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The α_{1C}-Adrenergic Receptor: Characterization of Signal Transduction Pathways and Mammalian Tissue Heterogeneity

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SUMMARY

We recently reported the cloning of a novel α_1-adrenergic receptor (AR), the α_{1C}AR. By transient and stable expression of the α_{1C}AR and the previously cloned α_{1B}AR in COS-7 and HeLa cells, respectively, we have now compared their ability to interact with major signal-transduction pathways (including polyphosphoinositide hydrolysis, intracellular calcium, and cAMP metabolism), as well as their mammalian tissue localization. Both α_{1C}- and α_{1B}ARs primarily couple to phospholipase C via a pertussis toxin-insensitive GTP-binding protein, leading to the release of calcium from intracellular stores. Even though α_{1C}- and α_{1B}ARs activate polyphosphoinositide hydrolysis by similar biochemical mechanisms, the α_{1C}AR couples to phospholipase C more efficiently than does the α_{1B}AR; activation of the α_{1C}AR results in a 2–3-fold greater increase in inositol phosphates, compared with the α_{1B}AR. Both α_1AR subtypes can also increase intracellular cAMP, by a mechanism that does not involve direct activation of adenylyl cyclase. In agreement with ligand binding data, the agonist methoxamine and the antagonist WB4101 are 10-fold more potent in activating or inhibiting, respectively, the ability of the α_{1C}AR to stimulate phospholipase C, compared with the α_{1B}AR. In addition, methoxamine is almost a full agonist at the α_{1C}AR, whereas it can only weakly activate the α_{1B}AR. Tissue localization, using Northern blot analysis of total and poly(A)$^+$-selected RNA from rabbit tissues, revealed striking mammalian species heterogeneity. As previously described, the α_{1B}AR is present in several rat tissues, including heart, liver, brain, kidney, lung, and spleen, whereas the α_{1C}AR is not present in any rat tissue studied. The α_{1B}AR is also present in rabbit aorta, heart, spleen, and kidney (and absent in rabbit liver), whereas the α_{1C}AR is present in rabbit liver. Our results indicate that the cloning and expression of different α_1AR subtypes represents a valuable tool to elucidate functional correlates of α_1AR heterogeneity.

Catecholamines such as NE and epinephrine mediate their effects by binding to different AR subtypes (α_1, α_2, β_1, β_2) (1, 2). These receptors are encoded by different genes and are members of the larger family of G protein-coupled receptors (3). Increasing heterogeneity of ARs has become apparent with the cloning of the genes encoding three β receptors (4–7), three α_2 receptors (8–11), and three α_1 receptors (12–14). Heterogeneity of α_1ARs (α_{1A}- and α_{1B}AR subtypes) was first suggested by pharmacological studies, based on differential sensitivity of α_1AR-mediated responses to a variety of agonists (e.g., oxymetazoline) and antagonists (e.g., WB4101 and phentolamine) and the alkylating agent CEC, as well as on different requirements of α_1AR-induced responses for extracellular calcium (15–21).

We previously reported the cloning of the cDNA that encodes the hamster α_1ARs purified from DDT$_1$-MF$_2$ cells (12). This receptor corresponds to the α_{1B}AR subtype, having relatively low affinity for the agonist oxymetazoline and antagonists WB4101 and phentolamine, as well as being totally inactivated by CEC. Recently, we reported the cloning of a cDNA that encodes a novel α_1AR subtype from bovine brain (13). Although pharmacologically similar to the α_{1A}AR subtype (relatively high affinity for the agonist oxymetazoline and antagonists WB4101 and phentolamine), this receptor is intermediate in sensitivity to CEC and is not expressed in rat tissues where the α_{1A}AR is

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ABBREVIATIONS: NE, norepinephrine; AR, adrenergic receptor; [^{125}I]HEAT, 2-{β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl}-tetralone; IP, inositol phosphate; IP$_{1(1)}$, inositol 1-monophosphate; IP$_{2(1,4)}$, inositol 1,4-bisphosphate; IP$_{3(1,3,4)}$, inositol 1,3,4-trisphosphate; IP$_{3(1,4,5)}$, inositol 1,4,5-trisphosphate; IP$_4$, inositol tetrakisphosphate; CEC, chlorethylclonidine; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; G protein, GTP-binding protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PI, polyphosphoinositide; PLC, phospholipase C; IBMX, 3-isobutyl-1-methylxanthine; fura-2/AM, fura-2/acetoxymethyl ester; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HPLC, high performance liquid chromatography.

found. Hence, this receptor has been designated the α_{1C}AR. In order to compare the functional properties of the α_{1C}AR with those of the previously cloned α_{1B}AR, we present here an analysis of the signal-transduction properties of both α_1AR subtypes stably expressed in HeLa cells and transiently expressed in COS-7 cells. In addition, we have explored expression of both the α_{1C}AR and α_{1B}AR in different rabbit tissues, by Northern analysis, and compared the results with those previously obtained in the rat (14).

