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Comparison of α_1-Adrenergic Receptor Subtypes and Signal Transduction in SK-N-MC and NB41A3 Neuronal Cell Lines

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SUMMARY

We compared the α_1-adrenergic receptor subtypes in two neuronal cell lines, SK-N-MC (human neuroepithelioma) and NB41A3 (murine neuroblastoma). ^{125}I-BE 2254 labeled α_1-adrenergic receptor binding sites in membranes from both cell lines. Pretreatment with the α_{1B}-selective alkylating agent chloroethylclonidine (CEC) completely eliminated these binding sites in NB41A3 cells but caused only a 50% loss in SK-N-MC cells. Displacement with subtype-selective antagonists suggested that NB41A3 cells express only the α_{1B} subtype, whereas SK-N-MC cells express a pharmacologically heterogeneous receptor population, including both α_{1A} and α_{1B} subtypes. Norepinephrine increased [^{3}H] inositol phosphate formation in both cell lines, but with different sensitivities to pertussis toxin and the presence of extracellular Ca^{2+}. CEC pretreatment eliminated this response in NB41A3 cells but caused a maximal 42% reduction in SK-N-MC cells. Use of subtype-selective antagonists showed that the [^{3}H]inositol phosphate response involved only the α_{1B} subtype in NB41A3 cells but a combination of subtypes in SK-N-MC cells. Norepinephrine induced both transient and sustained increases in intracellular Ca^{2+} concentrations in both cell lines, as measured with fura-2. CEC pretreatment abolished the Ca^{2+} response in NB41A3 cells but had little effect in SK-N-MC cells. In SK-N-MC cells the Ca^{2+} response was potently blocked by α_{1A}-selective antagonists. Chelation of extracellular Ca^{2+} eliminated the sustained component of the Ca^{2+} signal in both cell lines. Poly(A)$^+$ RNA from NB41A3, DDT$_1$MF-2, BC$_3$H1, and MDCK-D1 cell lines showed one or more prominent transcripts (2.2–4.2 kilobases) that strongly hybridized to the hamster α_{1B} cDNA probe but not to the bovine α_{1C} or rat α_{1D} cDNA probes. Poly(A)$^+$ RNA from SK-N-MC cells showed multiple transcripts (1.3–5.6 kilobases) that hybridized to both hamster α_{1B} and rat α_{1D} but not bovine α_{1C} cDNA probes. We conclude that NB41A3 cells contain exclusively α_{1B}-adrenergic receptors linked to inositol phosphate formation and mobilization of intracellular Ca^{2+}, whereas at least two α_1-adrenergic receptor forms, which resemble the α_{1A} and α_{1B} subtypes, coexist in SK-N-MC cells. The CEC-insensitive α_{1A}-like subtype in SK-N-MC cells is capable of increasing inositol phosphate formation and mobilizing intracellular Ca^{2+}.

The ARs mediating the effects of NE and epinephrine have been subclassified into three families (α_1, α_2, and β) (1). Pharmacological and molecular approaches suggest that each of these families contains at least three distinct subtypes (1–8), and there is evidence for additional subtypes (9–13).

Two distinct α_1-AR subtypes (α_{1A} and α_{1B}) have been distinguished pharmacologically. The α_{1A} subtype has a high affinity for the competitive antagonists 5-MU, (+)-niguldipine, and WB 4101 and is insensitive to inactivation by CEC (2, 14–21), whereas the α_{1B} subtype has a lower affinity for these competitive antagonists and is highly sensitive to CEC inactivation (2). It has been suggested that these two α_1-AR subtypes activate different signal transduction mechanisms. It is generally agreed that the α_{1B} subtype activates phospholipase C, resulting in formation of diacylglycerol and inositol-1,4,5-trisphosphate, which mobilizes Ca^{2+} from intracellular stores (22–24). In contrast, the α_{1A} subtype often activates influx of extracellular Ca^{2+} through voltage-gated channels (15, 18–21), which has been suggested to be the primary signaling mechanism for this subtype. However, the α_{1A} subtype can also increase InsP formation (22, 25) and the α_{1B} subtype can activate Ca^{2+} influx (24), so the relationship between specific subtypes and signaling mechanisms is still unclear.

Three distinct α_1-AR cDNAs have been cloned. The phar-

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ABBREVIATIONS: AR, adrenergic receptor; CEC, chloroethylclonidine; 5-MU, 5-methylurapidil; DMEM, Dulbecco's modified Eagle's medium; InsP, inositol phosphate; PBS, phosphate-buffered saline; BSS, balanced salt solution; NE, norepinephrine; BE, BE 2254 [[2β-(4-hydroxyphenyl)ethylaminomethyl]tetralone]; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AM, acetoxymethyl ester; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin; SSC, standard saline citrate; PTX, pertussis toxin; KRB, Krebs-Ringer bicarbonate buffer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; kb, kilobase(s); SSPE, standard saline phosphate EDTA.

macological profile of the receptor encoded by a cDNA isolated from a hamster DDT_1MF-2 cell library resembles that of the native α_{1B} subtype (26). Tissue distribution of this mRNA is similar to that expected for the α_{1B} subtype, and it seems likely that this clone encodes the α_{1B} subtype. A cDNA isolated from a bovine brain library encodes a receptor with a unique pharmacological profile and a very rare mRNA distribution (27) and has been designated α_{1C}. Two virtually identical cDNA clones that encode another rat α_1-AR subtype have been independently isolated from rat cerebral cortex (28) and rat hippocampus libraries (12) and designated rat α_{1A} and α_{1D} subtypes, respectively. The mRNAs for these clones show a tissue distribution similar to that expected for the natively expressed α_{1A} subtype. These cDNA clones differ from each other by only two nucleotides, resulting in differences in the amino acids at positions 37 and 306 of the expressed protein. Although these two differences in sequence may suggest that these two cDNA clones encode pharmacologically distinct α_1-AR subtypes, this does not appear to be the case. Indeed, the groups that have isolated these two cDNA clones concede that they are identical (12, 29). A comparison of the radioligand binding data for the expressed α_{1A}-AR in a recent study by Schwinn and Lomasney (29) with those for the α_{1D}-AR (12) shows that these two recombinant receptors possess almost exactly the same affinities for the most selective competitve antagonists used to distinguish α_1-AR subtypes. Perhaps more importantly, these studies also show that the pharmacological properties of the recombinant α_{1A}- and α_{1D}-ARs are markedly different from those of the pharmacologically defined, natively expressed α_{1A}-AR. Thus, none of the known cDNA clones encodes a receptor with the pharmacological properties expected of the native α_{1A} subtype.

To better understand the pharmacology and signaling mechanisms of α_1-AR subtypes, we compared these properties in clonal cell lines. Most cell lines express exclusively the α_{1B} subtype, including the DDT_1MF-2 hamster smooth muscle (24), BC_3H1 mouse brain tumor (24), MDCK-D1 canine kidney (30), and FRTL-5 rat thyroid (31) cell lines. Fisher and Landon (32) recently reported the presence of the α_{1A} subtype in human SK-N-MC neuroepithelioma cells. We screened a variety of cell lines and found that mouse NB41A3 neuroblastoma cells contain the α_{1B} subtype. Here we compare the pharmacological profiles and signal transduction pathways of α_1-AR subtypes in the SK-N-MC and NB41A3 neuronal cell lines.

Experimental Procedures

Materials. BC_3H1 cells were obtained from Dr. R. Dale Brown (University of Illinois, Chicago, IL) and MDCK-D1 cells from Dr. Paul Insel (University of California, San Diego, La Jolla, CA). SK-N-MC, NB41A3, and all other cell lines were obtained from the American Type Culture Collection (Rockville, MD). Materials were obtained from the following sources: phentolamine mesylate, Ciba-Geigy (Summit, NJ); CEC, 5-MU, (+)- and (−)-niguldipine, and WB 4101, Research Biochemicals Inc. (Natick, MA); prazosin hydrochloride, Pfizer (Groton, CT); oxymetazoline hydrochloride, Schering Corp. (Bloomfield, NJ); BE, Beiersdorf AG (Hamburg, Germany); [^{3}H]inositol (20–40 Ci/mmol), American Radiolabelled Chemicals (St. Louis, MO); carrier-free Na^{125}I, Amersham (Chicago, IL); fura-2/AM, Molecular Probes (Eugene, OR); fetal bovine serum, horse serum, and trypsin/EDTA, GIBCO (Gaithersburg, MD); carbachol, digitonin, (−)-NE bitartrate, yohimbine hydrochloride, DMEM, F-10 medium, penicillin, streptomycin, and all other chemicals, Sigma Chemical Co. (St. Louis, MO).

