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Increased Voltage-Dependent Calcium Influx Produced by α_{1B}-Adrenergic Receptor Activation in Rat Medullary Thyroid Carcinoma 6-23 Cells

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SUMMARY

We characterized norepinephrine (NE)-activated Ca^{2+} influx in the rat medullary thyroid carcinoma (rMTC) 6-23 cell line using fura-2. NE caused a sustained increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which was completely reversed by addition of nifedipine or removal of extracellular Ca^{2+}. Bay K8644, KCl-induced depolarization, and ATP also increased $[Ca^{2+}]_i$ in rMTC 6-23 cells, effects that were also reversed by nifedipine. Release of intracellular Ca^{2+} by thapsigargin was not blocked by nifedipine, and NE caused nifedipine-sensitive increases in $[Ca^{2+}]_i$ even in the presence of thapsigargin. NE-stimulated increases in $[Ca^{2+}]_i$ were mimicked by the α_1-adrenergic receptor (AR) agonist phenylephrine but not by the β-AR agonist isoproterenol. The response to NE was blocked by the α-AR antagonist phentolamine and by pretreatment with the α_{1B}-selective alkylating agent chloroethylclonidine (CEC) but was not blocked by α_{1A}-selective concentrations of the subtype-selective antagonist 5-methylurapidil. α_1-AR binding sites labeled by ^{125}I-BE 2254 in membranes from this cell line were highly sensitive to inactivation by CEC (>80%), and competition with subtype-selective antagonists suggested the presence of a homogeneous population of α_{1B}-ARs. NE, epinephrine, and phenylephrine, but not KCl, ATP, or isoproterenol, caused large increases in [^{3}H]inositol phosphate (InsP) formation in these cells. This [^{3}H]InsP response was greatly reduced by CEC pretreatment, and competitive antagonists blocked this response with an α_{1B}-like pharmacology. Northern blots of poly(A)$^+$ RNA from rMTC 6-23 cells showed single transcripts hybridizing to the hamster α_{1B}-AR (2.2-kilobase) and less prominently to the rat α_{1D}-AR (4.0-kilobase) cDNAs but no detectable hybridization to the bovine α_{1C}-AR cDNA. The phospholipase C inhibitor U-73122 reduced the [^{3}H]InsP response to NE in a concentration-dependent manner but had little or no effect on the NE-induced increases in $[Ca^{2+}]_i$. Phorbol myristate acetate also increased $[Ca^{2+}]_i$ in rMTC 6-23 cells, although this response was not blocked by nifedipine. We conclude that activation of α_{1B}-like ARs (including possibly both α_{1B}- and α_{1D}-ARs) increases voltage-dependent Ca^{2+} influx in rat rMTC 6-23 cells. This effect appears to be independent of release of intracellular Ca^{2+}, activation of phospholipase C, and/or activation of protein kinase C. This cell line should be very useful in defining the mechanisms underlying the known effects of α_1-ARs on voltage-gated Ca^{2+} influx, which plays an important functional role in vascular smooth muscle.

Pharmacological and molecular evidence has conclusively proven that the α_1-ARs comprise a heterogeneous family. Two subtypes of α_1-ARs (α_{1A} and α_{1B}) have been distinguished pharmacologically in a number of animal tissues (1-6). cDNAs for three α_1-AR subtypes have been isolated by molecular cloning (7-10). The α_{1B}-AR cDNA was cloned from the hamster DDT$_1$MF-2 cell line, and this cDNA encodes a receptor with properties and tissue distribution similar to those of the pharmacologically defined α_{1B}-AR (7). Another cDNA clone was isolated from a bovine brain library and encodes a novel α_1-AR subtype, called the α_{1C}-AR, with a relatively high affinity for α_{1A}-AR-selective competitive antagonists but partial sensitivity to CEC inactivation and a very rare mRNA distribution (8). Two other cDNA clones, with sequences differing by only two codons, were isolated independently from rat brain libraries (9, 10) and had been designated as α_{1A}- and α_{1D}-AR subtypes, respectively. More stringent pharmacological analyses (10, 11) suggested that these two clones were identical but that both express proteins with primarily α_{1B}-type pharmacology. This

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ABBREVIATIONS: AR, adrenergic receptor; BSA, bovine serum albumin; BSS, balanced salt solution; CEC, chloroethylclonidine; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BE, BE 2254 ([2β-(4-hydroxyphenyl)ethylaminomethyl]tetralone); InsP, inositol phosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; kb, kilobase(s); KRB, Krebs-Ringer bicarbonate buffer; NE, norepinephrine; PBS, phosphate-buffered saline; PMA, 4-β-phorbol-12-myristate-13-acetate; SSC, standard saline citrate; U-73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U-73343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione; rMTC, rat medullary thyroid carcinoma; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate.

clone has thus been suggested to be designated as the $\alpha_{1A/D}$-AR subtype (11). It is generally agreed that a cDNA encoding the pharmacologically defined α_{1A}-AR subtype has not yet been isolated.

The primary functional effect of α_1-AR activation is an increase in $[Ca^{2+}]_i$ in most cells (1). This increase in $[Ca^{2+}]_i$ may result from the release of Ca^{2+} sequestered in internal stores and/or influx of extracellular Ca^{2+} into the cell through either voltage-dependent (12) or voltage-independent (13) membrane channels. Shortly after the discovery of pharmacologically distinct α_1-AR subtypes, data were obtained that suggested that these subtypes might be linked to different mechanisms for increasing $[Ca^{2+}]_i$ (1, 14–18). It was proposed by Han *et al.* (14) that α_1-AR subtypes may preferentially activate different signal transduction pathways, with the α_{1A}-ARs activating Ca^{2+} influx through voltage-gated channels and the α_{1B}-ARs mobilizing intracellular Ca^{2+} stores. Although these conclusions have been supported by many other correlative studies in smooth muscle, they have also been questioned (19, 20).

