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Characterization of Differences Between Rapid Agonist-Dependent Phosphorylation and Phorbol Ester-Mediated Phosphorylation of Human Substance P Receptor in Intact Cells

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ABSTRACT

Substance P receptor (SPR), which plays a key role in pain transmission, is known to undergo rapid agonist-dependent desensitization and internalization. The present study shows that human SPR undergoes agonist-dependent phosphorylation in intact cells. Immunoprecipitation of SPR from $^{32}P_i$-labeled Chinese hamster ovary cells stably expressing human SPR (CHO-hSPR) indicates that substance P (SP) causes a rapid ($T_{1/2} < 1$ min), dose-dependent ($EC_{50} = 2$ nM), and pronounced (5-fold over basal) phosphorylation of SPR. Because SPR in CHO-hSPR couples to $G\alpha_q$, $G\alpha_s$, and $G\alpha_o$ (Roush and Kwatra, 1998), we examined the involvement of various second messenger-activated protein kinases in SPR phosphorylation. Although increases in intracellular cyclic AMP or treatment with the calcium ionophore A23187 do not cause SPR phosphorylation, treatment with the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) causes a 2.5-fold increase in SPR phosphorylation with a $T_{1/2}$ of <1 min. However, PKC inhibitor GF109203X has no effect on SP-dependent SPR phosphorylation. Furthermore, although SP treatment phosphorylates SPR on both serine and threonine residues equally, PMA treatment phosphorylates the receptor predominantly on serine residues. Two-dimensional phosphopeptide mapping data indicate that SP-dependent and PMA-dependent phosphorylations of SPR have some unique differences. Taken together, these data suggest that although activation of PKC by PMA can lead to SPR phosphorylation, PKC does not mediate SP-dependent phosphorylation of SPR. In conclusion, the present study represents the first demonstration and characterization of agonist-dependent and PMA-mediated phosphorylation of SPR in intact cells.

Substance P receptor (SPR) is a G protein-coupled receptor (GPCR) that mediates the effects of substance P (SP) – a neuropeptide of 11 amino acids believed to be involved in several physiological processes including pain transmission and inflammation (Otsuka and Yoshioka, 1993). Activation of SPR leads to stimulation of phosphoinositide hydrolysis (Weiss et al., 1982; Hunter et al., 1985), stimulation of adenylyl cyclase (AC; Nakajima et al., 1992; Yamashita et al., 1983), inhibition of AC (Laniyonu et al., 1988), and inhibition of an inward rectifying potassium channel (Nakajima et al., 1991). Furthermore, recent studies on rat SPR stably transfected in Chinese hamster ovary (CHO) cells have shown that SPR activation in these cells results in stimulation of phos-

phoinositide hydrolysis through phospholipase C (PLC), stimulation of AC, and stimulation of arachidonic acid metabolism (Nakajima et al., 1992; Garcia et al., 1994).

SPR is known to undergo agonist-dependent desensitization, which has been observed in cells naturally expressing SPR and with recombinant SPR stably expressed in CHO cells (McMillian et al., 1987; Menniti et al., 1991; Holland et al., 1993; Garland et al., 1996; Sanders and LeVine, 1996). Additionally, SPR undergoes a rapid agonist-dependent internalization (Mantyh et al., 1995; Sanders and LeVine, 1996). The molecular events that lead to SPR desensitization and internalization remain uncharacterized. Studies performed over the last several years on other GPCRs, such as β_2-adrenergic receptor and rhodopsin, indicate that agonist-dependent desensitization of GPCRs often involves phosphorylation of the activated receptor by GPCR kinases

ABBREVIATIONS: AC, adenylyl cyclase; CHO-hSPR, Chinese hamster ovary cells stably expressing human SPR; CHO-K1, Chinese hamster ovary cells, strain K1; DAG, diacylglycerol; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; hSPR, human substance P receptor; hSPR-Ab, human substance P receptor antibody; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; SP, substance P; SPR, substance P receptor; TLC, thin layer chromatography; TTBS, 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.02% Tween-20.

(GRKs) followed by binding of the phosphorylated receptor to a protein of the arrestin family, resulting in the disruption of receptor/G protein coupling (Freedman and Lefkowitz, 1996).

Although SPR has been shown to be a substrate for GRKs in vitro (Kwatra et al., 1993; Nishimura et al., 1998a), no information is available on SPR phosphorylation in intact cells. Therefore, we examined whether SPR undergoes agonist-dependent phosphorylation upon exposure to SP in intact cells. To this end, human SPR was stably expressed in Chinese hamster ovary cells and phosphorylation of the receptor was examined by immunoprecipitating the receptor from $^{32}P_i$-labeled Chinese hamster ovary cells stably expressing human SPR (CHO-hSPR). Our results show that within seconds of exposure to SP, SPR undergoes a rapid ($T_{1/2} < 1$ min) increase in phosphorylation.

Surprisingly, direct activation of protein kinase C (PKC) by phorbol esters also leads to rapid phosphorylation of SPR in intact cells, although PKC-mediated phosphorylation does not appear to be responsible for SP-dependent phosphorylation of SPR. Other second messenger-activated kinases are also not involved in SP-dependent SPR phosphorylation. We conclude that SP-dependent phosphorylation of SPR most likely occurs through the action of GRKs, although the possibility that PKC may play a role in phosphorylation of SPR under certain physiological conditions cannot be excluded.

Experimental Procedures

Materials. Aprotinin, bacitracin, chymostatin, isobutyric acid, leupeptin, *O*-phospho-DL-serine, *O*-phospho-DL-threonine, *O*-phospho-DL-tyrosine, phenylmethylsulfonyl fluoride, phorbol 12-myristate 13-acetate (PMA), soybean trypsin inhibitor, tosyl-lysine chloromethyl ketone-treated trypsin, and SP were obtained from Sigma (St. Louis, MO). [^{3}H]SR140,333 (27.8 Ci/mmol) and $H_3^{32}PO_4$ (specific activity 8500–9120 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Protein A Sepharose and unstained low molecular weight markers were purchased from Pharmacia (Piscataway, NJ). The PKC inhibitor GF109203X was obtained from Calbiochem (La Jolla, CA). An antibody against the 15 amino acids (KTMTESFSFSSNVLS) from the C-terminus of human SPR was raised in rabbits by Southern Biotechnology Associates, Inc. (Birmingham, AL). This human SPR antibody (hSPR-Ab) is suitable for immunoblotting as well as immunoprecipitating the receptor (Nishimura et al., 1998a). The nonpeptide SPR receptor antagonist CP99,994 was a gift from Dr. Saul Kadin (Pfizer, Inc., Groton, CT). The cDNA of human SPR in pBluescript was kindly provided by Dr. J. E. Krause (Washington University School of Medicine, St. Louis, MO). Chinese hamster ovary strain K1 (CHO-K1) cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in appropriate media at the cell culture facility of the Duke Comprehensive Cancer Center, Durham, NC. Generation of CHO-hSPR cells expressing approximately 200,000 receptors/cell has been described previously (Roush and Kwatra, 1998). Radioligand binding assays using SPR antagonist radioligand [^{3}H]SR140,333 were performed as described previously (Nishimura et al. 1998b).

