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Hamster α_{1B}-Adrenergic Receptor Directly Activates G_s in the Transfected Chinese Hamster Ovary Cells

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SUMMARY

A prototypic Ca^{2+}-mobilizing hormone receptor, α_1-adrenergic receptor (α_1AR), stimulates cAMP accumulation. The mechanism underlying this phenomenon was previously suggested to be secondary to phosphatidylinositol hydrolysis-protein kinase C activation in some cells. We transfected Chinese hamster ovary (CHO)-K1 cells with hamster α_{1B}AR cDNA and isolated cells stably expressing α_{1B}AR (CHOα_{1B} cells). We investigated the molecular mechanism underlying the α_1AR-mediated cAMP production in the CHOα_{1B} cells. Norepinephrine (NE) stimulated intracellular calcium mobilization and cAMP production through α_{1B}AR. Pretreatment with a phospholipase C inhibitor, U-73,122 (10 μM), abolished the NE-induced intracellular calcium response, whereas it did not affect the NE-stimulated cAMP production. Treatment with various agents (protein kinase C inhibitors, calcium ionophore, cyclo-oxygenase inhibitor, or pertussis toxin) had little effect on the NE-induced cAMP production. The parent CHO and CHOα_{1B} cells contained similar amounts of $G_{s\alpha}$ (42 and 45 kDa, respectively), as detected with immunoblot analysis, and exhibited similar extents of cAMP synthesis with cholera toxin and forskolin. Adenylyl cyclase activity in the CHOα_{1B} cell membranes was also enhanced by NE. Furthermore, incubation of CHOα_{1B} cell membranes with antiserum directed against the carboxyl-terminal portion of $G_{s\alpha}$ inhibited the NE-stimulated adenylyl cyclase activity. Taken together, the results indicate that the α_{1B}AR-mediated cAMP synthesis in CHOα_{1B} cells reflects direct stimulation of G_s-adenylyl cyclase. Therefore, the α_1AR-stimulated cAMP production observed in some native tissues may involve the multiple mechanisms of the direct activation of G_s-adenylyl cyclase and a secondary effect through activation of phosphatidylinositol hydrolysis.

α_1AR is a prototypic Ca^{2+}-mobilizing hormone receptor. α_1AR was pharmacologically subclassified into two receptor subtypes (α_{1A} and α_{1B}) (1), whereas three α_1AR cDNAs have been cloned (α_{1a}, α_{1b}, and α_{1d}) (2–6). α_{1B}AR is the best characterized among the α_1AR subtypes; thus, the α_{1b}AR clone was identified as the pharmacologically defined α_{1B}AR because the profile of the expressed α_{1b}AR clone and the tissue distribution of its mRNA were similar to those expected for the α_{1B}AR subtype. α_{1B}AR is widely distributed and in general coexpressed with other subtypes; however, several tissues, such as rat liver, spleen, and adipose tissue, exclusively express α_{1B}AR (7–9). α_{1A}AR is less well characterized, and the α_{1a}AR (previously defined as α_{1c}AR) clone is considered to encode the pharmacologically defined α_{1A}AR (10).

α_1ARs are usually coupled to G_q after the activation of PLC and PI turnover-Ca^{2+} signaling. However, it was found in some tissues (e.g., rat liver and cerebral cortex), that on activation, α_1AR was also able to stimulate cAMP production

Throughout the article, we use the standardized nomenclature system for α_1-adrenoceptor subtypes recently recommended by the International Union of Pharmacology Committee for Receptor Nomenclature and Drug Classification. With this system, the cloned subtypes are designated in lowercase letters as α_{1a}, α_{1b}, and α_{1d}, which correspond to the clones previously defined as α_{1c}, α_{1b}, and α_{1a} (or $\alpha_{1a/d}$ and α_{1d}), respectively. The corresponding pharmacological subtypes are designated in uppercase letters as α_{1A}, α_{1B}, and α_{1D}, respectively.

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ABBREVIATIONS: AR, adrenergic receptor; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CEC, chloroethylclonidine; CHO cells, Chinese hamster ovary cells; CTX, cholera toxin; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DTT, dithiothreitol; Fura-2/AM, Fura-2 tetrakis(acetoxymethyl)ester; GTPγS, guanosine 5′-*O*-3-thiotriphosphate; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; IBMX, 3-isobutyl-1-methylxanthine; [^{125}I]HEAT; ^{125}I-(2-β-(4-hydroxyphenyl)-ethylaminomethyl)-tetralone; NE, norepinephrine; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; PMSF, phenylmethylsulfonyl fluoride; PTX, pertussis toxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Triton X-100, polyoxyethylene(10)[octhylphenyl] ether; U-73,122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U-73,343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrolidine-2,5-dione; BSS, buffered salt solution; TBS, Tris-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(11–13). The molecular mechanism underlying the α_1AR-stimulated cAMP synthesis has recently been studied with transfected cells expressing α_{1b}AR and suggested to be secondary to the α_1AR-induced PLC pathway (14–16); thus, activation of α_{1B}AR first stimulates PI turnover, promoting PKC activation, and then promotes PKC "cross-talks" with the stimulation of adenylyl cyclase (17). The experimental evidence supporting this sequential mechanism is that PKC inhibitors such as H-7 and staurosporine can inhibit the α_{1B}AR-induced cAMP production in transfected cells (14–16).

We recently transfected CHO cells with hamster α_{1B}AR cDNA and observed that isolated cells stably expressed α_{1B}AR (CHOα_{1B} cells). The pharmacological properties of CHOα_{1B} suggested that α_{1B}AR expression in the cells is the same as that defined in native tissues (18). In the process, we observed that α_{1B}AR could induce both $[Ca^{2+}]_i$ and cAMP responses; however, a PLC inhibitor, U-73,122 (19–21), abolished the agonist-induced $[Ca^{2+}]_i$ response but had little inhibitory effect on the cAMP response. Also, unlike in previous studies (14–16), PKC inhibitors (H-7 and staurosporine) did not inhibit the agonist-induced cAMP production in this system. Therefore, the present study was designed to investigate in greater detail the relationship between α_{1B}AR and G_s-adenylyl cyclase activation. We first compared the effects of selective agents on the α_{1B}AR-induced cAMP response with those on the $[Ca^{2+}]_i$ response. We used antiserum directed against the carboxyl-terminal portion of $G_{s\alpha}$, which has an inhibitory effect on the coupling of G protein-coupled receptor with $G_{s\alpha}$. The linkage of α_{1B}AR with G_s-adenylyl cyclase activity was examined.

