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Cross Talk Between m3-Muscarinic and β_2-Adrenergic Receptors at the Level of Receptor Phosphorylation and Desensitization

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ABSTRACT

In this study we investigated cross talk between m3-muscarinic and β_2-adrenergic receptors coexpressed in Chinese hamster ovary (CHO-m3/β_2) cells, focusing on two possible mechanisms of regulation. The first mechanism is based on recent in vitro studies demonstrating that G protein-coupled receptor kinase (GRK) activity, the protein kinase responsible for β_2-adrenergic receptor homologous phosphorylation and desensitization, may be regulated by calcium/calmodulin and membrane phosphatidylinositol 4,5-bisphosphate. Stimulation of the phospholipase C signaling pathway via m3-muscarinic receptors in CHO-m3/β_2 cells increased intracellular free calcium by ~10 fold and membrane phosphatidylinositol 4,5-bisphosphate levels decreased by ~74%. However, despite these changes the ability of endogenous kinases, possibly the GRKs, to phosphorylate the β_2-adrenergic receptor was not altered. The second mechanism investigated involves a direct heterologous phosphorylation of the β_2-adrenergic receptor after muscarinic receptor stimulation. Activation of m3-muscarinic receptors did mediate heterologous phosphorylation of β_2-adrenergic receptors in a GRK-independent fashion, via protein kinase C. Heterologous β_2-adrenergic receptor phosphorylation correlated with receptor desensitization as measured by a loss in guanine-nucleotide sensitive-high affinity agonist binding and reduction in maximal cAMP response. This receptor cross talk may have a profound physiological importance in a wide variety of cell types, for example smooth muscle, where these two receptors are known to be coexpressed.

This study aimed to investigate the ability of phospholipase C (PLC)-coupled m3-muscarinic receptors to regulate β_2-adrenergic receptor function. This question has physiological relevance because many cell types, including smooth muscle such as airway smooth muscle (Eglen et al., 1994), coexpress these two G protein-coupled receptors. Our study focuses on two potential regulatory mechanisms. The first of which is based on recent in vitro data suggesting that changes in intracellular calcium (Chuang et al., 1996; Pronin et al., 1997) and membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) (Pitcher et al., 1996; DebBurman et al., 1996) levels may regulate the activity of G protein-coupled receptor kinases (GRKs) known to phosphorylate and desensitize β_2-adrenergic receptors. Because activation of the PLC-pathway will both increase intracellular free calcium and decrease membrane PIP_2 levels, this may be a potential mechanism by which m3-muscarinic receptors could influence homologous GRK-mediated β_2-adrenergic receptor phosphorylation. The second mechanism investigated addresses the possibility that m3-muscarinic receptors may mediate heterologous phosphorylation of the β_2-adrenergic receptor in a GRK-independent manner.

It is now well established that agonist-mediated phosphorylation of the β_2-adrenergic receptor results in receptor desensitization (for reviews see; Hausdorff et al., 1990; Lohse, 1993; Tobin, 1997; Carman and Benovic, 1998; Pitcher et al., 1998). β-adrenergic receptor kinase 1 and 2 (GRK-2 and GRK-3) are the principal kinases involved, phosphorylating the receptor on sites in the C-terminal tail (Hausdorff et al., 1990). Recent cloning studies have demonstrated that GRK-2 and GRK-3 are part of the GRK family that currently has six members (GRK-1 through GRK-6; Tobin, 1997; Pitcher et al., 1998). With the exception of GRK-1 (rhodopsin kinase) all of the GRKs bind PIP_2, thereby participating in the anchoring of the kinase to the plasma membrane in a manner that is either cooperative with (i.e., GRK-2 and GRK-3; Touhara et al., 1995; Premont et al., 1996), or independent of (i.e., GRK-4, GRK-5, and GRK-6; Stoffel et al., 1994; Pitcher et al., 1996, Premont et al., 1996) G protein $\beta\gamma$-subunit binding.

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ABBREVIATIONS: CHO, Chinese hamster ovary; G protein-coupled receptor kinase, $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKA amide inhibitor, myristoylated protein kinase A inhibitor (14–22) amide (*N*-Myr-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2); PKC, protein kinase C; PLC, phosphoinositide-specific phospholipase C.

Furthermore, in reconstitution studies GRK activity can be maintained by a number of phospholipids, including PIP_2 (Onorato et al., 1995; DebBurman et al., 1996; Pitcher et al., 1996). In vitro GRK activity is affected by PIP_2 in a concentration-dependent manner (DebBurman et al., 1996), suggesting that in intact cells changes in membrane levels of PIP_2 may regulate GRK activity.

Recent studies have also implicated Ca^{2+}/calmodulin in the regulation of GRK membrane localization. Ca^{2+}/calmodulin has been found to inhibit membrane localization of GRK-2 and GRK-3 by competing with $\beta\gamma$-subunit binding (Chuang et al., 1996). Ca^{2+}/calmodulin was also able to inhibit GRK-5 activity (Chuang et al., 1996) via a mechanism that may involve direct binding of Ca^{2+}/calmodulin to the conserved polybasic N terminus of the kinase common to GRK-4, GRK-5, and GRK-6 (Chuang et al., 1996). This either prevents membrane association directly or stimulates autophosphorylation that results in membrane dissociation (Chuang et al., 1996; Pronin et al., 1997).

These in vitro studies predict that changes in intracellular free calcium and PIP_2 membrane concentrations could affect GRK activity, although this proposal has never been tested in intact cells. If such a mechanism does exist in vivo then stimulation of coexpressed PLC-coupled receptors (for example the m3-muscarinic receptor) with associated changes in intracellular free calcium and PIP_2 concentrations would diminish the ability of GRKs to phosphorylate β_2-adrenergic receptors. In the present study we test whether endogenous receptor kinases, possibly GRKs, responsible for the non-protein kinase A (PKA)-mediated phosphorylation of β_2-adrenergic receptors are regulated by changes in intracellular calcium and PIP_2 in a Chinese hamster ovary (CHO) cell line that is cotransfected with the human β_2-adrenergic and m3-muscarinic receptors.

The second mechanism of receptor cross talk investigated in this study is the possibility that the m3-muscarinic receptor may mediate phosphorylation of the β_2-adrenergic receptor directly. A potential mechanism involving protein kinase C (PKC) has recently been suggested, where PKC stimulation was shown to phosphorylate the β_2-adrenergic receptor at sites in the third intracellular loop resulting in receptor desensitization (Johnson et al., 1990; Yuan et al., 1994). These studies, however, employed phorbol ester stimulation of PKC and as such only imply the potential for a PLC-coupled receptor-mediated regulatory process. In this study we investigated whether m3-muscarinic receptor stimulation can mediate GRK-independent phosphorylation/regulation of the β_2-adrenergic receptor.

Materials and Methods

Antibodies and Reagents. Antiserum to the β_2-adrenergic receptor was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-m3-muscarinic antibody (Ab-332) was raised against a glutathione-*S*-transferase fusion protein containing a region of the third intracellular loop of the muscarinic m3 receptor as previously described (Tobin and Nahorski, 1993). The pCEP plasmid was purchased from Invitrogen. [^{32}P]Orthophosphate (10 mCi/ml), [^{3}H]cAMP (49Ci/mmol), and [^{3}H]inositol (68Ci/mmol) were purchased from NEN (Boston, MA). [^{3}H]inositol 1,4,5-trisphosphate]Ins(1,4,5)P_3; 21Ci/mmol] and [^{3}H]CGP-12177 (44Ci/mmol) was obtained from New England Nuclear-DuPont Ltd. (Stevenage, Hertfordshire, UK). Protein A Sepharose was purchased from Pharmacia (Uppsala, Sweden). α Minimal essential medium, fetal calf serum, penicillin/streptomycin, fungizone, and tissue culture flasks were purchased from Life Technologies (Paisley, Renfrewshire, Scotland). Hygromycin B and phorbol-12,13-dibutyrate were purchased from Calbiochem (Nottingham, UK). Myristoylated protein kinase A inhibitor (14–22) amide (*N*-Myr-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2) was purchased from Calbiochem. All other reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK).