Experimental Procedures

Materials

Drugs and reagents were obtained from the following sources: alprenolol, epinephrine, EDTA, EGTA, forskolin, IBMX, indomethacin, isoproterenol, methoxamine, nifedipine, nordihydroguaiaretic acid, NE, oxymetazoline, phenylephrine, prazosin, propranolol, and sodium fluoride, Sigma; 5-methylurapidil, Research Biochemical Inc.; fura-2/AM, Calbiochem; and pertussis toxin, List Biological Laboratories, Inc. (Campbell, CA). For drugs that exist as racemic mixtures, the (−)-isomer (or active isomer) was used unless otherwise indicated.

Methods

Cell culture. HeLa and COS-7 cells were grown as monolayers in DMEM (GIBCO) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in 5% CO_2 at 37°.

Expression of recombinant DNAs encoding α_1AR subtypes. The DNAs encoding the α_{1B}AR and α_{1C}AR were subcloned into the expression vector pBC12-BI (22), as previously described (12, 13), to obtain pBCα_{1B} and pBCα_{1C}, respectively. For stable expression of the α_1ARs, HeLa cells (23, 24) were cotransfected with pBCα_{1B} or pBCα_{1C} and pRSVNeo, using calcium phosphate precipitation (22). Clones resistant to the antibiotic G418 (0.8 mg/ml) were selected and tested for their ability to bind the α_1 antagonist [^{125}I]HEAT. Transient expression of the α_1AR in COS-7 cells was accomplished as previously described (12, 13).

Ligand binding. Membrane preparation of HeLa cells stably expressing the α_{1B}- and α_{1C}ARs and ligand binding assays using [^{125}I] HEAT were performed as described (13, 25). Prazosin (10^{-6} M) was used to determine nonspecific binding. For competition curves, a final [^{125}I]HEAT concentration of 80 pM was used. For saturation curve analysis, [^{125}I]HEAT concentrations ranged from 10 to 500 pM. Data were analyzed by computer, using an iterative nonlinear regression program (26).

IP determination in intact cells. HeLa or COS-7 cells expressing α_1AR subtypes, grown in 30-mm dishes (1×10^6 cells/dish), were labeled with [^{3}H]inositol (DuPont-New England Nuclear) for 16 hr at 2.5 μCi/ml in DMEM supplemented with 3.3% fetal calf serum (GIBCO). After labeling, cells were washed and incubated in PBS (no added calcium) for 30 min, followed by a 30-min incubation in PBS with 20 mM LiCl. IP were extracted as described by Martin (27) and were separated on AG 1-X8 columns (0.8 ml packed, 100–200 mesh) in the formate phase (28). Total IPs were eluted with 1 M ammonium formate/0.1 M formic acid. Analysis of individual IP isomers using HPLC was performed as previously described (29). For CEC experiments, CEC was added to the first PBS wash (30 min) and the cells were then rinsed three times with PBS before the procedure was continued as described above.

PLC assay in membranes. HeLa cells expressing the α_{1C}AR and α_{1B}AR were grown in 75-cm^2 flasks (10×10^6 cells/flask) and were labeled with [^{3}H]inositol for 48 hr at 20 μCi/ml, in DMEM supplemented with 5% fetal calf serum. After labeling, the cells were washed in PBS containing 1 mM EGTA and were scraped with a rubber policeman, and membranes were prepared according to the method of Okajima *et al.* (30). These membranes (≈100 μg of protein), containing about 5×10^5 dpm, were incubated for 10 min with various agents at 37°, in a volume of 200 μl. The reaction was terminated with 200 μl of 0.8 M perchloric acid, and IPs were extracted as described by Martin (27). The IPs were then separated on AG1-X8 columns (28). Ten milliliters of 0.2 M ammonium formate/0.1 M formic acid were added, the eluate was discarded, and the fraction corresponding to IP_2 and IP_3 (28) was eluted with 3.5 ml of 1 M ammonium formate/0.1 M formic acid.

Cytosolic calcium. HeLa cells expressing α_1AR subtypes were grown to confluence on glass coverslips and loaded for 1 hr at 37° with 20 μM fura-2/AM, in Hanks' balanced salt solution containing 0.01% albumin and 10 mM HEPES, at pH 7.4. Coverslips were placed into a temperature-controlled cuvette and continuously superfused at 37° with Hanks' balanced salt solution. The cuvette was placed into a heated cuvette holder of a spectrofluorimeter (Kontron SFM 25), and the emission was monitored at 510 nm over the excitation spectrum from 310 to 410 nm. Excitation wavelengths were alternated by a microcomputer (5–6 sec/cycle), and the emission data ratio (350/380 nm excitation) was calculated. Cytosolic Ca^{2+} concentration was calculated as previously described (31).

cAMP and adenylyl cyclase assays. cAMP assays were performed using the Rianen radioimmunoassay system (DuPont-New England Nuclear), according to the manufacturers instructions. HeLa or COS-7 cells expressing the α_1AR subtype, grown in 30-mm dishes (1×10^6 cells/dish), were incubated at 37° for 30 min in serum-free DMEM with 20 mM HEPES, pH 7.5, and 0.5 mM IBMX. Alprenolol (10^{-5} M) was included to block endogenous βARs present in the cells. After a 10-min incubation at 37° with different drugs, the supernatant was aspirated and 100 mM HCl was added. Aliquots were taken and assayed for cAMP.