Experimental Procedures

Materials. Materials were obtained from the following sources: [^{125}I]HEAT (2200 Ci/mmol) and [^{32}P]NAD (800 Ci/mmol) from DuPont-New England Nuclear; an [^{125}I]cAMP radioimmunoassay kit from Yamasa (Chiba, Japan); Ham's F12 medium and fetal calf serum from GIBCO-BRL; (−)-NE bitartrate, (−)-isoproterenol bitartrate, (±)-propranolol HCl, PMA, IBMX, forskolin, A23187, GTPγS, and indomethacin from Sigma Chemical Co.; rauwolscine HCl and CEC from Research Biochemicals; H-7 from Seikagaku Kogyo Co. (Tokyo, Japan); staurosporine from Kyowa Medex Co. (Tokyo, Japan); CTX from Sanko Junyaku Co. (Tokyo, Japan); PTX from Kaken Pharmaceutical Co. (Shiga); Fura-2/AM from Dojindo Laboratories; and Vectastain ABC reagent and biotinated goat anti-rabbit IgG from Vector Laboratories. The antisera directed against the carboxyl-terminal portions of $G_{q\alpha}$ and $G_{11\alpha}$ (anti-$G_{q\alpha/11\alpha}$ antisera) were purchased from DuPont-New England Nuclear. Prazosin HCl was a gift from Pfizer; U-73,122 and U-73,343 were gifts from Upjohn; and phenoxybenzamine was a gift from SmithKline and Beecham Pharmaceuticals. The cDNA-encoding hamster α_{1b}AR was generously provided by Dr. S. Cotecchia (Duke University Medical Center, Durham, NC) (2). All other chemicals were from sources listed previously (18, 22).

Cell culture and stable expression. Cell culture and transfection of hamster α_{1b}AR cDNA into CHO cells and the pharmacological properties of the α_{1b}AR stably expressed in CHO cells (called CHOα_{1B} cells) have been described elsewhere (18). The CHOα_{1B} cells used in the present study had a B_{max} of 1.8 ± 0.3 pmol/mg protein and a K_d of 170 ± 10 pM (five experiments) for the α_1AR antagonist radioligand [^{125}I]HEAT.

Measurement of cAMP production. cAMP production in intact CHOα_{1B} cells was determined as described previously (23). Briefly, CHOα_{1B} cells were seeded onto six-well plates at a density of 5×10^5 cells/well and then cultured for 12–16 hr. The cells were washed twice with PBS, incubated for 30 min in a BSS (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 1 mM $NaHPO_4$, 5 mM HEPES, and 11 mM glucose, pH 7.4), and then placed in fresh BSS containing 1 mM IBMX for 10 min. The reaction was started by adding various concentrations of the test reagents. After incubation for the indicated time, the medium was aspirated off, and the reaction was stopped with 6% (w/v) trichloroacetic acid. cAMP levels were determined with a cAMP radioimmunoassay kit. As judged in preliminary experiments, NE-induced cAMP production in intact CHOα_{1B} cells was not affected by 1 μM propranolol, so propranolol was included in the following experiments. Prazosin and rauwolscine were added 10 min before agonist stimulation in BSS containing 1 mM IBMX and 1 μM propranolol. Pretreatment with CEC was performed as described previously (30 min at 37° in BSS) (18), and then the cells were washed twice with BSS and incubated in fresh BSS containing 1 mM IBMX and 1 μM propranolol for 10 min before agonist stimulation. Pretreatment with the PLC inhibitor U-73,122 (19–21); its inactive analogue, U-73,343; or the vehicle (DMSO, 0.1% v/v final) was performed for 10 min before agonist stimulation in BSS containing 1 mM IBMX and 1 μM propranolol.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured as described previously (18). Briefly, cells were incubated in BSS containing 4 μM Fura-2/AM for 30 min at 25°. The cells were then washed twice and resuspended in BSS without the dye. Mobilization of Ca^{2+} evoked by agonists was monitored with a JASCO CAF-110 fluorescence spectrophotometer (Nihon Bunkoh, Tokyo, Japan) with dual excitation at 340 and 380 nm and emission at 500 nm. NE induced an acute $[Ca^{2+}]_i$ increase in the transfected cells that was followed by a lower plateau $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ calibration was performed for every aliquot by equilibrating intracellular and extracellular Ca^{2+} with 5 μl of 10% Triton X-100 followed by the addition of 5 μl of 300 mM EGTA/3 M Tris buffer, pH 9.0. $[Ca^{2+}]_i$ was determined from the ratio of fluorescence at 340 and 380 nm, as described by Grynkiewicz *et al.* (24), with the following equation: $[Ca^{2+}]_i$ (nM) = $K_d \times [(R-R_{min})/(R_{max}-R)] \times \beta$, where β is the ratio of the fluorescence of Fura-2 at 380 nm in zero and saturating Ca^{2+}, and K_d is the dissociation constant of Fura-2 for Ca^{2+}, assumed to be 224 nM.

Phenoxybenzamine inactivation method. In some experiments, to delineate the linkage between α_1AR occupation and the $[Ca^{2+}]_i$ or cAMP response, we used the phenoxybenzamine inactivation method (22, 25). To examine the effect of receptor inactivation by phenoxybenzamine on [^{125}I]HEAT binding, transfectant cells were suspended in BSS containing specified phenoxybenzamine concentrations (1 pM–10 μM) for 30 min at 37°. After incubation, the cells were washed three times and then homogenized into membranes for binding studies. Radioligand binding studies were performed as described previously (6). Briefly, buffer B (50 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, and 10 mM EGTA) was used as the incubation medium. Measurement of specific [^{125}I]HEAT binding was performed by incubating 0.1 ml of membrane preparation (~10 μg protein) with [^{125}I]HEAT in a final volume of 0.25 ml of buffer B for 60 min at 25° in the presence or absence of phentolamine (10 μM). The incubation was terminated by the addition of ice-cold buffer B and immediate filtering through Whatmann GF/C glass-fiber filters in a Brandel cell harvester (Model 30; Gaithersburg, MD). The radioactivity of each filter was measured. Binding assays were always performed in duplicate. Data were expressed as percentage of B/B_{max}, where B is the amount of [^{125}I]HEAT specifically bound under the experimental conditions, and B_{max} is the amount of specific [^{125}I]HEAT binding in the absence of phenoxybenzamine.

To measure phenoxybenzamine inhibition of the NE-induced $[Ca^{2+}]_i$ and cAMP responses, we treated transfectant cells with phenoxybenzamine in the same way as for the binding studies. Data are expressed as percentages of the $[Ca^{2+}]_i$ or cAMP response induced by 1 μM NE alone.