Cell Culture. CHO-K1 cells, transfected with cDNA encoding the m1- or m3-muscarinic receptors were kind gifts from Dr. N. J. Buckley (Dept. of Pharmacology, University College, London, England). The CHO-β_2/m3 cells were generated by subcloning the cDNA encoding the β_2-adrenergic receptor into the pCEP plasmid, which contains the constituitively expressed hygromycin B resistance gene. The resultant vector was transfected into a CHO-K1 cell line previously transfected with cDNA encoding for the m3-muscarinic receptor (Tobin et al., 1992) using the calcium phosphate precipitation method. β_2-Adrenergic receptor-expressing clones were selected using 200 μg/ml hygromycin B and subsequent clones were tested for β_2-adrenergic receptor and m3-muscarinic receptor expression using [^{3}H]CGP-12177 and [^{3}H]*N*-methylscopolamine binding. The work described in this article was performed using CHO-β_2/m3 clone 27, which expressed 224 $\pm$ 33 fmol/mg protein β_2-adrenergic receptor and 1456 $\pm$ 240 fmol/mg protein m3-muscarinic receptor. Transfected CHO cells were grown in medium consisting of α minimal essential medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 225 IU/ml hygromycin B. Cells were incubated in a 5% CO_2, humidified incubator at 37°C.

Membrane Sample Preparation. Membranes were prepared from confluent monolayers of CHO cells by harvesting the cells from 175-cm^2 flasks in ice-cold PBS/0.5 mM EDTA pH 7.4 solution. The cells were centrifuged at 1000*g* and the resultant pellet resuspended in ice-cold TE (10 mM Tris-HCl/2.5 mM EDTA, pH 7.4) including 1 mM sodium orthovanadate, 100 μg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride. The cells were left in ice-cold TE for 10 min to promote cell swelling and subsequently homogenized by a 10-s pulse using a Polytron tissue homogenizer. The resulting cell lysate was centrifuged at 500*g* and the pellet discarded. The supernatant was diluted with 15 ml of ice-cold TE and further centrifuged at (15000*g* for 15 min, 4°C). The resultant crude membrane pellet was resuspended in the appropriate buffer ready for use.

Immunoblotting. The protein concentration of the membrane samples prepared from CHO cells expressing recombinant G protein-coupled receptors was adjusted to 1 mg/ml. Fifty micrograms of membrane sample/lane was resolved on a 10% SDS/polyacrylamide gel electrophoresis gel and resolved proteins transferred to nitrocellulose sheets. Nitrocellulose sheets were subsequently blocked with TBS-T [5% (w/v) dried milk, 10 mM Tris (pH 7.4), 0.15 M NaCl, 0.05% (v/v) Tween-20] overnight at 4°C. Nitrocellulose sheets were then probed with the anti-β_2-adrenergic receptor antiserum at 1:500 dilution for 90 min. The blots were then washes with TBS-T at room temperature. Detection of immunoreactivity was achieved by horseradish peroxidase-conjugated anti-rabbit antibody and a commercially available enhanced chemiluminescence detection kit (Amersham, UK).

Immunoprecipitation of Phosphorylated Receptors. Intact CHO cells grown in 6-well dishes were washed with 1 ml of phosphate-free Krebs/HEPES buffer (10 mM HEPES, 118 mM NaCl, 4.3 mM, 1.17 mM $MgSO_4$.7, 1.3 mM $CaCl_2$, 25.0 mM $NaHCO_3$, and 11.7 mM glucose (pH 7.4), and the cells were incubated in phosphate-free Krebs/HEPES supplemented with [^{32}P]orthophosphate (50 μCi/ml) for 1 h at 37°C. Drugs or vehicle were added for varying times according to the experiment and stimulations were terminated by rapid aspiration of the drug-containing media and application of 2 ml of ice-cold solubilisation buffer (10 mM Tris, 10 mM EDTA, 500 mM NaCl, 1% NP-40 (Nonidet P40), 0.1% SDS, 0.5% deoxycholate pH 7.4). Samples were left on ice for 15 min and then cleared by micro-

centrifugation. Antiserum (0.2 μg) was added and the samples left on ice for 60 to 90 min. Immunocomplexes were isolated on protein-A Sepharose beads and the beads were washed three times with 10× ice-cold TBS-T and 1× ice-cold TE. Isolated immunocomplexes were resolved on 10% SDS/PAGE gels. The gels were dried and subjected to autoradiography and the level of receptor phosphorylation assessed with a Bio-Rad model GS 670 densitometer (Bio-Rad, Hercules, CA).

Isoproterenol Displacement of [^{3}H]CGP 12177 Binding to CHO-β_2/m3 Cell Membranes. Isoproterenol displacement of [^{3}H]CGP 12177 binding was performed in membrane preparations from CHO-β_2/m3 cells in the presence and absence of the guanine nucleotide GppNHp (100 μM) to assess the proportion of β_2-adrenergic receptor in the high- and low-affinity states. Cell membranes were prepared as described above and resuspended in binding buffer (20 mM Tris, 10 mM $MgCl_2$, and 1 mM EDTA, pH 7.4). Membranes were freshly prepared before each experiment. Reaction tubes containing a range of concentrations of isoproterenol (10^{-10} -10^{-4} M), ~0.5 nM [^{3}H]CGP 12177 ± 100 μM Gpp[NH]*p*, and CHO-β_2/m3 cell membranes (30–50 μg of protein) in a final volume of 200 μl. Reactions were initiated by the addition of membranes and allowed to proceed for 45 to 60 min at room temperature. The reactions were terminated by rapid vacuum filtration through Whatman GF/B filters followed by 2 × 5 ml washes with ice-cold binding buffer. Filters were removed and membrane samples allowed to extract overnight in 5 ml of scintillant before being placed on the scintillation counter. Results were analyzed using the GraphPad Prism program (GraphPad Software Inc. San Diego, CA).

Isoproterenol Displacement of [^{3}H]CGP 12177 Binding to Intact CHO-β_2/m3 Cells. CHO-β_2/m3 cells grown on 6-well dishes were washed once with Krebs/HEPES buffer and left to equilibrate for 10 min. Medium was then replaced with Krebs/HEPES buffer containing isoproterenol (10^{-10} -10^{-4} M), ~0.5 nM [^{3}H]CGP 12177 plus either one of vehicle, PKA amide inhibitor [myristoylated protein kinase A inhibitor (14–22) amide (*N*-Myr-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2; 20 μM] or the PKA inhibitor H-89 (25 μM). The incubation was allowed to reach equilibrium at 4°C overnight. Cells were then rapidly washed three times with ice-cold Krebs/HEPES buffer and then solubilized in solubilization buffer. The radioactivity in the solubilized extract was then determined by scintillation counting.

Mass Ins(1,4,5)P_3 Determination. Cells grown in 24-well dishes were washed with Krebs/HEPES buffer and challenged with agonists for the appropriate times. Incubations were terminated by rapid aspiration, and addition of ice-cold 0.5 M trichloroacetic acid and transfer to an ice-bath. After 15 min, the supernatant was removed and neutralized by addition of EDTA and freon/tri-*n*-octylamine as described previously (Tobin et al., 1992). Extracts were brought to pH 7 by addition of $NaHCO_3$ and stored at 4°C until analysis. Ins(1,4,5)P_3 mass measurements were performed according to a standard method (Challiss et al., 1988).

