Protein Kinase A Induces Phosphorylation of the Human 5-HT_{1A} Receptor and Augments Its Desensitization by Protein Kinase C in CHO-K1 Cells†

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ABSTRACT: Protein kinase C has been previously shown both to phosphorylate and to desensitize the ability of the human 5-HT_{1A} receptor to inhibit adenylyl cyclase [Raymond, J. R. (1991) *J. Biol. Chem. 266,* 14747–14753]. In this study, we examined the effects of short-term treatment with protein kinase A activators on coupling to the inhibition of adenylyl cyclase and on phosphorylation of the human serotonin 5-HT_{1A} receptor in CHO cells that stably express 1200 fmol of receptor/mg of protein. Forskolin induced a concentration- and time-dependent phosphorylation of the receptor that was detectable at 5 min and maximal at 15–30 min with a half-maximal concentration of 10–20 μM. Phosphorylation was also induced by *S*p-cAMPS or dibutyryl-cAMP, and blocked by *R*p-cAMPS and a pseudosubstrate inhibitor of PKA, but not by heparin (inhibitor of receptor kinase) or sphingosine (inhibitor of PKC). The stoichiometry of phosphorylation induced by forskolin was 1 mol of phosphate per mole of receptor. PKA activators did not induce a measurable desensitization of 5-HT_{1A} receptor-inhibited adenylyl cyclase activity. However, forskolin augmented the desensitization caused by a submaximal concentration of phorbol 12-myristate 13-acetate (300 nM PMA) as evidenced by a rightward shift of the concentration–response curve for 5-HT, and approximately doubled the amount of phosphate incorporated into the receptor by PMA. Forskolin did not augment desensitization or increase the degree of phosphorylation induced by a maximal concentration of PMA (5 μM). These studies suggest a cooperativity between PKA and PKC in the phosphorylation and desensitization of the 5-HT_{1A} receptor.

Many G protein-coupled receptors demonstrate rapid desensitization in the continued presence of agonist or other stimulus, manifested by uncoupling from second messengers (Sibley et al., 1987). In most instances, this rapid desensitization phenomenon depends on the posttranslational modification of the receptor by phosphorylation reactions (Sibley et al., 1987; Hausdorff et al., 1990; Huganir et al., 1990). The prototypical receptor modulated by this phenomenon is the β_2-adrenergic receptor. In a similar fashion, serotonin receptors also desensitize. For example, 5-HT_2 receptor-mediated phosphatidylinositol hydrolysis in bovine aorta is markedly attenuated by brief pretreatment with agonist (Pauwels et al., 1990). 5-HT_2 receptor-induced Ca^{2+} mobilization in rat C6BU-1 glioma cells is rapidly diminished after agonist treatment (Kagaya et al., 1993). Among the serotonin receptors, the most is known about desensitization of the 5-HT_{1A} receptor. The 5-HT_{1A} receptor acutely decreases the firing activity of rat brain neurons, but this effect attenuates after 7 days, and is abolished after 14 days of treatment with the 5-HT_{1A} receptor agonist gepirone (Blier & de Montigny, 1990). The cloning of a human (Kobilka et al., 1987; Fargin et al., 1988) and rat (Albert et al., 1990) 5-HT_{1A} receptor has allowed the development of specific tools such as cell lines that permanently express the 5-HT_{1A} receptor (Fargin et al., 1989; Raymond et al., 1989a,b; Albert et al., 1990; Raymond, 1991; Liu & Albert, 1991; Varrault et al., 1993; Harrington et al., 1994) and anti-receptor antibodies (Fargin et al., 1988; Raymond et al., 1989a,b, 1993a; El Mestikawy et al., 1990; Azmitia et al., 1992) which allow a more precise examination of 5-HT_{1A} receptor desensitization. Using such tools, Liu and Albert have suggested a role for both protein kinase A (PKA, cAMP-dependent protein kinase)[1] and protein kinase C (PKC, calcium- and phospholipid-dependent kinase) in desensitization of rat 5-HT_{1A} receptor-mediated elevations of intracellular Ca^{2+} in LZD-7 cells (Liu & Albert; 1991). Recently, Harrington and colleagues demonstrated a potential role for both kinases in desensitizing cAMP inhibition by the 5-HT_{1A} receptor in HeLa cells (Harrington et al., 1994). In another study, PKC was shown to mediate a rapid desensitization of the inhibition of forskolin-stimulated adenylyl cyclase activity by the human 5-HT_{1A} receptor expressed in CHO-K1 cells. That desensitization was associated with a phosphorylation of the receptor with a stoichiometry of about 2 mol of phosphate per mole of receptor (Raymond, 1991).The work of Liu and Albert and Harrington and colleagues suggested a potential role for PKA in the desensitization of the 5-HT_{1A} receptor-mediated inhibition of adenylyl cyclase, possibly through phosphorylation of the receptor. The purposes of the current studies were to investigate the role of PKA in phosphorylation and desensitization of the 5-HT_{1A} receptor, and to ascertain if cooperativity with PKC occurs.