Immunoblot Analysis. Immunoblotting was performed according to a protocol provided with an alkaline phosphatase conjugate substrate kit (Bio-Rad, Hercules, CA). Briefly, 10 μg each of crude membrane proteins from CHO-K1 and CHO-hSPR cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE) followed by electrophoretic transfer onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h with 5% nonfat milk in TTBS (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.02% Tween-20), probed with a 1:500 dilution of hSPR-Ab in TTBS, then incubated with a 1:3000 dilution in TTBS of a goat-anti-rabbit secondary anti-

body coupled to alkaline phosphatase. The immunoreactive proteins were visualized by immersing in a bromochloroindolyl phosphate/nitro blue tetrazolium solution prepared according to the protocol provided by the manufacturer (Bio-Rad, Hercules, CA).

Phosphorylation of SPR in CHO-hSPR Cells. CHO-hSPR cells were harvested from 175 cm^2 tissue culture dishes with PBS + 0.5 mM EDTA, washed once with 10 mM HEPES, pH 7.4 containing 118 mM NaCl, 4.3 mM KCl, 1.17 mM MgSO$_4$, 1.3 mM CaCl$_2$, 0.34 mM NaHCO$_3$, 11.7 mM glucose (HEPES/Krebs buffer), and then resuspended in HEPES/Krebs buffer. The cells were counted and aliquoted into a 50-ml conical tube at a concentration of 5×10^6 cells/ml. $H_3^{32}PO_4$ was added to the cells to give a final concentration of 200 μCi/ml, and the cells were incubated for 60 min at 37°C with agitation.

To initiate phosphorylation, 0.4-ml aliquots of $^{32}P_i$-labeled cells (2×10^6 cells) were added to 1.5-ml screw top microcentrifuge tubes containing 0.1 ml HEPES/Krebs buffer and the desired concentration of the compounds to be tested for stimulation of SPR phosphorylation. Each sample was incubated for the desired time at 37°C with agitation. At the end of the incubation, reactions were stopped by brief centrifugation at 12,000g in a microcentrifuge, followed by removal of media and addition of 1 ml of ice-cold lysis buffer (50 mM Tris-Cl, pH 7.4; 150 mM NaCl; 5 mM MgCl$_2$; 0.1 mM Na$_3$VO$_4$; 10 mM Na$_4$P$_2$O$_7$; 1 mM EGTA; 10 mM NaF; 0.5% NP-40; 1 mM benzamidine; 10 μg/ml leupeptin; 5 μg/ml aprotinin; 10 μg/ml soybean trypsin inhibitor; and 0.1 mM phenylmethylsulfonyl fluoride). The samples were allowed to incubate on ice for 5 min, then pelleted by centrifugation at 12,000g in a microcentrifuge for 15 min at 4°C. The supernatants were collected and incubated with 25 μl of Protein A Sepharose pre-equilibrated in lysis buffer for 20 min at 4°C. The Protein A Sepharose was pelleted by brief centrifugation and discarded, whereas supernatants were collected and incubated for 1 h with 5 μl (1:200 dilution) of hSPR-Ab; this antibody is equally effective at recognizing phosphorylated and nonphosphorylated receptor as determined by immunoblotting the receptor from control and SP- or PMA-treated CHO-hSPR cells (Fig. 3; PMA data not shown). The mixture was then incubated for 1 h at 4°C with 50 μl of Protein A Sepharose. The Protein A Sepharose was isolated by brief centrifugation in a microcentrifuge, washed twice with lysis buffer, and twice with lysis buffer containing 1 M NaCl. The Protein A Sepharose was then resuspended in SDS-PAGE sample buffer (2% SDS; 60 mM Tris-HCl, pH 6.8; 10% glycerol; 10% β-mercaptoethanol; and 0.025% bromophenol blue) and phosphorylated proteins were visualized by SDS-PAGE followed by autoradiography. Phosphorylation was quantitated by excising the receptor band from dried gels and counting in a scintillation counter. Background phosphorylation was not subtracted from basal or stimulated phosphorylation of the receptor; hence, the reported fold increase in hSPR phosphorylation by SP or PMA may be an underestimation.

Phosphoamino Acid Analysis on SPR Phosphorylated upon Treatments with SP or PMA. SP- or PMA-dependent phosphorylation of SPR was performed as described above, except the sample size was increased to 2×10^7 cells in a total volume of 2 ml, and SPR was immunoprecipitated with hSPR-Ab at 1:100 dilution. After SDS-PAGE, SPR was electrophoretically transferred to PVDF membrane (Kamps and Sefton, 1989). The membrane was stained with 1 μl/ml India Ink in 50 mM Tris-HCl (pH 6.5), 150 mM NaCl, and 0.2% Tween-20, dried, and exposed to autoradiography film overnight. PVDF membrane sections containing phosphorylated receptor were excised from the blot, washed once with methanol and three times with deionized water, and incubated with 500 μl 5.7 N HCl for 1 h at 110°C. The mixture was centrifuged for 5 min at maximum speed in a microcentrifuge; the supernatants were recovered and lyophilized overnight. Lyophilized samples were resuspended in 10 μl of isobutyric acid: 0.5 M NH$_4$OH (5:3) mixture containing 1 μM each of *O*-phospho-DL-serine, *O*-phospho-DL-threonine, and *O*-phospho-DL-tyrosine; they were then applied onto a cellulose thin-layer chromatography (TLC) plate (Kodak, Rochester, NY). Ten nanomoles of each

phosphoamino acid standard were also run separately on the TLC plate as a control. After attaching a paper towel to the top of the TLC plate, the plate was run for 12 h in isobutyric acid/0.5 M NH$_4$OH (5:3) mobile phase (Duclos et al., 1991). Phosphoamino acids were developed by a ninhydrin/cupric nitrate spray followed by a 2-min incubation at 110°C. After development, ^{32}P-incorporated phosphoamino acids were detected by autoradiography.

Two-Dimensional Phosphopeptide Mapping of SPR Phosphorylated in Response to SP or PMA. Phosphopeptides were identified using an alkaline electrophoretic system (Cheng et al., 1991). Briefly, gel slices containing SPR were excised from dried polyacrylamide gels, rehydrated, and washed in 10% acetic acid, 10% isopropanol, followed by a wash in 50% methanol. The gel slices were lyophilized and then rehydrated in 500 μl 100 mM NH$_4$HCO$_3$, pH 8.9, containing 30 μg trypsin. Gel slices were minced into small pieces and proteolysis was allowed to proceed overnight at 37°C. After 15 h, an additional 30 μg of trypsin was added to the reaction and proteolysis was allowed to proceed for an additional 5 h at 37°C. The gel slices were pelleted by brief centrifugation in a microcentrifuge and washed three times with 100 μl 100 mM NH$_4$HCO$_3$, pH 8.9. The digest supernatants and washes were pooled and lyophilized, then resuspended in 10 μl 1% NH$_4$HCO$_3$, pH 8.9, and applied to 20- × 20-cm cellulose TLC plates (Kodak, Rochester, NY). The plates were lightly moistened with 1% NH$_4$HCO$_3$, pH 8.9, and electrophoresed at 250 V for 2 h in the same buffer. After electrophoresis, the TLC plates were allowed to dry; then ascending chromatography was performed in butanol/acetic acid/pyridine/water (15:3:12:10 v/v) for 4 h. Phosphopeptides were visualized using a Storm 860 PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and by autoradiography.

Results

Characterization of Human SPR in CHO-hSPR Cells. When membranes from CHO-hSPR cells were subjected to immunoblotting with hSPR-Ab, we detected a broad protein band centered around 65 kDa (Fig. 1, lane 2). Because this band was not detected in membranes from untransfected CHO cells (Fig. 1, lane 1) or in the presence of 1 μM hSPR-Ab antigen peptide, we conclude that it corresponds to human SPR. A similar broad protein band between 56 and 64 kDa has been observed in CHO cells transfected with rat SPR; this band shifts to 43 to 46 kDa upon treatment with tunicamycin (Raddatz et al., 1995), indicating that the receptor is glycosylated.