Mass PIP_2 Determination. Cells grown in 24- well dishes and labeled with [^{3}H]inositol (2.5 μCi/ml) for 48 h, were washed with Krebs/HEPES buffer and challenged with agonists for the appropriate times. Incubations were terminated by rapid aspiration, and addition of 0.5 ml of acidified chloroform/methanol ($CHCl_3$/CH_3OH/concentrated HCl, 40:80:1). Each well was scraped thoroughly and the lipid extract from duplicates collected and pooled. The combined lipid extract was then resolved by addition of $CHCl_3$ (0.31 ml) and 0.1 M HCl (0.56 ml), thorough vortex mixing, and centrifugation (1000*g*, 10 min). A known volume of the $CHCl_3$ phase was recovered, dried under N_2, deacylated, and the [^{3}H]glycerophosphoinositol (phosphates) resolved exactly as previously described (Challiss et al., 1993).

Single Cell Calcium Measurements. Cells grown for 16 to 24 h on coverslips were incubated in Krebs/HEPES buffer supplemented with 2 μM fura-2 acetoxymethyl ester and 1 mg/ml BSA for 1 h. The coverslips were then washed in Krebs/HEPES buffer and incubated for a further 30 min to allow for complete de-esterification of the dye before being mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. Krebs/HEPES was continuously perfused over the cells at the rate of 4 ml/min and agonists were applied, where indicated, via the perfusion buffer. Using an intensified charged-couple device camera (Photonic Science) contained in a quanticell 700 system (Applied Imaging), images at wavelengths above 510 nm were collected after excitation at 340 and 380 nm (40 ms at each wavelength). Ratiometric values were converted to approximate [Ca^{2+}]using the Grynkiewicz equation (Grynkiewicz et al., 1985).

Measurement of Intracellular cAMP in Permeabilized Cells. cAMP accumulation in permeabilized CHO-β_2/m3 cells was assessed using a cAMP binding protein purified from calf adrenal glands (Brown et al., 1971). One flask of confluent CHO-β_2/m3 cells was treated with either 1 mM carbachol or vehicle for 10 min and the drug-containing medium rapidly removed. Cells were harvested using ice-cold PBS/0.5 mM EDTA solution and cells were centrifuged at 210*g* for 2 min. The pellet was washed twice in Ca^{2+}-free Krebs/HEPES buffer and the cells centrifuged at 210*g* for 2 min. The cell pellet was resuspended in 3.2 ml of cytosol-like buffer [CLB: 120 mM KCl, 2 mM KH_2PO_4, 5 mM Na-succinate, 5 mM $MgCl_2$, and 20 mM HEPES, pH to 7.2 (using KOH)]. β-Escin was added to the cell suspension to give a final concentration of 50 μg/ml, and the cell suspension was left on ice for 2 min. The permeabilized cells were subsequently centrifuged at 210*g* for 2 min and the cell pellet resuspended in CLB (1 mg protein/ml). This procedure results in permeabilization of ~100% of the cells as assessed using the dye Azur-A. Reaction tubes contained a range of concentrations of isoproterenol (2×10^{-9}–2×10^{-4} M), 4 mM ATP, and permeabilized cells (25 μg protein) in a final volume of 100 μl. Reactions were initiated by the addition of cells and the reactions allowed to proceed for 10 min at 37°C. Stimulations were terminated and samples neutralized as described above for Ins(1,4,5)P_3 determination and cAMP content determined as previously described (Brown et al., 1971).

Results

Immunodetection of β-Adrenergic Receptor in CHO-β_2/m3 Cells. Western blots using an anti-β_2-adrenergic receptor antibody revealed an immunoreactive band of ~64 kDa in membranes prepared from CHO cells expressing the β_2-adrenergic receptor (CHO-β_2/m3 cells) but not in CHO cells expressing either the m1- or m3-muscarinic receptors (Fig. 1A). The apparent molecular mass of the β_2-adrenergic receptor corresponded to the glycosylated receptor, which has been reported previously (Benovic et al., 1984). In phosphorylation studies, the anti-β_2-adrenergic receptor antiserum was used to immunoprecipitate the β_2-adrenergic receptor from CHO-β_2/m3 cells labeled with [^{32}P]orthophosphate. In these studies the receptor ran as an ~64-kDa phosphoprotein that demonstrated a rapid (measured in seconds) increase in its phosphorylation state after agonist stimulation (1 μM isoproterenol; Fig. 1B). In control experiments, using immunoprecipitates from cells expressing only the m3-muscarinic receptor, the ~64-kDa band was not present (Fig. 1C). Note that in addition to the β_2-adrenergic receptor, the antiserum was able to immunoprecipitate a high molecular mass band (>200 kDa) and a band running at ~42 kDa. Both of these phosphoproteins were present in the control immunoprecipitation (Fig. 1C) and are therefore distinct from the β_2-adrenergic receptor.

Identification of PKA- and Non-PKA-Mediated Components of β_2-Adrenergic Receptor Phosphorylation in CHO-β_2/m3 Cells. The established mechanism of β-ad-

renergic receptor phosphorylation is that at low agonist concentrations the β-adrenergic receptor is phosphorylated solely by PKA and at high concentrations by both PKA and GRKs (Clark et al., 1988; Johnson et al., 1990; Pippig et al., 1993). It was important to confirm that there were two components in the phosphorylation of the β_2-adrenergic receptor in the CHO-β_2/m3 cells.

Stimulation of intact CHO-β_2/m3 cells with the β-adrenergic agonist isoproterenol resulted in elevated cAMP levels with a maximal response at 1 μM and an EC_{50} ~5 nM (data not shown). Stimulation of CHO-β_2/m3 cells with 1 μM isoproterenol resulted in rapid phosphorylation of the β_2-adrenergic receptor (Fig. 1B) that was blocked by ~75% by the specific PKA amide inhibitor peptide *N*-Myr-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2 (Glass et al., 1989) (Fig. 2).

In contrast to PKA-mediated phosphorylation, GRK activity closely corresponds to agonist occupation of the receptor (Lohse et al., 1990). Binding studies on CHO-β_2/m3 cells demonstrated that the β_2-adrenergic receptor was 100% agonist-occupied in the presence of 100 μM isoproterenol (see

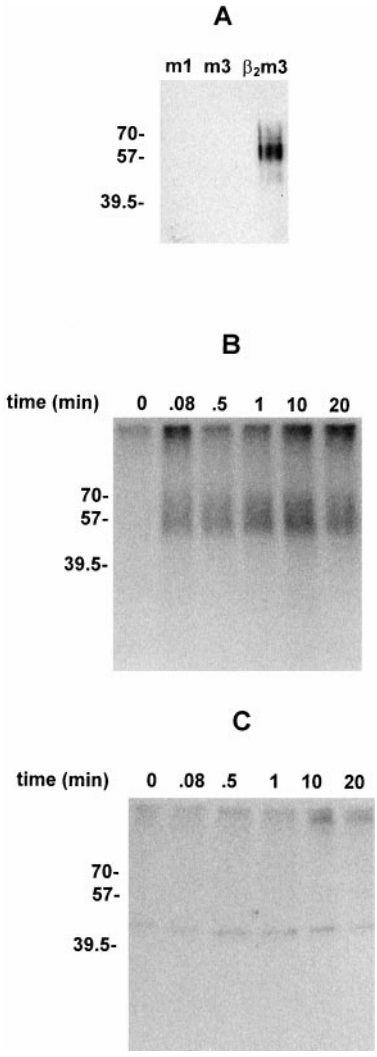

Fig. 1. Immunodetection of the β_2-adrenergic receptor expressed in CHO-β_2/m3 cells. A, Western blot of membranes prepared from CHO cells expressing the m1- or m3-muscarinic receptors or coexpressing the m3-muscarinic and β_2-adrenergic receptors (β_2m3). Fifty micrograms of membrane protein was loaded per well and the blot probed with anti-β_2-adrenergic receptor antiserum at a 1:500 dilution. B, time course for phosphorylation of the β_2-adrenergic receptor immunoprecipitated from CHO-β_2/m3 cells labeled with [^{32}P]-orthophosphate. The cells were stimulated with 1 μM isoproterenol for the times indicated. The receptors were then solubilized and immunoprecipiated using the anti-β_2-adrenergic receptor antiserum. C, control immunoprecipation using the β_2-adrenergic receptor antiserum and CHO cells expressing only the m3-muscarinic receptor. The positions of molecular mass markers are shown in kilodaltons.