Cell culture. SK-N-MC cells were propagated in 75-cm^2 flasks in a humidified 7% CO_2 incubator, in DMEM containing glucose (4.5 g/liter), streptomycin (100 mg/liter), and penicillin (10^5 units/liter) and supplemented with 10% fetal bovine serum. NB41A3, DDT_1MF-2, and BC_3H1 cells were propagated in 75-cm^2 flasks in a humidified 5% CO_2 incubator in the following media: NB41A3, F-10 medium containing streptomycin (20 mg/liter) and penicillin (3×10^4 units/liter) supplemented with 15% horse serum and 2.5% fetal bovine serum; DDT_1MF-2, DMEM containing glucose (4.5 g/liter), streptomycin (100 mg/liter), and penicillin (10^5 units/liter) supplemented with 2.5% calf serum and 2.5% horse serum; and BC_3H1, DMEM containing glucose (1 g/liter), streptomycin (100 mg/liter), and penicillin (10^5 units/liter) supplemented with 10% calf serum. All other cell lines were propagated under conditions recommended by the American Type Culture Collection. Upon reaching confluency, the cells were detached by mild trypsinization (0.25%) in the presence of 2.6 mM EDTA and were subcultured at a ratio of 1:10 (SK-N-MC, DDT_1MF-2, BC_3H1, and MDCK-D1 cell lines) or 1:4 (NB41A3 cells) in the indicated media.

For measurements of [^{3}H]InsP formation, 35-mm Primaria dishes were seeded at a density of 300,000 cells/2 ml for SK-N-MC cells and 150,000 cells/2 ml for NB41A3 cells. In studies involving radioligand binding, Ca^{2+}, and mRNA measurements, 100-mm dishes were seeded at a density of 3×10^6 cells/10 ml for SK-N-MC cells and 1.5×10^6 cells/10 ml for NB41A3 cells. Cells were grown to confluency before use.

^{125}I-BE binding. ^{125}I-BE binding was performed in membrane preparations as described previously (33). Cells were washed twice in PBS (20 mM $NaPO_4$, 154 mM NaCl, pH 7.6) and harvested by scraping, and the contents of eight to 20 confluent 100-mm plates were pooled. The cells were centrifuged for 10 min at 30,000 × *g* and the pellet was resuspended and homogenized with a Polytron (setting 7, 10 sec) in 10 ml of PBS. Membranes were collected by centrifugation at 30,000 × *g* for 10 min, resuspended by Polytron homogenization in 10 ml of PBS, and centrifuged again at 30,000 × *g*. The pellets were resuspended in PBS (one confluent 100-mm plate/0.5 ml). In experiments involving CEC, cell membranes were suspended in 10 mM Na-HEPES, pH 7.4, and incubated for 10 min at 37° with or without 10–100 μM CEC (33). Incubations were terminated by dilution with cold PBS and centrifugation at 30,000 × *g* for 10 min. All CEC not irreversibly bound was removed by washing the pellets twice, and the membranes were finally resuspended in PBS as described above.

BE was radioiodinated to theoretical specific activity (34) and stored at −20° in methanol. Specific ^{125}I-BE binding was measured by incubating 0.1 ml of cell membranes with ^{125}I-BE in a final volume of 0.25 ml of PBS, in the presence or absence of competing drugs, for 20 min at 37° (33). The incubation was terminated by addition of 10 ml of 10 mM Tris·HCl, pH 7.4, and filtration through a glass fiber filter (Schleicher and Schuell no. 30) under vacuum. The filters were washed once with 10 ml of 10 mM Tris·HCl, pH 7.4, and dried, and radioactivity was measured. Nonreceptor binding was determined in the presence of 10 μM phentolamine.

Saturation curves were obtained by incubating cell membranes with increasing concentrations of ^{125}I-BE and were analyzed by the method of Scatchard (35). Displacement by competitive antagonists was determined by incubating a single concentration of ^{125}I-BE (40–50 pM) in the presence or absence of 14 to 16 concentrations of drug. Hill plots were then constructed and the IC_{50} value was determined as the *x*-intercept. A nonlinear regression analysis that minimized the sum of squares of the errors was used to determine the best two-site fit for binding curves. These two-site models were then compared with a one-site model to determine whether the increase in the goodness of fit was significantly more than would be expected by chance alone (36), using a partial *F* test. Significant differences were considered those with $p < 0.05$.

Measurement of [^{3}H]InsPs. Accumulation of [^{3}H]InsPs was determined in confluent 35-mm dishes. Cells were prelabeled with *myo*-[^{3}H]inositol for 3–4 days and the production of [^{3}H]InsPs was determined as described previously (37). In brief, medium containing [^{3}H] inositol was removed, and the plates were washed twice with 1 ml of

KRB (in mM: NaCl, 120; KCl, 5.5; $CaCl_2$, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $NaHCO_3$, 20; glucose, 11; Na_2EDTA, 0.029) and incubated with or without drugs for 1 hr at 37° in KRB containing 10 mM LiCl. The incubation buffer was removed, the reaction was stopped with ice-cold methanol, and [^{3}H]InsPs were isolated by extraction and anion exchange chromatography. In experiments using CEC, cells were incubated with or without CEC (10 or 100 μM) for 30 min in KRB, the cells were washed three times, and [^{3}H]InsPs were determined as described above. In studies that determined the dependence of [^{3}H]InsP accumulation on extracellular Ca^{2+}, Ca^{2+} concentrations were buffered by the addition of EGTA.

$[Ca^{2+}]_i$ determinations. $[Ca^{2+}]_i$ was determined in both cell lines with fura-2, essentially as described by Berk *et al.* (38). Confluent 100-mm plates were washed with BSS (in mM: NaCl, 130; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1.5; HEPES, 20; glucose, 10; with 0.1% BSA) and pretreated with or without 100 μM CEC for 30 min. Cells were then washed with HBSS and detached by incubation with 0.05% trypsin/0.53 mM EDTA in HBSS for 1 min, followed by incubation for 5–10 min in HBSS. Cells were centrifuged, resuspended (3–4 × 10^6 cells/ml) in DMEM containing 0.05% BSA, and incubated with 1 μM fura-2/AM for 15 min (SK-N-MC) or with 5 μM fura-2/AM for 30 min (NB41A3) at 37°. After a 5-fold dilution of the cells with DMEM containing 0.05% BSA and an additional 5-min incubation, the cells were centrifuged at 300 × *g*. These fura-2-loaded cells were resuspended (2 × 10^6 cells/ml) in BSS, divided into 3-ml aliquots, and placed on ice.

Aliquots of cells were warmed immediately before use by incubation at 37° and were pelleted at 300 × *g*. The pellet was resuspended in 3 ml of oxygenated BSS, transferred to a cuvette, and placed in a Perkin-Elmer (Beaconsfield, Buckingshamshire, England) LS 50 luminescence spectrofluorometer with a thermostatted (37°) stirred cell holder. The excitation wavelengths were 340 and 380 nm and the emission wavelength was 510 nm, with 5-nm bandwidths for each. Calibration of the fluorescence signals for calculation of $[Ca^{2+}]_i$ was performed for every aliquot by equilibrating intracellular and extracellular Ca^{2+} with 30 μM digitonin (R_{max}), followed by addition of 300 mM EGTA, 1 M Tris, pH 9.0 (R_{min}), and using a K_d of 225 nM for fura-2 (39).

mRNA analysis. The cDNAs encoding the hamster α_{1B}-AR (2 kb subcloned in SP65) and bovine α_{1C}-AR (3 kb subcloned in pBluescript) were kindly provided by Dr. Jon Lomasney (Duke University, Durham, NC). The rat α_{1D}-AR cDNA (1.8 kb cloned into pMT2′) was kindly provided by Dr. R. Graham (Case Western University, Cleveland, OH). The α_{1B} probe used in this study was a 1.2-kb *Xho*I-*Sma*I fragment, the α_{1C} probe was a 1.1-kb *Pst*I-*Nco*I fragment, and the α_{1D} probe was a 1.2-kb *Apa*I-*Sst*I fragment. All of these cDNA probes encompass predominantly the coding regions for the seven transmembrane regions of the receptors. Before conducting these mRNA studies, we had already determined the pharmacological properties of the α_1-AR subtype present in the NB41A3 cell line to be α_{1B} and those in SK-N-MC cells to be like those of the pharmacologically defined α_{1B}- and α_{1A}-AR subtypes. Because the cloned α_{1A}- and α_{1D}-AR cDNAs are essentially identical and neither encodes a receptor with the pharmacological characteristics found in the SK-N-MC cell line, the cDNAs could be used interchangeably to determine the expression of mRNA of α_1-AR subtypes in the different cell lines and tissues. We chose to use the α_{1D}-AR cDNA probe throughout these studies. Probes were labeled using the random primer method with [α-^{32}P]dCTP (6000 Ci/mmol; Amersham, Chicago, IL), using the Prime-It kit (Stratagene, La Jolla, CA).