For adenylyl cyclase assays, HeLa cells stably expressing either the α_{1B}- or α_{1C}AR subtype, grown in 75-cm^2 flasks, were rinsed with PBS and incubated in the flask with 10 ml of serum-free DMEM for 30 min, followed by a second rinse with ice-cold PBS. Membrane preparation and adenylyl cyclase activity were measured as previously described (25). Incubation was at 37° for 30 min in 30 mM Tris, 5 mM $MgCl_2$, 0.8 mM EDTA, alprenolol (10^{-5} M). The reaction was stopped with the addition of 1 ml of 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (25,000 cpm) per assay tube. [α-^{32}P]cAMP was isolated using Dowex and alumina columns, as previously described (32).

Northern analysis. Isolation of total RNA from rabbit tissues, preparation of poly(A)$^+$-selected RNA, and Northern analysis were performed as previously described (13, 14).

Statistics. Analysis of ligand binding was determined by nonlinear least squares regression analysis (LIGAND) (26). Statistical comparisons were performed using paired or unpaired Student *t* test as appropriate.

Results

Permanent expression of α_1AR subtypes. HeLa cells were chosen for expression of α_1AR subtypes because of the ease of transfection and their lack of endogenous α_1ARs. Two clonal cell lines stably expressing either the α_{1B}- or α_{1C}AR subtype were chosen and characterized by ligand binding. Saturation analysis of the binding of the α_1AR antagonist [^{125}I] HEAT gave receptor densities of 1500 and 700 fmol/mg of protein for the α_{1B}- and α_{1C}AR, respectively, with similar affinities ($K_D = 7 \times 10^{-11}$ M). Analysis of competition studies with selected ligands (Table 1) showed that the α_{1C} receptor subtype has higher affinity for the agonists methoxamine and oxymetazoline, as well as the antagonist WB4101. These results are consistent with previous data obtained after transient expression of these receptors in COS-7 cells (12, 13). In addition, the antagonist 5-methylurapidil, which has been described to be selective for the α_{1A}AR subtype, has 50-fold higher affinity for the α_{1C}- than the α_{1B}AR (Table 1). These results confirm that

TABLE 1

Pharmacological profile of α_{1C}- and α_{1B}ARs expressed in HeLa cells

Competition by selected agonists and antagonists for the binding of [^{125}I]HEAT to membranes prepared from HeLa cells expressing the α_{1C}- and the α_{1B}AR subtypes was measured. Each concentration of ligand was tested in triplicate; (−)-isomers were used. Ratios (α_{1B}/α_{1C}) are rounded to the nearest digit. Results shown are the mean of two to four experiments, which agreed within 10%.

	K_i		Ratio (B/C)
	α_{1C}	α_{1B}	
	nM		
Agonist			
Oxymetazoline	13	190	14
Epinephrine	6,400	1,800	0.3
NE	17,200	2,900	0.2
Phenylephrine	12,600	13,400	1
Methoxamine	85,300	527,000	6
Antagonist			
Prazosin	0.06	0.11	2
WB4101	0.70	12	17
5-Methylurapidil	6.8	340	50

TABLE 2

Stimulation of PI hydrolysis via α_1AR subtypes in HeLa cells

HeLa cells expressing the α_{1B}AR and α_{1C}AR were labeled with [^{3}H]inositol (2.5 μCi/ml) as described in Experimental Procedures and were stimulated with different concentrations of agonist for 20 min. Increase (%) indicates the percentage increase of total IPs over basal induced by 10^{-3} M agonist. The ratio of the percentage increase determined with different agonists to that with NE is indicated. Values are mean ± standard error; *n* represents the number of dose-response curves done in triplicate.

Drug	EC_{50}	Increase	Ratio to NE	*n*
	μM	%		
α_{1C}AR				
NE	22 ± 5.8[a]	700 ± 97[b]	1.00	11
Epinephrine	5.8 ± 4.1	740 ± 10[c]	1.06	5
Methoxamine	180 ± 29	490 ± 42[b]	0.70	6
Oxymetazoline	7.4 ± 5.7	130 ± 42[a]	0.18	3
Phenylephrine	64 ± 1.9[c]	550 ± 25[c]	0.78	2
α_{1B}AR				
NE	7.4 ± 3.1	200 ± 34	1.00	11
Epinephrine	1.9 ± 1.4	290 ± 25	1.45	5
Methoxamine	86 ± 29	42 ± 12	0.21	6
Oxymetazoline	5.5 ± 5.2	40 ± 26	0.20	3
Phenylephrine	19 ± 0.1	150 ± 25	0.74	2

[a] $p < 0.05$; compared with the α_{1B}AR.
[b] $p < 0.001$.
[c] $p < 0.01$.

the α_{1C}AR has pharmacological properties very similar to those described for the α_{1A} subtype.