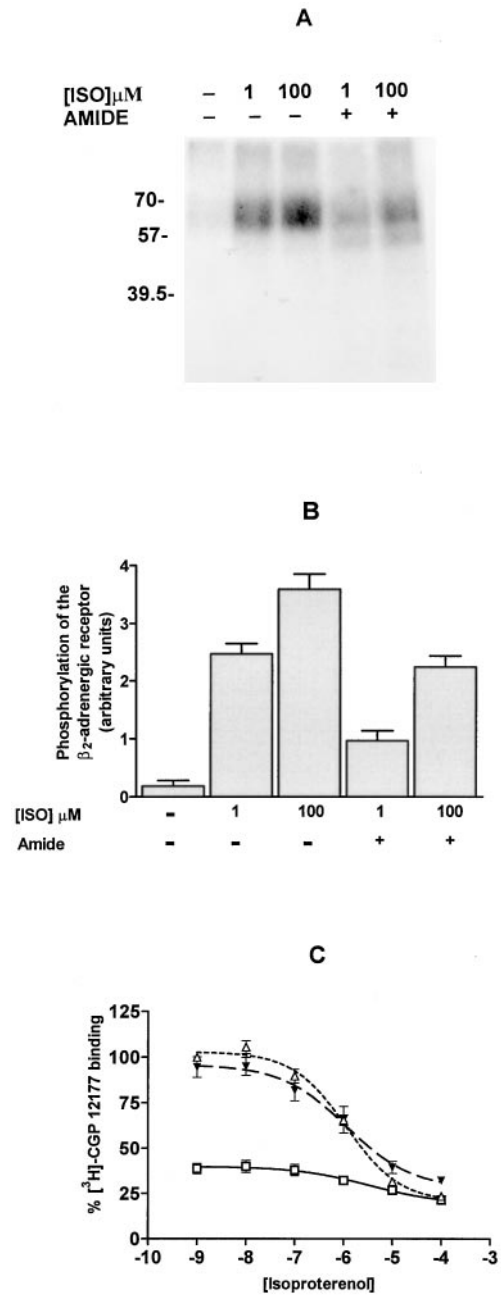

Fig. 2. Agonist-mediated phosphorylation of the β_2-adrenergic receptor. CHO-β_2/m3 cells were labeled with [^{32}P]orthophosphate and stimulated with vehicle or isoproterenol (ISO; 1 μM or 100 μM) for 10 min. In the appropriate experiments the PKA amide inhibitor (20 μM) was added 10 min before agonist. A, a representative autoradiograph. The position of molecular mass markers are shown in kilodaltons. B, cumulative data form three experiments in which the data have been normalized to the basal phosphorylation of the receptor. C, displacement of [^{3}H]CGP 12177 (~0.5 nM) binding by isoproterenol from intact CHO-β_2/m3 cells was conducted in the presence of vehicle (△), PKA amide inhibitor (20 μM; ▼), or the PKA inhibitor H-89 (25 μM; □). Data are means (±S.E.M.) of three experiments carried out in duplicate.

below). Increasing the concentration of agonist to 100 μM resulted in an increase in receptor phosphorylation over that observed at the lower (1 μM) agonist concentration (Fig. 2). Furthermore, phosphorylation at 100 μM isoproterenol was only partially inhibited (~30%) by the PKA amide inhibitor (Fig. 2). These data are consistent with a dual phosphorylation of the β_2-adrenergic receptor via PKA and a protein kinase(s) distinct form PKA, possibly a GRK, in CHO-β_2/m3 cells at high agonist concentrations.

Recent studies have demonstrated that the widely used PKA inhibitor H-89 was able to inhibit β-adrenergic receptor ligand binding (Penn et al., 1999). We therefore tested whether the PKA inhibitor used in the present study also affected ligand binding. In contrast to H-89, which dramatically reduced [^{3}H]CGP 12177 binding (Fig. 2C), the PKA amide inhibitor had no significant effect on [^{3}H]CGP 12177 binding to the β_2-adrenergic receptor nor the ability of isoproterenol to displace [^{3}H]CGP 12177 binding (Fig. 2C).

Can Changes in Intracellular Free Calcium and PIP_2 Levels Influence Homologous β_2-Adrenergic Receptor Phosphorylation? Previous work from our laboratory and others (Tobin et al., 1992; Fisher et al., 1994; Willars et al., 1996) have demonstrated that stimulation of the PLC-coupled m3-muscarinic receptor results in a biphasic increase in $Ins(1,4,5)P_3$ and a corresponding fall in PIP_2. The biphasic rise in $Ins(1,4,5)P_3$ also correlates with a biphasic increase in intracellular free calcium (Tobin et al., 1992). The experimental protocol employed in this study was to stimulate the CHO-β_2/m3 cells for 30 s with a maximally effective concentration of the muscarinic agonist carbachol (1 mM) followed by stimulation with 100 μM isoproterenol to induce GRK-mediated β_2-adrenergic receptor phosphorylation.

We first tested the ability of the m3-muscarinic receptor to couple to the PLC pathway in CHO-β_2/m3 cells, and that application of the β-adrenergic agonist did not compromise muscarinic receptor signaling. Figure 3 demonstrates that after agonist stimulation of CHO-β_2/m3 cells there is a rapid increase in $Ins(1,4,5)P_3$ that peaks within 20 s of agonist application and then reaches a maintained plateau phase (Fig. 3A). This correlates with a rapid fall in membrane PIP_2 levels, which are reduced by 74.3 $\pm$ 2.2% (n = 4) within 20 s of agonist stimulation (Fig. 3B). Furthermore, intracellular calcium increases from a basal value of 20.2 $\pm$ 0.5 to 382.9 $\pm$ 15.3 nM (n = 17) within seconds of agonist stimulation and then reaches a plateau phase that is maintained for at least 10 min (Fig. 3C). The level of calcium after 10 min was 238.5 $\pm$ 23.4 nM (n = 17). Application of isoproterenol (100 μM) after 30 s of carbachol stimulation has no effect on the $Ins(1,4,5)P_3$ and PIP_2 responses (Fig. 3). In contrast, the peak muscarinic calcium response appears to be elevated in the