Poly(A)$^+$-selected RNA from cell lines and male Sprague-Dawley rat tissues was prepared by oligo(dT)-cellulose chromatography using the FastTrack kit (Invitrogen, San Diego, CA). Confluent plates of cells were washed with 5 ml of PBS, which was aspirated. Five milliliters of 0.025% trypsin/0.26 mM EDTA (GIBCO) were added and plates were incubated for 1–5 min. After cells began to detach, the trypsin solution was aspirated and 3 ml of Ca^{2+}-free (HBSS) buffer were added. Cells were briefly triturated, transferred to a 50-ml sterile centrifuge tube, and put on ice while remaining cells were harvested. Cells were collected by centrifugation and counted, and 15 ml of lysis buffer were added to cell pellets. Total numbers of cells were as follows: BC_3H1, 100 × 10^6 (15 plates); DDT_1MF-2, 40 × 10^6 (15 plates); NB41A3, 44 × 10^6 (40 plates); MDCK-D1, 54 × 10^6 (25 plates); and SK-N-MC, 100 × 10^6 (five plates). Rat tissues were dissected, frozen on dry ice, and homogenized with a Polytron in lysis buffer. After isolation, the mRNA was denatured, electrophoresed on 1.2% agarose-0.7% formaldehyde gels, and transferred by capillary blotting in 20× SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) to Hybond N nylon membranes (Amersham, Chicago, IL). RNA was immobilized on the membranes by baking at 90° for 3 hr. The size of the mRNA was determined by comparison with a 0.24–9.5-kb RNA ladder (GIBCO), and quality was assessed by examining the ribosomal bands after staining with ethidium bromide.

The membranes were prehybridized for 2 hr at 42° in 6× SSC, 5× Denhardt's reagent, 50% formamide, 1% sodium dodecyl sulfate, 100 μg/ml sheared salmon sperm DNA. The prehybridization solution was removed and replaced with the identical solution containing the appropriate ^{32}P-labeled cDNA probe, and membranes were hybridized for 16 hr at 42°. After hybridization, the blots were washed twice at room temperature with 2× SSC for 8 min each, followed by two 15-min washes with 0.1× SSC at 55°. Autoradiographs were then prepared by exposure of the blots to Kodak X-Omat AR film (Kodak, Rochester, NY) for 1–8 days. Blots were stripped by incubating them twice in sequence for 1 hr in formamide/20× SSPE (1 × SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA, pH 7.4)/water (5:1:4) at 65°. Blots were reexposed to film and restripped if necessary before reprobing.

Results

Screening of cell lines. A variety of cell lines derived from tissues that might be expected to express α1-ARs were screened for the presence of α_1-AR binding sites (labeled with ^{125}I-BE) and NE-stimulated [^{3}H]InsP formation. We were particularly interested in identifying human cell lines that possessed α_{1A}- and/or α_{1B}-AR subtypes, so that comparisons of the pharmacological properties of the subtypes could be made without concern for the effects of species variation on α_1-AR subtypes and their signaling mechanisms. The following cell lines showed no evidence of either ^{125}I-BE binding sites or NE-stimulated [^{3}H]InsP formation: A10 rat thoracic aorta, A7r5 rat embryonic thoracic aorta, KNRK rat kidney, NRK-52E rat kidney, HISM human intestinal smooth muscle, DU 145 human prostate, RPMI 2650 human nasal septum, and C_6 rat glioma. However, significant α_1-AR binding sites and InsP responses were observed in human SK-N-MC neuroepithelioma and mouse NB41A3 neuroblastoma cells (see below).

Radioligand binding. The α_1AR-selective antagonist radioligand ^{125}I-BE (33) labeled a homogeneous population of binding sites in membrane preparations from both SK-N-MC and NB41A3 cells (Fig. 1). Scatchard analysis of saturation isotherms gave B_{max} values of 33 and 23 fmol/mg of protein in SK-N-MC and NB41A3 cells, respectively. K_d values were 45 and 34 pM in SK-N-MC and NB41A3 cells, respectively.

CEC pretreatment caused different effects in the two cell lines (Fig. 1). Pretreatment of membrane preparations with 10 μM CEC for 10 min inactivated essentially all of the binding sites in NB41A3 membranes but only half of the sites in SK-N-MC membranes.

Inhibition of specific ^{125}I-BE binding by selected competitive antagonists in membranes from the two cell lines is shown in Fig. 2. In NB41A3 cells, all drugs tested displaced ^{125}I-BE with Hill coefficients close to 1.0 and a relatively low affinity (Fig. 2; Table 1). These data are consistent with the presence of a single population of α_1-ARs, with properties resembling those of the α_{1B} subtype, in NB41A3 cells. In SK-N-MC cells a

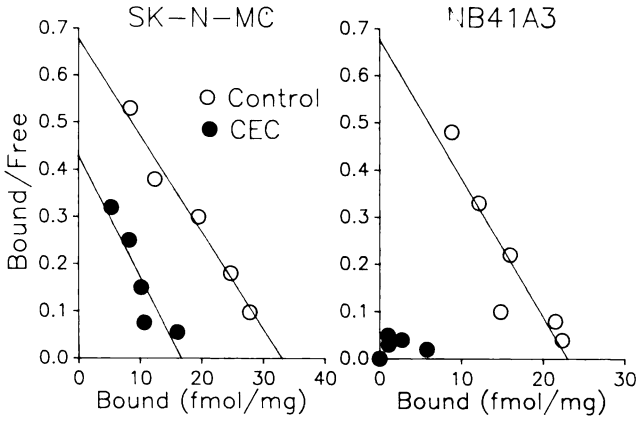

Fig. 1. Effect of CEC pretreatment on α_1-AR binding sites in membrane preparations of SK-N-MC (*left*) and NB41A3 (*right*) cells. Membrane preparations were pretreated with (●) or without (○) 10 μM CEC for 10 min at 37° in hypotonic buffer. Each *point* is the mean of duplicate determinatons from five (SK-N-MC) or three (NB41A3) experiments.

different pattern was observed. The α_{1A}-selective competitive antagonists 5-MU, (+)-niguldipine, oxymetazoline, and phentolamine all exhibited relatively shallow displacement curves for inhibition of ^{125}I-BE binding (Fig. 2), with Hill coefficients (n_H) that ranged from 0.5 to 0.65 (Table 1). Displacement curves for these antagonists significantly better fit a two-site model (Table 2) in which approximately 50% of the binding sites had affinities for these drugs that are normally associated with the α_{1B} subtype. Additionally, the K_H values for the high affinity site revealed by these drugs closely resemble those reported for the α_{1A} subtype.

[^{3}H]InsP formation. NE increased formation of [^{3}H]InsP in both SK-N-MC (EC_{50}, 700 nM) and NB41A3 cells (EC_{50}, 94 nM) (Fig. 3). Maximal stimulation by NE averaged 1.9- and 2.2-fold over basal in SK-N-MC and NB41A3 cells, respectively. NE-stimulated [^{3}H]InsP formation was inhibited in a concentration-dependent manner by subtype-selective competitive antagonists (Fig. 4; Table 3). Prazosin was >100-fold more potent than yohimbine in blocking this response in both cell lines. Subtype-selective competitive antagonists inhibited [^{3}H]InsP formation in NB41A3 cells in a single-site manner with fairly low potencies, also suggesting that the receptors in these cells are predominantly of the α_{1B} subtype. In SK-N-MC cells, (+)-niguldipine, 5-MU, oxymetazoline, phentolamine, and WB 4101 exhibited relatively flat inhibition curves (n_H = 0.4–0.7) for the blockade of [^{3}H]InsP formation caused by 10 μM NE (Fig. 4), suggesting the existence of two α_1-AR subtypes involved in this response.

CEC pretreatment decreased NE-stimulated [^{3}H]InsP formation in a concentration-dependent manner in NB41A3 cells (Fig. 5), with complete elimination of the response being observed after treatment with 3 μM CEC for 30 min. In contrast, CEC pretreatment maximally inhibited this response by only 42% in SK-N-MC cells, even at concentrations of CEC up to 100 μM (Fig. 5).

PTX pretreatment of NB41A3 cells caused no inhibition of the [^{3}H]InsP formation response elicited by 100 μM NE (Table 4). Pretreatment of SK-N-MC cells with PTX caused a 50% decrease in the level of [^{3}H]InsP formation stimulated by 100 μM NE. However, this decrease was not statistically significant. CEC (100 μM) pretreatment also caused a 50% decrease in the level of [^{3}H]InsP formation. Pretreatment of SK-N-MC cells with a combination of PTX and CEC produced no greater inhibition of the [^{3}H]InsP formation response than did treatment with either compound alone.