Treatment with PTX and CTX and ADP-ribosylation. The pretreatment of cells with PTX (1 μg/ml) or CTX (20 μg/ml) was

performed in monolayers on Ham's F12 medium without fetal calf serum for 24 hr. To assess the adequacy of the PTX treatment, we quantified the ADP-ribosylation of substrates derived from membranes of nontreated CHOα_{1B} cells and of those treated with PTX, as described previously (26). Membranes were prepared from control cells and cells treated with PTX (1 μg/ml) for 24 hr and used for [^{32}P]ADP-ribosylation with preactivated PTX following the method of Bokoch *et al.* (27). They were then incubated with [^{32}P]NAD (10 μM, 5,000–15,000 cpm/pmol) in the presence of 100 mM Tris·HCl, pH 8.0, 10 mM thymidine, 1 mM ATP, 100 μM GTP, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 50 μg/ml PTX for 30 min at 30°. At the end of the labeling period, samples were diluted 5-fold in Laemmli sample buffer (28) and then incubated at 99° for 1 min to stop the reaction. Next, 10% SDS-PAGE and autoradiography were carried out according to the method of Schleifer *et al.* (29). Autoradiography revealed a single ^{32}P-labeled band corresponding to molecular mass of ~40 kDa. The ^{32}P content was determined with a BAS2000 bioimaging analysis system (Fuji Co., Tokyo, Japan).

Immunological studies. Antiserum directed against the carboxyl-terminal portion of $G_{s\alpha}$ (anti-$G_{s\alpha}$ antiserum) was produced in a New Zealand White rabbit by immunization with a conjugate of keyhole-limpet hemocyanin (Calbiochem) and a synthetic peptide, RMHLRQYELL, which corresponds to the carboxyl-terminal decapeptide of all forms of $G_{s\alpha}$. The methods used have been described in detail (30). The specificities of the anti-$G_{s\alpha}$ and anti-$G_{q\alpha/11\alpha}$ antisera were investigated with immunoblot analysis using the recombinant bovine $G_{s\alpha}$ short-form ($G_{s\alpha\text{-}s}$) protein prepared with an *Escherichia coli* expression system (31) and a membrane preparation derived from human embryonic kidney 293 cells transiently expressing $G_{s\alpha\text{-}s}$ or mouse $G_{11\alpha}$. A plasmid containing bovine $G_{s\alpha\text{-}s}$ cDNA was used as described previously (32). Mouse $G_{11\alpha}$ cDNA was cloned by reverse-transciptase polymerase chain reaction and confirmed by sequencing.

With anti-$G_{s\alpha}$ antiserum, immunoblot analysis of $G_{s\alpha}$ expression in membranes from wild-type CHO-K1 cells and CHOα_{1B} cells and from wild-type and *cyc*$^-$ S49 murine lymphoma cells was performed. Briefly, 25 μg of a membrane sample was resolved by 10% SDS-PAGE under denaturing conditions. The proteins were transferred to an Immobilon-P transfer membrane (Nihon Millipore, Tokyo, Japan) in a Trans-Blot semidry transfer cell (Bio-Rad), followed by blocking with 5% BSA in TBS (20 mM Tris·HCl, pH 7.5, and 500 mM NaCl) and incubation with a 1:500 dilution of anti-$G_{s\alpha}$ antiserum in 5% BSA-TBS overnight at 4°. The membrane was then exclusively washed with TBS containing 0.1% (v/v) Tween 20, incubated with biotinated goat anti-rabbit IgG (1:3000 dilution) for 1 hr, exclusively washed again, and finally incubated with Vectastain ABC reagent for 1 hr. After extensive washes, the blotting membrane was developed with DAB with $NiCl_2$ enhancement. The protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce).

Adenylyl cyclase assay. CHOα_{1B} cells were cultured in 15-cm dishes, suspended in a lysis buffer (62.5 mM Tris·HCl, pH 7.4, 2 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine, 5 μg/ml pepstatin A, and 1 μg/ml leupeptin), and then homogenized in a Cell Disruption Bomb (Parr Instrument Co.) at 400 psi for 30 min. The homogenate was centrifuged at 35,000 × *g* for 20 min at 4°, and the pellet was resuspended in 10 mM Tris·HCl, pH 7.4, (1 mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine, 5 μg/ml pepstatin A, and 1 μg/ml leupeptin) and then used as the membrane preparation. The standard assay mixture comprised 20 μg of membrane proteins in 100 μl of 50 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM IBMX, 0.5 mM ATP, 1 mM DTT, 1 μM GTP, and 1 μM propranolol. Membranes from wild-type and *cyc*$^-$ S49 murine lymphoma cells were prepared in the same manner.

Reactions were started by the addition of the agonists, carried out for 15 min at 37°, and terminated by being boiled at 90 sec. The cAMP levels were determined with a cAMP radioimmunoassay kit. For the experiments involving anti-$G_{s\alpha}$ and anti-$G_{q\alpha/11\alpha}$ antisera, membranes were resuspended in the standard assay mixture and then incubated with anti-$G_{s\alpha}$ antiserum (1:50 dilution), anti-$G_{q\alpha/11\alpha}$ antiserum (1:50 dilution), preimmune serum (1:50 dilution), or buffer alone for 1 hr on ice. Membranes were added to the reaction mixture with various drugs, and adenylyl cyclase activity was determined.

Statistical analysis. Values are expressed as mean ± standard error. Two-way analysis of variance with 95% or 99% confidence limits, followed by a Student's *t* test on individual sets of data, was performed with the analytical software StatView 4.0 (BrainPower Inc.).

Results

α_{1B}AR-stimulated $[Ca^{2+}]_i$ response and cAMP synthesis. The pharmacological properties of the α_{1B}AR-stimulated $[Ca^{2+}]_i$ response have been described in detail (18). NE elicited a rapid increase in $[Ca^{2+}]_i$ in CHOα_{1B} cells consisting of a quick transient peak and then a more-sustained component. The NE-stimulated $[Ca^{2+}]_i$ response was abolished by the α_1AR antagonist prazosin (100 nM). Treatment with the α_{1B}AR-selective alkylating agent CEC (100 μM) eliminated 84.6% of the NE (100 nM)-induced $[Ca^{2+}]_i$ response (two experiments). The dose-response relationship between NE and the $[Ca^{2+}]_i$ response in CHOα_{1B} cells demonstrated an EC_{50} value of 43.0 ± 5.5 nM (five experiments), with maximal stimulation achieved with 10 μM NE (Fig. 1). The time course of NE (1 μM)-stimulated cAMP accumulation in intact CHOα_{1B} cells demonstrated that the cAMP level increased linearly and reached the maximal level at 10 min after the addition of NE (data not shown); therefore, this incubation time was used for all experiments. The dose-response relationship between NE and cAMP production in CHOα_{1B} cells demonstrated an EC_{50} value of 331 ± 45 nM (four experiments), indicating that maximal stimulation was achieved at 10 μM (Fig. 1). NE did not increase $[Ca^{2+}]_i$ or cAMP accumulation in wild-type CHO-K1 cells or in CHO cells transfected with the vector DNA alone (data not shown).