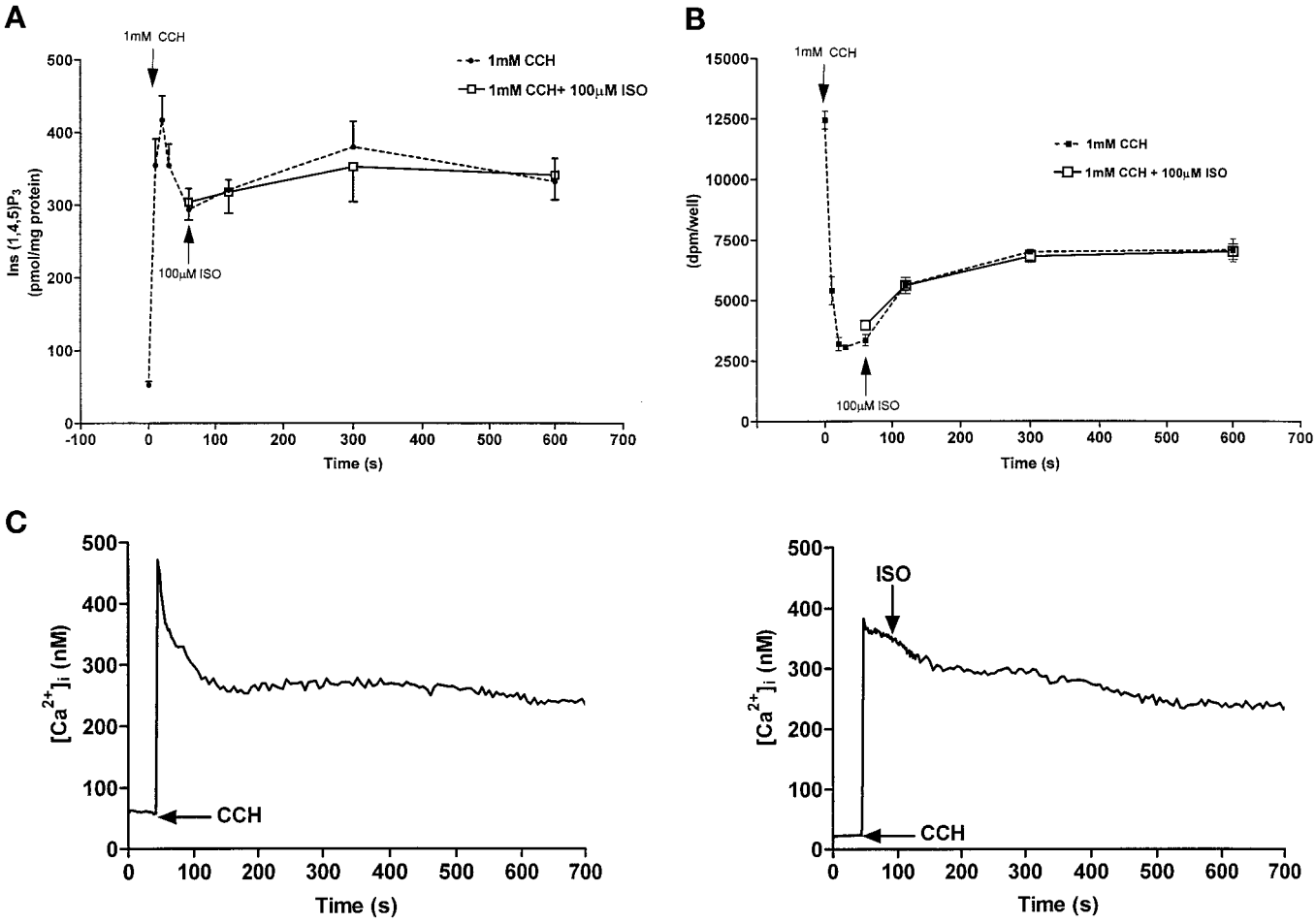

Fig. 3. Signaling through the m3-muscarinic receptor. Determination of $Ins(1,4,5)P_3$ (A), PIP_2 (B), and intracellular free calcium (C) in CHO-β_2/m3 cells stimulated with 1 mM carbachol (●) or 1 mM carbachol and 100 μM isoproterenol (□). The isoproterenol was added 30 s after carbachol addition. The $Ins(1,4,5)P_3$ and PIP_2 data are the means (±S.E.M) of four experiments carried out in duplicate. The PIP_2 data is expressed as disintegrations per minute associated with the ^{3}H-deacylated product of PIP_2 per well. The calcium data are the means of 17 cells. CCH, carbachol; ISO, isoproterenol.

presence of isoproterenol 471.4 ± 26.6 nM, however, the plateau phase of the calcium response is not affected by isoproterenol costimulation with levels of 239.4 ± 23.4 nM after 10 min (Fig. 3C).

Analysis of the phosphorylation of the β_2-adrenergic receptor demonstrated that activation of the m3-muscarinic receptor had no affect on the ability of 100 μM isoproterenol to stimulate β_2-adrenergic receptor phosphorylation (Fig. 4A). However, interpretation of the data was difficult due to the unexpected finding (which is fully explained below) that muscarinic receptor stimulation alone was able to mediate phosphorylation of the β_2-adrenergic receptor (Fig. 4A, lane 2). By using the specific PKC inhibitor Ro 31-8220 (Davis et al., 1989) the ability of the m3-muscarinic receptor to phosphorylate the β_2-adrenergic receptor could be completely inhibited (Fig. 4B; also see below for further explanation). The above experiment was, therefore, repeated in the presence of Ro 31-8220. Under these conditions muscarinic receptor stimulation still did not affect the ability of 100 μM isoproterenol to mediate phosphorylation of the β_2-adrenergic receptor (Fig. 4B). Importantly, the presence of the PKC inhibitor had no effect on the signaling properties of the m3-

