forskolin plus 200 μM IBMX (added at $t = 0.5$ min). Histogram (vii) of $[Ca^{2+}]_i$ increases in DT40 or 3KO cell lines in response to 5, 1, or 0.05 $\mu\text{g/ml}$ anti-IgM in the absence (clear bars) or presence

(solid bars) of forskolin plus IBMX. (C) Typical traces of Ca^{2+} responses in 3KO-SSS cells (i) exposed to 5mM EGTA (added at $t = 10$ min) and 1 $\mu\text{g/ml}$ anti-IgM (added at $t = 10.5$ min) in the absence (solid line) or presence (broken line) of 1 μM forskolin plus 200 μM IBMX (added at $t = 0.5$ min). Histogram (ii) of $[\text{Ca}^{2+}]_i$ increases in 3KO-SSS and 3KO-AAA cells in the absence (clear bars) or presence (solid bars) of forskolin plus IBMX.

Figure 5. Effect of PKA activation on IP₃R1-mediated Ca²⁺ mobilization in response to BCR stimulation

(A) HEK cells transfected with empty vector (lanes 1-2), or cDNA encoding wild-type IP₃R1 (IP₃R3^{SS}; lanes 3-4), IP₃R1 mutated at S¹⁷⁵⁵ (IP₃R3^{SA}; lanes 5-6), mutated at S¹⁵⁸⁸ (IP₃R3^{AS}; lanes 7-8), mutated at S¹⁵⁸⁸ and S¹⁷⁵⁵ (IP₃R1^{AA}; lanes 9-10), or wild-type IP₃R3 (IP₃R3^{SSS}; lanes 11-12) were exposed to 10 µM forskolin for 5 min as indicated, lysates were prepared, and IP₃R1 phosphorylated at S¹⁷⁵⁵ were immunoprecipitated with anti-pS1755 and visualized in immunoblots with CT1. (B) DT40 (lanes 1-2), or IP₃R1-expressing 3KO (lanes 3-6) cells were exposed to 1 µM forskolin plus 200 µM IBMX for 10 min as indicated, cell lysates were prepared, and IP₃R1 phosphorylated at S¹⁷⁵⁵ was immunoprecipitated with anti-pS1755 and visualized in immunoblots with CT1 (*lower panel*). IP₃R1 levels in lysates prior to immunoprecipitation were assessed with CT1 (*upper panel*). (C) 3KO-SS cells were incubated with 1 µM forskolin plus 200 µM IBMX for 10 min and levels of IP₃R1 in cell lysates prior to (pre) and following (post) immunoprecipitation of phosphorylated IP₃R1 with anti-pS1755 were assessed in immunoblots with CT1. The ~50% reduction in signal intensity post immunoprecipitation indicates that at least 50% of IP₃R1 is phosphorylated. (D) Traces of typical Ca²⁺ responses to 5 µg/ml anti-IgM (added at *t* = 10.5) in the absence (solid line) or presence (broken line) of 1 µM forskolin plus 200 µM IBMX (added at *t* = 0.5 min) in 3KO-SS cells. (E) Histogram of [Ca²⁺]_i increases in 3KO-SS and 3KO-AA cells in response to 5 µg/ml or 1 µg/ml anti-IgM in the absence (clear bars) or presence (solid bars) of forskolin plus IBMX.

Figure 6. Effect of PKA activation on trypsin-induced Ca^{2+} mobilization

(A) Trace showing a typical Ca^{2+} response to a maximal dose of trypsin (1000 U/ml) in DT40 cells. (B) Histogram of $[Ca^{2+}]_i$ increases in DT40 and 3KO cell lines in response to 1000 U/ml or 50 U/ml trypsin in the absence (clear bars) or presence (solid bars) of 1 μ M forskolin plus 200 μ M IBMX.