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*

(Received for publication, September 8, 1997, and in revised form, December 5, 1997)

Richard J. H. Wojcikiewicz‡ and Su Ge Luo

From the Department of Pharmacology, College of Medicine, State University of New York Health Science Center at Syracuse, Syracuse, New York 13210-2339

The ability of cAMP-dependent protein kinase (PKA) to phosphorylate type I, II, and III inositol 1,4,5-trisphosphate (InsP₃) receptors was examined. The receptors either were immunopurified from cell lines and then phosphorylated with purified PKA or were phosphorylated in intact cells after activating intracellular cAMP formation. The former studies showed that the type I receptor was a good substrate for PKA (0.65 mol Pi incorporated/mol receptor), whereas type II and III receptors were phosphorylated relatively weakly. The latter studies showed that despite these differences, each of the receptors was phosphorylated in intact cells in response to forskolin or activation of neurohormone receptors. Detailed examination of SH-SY5Y neuroblastoma cells, which express >99% type I receptor, revealed that minor increases in cAMP concentration were sufficient to cause maximal phosphorylation. Thus, VIP and pituitary adenyl cyclase activating peptide (acting through Gₛ-coupled pituitary adenyl cyclase-activating peptide-I receptors) were potent stimuli of type I receptor phosphorylation, and remarkably, even slight increases in cAMP concentration induced by carbachol (acting through Gₛ-coupled muscarinic receptors) or other Ca²⁺ mobilizing agents were sufficient to cause phosphorylation. Finally, PKA enhanced InsP₃-induced Ca²⁺ mobilization in a range of permeabilized cell types, irrespective of whether the type I, II, or III receptor was predominant. In summary, these data show that all InsP₃ receptors are phosphorylated by PKA, albeit with marked differences in stoichiometry. The ability of both Gₛ- and Gₛ-coupled cell surface receptors to effect InsP₃ receptor phosphorylation by PKA suggests that this process is widespread in mammalian cells and provides multiple routes by which the cAMP signaling pathway can influence Ca²⁺ mobilization.

Inositol 1,4,5-trisphosphate (InsP₃) receptors form tetrameric channels in endoplasmic reticulum membranes that conduct Ca²⁺ in an InsP₃-sensitive manner (1–3). Thus, they link cell surface receptor-mediated increases in InsP₃ formation to increases in cytoplasmic free Ca²⁺ concentration ([Ca²⁺]), To date, the coding regions of three mammalian InsP₃ receptor genes have been sequenced (4–11). Their products, termed type I, II, and III receptors, are ~2700 amino acids in length and are 60–70% identical at the amino acid level (2–11). Each receptor is thought to have the same overall structure, being divided into three domains: an N-terminal ligand binding domain, a C-terminal channel forming domain, and an intervening sequence, termed the coupling domain, that contains sites either known or hypothesized to be involved in receptor regulation (2–4, 6, 12). Type I, II, and III receptors are expressed in different amounts in different cell types, and some cells co-express all three receptors (13, 14). InsP₃ receptors form heterotetramers in intact cells, and such associations persist after receptor solubilization (14–17).

Characterization of differences between type I, II, and III receptors is at a preliminary stage, and it is not yet clear what properties are conferred upon a cell by the selective expression of a particular receptor. Recent studies have indicated, however, that type I, II, and III receptors bind InsP₃ with different affinities (8, 18, 19), raising the possibility that this could influence the potency of InsP₃ as a Ca²⁺ mobilizing agent. Also, from sequence analysis it is considered likely that the receptors will differ in other ways; for example, in their ability to be phosphorylated by cAMP-dependent protein kinase (PKA) (2, 3, 8–11). Two serines within the PKA consensus sequence (R/K)(R/K)(S/T) (20) are present in the rat type I receptor coupling domain (serines 1589 and 1755 in the sequences RRDS and K)(R/K)(S/T). To date, the coding regions of three mammalian InsP₃ receptor genes have been sequenced (4–11). Their products, termed type I, II, and III receptors, are ~2700 amino acids in length and are 60–70% identical at the amino acid level (2–11). Each receptor is thought to have the same overall structure, being divided into three domains: an N-terminal ligand binding domain, a C-terminal channel forming domain, and an intervening sequence, termed the coupling domain, that contains sites either known or hypothesized to be involved in receptor regulation (2–4, 6, 12). Type I, II, and III receptors are expressed in different amounts in different cell types, and some cells co-express all three receptors (13, 14). InsP₃ receptors form heterotetramers in intact cells, and such associations persist after receptor solubilization (14–17).