Selectivity of agonists and antagonists in α_1AR subtype-mediated activation of phosphoinositide metabolism. In order to ensure that the two HeLa cell lines stably expressing the α_{1C}AR and α_{1B}AR subtypes had similar metabolic properties, we compared the ability of histamine to stimulate PLC via endogenous histamine receptors in the two cell lines. Stimulation of HeLa cells with histamine (10^{-4} M) for 20 min resulted in ≈35% increase of IPs in both cell lines (data not shown).

Table 2 shows that stimulation of HeLa cells, expressing either the α_{1C}AR or α_{1B}AR subtype, with a variety of agonists results in the activation of PI hydrolysis, indicating that both α_1AR subtypes are coupled to PLC activation. Maximal increase of agonist-induced IP release was 2–3-fold greater for the α_{1C}AR than the α_{1B}AR for every agonist studied. These results indicate that the α_{1C}AR is coupled to PLC with greater efficiency than the α_{1B}AR. Similar results were obtained in COS-7 cells expressing α_1AR subtypes, where stimulation of the α_{1C}AR with NE (10^{-4} M) for 30 min resulted in a greater (2-fold) increase of IPs, compared with the α_{1B}AR (data not shown). Despite the larger IP release induced by the activation of the α_{1C}AR, compared with the α_{1B}AR, the ratios of different agonist-induced PI hydrolysis, compared with that induced by NE, are similar for the two receptors, with the exception of methoxamine. In fact, methoxamine is only a weak partial agonist at the α_{1B}AR, whereas it is almost a full agonist at the α_{1C}AR. Oxymetazoline is a weak partial agonist at both receptors. EC_{50} values (Table 2) for activation of PI metabolism by different agonists were similar between α_1AR subtypes, with the exception of a slightly lower EC_{50} values for NE and phenylephrine for the α_{1B}AR.

In order to assess the effect of α_1AR antagonists on α_1AR-mediated activation of PI hydrolysis, HeLa cells expressing the α_{1C}AR and α_{1B}AR, respectively, were treated with different concentrations of prazosin and WB4101. Dose-response curves of NE for stimulation of IP release were generated in the absence and presence of varying concentrations of antagonists. Using Schild plot analysis (33, 34), pA_2 values for prazosin and WB4101 were 0.1 and 6 nM for the α_{1B}AR and 0.1 and 0.9 nM for the α_{1C}AR, respectively (mean of two experiments, which agree within 10%). In agreement with our previous binding data, these results indicate that WB4101 is approximately 10-fold more potent in antagonizing the effect of NE at the α_{1C}AR than at the α_{1B}AR, whereas prazosin is equally potent at the two receptors.

We have previously reported that the α_{1C}AR is less sensitive to the alkylating clonidine derivative CEC than is the α_{1B}AR. In order to explore the effect of CEC on α_1AR subtype-mediated activation of PI hydrolysis, HeLa cells expressing either α_{1B}- or α_{1C}AR were treated with 100 μM CEC for 30 min at 37° and then washed four times with PBS before measurement of IPs. CEC inhibited NE-activated PI hydrolysis by 77 ± 3% at the α_{1B}AR and 39 ± 3% at the α_{1C}AR. Binding of [^{125}I]HEAT was measured in membranes derived from HeLa cells treated with CEC, under identical conditions as used for PI hydrolysis. CEC inactivated 88 ± 0.2% of α_{1B}AR and 32 ± 3% of α_{1C}AR binding. This effect of CEC is smaller than the 95% and 68% inhibition of α_{1B}AR and α_{1C}AR previously reported after treatment of COS membranes with CEC (13). This discrepancy may reflect differences in the conditions of CEC treatment in whole cells versus membrane preparations. However, these results confirm that the sensitivity of the α_{1C}AR to CEC is intermediate between that described for the α_{1B}- and α_{1A}AR.

Kinetic analysis of IP isomer accumulation via α_1AR subtypes. In order to characterize the IP isomers released after α_1AR subtype activation, HeLa cells stably expressing the α_{1C}- and α_{1B}AR were stimulated with NE (10^{-4} M), and the IPs generated were separated by HPLC. Table 3 shows that within 10 sec $IP_{1,4,5}$ is the major product resulting from NE stimulation of both α_{1C}- and α_{1B}ARs. This result indicates that both receptors primarily activate phosphatidylinositolbisphosphate-specific PLC in HeLa cells. After 1, 5, and 20 min of stimulation with NE, $IP_{(1,4,5)}$ levels decrease, while the levels of $IP_{1(1)}$, $IP_{2(1,4)}$, $IP_{3(1,3,4)}$, and IP_4 progressively increase. The pattern of IPs generated after NE stimulation is very similar between the two α_1AR subtypes. Similar results were also obtained in COS-7 cells expressing the α_{1C}AR and α_{1B}AR, where stimulation with NE for 30 sec and 5 min resulted in a pattern of IPs that was

TABLE 3

HPLC analysis of IP isomers released via α_1AR subtypes

HeLa cells expressing each α_1AR subtype were labeled with [^{3}H]inositol (20 μCi/ml) as described and were incubated with NE (10^{-4} M) for varying time periods. IP isomers were analyzed by HPLC as described (29). Results from a representative experiment done in triplicate are shown (two total experiments). Numbers represent the area under each radioactive HPLC peak corresponding to different IP isomers. The IP_4 peak was broad and, therefore, the specific IP_4 isomers cannot be identified with certainty.