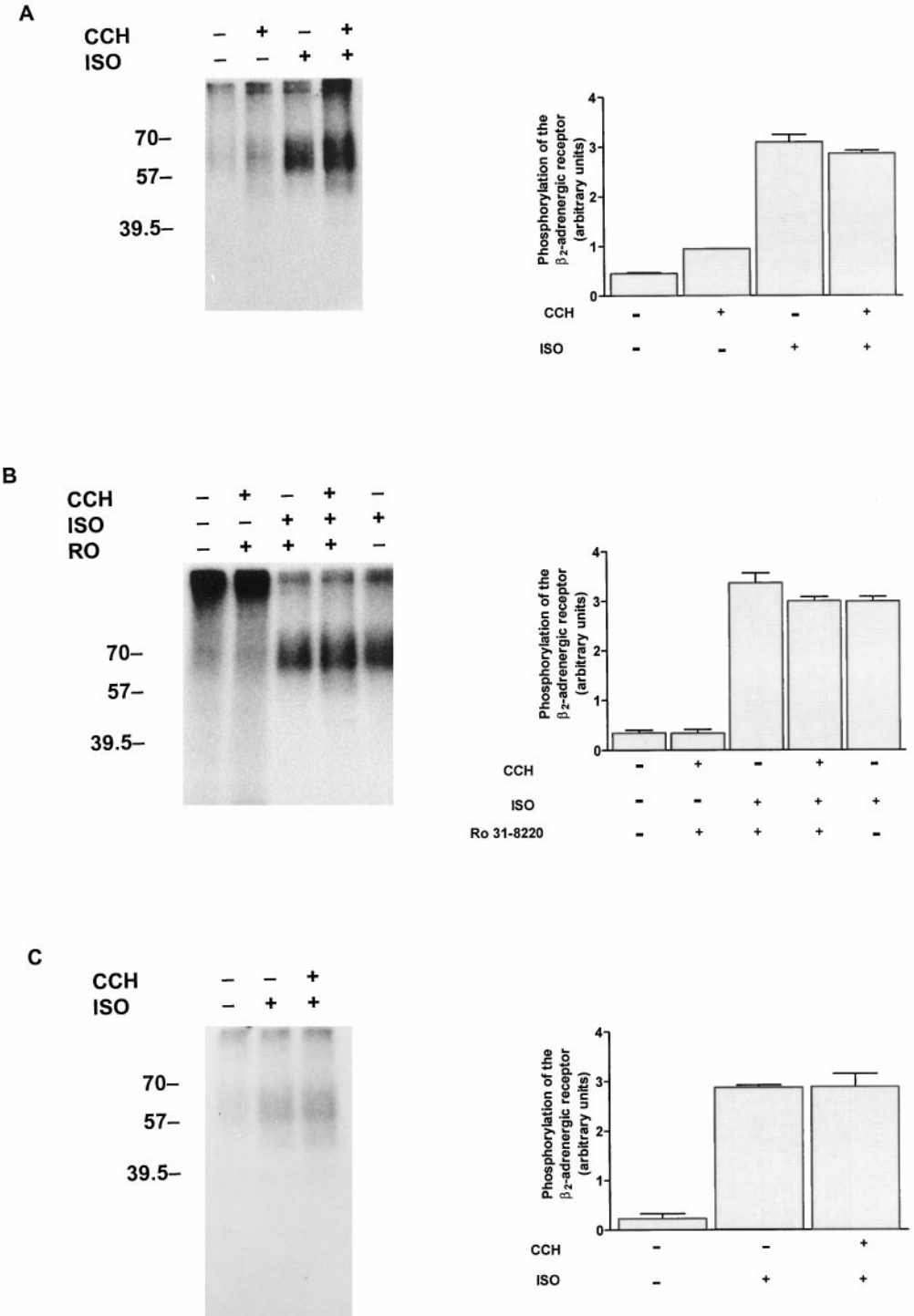

Fig. 4. Effects of muscarinic receptor stimulation on GRK-mediated β_2-adrenergic receptor phosphorylation. CHO-β_2/m3 cells labeled with [^{32}P]orthophosphate were stimulated with carbachol (1 mM) or isoproterenol (100 μM) only, or with carbachol (1 mM) for 30 s then isoproterenol (100 μM). The reactions were allowed to continue for 10 mins after which receptors were solubilized and immunoprecipitated with the anti-β_2-adrenergic receptor antiserum. A, the experiment was conducted in the absence of the PKC inhibitor Ro 31-8220. B, in the experiments indicated, Ro 31-8220 (RO; 10 μM) was added 10 min before agonist stimulation. C, cells were treated with Ro 31-8220 (10 μM) and the PKA amide inhibitor (20 μM) 10 min before agonist stimulation. The gels shown are representive of at least three experiments. Next to each autoradiograph is the cummulative data (±S.E.M) as determined by densitometric anaylsis. The positions of molecular mass markers are shown in kilodaltons.

muscarinic receptor (data not shown) nor did it effect the ability of 100 μM isoproterenol to stimulate β_2-adrenergic receptor phosphorylation in the absence of carbachol (Fig. 4B, compare lanes 3 and 5).

Phosphorylation of the β_2-adrenergic receptor after addition of 100 μM isoproterenol is mediated by both PKA and potentially GRK (see above). It was therefore decided to further assess whether m3-muscarinic receptor activation affected the PKA-independent β_2-adrenergic receptor phosphorylation by inhibiting the PKA component of phosphorylation with the PKA amide inhibitor. Hence, the effects of m3-muscarinic receptor stimulation on β_2-adrenergic receptor phosphorylation (mediated by 100 μM isoproterenol) was tested in cells that had been treated with Ro 31-8220 to inhibit PKC and the PKA amide inhibitor. Under conditions in which PKA and PKC were inhibited, activation of the m3-muscarinic receptor still had no affect on the level of phosphorylation stimulated by 100 μM isoproterenol (Fig. 4C).

Characterization of m3-Muscarinic Receptor-Mediated Phosphorylation of β_2-Adrenergic Receptor. The second possible mechanism of receptor cross talk under investigation in this study was the possibility of direct β_2-adrenergic receptor phosphorylation after m3-muscarinic receptor activation. As demonstrated above, stimulation of m3-muscarinic receptors mediated heterologous phosphorylation of the β_2-adrenergic receptor via PKC (Fig. 4).

The m3-muscarinic receptor-mediated heterologous phosphorylation could be blocked by the muscarinic receptor antagonist atropine (10 μM) and not by the β-adrenergic antagonist timolol (5 μM; Fig. 5A). In these experiments cells were stimulated with carbachol (1 mM) for 10 min, which resulted in an increase (2.1 $\pm$ 0.2-fold over basal; $n = 3$) in phosphorylation of the β_2-adrenergic receptor. Furthermore, the muscarinic response was completely inhibited by the PKC inhibitor Ro 31-8220 and was partially mimicked by the phorbol ester, phorbol 12,13-dibutyrate (Fig. 5A). Time-course studies indicated that the stimulation of the m3-muscarinic receptor resulted in a rapid but transient β_2-adrenergic receptor phosphorylation peaking at 30 to 60 s and returning to basal by 20 min (Fig. 5B). The level of phosphorylation observed at 60 s was 2.7 $\pm$ 0.13-fold over basal ($n = 3$). The phosphorylation was concentration dependent with an EC_{50} of <1 μM (Fig. 6), although these results should be viewed in the light of the semiquantitative nature of immunoprecipitation experiments of this type.

Studies were also conducted to investigate the possibility that stimulation of the β_2-adrenergic receptor could result in heterologous phosphorylation of the m3-muscarinic receptor. We found no evidence that this occurred (data not shown).

Functional Significance of Heterologous Phosphorylation of β_2-Adrenergic Receptor. To test whether PKC-mediated phosphorylation of the β_2-adrenergic receptor driven by the m3-muscarinic receptor resulted in receptor desensitization, we analyzed agonist binding curves generated from membranes derived from CHO-β_2/m3 cells. In control membranes isoproterenol displaced [^{3}H]CGP-12177 binding in a biphasic fashion with computer-assisted curve fitting revealing two sites with a high affinity (K_H ~8.4 nM) and a low affinity (K_L ~1.34 μM). In the presence of the nonhydrolyzable GTP analog (GppNHp, 100 μM), the agonist binding curves shifted exclusively to the low-affinity binding state (K_L ~1.2 μM; Fig. 7A). Membranes prepared from CHO-β_2/m3 cells pre-exposed to carbachol (1 mM, 10 min) exhibited ligand-binding curves that only had a low-affinity component (K_L ~1.6 μM), and no significant shift in the binding curve was observed after addition of GppNHp (Fig. 7B). These results demonstrate the inability of the receptor to form a high-affinity ternary complex and is indicative of a reduced coupling efficiency (or desensitization; e.g., Strasser and Lefkowitz, 1985; Samama et al., 1993). The ability of carbachol to desensitize the β_2-adrenergic receptor could be prevented by inhibition of PKC with Ro 31-8220 in intact cells during agonist treatment. The ligand binding curve of membranes prepared from cells exposed to carbachol and Ro-318220 showed both high- and low-affinity components together with a guanine nucleotide induced rightward shift (Fig. 7C).

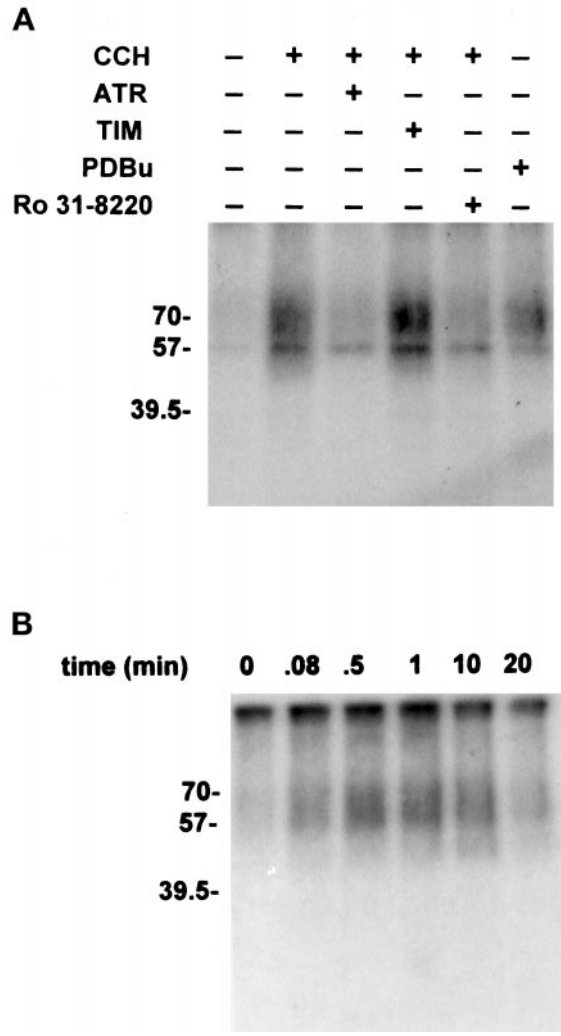

Fig. 5. Characterization of the m3-muscarinic receptor-mediated phosphorylation of the β_2-adrenergic receptor. A, effects of the muscarinic receptor antagonist atropine (10 μM), the β-adrenergic receptor antagonist timolol (5 μM), and the PKC inhibitor Ro 31-8220 (10 μM) on carbachol (1 mM) -mediated phosphorylation of the β_2-adrenergic receptor. The effect of phorbol 12,13,-dibutyrate (1 μM) on β_2-adrenergic receptor phosphorylation is also shown. B, time course for carbachol (1 mM)-stimulated phosphorylated of the β_2-adrenergic receptor. The gels are representive of at least two experiments. Stimulation with agonists and phorbol esters were for 10 min. Antagonists and Ro 31-8220 were added 10 min before agonist stimulation. The positions of molecular mass markers are shown in kilodaltons. ATR, atropine; TIM, timolol.