Characterization of differences between type I, II, and III receptors is at a preliminary stage, and it is not yet clear what properties are conferred upon a cell by the selective expression of a particular receptor. Recent studies have indicated, however, that type I, II, and III receptors bind InsP₃ with different affinities (8, 18, 19), raising the possibility that this could influence the potency of InsP₃ as a Ca²⁺ mobilizing agent. Also, from sequence analysis it is considered likely that the receptors will differ in other ways; for example, in their ability to be phosphorylated by cAMP-dependent protein kinase (PKA) (2, 3, 8–11). Two serines within the PKA consensus sequence (R/K)(R/K)(S/T) (20) are present in the rat type I receptor coupling domain (serines 1589 and 1755 in the sequences RRDS and KRES, respectively), and in vitro studies on purified rat type I receptor (21) have shown that both residues can be phosphorylated. These sites are also conserved in mouse and human type I receptors (4, 7), testifying to their importance. In contrast, neither consensus sequence is conserved in type II and III receptors (8–11), and although other serines in PKA consensus sequences are present elsewhere (8–11), it is not yet known whether type II and III receptors are substrates for PKA. In the present study we have examined the ability of PKA to phosphorylate type I, II, and III receptors by analyzing PKA-induced phosphorylation of purified receptors and the ability of agents that raise cAMP to cause receptor phosphorylation in intact cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Antisera—SH-SY5Y human neuroblastoma cells, AR4–2J rat pancreatoma cells, and RINm5F rat insulinoma cells were obtained and cultured as monolayers in dishes (15 cm in diameter) as described (14). Rabbit polyclonal antisera termed CT1, CT2, and CT3 were raised against the C termini of rat type I, II, and III receptors,
were aliquotted into 100-m
Triton X-100, 0.3 mM MgCl₂, pH 7.2), and were finally resuspended in
by immunoprecipitation, were phosphorylated with
activity of the
amounts of phosphorylated type I, II, and III receptor were electro-
were centrifuged (500 × g for 2 min), were washed twice with 1.5 ml of phosphorylation buffer plus ATP and were finally resuspended in 2× gel loading buffer (14).

Electrophoresis, Immunoblotting, and Autoradiography—To assess
and concentration of receptors being established by comparison to stand-
ards of myosin (molecular mass, 205 kDa) and β-galactosidase (molecu-
lar mass, 116 kDa). Radioactivity associated with electrophoresed
receptors was assessed initially by autoradiography of dried gels
and then quantitated by excision and scintillation counting of the
−240–280-kDa region.

Phosphorylation Stoichiometry—Based on silver staining, equal
amounts of phosphorylated type I, II, and III InsP₃ receptors and the composition of immunoprecipitates, samples of
were electrophoresed in 4% gels and were either silver-
ored or immunoblotted as described (14, 16), the molecular mass and
centration of receptors being established by comparison to stand-
ards of myosin (molecular mass, 205 kDa) and β-galactosidase (molecu-
lar mass, 116 kDa). Radioactivity associated with electrophoresed
receptors was assessed initially by autoradiography of dried gels
and then quantitated by excision and scintillation counting of the
−240–280-kDa region.

Phosphorylation of Immunoprecipitated Type I, II, and III
InsP₃ Receptors—Previous studies have shown that enrichment
of type I, II, and III InsP₃ receptors in SH-SY5Y, AR4–2J, and
m5F cells, respectively, makes these cells convenient
starting points for InsP₃ receptor purification (14, 16). Because
SH-SY5Y cells contain ≥99% type I receptor, a preparation
comprised solely of type I receptor can be immunoprecipitated
from these cells with antisera CT1 (14, 16). Type II and III
receptor preparations were immunoprecipitated from AR4–2J
and m5F cells with antisera CT2 and CT3 are, in contrast, not
homogeneous because they are “contaminated” with traces of co-immunoprecipitating type I receptor, which represents 12
and 4% of total receptor in these cell lines (14, 16).