Stimulation of α_{1B}-ARs is known to activate phospholipase C, increasing the formation of diacylglycerol, an activator of protein kinase C, and Ins(1,4,5)P_3, which mobilizes Ca^{2+} from intracellular stores (13, 21–24). This increase in $[Ca^{2+}]_i$ often activates Ca^{2+} influx, usually of a capacititative type, occurring through voltage-independent channels (13). However, the signaling mechanism activated by the α_{1A}-AR subtype and the mechanisms involved in α_1-AR-mediated activation of voltage-dependent Ca^{2+} influx are much less clear (1, 14–18). Indeed, α_{1A}-ARs can also increase InsP formation (25, 26), and α_1-ARs can activate other signaling pathways such as phospholipases A_2 (27–29) and D (30) and can potentiate adenylate cyclase activity (31, 32), all of which may also regulate $[Ca^{2+}]_i$. The relationship between specific α_1-AR subtypes and signaling mechanisms is still unclear.

One factor contributing to this confusion has been the lack of a suitable system in which the link between α_1-AR activation and stimulation of voltage-gated Ca^{2+} influx could be easily studied (24). Zink and Raue (33) recently demonstrated that NE increased $[Ca^{2+}]_i$ in the rMTC 6–23 cell line and that this effect could be blocked by verapamil but not by pertussis toxin treatment. We show here that this cell line contains α_1-ARs linked to voltage-gated Ca^{2+} influx and, surprisingly, that these are predominantly of the α_{1B} subtype. Preliminary analysis of the mechanisms involved suggests that they are independent of known effects on phospholipase C activity.

Experimental Procedures

Materials. The rMTC 6–23 cells were obtained from the American Type Culture Collection (Rockville, MD). Materials were obtained from the following sources: U-73122 and U-73343, kindly provided by Dr. J. Bleasdale, Upjohn Co. (Kalamazoo, MI); phentolamine mesylate, Ciba-Geigy (Summit, NJ); CEC, 5-methylurapidil, (+)-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/acetoxymethyl ester, Molecular Probes (Eugene, OR); fetal bovine serum, horse serum, and trypsin/EDTA, GIBCO (Gaithersburg, MD); carbachol, digitonin, (−)-NE bitartrate, yohimbine hydrochloride, DMEM, penicillin, streptomycin, and all other chemicals, Sigma Chemical Co. (St. Louis, MO).

Cell culture. rMTC 6–23 cells were propagated in 75-cm^2 flasks in a humidified 5% CO_2 incubator, in DMEM containing 4.5 g/liter glucose, 1.4% glutamine, 1.4% sodium pyruvate, 20 mM HEPES, 100 mg/liter streptomycin, 10^5 units/liter penicillin, 15% horse serum, and 2.5% fetal bovine serum. The cells were detached by mild trypsinization (0.25%) in the presence of 2.6 mM EDTA and were subcultured at a ratio of 1:3 upon reaching confluency. For measurements of [^{3}H]InsP formation, 35-mm Falcon Primaria dishes were seeded at a density of 600,000 cells/2 ml. For studies involving radioligand binding and Ca^{2+} and mRNA measurements, 100-mm dishes were seeded at a density of 6×10^6 cells/10 ml. Cells were grown to confluency before use.

^{125}I-BE binding. ^{125}I-BE binding was performed in membrane preparations as described previously (34). rMTC 6–23 cells were washed two times in PBS (20 mM $NaPO_4$, 154 mM NaCl, pH 7.6), and membranes were prepared and resuspended in PBS (one confluent 100-mm plate/3.5 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 μM CEC (34). The incubations were terminated by dilution with cold PBS and centrifugation at 30,000 × *g* for 10 min. The pellets were washed twice to remove all CEC not irreversibly bound and the membranes were finally resuspended in PBS as described above.

BE was radioiodinated to theoretical specific activity (35) 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 for 20 min at 37°, in the presence or absence of competing drugs, in a final volume of 0.25 ml of PBS, as described previously (33). Non-receptor binding was determined in the presence of 10 μM phentolamine. Saturation plots were analyzed by the method of Scatchard (36), and displacement by competitive antagonists was analyzed with Hill plots.

Measurement of [^{3}H]InsPs. Accumulation of total [^{3}H]InsPs was determined in confluent 35-mm dishes. Cells were prelabeled with *myo*-[^{3}H]inositol (2 μCi/plate) 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 (120 mM NaCl, 5.5 mM KCl, 0.625 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 20 mM $NaHCO_3$, 11 mM glucose, 0.029 mM $CaNa_2EDTA$) containing 10 mM LiCl and were incubated with or without drugs, in KRB with 10 mM LiCl, for 1 hr at 37°. 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 utilizing CEC, cells were incubated for 30 min in KRB with or without CEC (100 μM), the cells were washed three times, and [^{3}H]InsPs were determined as described above.

$[Ca^{2+}]_i$ determinations. $[Ca^{2+}]_i$ transients were determined using fura-2, as described previously (13, 24). Confluent 100-mm plates were washed with BSS (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, 0.1% BSA) and were pretreated with or without 100 μM CEC for 30 min. Cells were then washed with HBSS and detached by incubation for 1 min with 0.05% trypsin/0.53 mM EDTA in HBSS, 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 5 μM fura-2/acetoxymethyl ester for 30 min 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 stored 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 (all with 5-nm bandwidths). 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 (38).

mRNA analysis. The cDNAs encoding the hamster α_{1B}-AR (7) and bovine α_{1C}-AR (8) were kindly provided by Dr. Jon Lomasney (Duke University, Durham, NC). The rat α_{1D}-AR cDNA (10) was kindly provided by Dr. R. Graham (Case Western University, Cleveland, OH). The full length cDNAs were labeled with [α-^{32}P]dCTP (6000 Ci/mmol; Amersham) by the random primer method, using the Prime-It II kit (Stratagene, La Jolla, CA).

