The contribution of serine residues 1588 and 1755 to phosphorylation of the type I inositol 1,4,5-trisphosphate receptor by PKA and PKG

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Abstract Type I inositol 1,4,5-trisphosphate receptors can be phosphorylated by cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG). To define the site-specificity of these events we analyzed the phosphorylation of mutant receptors expressed in intact cells. These studies showed that S^{1588} and S^{1755}, the serine residues within kinase consensus sequences, are equally sensitive to PKA, that phosphorylation events at these sites are independent of each other, and that PKG predominantly phosphorylates S^{1588}. These findings provide the basis for understanding the functional consequences of type I inositol 1,4,5-trisphosphate receptor phosphorylation.

Key words: Inositol 1,4,5-trisphosphate receptor; Phosphorylation; cAMP-dependent protein kinase; cGMP-dependent protein kinase

1. Introduction

Type I, II and III inositol 1,4,5-trisphosphate ($InsP_3$) receptors are a family of proteins which form Ca^{2+} channels in endoplasmic reticulum membranes and which govern Ca^{2+} release from this organelle by opening in response to $InsP_3$ and Ca^{2+} binding [1–3]. The type I $InsP_3$ receptor is the most widely expressed and most extensively studied of the family [2–5], and in addition to its activation by $InsP_3$ and Ca^{2+}, several modes of regulation have been established [2], including phosphorylation by cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) [6–13]. These kinases have been reported to phosphorylate the receptor at two serine residues, designated S^{1588} and S^{1755} in the mouse type I receptor [4,5,7,11,13]. The position of these sites, between the ligand-binding domain and the channel domain of the receptor [2,3,14,15], suggested that their phosphorylation might be involved in channel regulation. This was later confirmed by findings that PKA-dependent phosphorylation enhanced $InsP_3$-induced Ca^{2+} mobilization [10,12,16–19].

Despite this knowledge, information on how S^{1588} and S^{1755} contribute to overall receptor phosphorylation is incomplete and often contradictory. For example, early in vitro studies on purified cerebellar type I $InsP_3$ receptors demonstrating that S^{1588} and S^{1755} were phosphorylated by PKA also indicated that S^{1755} was considerably more susceptible to phosphorylation than S^{1588} [7], but as yet, this has not been verified in intact cells. Further, PKG was shown to phosphorylate purified cerebellar receptor only at S^{1755} [11], but this was later contradicted by findings from intact cells indicating that S^{1588} was the predominant site [13]. In order to clarify this situation we have, for the first time, used a mutagenic approach to characterize phosphorylation of the type I $InsP_3$ receptor, allowing us to directly measure and compare phosphorylation at S^{1588} and S^{1755} in intact cells.

2. Materials and methods

2.1. Materials

Okadaic acid and sodium fluoride were from Alexis Corp, $^{32}P_i$ (H_3PO_4, carrier free) and [^{3}H]$InsP_3$ (23 Ci/mmol) were from Perkin Elmer (NEN), [γ-^{32}P]ATP (4500 Ci/mmol) was from ICN Biomedicals, and PKA catalytic subunit was from Promega. All other chemicals were from Sigma.

2.2. Mutagenesis and transfection

Mutation of S^{1588} and S^{1755} to alanine in the SI^+/SII^+ (neuronal) form of the mouse type I $InsP_3$ receptor [4] was achieved as described [20]. Briefly, cDNA encoding mouse type I $InsP_3$ receptor [21] was mutagenized using the QuikChange kit (Stratagene) and pairs of mutagenic primers [19]. The *Sac*II/*Sbf*I fragments containing the mutated sites were then transferred into the correspondingly cut pcWI [21] and correct introduction of the desired mutations was confirmed by cDNA sequencing. Human embryonic kidney (HEK) cells, grown in monolayer as described [22], were seeded (750 000 cells/9.6 cm^2 well) and were transfected 24 h later with ~1 μg $InsP_3$ receptor cDNA (or empty vector) and 6 μl Superfect (Qiagen) [20] and were used in experiments 18–20 h later.