Fig. 1 (lanes 1–9) reveals the extent to which the type I, II, and
III InsP₃ receptor preparations are phosphorylated by PKA in vitro. Because equal amounts of type I, II, and III receptors
were loaded, it is clear that the type I receptor (lanes 1, 4, and
7) is phosphorylated more readily than type II (lanes 2, 5, and
8) or type III (lanes 3, 6, and 9) receptors. The phosphorylation
seen is totally PKA-dependent, because none is detected if the
kinase is omitted (lanes 10–12). Fig. 1A also shows that two proteins are phosphorylated by the type II and III preparations,
one of which (the upper band) migrates with the type I receptor.
Immunoblotting was performed to identify the various proteins
(Fig. 1, B–D). Fig. 1B (lanes 2 and 3) confirms the presence of the type I receptor in the type II and III receptor preparations
and that the upper phosphorylated band in the type II and III receptor preparations is indeed type I receptor.
phosphorylated bands in the type II and III receptor preparations (Fig. 1A). Regarding the efficiency of phosphorylation, it is remarkable that although type II receptor comprises ~90% of the type II receptor preparation (14, 16), it accounts for only ~20% of the total 32P incorporated (Fig. 1A, lanes 2, 5, and 8), showing that the type II receptor is phosphorylated very poorly. For the type III receptor preparation, however, ~90% of 32P incorporated is type III receptor-associated (Fig. 1A, lanes 3, 6, and 9), confirming that the type III receptor is phosphorylated relatively well. For each receptor, phosphorylation was maximal at 15 min, at which point 0.65 ± 0.06, 0.04 ± 0.01, and 0.14 ± 0.03 mol of P_i were incorporated/mol type I, II, and III receptor, respectively (Fig. 2).

InsP_3 Receptor Phosphorylation in Intact SH-SY5Y Cells—Because the type I receptor is a good substrate for PKA in vitro (Figs. 1 and 2), we examined whether it is phosphorylated in intact SH-SY5Y cells in response to cAMP elevation using either a back-phosphorylation procedure (23, 25) or 32P labeling of intact cells. Fig. 3A shows that vasoactive intestinal peptide (VIP), which stimulates cAMP levels in SH-SY5Y cells (27), causes type I receptor phosphorylation in intact cells because back-phosphorylation was inhibited (lanes 1–5). Pituitary adenylyl cyclase activating peptide (PACAP), which belongs to the same neurohormone family as VIP (28), had a similar effect (lanes 6–8). Because IC_{50} values for VIP and PACAP were 7 and 0.15 nM, respectively (Fig. 3B), and PACAP-I receptors bind PACAP with ~100-fold higher affinity than VIP (28, 29) and are present in SH-SY5Y cells (29), it is likely that the effects of both PACAP and VIP are mediated by these receptors. Fig. 3C shows that forskolin, which also elevates cAMP levels in SH-SY5Y cells (30, 31), similarly inhibited back-phosphorylation (maximally by ~60%). This indicates that phosphorylation of the type I receptor results from cAMP elevation.

FIG. 1. PKA-mediated phosphorylation of type I, II, and III InsP_3 receptor preparations. InsP_3 receptors were immunoprecipitated from unstimulated SH-SY5Y, AR4–2J, and RINm5F cell lysates with CT1, CT2, and CT3, respectively. The type I, II, and III receptor content of these preparations was then quantified by silver staining of electrophoresed samples so that equal amounts of receptor could be loaded onto gels. The positions to which type I, II, and III receptors migrated (approximately 270, 255, and 260 kDa, respectively) are indicated. Data shown are representative of two identical experiments. A, autoradiograph showing phosphorylation that results from incubation of type I, II, or III receptor preparations with [γ-32P]ATP (5 μM) in the presence (lanes 1–9) or the absence (lanes 10–12) of 100 units/ml PKA. B–D, immunoblots of type I, II, and III receptor preparations probed with CT1 (B), CT1 + CT2 (C), or CT1 + CT3 (D). Antibody dilutions were adjusted so that equal amounts of type I, II, and III receptor produced approximately equal amounts of chemiluminescence. inc., incubation; prep., preparation.