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[1] Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; PKC, protein kinase C (calcium- and phospholipid-dependent kinase); PKA, protein kinase A (cAMP-dependent protein kinase; 5-HT, serotonin; PMA, phorbol 12-myristate, 13-acetate; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfate; βARK, β-adrenergic receptor kinase; Me_2SO, dimethyl sulfoxide, PKI, peptide pseudosubstrate inhibitor of PKA (TTYADFIASGRTGPRNAIHD); *R*p-cAMPS, *R*p-diastereomer of adenosine 3′,5′-cyclic monophosphothioate; *S*p-cAMPS, *S*p-diastereomer of adenosine 3′,5′-cyclic monophosphothioate; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)-1,2,3,4-tetrahydronaphthalene.

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MATERIALS AND METHODS

Materials. [α^{32}-P]ATP (30 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from DuPont New England Nuclear, and [^{3}H]-8-OH-DPAT was from Research Products International (Mt. Prospect, IL). Cell culture supplies were from GIBCO (Grand Island, NY). All other reagents were of the highest quality available from Sigma (St. Louis, MO) or Calbiochem (San Diego, CA). CHO-5-HT_{1A}/WT-27 cells which expressed ≈1200 fmol of 5-HT_{1A} receptors per milligram of protein during these studies, and anti-receptor IgG fraction JWR21, were obtained as previously described (Fargin et al., 1989; Raymond, 1991).

Cell Culture. Cells were grown in Ham's F12 supplemented with 10% fetal calf serum and penicillin (100 units/mL) and G-418 (400 μg/mL) at 37 °C in 95% air/5% CO_2.

Adenylyl Cyclase Assays. Adenylyl cyclase activity in membranes was measured by the method of Salomon et al. (1974) with modifications as previously described (Raymond, 1991). Data presented were obtained in the presence of 100 μM forskolin, except for basal values, which were derived in the presence of vehicle (0.5% methanol). For some studies, a radioimmunassay was used to measure intracellular cAMP in cell monolayers (Raymond et al., 1994).

Desensitization Assay. Cell monolayers were treated with vehicle or various concentrations of agents for various times, washed 5 × with PBS (37 °C), and then lysed and processed for adenylyl cyclase assays as previously described (Raymond, 1991) or cAMP radioimmunoassay (Raymond et al., 1994).

Protein Determinations. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Permeabilized Cell Phosphorylations. Cells were treated using a modification of the protocol of Lohse et al. (1990) as previously described (Raymond, 1991). This procedure involved permeabilization of cells with digitonin, followed by treatment with various agents in the presence of [γ-^{32}P]ATP. Because the permeabilization procedure allows ready diffusion of small molecules such as ATP across the plasma membrane, intracellular and extracellular concentrations of ions and other small molecules were assumed to be equal.