Ligand binding studies indicated that the SPR antagonist radioligand [^{3}H]SR140,333 binds to CHO-hSPR cells with a K_d of 1.6 nM ± 0.2 nM (S.E.M.; n = 3), similar to the K_d reported for this radioligand with human SPR in Sf9 membranes (Nishimura et al., 1998b). Competition of [^{3}H]SR140,333 binding by SP indicates binding of SP to a single site with a K_d of 1.4 nM ± 0.6 nM (S.E.M.; n = 3). As described previously (Roush and Kwatra, 1998), agonist stimulation of SPR in CHO-hSPR cells activates multiple G proteins and stimulates phosphoinositide hydrolysis, cAMP formation, and arachidonic acid release. Furthermore, SPR in CHO-hSPR cells exhibits agonist-dependent desensitization (E.D.R. and M.M.K., unpublished observations). These results are similar to those obtained with rat SPR stably expressed in CHO cells (Nakajima et al., 1992; Garland et al., 1996; Sanders and LeVine, 1996).

Agonist-Dependent Phosphorylation of SPR in Intact Cells. CHO-hSPR cells were equilibrated with ^{32}P$_i$ to label cellular adenosine triphosphate (ATP) and exposed to SP in the presence and absence of the SPR antagonist CP99,994; the receptor was then immunoprecipitated with

hSPR-Ab. As seen in Fig. 2, a broad protein band centered at approximately 65 kDa is phosphorylated upon stimulation of CHO-hSPR cells with SP. This band corresponds to SPR immunoreactivity detected in CHO-hSPR membranes (Fig. 1). In addition, immunoprecipitation of this phosphoprotein can be blocked by incubation with 1 μM of the hSPR-Ab antigen peptide (data not shown). Therefore, we conclude that the phosphorylated 65-kDa band is SPR. The phosphorylation of SPR by SP is blocked by the SPR antagonist CP99,994 (Fig. 2, lane 3) indicating that SPR phosphorylation requires receptor activation.

Because hSPR-Ab is derived from the C-terminus of human SPR, which contains potential phosphorylation sites, it is important to know whether hSPR-Ab is competent to recognize and immunoprecipitate phosphorylated SPR. To this end, we examined the ability of hSPR-Ab to recognize phosphorylated SPR. Figure 3 (left) shows immunoblotting of SPR in cell lysates from control, SP-treated, and CP99,994/SP-treated CHO-hSPR cells. As can be seen, hSPR-Ab is equally effective in recognizing phosphorylated (Fig. 3, lane 2) and unphosphorylated SPR (Fig. 3, lanes 1 and 3). Of note, phosphorylated SPR (Fig. 3, lane 2) exhibits a striking decrease in electrophoretic mobility due to receptor phosphorylation. Furthermore, after immunoprecipitation with hSPR-Ab, no SPR immunoreactivity is seen in control, SP-treated, and CP99,994/SP-treated groups, demonstrating that hSPR-Ab is
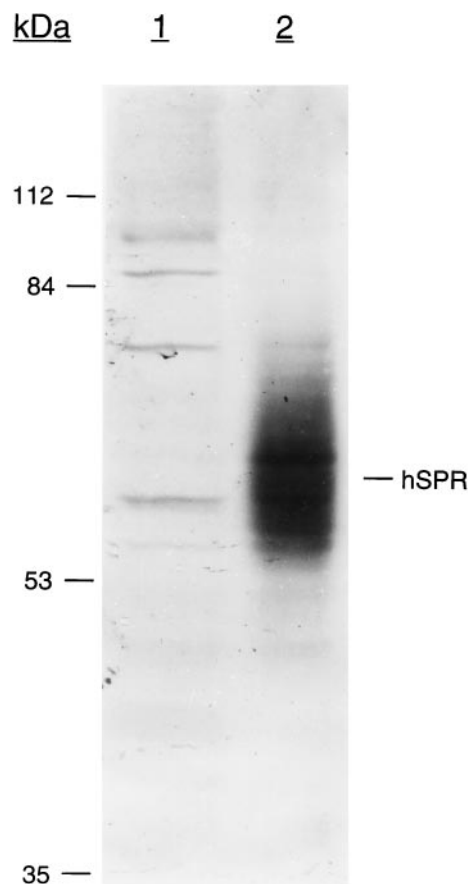

Fig. 1. Immunoblot analysis of human SPR expression in CHO cells. Ten micrograms of crude membrane proteins from both untransfected CHO-K1 cells (lane 1) and CHO-hSPR cells (lane 2) were subjected to SDS-PAGE, electrophoretically transferred to PVDF membrane, and immunoblotted with hSPR-Ab (1:500) as described in *Experimental Procedures*.

equally effective in immunoprecipitation of phosphorylated and unphosphorylated SPR (Fig. 3, lanes 4–6). Therefore, it is clear that the SP-induced increase in SPR phosphorylation

Fig. 2. SP-dependent phosphorylation of human SPR in intact cells. $^{32}P_i$-labeled CHO-hSPR cells were treated with or without SPR ligands for 20 min at 37°C. The receptor was immunoprecipitated, resolved on 10% SDS-PAGE, and visualized by autoradiography. A representative autoradiogram is shown. Lane 1, control; lane 2, 0.1 μM SP; lane 3, 0.1 μM SP + 0.1 mM CP99,994.
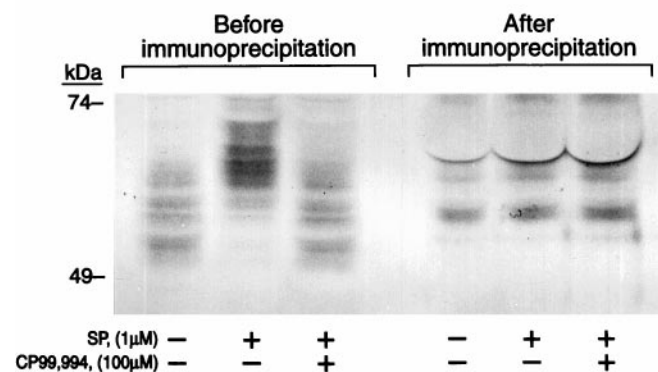
Fig. 3. Effectiveness of hSPR-Ab to recognize phosphorylated and unphosphorylated SPR. $^{32}P_i$-labeled CHO-hSPR cells (7 × 10^6 cells/group) were treated with or without SPR ligands for 10 min at 37°C and taken in 1 ml of lysis buffer. Eighty microliters of cell lysates from each group were analyzed for SPR immunoreactivity before (lanes 1–3) and after (lanes 4–6) immunoprecipitation with hSPR-Ab as described in *Experimental Procedures*. The samples were subjected to SDS-PAGE, electrophoretically transferred to PVDF, and immunoblotted with hSPR-Ab as described in *Experimental Procedures*.

shown in Fig. 2 is due to an increase in receptor phosphorylation and not due to significant differences in the amount of receptor between control and SP-treated groups.