Ca^{2+} dependence of [^{3}H]InsP formation. The effect of NE on [^{3}H]InsP formation in NB41A3 cells showed a complex dependence on extracellular Ca^{2+} (Fig. 6). Chelation of extracellular Ca^{2+} by EGTA reduced the response to 3 μM NE to

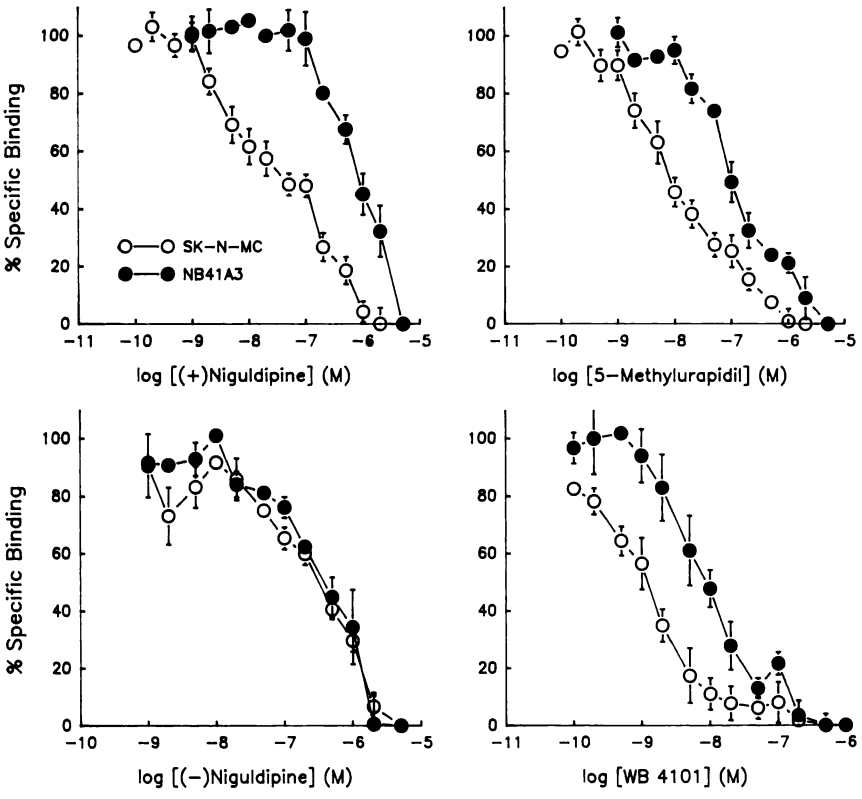

Fig. 2. Inhibition of specific ^{125}I-BE binding by selective antagonists in membranes from SK-N-MC and NB41A3 cells. Each *point* is the mean ± standard error from three to nine experiments, each performed in duplicate.

TABLE 1

Inhibition of specific ^{125}I-BE binding by selected α-AR antagonists in SK-N-MC and NB41A3 cell lines

IC_{50} values and Hill coefficients were determined from Hill plots. Each value is the mean ± standard error of the number of observations indicated (*n*).

Drug	SK-N-MC			NB41A3		
	n	−log IC_{50}	n_H	*n*	−log IC_{50}	n_H
5-MU	8	8.00 ± 0.16	0.60 ± 0.05	3	6.78 ± 0.14	0.92 ± 0.05
(+)-Niguldipine	9	7.45 ± 0.18	0.50 ± 0.03	3	6.06 ± 0.08	1.02 ± 0.08
WB 4101	4	8.96 ± 0.16	0.79 ±0.07	3	8.12 ± 0.12	0.94 ± 0.15
Phentolamine	8	7.51 ± 0.07	0.65 ± 0.03	3	7.00 ± 0.06	0.88 ± 0.18
Oxymetazoline	4	7.11 ± 0.19	0.50 ± 0.04	3	6.59 ± 0.21	0.93 ± 0.22
Prazosin	3	9.06 ± 0.08	0.80 ± 0.05	3	9.47 ± 0.08	1.24 ± 0.06
(−)-Niguldipine	4	6.63 ± 0.07	0.65 ± 0.02	3	6.44 ± 0.05	0.93 ± 0.10
Yohimbine	4	5.92 ± 0.11	1.17 ± 0.13	3	5.27 ± 0.33	0.72 ± 0.09

TABLE 2

Two-site analysis of the displacement of specific ^{125}I-BE binding in membranes of SK-N-MC cells by selective α_{1A}-AR antagonists

Composite inhibition curves were subjected to two-site analysis as described in the text. The equilibrium binding constants for high (K_H) and low (K_L) affinity sites were determined, as was the percentage of low affinity sites (R_L), if a two-site model was significantly better ($p < 0.05$) fit than a one-site model. The significance of the two-site fit, compared with the one-site fit, is given in parentheses.

Drug	K_H	K_L	R_L
	nM	nM	%
5-MU	4.0	154	31 ($p < 0.001$)
(+)-Niguldipine	3.9	212	56 ($p < 0.001$)
Oxymetazoline	7.0	550	56 ($p < 0.001$)
Phentolamine	3.8	54	78 ($p < 0.05$)
WB 4101	0.7	6.2	21 (NS*)

* NS, not significant.

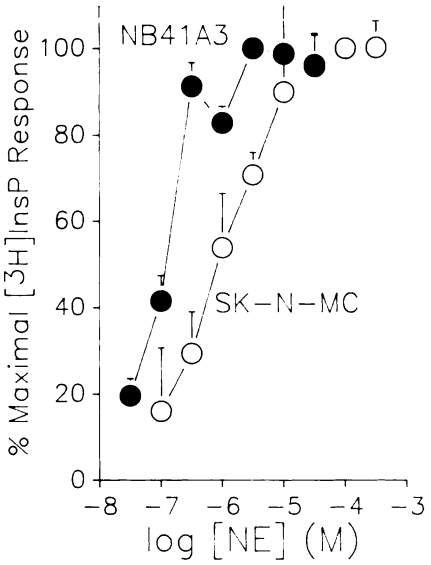

Fig. 3. Concentration dependence of NE-stimulated [^{3}H]InsP formation in NB41A3 (EC_{50} = 94 nM) and SK-N-MC (EC_{50} = 700 nM) cells. The data are expressed as percentage of maximum response. Basal and NE-stimulated hydrolysis was 2.2 ± 0.8% and 4.8 ± 2.3% in NB41A3 cells and 6.9 ± 0.8% and 13.0 ± 2.1% in SK-N-MC cells, respectively. Each *point* represents the mean ± standard error of three (NB41A3) or five (SK-N-MC) experiments performed in duplicate.

half of that observed in the presence of normal (2.5 mM) Ca^{2+} concentrations. In SK-N-MC cells, however, this response showed a greater dependence on extracellular Ca^{2+} (Fig. 6). At low concentrations (30 μM) of extracellular Ca^{2+}, no response to 100 μM NE was observed. The response could be reconstituted by increasing the amount of extracellular Ca^{2+}, with an EC_{50} around 300 μM.

Effect of NE on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was monitored with fura-2 in both cell lines (Fig. 7). As shown in Fig. 7, a rapid 3-fold increase in $[Ca^{2+}]_i$ occurred in both cell lines in response to 30 μM NE (1.5 mM free extracellular Ca^{2+}). This rapid rise in $[Ca^{2+}]_i$ was followed by a sustained level of increased $[Ca^{2+}]_i$, which declined slowly over a 2-min period to about twice the basal $[Ca^{2+}]_i$ in SK-N-MC cells. NB41A3 cells also exhibited a sustained component of the Ca^{2+} transient induced by NE, but it appeared to decline more rapidly to near base-line values after a 2-min period. Pretreatment of NB41A3 cells with 100 μM CEC for 30 min, with subsequent washing of the cells, resulted in complete inhibition of the Ca^{2+} transient response induced by NE in NB41A3 cells (Fig. 7, *upper right*). In contrast, pretreatment of SK-N-MC cells with 100 μM CEC for 30 min caused only a 20% reduction in the increase in $[Ca^{2+}]_i$ stimulated by 30 μM NE (Figs. 7, *upper left*, and 8). CEC did not greatly affect the Ca^{2+} transients induced by the non-AR agonists carbachol and ATP in SK-N-MC and NB41A3 cells, respectively (Fig. 7, *upper*).

Dependence of $[Ca^{2+}]_i$ on extracellular Ca^{2+}. Increases in $[Ca^{2+}]_i$ could also be seen in both cell lines in the absence of extracellular Ca^{2+} (Ca^{2+}-free buffer with 2 mM EGTA), although the increases in $[Ca^{2+}]_i$ in SK-N-MC cells were consistently larger than those in the NB41A3 cell line (Fig. 7, *lower*). NE (30 μM) elicited 4- and 2-fold increases in $[Ca^{2+}]_i$ in SK-N-MC and NB41A3 cells, respectively. However, under these conditions the sustained component of the NE-stimulated increase in $[Ca^{2+}]_i$ seen in the presence of extracellular Ca^{2+} disappeared in both cell lines. Preincubation of NB41A3 cells with 100 μM CEC eliminated the increase in $[Ca^{2+}]_i$ induced by NE in the absence of extracellular Ca^{2+} (Fig. 7, *lower right*), as would be predicted for an α_{1B} subtype. Surprisingly, however, the elevation of $[Ca^{2+}]_i$ caused by NE in the absence of extracellular Ca^{2+} in SK-N-MC cells was decreased by only about 20% by preincubation with 100 μM CEC (Figs. 7, *lower left*, and 8), suggesting the presence of CEC-insensitive α_1-ARs capable of mobilizing Ca^{2+} from intracellular stores.