The NE-stimulated cAMP production in CHOα_{1B} cells was confirmed to be mediated by the α_{1B}AR subtype. As summarized in Fig. 2, both the α_1AR antagonist prazosin (1 μM) and pretreatment with CEC (10 μM) markedly inhibited the NE-

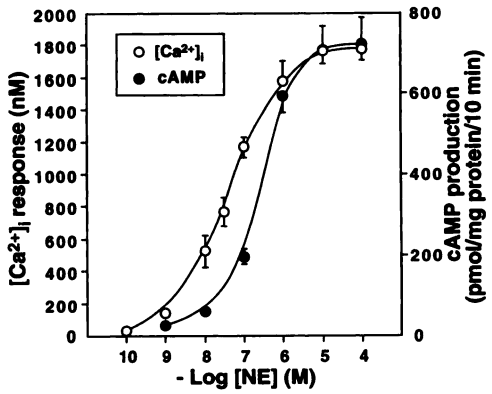

Fig. 1. Concentration-dependent stimulation by NE of $[Ca^{2+}]_i$ and cAMP responses in CHOα_{1B} cells. $[Ca^{2+}]_i$ responses were determined by subtracting basal $[Ca^{2+}]_i$ levels from peak $[Ca^{2+}]_i$ levels induced by NE. The basal $[Ca^{2+}]_i$ level was 76 ± 14 nM (five experiments), and the basal cAMP level was 25.2 ± 2.6 pmol/mg protein/10 min (four experiments), respectively. Details are given in Experimental procedures. Values are given as mean ± standard error of five independent experiments for $[Ca^{2+}]_i$ response and of four independent experiments for cAMP production, which were performed in duplicate.

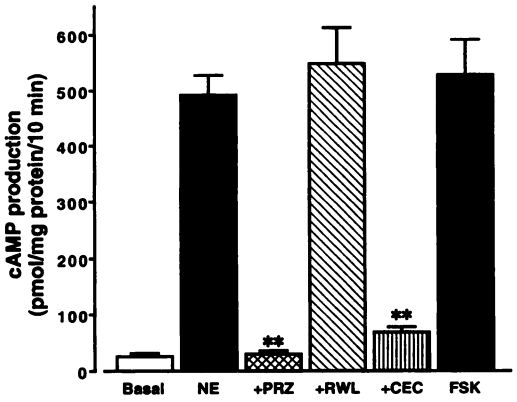

Fig. 2. Effects of various agents on cAMP production in CHOα_{1B} cells. Cells were pretreated without or with 1 μM prazosin (+*PRZ*) or 100 nM rauwolscine (+*RWL*) for 10 min or with CEC (10 μM) (+*CEC*) for 30 min. Then, the cells were incubated with NE (1 μM). Also, cells were stimulated by 5 μM forskolin for 10 min (*FSK*). cAMP levels were measured as described in Experimental procedures. Values are given as mean ± standard error of 6–14 independent experiments, which were performed in duplicate. Basal cAMP level was 27.1 ± 1.8 pmol/mg protein/10 min (14 experiments). **, $p < 0.01$ versus NE alone.

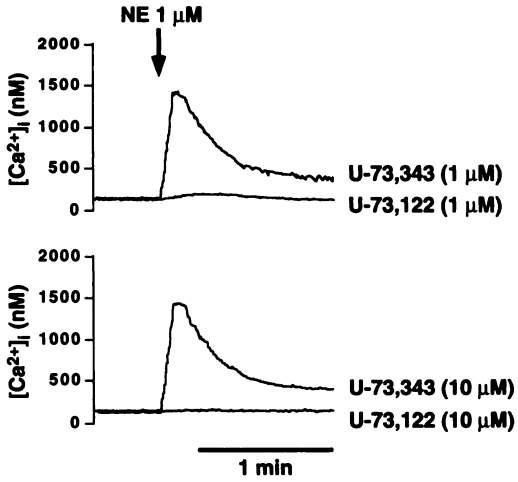

Fig. 3. Inhibitory effects of PLC inhibitor U-73,122 on NE-stimulated $[Ca^{2+}]_i$ responses in CHOα_{1B} cells. Cells were loaded with Fura-2/AM as described in Experimental procedures and then incubated (1×10^6 cells/ml) for 3 min at 25° with either U-73,122, its inactive analogue U-73,343, or vehicle (DMSO, 0.1% v/v final) before NE stimulation. When cells were exposed to NE alone, $[Ca^{2+}]_i$ increased from 152 ± 21 nM to 1475 ± 137 nM (three experiments). Neither U-73,122, U-73,343, nor vehicle alone significantly altered the fluorescence emission ratio in unstimulated cells. The results presented are representative of at least three similar experiments.

stimulated cAMP production—by 93.8 ± 0.1% (six experiments) and 86.1 ± 1.8% (six experiments), respectively. The α_2AR antagonist rauwolscine (100 nM), however, had no effect on NE-stimulated cAMP production. Neither rauwolscine, prazosin, nor CEC treatment had a significant effect on the basal level of cAMP in CHOα_{1B} cells (data not shown). Forskolin (5 μM) elicited approximately 12- and 14-fold cAMP production above basal levels in wild-type CHO-K1 and CHOα_{1B} cells, respectively.

Effects of the PLC inhibitor U-73,122, a Ca^{2+} ionophore, and indomethacin on α_{1B}AR-stimulated cAMP production. To determine whether the two α_{1B}AR-mediated responses of $[Ca^{2+}]_i$ and cAMP synthesis in CHOα_{1B} cells were independent or sequential events, we examined the effects of a PLC inhibitor, U-73,122, on the two responses. Although U-73,122 (1–10 μM) did not cause a change in the basal $[Ca^{2+}]_i$ level, pretreatment with U-73,122 (3 min) almost abolished the NE-induced $[Ca^{2+}]_i$ response (treatment with 1 μM, 93.6 ± 1.3%, three experiments; with 10 μM, 100%, three experiments) (Fig. 3). On the other hand, neither an inactive analogue, U-73,343 (Fig. 3), nor the vehicle alone (DMSO, 0.1% v/v final; data not shown) had an effect on the NE-induced $[Ca^{2+}]_i$ response. In contrast to the effect on the $[Ca^{2+}]_i$ response, pretreatment with U-73,122 (10 μM) did not affect the NE-stimulated cAMP production (623.6 ± 65.3 versus 556.0 ± 47.8 pmol/mg protein/10 min, seven experiments each, with and without U-73,122 treatment, respectively). Similarly, neither U-73,343 nor the vehicle alone had an effect (data not shown)

We also examined the effect of the $[Ca^{2+}]_i$ increase on the NE-stimulated cAMP production in CHOα_{1B} cells. Cells were pretreated with 10 μM Ca^{2+} ionophore A23187 or the vehicle (DMSO, 0.1% v/v final) for 15 min and then stimulated with NE (1 μM). Neither A23187 nor the vehicle had an effect on the basal level of cAMP production (data not shown) or on the NE-stimulated cAMP production (650.1 ± 18.1 versus 647.7 ± 67.6 pmol/mg protein/10 min, four experiments each, with and without A23187 treatment, respectively). Although a previous study showed that α_1AR-stimulated cAMP production in the rat brain was blocked by an inhibitor of prostaglandin synthesis (33), treatment with indomethacin (50 μM, 1 hr) had no effect on the NE-stimulated cAMP production in CHOα_{1B} cells (557.2 ± 76.5 versus 508.8 ± 25.8 pmol/mg protein/10 min, four experiments each, with and without indomethacin treatment, respectively).