	Isomer				
	$IP_{1(1)}$	$IP_{2(1,4)}$	$IP_{3(1,3,4)}$	$IP_{3(1,4,5)}$	IP_4
	arc cpm × 10^{-3}*a*				
α_{1C}AR					
10 sec	0.29	2.04	0	3.10	0.12
1 min	0.34	6.25	0.27	2.42	0.49
5 min	7.90	19.31	3.16	0.58	1.03
20 min	37.51	26.93	6.03	0.91	1.14
α_{1B}AR					
10 sec	0	0.24	0	0.53	0
1 min	0.18	1.78	0.14	0.36	0.21
5 min	2.29	4.46	1.12	0	0.19
20 min	17.15	6.80	2.53	0.22	0.59

similar for the two α_1AR subtypes (data not shown). These results indicate that the α_{1C}AR and α_{1B}AR, when compared in HeLa or COS-7 cells, activate the same PLC or different phospholipases with very similar phospholipid specificities. However, as previously observed with the total IP content, stimulation of the α_{1C}AR results in a 2–3-fold greater increase of each IP isomer, compared with the α_{1B}AR.

Effect of extracellular calcium on α_1AR subtype-mediated activation of PLC. In order to identify potential differences in the biochemical mechanism of α_{1C}AR- and α_{1B}AR-mediated activation of PLC, we investigated the effect of extracellular calcium on α_1AR-induced PI hydrolysis. In fact, activation of PLC may also occur as a consequence of raising intracellular calcium by increasing its influx (35). In the experiments previously described, IP release was measured in HeLa cells incubated in PBS without the addition of calcium. Incubation of HeLa cells for 30 min with EGTA at concentrations of 0.2, 1, and 2 mM did not affect NE-induced stimulation of PI hydrolysis mediated by either the α_{1C}AR or α_{1B}AR (data not shown). When HeLa cells were incubated in the presence of 0.9 mM $CaCl_2$, stimulation with NE (10^{-4} M) for 20 min resulted in 2-fold greater release of total IPs for both the α_{1C}AR and α_{1B}AR, compared with the absence of extracellular calcium (Fig. 1). Such an effect of Ca^{2+} was only observed after 5- and 20-min stimulation with NE, whereas the amount of individual IP isomers released at shorter times of stimulation with NE (5, 10, 30, and 60 sec) was similar in the absence or presence of extracellular Ca^{2+} (data not shown). The effect of calcium on NE-induced PI hydrolysis was not blocked by nifedipine (1 μM), ruling out a role of dihydropyridine-sensitive calcium channels in both α_{1C}- and α_{1B}-mediated responses in HeLa cells (data not shown). These results suggest a bimodal requirement for cytosolic calcium by PLC; first, the physiological intracellular Ca^{2+} is sufficient for initial PLC activation and, second, entry of extracellular Ca^{2+} may play a permissive role in long term activation of PLC (36).

Role of a G protein in α_1AR subtype-mediated activation of PLC. In order to demonstrate that both α_{1C} and α_{1B} activate PI metabolism via a G protein, receptor-mediated activation of PLC was measured in a cell-free membrane preparation from HeLa cells expressing either α_{1B}- or α_{1C}ARs. Incubation of membranes derived from HeLa cells with increasing concentrations of free calcium resulted in the increase of IP_2 and IP_3, with an EC_{50} of calcium of about 50 μM (data not shown). These results indicated that a functional PLC activity was present in membranes from HeLa cells and it was calcium dependent. A physiological concentration of free calcium (50 nM) did not produce any appreciable formation of IP_2 and IP_3 above basal levels. In the presence of this concentration of calcium, GTPγS activated PLC in a dose-dependent manner in membranes derived from HeLa cells expressing the α_{1C}- and α_{1B}AR (Fig. 2). In the absence of GTPγS, NE did not increase IP release. However, NE was able to potentiate the effect of GTPγS on PLC activation, thus shifting the dose-response of GTPγS to the left by approximately 1 order of magnitude (Fig. 2). These results indicate that both the α_{1C}AR and α_{1B}AR require the intermediary role of a G protein in order to activate PLC.

In order to characterize the G protein involved in α_1AR-mediated activation of PLC, HeLa cells expressing either the α_{1C}- or α_{1B}AR were treated with increasing concentrations (10–1000 ng/ml) of pertussis toxin for 18 hr. This treatment had no effect on α_1AR-mediated activation of PI metabolism (data not shown). Similar results were also obtained in COS-7 cells transiently expressing the α_{1C}- or α_{1B}AR (data not shown). These results indicate that the G protein that mediates the effect of both α_1AR subtypes on PLC activation is pertussis toxin insensitive.