Because the effects of the absence of Ca^{2+} on [^{3}H]InsP formation were determined after a 1-hr exposure of SK-N-MC cells to a Ca^{2+}-free environment, similar conditions were used to evaluate the effect on $[Ca^{2+}]_i$ in these cells. Cells incubated for 1 hr in a Ca^{2+}-free buffer had very low basal $[Ca^{2+}]_i$, and 30 μM NE failed to increase $[Ca^{2+}]_i$. However, cells exposed to 2 mM EGTA in the absence of Ca^{2+} for 30 sec before the addition of NE exhibited higher basal $[Ca^{2+}]_i$, and NE elicited an increase in $[Ca^{2+}]_i$. SK-N-MC cells incubated in buffer containing 1.5 mM Ca^{2+} exhibited higher basal $[Ca^{2+}]_i$ and stimulation by

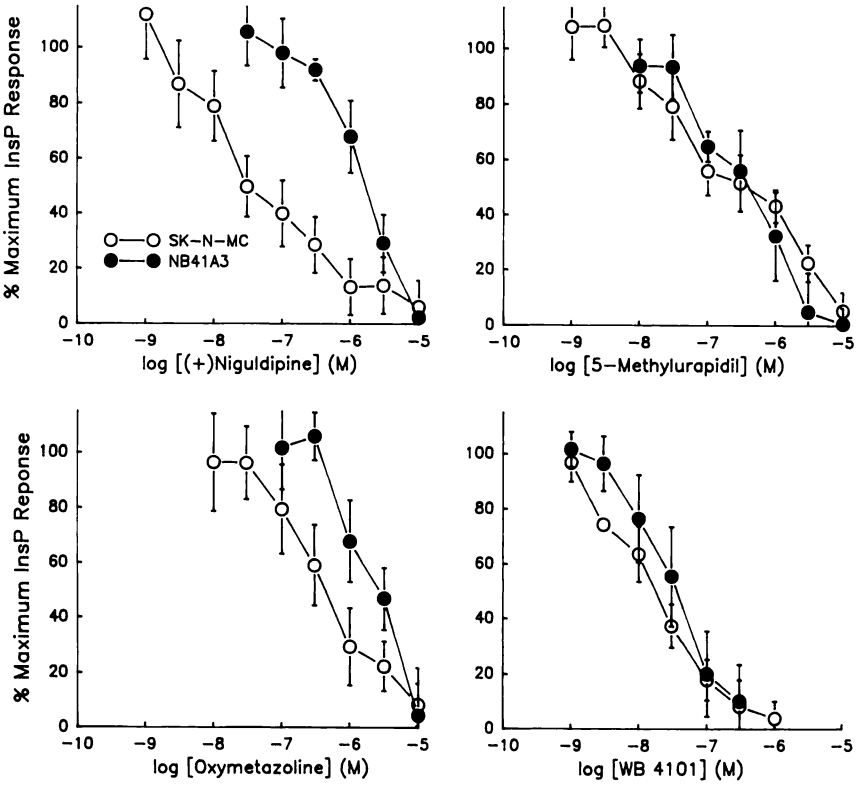

Fig. 4. Inhibition of NE-stimulated [^{3}H]InsP formation by selective α_{1A}-AR antagonists in SK-N-MC and NB41A3 cells. Concentration-response curves for inhibition of responses to 10 μM (SK-N-MC) and 3 μM (NB41A3) NE were generated as described in the text. Results are expressed as percentage of maximum response in the absence of antagonists. Each *point* is the mean ± standard error from three to five experiments performed in duplicate.

TABLE 3

Inhibition of [^{3}H]InsP formation by selected α-AR antagonists in SK-N-MC and NB41A3 cell lines

Cells were exposed to various concentrations of the indicated antagonists and then stimulated with 10 μM NE (SK-N-MC) or 3 μM NE (NB41A3) in the presence of the antagonists. IC_{50} values and Hill coefficients were determined from Hill plots of the inhibition curves. Each value is the mean ± standard error of the number of observations indicated (*n*).

Drug	SK-N-MC			NB41A3		
	n	$-\log IC_{50}$	n_H	*n*	$-\log IC_{50}$	n_H
5-MU	4	6.54 ± 0.29	0.60 ± 0.14	4	6.40 ± 0.24	0.97 ± 0.19
(+)-Niguldipine	5	7.25 ± 0.40	0.39 ± 0.04	4	5.78 ± 0.15	1.21 ± 0.20
WB 4101	4	7.84 ± 0.12	0.68 ± 0.07	5	7.61 ± 0.28	0.98 ± 0.10
Phentolamine	3	7.19 ± 0.16	0.68 ± 0.09	4	6.78 ± 0.24	0.94 ± 0.12
Oxymetazoline	4	6.38 ±0.23	0.59 ± 0.16	3	5.72 ± 0.31	1.01 ± 0.24
Prazosin	3	8.05 ± 0.29	0.65 ± 0.12	3	9.86 ± 0.41	0.95 ± 0.45
(−)-Niguldipine	3	5.73 ± 0.95	0.54 ± 0.06		ND[a]	
Yohimbine	3	5.70 ±0.40	0.77 ± 0.01	3	5.46 ± 0.15	0.97 ± 0.12

[a] ND, not determined.

NE resulted in an increase in $[Ca^{2+}]_i$ with a sustained component that was blocked by 0.1 μM prazosin (data not shown).

Pharmacology of the Ca^{2+} response. To further evaluate the α_1 subtypes that mediate the mobilization of Ca^{2+} in SK-N-MC cells, the effects of the α_{1A} subtype-selective competitive antagonists 5-MU and (+)-niguldipine on changes in $[Ca^{2+}]_i$ induced by NE were examined. When added to buffer 30 sec before NE administration, both 5-MU and (+)-niguldipine (at 100 nM) essentially eliminated the increase in $[Ca^{2+}]_i$ elicited by 30 μM NE (Fig. 9, *upper*). Similarly, both antagonists also reversed the sustained component of elevated $[Ca^{2+}]_i$ caused by NE before addition of the antagonists (Fig. 9, *lower*). Neither 5-MU nor (+)-niguldipine had any effect on the $[Ca^{2+}]_i$ changes induced by carbachol.

mRNA analysis. To further evaluate the subtypes of α_1-AR present in the SK-N-MC and NB41A3 cell lines, Northern blot hybridizations of poly(A)$^+$ RNA prepared from these cell lines as well as other cell lines and rat tissues known to contain α_1-ARs were performed (Fig. 10). When the α_{1B}-AR cDNA probe was used, a single transcript of 3.0 kb was seen in mRNA prepared from the NB41A3 cell line. In contrast, multiple transcripts of 1.3–5.6 kb that hybridized to the same probe were seen in SK-N-MC mRNA, with the 5.6-kb species being predominant. In cell lines that have been demonstrated by pharmacological and molecular biological approaches to contain the α_{1B} subtype, mRNA species that hybridized with the α_{1B}-AR cDNA probe were seen. The mRNA from DDT_1MF-2 cells (from which the α_{1B}-AR cDNA was cloned) (26) contained two distinct species, of 2.2 and 3.0 kb, that hybridized to the α_{1B}-AR cDNA probe and were approximately equal in abundance. One 3.0-kb mRNA transcript from BC_3H1 cells and two (2.7- and 4.2-kb) mRNA species from the MDCK-D1 cell line hybridized to the α_{1B} probe. In mRNA prepared from rat cortex, heart, spleen, and lung, a 3.0-kb transcript hybridized to the α_{1B}-AR cDNA probe; those from the heart and lung appeared to be in greatest abundance.

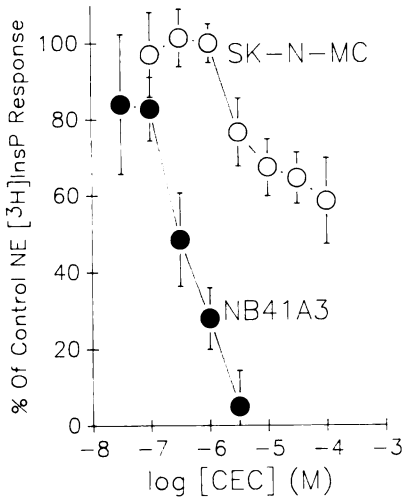

Fig. 5. Inhibition of NE-stimulated [^{3}H]InsP formation by CEC pretreatment in NB41A3 and SK-N-MC cells. Cells were exposed to varying concentrations of CEC for 30 min, washed, and then exposed to 3 μM (NB41A3) or 100 μM (SK-N-MC) NE. Basal and NE-stimulated hydrolysis was 1.6 ± 0.1% and 2.8 ± 0.4% in NB41A3 cells and 4.7 ± 0.7% and 11.2 ± 2.1% in SK-N-MC cells without CEC, respectively. Each *point* represents the mean ± standard error of duplicate determinations from three experiments.

TABLE 4

Inhibition of [^{3}H]InsP formation by PTX in SK-N-MC and NB41A3 cell lines

[^{3}H]InsP formation stimulated by NE (100 μM, SK-N-MC; 3 μM, NB41A3) was determined as described. Cells treated with PTX were exposed to 300 ng/ml for 16 hr and washed, and [^{3}H]InsP formation was determined. Other cells were pretreated with 100 μM CEC for 30 min and washed, and [^{3}H]InsP formation was determined. Some cells were exposed to both PTX and CEC (PTX + CEC). Results are expressed as the mean ± standard error of duplicate determinations from two to four experiments. None of the treatments caused a significant decrease in [^{3}H]InsP formation caused by 100 μM NE in either cell line.