Poly(A)$^+$-selected RNA from rMTC 6-23 cells was prepared by oligo(dT)-cellulose chromatography using the FastTrack kit (Invitrogen, San Diego, CA). mRNA was denatured, electrophoresed on 1.2% agarose/0.7% formaldehyde gels, and transferred to Hybond M nylon membranes (Amersham) by capillary blotting with 20× SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The membranes were hybridized with the appropriate ^{32}P-labeled cDNA probe for 16 hr at 42°, the blots were washed twice at room temperature with 2× SSC/0.1% sodium dodecyl sulfate for 7 min each time, followed by two 10-min washes with 0.2× SSC/0.1% sodium dodecyl sulfate at 60°, and autoradiographs were prepared.

Results

Effect of NE on $[Ca^{2+}]_i$. Fura-2 was used to monitor $[Ca^{2+}]_i$ in suspensions of rMTC 6-23 cells. NE (30 μM) stimulated a rapid 2-fold increase in $[Ca^{2+}]_i$ in the presence of 1.5 mM free extracellular Ca^{2+}, as shown in Fig. 1. After this rapid increase, the $[Ca^{2+}]_i$ was maintained at a sustained level, which slowly declined over time. Upon addition of the dihydropyridine Ca^{2+} channel antagonist nifedipine (1 μM), $[Ca^{2+}]_i$ returned to the basal level. Administration of the Ca^{2+}/ATPase inhibitor thapsigargin (1 μM), which blocks Ca^{2+} uptake into the endoplasmic reticulum and thus depletes this intracellular store of Ca^{2+}, caused an approximately 2-fold increase in $[Ca^{2+}]_i$, which was sustained (Fig. 1, *right*). Exposure of these thapsigargin-treated cells to 30 μM NE stimulated an additional increase in $[Ca^{2+}]_i$, which was completely reversed, to the level stimulated by thapsigargin, by the addition of 1 μM nifedipine. In addition, nifedipine had no effect on the thapsigargin response in this cell line.

Pharmacology of the $[Ca^{2+}]_i$ response. The α_{1B}-AR-selective alkylating agent CEC and subtype-selective agonists and competitive antagonists were used to evaluate the pharmacology of the AR that activates voltage-dependent Ca^{2+} channels in fura-2-loaded rMTC 6-23 cells. As described above, cells treated with 30 μM NE caused an approximately 2-fold increase in $[Ca^{2+}]_i$, which could be reversed to the basal level of $[Ca^{2+}]_i$ by the addition of the α-AR antagonist phentolamine (100 μM). Pretreatment of the cells for 30 min with 100 μM CEC completely inactivated the response to 30 μM NE (Fig. 2, *upper left*). However, the α_{1A}-AR-selective competitive antagonist 5-methylurapidil (100 nM) did not antagonize the increase in $[Ca^{2+}]_i$ induced by 30 μM NE (Fig. 2, *upper right*). The β-AR-selective agonist isoproterenol (10 μM) did not increase $[Ca^{2+}]_i$ in rMTC 6-23 cells (Fig. 2, *lower left*). The α_1-AR agonist phenylephrine (100 μM) produced an almost 2-fold increase in $[Ca^{2+}]_i$, which was quite similar to the response caused by 30 μM NE. The response to phenylephrine, like that to NE, was also completely blocked by the addition of 1 μM nifedipine (Fig. 2, *lower right*).

Further characterization of the $[Ca^{2+}]_i$ response in rMTC 6-23 cells. The voltage-dependent Ca^{2+} channel activator Bay K8644 (10 nM) caused a small sustained increase in $[Ca^{2+}]_i$ in rMTC 6-23 cells loaded with fura-2 (Fig. 3, *left*). After this small increase in $[Ca^{2+}]_i$, addition of 30 μM NE produced the familiar rapid increase in $[Ca^{2+}]_i$. Further addition

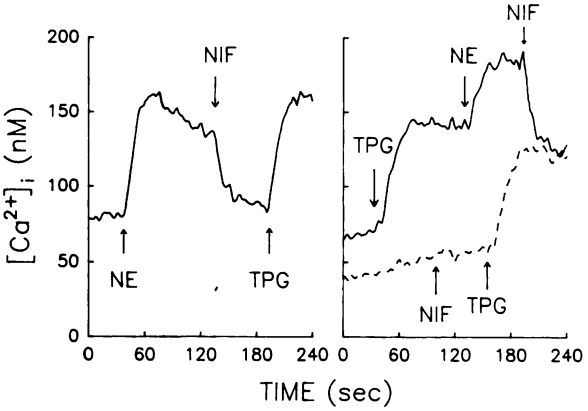

Fig. 1. Effect of thapsigargin (*TPG*) on $[Ca^{2+}]_i$ in fura-2-loaded rMTC 6-23 cells. *Left*, cells were exposed to NE (30 μM) at 30 sec and the elevated $[Ca^{2+}]_i$ was reversed by 1 μM nifedipine (*NIF*) 100 sec later. Thapsigargin (2 μM) was administered 60 sec later. *Right*, thapsigargin (1 μM) administered to cells at 30 sec caused an elevation in $[Ca^{2+}]_i$ and NE (30 μM) administered at 130 sec caused an additional increase in $[Ca^{2+}]_i$, which was reversed by 1 μM nifedipine, 60 sec later, to the level induced by thapsigargin (*solid line*). Nifedipine (1 μM) had no effect on $[Ca^{2+}]_i$ induced by 1 μM thapsigargin (*dashed line*). Dimethylsulfoxide (0.1% final concentration) had no effect on $[Ca^{2+}]_i$ in rMTC 6-23 cells. Data are from a single experiment representative of two or three experiments.