FIG. 2. Stoichiometry of InsP_3 receptor phosphorylation. The number of moles of P_i incorporated into type I, II, or III receptors was calculated from the radioactive content of the ~240–280-kDa region of gels and the estimate that 20 and 90% of 32P is type II and III receptor-associated in the type II and III receptor preparations. The number of moles of receptor loaded was calculated from the number of InsP_3 binding sites in the preparations. Data shown are the means ± range of two independent experiments.
cates that the effects of VIP and PACAP are mediated by increases in cAMP alone, a view supported by findings that 1 mM dibutryl cAMP inhibited back-phosphorylation by 65 ± 4%, whereas 1 mM dibutryl cGMP inhibited by only 20 ± 4% (mean ± S.E., n = 4).

Surprisingly, carbachol also inhibited back-phosphorylation, maximally by ~45% (Fig. 3D). This was unexpected because the predominant muscarinic receptor type in SH-SY5Y cells is m3 (32, 33), which via phosphoinositide C generates InsP3 and diacylglycerol and thus elevates [Ca2+]i, and protein kinase C activity (33). The effect of carbachol (IC50 = ~1 μM) was blocked by atropine and was not additive with a maximal concentration of VIP, suggesting that VIP and carbachol act via the same pathway (Fig. 3D). To probe this effect, cells were exposed to 0.1 μM phorbol 12-myristate 13-acetate to activate protein kinases C (30, 33), 0.2 μM ionomycin to raise [Ca2+]i, to a similar extent as 100 μM carbachol, and thapsigargin to discharge intracellular Ca2+ stores (26). Although phorbol 12-myristate 13-acetate was without effect, both ionomycin and thapsigargin inhibited back-phosphorylation to a similar extent as carbachol (Fig. 3D). Thus, mere elevation of [Ca2+]i causes InsP3 receptor phosphorylation at PKA consensus sequences, suggesting that increases in [Ca2+]i, that accompany m3 receptor activation (33) mediate the effect of carbachol.

Analysis of 32P-labeled intact cells (Fig. 3E) showed that VIP, PACAP, forskolin, and carbachol all increased 32P incorporation into the type I receptor and that the effect of carbachol was blocked by atropine. Fold increases were relatively small, however, perhaps because the receptor already contains significant amounts of 32P prior to stimulus (lane 1). Nevertheless, Fig. 3E establishes that the back-phosphorylation procedure reliably reflects events in intact cells and, indeed, that it is the more sensitive way of measuring PKA-mediated effects. The radioactivity seen in Fig. 3E (lane 1) reflects metabolic incorporation of 32P was demonstrated by the fact that addition of 32P to cells immediately prior to cell lysis did not lead to the recovery of radioactive receptors.

Phosphorylation of InsP3 Receptors by PKA

Figure 3. Type I InsP3 receptor phosphorylation in intact SH-SY5Y cells. In panels A–D, type I InsP3 receptors were immunoprecipitated from control or stimulated cells and then exposed to PKA and [γ-32P]ATP (back-phosphorylated). In panel E, type I receptors were immunoprecipitated from control or stimulated 32P-labeled cells. The arrows mark the type I receptor migration positions. A, autoradiograph showing inhibition of back-phosphorylation after 2 min of incubation with VIP (lanes 2–5) or PACAP (lanes 6–8); representative of three independent experiments. B, dose dependence of VIP (○) and PACAP (●) effects (means ± S.E., n ≥ 3). CONC., concentration. C, time dependence of 1 μM VIP (○), 0.1 μM PACAP (●), and 10 μM forskolin (▲) effects (means, n ≥ 2). D, effects of 1–100 μM carbachol (C) in the absence or the presence of 1 μM VIP or 10 μM atropine and of 1 μM PACAP (P). Means ± S.E., n ≥ 3. E, autoradiograph showing receptor phosphorylation in 32P-labeled cells after 2 min of incubation without stimulus (—, lane 1) or with 0.1 μM VIP (V, lane 2), 10 μM forskolin (F, lane 3), 10 μM PACAP (P, lane 4), 100 μM carbachol (C, lane 5), and 100 μM carbachol plus 10 μM atropine (C+A, lane 6); representative of four independent experiments.