Characterization of SP-Dependent Phosphorylation of SPR in CHO-hSPR Cells. Stimulation of SPR phosphorylation by SP is 5.0 ± 1.0-fold (S.E.M., n = 34) over the basal levels and occurs rapidly after SP exposure; the $T_{1/2}$ of SP-dependent phosphorylation of SPR is <1 min (Fig. 4A). This time course of SP-dependent phosphorylation of SPR precedes agonist-dependent desensitization and internalization
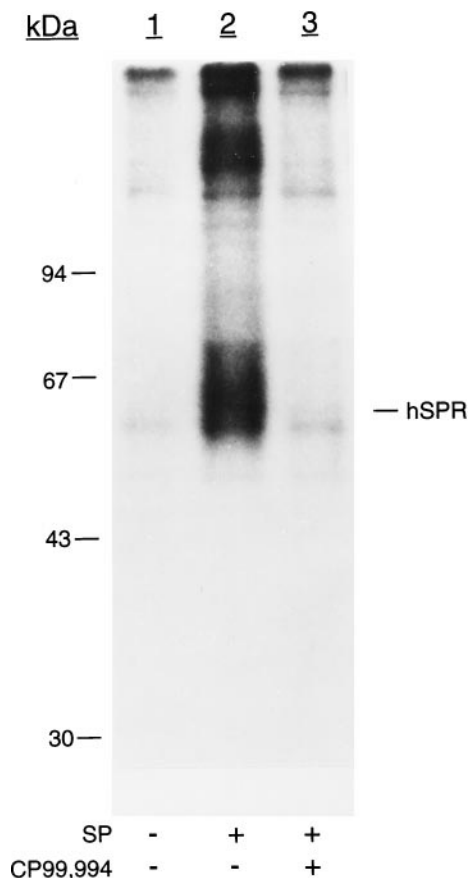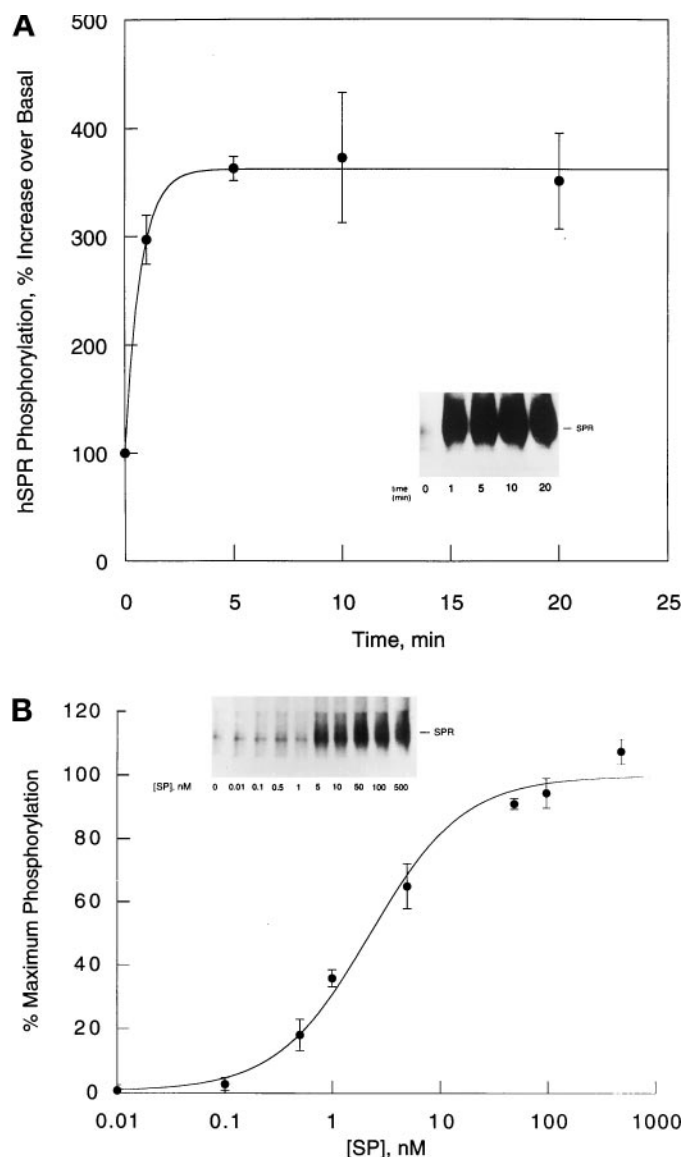

Fig. 4. Characterization of SP-dependent phosphorylation of human SPR in CHO-hSPR cells. A, time course of SP-dependent SPR phosphorylation. $^{32}P_i$-labeled CHO-hSPR cells were stimulated with 1 μM SP at 37°C for the indicated times. Immunoprecipitated receptor was visualized by SDS-PAGE followed by autoradiography. Quantitation of results was achieved as described in *Experimental Procedures*. The results shown are the mean ± S.E.M. of three experiments; a representative autoradiogram is shown in the insert. B, concentration dependence of SP-dependent SPR phosphorylation. $^{32}P_i$-labeled CHO-hSPR cells were stimulated with the indicated concentrations of SP for 20 min at 37°C. Immunoprecipitated receptor was visualized by SDS-PAGE followed by autoradiography. Quantitation of results was achieved as described in *Experimental Procedures*. The results shown are the mean ± S.E.M. of three experiments; a representative autoradiogram is shown in the inset.

of SPR as reported in the literature (McMillian et al., 1987; Menniti et al., 1991; Holland et al., 1993; Mantyh et al., 1995; Garland et al., 1996; Sanders and LeVine, 1996). Furthermore, SP-dependent phosphorylation is dependent on the concentration of SP, occurring with an EC_{50} of 2 nM (Fig. 4B); this EC_{50} is consistent with the K_d of SP binding to SPR in intact CHO-hSPR cells.

Mechanism of SP-Dependent Phosphorylation of SPR. As we reported recently (Roush and Kwatra, 1998), stimulation of SPR in CHO-hSPR cells activates multiple G proteins ($G\alpha_q$, $G\alpha_s$, $G\alpha_o$) and stimulates AC, PLC, and arachidonic acid release. Activation of these pathways can lead to the activation of several protein kinases. For example, stimulation of AC results in increased levels of intracellular cAMP, leading to the activation of protein kinase A (PKA), whereas stimulation of PLC results in the hydrolysis of membrane phosphoinositides into inositol triphosphate and diacylglycerol (DAG). When inositol triphosphate is formed, it releases Ca^{2+} from intracellular stores, leading to the activation of Ca^{2+}-dependent protein kinases. DAG, on the other hand, activates PKC. To determine if any of these kinases play a role in SP-dependent phosphorylation of human SPR, we increased the levels of various second messengers by pharmacological means. As shown in Fig. 5, SPR phosphorylation is not increased by dibutyryl cAMP, a cell permeable analog of cAMP, or forskolin, which directly activates AC and increases cAMP levels (Laurenza et al., 1989). These results indicate that SP-dependent phosphorylation of SPR does not involve PKA. Furthermore, SP-dependent phosphorylation of SPR does not involve Ca^{2+}-dependent protein kinases since the calcium ionophore A23187 does not increase SPR phosphorylation (Fig. 5).