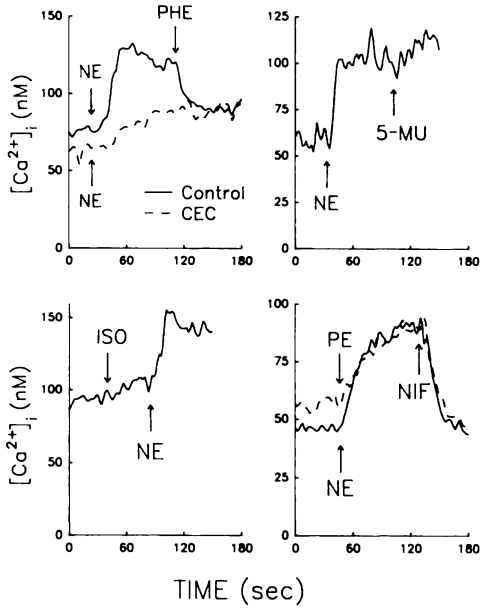

Fig. 2. Pharmacological profile of NE-stimulated Ca^{2+} transients measured by fura-2 fluorescence in rMTC 6-23 cells. *Upper left*, cells were incubated for 30 min with (*dashed line*) or without (*solid line*) 100 μM CEC, harvested, and prepared for measurements of $[Ca^{2+}]_i$ with fura-2 as described in the text. NE (30 μM) was added at 30 sec and 100 μM phentolamine (*PHE*) was added 90 sec later as indicated. *Upper right*, cells were stimulated with 30 μM NE at 30 sec and 100 nM 5-methylurapidil (*5-MU*) was added 90 sec later as indicated. *Lower left*, rMTC 6-23 cells were exposed to 10 μM isoproterenol (*ISO*) at 50 sec and then stimulated with 30 μM NE 30 sec later. *Lower right*, cells were stimulated with 30 μM NE (*solid line*) or 100 μM phenylephrine (*PE*) (*dashed line*) at 40 sec and then exposed to 1 μM nifedipine (*NIF*) 90 sec later as indicated. Data are from a single experiment, representative of two or three experiments.

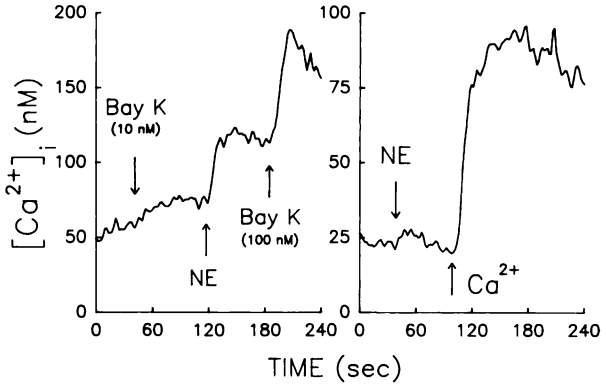

Fig. 3. Role of extracellular Ca^{2+} in $[Ca^{2+}]_i$ fluxes in fura-2-loaded rMTC 6–23 cells. *Left*, cells were exposed sequentially to 10 nM Bay K8644 (*Bay K*), 30 μM NE, and 100 nM Bay K8644 at 60-sec intervals. *Right*, cells were incubated in Ca^{2+}-free BSS containing 2 mM EGTA for 30 sec before addition of 30 μM NE, and 3.5 mM Ca^{2+} was added 60 sec later. Data are from a single experiment, representative of two or three experiments.

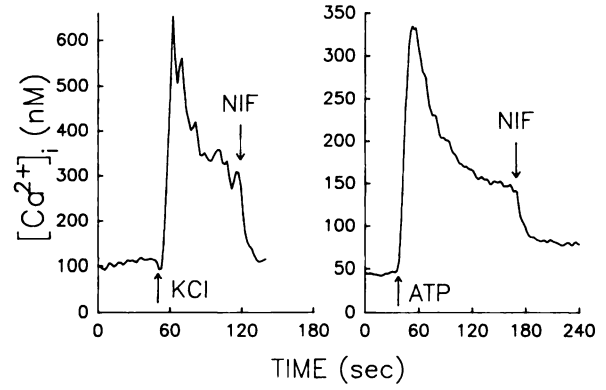

Fig. 4. Fura-2 measurements of $[Ca^{2+}]_i$ in rMTC 6–23 cells stimulated with KCl and ATP. *Left*, cells were exposed to 50 mM KCl at 60 sec and then to 1 μM nifedipine (*NIF*) 60 sec later. *Right*, cells were exposed to 1 mM ATP at 30 sec and then to 1 μM nifedipine 150 sec later. Results are from a single experiment, representative of two or three experiments.

of a higher concentration (100 nM) of Bay K8644 caused an even greater increase in the $[Ca^{2+}]_i$ in these cells. When fura-2-loaded rMTC 6–23 cells were incubated in buffer containing no extracellular Ca^{2+} and 2 mM EGTA for 1 min before the addition of NE, the basal level of $[Ca^{2+}]_i$ decreased by approximately 50% (Fig. 3, *right*). Cells incubated under this condition showed little or no increase in $[Ca^{2+}]_i$ when exposed to 30 μM NE. However, upon the addition of 1.5 mM free extracellular Ca^{2+}, the $[Ca^{2+}]_i$ increased by >3-fold. When fura-2-loaded rMTC cells were exposed to a depolarizing concentration (50 mM) of KCl, the $[Ca^{2+}]_i$ increased rapidly, peaked at a level >6-fold greater than the basal level, and then exhibited a sustained, approximately 3-fold increase in $[Ca^{2+}]_i$, which was completely reversed by the addition of 1 μM nifedipine (Fig. 4, *left*). Similarly, ATP (1 mM) caused an almost 6-fold increase in peak $[Ca^{2+}]_i$ and then a sustained 3-fold increase in $[Ca^{2+}]_i$ that was blocked by the addition of 1 μM nifedipine.