Measurement of cytosolic calcium. Fig. 3 shows that treatment of HeLa cells expressing the α_{1B}AR (Fig. 3A) and

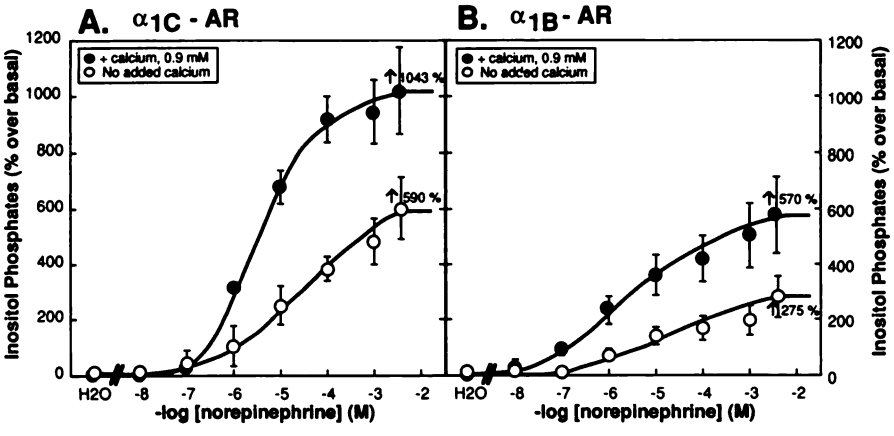

Fig. 1. Role of extracellular calcium in NE-stimulated PI hydrolysis via α_1AR subtypes. HeLa cells stably expressing either the α_{1C}- or the α_{1B}AR (A and B, respectively) were incubated in the presence of varying concentrations of NE for 20 min, in the absence or presence of added extracellular Ca^{2+} (0.9 mM). Mean values of several experiments (three to six), done in triplicate, are shown.

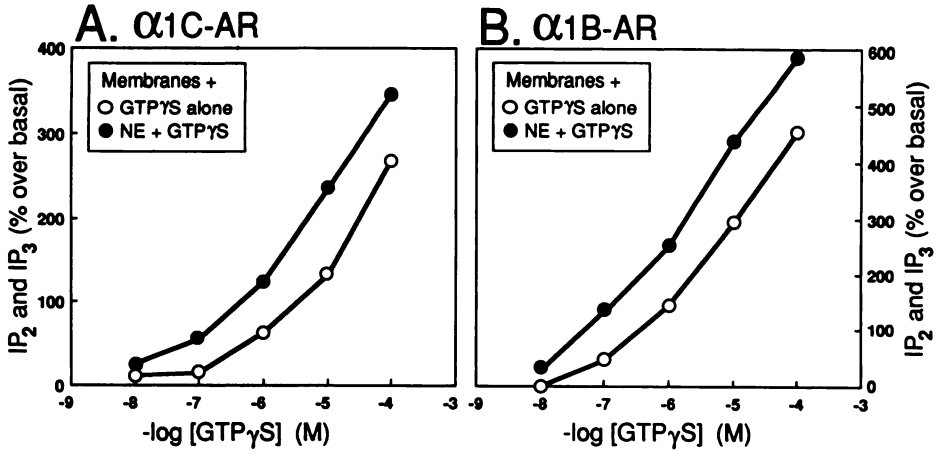

Fig. 2. Activation of PLC in membranes via α_1AR subtypes. HeLa cells stably expressing the α_{1C}- or the α_{1B}AR were labeled with [^{3}H] inositol, and membranes were prepared as described in Experimental Procedures. ^{3}H-labeled membranes were incubated with increasing concentrations of GTPγS, in the absence (○) or presence (●) of 10^{-4} M NE, for 20 min. The concentration of free calcium was 50 nM, as calculated by a computer program.

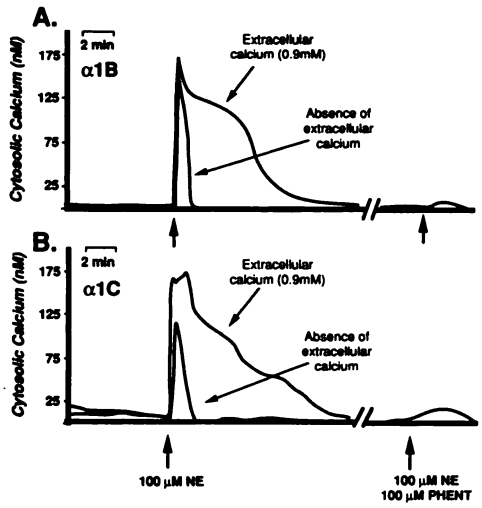

Fig. 3. Cytosolic calcium response to NE in HeLa cells stably expressing individual α_1AR subtypes. Cells expressing the α_{1C}AR (A) and the α_{1B}AR (B) were loaded with fura-2/AM, and cytosolic calcium was determined with a spectrofluorimeter, using the ratio of emission (510 nm) after excitation at 350 and 385 nm, as described in the text. The *traces* represent the net increase of calcium concentration above basal induced by 100 μM NE, in the presence and absence of 0.9 mM extracellular calcium. The response to NE in cells pretreated with 100 μM phentolamine (*PHENT*) is also indicated.