Other Procedures. Stable expression of the human 5-HT_{1A} receptor in CHO cells, generation of peptide antibodies, immunoprecipitation of receptors, and calculation of the stoichiometry of the phosphorylation of the human 5-HT_{1A} receptor by PMA were performed exactly as described previously (Raymond, 1991). For calculation of stoichiometry, the following assumptions were made: (i) intracellullar and extracellular ATP concentrations were assumed to be equal (2.2 mM); (ii) all counts of ^{32}P in the immunoprecipitates were assumed to be incorporated into the receptors; (iii) receptor binding was assumed to be nearly maximal at 20 nM [^{3}H]-8-OH-DPAT; and (iv) immunoprecipitation of liganded and phosphorylated receptors was assumed to be equally efficient. Assumption i is based on the fact that >95% of the cells were permeabilized as assessed by trypan blue uptake, and that the added extracellular volume greatly exceeded the intracellular volume. Assumption ii was directly tested by SDS–PAGE, and was confirmed (see Results). Assumption iii was based on affinity constants of 0.5 and 3.5 nM for [^{3}H]-8-OH-DPAT in these cells as revealed by saturation binding experiments (n = 3 in triplicate, not shown).

Statistical Analyses. Paired one-tailed t-tests were used to compare the data sets. A modified Bonferroni correction was used to correct for multiple comparisons as follows: the calculated level of significance was multiplied by the number of comparisons made for each figure. This allowed us to maintain a 95% confidence interval for $p \leq 0.05$.

RESULTS

Exposure of CHO-5-HT_{1A}/WT-27 to 100 μM forskolin resulted in an ≈15-fold increase in membrane adenylyl cyclase activity compared to untreated values [≈500 and ≈7500 pmol of cAMP (mg of membrane protein)$^{-1}$ (30 min)$^{-1}$]. Pooled (sham) clones cotransfected with pSV-neo and PBC12BI vectors not containing 5-HT_{1A} receptor sequence were used as controls. In those cells, 5-HT did not inhibit cAMP accumulation (n = 5, not shown). Because the human 5-HT_{1A} receptor has at least two potential sites for PKA-activated phosphorylation (R^{226}KT and R^{340}KT within the putative third intracellular loop that conform to the PKA recognition motif R-X-S/T) (Kemp & Pearson, 1990), the effects of pretreatment with activators of PKA on the 5-HT-mediated inhibition of membrane adenylyl cyclase activity and on receptor phosphorylation were assessed.

Effects of Activators of PKA on 5-HT_{1A} Receptor-Mediated Inhibition of Membrane Adenylyl Cyclase Activity. Pretreatment of cell monolayers with 100 μM forskolin or dibutyryl-cAMP (Bt_2-cAMP) for 15 min did not result in a statistically significant desensitization of the subsequent 5-HT-induced inhibition of adenylyl cyclase activity in membranes derived from these cells [IC_{50}(control) = 133 ± 13 nM; IC_{50}(forskolin) = 172 ± 25 nM; IC_{50}(Bt_2-cAMP) = 163 ± 19 nM; n = 6 for each], or in the efficacy of 5-HT to inhibit cAMP (control = 62 ± 4%; forskolin = 55 ± 3%; Bt_2-cAMP = 53 ± 5 nM), although there were nonsignificant trends toward reduction in both potency and efficacy. Treatment with the PKC activator phorbol 12-myristate 13-acetate (PMA) caused a detectable change in the potency of 5-HT to inhibit adenylyl cyclase activity that was manifested as a shift to the right of the IC_{50} values for the 5-HT effect on adenylyl cyclase [IC_{50}(control) = 143 ± 16 nM; IC_{50}(5 μM PMA) = 430 ± 37 nM; $n = 6, p \leq 0.02$], consistent with our previous report (Raymond, 1991). We then tested the ability of forskolin to augment the desensitization of 5-HT-inhibited adenylyl cyclase activity induced by PMA. Forskolin did not significantly alter the effect of pretreatment with a high concentration (5 μM) of PMA [IC_{50}(forskolin + 5 μM PMA) = 426 ± 43 nM; n = 6]. Neither treatment significantly altered the efficacy of 5-HT to inhibit cAMP accumulation (control = 65 ± 3%; 5 μM PMA = 54 ± 6%; forskolin + 5 μM PMA = 50 ± 9%). However, forskolin did augment the desensitization induced by submaximal PMA [IC_{50}(control) = 127 ± 5 nM; IC_{50}(300 nM PMA) = 210 ± 15 nM; IC_{50}(forskolin + 300 nM PMA) = 457 ± 66 nM; n = 7]. The IC_{50} value for 300 nM PMA was significantly different ($p \leq 0.02$) than either control or forskolin + 300 nM PMA, whereas that of forskolin + 300 nM PMA was also signficantly different than control ($p \leq 0.03$). There was no significant change in the efficacy of 5-HT to inhibit adenylyl cyclase activity with any of the pretreatment conditions (control = 61 ± 8%; 300 nM PMA = 57 ± 5%; forskolin + 300 nM PMA = 51 ± 3%). These studies suggest that activation of PKA augments PKC-induced rapid desensitization (as manifested by a right shift in the concentration–response curve for 5-HT) of the 5-HT_{1A} receptor at submaximal, but not at maximal, concentrations of PMA.