Radioligand binding. ^{125}I-BE, an α_1-AR-selective antagonist radioligand (35), was used to label α_1-ARs in membranes prepared from rMTC 6–23 cells. Fig. 5 shows that a homogeneous population of binding sites was labeled by ^{125}I-BE. Saturation binding isotherms analyzed by the method of Scatchard (36) showed that the density of binding sites (B_{max}) was 47

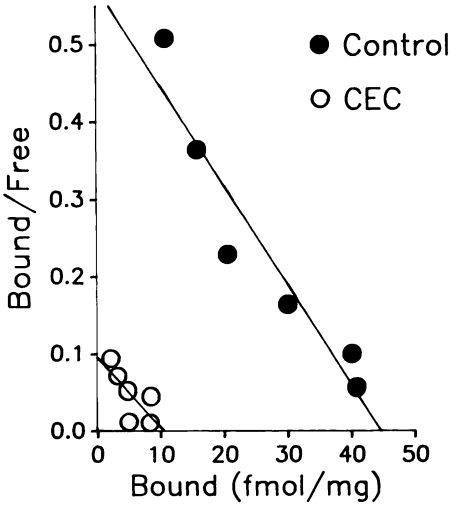

Fig. 5. Effect of CEC pretreatment on α_1-AR binding sites in membrane preparations of rMTC 6–23 cells. Membrane preparations were pretreated for 10 min at 37° with (○) or without (●) 10 μM CEC in hypotonic buffer. Each *point* is the mean of duplicate determinations from five experiments.

TABLE 1

Inhibition by selected α-AR antagonists of specific ^{125}I-BE binding to rMTC 6-23 cell membranes

K_i values are presented as the mean ± standard error from three or four experiments. Hill coefficients were calculated from the combined mean data.

Drug	K_i	n_H
	nM	
Prazosin	0.19 ± 0.09	1.28
WB-4101	6.20 ± 2.6	1.00
Benoxathian	16.7 ± 2.3	1.03
Phentolamine	18.3 ± 5.4	0.86
5-Methylurapidil	54.0 ± 10.5	0.82
Oxymetazoline	113 ± 41.2	1.16
(+)-Niguldipine	137 ± 44.6	1.31
Yohimbine	402 ± 92.2	0.83

fmol/mg of protein and that the K_d value was 105 pM. Pretreatment of the membranes with 10 μM CEC for 10 min in hypotonic buffer, with subsequent washing, inactivated nearly 85% of the α_1-AR binding sites.

Inhibition of specific ^{125}I-BE (50 pM) binding by selective competitive antagonists was performed in membranes from rMTC 6–23 cells. The affinities and Hill coefficients of these drugs are displayed in Table 1. All of the compounds displaced ^{125}I-BE with relatively low affinities and Hill coefficients that were close to 1.0. These results suggest the presence of a single homogeneous population of binding sites, with properties that resemble those of the α_{1B}-AR subtype.

[^{3}H]InsP formation. NE and the α_1-AR-selective agonists phenylephrine and methoxamine increased [^{3}H]InsP formation in a concentration-dependent manner in rMTC 6–23 cells (Fig. 6, *upper*). NE caused the greatest maximal stimulation (average of 3.6-fold over basal) of [^{3}H]InsP formation among the agonists tested. Phenylephrine also produced a substantial [^{3}H]InsP response, but the maximal response to the highest concentration of phenylephrine used (100 μM) was only about 75% of that caused by NE. Methoxamine stimulated much less [^{3}H]InsP formation than did either NE or phenylephrine and, at 1 mM, the [^{3}H]InsP formation response was only 35% of the maximal response to NE.

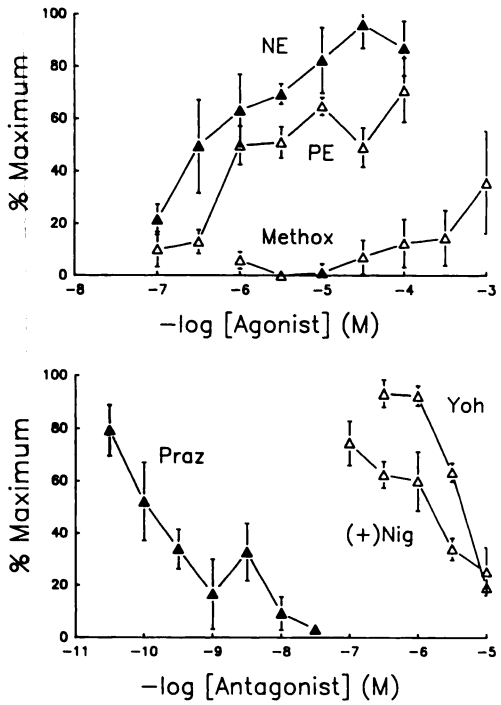

Fig. 6. α_1-AR-mediated [^{3}H]InsP formation in rMTC 6–23 cells. *Upper*, NE, phenylephrine (*PE*), and methoxamine (*Methox*) stimulated [^{3}H]InsP formation in a concentration-dependent manner in rMTC 6–23 cells. The data are expressed as a percentage of the maximal NE response. Basal and NE-stimulated percentages of hydrolysis were 2.5 ± 0.4% and 9.1 ± 1.2%, respectively. Each *point* represents the mean ± standard error of three experiments performed in duplicate. *Lower*, the NE (30 μM)-stimulated [^{3}H]InsP formation response was blocked by prazosin (*Praz*), (+)-niguldipine [*(+)Nig*], and yohimbine (*Yoh*) in a concentration-dependent manner in rMTC 6–23 cells. Results are expressed as percentage of the maximal response to NE in the absence of antagonists. Basal and NE-stimulated percentages of hydrolysis were 2.5 ± 0.4% and 7.2 ± 0.9%, respectively. Each *point* is the mean ± standard error from three experiments performed in duplicate.