2.3. Phosphorylation of purified $InsP_3$ receptors

Transfected cells were solubilized with 1 ml ice-cold lysis buffer [12] supplemented with the phosphatase inhibitors Na_3VO_4 (1 mM), NaF (100 mM) and okadaic acid (100 nM), and type I $InsP_3$ receptors were immunoprecipitated at 4°C with type I $InsP_3$ receptor-specific CT1 [22] for 2 h and protein A-Sepharose CL-4B beads for 1 h. In vitro phosphorylation of the purified receptors was then performed in 400 μl phosphorylation buffer [12] supplemented with ~20 μCi [γ-^{32}P] ATP, 5 μM non-radioactive ATP, and 40 units of PKA catalytic subunit for 15 min at 30°C. The immune complexes were then washed three times with phosphorylation buffer supplemented with 1 mM ATP and finally resuspended in 2× gel loading buffer [22].

2.4. Phosphorylation of $InsP_3$ receptors in intact cells

Wells of transfected HEK cells were incubated with ~100 μCi $^{32}P_i$ for 2 h in 750 μl serum-free, phosphate-free Dulbecco's modified Eagle's medium and then stimulated to induce $InsP_3$ receptor phosphorylation. Cells were then solubilized with inhibitor-supplemented

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Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PGE_1, prostaglandin E_1; PKC, protein kinase C; PMA, phorbol-12-myristate 13-acetate; HEK, human embryonic kidney

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lysis buffer, type I $InsP_3$ receptors were immunoprecipitated, immune complexes were washed three times with the same buffer and resuspended in 2× gel loading buffer. Alternatively, in a 'back-phosphorylation' procedure [12], non-labeled transfected cells were incubated with or without forskolin in 750 μl serum-free Dulbecco's modified Eagle's medium, and were then solubilized, and type I $InsP_3$ receptors were immunoprecipitated and phosphorylated with [γ-^{32}P]ATP in vitro.

2.5. Electrophoresis, staining, and autoradiography

Type I $InsP_3$ receptors (~260 kDa) were electrophoresed in 5% gels as described [22]. Gels were then stained with Coomassie blue and radioactivity associated with electrophoresed $InsP_3$ receptors was assessed initially by autoradiography of dried gels and then quantified by excision and scintillation counting of the ~240–270 kDa region. Phosphorylation stoichiometry was determined as described [12]. Briefly, the number of moles of phosphate incorporated into $InsP_3$ receptor was calculated from receptor radioactivity and [γ-^{32}P]ATP specific activity, and the number of moles of $InsP_3$ receptor was calculated by measuring the number of [^{3}H]$InsP_3$ binding sites in receptor preparations destined for phosphorylation.

2.6. [^{3}H]$InsP_3$ binding

Transfected HEK cells were harvested and homogenized [22], centrifuged (16 000×*g* for 10 min at 4°C), resuspended in ice-cold 25 mM Tris, 1 mM EDTA, pH 8.0, and were incubated at 4°C with 1.3 nM [^{3}H]$InsP_3$ for 20 min. Bound and free ligand were separated by filtration through Whatman GF/B filters and specific binding was defined using 10 μM non-radiolabeled $InsP_3$.

3. Results and discussion

3.1. S^{1588} and S^{1755} contribute equally to $InsP_3$ receptor phosphorylation following maximal PKA activation in intact cells

We first examined phosphorylation in response to 10 μM forskolin, a condition that causes maximal activation of adenylyl cyclase and PKA in HEK cells [23]. Treatment of HEK cells expressing exogenous wild-type type I $InsP_3$ receptor ($InsP_3R^{SS}$) with forskolin for 5 min (sufficient time for maximal phosphorylation; data not shown) caused an approximately four-fold increase in receptor phosphorylation over basal levels (Fig. 1A, lanes 3 and 4). The signals obtained (lanes 3 and 4) were dramatically greater than those seen in vector-transfected cells (lanes 1 and 2), consistent with the paucity of type I receptors in HEK cells [22], and indicating that exogenous receptors account for essentially all of the signal obtained from $InsP_3$ receptor-transfected cells. Mutation of both S^{1588} and S^{1755} to alanine ($InsP_3R^{AA}$) greatly reduced basal phosphorylation and abolished forskolin-induced phosphorylation (lanes 5 and 6), while mutation of S^{1588} ($InsP_3R^{AS}$) or S^{1755} ($InsP_3R^{SA}$) reduced both basal and forskolin-induced phosphorylation by ~50% (lanes 7–10). Erroneous expression of the mutant receptors did not account for this reduction, as both $InsP_3R^{SS}$ and $InsP_3R^{AA}$ bound $InsP_3$ identically (Fig. 1B) and were similarly localized, as indicated by immunofluorescence microscopy (data not shown). Thus, S^{1588} and S^{1755} contribute equally to type I receptor phosphorylation under both resting conditions and during maximal stimulation and are the only sites phosphorylated by PKA; a third potential site (S^{2556}) [4,5] within the consensus sequence RKPS is clearly not a valid site for PKA.