2 R. J. H. Wojcikiewicz and S. G. Luo, unpublished data.

3 That the radioactivity seen in Fig. 3E (lane 1) reflects metabolic incorporation of 32P was demonstrated by the fact that addition of 32P to cells immediately prior to cell lysis did not lead to the recovery of radioactive receptors.
**Phosphorylation of InsP₃ Receptors by PKA**

**Agonist-induced Changes in cAMP Concentration**—We next examined cAMP levels in the three cell types to establish the extent to which VIP, PACAP, and forskolin were stimulatory and whether $[\text{Ca}^{2+}]_i$-elevating agents might also raise cAMP levels, because this could account for the effects of carbachol, ionomycin and thapsigargin on phosphorylation (Fig. 3D).

In SH-SY5Y cells in the absence of IBMX (Fig. 5A, lower panel), VIP and PACAP increased cAMP levels only modestly (by $\sim$100%). Thus, IBMX was included to amplify agonist effects and facilitate accurate measurement of potency. In the presence of IBMX (Fig. 5A, upper panel), VIP and PACAP produced $\sim$1,000 and 1,200% increases with EC₅₀ values of 580 and 17 nM, respectively. Interestingly, VIP and PACAP exhibited the same $\sim$50-fold potency difference as that seen for phosphorylation (Figs. 3B), but the absolute IC₅₀ values for inhibition of back-phosphorylation were $\sim$100-fold lower than the absolute EC₅₀ values for stimulation of cAMP formation. Thus, a receptor reserve exists for phosphorylation and submaximal increases in cAMP concentration are sufficient to cause maximal phosphorylation. Carbachol also elevated cAMP levels (Fig. 5A), by $\sim$35% in the absence of IBMX and by $\sim$130% in the presence of IBMX (EC₅₀ = 0.27 $\mu$M). This modest effect was blocked by atropine (Fig. 5A) and was mimicked by ionomycin (0.2 $\mu$M) and thapsigargin (1 $\mu$M), which produced $\sim$170 and $\sim$180% increases, respectively. Thus, the effect of carbachol on cAMP levels appears to result from elevation of $[\text{Ca}^{2+}]_i$, and InsP₃ receptor phosphorylation correlates with increases in cAMP concentration.

Similarly, in AR4–2J cells, the extent to which VIP, forskolin and PACAP elevate cAMP levels (Fig. 5B) correlates with the extent to which these agents cause InsP₃ receptor phosphorylation (Fig. 4). In RINm5F cells, only forskolin elevated cAMP levels substantially (Fig. 5B, consistent with it being the only test substance to markedly stimulate phosphorylation (Fig. 4).

**Consequences of Receptor Phosphorylation**—To establish whether InsP₃ receptor function was modulated by PKA, we examined whether PKA altered InsP₃-induced Ca²⁺ mobilization in permeabilized cells. Firstly, however, we sought to establish that PKA could enter and phosphorylate InsP₃ receptors in permeabilized cells. This was found to be the case (Fig. 6A, inset), because back-phosphorylation of type I receptor was much greater in control permeabilized SH-SY5Y cells (lane 1) than in permeabilized cells exposed to PKA (lane 2), and analogous results were obtained for permeabilized AR4–2J and RINm5F cells. In each cell type, PKA significantly enhanced the potency of InsP₃ by $\sim$20% (Fig. 6, A–C) and in SH-SY5Y cells also caused an $\sim$10% increase in maximal response (Fig. 6A). These effects on InsP₃ action were truly PKA-dependent, because they were blocked if PKA was denatured by heating (see Fig. 6A legend), a manipulation that also blocked the kinase activity of PKA (Fig. 6A, inset, lane 3). Further, PKA did not enhance ionomycin-induced $[\text{Ca}^{2+}]_i$ release (Fig. 6, A–C) or alter Ca²⁺ uptake (see Experimental Procedures), showing that the PKA-mediated modification of InsP₃ action was not due to a nonspecific change in Ca²⁺ store characteristics.

Finally, combination of the data in Fig. 6 (n = 4) with other independent determinations of InsP₃ potency yielded the following EC₅₀ values for InsP₃ in the absence or the presence of PKA: for SH-SY5Y cells, 82 ± 4 and 68 ± 3 nm (n = 12), for AR4–2J cells, 72 ± 3 and 56 ± 2 nm (n = 11), and for RINm5F cells, 400 ± 35 and 301 ± 18 nm (n = 10), respectively, the effect of PKA being significant in all cell types (p < 0.05, by unpaired t test). That InsP₃ exhibits relatively low potency in RINm5F cells (Fig. 6C) appears to reflect the predominance in this cell type of type III receptor, which has a lower affinity for InsP₃ than type I or II receptors (8, 18, 19, 45).