The ability of m3-muscarinic receptor activation to mediate β_2-adrenergic receptor desensitization was further investigated by measuring cAMP accumulation in permeabilized CHO-β_2/m3 cells. Prestimulation of CHO-β_2/m3 cells with carbachol before β-escin permeabilization did not affect the potency of isoproterenol-mediated cAMP accumulation (EC_{50} values of control and carbachol pretreated cells were 6 and 10 μM, respectively) but significantly reduced the maximal cAMP response by 41% ($P < .001$; two-way ANOVA; Fig. 8).

Discussion

The present study investigated two possible mechanisms of receptor cross talk between m3-muscarinic and β_2-adrenergic receptors coexpressed in CHO cells. The first mechanism investigated addresses the possibility that homologous phosphorylation of β_2-adrenergic receptors may be regulated by changes in PIP_2 and intracellular free calcium (Chuang et al., 1996; DebBurman et al., 1996; Pitcher et al., 1996; Pronin et al., 1997). The second mechanism studied centers on the possibility that m3-muscarinic receptor stimulation activates heterologous phosphorylation of the β_2-adrenergic receptor in a GRK-independent manner. We clearly demonstrate that elevation of intracellular free calcium and decrease in PIP_2 pools mediated by m3-muscinic receptor activation has no effect on the ability of endogenous receptor kinases, possibly GRKs, to mediate β_2-adrenergic receptor phosphorylation. In contrast, m3-muscarinic receptor stimulation does regulate β_2-adrenergic receptor function via heterologous phosphorylation of the receptor by PKC.

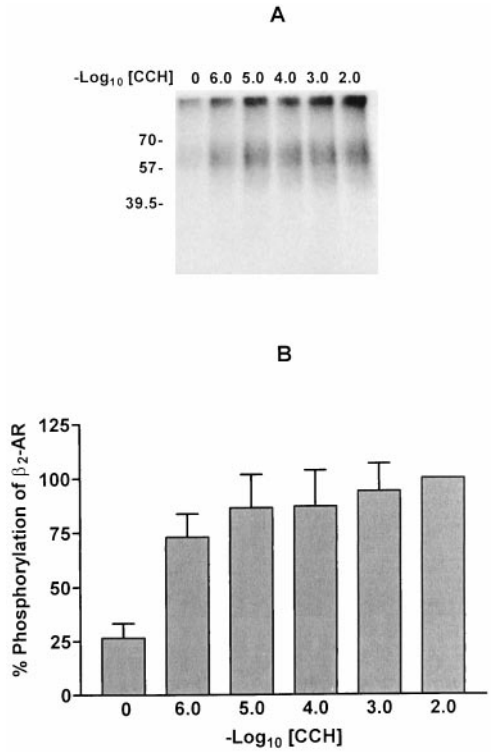

Fig. 6. Concentration dependence of muscarinic receptor-mediated phosphorylation of the β_2-adrenergic receptor. A, representive gel of carbachol-mediated phosphorylation of the β_2-adrenergic receptor. B, cumulative results from three experiments. The data shown represent the means ± S.E.M. The positions of molecular mass markers are shown in kDa. CCH=carbachol.

Recent in vitro studies have demonstrated that GRK activity has an absolute requirement for phospholipids (Onorato et al., 1995; DebBurman et al., 1996; Pitcher et al., 1996) and that PIP_2 levels can modulate purified GRK activity in a concentration-dependent manner (DebBurman et al., 1996). In addition, studies on GRK-2, GRK-3, and GRK-5 have demonstrated that Ca^{2+}/calmodulin is a potent inhibitor of GRK activity in vitro (Chuang et al., 1996; Pronin, et al., 1997). These studies suggest that cellular changes in free calcium and membrane PIP_2 levels may be physiological regulators of GRK activity, however, this has never been tested in intact cells. Using a CHO cell line coexpressing the β_2-adrenergic and the m3-muscarinic receptors we were able to induce rapid changes in intracellular free calcium and PIP_2 by stimulation of the m3-muscarinic receptor. Despite elevating intracellular free calcium by ~10-fold and decreasing membrane PIP_2 levels by >70% there was no change in the ability of endogenous kinases to phosphorylate the β_2-adrenergic receptor in CHO-β_2/m3 cells. Our data, therefore,

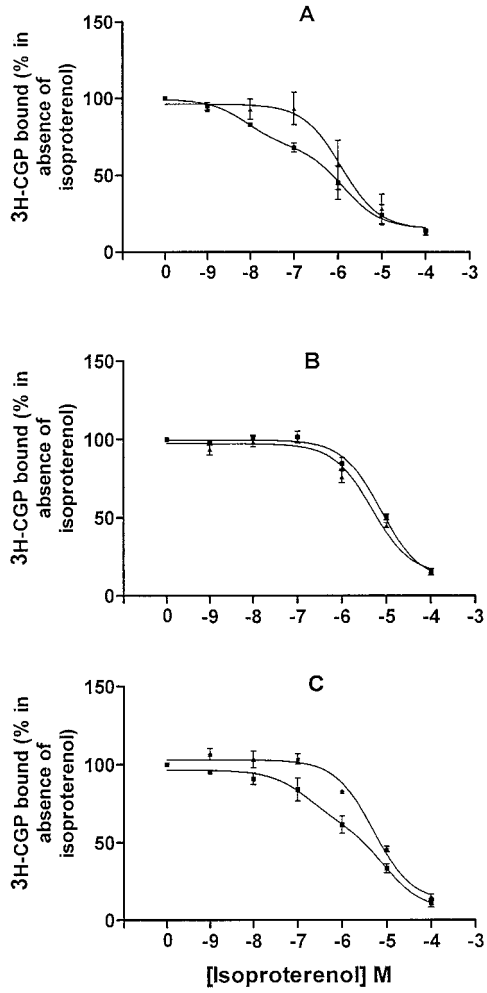

Fig. 7. Effects of muscarinic receptor stimulation on agonist binding curves for isoproterenol at the β_2-adrenergic receptor. Intact CHO-β_2/m3 cells were incubated with vehicle (A), 1 mM carbachol (B), or 1 mM carbachol + 10 μM Ro 31-8220 (C). Reactions were stopped by washing cells in ice-cold PBS/EDTA. Cells were harvested and membranes prepared. Displacement of [^{3}H]CGP 12177 (~0.5 nM) binding by isoproterenol was conducted in the presence (▲) or absence (■) of GppNHp (100 μM). The data shown are the means (±S.E.M) of four experiments carried out in duplicate.

demonstrates that the non-PKA component of β_2-adrenergic receptor phosphorylation, which is possibly mediated by the GRKs, is not regulated by changes in intracellular calcium or PIP_2.

Furthermore, our data dispel the notion that PLC-coupled receptors, by virtue of the ability to mobilize intracellular calcium stores and decrease PIP_2, may regulate β_2-adrenergic receptor phosphorylation. This is particularly important because PLC-coupled receptors are coexpressed with β_2-adrenergic receptors in many cell types, for example, m3-muscarinic and β_2-adrenergic receptors in smooth muscle (Eglen et al., 1994).