3.2. Phosphorylation of purified receptors

We next sought to determine the stoichiometry of the phosphorylation seen in Fig. 1A, since only if a large proportion of the exogenous receptors were efficiently phosphorylated,

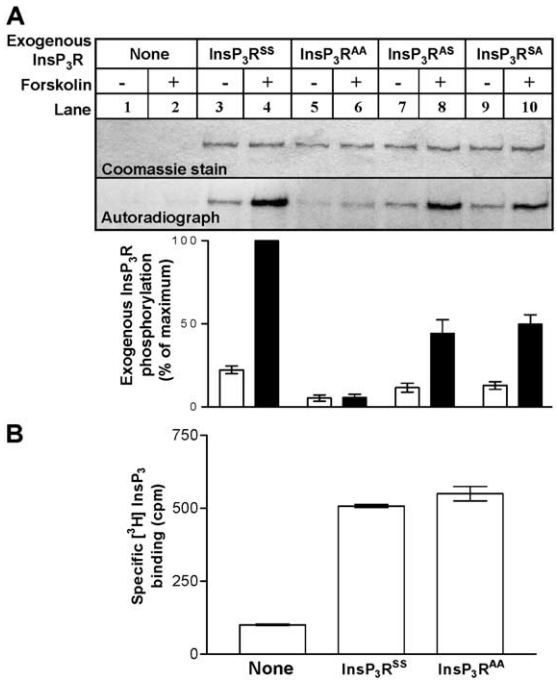

Fig. 1. Phosphorylation of exogenous type I $InsP_3$ receptors in intact HEK cells. A: $^{32}P_i$-labeled cells transfected with empty vector (lanes 1 and 2) or $InsP_3$ receptor cDNAs (lanes 3–10) were exposed to 10 μM forskolin for 5 min as indicated and type I $InsP_3$ receptors were immunoprecipitated, electrophoresed and assessed for staining capacity (upper panel) and associated radioactivity (lower panel). The ~200–300 kDa region of a gel is shown. Exogenous $InsP_3$ receptor phosphorylation (histogram, mean ± S.E.M., $n = 3$) was calculated by subtracting radioactivity associated with endogenous receptor (lane 1 or 2) from that in lanes 3–10 and is expressed as a percentage of the radioactivity associated with $InsP_3R^{SS}$ in the presence of forskolin (lane 4). B: Specific [^{3}H]$InsP_3$ binding to membranes prepared from cells expressing exogenous receptor as in A (mean ± S.E.M., $n = 3$).

would our conclusions be valid. Purified $InsP_3R^{SS}$ from unstimulated cells was phosphorylated by PKA in vitro with a stoichiometry of 0.74 ± 0.13 mol ^{32}P/mol $InsP_3$ receptor (Fig. 2A, lane 3; $n = 4$), and this was reduced ~90% by pretreatment of intact cells with forskolin (lane 4). Thus, forskolin appears to cause the incorporation of ~0.7 mol phosphate/mol $InsP_3$ receptor in intact cells. However, since ~25% of exogenous receptors are already phosphorylated under basal conditions (Fig. 1A, lane 3), this value is an underestimate of the true stoichiometry. Furthermore, and surprisingly, analysis of the phosphorylation of purified receptors showed that S^{1755} (Fig. 2B, lane 4) was phosphorylated much less well than S^{1588} (lane 5). Since the two sites are phosphorylated equally well in intact cells (Fig. 1A), it appears that receptor immunopurification suppresses S^{1755} phosphorylation, perhaps due to distortion of the linker region between the channel-forming and cytoplasmic domains of the receptor in which S^{1755} is thought to reside [14], or due to the loss of required proteins. These factors are likely to explain why our estimate and many previous estimates [6,10–13] of type I receptor stoichiometry are less than the 2 mol ^{32}P/mol $InsP_3$ receptor one would expect if both S^{1588} and S^{1755} were phosphorylated efficiently. In view of these factors, we conclude that $InsP_3R^{SS}$ is phosphorylated with a stoichiometry of at least 1 mol phosphate/mol $InsP_3$ receptor in intact cells. Thus, exogenous $InsP_3$ re-