**Fig. 4.** InsP₃ receptor phosphorylation in intact AR4–2J and RINm5F cells. In panels A and B, InsP₃ receptors were immunoprecipitated from control or stimulated AR4–2J and RINm5F cells with CT2 and CT3, respectively, and were then exposed to PKA and γ-32P]ATP (back-phosphorylated). In panel C, receptors were immunoprecipitated from control or stimulated ³²P-labeled cells. The arrows mark the migration positions of the type I, II, and III receptors. A, autoradiograph showing back-phosphorylation after 2 min of incubation without stimulus (lanes 1 and 5) or with 1 $\mu$M VIP (V, lanes 2 and 6), 10 $\mu$M forskolin (F, lanes 3 and 7), and 0.1 $\mu$M PACAP (P, lanes 4 and 8); representative of more than three independent experiments. B, quantitation of inhibition of back-phosphorylation. Note that the values shown are derived from the ³²P content of the 240–280-kDa region of gels and thus are the total of type I plus type II receptor for AR4–2J cells and type I plus type III receptor for RINm5F cells (means ± S.E., n = 3). C, autoradiograph showing phosphorylation of InsP₃ receptors in ³²P-labeled cells incubated as in panel A; representative of two independent experiments.
DISCUSSION

The major findings presented herein are (i) that all of the known InsP₃ receptor types are phosphorylated by PKA *in vitro*, albeit inefficiently in the case of type III and particularly type II receptors, (ii) that each of the receptors is phosphorylated in intact cells in response to neurohormone receptor activation, (iii) that slight changes in intracellular cAMP concentration cause maximal phosphorylation, and (iv) that PKA-dependent phosphorylation enhances Ca²⁺ mobilization irrespective of which InsP₃ receptor type is predominant.

It is clear from these and previous findings (2, 3), that the type I receptor is an excellent substrate for PKA. Previous measurements of phosphorylation stoichiometry *in vitro* using column chromatography-purified cerebellar type I receptors showed that although relatively high PKA concentrations incorporated 2 mol P/mol receptor, consistent with the presence of two serines within PKA consensus sequences (2, 3), lower PKA concentrations phosphorylated only serine 1755 and thus incorporated only 1 mol P/mol receptor (21, 36). Recent studies have also indicated that serine 1755 is phosphorylated preferentially in intact cells (37). Our value of 0.65 mol P/mol SH-SY5Y cell type I receptor is consistent with the view that only one serine, presumably serine 1755, is phosphorylated. It is possible that the incorporation is <1 mol/mol in our studies because some of the PKA sites are already occupied by nonradioactive phosphate, an argument supported by the fact that the type I receptor is clearly a phosphoprotein in unstimulated cells (Fig. 3E, lane 1). These phosphates remain receptor-associated during immunoprecipitation (Figs. 1–4) but may not survive purification by column chromatography (21, 36).

Our work also shows, for the first time, that PKA phosphorylates type II and III receptors, albeit with low efficiency (0.04 and 0.14 mol P/mol receptor, respectively). This was not an artifact resulting from the incubation of purified receptors and kinases, because both receptors were phosphorylated in intact cells, most notably in response to forskolin. The identity of the sites phosphorylated and the reason why the phosphorylation stoichiometry is so low were not examined in the present study. Regarding the first point, however, although the sites phosphorylated in the type I receptor are not conserved in type II or III receptors, other serines within the PKA consensus sequence (R/K)(R/K)/X(S) (20) are present in the coupling domains of type II and III receptors: in rat and human type II receptors at serine 1687 (8, 10), in rat and human type III receptors at serines 934 and 1133, and in rat type III receptor at serine 1460 (9–11). Thus, it is quite plausible that type II and III receptors are PKA substrates. Regarding the stoichiometry, the low values for type II and III receptors cannot be explained by occupation of phosphorylation sites by nonradioactive phosphate, because the receptors were not heavily phosphorylated in ³²P-labeled resting cells. Rather, either the conformation of the type II and III receptors or their orientation when tetramerized may make phosphorylation of the consensus sequences unfavorable.