[^{3}H]InsP formation stimulated by 30 μM NE was inhibited by selective competitive antagonists in a concentration-dependent manner in rMTC 6–23 cells (Fig. 6, *lower*). The α_1-AR-selective competitive antagonist prazosin inhibited the response most potently, with an IC_{50} of approximately 1 nM, whereas the α_2-AR selective antagonist yohimbine was least potent in inhibiting the response, with an IC_{50} of about 5 μM. The α_{1A}-AR-selective competitive antagonist (+)-niguldipine also inhibited the response with relatively low potency (IC_{50} = 2 μM), suggesting the involvement of the α_{1B}-AR in this response.

The [^{3}H]InsP formation response elicited by maximally stimulating concentrations of α_1-AR agonists was greatly reduced when rMTC 6–23 cells were pretreated for 30 min with 100 μM CEC (Fig. 7). The responses to NE and epinephrine were decreased by 75–80%, which is similar to the decrease in α_1-AR binding sites caused by pretreatment of rMTC cell membranes with CEC. The [^{3}H]InsP response to phenylephrine was almost completely blocked by CEC pretreatment. No increases in [^{3}H]InsP formation were seen with the β-AR agonist isoproterenol, the purinergic agonist ATP, the muscarinic agonist carbachol, or a depolarizing concentration (50 mM) of KCl.

mRNA analysis. Northern blot hybridizations of poly(A)$^+$-selected RNA prepared from rMTC 6–23 cells with probes prepared from full length cDNA clones of the hamster α_{1B}-AR, bovine α_{1C}-AR, and rat α_{1D}-AR were performed (Fig. 8). The

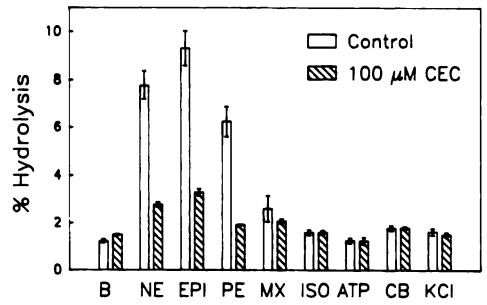

Fig. 7. Inhibition of agonist-stimulated [^{3}H]InsP formation in 100 μM CEC-pretreated rMTC cells. Cells were exposed to 100 μM CEC in KRB for 30 min, washed, and then exposed to agonists for 1 hr, as described in Experimental Procedures. The treatments were basal (*B*), 100 μM NE (*NE*), 100 μM epinephrine (*EPI*), 100 μM phenylephrine (*PE*), 1 mM methoxamine (*MX*), 10 μM isoproterenol (*ISO*), 1 mM ATP (*ATP*), 1 mM carbachol (*CB*), and 50 mM KCl (*KCl*). Data are expressed as percentage of hydrolysis of total [^{3}H]inositol incorporated into lipid. Each *bar* represents the mean ± standard error of duplicate determinations from three experiments.

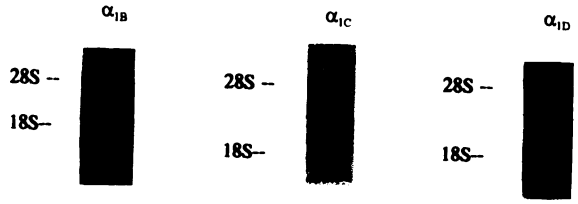

Fig. 8. Northern blot hybridizations of poly(A)$^+$ RNA from rMTC 6–23 cells with α_{1B}-AR (*left*), α_{1C}-AR (*middle*), and α_{1D}-AR (*right*) cDNA probes. Each lane contained 10 μg of poly(A)$^+$ RNA. Hybridization and high stringency washing conditions were as described in the text. Autoradiographs were exposed for 24 hr. These results are representative of two individual Northern blots.

α_{1B}-cDNA probe hybridized to a single transcript of 2.2 kb, whereas the α_{1D}-cDNA probe hybridized to a single transcript of 4.0 kb. No transcripts hybridized to the α_{1C}-cDNA probe.

Role of phospholipase C in the $[Ca^{2+}]_i$ responses. To further evaluate the role that activation of phospholipase C may play in the $[Ca^{2+}]_i$ responses elicited by NE in the rMTC 6–23 cells, U-73122 was used to selectively inhibit phospholipase C activity (39). U-73122 inhibited [^{3}H]InsP formation stimulated by 30 μM NE in a concentration-dependent manner, with an IC_{50} of approximately 1 μM, and caused a maximal 75% decrease in the response at 10 μM, the highest concentration used (Fig. 9). The less active structural analog U-73343 inhibited [^{3}H]InsP formation by only 35% at the highest concentration used (10 μM).

In fura-2-loaded rMTC 6–23 cells, 10 μM U-73122 had little effect on the increase in $[Ca^{2+}]_i$ stimulated by 30 μM NE (Fig. 10, *left*). Similarly, U-73343 had little effect on the $[Ca^{2+}]_i$ response (data not shown). When cells were exposed to 1 μM PMA, an activator of protein kinase C, $[Ca^{2+}]_i$ was increased approximately 2-fold over the basal level (Fig. 10, *right*). However, 1 μM nifedipine did not block this increase in $[Ca^{2+}]_i$.

α_1-AR-activated Ca^{2+} influx in rMTC 44–2 cells. NE has also been shown to increase $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$ in another rMTC clonal cell line, designated rMTC 44–2. We have also found that rMTC 44–2 cells possess α_1-AR-mediated $[Ca^{2+}]_i$ and [^{3}H]InsP formation responses and ^{125}I-BE-binding characteristics quite similar to those of the rMTC 6–23 cell line (data not shown).