ceptor phosphorylation in intact cells (Fig. 1A) reflects modification of the majority of the receptors, rather than an insignificant fraction.

3.3. Phosphorylation of S^{1588} and S^{1755} at submaximal PKA activation in intact cells

We next sought to determine whether S^{1755} is preferentially phosphorylated at submaximal PKA activation, as was concluded from previous work in vitro [7]. For this study, we examined phosphorylation in response to prostaglandin E_1 (PGE_1), which activates adenylyl cylclase through an endogenous G protein-coupled receptor [23], and produces more moderate increases in cAMP levels than forskolin [24]. Fig. 3 shows that $InsP_3R^{AS}$ and $InsP_3R^{SA}$ were phosphorylated in response to PGE_1 with identical kinetics (A) and dose dependence (B), and that at all times and PGE_1 concentrations, the mutants were phosphorylated to ~50% of the level seen with $InsP_3R^{SS}$. Thus, in contrast to previous results [7], these data indicate that S^{1588} and S^{1755} are equally sensitive to PKA. Furthermore, that $InsP_3R^{AS}$ and $InsP_3R^{SA}$ were phosphorylated equally well and addition of their phosphorylation signals equaled that seen with $InsP_3R^{SS}$ (Figs. 1A and 3) indicates that the two sites are non-interacting; phosphorylation at position 1755 is unaffected by events at position 1588, and vice versa.

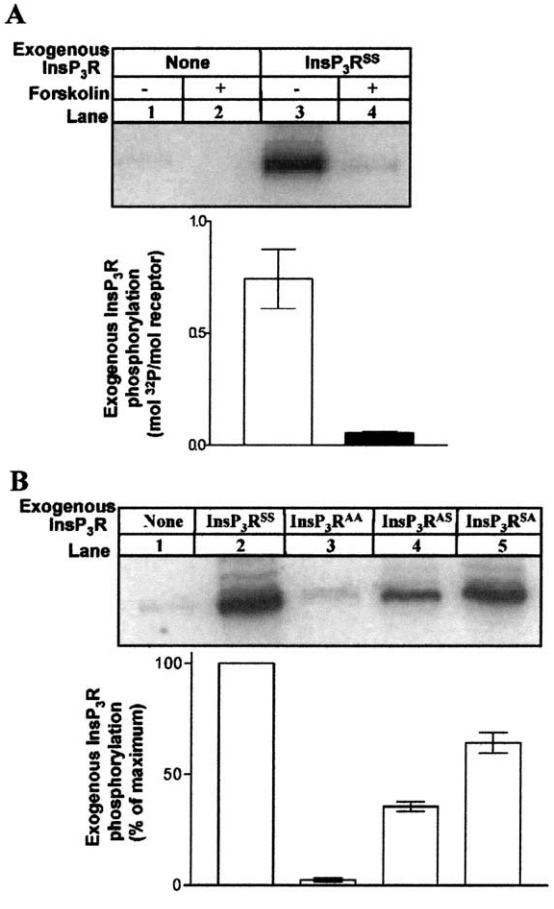

Fig. 2. Phosphorylation of purified type I $InsP_3$ receptors. A: Cells transfected with empty vector (lanes 1 and 2) or $InsP_3R^{SS}$ cDNA (lanes 3 and 4) were exposed to 10 μM forskolin for 5 min as indicated, type I $InsP_3$ receptors were immunoprecipitated and phosphorylated in vitro, and the stoichiometry of phosphorylation was calculated. B: Type I $InsP_3$ receptors were immunoprecipitated from non-stimulated transfected cells and were phosphorylated in vitro. Exogenous $InsP_3$ receptor phosphorylation was calculated by subtracting radioactivity incorporated into endogenous receptor (lane 1) and expressed as a percentage of that seen with $InsP_3R^{SS}$. Data shown are mean ± S.E.M., $n = 4$.