In order to ascertain whether Bt_2-cAMP could induce a desensitization in intact cells (rather than membranes), we used a radioimmunoassay technique to measure intracellular cAMP in the presence and absence of various concentrations

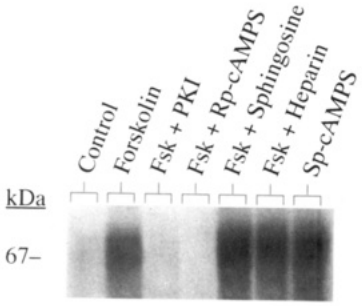

FIGURE 1: PKA-induced phosphorylation of the human 5-HT_{1A} receptor. Experiments were performed after pretreatment of cell monolayers with various concentrations of agents for 30 min at 37 °C. Immunoprecipitates were run in Laemmli buffer using reducing conditions and a discontinuous 10% SDS–PAGE gel. The experiment depicted is representative of three performed. Concentrations of agents used were as follows: forskolin (50 μM), PKI (1 μM), *R*p-cAMPS (300 μM), sphingosine (10 μM), heparin (10 μM), and *S*p-cAMPS (100 μM). The gel was exposed to Kodak X-AR film for 29 days at –80 °C.

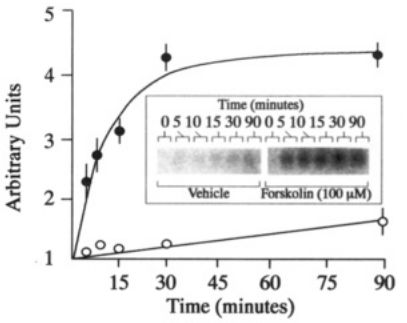

FIGURE 2: Time course of forskolin-induced phosphorylation of the recombinant human 5-HT_{1A} receptor expressed in CHO cells. Experiments were performed as described under Materials and Methods. The kinetics of phosphorylation were derived by scanning densitometry of immunoprecipitated phosphorylated 5-HT_{1A} receptors. Each point ± SE is derived from three separate experiments (unstimulated cells = open circles, 50 μM forskolin-stimulated cells = dark circles). The inset is from a single representative experiment. All products were run in Laemmli buffer using reducing conditions on a 10% SDS–PAGE gel. The gel was exposed to Kodak X-AR film for 26 days at –80 °C.

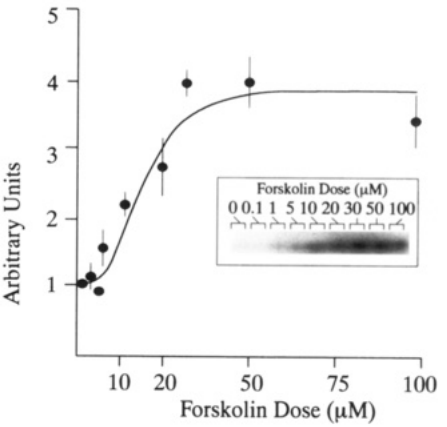

FIGURE 3: Concentration-dependent phosphorylation of the human 5-HT_{1A} receptor by PMA. Permeabilized cell phosphorylation and immunoprecipitation experiments were performed as in Figure 2. Cells were treated for 30 min. The inset of phosphorylated 5-HT_{1A} receptors is from one representative experiment. The graph is a composite of values ± SE obtained from scanning densitometry from three separate experiments. Gels were exposed to Kodak X-AR film for 28–35 days at –80 °C.