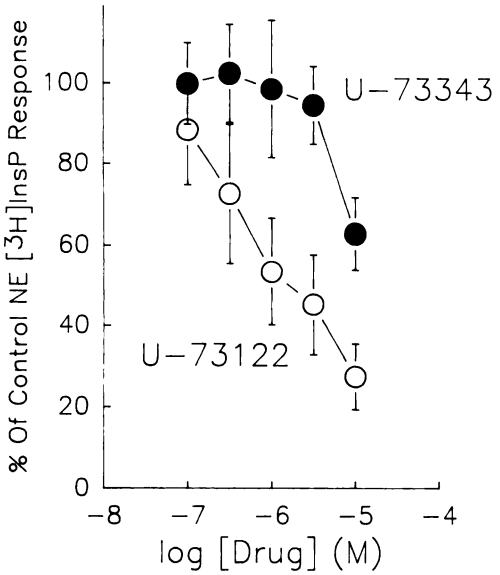

Fig. 9. Effect of U-73122 and U-73343 on [^{3}H]InsP formation stimulated by 30 μM NE in rMTC 6–23 cells. The cells were pretreated for 3 min in the presence of the indicated concentrations of U-73122 and U-73343 and then stimulated for 1 hr with 30 μM NE in the continued presence of these compounds. Results are expressed as the percentage of the maximal response to 30 μM NE in the absence of U-73122 or U-73343. Basal and NE-stimulated percentages of hydrolysis were 2.4 ± 0.5% and 11.1 ± 1.0%, respectively. Each *point* is the mean ± standard error from three or four experiments performed in duplicate.

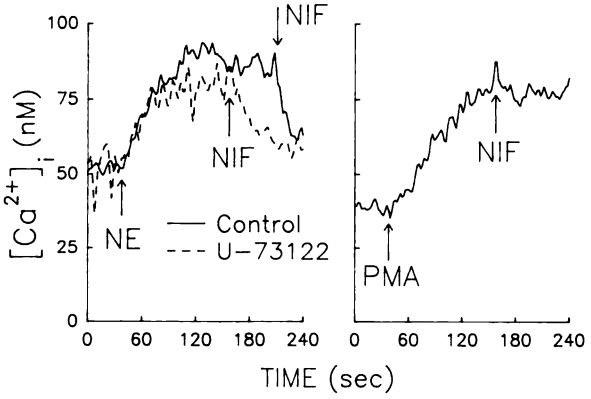

Fig. 10. Effect of U-73122 and PMA on $[Ca^{2+}]_i$ in fura-2-loaded rMTC 6–23 cells. *Left*, cells were preincubated for 3 min with (*dashed line*) or without (*solid line*) 10 μM U-73122 before the addition of 30 μM NE. Nifedipine (*NIF*) (1 μM) was added later, at the indicated times. *Right*, cells were exposed to 1 μM PMA at 30 sec and then to 1 μM nifedipine 120 sec later. Data are from a single experiment, representative of two or three experiments.

Discussion

The experiments presented here demonstrate that NE stimulates Ca^{2+} influx into rMTC 6–23 cells through α_1-AR-mediated activation of nifedipine-sensitive Ca^{2+} channels. This cell line, therefore, provides a good model system for unequivocally identifying α_1-AR subtype(s) involved in this response and the mechanism(s) through which activation of these receptors leads to dihydropyridine-sensitive Ca^{2+} influx. Surprisingly, it appears that the α_{1B}-AR subtype (with possibly some participation by the α_{1D}-AR subtype) activates this response, and preliminary evidence suggests that it is unrelated to phospholipase C activation, release of intracellular Ca^{2+} stores, or formation of diacylglycerol.

The rMTC 6–23 cell line has been used as a model for studying calcitonin-secreting C cells (40, 41). Voltage-dependent Ca^{2+} channels play a major role in maintaining $[Ca^{2+}]_i$ oscillations in cells treated with glucagon or 8-bromo-cAMP (42). Recently, Zink and Raue (33) have shown that NE can increase $[Ca^{2+}]_i$ in these cells and that this effect can be blocked by verapamil but not by pertussis toxin. A similar cell line (rMTC 44–2) (43), with NE-activated voltage-dependent Ca^{2+} channels (44, 45), has also been developed. Our goal was to characterize the AR and signal transduction mechanisms associated with the activation of voltage-dependent Ca^{2+} channels by NE.

In rMTC 6–23 cells, NE caused a doubling in $[Ca^{2+}]_i$, which was sustained for several minutes. This increase was completely reversed by addition of 1 μM nifedipine and was eliminated by removal of extracellular Ca^{2+} or pretreatment with nifedipine (data not shown). Release of intracellular Ca^{2+} stores by thapsigargin was unaffected by nifedipine, suggesting that Ca^{2+} release alone is not sufficient for activation of voltage-gated Ca^{2+} influx, an effect also seen in rat vascular smooth muscle cells (46). Also, the effect of NE on $[Ca^{2+}]_i$ was additive with that of thapsigargin, and only the NE-stimulated influx was reversed by nifedipine. These data suggest that the primary effect of NE in these cells is to increase Ca^{2+} influx through dihydropyridine-sensitive channels. There appears to be little or no effect of NE on release of stored Ca^{2+} in these cells.

The effects of NE were mimicked by addition of the voltage-dependent Ca^{2+} channel agonist Bay K8644, although there was no synergistic interaction between these compounds. Both depolarization with KCl and addition of ATP also caused large increases in $[Ca^{2+}]_i$, which were fully or partially blocked by nifedipine. These data suggest that rMTC 6–23 cells will be useful for studying the link between G protein-linked receptors and increases in voltage-gated Ca^{2+} influx.