We next studied the effect of PMA, a phorbol ester that directly activates PKC in a manner similar to DAG (Castagna et al., 1982). We find that PMA causes a 2.5 ± 0.4-fold (S.E.M.; $n = 32$) increase in SPR phosphorylation over basal levels; the time course of PMA-dependent phosphorylation of SPR (Fig. 6A) is similar to that seen for SP-dependent phosphorylation of SPR (Fig. 4A). Furthermore, PMA-induced phosphorylation of SPR is dependent on the concentration of PMA and occurs with an EC_{50} of 190 nM (Fig. 6B); this value is similar to the EC_{50} of PMA to activate PKC (Evans et al., 1991). These results indicate that PMA-mediated phosphorylation of SPR proceeds through PKC. Consistent with this interpretation, treatment with 4α-phorbol-12,13-didecano-

ate, a phorbol ester that does not activate PKC (Castagna et al., 1982), has no effect on SPR phosphorylation (data not shown).

Role of PKC in SP-Induced Phosphorylation of Human SPR in CHO-hSPR Cells. Because PMA substantially increases SPR phosphorylation with a time course similar to SP-dependent phosphorylation, it follows that SP-dependent phosphorylation of SPR may involve PKC. To test this possibility, we studied the effect of GF109203X, a selective inhibitor of PKC (Toullec et al., 1991) on SP-dependent phosphorylation of SPR. As shown in Fig. 7, GF109203X inhibits
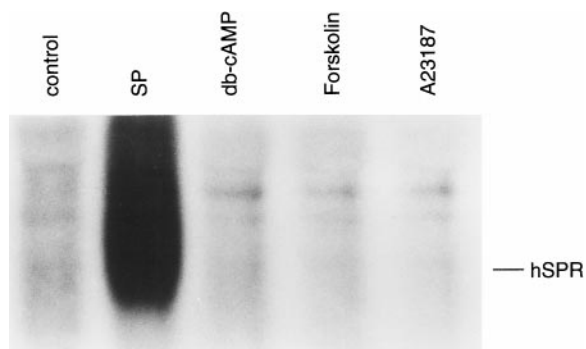
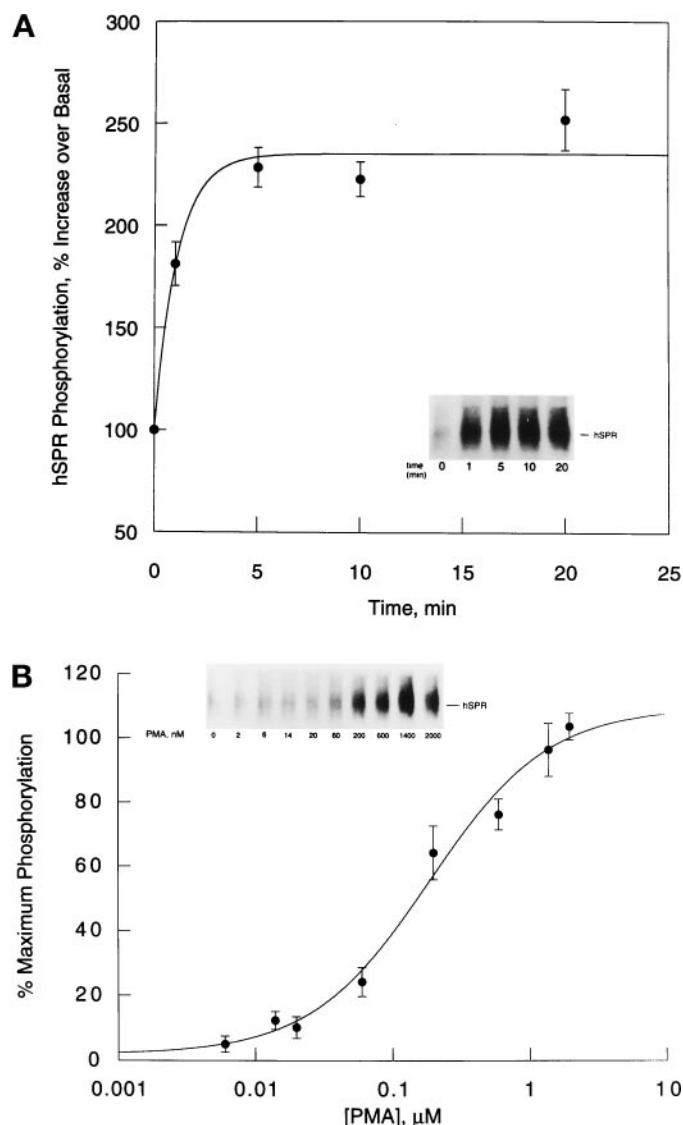**Fig. 6.** Characterization of PMA-stimulated phosphorylation of human SPR. A, time course of PMA-stimulated SPR phosphorylation. $^{32}P_i$-labeled CHO-hSPR cells were stimulated with 2 μM PMA at 37°C for the indicated times. Immunoprecipitated receptor was visualized by SDS-PAGE/autoradiography. Quantitation of results was achieved as described in *Experimental Procedures*. The results shown are the mean $\pm$ S.E.M. of two experiments; a representative autoradiogram is shown in the inset. B. concentration dependence of PMA-stimulated SPR phosphorylation. $^{32}P_i$-labeled CHO-hSPR cells were stimulated with the indicated concentrations of PMA for 20 min at 37°C. Immunoprecipitated receptor was visualized by SDS-PAGE/autoradiography. Quantitation of results was achieved as described in *Experimental Procedures*. The results shown are the mean $\pm$ S.E.M. of three experiments; a representative autoradiogram is shown in the insert.

Fig. 5. Second messengers and phosphorylation of human SPR. $^{32}P_i$-labeled CHO-hSPR cells were stimulated with the indicated agents for 20 min at 37°C. Immunoprecipitated receptor was visualized by SDS-PAGE/autoradiography. Lane 1, control; lane 2, 1 μM SP; lane 3, 2 mM dibutyrl-cAMP; lane 4, 50 μM Forskolin; lane 5, 10 μM A23187.

PMA-dependent phosphorylation of SPR, providing additional evidence that PMA-dependent phosphorylation of SPR occurs through PKC. However, GF109203X has no effect on SP-dependent phosphorylation of SPR, suggesting that SP-dependent phosphorylation of SPR does not involve PKC. Thus, although PKC may be activated upon agonist-stimulation of SPR, it does not appear to be the kinase responsible for the SP-dependent phosphorylation of SPR. Interestingly, the extent of SPR phosphorylation in the presence of both SP and PMA, with or without GF109203X, is not statistically different from the extent of SPR phosphorylation observed with SP alone. These results suggest two possibilities: either the protein kinases activated by SP and PMA phosphorylate SPR on overlapping sites, or PKC does not act on SPR phosphorylated during treatment with SP.

Because SPR phosphorylation can occur through the activation of PKC, we examined whether PKC activation through stimulation of other PLC-coupled receptors leads to SPR phosphorylation. To this end, we stimulated thrombin and ATP receptors endogenously present in CHO cells (Carney, 1983; Freund et al., 1994). Activation of thrombin and ATP receptors in CHO-hSPR cells increases inositol phosphate levels 2- and 8-fold respectively (data not shown). However, activation of these receptors has no effect on SPR phosphorylation (Fig. 8).