Effects of PKC modulators on the α_{1B}AR-stimulated $[Ca^{2+}]_i$ and cAMP responses. The effects of the PKC inhibitors staurosporine and H-7 were examined in CHOα_{1B} cells. Cells were treated with 100 nM staurosporine or 50 μM H-7 for 1 or 12 hr before the NE stimulation. Neither of the PKC inhibitors (1-hr treatment) had a significant effect on the NE-stimulated cAMP production in CHOα_{1B} cells (552.7 ± 48.2 versus 461.4 ± 41.2 pmol/mg protein/10 min, seven experiments each, with and without H-7 treatment, respectively; 520.4 ± 51.7 versus 461.4 ± 41.2 pmol/mg protein/10 min, seven experiments each, with and without staurosporine treatment, respectively). Similarly, longer incubation (12 hr) with H-7 had no significant effect (data not shown); however, longer treatment with staurosporine (100 nM, 12 hr) significantly ($p < 0.01$) increased the NE-stimulated cAMP production, which might be due to its cytotoxic effect (1076.8 ± 161.5 versus 262.0 ± 20.0 pmol/mg protein/10 min, four experiments each, with and without staurosporine treatment, respectively). The longer treatment with staurosporine markedly affected cell viability (number of living cells, 5.1×10^5 and 0.8×10^5 cells/well in the control and staurosporine-treated groups, respectively, two experiments each; 5.0×10^5 cells/well were initially seeded), whereas H-7 did not have such an effect (data not shown).

The effect of the PKC activator PMA was also examined in CHOα_{1B} cells. Treatment with PMA (100 nM, 10 min) did not affect the basal level of either $[Ca^{2+}]_i$ or cAMP in CHOα_{1B} cells; however, PMA (100 nM, 10 min) significantly ($p < 0.01$) attenuated both the NE (1 μM)-induced $[Ca^{2+}]_i$ (121 ± 4 versus 1022 ± 130 nM, four experiments each, with and

without PMA treatment for 10 min, respectively) and cAMP (242.4 ± 28.5 versus 656.3 ± 21.2 pmol/mg protein/10 min, four experiments each, with and without PMA treatment for 10 min, respectively) responses. Similarly, longer treatment with PMA (100 nM, 24 hr) significantly attenuated both the NE (1 μM)-induced $[Ca^{2+}]_i$ and cAMP responses (data not shown). The attenuation of α_1AR-mediated responses by the phorbol ester may be associated with phosphorylation of α_1AR (34).

Effects of PTX and CTX treatments. To determine whether a PTX-sensitive G protein is involved in the NE-stimulated cAMP production, we incubated CHOα_{1B} cells with 1 μg/ml PTX for 24 hr. The PTX treatment significantly ($p < 0.01$) inhibited the thrombin-induced (0.5 units/ml) $[Ca^{2+}]_i$ response in CHOα_{1B} cells (70.7 ± 3.3% inhibition, eight experiments), confirming the previous finding that the thrombin-stimulated PI hydrolysis in CHO-K1 cells is PTX sensitive (35). Also, the PTX treatment was sufficient for complete ADP-ribosylation of all of the substrates present in the cells (Fig. 4; autoradiography revealed a single ^{32}P-labeled band corresponding to a molecular mass of ~40 kDa). The PTX treatment, however, did not affect either the NE-stimulated $[Ca^{2+}]_i$ response (1052 ± 37 versus 1110 ± 81 nM, four experiments each, with and without PTX treatment, respectively) or the cAMP response (416.5 ± 27.9 versus 428.9 ± 18.0 pmol/mg protein/10 min, four experiments each, with and without PTX treatment, respectively) in CHOα_{1B} cells, indicating that none of the PTX-sensitive G proteins are involved in either the α_{1B}AR-mediated $[Ca^{2+}]_i$ or cAMP response in CHOα_{1B} cells.

When CHOα_{1B} cells were pretreated with CTX (20 μg/ml, 24 hr), the basal level of cAMP increased 50-fold compared with control CHOα_{1B} cells (2855 versus 57 pmol/mg protein/10 min, two experiments each, with and without CTX treatment, respectively), suggesting that CHOα_{1B} cells contain a CTX-sensitive G_s protein or proteins that couple to adenylyl cyclase.

Immunoblot analysis of $G_{s\alpha}$ expression with anti-$G_{s\alpha}$ antisera. The specificities of the anti-$G_{s\alpha}$ and anti-$G_{q\alpha/11\alpha}$ antisera were examined with immunoblot analysis of the recombinant $G_{s\alpha}$ short-form ($G_{s\alpha\text{-}s}$) protein and $G_{11\alpha}$. As shown in Fig. 5A, the results of immunoblotting clearly show that the anti-$G_{s\alpha}$ antiserum is highly specific for $G_{s\alpha}$ and exhibits little cross-reactivity to $G_{11\alpha}$, whereas the anti-$G_{q\alpha/11\alpha}$ antiserum is highly specific for $G_{11\alpha}$ and exhibites little cross-reactivity to $G_{s\alpha}$. Next, membranes of wild-type CHO-K1 and CHOα_{1B} cells were immunoblotted with the anti-$G_{s\alpha}$ antiserum. The antiserum used is directed against a decapeptide corresponding to the carboxyl-terminus of $G_{s\alpha}$, a region critical for receptor-G_s coupling (36, 37). As shown in Fig. 5B, the antisera recognized almost similar amounts of two proteins (molecular masses of 42 and 45 kDa) on immunoblots of the two types of cell membranes. By using this antiserum, the same species of membrane proteins were identified in wild-type S49 murine lymphoma cells, whereas they were not detected in the *cyc*$^-$ mutant of S49 cells, which lacks $G_{s\alpha}$ (Fig. 5B).