α_{1C}AR (Fig. 3B) with 100 μM NE in the presence of extracellular calcium resulted in a rapid increase in cytosolic calcium. The concentration of intracellular calcium in the presence of NE was increased by 107.9 ± 25.9 nM and 114.8 ± 19.4 nM above basal levels in HeLa cells expressing the α_{1C}AR and α_{1B}AR, respectively (mean ± standard error; four or five experiments). This early calcium peak response was followed by a plateau and then slowly declined towards basal levels within 10–15 min. The responses were α_1-mediated, as shown by the fact that the effect of NE was abolished by pretreatment of the cells with 100 μM phentolamine. The effect of NE was then studied in calcium-free medium, a condition in which calcium release from intracellular stores is maintained while calcium influx is no longer possible. Under these conditions (Fig. 3), the early calcium peak response to NE was not significantly changed, whereas the plateau phase was abolished, resulting in the return of the calcium concentration to basal levels within 2 min. As previously shown for several other PLC-coupled receptors, the early calcium response to NE might result from calcium release from intracellular stores mediated by IP_3 produced after α_1AR-mediated activation of PLC (36). On the other hand, the plateau phase of calcium increase seems to be dependent on calcium influx from the extracellular compartment. The entry of calcium across the plasma membrane has been proposed to be regulated by a receptor-operated channel in one case (37) and by second messengers, such as calcium itself or the phosphorylated products of IP_3, in other cases (36, 38).

Treatment of HeLa cells expressing α_1AR subtypes with nifedipine (10 μM) did not change the intracellular calcium response in the presence of extracellular calcium, ruling out a role of nifedipine-sensitive calcium channels in α_1AR-mediated calcium mobilization in HeLa cells. In addition, electrophysiological measurements of plasma membrane conductance did not provide any convincing evidence in favor of α_1AR-mediated calcium channel activity in HeLa cells.[1] In conclusion, our results indicate that the cytosolic calcium responses to α_{1C}AR and α_{1B}AR are similar and are contributed to by both intra- and extracellular stores of calcium.

Effects of α_{1C}- and α_{1B}AR on cAMP metabolism. Recently it has been shown that agonist stimulation of the α_{1B}AR increased cAMP levels in COS-7 cells transiently expressing the α_{1B}AR (25). In order to compare the α_{1C}AR-mediated effect on cAMP metabolism with that mediated by the α_{1B}AR, cAMP levels were measured in COS-7 and HeLa cells transiently and stably expressing α_{1C}- and α_{1B}ARs, respectively. Stimulation with NE (100 μM) of COS-7 cells transiently expressing the α_{1C}AR and α_{1B}AR induced a 2-fold increase of cAMP levels for each receptor subtype (Fig. 4). In HeLa cells stably expressing each α_1AR subtype, an approximately 20% increase of cAMP levels was observed after stimulation with NE (data not shown). In both COS-7 and HeLa cells expressing α_{1C}AR and α_{1B}AR subtypes, prazosin (1–10 μM) completely abolished the effect of NE on cAMP metabolism. When adenylyl cyclase activity was measured in membranes derived from COS-7 or HeLa cells expressing each α_1AR subtype, NE did not have any effect on cAMP production via either the α_{1C}AR or the α_{1B}AR subtype. These findings suggest that, as already proposed for the α_{1B}AR (25), the α_{1C}AR can regulate cAMP metabolism via an indirect mechanism that does not involve direct activation of adenylyl cyclase.

Distribution of α_{1C}- and α_{1B}AR in mammalian tissue. We previously reported (14) that the α_{1B}AR is expressed in

[1]J. Rendt and G. Oxford, Department of Physiology, University of North Carolina at Chapel Hill, personal communication.

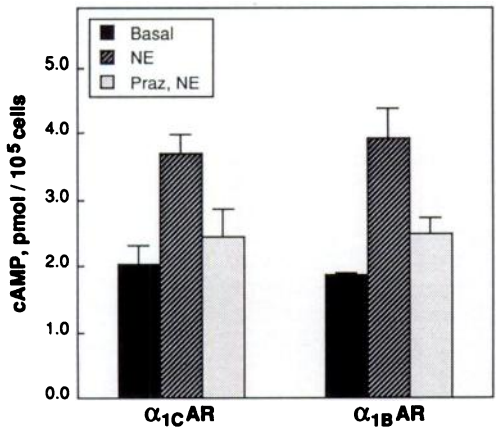

Fig. 4. Effects of α_1AR subtypes on cAMP metabolism in COS-7 cells. COS-7 cells transiently expressing either the α_{1C}AR (256 fmol/mg of protein) or α_{1B}AR (462 fmol/mg of protein) were stimulated with 100 μM NE, in the absence or presence of 1 μM prazosin (*Praz*). Means ± standard errors of three experiments are shown.