of 5-HT and 100 μM forskolin. Concentration–response curves for 5-HT were modeled after 30 min exposure to vehicle, 100 μM Bt_2-cAMP, or 5 μM PMA. Results were similar to those obtained using a membrane adenylyl cyclase assay. Prior exposure to Bt_2-cAMP did not significantly alter the half-effective dose of 5-HT, but prior PMA treatment did shift the curve rightward [IC_{50}(control = 84 ± 33 nM; IC_{50}(Bt_2-cAMP) = 105 ± 16 nM, not significantly different from control; IC_{50}-(PMA) = 374 ± 49 nM, $p \leq 0.02$ vs control; $n = 3$ for each]. Efficacy did not change with either treatment (control = 46 ± 5%; Bt_2-cAMP = 51 ± 9%; PMA = 34 ± 9%).

PKA-Induced Phosphorylation of the Recombinant Human 5-HT_{1A} Receptor Expressed in CHO Cells. As shown in Figure 1, affinity purified-JWR21 immunoprecipitated a single broad phosphorylated band of ≈65–75 kDa from stable transformants, but not from sham-transfected cells. This particular antiserum has been previously characterized as able to immunoprecipitate photoaffinity-labeled, liganded, and phosphorylated human 5-HT_{1A} receptors (Fargin et al., 1988; Raymond, 1991; Raymond et al., 1993a). The density of this band was increased ≈4-fold over untreated levels by treatment for 15 min with the PKA activators forskolin or *S*p-cAMPS. Increased phosphorylation of 5-HT_{1A} receptors could be blocked by the PKA pseudosubstrate inhibitor PKI and the PKA competitive antagonist *R*p-cAMPS, but not by the PKC inhibitor sphingosine (Hannun & Bell, 1989a,b) or by the polyanion receptor kinase inhibitor heparin. These results suggest that PKA activation causes a rapid phosphorylation of 5-HT_{1A} receptors expressed in CHO cells, and that this phosphorylation does not require activation of PKC or a specific receptor kinase.

Concentration- and Time-Dependent Phosphorylation of the Human 5-HT_{1A} Receptor by Forskolin. The phosphorylation of the 5-HT_{1A} receptor was rapid and time-dependent at 50 μM forskolin (Figure 2). Phosphorylation was detectable after as little as 5 min of incubation, half-maximal by 5–10 min, and maximal at 15–30 min. The maximal ≈4-fold increase in phosphorylation was sustained for up to 90 min. The time course of phosphorylation was somewhat slower than that induced by PKC (Raymond, 1991), but is consistent with the involvement of a kinase. Data presented in Figure 3 demonstrate that the phosphorylation of the 5-HT_{1A} receptor was dependent on the concentration of forskolin. Moreover, phosphorylation occurred within the range of concentrations known to be effective for PKA activation in many systems; half-maximal phosphorylation was apparent between 10 and 20 μM, and was maximal at 30 μM forskolin. Increasing the forskolin concentration to as high as 100 μM did not further increase the degree of phosphorylation of immunoprecipitated 5-HT_{1A} receptors.

Interactions between PKA- and PKC-Dependent Phosphorylation of the Human 5-HT_{1A} Receptor. We examined the effects of forskolin on the phosphorylation of 5-HT_{1A} receptors by "high" and "low" concentrations of PMA. Figure 4 shows that the effects of forskolin on PMA-induced phosphorylation of the 5-HT_{1A} receptors were similar to those on desensitization. Forskolin (50 μM) or a submaximal concentration of PMA (300 nM) alone phosphorylated the 5-HT_{1A} receptors equally well (≈4-fold increase). Treatment with forskolin (50 μM) + 300 nM PMA caused a more marked increase in phosphorylation of the receptors (≈8-fold increase) than either single treatment. This increased degree of phosphorylation was equal to that induced by a maximal concentration (5 μM) of PMA. However, forskolin did not further augment the phosphorylation induced by 5 μM PMA. On the basis of our previous study, these findings suggested that the maximal stoichiometry of phosphorylation of the 5-HT_{1A} receptor in CHO-K1 cells by PKA is ≈1 mol of