Treatment	Hydrolysis	
	Basal	100 μM NE
	%	
SK-N-MC cells		
Control	5.4 ± 0.5	16.1 ± 4.0
PTX	6.0 ± 1.0	10.8 ± 1.6 (NS*)
CEC	5.5 ± 0.9	10.9 ± 2.3 (NS)
PTX + CEC	5.9 ± 0.8	9.6 ± 1.4 (NS)
NB41A3 cells		
Control	2.3 ± 0.1	3.4 ± 0.1
PTX	2.1 ± 0.1	3.5 ± 0.1 (NS)

* NS, not significant ($p > 0.05$, Student t test).

The same poly(A)$^+$ RNA blot used for the analysis of mRNA for the α_{1B} subtype (after stripping) was also used for the determination of the presence of mRNA transcripts for the α_{1D}-AR, to facilitate comparison of the mRNA transcript sizes that hybridized to the two cDNA probes. Multiple mRNA transcripts from the SK-N-MC cell line, ranging in size from 1.3 kb to the predominant 5.6-kb species, hybridized to the α_{1D}-AR cDNA probe. No apparent mRNA transcripts from the other cell lines that were enriched in mRNA for the α_{1B}-AR hybridized to the α_{1D}-AR probe, and there was little cross-hybridization of the α_{1B}-AR mRNA with the α_{1D}-AR cDNA probe. Some cross-hybridization was observed in the NB41A3 cell lines; however, no distinct bands were observed, as was seen with the α_{1B}-AR cDNA probe and shorter film exposure times. Rat cortex, heart, spleen, and lung all contained mRNA of the same size (3.0 kb) that hybridized to the α_{1D}-AR cDNA probe, and this message appeared to be most abundantly expressed in the cortex and heart. No expression of the α_{1C}-AR mRNA was observed in any cell line or tissue examined (data not shown). We have performed at least six Northern hybridizations of poly(A)$^+$ RNA from the five different cell lines using the indicated cDNA probes alone (no stripping and reprobing) or in orders of hybridization with the cDNA probes different from the one presented here. In all cases, the hybridization signals were the same as those presented here, regardless of the order in which the Northern blots were hybridized with different probes. Additionally, we made certain that all of the blots were completely stripped before hybridization with another cDNA probe was performed.

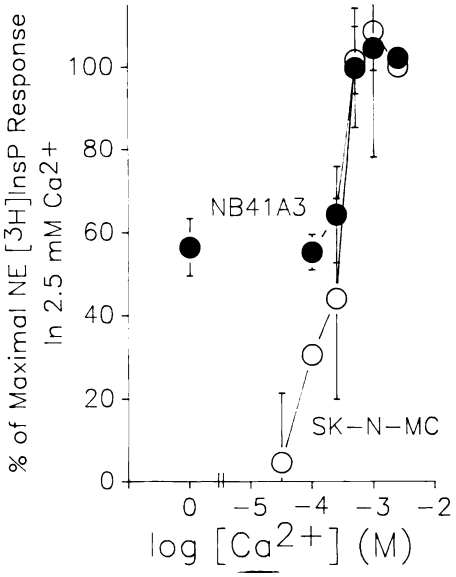

Fig. 6. Dependence of NE-stimulated [^{3}H]InsP formation on extracellular Ca^{2+} in NB41A3 and SK-N-MC cells. Cells were incubated for 1 hr in KRB (containing 1 mM Li^+) with various concentrations of free Ca^{2+} (buffered by 1 mM EGTA) in the presence or absence of 3 μM (NB41A3) or 100 μM (SK-N-MC) NE. Results are expressed as a percentage of the respective total [^{3}H]InsP produced by stimulation with NE in the presence of 2.5 mM free Ca^{2+}. The basal and 100 μM NE-stimulated hydrolysis in SK-N-MC cells was 5.1 ± 0.4% and 7.8 ± 0.2% in Ca^{2+}-free buffer and 6.4 ± 0.4% and 15.6 ± 6.1% in 2.5 mM Ca^{2+}-containing buffer, respectively. For NB41A3 cells the basal and 3 μM NE-stimulated hydrolysis was 1.8 ± 0.1% and 3.1 ± 0.6% in Ca^{2+}-free buffer and 1.2 ± 0.1% and 4.8 ± 1.3% in 2.5 mM Ca^{2+}-containing buffer, respectively. Each *point* represents the mean ± standard error of three experiments performed in duplicate.

Discussion

These results show that NB41A3 and SK-N-MC cell lines express pharmacologically distinct α_1-AR subtypes. Murine NB41A3 cells appear to express a single subtype, whereas human SK-N-MC cells express at least two subtypes. We have compared the pharmacological properties and signal transduction pathways activated by each receptor subtype in these cell lines to directly test the hypothesis that different receptor subtypes activate different signaling mechanisms (15).

Studies of radioligand binding, second messenger production, and mRNA expression suggest that the receptors in NB41A3 cells are exclusively of the α_{1B} subtype. These receptors have relatively low affinities for the α_{1A}-selective competitive antagonists 5-MU, (+)-niguldipine, and WB 4101 and are potently and completely inactivated by the α_{1B}-selective alkylating agent CEC. The presence of a distinct 3.0-kb mRNA transcript that

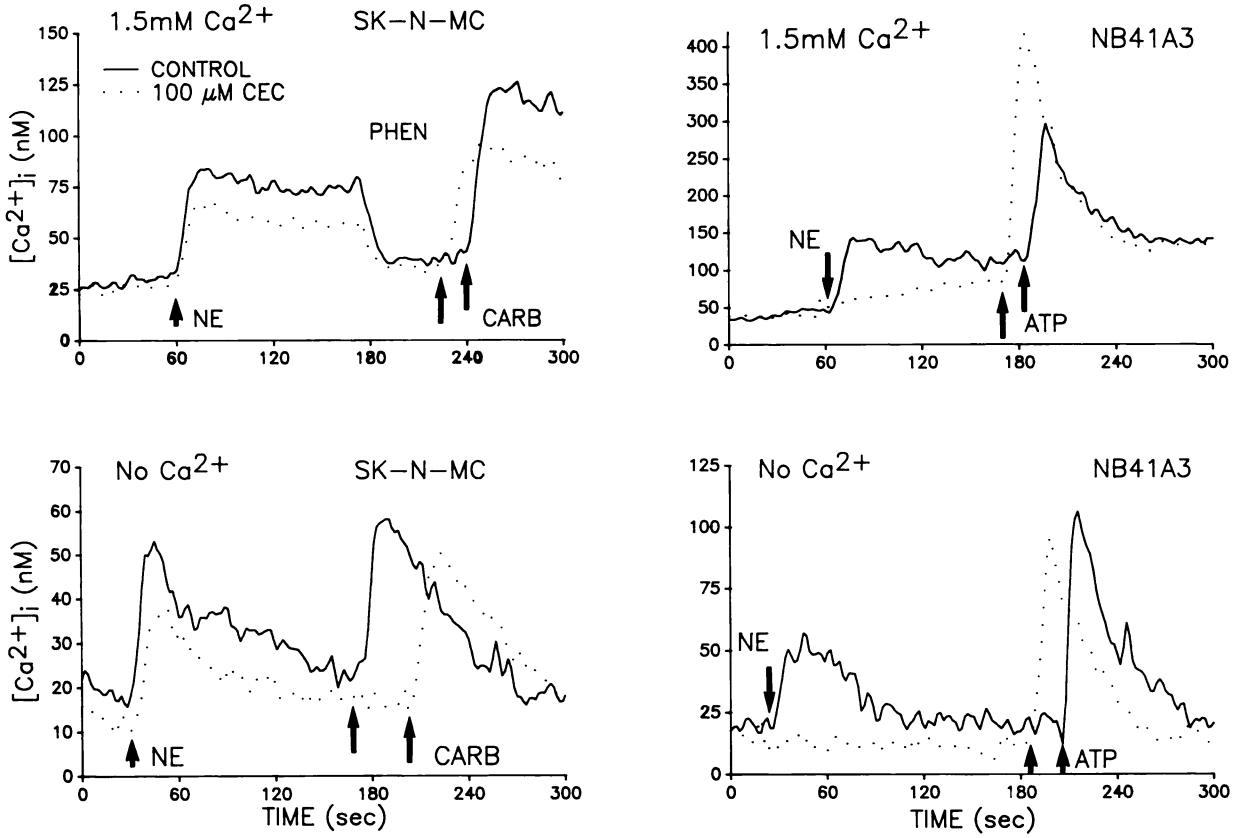

Fig. 7. Fura-2 measurements of NE-induced Ca^{2+} transients in SK-N-MC (*left*) and NB41A3 (*right*) cells. Cells were incubated with (· · · · ·) or without (——) 100 μM CEC for 30 min, harvested, and prepared for measurement of $[Ca^{2+}]_i$ with fura-2 as described in the text. *Upper*, measurement of $[Ca^{2+}]_i$ in cells incubated in the presence of extracellular Ca^{2+} (1.5 mM). In SK-N-MC cells, NE (30 μM) was added at 60 sec and 30 μM phentolamine (*PHEN*) and 1 mM carbachol (*CARB*) were added 120 and 180 sec later, respectively, as indicated. For NB41A3 cells, 30 μM NE was added at 60 sec and 10 μM ATP was added at 180 sec. *Lower*, measurement of $[Ca^{2+}]_i$ in cells incubated in Ca^{2+}-free BSS, containing 2 mM EGTA, for 30–60 sec before addition of NE (30 μM) and carbachol (1 mM) or ATP (10 μM). Multiple arrows by CARB and ATP indicate the different times these agonists were exposed to the different cell batches. Data are from a single experiment representative of five or six experiments.