Characterization of SP- and PMA-Dependent Phosphorylation of Human SPR. We identified the amino acids of SPR phosphorylated in response to SP or PMA. As shown in Fig. 9, both SP- and PMA-dependent phosphorylation of SPR occur only on serine and threonine residues. However, SP-dependent phosphorylation occurs almost equally on both serine and threonine residues, whereas PMA-dependent phosphorylation primarily occurs on serine residues. We next examined 2-dimensional phosphopeptide maps of tryptic digests of SPR phosphorylated in response to stimulation with

SP (Fig. 10A) or PMA (Fig. 10B). The phosphopeptide maps of SPR phosphorylated upon stimulation with SP or PMA are quite similar with two significant differences. First, phosphopeptide 7 is absent in maps from PMA-treated cells; this finding is consistent with the observation that the extent of SPR phosphorylation with PMA is less than that seen with SP. Second, despite the lower total phosphorylation of SPR from PMA-treated cells, the intensity of phosphopeptide 2 is greater in SPR from PMA-treated cells than from SP-treated cells, suggesting that this peptide contains predominantly PKC sites. Taken together, these data provide further evidence that protein kinases distinct from PKC are involved in catalyzing SP-dependent phosphorylation of SPR.

Discussion

While previous studies from our laboratory have documented that rat and human SPR undergo agonist-dependent phosphorylation by GRKs in vitro (Kwatra et al., 1993; Nish-
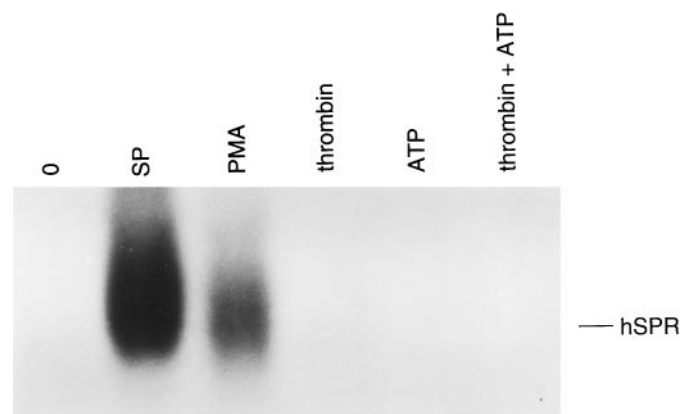
Fig. 8. Activation of endogenous thrombin and ATP receptors in CHO-hSPR cells and SPR phosphorylation. $^{32}P_i$-labeled CHO-hSPR cells were stimulated with the indicated agents for 20 min at 37°C. Immunoprecipitated receptor was visualized by SDS-PAGE/autoradiography. A representative autoradiogram is shown. Lane 1, control; lane 2, 0.1 μM SP; lane 3, 10 μM PMA; lane 4, 1 U/ml thrombin; lane 5, 10 μM ATP; lane 6, 1 U/ml thrombin + 10 μM ATP. The experiment was repeated four times with similar results.
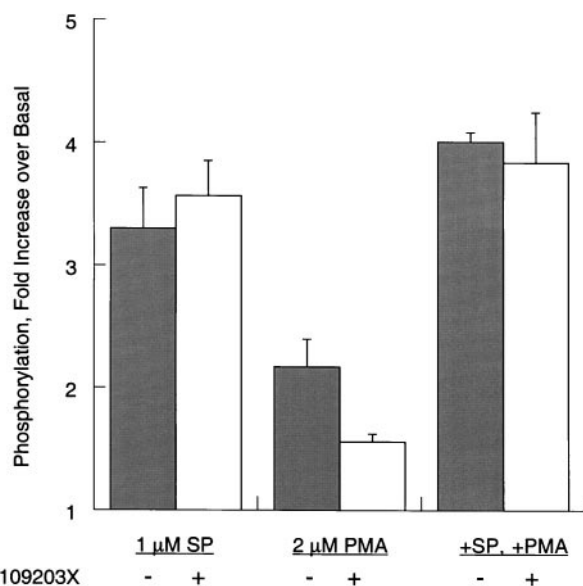
Fig. 7. Effect of PKC inhibitor GF109203X on SP-stimulated and PMA-stimulated phosphorylation of human SPR. $^{32}P_i$-labeled CHO-hSPR cells were stimulated with SP (1 μM) and/or PMA (2 μM) in the presence or absence of the specific PKC inhibitor GF109203X (5 μM). Immunoprecipitated receptor was visualized by SDS-PAGE/autoradiography and quantitated as described in *Experimental Procedures*. The results shown are the mean ± S.E.M. of three experiments.
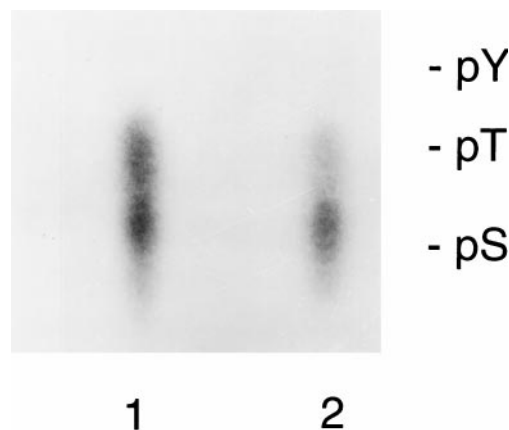
Fig. 9. Phosphoamino acid analysis of human SPR phosphorylated in response to SP or PMA. $^{32}P_i$-labeled CHO-hSPR cells were treated with 1 μM SP (lane 1) or 2 μM PMA (lane 2) for 20 min at 37°C. Phosphorylated SPR was immunoprecipitated, subjected to SDS-PAGE, transferred to PVDF, then hydrolyzed by treatment with 5.7 N HCl for 1 h at 110°C. Phosphoamino acids were resolved by TLC in isobutyric acid/500 mM NH$_4$OH (5:3) and visualized by autoradiography.

imura et al., 1998a), the present study represents the first demonstration and characterization of agonist-dependent phosphorylation of SPR in intact cells. Furthermore, the present study also represents the first demonstration that SPR undergoes PKC-mediated phosphorylation.

Studies performed over the last 15 years on SPR in a variety of systems have shown that within minutes of exposure to SP, SPR is desensitized and internalized (McMillian et al., 1987; Menniti et al., 1991; Holland et al., 1993; Mantyh et al., 1995; Garland et al., 1996; Sanders and LeVine, 1996). The present study shows that human SPR undergoes a rapid agonist-dependent phosphorylation in intact cells. Because this event occurs very rapidly ($T_{1/2} < 1$ min), it is possibly linked to receptor desensitization and internalization. This notion is supported by studies showing that the removal of the carboxyl tail of SPR, a region that carries most of the potential phosphorylation sites, makes the receptor more resistant to desensitization (Sasakawa et al., 1994; Li et al., 1997). However, it should be mentioned that although agonist-dependent phosphorylation is considered a key step in the desensitization of GPCRs, agonist-dependent desensiti-

Fig. 10. Two-dimensional phosphopeptide maps of human SPR phosphorylated in response to SP or PMA. $^{32}P_i$-labeled CHO-hSPR cells were treated with 1 μM SP (A) or 2 μM PMA (B) for 20 min at 37°C. Phosphopeptides were generated by digestion of phosphorylated SPR with trypsin, mapped in the first dimension by thin layer electrophoresis in 1% NH_4HCO_3, pH 8.9, followed by ascending chromatography in butanol/acetic acid/pyridine/water (15:3:12:10 v/v). These maps are representative of three similar experiments.

zation has been noted in chemoattractant receptors in the absence of phosphorylation (Kim et al., 1997). Therefore, further work is needed to delineate the precise role of agonist-dependent phosphorylation of SPR in agonist-dependent desensitization and internalization of SPR.