The effect of NE on $[Ca^{2+}]_i$ in rMTC 6–23 cells appears to be mediated by an α_1-AR with pharmacological properties similar to those of the α_{1B} subtype. This is surprising, because the α_{1B} subtype is well known to increase $[Ca^{2+}]_i$ by activating Ins(1,4,5)P_3 formation and release of intracellular Ca^{2+} (13, 22–24, 47) but does not appear to do so through this pathway in these cells. Rather, α_{1B}-AR activation of rMTC 6–23 cells causes influx of Ca^{2+} through voltage-gated channels, an effect that has previously been associated primarily with the α_{1A} subtype (1, 14–18). These results are supported by a recent study (48) that showed that both α_{1B}- and α_{1D}-ARs expressed in COS-1 cells can increase arachidonic acid release through a nifedipine-sensitive mechanism.

Radioligand binding studies provided additional evidence for α_{1B}-like ARs in these cells. Most (85%) α_1-AR binding sites were eliminated by pretreatment with CEC for 10 min, and subtype-selective competitive antagonists all showed relatively low affinities, consistent with an α_{1B}-type pharmacology. Hill coefficients for inhibition of specific ^{125}I-BE binding by these selective drugs were generally close to 1.0, consistent with a pharmacologically homogeneous population of α_{1B}-like binding sites.

Because NE seemed to have little or no effect on intracellular Ca^{2+} stores in rMTC 6–23 cells, it was of interest to determine whether it could increase InsP formation in these cells. We

found that NE, epinephrine, and phenylephrine all stimulated substantial increases in formation of inositol-1-phosphate in the presence of LiCl, although another α_1-selective agonist, methoxamine, was much less effective. This effect was also mediated through an α_{1B}-like subtype, because it was blocked with very low affinity by the α_{1A}-selective antagonist (+)-niguldipine and was mostly inhibited by pretreatment with CEC. However, very high CEC concentrations (100 μM, for 30 min) were necessary to block the InsP responses, and only about 75% maximal inhibition of these responses could be achieved. This is in contrast to NB 41A3 cells, in which complete inhibition of NE-stimulated InsP formation was observed with 3 μM CEC pretreatment (24). This suggests that the response is not completely typical of the α_{1B} subtype.

Depolarization with KCl did not alter InsP formation, suggesting that this response is not subsequent to Ca^{2+} influx. Interestingly, ATP, which activates InsP formation in most cells through P_2 receptors and has very dramatic effects on Ca^{2+} influx in rMTC cells, did not activate InsP formation. This is additional evidence for the separation between the effects on InsP formation and Ca^{2+} influx through voltage-gated channels.

Northern blots of poly(A)$^+$ RNA from rMTC 6–23 cells with cDNAs for the three cloned α_1-AR subtypes showed clear evidence of the presence of transcripts for the α_{1B} and α_{1D} (but not α_{1C}) subtypes. The α_{1B} (2.2-kb) and α_{1D} (4.0-kb) transcripts were of different sizes, suggesting that they are not due to nonspecific hybridization, and the α_{1B} transcript was clearly present at a higher density. The presence of low levels of α_{1D} transcript is interesting, however, because this clone expresses primarily α_{1B}-type pharmacology but is less sensitive to CEC (10, 11). Contribution by the α_{1D} subtype might explain the inability of CEC pretreatment to completely inactivate NE-stimulated InsP formation. Potential interactions between these coexisting subtypes, leading to novel signaling responses, will be interesting to explore.

Because α_{1B}-ARs are known to stimulate phospholipase C activity, we wanted to determine whether this response contributed to voltage-gated Ca^{2+} influx. Because the entire NE-stimulated increase in Ca^{2+} in these cells was blocked by nifedipine, we found no evidence for release of stored Ca^{2+}. Also, release of intracellular Ca^{2+} by thapsigargin did not activate voltage-gated Ca^{2+} influx. We used the structurally related drugs U-73122 and U-73343, which have 10-fold or greater differences in potency as inhibitors of phospholipase C (39). The active compound U-73122 was about 10-fold more potent in inhibiting NE-stimulated InsP formation in rMTC 6–23 cells, with an IC_{50} of approximately 1 μM. Pretreatment of rMTC 6–23 cells with 10 μM U-73122 for 3 min had no effect on NE-stimulated Ca^{2+} influx, and addition of this compound after NE stimulation of $[Ca^{2+}]_i$ did not reverse the response (data not shown). Although it is impossible to directly compare NE-stimulated InsP formation and Ca^{2+} influx under the same conditions, these data further support the hypothesis that the two events are unrelated. Finally, phorbol esters that mimic diacylglycerol to activate protein kinase C are also known to have both inhibitory (49, 50) and stimulatory (51) effects on voltage-dependent Ca^{2+} channels in excitable cells and inhibitory effects on agonist-induced $[Ca^{2+}]_i$ responses in nonexcitable cells (52). We found that PMA increased $[Ca^{2+}]_i$ in rMTC 6–23 cells but that this response was not blocked by nifedipine. Thus, it seems unlikely that NE-induced stimulation of voltage-gated Ca^{2+} influx is due to protein kinase C activation.

In summary, we have identified a cell line in which stimulation of α_1-ARs activates nifedipine-sensitive, apparently voltage-gated, Ca^{2+} influx. This response appears to be mediated by primarily α_{1B}-like ARs and does not appear to be due to release of intracellular Ca^{2+} stores or activation of phospholipase C or protein kinase C. The presence of mRNA transcripts for both α_{1B}- and α_{1D}-ARs raises the interesting possibility that these two closely related subtypes may coexist in these cells and that such coexistence may be important in determining coupling specificity of these subtypes. Determination of the precise roles of each subtype and the coupling mechanisms involved in activation of voltage-sensitive Ca^{2+} influx should be greatly facilitated by the availability of this cell line. This cell line may also serve as a convenient transfection vehicle for monitoring the ability of different cloned α_1-AR subtypes to activate voltage-dependent Ca^{2+} channels.

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