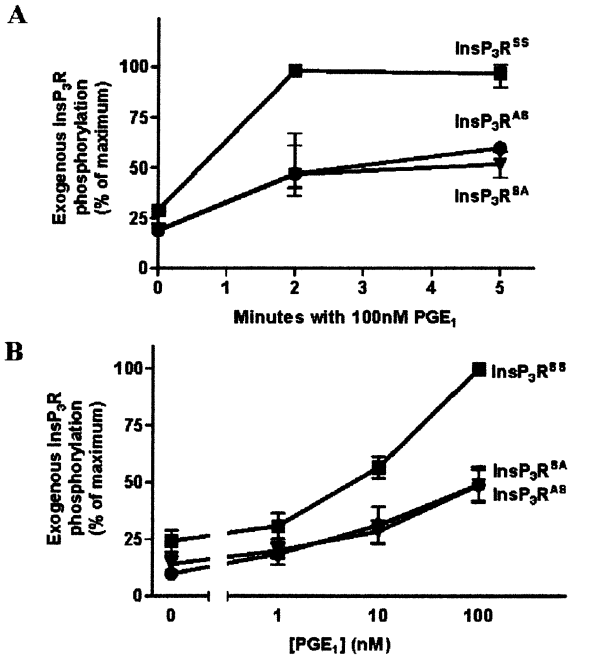

Fig. 3. PGE_1-induced phosphorylation of S^{1588} and S^{1755}. $^{32}P_i$-labeled cells transfected with cDNAs encoding $InsP_3R^{SS}$ (■), $InsP_3R^{AS}$ (●) or $InsP_3R^{SA}$ (▼) were exposed to PGE_1 as indicated. Exogenous $InsP_3$ receptor phosphorylation was calculated as in Fig. 1 (mean ± S.E.M., $n = 3$). A: Time course of phosphorylation. B: Dose dependence of phosphorylation after 5 min incubations.

3.4. Effects of protein kinase C (PKC) and PKG activation on $InsP_3$ receptor phosphorylation in intact cells

Finally, we sought to define the effects of PKC and PKG. Activation of PKC, and the subsequent phosphorylation of cellular substrates (Fig. 4A, lanes 3 and 4), with 1 μM phorbol-12-myristate 13-acetate (PMA) did not cause phosphorylation of $InsP_3R^{SS}$ (Fig. 4A, lanes 1 and 2), indicating that, in intact cells, the type I receptor is not a substrate for PKC. Previous results showing PKC-dependent phosphorylation of purified type I receptors in vitro [8], therefore, appear to reflect a non-physiological process. Activation of PKG with 8-Br-cGMP (Fig. 4B) resulted in a modest increase in $InsP_3R^{SS}$ phosphorylation, ~30% of that seen in response to PKA activation with 8-Br-cAMP. PKG-mediated phosphorylation was almost exclusively on S^{1588}, as $InsP_3R^{SA}$ was phosphorylated equivalently to $InsP_3R^{SS}$, and much more than $InsP_3R^{AS}$. The effects of 8-Br-cGMP were not due to cross-activation of PKA, as PKA activation led to phosphorylation of both $InsP_3R^{AS}$ and $InsP_3R^{SA}$ (Fig. 4B). Thus, in intact cells, PKG phosphorylates the type I $InsP_3$ receptor predominately at S^{1588}.