Overall, our studies in intact cells show that modest increases in cAMP concentration result in type I, II, and III InsP₃ receptor phosphorylation. That the type I receptor is phosphorylated in SH-SY5Y cells is, to our knowledge, the first demonstration of this modification in intact neuronal cells and a number of aspects of this work are worthy of comment. Firstly, very modest increases in cAMP concentration are sufficient to maximally stimulate InsP₃ receptor phosphorylation. Thus, a receptor reserve is apparent for VIP and PACAP, which via Gₛ-coupled PACAP-I receptors activate adenylyl cyclase (28, 29) to produce >10-fold increases in cAMP concentration. In contrast, activation of m3 receptors produced much smaller increases in cAMP concentration, and thus, half-maximal values for carbachol-induced phosphorylation and cAMP formation were similar. The mechanism by which carbachol increases cAMP levels is intriguing because m3 receptors are...
Phosphorylation of InsP₃ Receptors by PKA

**Fig. 6.** Effects of PKA on InsP₃-induced Ca²⁺ mobilization in permeabilized SH-SY5Y, AR4–2J, and RINm5F cells. Electrically permeabilized cells were preincubated without PKA (open symbols) or with 100 units/ml PKA (closed symbols) or with an equivalent amount of denatured PKA (PKA that was heated at 140 °C for 1–3 h) and then with 45Ca²⁺. Cells were then incubated with InsP₃ (○, ○) or ionomycin (■, ■), and the amount of 45Ca²⁺ remaining sequestered (45Ca²⁺ content) was determined. Data shown and quoted below are the means ± S.E. of four independent experiments, and the significance of PKA (open triangles) was determined. Data shown and quoted below are the means ± S.E. of four independent experiments, and the significance of PKA (open triangles) determined. Data shown and quoted below are the means ± S.E. of four independent experiments, and the significance of PKA (open triangles) determined.

**REFERENCES**

1. Berridge, M. J. (1993) Nature 361, 315–325
2. Furuichi, T., and Sudhof, T. C. (1991) J. Neurochem. 56, 953–960
3. Joseph, S. K. (1996) Cell. Signaling 8, 1–7
4. Furuchi, T., Yashikawa, S., Tani, M., Wada, K., Maeda, N., and Mikoshiba, K. (1989) Nature 342, 32–38
5. Mignery, G. A., Sudhof, T. C., Takei, K., and De Camilli, P. (1989) Nature 342, 192–195
6. Mignery, G. A., Newton, C. L., Archer, B. T., III, and Sudhof, T. C. (1990) J. Biol. Chem. 265, 12679–12685
7. Yamada, M., Makino, Y., Clark, R. A., Pearson, D. W., Mattei, M.-G., Guenet, C.-L., Ohama, E., Fujino, I., Miyawaki, A., Furuchi, T., and Mikoshiba, K. (1994) Biochem. J. 302, 781–790
8. Sudhof, T. C., Newton, C. L., Archer, B. T., III, Ushkaryov, Y. A., and Mignery, G. A. (1991) EMBO J. 10, 3199–3206
9. Blondel, O., Takeda, J., Janssen, H., Seino, S., and Bell, G. I. (1993) J. Biol. Chem. 268, 11356–11363
10. Yamamoto-Hino, M., Sugiyama, Y., Hikichi, K., Mattei, M.-G., Hasegawa, K., Sekine, S., Sakurada, K., Miyawaki, A., Furuchi, T., and Mikoshiba, K. (1991) Recombinant Channels 2, 9–22
11. Maranto, A. R. (1994) J. Biol. Chem. 269, 1222–1230
12. Mignery, G. A., and Sudhof, T. C. (1990) EMBO J. 9, 3893–3898

**Acknowledgments**—We thank Carol Jones for performing cAMP assays and Grant Kelley, Jon Oberdorf, and Chang-Cheng Zhu for many helpful discussions.
Phosphorylation of InsP₃ Receptors by PKA