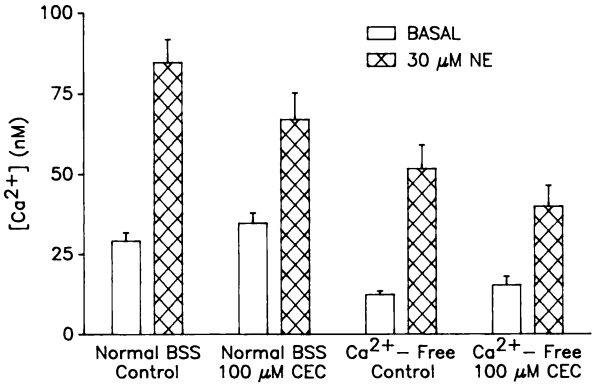

Fig. 8. Effect of extracellular Ca^{2+} and CEC (100 μM) pretreatment on $[Ca^{2+}]_i$ in SK-N-MC cells. Cells were treated and $[Ca^{2+}]_i$ was determined as described in the legend to Fig. 7. Basal and stimulated $[Ca^{2+}]_i$ values were determined as the average $[Ca^{2+}]_i$ over a 6-sec interval before the addition of NE and during the time of peak $[Ca^{2+}]_i$, respectively. Results are expressed as the mean ± standard error of data from five or six experiments.

hybridizes under high stringency conditions to a hamster α_{1B} cDNA probe but not to bovine α_{1C} or rat α_{1D} cDNA probes also supports the conclusion that this cell line expresses only the α_{1B} subtype.

The pharmacological properties of the α_1-ARs in SK-N-MC cells are more complex. The presence of at least two pharmacologically distinct subtypes is suggested by the incomplete inactivation of both α_1-AR binding sites and NE-stimulated InsP formation by CEC. Additionally, the ability of α_{1A}-selective competitive antagonists to inhibit both radioligand binding and InsP formation suggests the presence of at least two pharmacologically distinct subtypes. Curves for inhibition of specific ^{125}I-BE binding by 5-MU, (+)-niguldipine, oxymetazoline, and phentolamine were significantly better fit by a two-site model. Similar results were obtained by studying NE-stimulated InsP formation.

One of the subtypes in SK-N-MC cells has pharmacological properties resembling those of the α_{1A}-AR, including a high affinity for α_{1A}-selective antagonists and a resistance to inactivation by CEC. The K_d values for 5-MU (4 nM), (+)-niguldipine (4 nM), oxymetazoline (7 nM), and WB 4101 (0.7 nM) determined by two-site analysis of displacement of ^{125}I-BE binding are in close agreement with those expected for the α_{1A} subtype (11). They are also in agreement with the K_i values for inhibition of NE-stimulated InsP formation in this cell line previously reported by Fisher and Landon (32). However, those authors found an apparently homogeneous population of this subtype, based on two-site analysis of the inhibition curves, whereas we found that both binding and functional studies indicated the presence of at least two subtypes. We found that an α_1-AR subtype with characteristics of the α_{1B} subtype (low affinity for selective antagonists and inactivation by CEC) comprised about half of the total α_1-AR population. It is possible that technical differences might explain this discrepancy

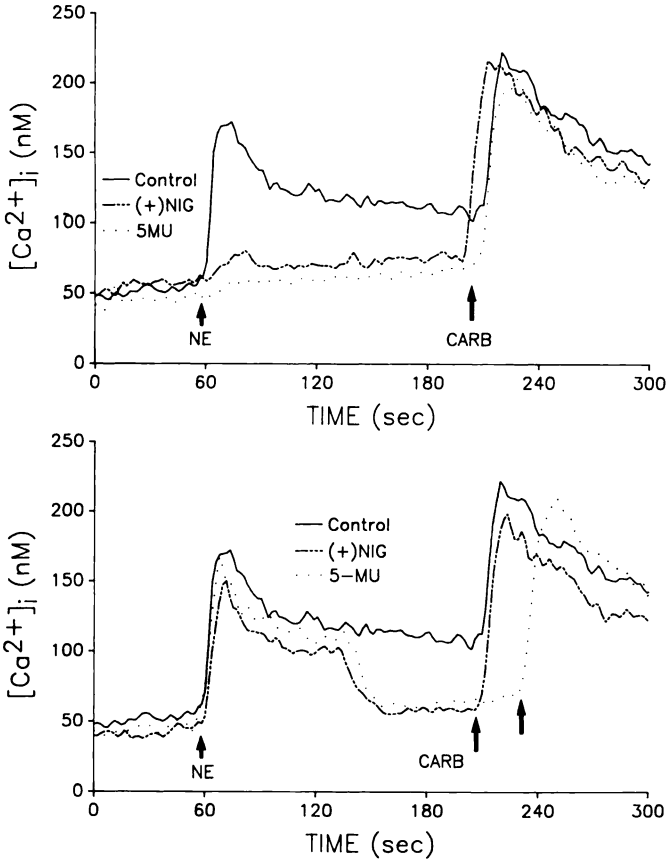

Fig. 9. Effect of α_{1A}-AR subtype-selective antagonists on Ca^{2+} transients stimulated by NE in SK-N-MC cells. Cells were harvested and prepared for measurements of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (1.5 mM) as described in the text. *Upper*, cells were exposed to 100 nM 5-MU or 100 nM (+)-niguldipine [*(+)NIG*] 30 sec before administration of 10 μM NE. Carbachol (*CARB*) (1 mM) was added 120 sec after NE. *Lower*, cells were exposed to 10 μM NE after 60 sec and 100 nM 5-MU or 100 nM (+)-niguldipine was added 60 sec later, followed by the addition of 1 mM carbachol. Multiple arrows by CARB indicate the different times CARB was exposed to the different cell batches. Data are from a single experiment representative of three experiments.

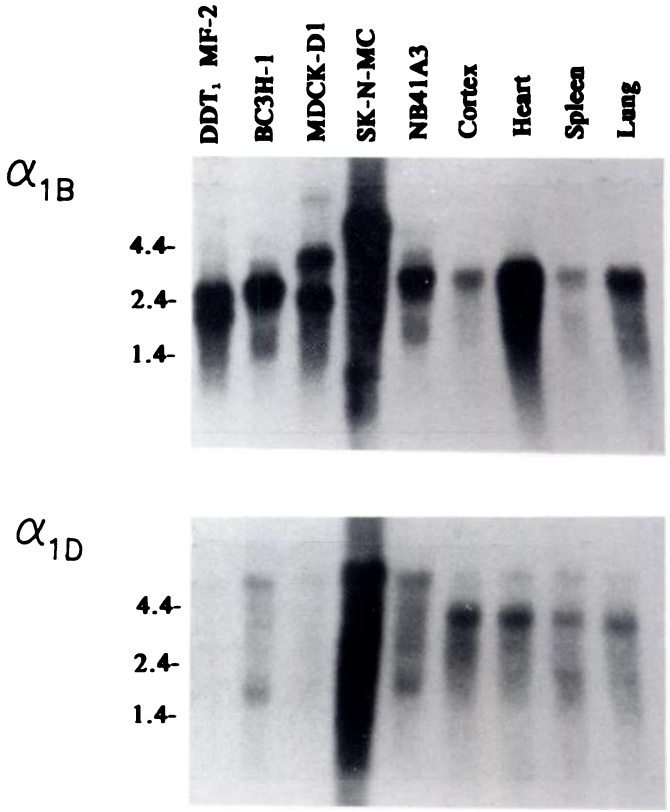

Fig. 10. Northern blot hybridizations to α_{1B}-AR (*upper*) and α_{1D}-AR (*lower*) cDNA probes of poly(A)$^+$ RNA from cell lines and rat tissues. Lanes for all cell lines and rat tissues contained 10 μg of poly(A)$^+$ RNA, except for the DDT_1MF-2 and MDCK-D1 cells, where only 2 μg of poly(A)$^+$ RNA were used because of the extremely strong signals. Hybridization and high stringency washing conditions were as described in the text. Autoradiographs were exposed for 18 hr (α_{1B}) or 9 days (α_{1D}). Data are representative of a number of similar blots from different gels.

or that the cells we are using have been phenotypically transformed. In agreement with our results, however, Fisher and Landon (32) found that pretreatment with 50 μM CEC decreased the InsP response by 36%.