4. Summary and conclusions

Analysis of exogenous $InsP_3R^{SS}$ and its mutants in intact cells has revealed that S^{1588} and S^{1755} are equally sensitive to PKA, that phosphorylation at S^{1588} and phosphorylation at S^{1755} are independent events, that PKG predominantly phos-

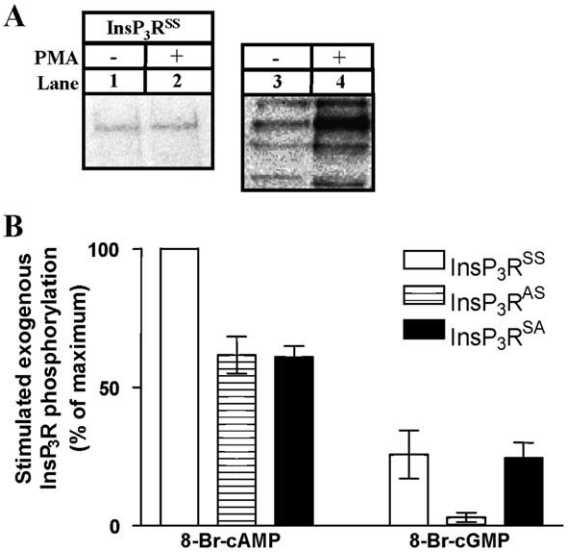

Fig. 4. Effects of PKC and PKG activation. $^{32}P_i$-labeled cells transfected with $InsP_3$ receptor cDNAs were exposed to 1 μM PMA, 1 mM 8-Br-cAMP or 8-Br-cGMP for 30 min as indicated and type I $InsP_3$ receptors were immunoprecipitated, electrophoresed and assessed for associated radioactivity. A: Lanes 1 and 2 show an autoradiograph of immunoprecipitated $InsP_3R^{SS}$. Lanes 3 and 4 show lysates from unstimulated or PMA-treated cells electrophoresed in 12% gels and probed in immunoblots with an antibody against phospho-serine/threonine (#9621; Cell Signaling Technology) to demonstrate that PKC was activated by PMA. The ~65–150 kDa region of a gel is shown. Data shown are representative of three independent experiments. B: Stimulated exogenous $InsP_3$ receptor phosphorylation was calculated by subtracting basal (unstimulated) phosphorylation from stimulated levels for each receptor and is expressed as a percentage of the radioactivity associated with $InsP_3R^{SS}$ in response to 8-Br-cAMP. Data shown are mean ± S.E.M., $n = 3$.

phorylates S^{1588}, and that PKC does not phosphorylate the type I receptor. Overall, these findings disagree with the majority of in vitro studies on purified receptors using PKA and PKG, in which preferential phosphorylation at S^{1755} has most often been observed [7,11,13], and with findings that PKC phosphorylates the type I receptor [8]. These discrepancies may reflect disruption of $InsP_3$ receptors during purification and/or the loss of associated proteins, and indicates that extrapolation of results from in vitro studies to intact cells may be invalid. In contrast, our data broadly agree with the only study in which phosphorylation of S^{1588} and S^{1755} has been mapped in intact cells; Haug et al. [13] found PKA-dependent phosphorylation at both S^{1588} and S^{1755} (with some preference for S^{1755}) and PKG-dependent phosphorylation predominantly at S^{1588}. Thus, exogenous receptors in HEK cells are phosphorylated similarly to endogenous receptors, indicating for the first time that analyses of the functional consequences of phosphorylation of exogenously expressed receptors will yield physiologically relevant data. In this regard, a recent study in chicken DT40 cells expressing exogenous wild-type SII^+ type I receptor showed that activation of both PKA and PKG enhanced $InsP_3$ receptor-mediated Ca^{2+} mobilization and, based on studies with mutant receptors, that this resulted from phosphorylation at S^{1755} [19]; however, receptor phosphorylation was not assessed directly [19]. Thus, our data broadly agree with these findings in that we demonstrate phosphorylation of exogenously expressed type I $InsP_3$ receptors by both PKA and PKG. Paradoxically, however, while the studies in DT40 cells suggest that PKG phosphorylates S^{1755} [19], our data demonstrate that PKG phosphorylates the type I receptor only at S^{1588} (Fig. 4B). Reconciliation of this paradox may be attained when phosphorylation is measured in DT40 cells and, indeed, it is plausible that the site-specificity of type I $InsP_3$ receptor phosphorylation differs between cells types. This could reflect differential expression of proteins that associate with the type I receptor, e.g. phosphatases [16,17].

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