Having demonstrated agonist-dependent phosphorylation of SPR, the next goal of our studies was to examine which, if any, of the several signal transduction pathways stimulated by SPR activation contribute to receptor phosphorylation (Roush and Kwatra, 1998). By activating individual components of signal transduction pathways, we have ruled out the involvement of PKA and Ca^{2+}-dependent protein kinases in SPR phosphorylation. Interestingly, PKC activation via PMA leads to substantial SPR phosphorylation with a time course similar to that seen with SP-dependent phosphorylation. Although these results suggest a role for PKC in SP-dependent phosphorylation of SPR, this possibility is ruled out because PKC inhibitor GF109203X has no effect on SP-dependent phosphorylation of SPR. Therefore, we suggest that SP-dependent phosphorylation of SPR in intact cells may involve GRK2, a widely distributed member of the GRK family. This proposal is consistent with our in vitro data showing that human and rat SPR are good substrates for GRK2 (Kwatra et al., 1993: Nishimura et al., 1998a). Furthermore, preliminary screening of CHO-hSPR cell homogenates with a panel of anti-GRK antibodies indicates the presence of GRK2 in CHO cells (data not shown). However, our data do not rule out the involvement of protein kinases other than GRK2 in SP-dependent phosphorylation of SPR. In this connection, it is important to note that casein kinase 1α has recently been shown to phosphorylate m3-muscarinic receptor in an agonist-dependent manner (Tobin et al., 1997).

An intriguing finding of the present study is that PKC-mediated phosphorylation of human SPR occurs when PKC is activated with PMA but not with DAG formed by receptor activation. One explanation for this observation could be that DAG formed by SPR stimulation is not sufficient to fully activate PKC. This explanation is consistent with our observing no effect on SPR phosphorylation after stimulation of thrombin and ATP receptors. One should also consider the possibility that if stimulation of SPR activates both GRK2 and PKC, the receptor will be phosphorylated by the enzyme for which it has the highest affinity. Because human SPR is a very good substrate of GRK2 (Nishimura et al., 1998a), it will likely be phosphorylated mainly by GRK2 even though PKC may also be activated. Further work is clearly needed to explain why PKC is not involved in SP-dependent phosphorylation of SPR. However, it should be noted that several other PLC-coupled receptors including m3-muscarinic (Tobin and Nahorski, 1993) and thromboxane receptor (Habib et al., 1997) have been shown to undergo PKC-mediated phosphorylation when stimulated with PMA, but PKC does not play a role in their agonist-dependent phosphorylation.

The finding that human SPR undergoes PMA-dependent phosphorylation mediated through PKC suggests a role for PKC in SPR function. This finding may explain the reported PMA-induced desensitization of rat SPR in parotid acinar cells (Sugiya et al., 1988; Sugiya and Putney, 1988) and human SPR in UC11 astrocytoma cells (Barr and Watson, 1994). PMA-induced desensitization of SPR, however, has been reported to be weaker than agonist-induced desensitization of SPR (Sugiya et al., 1988). This difference between
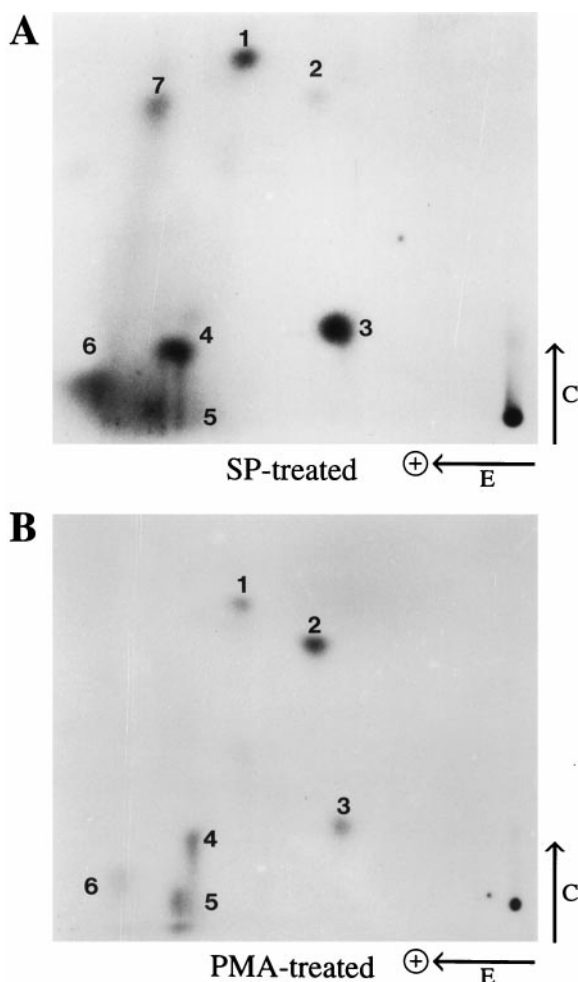
SP and PMA-dependent desensitization of SPR is consistent with our observation that treatment with PMA results in reduced phosphorylation of the receptor relative to treatment with SP. The findings that phosphopeptide 7 is absent from 2-dimensional maps on SPR from PMA-treated cells and phosphopeptide 2 is more heavily phosphorylated (Fig. 10) support this hypothesis; future studies will attempt to identify these phosphopeptides to better understand SP- and PMA-dependent desensitization of SPR.

Although PMA-dependent phosphorylation of SPR clearly occurs through PKC, we do not yet know whether PKC acts directly or through another kinase. A direct action of PKC on SPR is possible because an examination of the amino acid sequence of SPR indicates the presence of several potential PKC sites. Alternatively, PKC may be phosphorylating SPR through another kinase. In this connection, it is pertinent to note that PKC has been shown to phosphorylate and activate GRK2 (Chuang et al., 1995; Winstel et al., 1996).

In summary, the results of the present study show that human SPR undergoes a rapid agonist-dependent phosphorylation in intact cells under conditions known to result in receptor desensitization and internalization. Furthermore, SPR also undergoes an equally rapid PKC-mediated phosphorylation, but the extent of PKC-mediated phosphorylation of SPR is about 50% of SP-dependent phosphorylation. Although PKC-mediated phosphorylation of SPR clearly occurs, PKC does not catalyze SP-dependent phosphorylation of SPR. Further work is needed to determine whether phosphorylation of SPR by PKC has physiological significance.

Acknowledgments

References

Barr AJ and Watson SP (1994) Protein kinase C mediates delayed inhibitory feedback regulation of human neurokinin type 1 receptor activation of phospholipase C in UC11 astrocytoma cells. *Mol Pharmacol* **46:**266–273.

Carney DH (1983) Immunofluorescent visualization of specifically bound thrombin reveals cellular heterogeneity in number and density of preclustered receptors. *J Cell Physiol* **117:**297–307.

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U and Nishizuka Y (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* **257:**7847–7851.

Cheng SH, Rich DP, Marshall J, Gregory RJ, Welsh MJ and Smith AE (1991) Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell* **66:**1027–1036.

Chuang TT, LeVine III H and De Blasi A (1995) Phosphorylation and activation of β-adrenergic receptor kinase by protein kinase C. *J Biol Chem* **270:**18660–18665.

Duclos B, Marcandier S and Cozzone AJ (1991) Chemical properties and separation of phosphoamino acids by thin-layer chromatography and/or electrophoresis. *Methods Enzymol* **201:**10–21.

Evans FJ, Parker PJ, Olivier AR, Thomas S, Ryves WJ, Evans AT, Gordge P and Sharma P (1991) Phorbol ester activation of the isotypes of protein kinase C from bovine and rat brain. *Biochem Soc Trans* **19:**397–402.