In an attempt to further identify the subtypes of α_1-AR expressed in the SK-N-MC cell line, Northern hybridizations of poly(A)$^+$ RNA from these cells with various α_1 subtype cDNA probes were conducted. We identified multiple transcripts of various sizes (1.3–5.6 kb) that hybridized to both the hamster α_{1B} and rat α_{1D} but not the bovine α_{1C} cDNA probes. However, the identity and function of these multiple mRNA transcripts are not clear, although it does seem apparent that these cells (like most mammalian tissues so far examined) do not express mRNA for the α_{1C} subtype at levels detectable by Northern hybridization. The much longer length of exposure time required for the autoradiographs for the α_{1D}-AR cDNA hybridizations (9 days), compared with those for the α_{1B}-AR cDNA probe (18 hr), may suggest that the α_{1B}-AR mRNA is expressed at a higher level than is the α_{1A}-AR mRNA in the SK-N-MC cell line. However, it appears that the α_{1A}- and α_{1B}-AR proteins are expressed about equally in these cells, based upon the inactivation of one half of the α_1-AR binding sites and InsP response by CEC. Additional studies are necessary to determine the relationship between expression of the mRNA for these receptor subtypes and the actual expression of the respective receptor proteins.

The signal transduction pathway utilized by the α_{1B}-AR subtype in NB41A3 cells appears to be that commonly ascribed to this subtype. NE stimulates [^{3}H]InsP formation in these cells, and pretreatment with CEC eliminates this response. This is similar to the situation in other cell lines such as DDT_1MF-2 (24), BC_3H1 (24), and MDCK-D1 (30), which also express only the α_{1B}-AR. The ability of NE to stimulate [^{3}H]InsP formation even in the complete absence of extracellular Ca^{2+} suggests that the primary signaling mechanism used by this subtype is via phospholipase C-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate. Finally, pertussis toxin had no effect on NE-stimulated [^{3}H]InsP formation in this cell line, consistent with findings in other α_{1B}-AR-containing cells (40–42).

Activation of α_{1B}-ARs in NB41A3 cells was shown to increase $[Ca^{2+}]_i$. This may be due to inositol-1,4,5-trisphosphate-induced mobilization of intracellular stores (43). CEC pretreatment abolished this increase both in the presence and in the absence of extracellular Ca^{2+}. However, some of the α_{1B}-AR-mediated increase in $[Ca^{2+}]_i$ was also dependent upon extracellular Ca^{2+} influx, because removal of extracellular Ca^{2+} eliminated the sustained component of the Ca^{2+} transient. This is consistent with results seen in DDT_1MF-2 cells (24) and may represent a

type of 'capacitative' Ca^{2+} influx for refilling of internal pools of Ca^{2+} (44).

The signaling mechanisms described above for the homogeneous α_{1B}-AR subtype population present in NB41A3 cells provide a good model for directly comparing the signal transduction pathway used by the α_1-AR subtypes present in SK-N-MC cells, despite differences in the species origins of the two cell lines. Indeed, recent evidence suggests that the radioligand-binding properties of the recombinant human α_{1B}-AR do not differ significantly from those of the expressed hamster α_{1B}-AR (45). In addition, the pharmacological properties of α_{1B}-ARs expressed in cell lines that originate from a variety of species, such as hamster (DDT_1MF-2), mouse (BC_3H1), rat (FRTL-5), and dog (MDCK-D1), are all quite similar to those of the NB41A3 cell line.

Our results indicate that both pharmacologically defined α_{1A}- and α_{1B}-AR subtypes are involved in NE-stimulated [^{3}H]InsP formation in SK-N-MC cells. An interesting observation is that the [^{3}H]InsP response in SK-N-MC cells showed a greater dependence on extracellular Ca^{2+} than was observed in NB41A3 cells. It was suggested previously that α_{1A}-AR-mediated InsP responses are more dependent upon extracellular Ca^{2+} than are those of the α_{1B} subtype (22). In addition, treatment with pertussis toxin caused a 50% decrease in NE-stimulated [^{3}H] InsP formation in SK-N-MC cells, although this was not statistically significant. Additionally, PTX had no effect on the elevation of $[Ca^{2+}]_i$ (data not shown). Previous work in primary rat brain cell cultures has suggested that stimulation of [^{3}H] InsP formation by the α_{1A} subtype occurs through a pertussis toxin-sensitive guanine nucleotide-binding protein and is heavily dependent upon extracellular Ca^{2+} (46).

The profiles of intracellular Ca^{2+} transients stimulated by NE in SK-N-MC cells were similar to those observed in NB41A3 cells and consisted of a rapid transient increase in $[Ca^{2+}]_i$ as well as a smaller, more sustained component that was dependent upon the presence of extracellular Ca^{2+}. Pretreatment with CEC did not significantly alter the NE-stimulated Ca^{2+} response in SK-N-MC cells, although it caused a loss of about half of the α_1-AR binding sites and NE-stimulated InsP formation. This may suggest that each of the α_1-AR subtypes present in this cell line can maximally increase $[Ca^{2+}]_i$ by a similar signaling mechanism (a redundant response) or that the α_{1B} subtype contributes little to the elevation of $[Ca^{2+}]_i$ in these cells. Pretreatment of SK-N-MC cells with pertussis toxin had no effect on the Ca^{2+} transients induced by NE (data not shown), despite the small effects on NE-stimulated InsP formation. This is in contrast to the results seen with muscarinic and neuropeptide Y receptor activation in this same cell line, where pertussis toxin treatment inhibited Ca^{2+} influx stimulated by carbachol and intracellular Ca^{2+} mobilization induced by neuropeptide Y (47).

Perhaps the most surprising finding is that, when SK-N-MC cells are pretreated with CEC (to remove the α_{1B} subtype) and incubated in the absence of extracellular Ca^{2+}, stimulation of the α_{1A} subtype is clearly capable of mobilizing intracellular Ca^{2+} stores. This provides direct evidence that a pharmacologically defined α_{1A}-like subtype can be directly linked to InsP formation in the absence of Ca^{2+} influx and can mobilize intracellular Ca^{2+}. Similar results have been reported in rat parotid acinar cells (48). Whether these α_{1A}-like subtypes are identical to the subtypes linked to influx of extracellular Ca^{2+} through voltage-gated channels in smooth muscle (2) remains to be determined. However, at least in these systems, it is clear that α_{1A}-like subtypes can directly activate InsP formation and mobilize intracellular Ca^{2+}.

Although our results are consistent for the most part with the coexistence of the α_{1A}- and α_{1B}-AR subtypes in the SK-N-MC cell line, there are several interesting observations that may be inconsistent with the pharmacology defined for the α_1-AR subtypes. For example, when examining the InsP response data, one can see the ability of the α_{1A}-selective competitive antagonists (+)-niguldipine and oxymetazoline to more clearly distinguish the α_1-AR subtypes, compared with 5-MU or WB 4101, in SK-N-MC cells, even though all four antagonists easily discriminate these subtypes in the radioligand binding assays. In addition, both (+)-niguldipine and 5-MU completely inhibited NE-induced increases in $[Ca^{2+}]_i$ in SK-N-MC cells at a concentration (100 nM) that should block predominately the α_{1A} subtype. This suggests that the α_{1B} subtype is also sensitive to these antagonists at this concentration or that the α_{1B} subtype contributes little to the $[Ca^{2+}]_i$ responses. Because NE can stimulate multiple α_1-AR subtypes, all of which may contribute to the increases in InsP formation and $[Ca^{2+}]_i$, the resultant pharmacology seen with these selective antagonists in these functional assays may reflect an inability of these compounds to adequately discriminate between α_1-AR subtypes that may be stimulated differentially by NE. Alternatively, the pharmacology of the response may reflect a type of 'hybrid' pharmacology that results from the presence on the same cell of two α_1 subtypes that may behave differently when stimulated simultaneously, as opposed to the responses when only a single subtype is expressed. Although a number of cell lines express only the α_{1B}-AR, there is currently no known cell line that expresses exclusively the α_{1A}-AR subtype. Indeed, the SK-N-MC cell line is presently the only cell line that coexpresses two α_1-AR subtypes, which allowed for the description above of the pharmacology and signaling mechanisms of the α_{1A} subtype after selective inactivation of the α_{1B} subtype with CEC. Finally, our results may indicate that there exists another α_1-AR subtype with pharmacological characteristics that differ from those described for the α_{1A} and α_{1B} subtypes. Development of more selective compounds that discriminate among these α_1-AR subtypes and further molecular characterization of these receptors would help to clarify further the pharmacological profiles of the α_1-AR subtypes.

In summary, we have compared two neuronal cell lines expressing α_1-ARs. Murine NB41A3 cells express only the α_{1B} subtype, which is linked to the expected signaling mechanism of InsP formation and mobilization of intracellular Ca^{2+}. SK-N-MC cells appear to express at least two subtypes, one of which pharmacologically resembles the α_{1A} subtype. This subtype also appears to be linked to InsP formation and mobilization of intracellular Ca^{2+}, although with a different sensitivity to extracellular Ca^{2+} and apparently through a different guanine nucleotide-binding protein. This cell line may provide a useful source for attempting to clone the α_{1A} subtype.

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