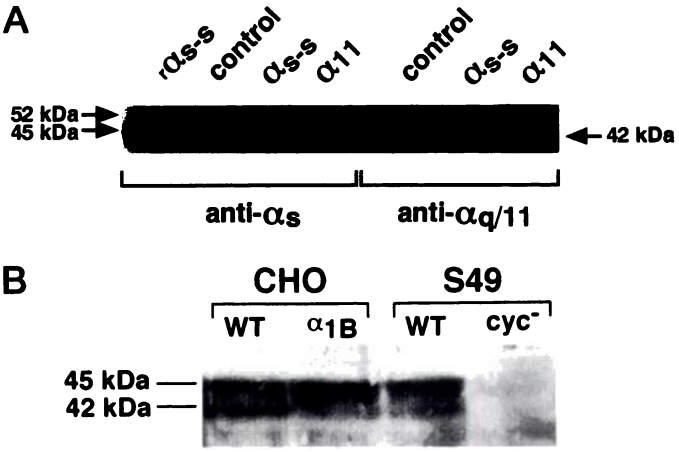

Fig. 5. A, Immunoblot analysis with anti-$G_{s\alpha}$ and anti-$G_{q\alpha/11\alpha}$ antisera. One hundred nanograms of recombinant $G_{s\alpha}$ short-form protein prepared from *E. coli* expression system ($r\alpha_{s\text{-}s}$) or 30 μg of membrane proteins prepared from wild-type HEK 293 cells (control) and transfected cells ($\alpha_{s\text{-}s}$ and α_{11}) was separated by 11% SDS-PAGE, transferred to filter, and immunoblotted with indicated antibodies. B, Immunoblot analysis with anti-$G_{s\alpha}$ antisera. Twenty-five micrograms of membrane proteins from wild-type CHO cells (*CHO, WT*), CHOα_{1B} cells (*CHO, α_{1B}*), wild-type S49 murine lymphoma cells (*S49, WT*), or *cyc*$^-$ S49 cells (*S49, cyc*$^-$) were separated by SDS-PAGE, transferred to filter, and immunoblotted with anti-$G_{s\alpha}$ antisera.

Effects of anti-$G_{s\alpha}$ antisera on adenylyl cyclase activity in membranes. NE dose-dependently increased the adenylyl cyclase activity in the CHOα_{1B} cell membranes (Fig. 6). The NE (10 μM)-stimulated adenylyl cyclase activity was

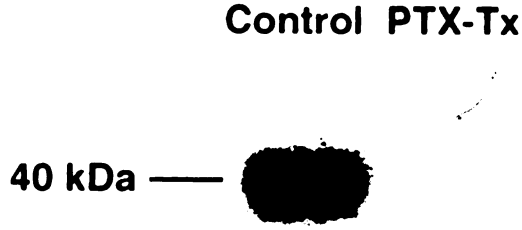

Fig. 4. ADP-ribosylation of CHOα_{1B} cell membranes obtained from control and PTX-treated cells. A typical autoradiograph is shown. Pretreatment with vehicle (*Control*) or 1 μg/ml PTX (*PTX-Tx*) for 24 hr was performed as described in Experimental procedures. In our experimental condition, ADP-ribosylation was observed of a major protein with a molecular mass of ~40 kDa.

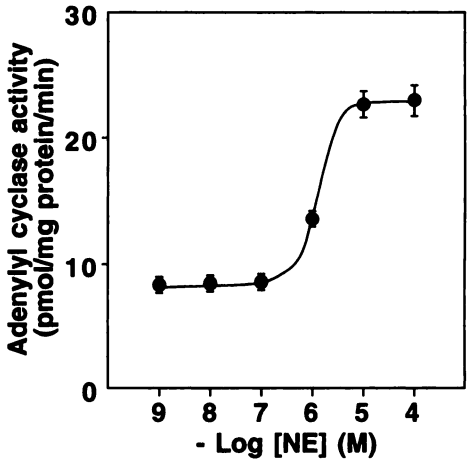

Fig. 6. Concentration-dependent stimulation by NE of adenylyl cyclase activity in CHOα_{1B} cell membranes. Membrane preparation derived from CHOα_{1B} cells were incubated with indicated concentrations of NE for 15 min at 37° and then used to determine cAMP production. Values are given as mean ± standard error of three independent experiments, which were performed in duplicate.

completely inhibited by 1 μM prazosin (Fig. 7). Both forskolin (5 μM) and a GTP analogue, GTPγS (10 μM), elicited an approximately 15-fold increase in adenylyl cyclase activity above the basal level in CHOα_{1B} cells (Fig. 7). The NE-stimulated adenylyl cyclase activation was not observed in the membrane preparation derived from wild-type CHO-K1 or CHO cells transfected with the vector DNA alone (data not shown).

Next, we examined the effects of the anti-$G_{s\alpha}$ and anti-$G_{q\alpha/11\alpha}$ antisera on the NE-stimulated adenylyl cyclase activation in CHOα_{1B} cell membranes. As shown in Fig. 8, incubation of a CHOα_{1B} cell membrane preparation with the anti-$G_{s\alpha}$ antiserum significantly ($p < 0.01$) attenuated the increase in NE (10 μM)-stimulated adenylyl cyclase activation by 77.6 ± 6.3% (14 experiments) compared with the corresponding increase in NE-stimulated adenylyl cyclase activity on incubation with preimmune serum. Incubation of CHOα_{1B} cell membranes with the anti-$G_{q\alpha/11\alpha}$ antiserum, however, did not significantly attenuate the increase in NE-stimulated adenylyl cyclase activation compared with incubation with preimmune serum (increase, 28.9 ± 9.3%; three experiments). Under the same experimental condition, an inhibitory effect of the anti-$G_{s\alpha}$ antiserum was also observed for the isoproterenol (1 μM)-stimulated adenylyl cyclase activation in wild-type S49 cells (41.4 ± 9.4% inhibition, three experiments, compared with the corresponding increase in isoproterenol-stimulated adenylyl cyclase activity on incubation with preimmune serum, $p < 0.05$). The inhibitory effect of the anti-$G_{s\alpha}$ antiserum observed in CHOα_{1B} or wild-type S49 cells was blocked by coincubation with the antigen of the relevant peptide (25 μg/ml), whereas a nonspecific decapeptide (25 μg/ml) had no effect (data not shown). The anti-$G_{s\alpha}$ antiserum had no effect on the GTPγS-induced activation of adenylyl cyclase.

α_{1B}AR occupancy-response relationships for $[Ca^{2+}]_i$ and cAMP. We examined the quantitative relationship between the fractional occupation of α_{1B}AR and the NE-induced $[Ca^{2+}]_i$ or cAMP response by the phenoxybenzamine inactivation method. The maximal [^{125}I]HEAT binding capacity progressively decreased with exposure to increasing concentrations of phenoxybenzamine in CHOα_{1B} cells, with the minimal level of 1.5 ± 0.5% (four experiments) being reached at a phenoxybenzamine concentration of 10 μM (Fig. 9A). The phenoxybenzamine concentration necessary to block half of the [^{125}I]HEAT sites was 17 ± 4 nM (four experiments). The effects of partial α_{1B}AR inactivation on the maximal NE-induced $[Ca^{2+}]_i$ and cAMP responses in CHOα_{1B} cells are shown in Fig. 9B. The IC_{50} for the NE-induced $[Ca^{2+}]_i$ response is 46 ± 4 nM phenoxybenzamine (five experiments), whereas that for NE-induced cAMP production is 9.6 nM phenoxybenzamine (two experiments), respectively. As summarized in Fig. 9C, the relationship between the receptor occupancy and the responses was markedly different between the two responses; the relationship between receptor occupancy and the $[Ca^{2+}]_i$ response was markedly nonlinear, whereas that for cAMP production was linear.