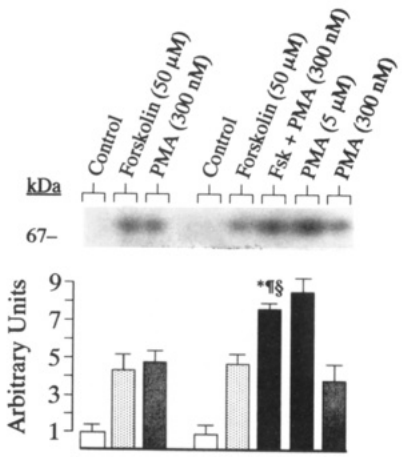

FIGURE 4: Effects of forskolin on phosphorylation of the human 5-HT1A receptor by PMA. Permeabilized cell phosphorylation and immunoprecipitation experiments were performed as described under Materials and Methods. Immunoprecipitates were run in Laemmli buffer using reducing conditions on a 10% SDS–PAGE gel. The gels were exposed to Kodak X-AR film for 25–29 days at –80 °C. The graph is a composite of values ± SE obtained from scanning densitometry from three identical experiments. Controls were performed with vehicle (0.1% Me_2SO + 0.1% ethanol). * indicates $p \leq 0.05$ vs 50 μM forskolin; § indicates $p \leq 0.05$ vs 300 nM PMA; ¶ indicates not significant vs 5 μM PMA.

phosphate per mole of receptor, and that the combination of forskolin and 300 nM PMA causes phosphorylation of the 5-HT$_{1A}$ receptor that is equal to, but not greater than, that induced by 5 μM PMA (≈2 mol of phosphate per mole of receptor) (Raymond, 1991). Accordingly, we directly tested the molar ratio of phosphorylation of the 5-HT$_{1A}$ receptor by PKA as described in the next section.

Stoichiometry of Phosphorylation of the Human 5-HT$_{1A}$ Receptor by PKA. In CHAPS-solubilized membrane fractions from CHO-5-HT$_{1A}$/WT-27 cells, the stoichiometry of 5-HT$_{1A}$ receptor phosphorylation was calculated by comparing the amount of ^{32}P immunoprecipitated, and the amount of [^{3}H]-8-OH-DPAT binding in immunoprecipitates treated in the same manner but not exposed to [γ-^{32}P]ATP. In those studies ($n = 3$), 12 351 ± 502 specific dpm of [^{3}H]-8-OH-DPAT and 55 ± 12 dpm of ^{32}P (over background) were immunoprecipitated. These values calculate to ≈62 fmol of receptor and 55 fmol of phosphate, and a ratio of 0.9 mol of phosphate/mol of 5-HT$_{1A}$ receptor. This ratio is about half of that reported for PKC (≈2:1) (Raymond, 1991). Nonspecific serum was used in control immunoprecipitations to establish the level of background "trapping" of ^{32}P and [^{3}H]-8-OH-DPAT, which were 10 ± 5 dpm of [^{3}H]-8-OH-DPAT and 23 ± 5 dpm of ^{32}P. We performed additional studies to confirm that the phosphorylated products were in fact derived from the 5-HT$_{1A}$ receptor. First, we repeated the studies with another anti-5-HT$_{1A}$ receptor serum, JWR41, at 1:100 dilution (Raymond et al., 1993a). That serum precipitated 19 050 ± 1030 specific dpm of [^{3}H]-8-OH-DPAT (≈95 fmol of receptor), and 106 ± 5 dpm of ^{32}P (≈106 fmol of phosphate) ($n = 3$). In the presence of 10 μM specific blocking peptide against which the antiserum was raised, no counts above background were precipitated. The stoichiometry of phosphorylation estimated by that technique was ≈1.1. Second, in order to more firmly establish that the precipitated counts were truly covalently bound into the 5-HT$_{1A}$ receptor, identical aliquots were run on 10% SDS–PAGE, and the areas corresponding to the 65–75 kDa range were excised from the gels, solubilized, and subjected to scintillation counting. Those experiments yielded 112 ± 9 dpm of ^{32}P over background, for an estimated stoichiometry of ≈1.2. Therefore, the four methods of calculating stoichiometry (densitometric comparison with PKC-phosphorylated receptors, direct immunoprecipitation with two different receptor antisera, and SDS–PAGE purification of products obtained from precipitation with one of the sera) were in close agreement with a stoichiometry of ≈1 mol of phosphate per mole of receptor.