The large reserves of the lipid precursors phosphatidylinositol 4-phosphate and phosphatidylinositol ensure that on PLC-coupled receptor stimulation PIP_2 is never completely depleted (Willars et al., 1998). It is therefore possible that there is always sufficient PIP_2 to enable proteins that have an absolute requirement for this low abundance phospholipid to operate. In addition to the GRKs there is now a growing number of signaling proteins such as protein kinases. (e.g., Bruton's tyrosine kinase), exchange factors, and GTPase-activating proteins (e.g., Ras GTPase-activating protein) that have been shown to interact with PIP_2, either through pleckstrin homology domains or other protein motifs (e.g., the phosphotyrosine binding-domain on Shc; Harlan et al., 1994; Rameh et al., 1997). The notion that all of these proteins may be sensitive to receptor-mediated changes in membrane PIP_2 levels seems unlikely. The data we present here demonstrate that despite there being a potential for regulating these proteins by receptor-mediated changes in PIP_2, certainly for the GRKs this appears not to be the case. Further studies to establish the concentration of cellular PIP_2 necessary to sustain GRK activity and the size of the "PLC-insensitive" PIP_2 pool will be needed to further address this question. An additional explanation for our results is that GRK activity in intact cells is maintained by phospholipids other than PIP_2 for which the concentrations do not change after PLC-coupled receptor stimulation. For example, in vitro studies have demonstrated that phosphatidylserine and phosphatidylinositol can support GRK activity in vitro (Onorato et al., 1995; DebBurman et al., 1996). This explanation does not, however, detract from the fact that changes in the cellular levels of PIP_2 appear not to regulate the phosphorylation of β_2-adrenergic receptors.

The possibility that changes in intracellular free calcium might regulate GRK activity has a precedent in the interaction of GRK-1 with recoverin. GRK-1 is localized exclusively to the retinal rod outer segments and phosphorylates and desensitizes rhodopsin in response to light stimulation (Lorenz et al., 1991). Recoverin, a calcium binding protein almost exclusively found in photoreceptors, has been shown to bind to and inhibit the activity of GRK-1 in a calcium-sensitive manner, and that this binding and inhibition has a physiological role in photoreceptor light adaptation (Chen et al., 1995). Recent studies have demonstrated that Ca^{2+}/calmodulin can inhibit GRK-2, GRK-3, and GRK-5 activity in vitro (Chuang et al., 1996; Pronin et al., 1997), raising the possibility that, like GRK-1, the other GRKs may be physiologically regulated by changes in free intracellular calcium. This, however, has never been tested in intact cells. We report here that m3-muscarinic receptor-mediated increases in intracellular free calcium concentrations have no effect on β_2-adrenergic receptor phosphorylation mediated by endogenous receptor kinase(s), possibly the GRKs. Our results indicate, therefore, that despite in vitro experiments demonstrating the ability of Ca^{2+}/calmodulin to regulate GRK activity, changes in intracellular calcium concentrations in the CHO-β_2/m3 cell line have no regulatory effect on homologous β_2-adrenergic receptor phosphorylation.

In drawing conclusions from the data present here, we have made the assumption that the PKA-independent phosphorylation of the β_2-adrenergic receptor is mediated via the GRKs. This is based on a very extensive literature including a number of studies that have focused on endogenous GRK activity in intact CHO cells (e.g., Bouvier et al., 1988; Moffett et al., 1993; Pippig et al., 1993). Due to the lack of specific inhibitors to the GRKs, it is not, however, possible to categorically assign the PKA-independent phosphorylation identified in this study to the GRKs. As such we cannot discount the possibility that in our cells the β_2-adrenergic receptor may be phosphorylated by a kinase distinct from the GRKs. We have, for example, recently reported that rhodopsin and the m3-muscarinic receptor is phosphorylated in an agonist-sensitive manner by casein kinase 1α (Tobin et al., 1996, 1997). It is possible that the β_2-adrenergic receptor is also phosphorylated by casein kinase 1α, or another kinase that is distinct from the GRKs and is not regulated by calcium nor PIP_2. At present, however, the overwhelming evidence from the literature (Pitcher et al., 1998) is that this is not the case and that GRKs represent the receptor-specific kinases responsible for β_2-adrenergic receptor phosphorylation in cell lines and intact tissues.

Although activation of the PLC pathway appears to have no effect on homologous β_2-adrenergic receptor phosphorylation, we show in this study that stimulation of the m3-muscarinic receptor can influence β-adrenergic receptor function through heterologous receptor phosphorylation via PKC. It has been shown previously that the β-adrenergic receptor can be desensitized by phorbol ester treatment (Johnson et al., 1990; Yuan et al., 1994), however the present study is the first to demonstrate that β_2-adrenergic receptors are phosphorylated in a heterologous fashion after PLC-coupled receptor activation through a mechanism that involves

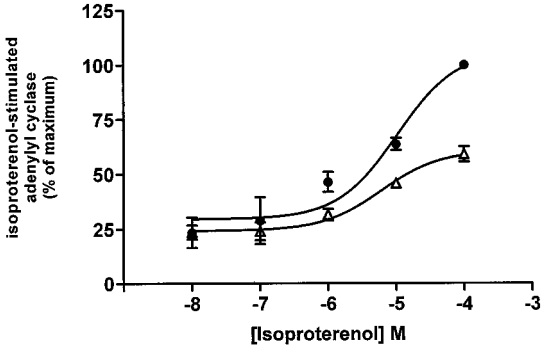

Fig. 8. Concentration-response curves for cAMP production in permeabilized CHO-β_2/m3 cells prestimulated with carbachol (△) or vehicle (●). Intact CHO-β_2/m3 cells were stimulated with vehicle or carbachol (1 mM) for 10 mins before permeabilization. After β-escin permeabilization, cells were stimulated with isoproterenol for 10 min and cAMP levels determined as described in the text. The data shown are the means ±S.E.M of three experiments carried out in duplicate.

PKC. Because receptor-mediated PKC activation represents the physiologically relevant signaling pathway for this kinase, our data indicate that PKC may play a physiological role in β_2-adrenergic receptor regulation. Furthermore, the rapid time course of m3-muscarinic receptor-evoked phosphorylation of the β_2-adrenergic receptor suggests a rapid functional role for phosphorylation.

Previous studies have demonstrated that PKC can phosphorylate and, by promoting membrane translocation, activate GRK-2 (Winstel et al., 1996). This may, therefore, provide for an indirect mechanism of stimulating β_2-adrenergic receptor phosphorylation. We, however, do not favor this possibility in the context of m3-muscarinic receptor stimulation of β_2-adrenergic receptor phosphorylation because the GRKs are known to phosphorylate only the agonist-occupied β_2-adrenergic receptor (Pitcher et al., 1998) and m3-muscarinic receptors are able to induce phosphorylation of the agonist unoccupied β_2-adrenergic receptor. It appears more likely that PKC is able to directly phosphorylate the β_2-adrenergic receptor.

The functional consequence of m3-muscarinic receptor-mediated phosphorylation of the β_2-adrenergic receptor was to reduce the coupling efficiency of the β_2-adrenergic receptor, as determined by a loss of high-affinity agonist binding and guanine nucleotide-induced shift. This desensitization response could be prevented by pharmacological inhibition of PKC activity, indicating that the loss of coupling and heterologous receptor phosphorylation are linked. Furthermore, the maximal adenylyl cyclase response was reduced after m3-muscarinic receptor pretreatment, indicative of a partial desensitization of the receptor. This may be of profound physiological and pathophysiological importance because m3-muscarinic and β_2-adrenergic receptors are coexpressed in many smooth muscle types, for example airway smooth muscle (Eglen et al., 1994), where they regulate smooth muscle tone. Furthermore, the β_2-adrenergic receptor in airway smooth muscle is the therapeutic site for β-adrenergic receptor agonists in obstructive airway diseases such as asthma. The ability of the m3-muscarinic receptor to phosphorylate and desensitize the β_2-adrenergic receptor via PKC may, therefore, have a significant role in the control of smooth muscle tone under normal and pathological conditions.

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