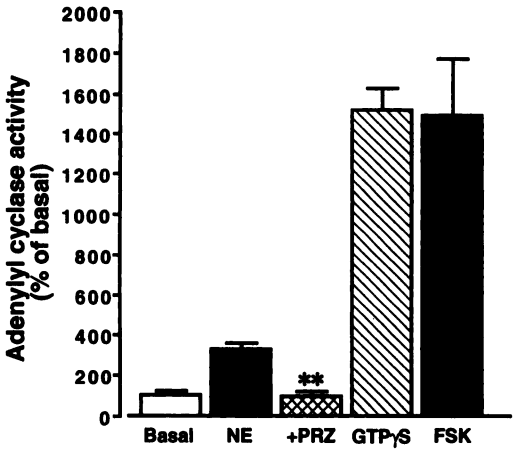

Fig. 7. Effects of various agents on adenylyl cyclase activity in CHOα_{1B} cell membranes. Adenylyl cyclase activities in membranes prepared from CHOα_{1B} cells were determined as described in Experimental procedures. Twenty micrograms of membrane proteins from CHOα_{1B} cells were incubated for 15 min at 37° with vehicle (*Basal*) or 10 μM NE without (*NE*) or with pretreatment with 1 μM prazosin (*+PRZ*) or with 10 μM GTPγS (*GTPγS*) or 5 μM forskolin (*FSK*). Values are given as mean ± standard error of 4–11 independent experiments, which were performed in duplicate. Basal adenylyl cyclase activity was 8.0 ± 0.5 pmol/mg protein/min. **, $p < 0.01$ versus NE alone.

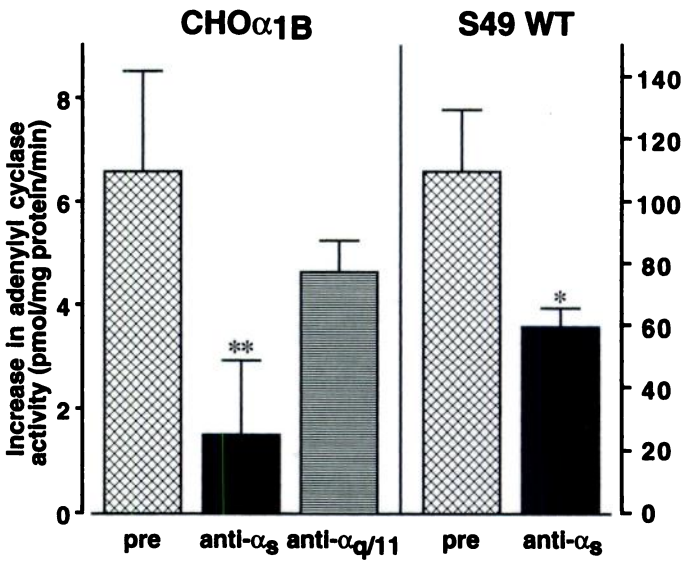

Fig. 8. Functional attenuation of G_s coupling by anti-$G_{s\alpha}$ antisera. Membranes prepared from CHOα_{1B} cells or wild-type S49 murine lymphoma cells were preincubated with preimmune sera (*pre*), anti-$G_{s\alpha}$ antisera (1:50 dilution) (*anti-*α_s), or anti-$G_{q\alpha/11\alpha}$ antisera (1:50 dilution) (*anti-*$\alpha_{q/11}$) for 1 hr on ice and stimulated for 15 min by 10 μM NE for CHOα_{1B} cells or by 1 μM isoproterenol for S49 cells, respectively. Adenylyl cyclase activities were determined as described in Experimental procedures. Values are given as mean ± standard error of 3–14 independent experiments, which were performed in duplicate. Basal adenylyl cyclase activities were 7.9 ± 1.7 pmol/mg protein/min (14 experiments, CHOα_{1B} cells) and 16.2 ± 1.2 pmol/mg protein/min (three experiments, S49 cells), respectively. **, $p < 0.01$ compared with corresponding increase in NE (10 μM)-stimulated adenylyl cyclase activity of CHOα_{1B} cells incubated with preimmune serum. *, $p < 0.05$ compared with corresponding increase in isoproterenol (1 μM)-stimulated adenylyl cyclase activity of S49 cells incubated with preimmune serum.

Discussion

Using CHOα_{1B} cells as a model, we examined whether α_{1B}AR-mediated cAMP production reflects direct stimulation of G_s/adenylyl cyclase or a secondary effect to its PI hydrolysis/Ca^{2+}-PKC signaling. In CHOα_{1B} cells, NE elicited both $[Ca^{2+}]_i$ and cAMP responses; however, a PLC inhibitor, U-73,122, abolished the NE-induced $[Ca^{2+}]_i$ response but

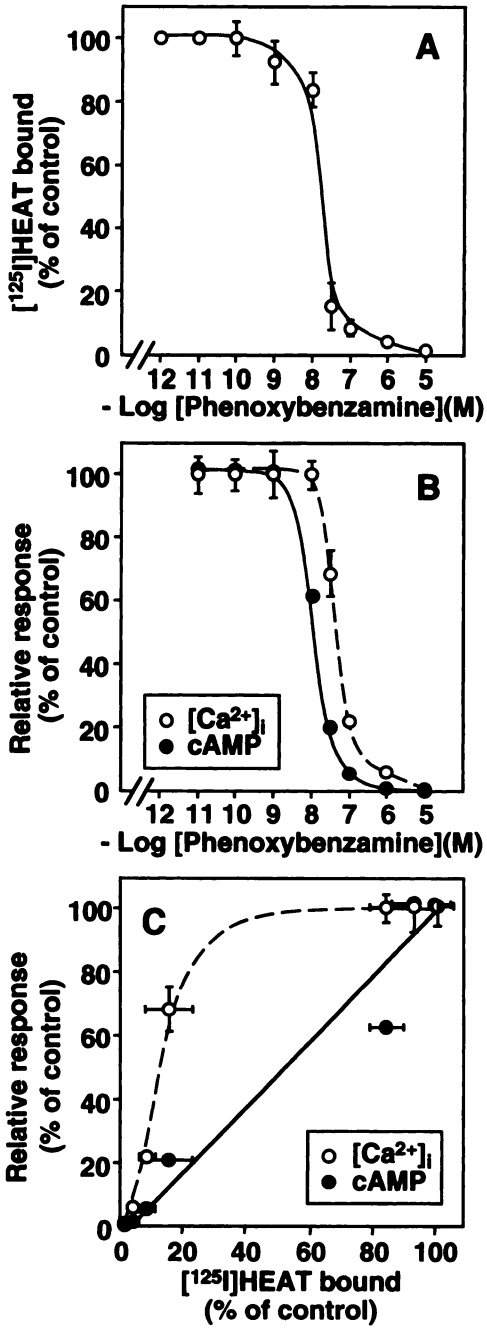

Fig. 9. Effects of treatment of CHOα_{1B} cells with phenoxybenzamine on [^{125}I]HEAT binding capacity (A) and 1 μM NE-stimulated $[Ca^{2+}]_i$ and cAMP responses (B). Details are given in Experimental procedures. Maximal specific [^{125}I]HEAT binding capacity was 1.8 ± 0.3 pmol/mg protein (five experiments). Maximal $[Ca^{2+}]_i$ response induced by 1 μM NE was 1535 ± 186 nM (five experiments). Maximal cAMP production induced by 1 μM NE was 556.3 pmol/mg protein/10 min (two experiments). Relationship between [^{125}I]HEAT binding capacity and 1 μM NE-stimulated $[Ca^{2+}]_i$ and cAMP responses after incubation with phenoxybenzamine (C). Data depicted are replotted from Fig. 9, A and B.