5677

13. De Smedt, H., Missiaen, L., Parys, J. B., Bootman, M. D., Mertens, L., Van Den Bosch, L., and Casteels, R. (1994) J. Biol. Chem. 269, 21691–21698
14. Wojcikiewicz, R. J. H. (1995) J. Biol. Chem. 270, 11678–11683
15. Monkawa, T., Miyawaki, A., Sugiyama, T., Yoneshima, H., Yamamoto-Hino, M., Furuichi, T., Saruta, T., Hasegawa, M., and Mikoshiba, K. (1995) J. Biol. Chem. 270, 14700–14704
16. Wojcikiewicz, R. J. H., and He, Y. (1995) Biochem. Biophys. Res. Commun. 213, 334–341
17. Joseph, S. K., Lin, C., Pierson, S., Thomas, A. P., and Maranto, A. R. (1995) J. Biol. Chem. 270, 23310–23316
18. Newton, C. L., Mignery, G. A., and Sudhof, T. C. (1994) J. Biol. Chem. 269, 28613–28619
19. Yoneshima, H., Miyawaki, A., Michikawa, T., Furuichi, T., and Mikoshiba, K. (1997) Biochem. J. 322, 591–596
20. Walsh, D. A., and Van Patten, S. M. (1994) FASEB J. 8, 1227–1236
21. Ferris, C. D., Cameron, A. M., Bredt, D. S., Huganir, R. L., and Snyder, S. H. (1991) Biochem. Biophys. Res. Commun. 175, 192–198
22. Wojcikiewicz, R. J. H., Furuichi, T., Nakade, S., Mikoshiba, K., and Nahorski, S. R. (1994) J. Biol. Chem. 269, 7693–7699
23. Joseph, S. K., and Ryan, S. V. (1993) J. Biol. Chem. 268, 23059–23065
24. Joseph, S. K., and Samanta, S. (1993) J. Biol. Chem. 268, 6477–6486
25. Komalavilas, P., and Lincoln, T. M. (1996) J. Biol. Chem. 271, 21933–21938
26. Wojcikiewicz, R. J. H., and Nahorski, S. R. (1991) J. Biol. Chem. 266, 22234–22241
27. Waschek, J. A., Muller, J.-M., Duan, D.-S., and Sudee, W. (1989) FEBS Lett. 250, 611–614
28. Harmer, A., and Lutz, E. (1994) Trends Pharmacol. Sci. 15, 97–99
29. Vertongen, P., Devalck, C., Sariban, E., De Laet, M.-H., Martelli, H., Paraf, F., Helardot, P., and Robberecht, P. (1996) J. Cell. Physiol. 167, 36–46
30. Baumgold, J., and Fishman, P. H. (1988) Biochem. Biophys. Res Commun. 154, 1137–1143
31. Hirst, R. A., and Lambert, D. G. (1995) Biochem. Pharmacol. 49, 1633–1640
32. Wall, S. J., Yasuda, R. P., Li, M., and Wolfe, B. B. (1991) Mol. Pharmacol. 40, 783–789
33. Vaughan, P. F. T., Peers, C., and Walker, J. H. (1995) Gen. Pharmacol. 26, 1191–1201
34. Simeone, D. M., Yule, D. I., Logsdon, C. D., and Williams, J. A. (1995) Regul. Pept. 55, 197–206
35. Andersson, M., Sillard, R., and Rokaeus, A. (1992) Regul. Pept. 40, 41–49
36. Komalavilas, P., and Lincoln, T. M. (1994) J. Biol. Chem. 269, 8701–8707
37. Sharma, K., Wang, L., Zhu, Y., Bokkala, S., and Joseph, S. K. (1997) J. Biol. Chem. 272, 14617–14622
38. Fagan, K. A., Mahey, R., and Cooper, D. M. F. (1996) J. Biol. Chem. 271, 12438–12444
39. Cooper, D. M. F., Mons, N., and Karpen, J. W. (1995) Nature 374, 421–424
40. Nakade, S., Rhee, S. K., Hamaana, H., and Mikoshiba, K. (1994) J. Biol. Chem. 269, 6735–6742
41. Bird, G. St. J., Burgess, G. M., and Putney, J. W., Jr. (1993) J. Biol. Chem. 268, 17917–17923
42. Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V., and Snyder, S. H. (1995) Cell 83, 463–472
43. Cavalli, L., Coassin, M., Borean, A., and Alexandre, A. (1996) J. Biol. Chem. 271, 5545–5551
44. Schramm, C. M., Chuang, S. T., and Grunstein, M. M. (1995) Am. J. Physiol. 269, L715–L719
45. Wojcikiewicz, R. J. H., and Luo, S. G. (1988) Mol. Pharmacol., in press.