DISCUSSION

Desensitization is an intricate process by which exposure to an agonist results in a decreased responsiveness to that agonist. Short-term desensitization usually results from the modification of the receptor by phosphorylation reactions. These mechanisms have been best elucidated for the β-adrenergic receptors, where desensitization is mediated by both βARK and PKA (Sibley et al., 1987). Both βARK and PKC have been implicated in phosphorylation and desensitization of muscarinic (Kwatra et al., 1989; Scherer & Nathanson, 1990; Richardson & Hosey, 1990) and adrenergic receptors (Sibley et al., 1984; Kelleher et al., 1984; Leeb-Lundberg et al., 1987; Bouvier et al., 1987; Kurose & Lefkowitz, 1994). Although the picture for 5-HT receptors has been less clear, recent studies have allowed elucidation of some of the molecular mechanisms of 5-HT$_{1A}$ receptor desensitization (Raymond, 1991; Liu & Albert, 1991; Harrington et al., 1994).

We have previously shown that PKC can phosphorylate and desensitize the human 5-HT$_{1A}$ receptor (Raymond, 1991), although a causal link between the two processes remains to be proven. Liu and Albert contemporaneously suggested that PKA could augment PKC-induced desensitization of rat 5-HT$_{1A}$ receptor-stimulated elevations of intracellular Ca^{2+} levels (Liu & Albert, 1991). Harrington et al. more recently showed that forskolin pretreatment could rapidly eliminate the ability of the 5-HT$_{1A}$ receptor to inhibit adenylyl cyclase in HeLa cells (Harrington et al., 1994). Because the human 5-HT$_{1A}$ receptor contains two putative sequences which roughly conform to the motif **RXS/T*** [2] (R^{226}KT and R^{340}-KT within the putative third intracellular loop),[3] the current studies examined the effects of short-term treatment with activators of PKA on the phosphorylation state and functional coupling to adenylyl cyclase of that receptor in CHO-K1 cells.

The data from the current studies suggest a potential subtle role for PKA in the modification by phosphorylation, and functional regulation by desensitization of the 5-HT$_{1A}$ receptor coupling to the inhibition of adenylyl cyclase. Although PKA activation is associated with phosphorylation of the 5-HT$_{1A}$ receptor with a molar ratio of ≈1 (Figures 1–4), no statistically significant desensitization apparently results from this phosphorylation. However, PKA activation augments both the phosphorylation and desensitization of the receptor induced by 300 nM (submaximal) PMA. The time course and concentration–response curve of phosphorylation of the 5-HT$_{1A}$ receptor are consistent with the involvement of PKA (Figures 2 and 3).

The stoichiometry of phosphorylation by 300 nM PMA appears to approximately double with cotreatment with forskolin. Cotreatment with forskolin and 5 μM PMA does not further increase the degree of phosphorylation (stoichiometry of ≈2; Figure 4) or desensitization of the receptor.

[2] The asterisk denotes the amino acid residue which is phosphorylated.

[3] The consensus site for PKA phosphorylation is less restrictive than that for PKC (Kemp & Pearson, 1990). The sequences **RXS***, **RRXS***, **RXXS***, and **KRXXS*** are the most common PKA recognition motifs.

Therefore, PKA activation can augment the actions of submaximally activated PKC on the 5-HT_{1A} receptor, but does not apparently alter the actions of maximally activated PKC. One potential pathway through which such effects might occur would be that PKA augments PMA-induced PKC activation. However, the PKC antagonist sphingosine (Hannun & Bell, 1989a,b) does not block the phosphorylation of the receptor by forskolin, suggesting that PKA does not indirectly cause phosphorylation of the 5-HT_{1A} receptor by activating PKC. Neither does the polyanion βARK inhibitor heparin block the forskolin-induced phosphorylation of the 5-HT_{1A} receptor. Because these studies were performed in relatively intact (permeabilized) cells, they do not allow us to distinguish between a direct role for PKA in phosphorylation of the 5-HT_{1A} receptor, or an indirect role of PKA by activating or inhibiting another kinase or phosphatase which has the 5-HT_{1A} receptor as a substrate. Therefore, we cannot yet unequivocally state that the 5-HT_{1A} receptor is a direct substrate for PKA. Mutagenesis and reconstitution studies will be necessary to address this issue.