had little effect on the cAMP production. Also, treatment with PKC inhibitors had little effect on the NE-induced cAMP production. PTX-sensitive G proteins were not involved in the α_{1B}AR-mediated cAMP production in CHOα_{1B} cells; however, the antiserum against the carboxyl-terminus of $G_{s\alpha}$ inhibited the NE-stimulated adenylyl cyclase activity. The results show that the α_{1B}AR-mediated cAMP production in CHOα_{1B} cells is not secondary to the PI hydrolysis/PKC signaling activation but rather reflects direct stimulation of G_s-adenylyl cyclase.

As described in the introductory paragraphs, α_{1B}AR-mediated cAMP production was believed to be secondary to the activation of PKC in α_{1B}AR-expressing COS or HeLa cells because PKC inhibitors (H-7, sphingosine, and staurosporine) reduced the α_{1B}AR-mediated cAMP production (14–16). Data from the present study, however, show that these PKC inhibitors had little effect on the α_{1B}AR-stimulated cAMP production in CHO cells stably expressing α_{1B}AR. The reason for the different observations between the COS and HeLa cells and the CHO cells is not clear. Factors that could be related to this disparity are the different adenylyl cyclase and PKC isoforms in the cells. For adenylyl cyclase, at least six mammalian isozymes, which have different properties, have been identified (38). The enzymes are activated by the α subunit of G_s and forskolin, but type-specific regulation of enzymatic activity is known (39); thus, types II and IV adenylyl cyclases are activated by the $\beta\gamma$ subunit complex of G proteins, types I and III are known to be activated by Ca^{2+}-calmodulin, and type II (and possibly types I and VI) (40, 41) can be activated by PKC. A difference in the type-specific regulation of adenylyl cyclase isozymes was found to play an important role in the cellular difference of cAMP synthesis (40). α_2AR can inhibit G_s-mediated cAMP production when transfected into human embryonic kidney 293 cells; however, cotransfection with adenylyl cyclase type II changed the α_2AR-mediated inhibitory action into a stimulatory one through the activation of G protein $\beta\gamma$ subunits (possibly from G_s and G_i proteins) (42). The effects of $\beta\gamma$ dimers of G_q proteins released on α_1AR activation on other adenylyl cyclase isozymes are presently not well known, and the possible effects on the adenylyl cyclase isozymes in CHO cells need to be clarified. Furthermore, for regulation of cAMP synthesis by PKC, various isozymes of PKC have been postulated to play a modulatory role, in that the α isozyme of PKC may inhibit forskolin-mediated cAMP production, whereas the γ isozyme may be facilitatory (43). More recently, it was shown that two PKC isozymes (α and ζ) phosphorylate and activate the type V adenylyl cyclase *in vitro* (44). Therefore, the isozyme profiles of adenylyl cyclases and PKC may be important factors in the conditional stimulation of cAMP synthesis.

Another important factor that should be considered when studying G protein-coupled receptor signaling is receptor-G protein promiscuity. The EC_{50} values and the results of phenoxybenzamine experiments on the α_{1B}AR-mediated $[Ca^{2+}]_i$ response and cAMP production demonstrated what has been reported for other G protein-coupled receptor systems (45)—that overexpression of receptors can lead to receptor-G protein promiscuity. Therefore, the α_{1B}AR-G_s protein interaction might appear with a high receptor number. In fact, the native tissues in which α_1AR is able to stimulate cAMP synthesis have relatively high receptor numbers (e.g., rat brain, ~0.4–1.0 pmol/mg protein; rat liver, ~0.7–0.9 pmol/mg protein) (12, 46). Because the receptor-G protein interaction is in large part (if not totally) determined by the affinities of the two components for each other, then by manipulating the concentrations of reagents, one can drive the reaction in either direction. This simple explanation may be all that is necessary (in most instances) to account for the different effects in different tissues and cell lines. In addition

to receptor-G protein promiscuity, G protein-effector promiscuity can be considered in α_{1B}AR-mediated stimulation of adenylyl cyclase. Thus, $G_{q\alpha/11\alpha}$ can directly activate adenylyl cyclase in the transfected CHO cell system. As shown in Fig. 8, however, the anti-$G_{q\alpha/11\alpha}$ antiserum was found not to inhibit α_{1B}-AR-mediated adenylate cyclase activation. Also, using the recombinant G_α proteins expressed in the Sf9 system, Hepler *et al.* (47) showed that only $G_{q\alpha/11\alpha}$, i.e., not $G_{s\alpha}$, specifically and selectively stimulates PLCβ. The study also showed that neither $G_{q\alpha}$ nor $G_{11\alpha}$ activates adenylyl cyclase; only $G_{s\alpha}$ is clearly effective. Overall, we consider that the G_q subfamily, all of which couple to α_1-AR, would not directly activate adenylyl cyclase, if at all.

It is interesting to consider the present findings in the light of studies that revealed the molecular determinants for G_s coupling in receptors that primarily couple to G_s. Using chimeric $\alpha_2\beta_2$AR, Kobilka *et al.* (48) showed that the third intracellular loop was required for G_s coupling. Additional studies involving site-directed mutagenesis of human (49, 50) and hamster (51) β_2AR revealed that the amino-terminal portion and, in particular, the carboxyl-terminal portion of the third intracellular loop and the carboxyl-terminal portion of the cytoplasmic tail are major determinants for G_s coupling. However, examination of the amino acid sequences of the third intracellular loops of the three α_1AR subtypes did not reveal a similar sequence that might be a specific region for G_s coupling. We therefore cannot conjecture as to what are the molecular determinants for G_s coupling of the α_1AR.

In conclusion, the results of the present study suggest that α_1AR-mediated cAMP synthesis in CHOα_{1B} cells could reflect the direct activation of G_s-adenylyl cyclase. Thus, the α_1AR-stimulated cAMP production observed in some native tissues may involve multiple mechanisms of the direct activation of G_s/adenylyl cyclase and a secondary effect through activation of PI hydrolysis. Further studies on the receptor number and subtype profiles, isozymes of adenylyl cyclase and PKC involved, and their interactions with subunits of different G proteins will be required to clarify the cell- and tissue-specific pathways that control cAMP synthesis in response to α_1AR stimulation.

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