Freedman NJ and Lefkowitz RJ (1996) Desensitization of G protein-coupled receptors. *Recent Prog Horm Res* **51:**319–351.

Freund S, Ungerer M and Lohse MJ (1994) A_1 Adenosine receptors expressed in CHO-cells couple to adenylyl cyclase and to phospholipase C. *Naunyn-Schmiedeberg's Arch Pharmacol* **350:**49–56.

Garcia M, Sakamoto K, Shigekawa M, Nakanishi S and Ito S (1994) Multiple mechanisms of arachidonic acid release in Chinese hamster ovary cells transfected with cDNA of substance P receptor. *Biochem Pharmacol* **48:**1735–1741.

Garland AM, Grady EF, Lovett M, Vigna SR, Frucht MM, Krause JE and Bunnett NW (1996) Mechanism of desensitization and resensitization of G protein-coupled neurokinin₁ and neurokinin₂ receptors. *Mol Pharmacol* **49:**438–446.

Habib A, Vezza R, Creminon C, Maclouf J and FitzGerald GA (1997) Rapid, agonist-dependent phosphorylation *in vivo* of human thromboxane receptor isoforms. *J Biol Chem* **272:**7191–7200.

Holland LN, Goldstein BD and Aronstam RS (1993) Substance P receptor desensitization in the dorsal horn: Possible involvement of receptor-G protein complexes. *Brain Res* **600:**89–96.

Hunter JC, Goedert M and Pinnock RD (1985) Mammalian tachykinin-induced hydrolysis of inositol phospholipids in rat brain slices. *Biochem Biophys Res Commun* **127:**616–622.

Kamps MP and Sefton BM (1989) Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates analysis of phosphoamino acids is gel-fractionated proteins. *Anal Biochem* **176:**22–27.

Kim J-Y, Soede RDM, Schaap P, Valkema R, Borleis JA, Van Haastert PJM, Devreotes PN and Hereld D (1997) Phosphorylation of chemoattractant receptors is not essential for chemotaxis or termination of G protein-mediated responses. *J Biol Chem* **272:**27313–27318.

Kwatra MM, Schwinn DA, Schreurs J, Blank JL, Kim CM, Benovic JL, Krause JE, Caron MG and Lefkowitz RJ (1993) The substance P receptor, which couples to $G_{q/11}$, is a substrate of β-adrenergic receptor kinase 1 and 2. *J Biol Chem* **268:**9161–9164.

Laniyonu A, Sliwinski-Lis E and Fleming N (1988) Different tachykinin receptor subtypes are coupled to the phosphoinositide or cyclic AMP signal transduction pathways in rat submandibular cells. *FEBS Lett* **240:**186–190.

Laurenza A, McHugh Sutkowski E and Seamon KB (1989) Forskolin: A specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends Pharmacol Sci* **10:**442–447.

Li H, Leeman SE, Slack BE, Hauser G, Saltsman WS, Krause JE, Blusztajn JK and Boyd ND (1997) A substance P (neurokinin-1) receptor mutant carboxyl-terminally truncated to resemble a naturally occurring receptor isoform displays enhanced responsiveness and resistance to desensitization. *Proc Natl Acad Sci USA* **94:**9475–9480.

Mantyh PW, Allen CJ, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR and Maggio JE (1995) Rapid endocytosis of a G protein-coupled receptor: Substance P evoked internalization of its receptor in the rat striatum *in vivo. Proc Natl Acad Sci USA* **92:**2622–2626.

McMillian MK, Soltoff SP and Talamo BR (1987) Rapid desensitization of substance P- but not carbachol-induced increases in inositol triphosphate and intracellular Ca^{++} in rat parotid acinar cells. *Biochem Biophys Res Commun* **148:**1017–1024.

Menniti FS, Takemura H, Oliver KG and Putney Jr. JW (1991) Different modes of regulation for receptors activating phospholipase C in the rat pancreatoma cell line AR4–2J. *Mol Pharmacol* **40:**727–733.

Nakajima Y, Nakajima S and Inoue M (1991) Substance P induced inhibition of potassium channels via a pertussis toxin-insensitive G protein. *Ann NY Acad Sci* **632:**103–111.

Nakajima Y, Tsuchida K, Negishi M, Ito S and Nakanishi S (1992) Direct linkage of three tachykinin receptors to stimulation of both phosphatidylinositol hydrolysis and cyclic AMP cascades in transfected Chinese hamster ovary cells. *J Biol Chem* **267:**2437–2442.

Nishimura K, Frederick J and Kwatra MM (1998b) Human substance P receptor expressed in Sf9 cells couples with multiple endogenous G proteins. *J Recept Signal Transduct Res* **18:**51–65.

Nishimura K, Warabi K, Roush ED, Frederick J, Schwinn DA and Kwatra MM (1998a) Characterization of GRK-2-catalyzed phosphorylation of the human substance P receptor in Sf9 membranes. *Biochemistry* **37:**1192–1198.

Otsuka M and Yoshioka K (1993) Neurotransmitter functions of mammalian tachykinins. *Physiol Rev* **73:**229–308.

Raddatz R, Crankshaw CL, Snider RM and Krause JE (1995) Similar rates of phosphatidylinositol hydrolysis following activation of wild-type and truncated rat neurokinin-1 receptors. *J Neurochem* **64:**1183–1191.

Roush ED and Kwatra MM (1998) Human substance P receptor expressed in Chinese hamster ovary cells directly activates Gαq/ll, Gαs, and Gαo. *FEBS Lett* **428:**291–294.

Sanders MA and LeVine III H (1996) Desensitization of the neurokinin 1 receptor is mediated by the receptor carboxy-terminal region but is not caused by receptor internalization. *J Neurochem* **67:**2362–2372.

Sasakawa N, Sharif M and Hanley MR (1994) Attenuation of agonist-induced desensitization of the rat substance P receptor by progressive truncation of the C-terminus. *FEBS Lett* **347:**181–184.

Sugiya H, Obie JF and Putney JW Jr (1988) Two modes of regulation of the phospholipase C-linked substance P receptor in rat parotid acinar cells. *Biochem J* **253:**459–466.

Sugiya H and Putney JW Jr (1988) Protein kinase C-dependent and -independent mechanisms regulating the parotid substance P receptor as revealed by differential effects of protein kinase C inhibitors. *Biochem J* **256:**677–680.

Tobin AB and Nahorski SR (1993) Rapid agonist-mediated phosphorylation of m3-muscarinic receptors revealed by immunoprecipitation. *J Biol Chem* **268:**9817–9823.

Tobin AB, Totty NF, Sterlin AE and Nahorski SR (1997) Stimulus-dependent phosphorylation of G protein-coupled receptors by casein kinase 1α. *J Biol Chem* **272:**20844–20849.

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D and Kirilovsky J (1991) The bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **266:**15771–15781.

Weiss SJ, McKinney JS and Putney JW Jr (1982) Receptor-mediated net breakdown of phosphatidylinositol 4,5-bisphosphate in parotid acinar cells. *Biochem J* **206:**555–560.

Winstel R, Freund S, Krasel C, Hoppe E and Lohse MJ (1996) Protein kinase cross-talk: Membrane targeting of the β-adrenergic receptor kinase by protein kinase C. *Proc Natl Acad Sci USA* **93:**2105–2109.

Yamashita K, Koide Y and Aiyoshi Y (1983) Effects of substance P on thyroidal cyclic AMP levels and thyroid hormone release from canine thyroid slices. *Life Sci* **32:**2163–2166.

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