The augmentation by forskolin on the actions of a submaximal concentration of PMA (300 nM) *vis à vis* the lack of effect on those of a maximal concentration of PMA (5 μM) suggests either that PKA activation and PKC activation result in phosphorylation of an overlapping site within the 5-HT_{1A} receptor or that some form of hierarchal phosphorylation occurs within the receptor (Roach, 1991). An alternative hypothesis is that PKA and/or the receptors are compartmentalized, and activated PKA has access to a limited portion of the total cellular receptors when compared with PKC. Pertinent to that conjecture are recent data showing that kinases (Carr et al., 1992; Rosenmund et al., 1994) and receptors (Keefer & Limbird, 1993) may be anchored within cellular microdomains. In fact, 5-HT_{1A} receptors have been proposed to be compartmentalized into plasma membrane microdomains (Varrault et al., 1992). Reconstitution studies will probably be necessary to distinguish between the two possiblities discussed in this paragraph.

Our results must be compared to those of Harrington et al., who showed a profound desensitization by PKA of 5-HT_{1A} receptors expressed at $\approx$500 fmol/mg of protein in HeLa cells (Harrington et al., 1994). In our studies, the effect of PKA activators alone was much less dramatic in CHO-K1 cells expressing at least twice as many receptors as in the HA-7 HeLa cell clones used by Harrington et al. Three speculative hypotheses for those differences are as follows: (i) higher receptor numbers or receptor "reserve" may lead to a relative resistance to desensitization; (ii) higher amounts of $G_{i\alpha}$ proteins in CHO-K1 cells vs HeLa cells [5.7 vs 1.1 pmol/ mg of membrane protein; see Raymond et al. (1993b)] may confer relative resistance to desensitization, and (iii) other host cell-specific factors (phosphatases, etc.) may be important regulators of the desensitization process. Another possible explanation for the different results from Harrington et al. and the current report might involve different methodologies used by the two groups. In contrast to the use of cell monolayers in the current study, Harrington et al. detached cells with trypsin and then performed subsequent steps in cell suspensions. The buffers used by the two groups were different, and membranes were also harvested for use in a very different manner for the two studies. Finally, Harrington's results were obtained over a shorter time period than ours, which might have resulted in potential differences in *re*sensitization. If the results between the two studies are not secondary to methodological differences, they would indicate that there might indeed be cell-specific differences in the susceptibility of the 5-HT_{1A} receptor to desensitization. Along those lines, Falck-Pedersen et al. recently showed host cell specificity in agonist- and phorbol ester-induced desensitization of the thyrotropin-releasing hormone receptor (Falck-Pedersen et al., 1994).

As a cautionary note, we should emphasize that the current study only shows an association between phosphorylation and desensitization of the receptor by PKA and PKC. We do not address the effects of those kinases on other potential targets for desensitization in the signaling cascade. Indeed, Bauer et al. (1992) suggested a potential role for PKA in regulating the inhibitory effect of phosducin on $G_{s\alpha}$. Moreover, phosphorylation of several G proteins by PKC may inhibit their function (Katada et al., 1985; Carlson et al., 1989; Lounsbury et al., 1993; Strassheim, 1994). Those possibilities were not examined in this study.

It seems counterintuitive that PKA would participate in the desensitization of the 5-HT_{1A} receptor, which typically inhibits adenylyl cyclase. One speculation is that this pathway may provide the cell with a means of "turning-off" receptors when both PKA and PKC are activated. In any case, cooperative cross-talk at the level of serine-threonine kinases such as PKA and PKC may play an important role in receptor and cell physiology. The working hypothesis generated by these studies is that PKA phosphorylates the 5-HT_{1A} receptor at one site within the third intracellular loop. This PKA site may also be a substrate for PKC, or may participate in a hierarchal phosphorylation relationship with the three putative PKC sites within the receptor. An alternative possibility is that PKA has access to a limited number of the total cellular receptors through some form of compartmentalization into microdomains when compared with PKC. Further studies utilizing site-directed mutagenesis to alter those three sites will be necessary to elucidate their respective roles in the desensitization of the human 5-HT_{1A} receptor.

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