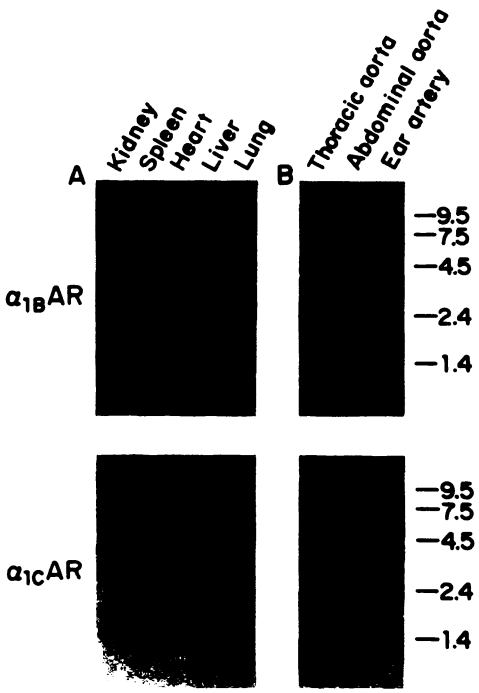

Fig. 5. Tissue distribution of α_1AR subtypes in the rabbit. Northern analysis of total and poly(A)$^+$-selected mRNA was performed by hybridizing radioactively labeled probes from either the α_{1B}AR or α_{1C}AR to various rabbit tissues. A, Poly(A)$^+$-selected mRNA from rabbit kidney, spleen, heart, liver, and lung (10 μg each), hybridized with α_1AR subtype-selective probes (24-hr exposure). B, Total mRNA from rabbit thoracic aorta (20 μg), abdominal aorta (5 μg), and ear artery (5 μg), hybridized with α_1AR subtype-selective probes (5-day exposure). *Top* and *bottom*, rehybridization of the same blot.

several rat tissues, including heart, liver, brain, kidney, lung, and spleen, whereas the α_{1C}AR is not present in any rat tissue studied. In order to assess whether the tissue distribution of the α_1AR subtypes might vary among mammalian species, Northern analysis of total and poly(A)$^+$-selected RNA from various rabbit tissues was performed and compared with previous results obtained in the rat. Fig. 5 shows that the α_{1B}AR is expressed in several rabbit tissues, including heart, spleen, kidney, thoracic aorta, abdominal aorta, and ear artery. The α_{1C}AR did not hybridize to the RNA of any of these tissues, whereas it was expressed in rabbit liver, where the RNA for the α_{1B}AR was not found (Fig. 5). A DNA probe derived from the rat α_{1A}AR (14) did not hybridize to the RNA from any of these rabbit tissues (data not shown). These data indicate that the distribution of different α_1AR subtypes varies among different tissues, as well as different mammalian species.

Discussion

The cloning of different AR subtypes has provided new approaches for studying functional properties of ARs. Recently, we described the cloning of the α_{1C}AR, a novel α_1AR subtype that differs from previously described α_{1B}- and α_{1A}AR subtypes in pharmacological properties as well as tissue distribution in the rat (13). Now, utilizing transient and stable expression of recombinant DNAs encoding the α_{1C}AR and α_{1B}AR, we examined the ability of each receptor subtype to interact with major signal transduction pathways involving PI hydrolysis, intracellular calcium, and cAMP metabolism. In addition, we compared the distribution of α_1AR subtypes in two mammalian species (rat and rabbit). We report that the newly discovered α_{1C}AR primarily activates PLC via a pertussis toxin-insensitive G protein, similarly to the α_{1B}AR. However, differences in the potency ratio of agonists and antagonists, as well as in efficiency of receptor coupling to PLC, exist between the two α_1AR subtypes. Intriguing differences are also apparent in tissue distribution of α_1AR subtypes in rat and rabbit.

As previously reported after transient expression in COS-7 cells (13), the α_{1C}AR stably expressed in HeLa cells is characterized by higher affinity for the agonists methoxamine and oxymetazoline and the antagonist WB4101, compared with the α_{1B}AR. In addition, the α_{1C}AR has higher affinity for the α_1AR antagonist 5-methylurapidil. In agreement with ligand binding data, methoxamine and WB4101 are 10-fold more potent in activating or inhibiting, respectively, the ability of the α_{1C}AR to stimulate PLC, compared with the α_{1B}AR. In addition, methoxamine can almost fully activate the α_{1C}AR, whereas it is only a weak agonist at the α_{1B}AR; oxymetazoline is a weak agonist at both receptor subtypes. The alkylating agent CEC inhibits α_{1B}AR binding almost completely and α_{1C}AR binding partially; inhibition of α_1AR subtype stimulation of PLC by CEC parallels binding. Because in this study we used clonal HeLa cells expressing similar numbers of each α_1AR subtype (and also repeated each experiment using lower and exactly equal receptor numbers, with comparable results), the different efficacy of methoxamine at the α_{1C} and α_{1B}AR is not due to a difference in receptor number but, rather, reflects a true difference in intrinsic activity of methoxamine at the two α_1AR subtypes.

Differing efficacy values for various α_1AR agonists have been described in a variety of tissues; these findings have often been interpreted as favoring the existence of distinct receptor subtypes in different tissues (19). For example, a previous report by Tsujimoto *et al.* (39) indicated that methoxamine is a weak α_1AR agonist in rat hepatocytes while being much more efficacious in rabbit aorta, suggesting that rat hepatocytes and rabbit aorta express different α_1AR subtypes. By Northern analysis, we observe that the α_1AR expressed in both rat liver and rabbit aorta is predominantly the α_{1B}AR subtype. This finding seems to indicate that differences in α_1AR-mediated responses in rat liver and rabbit aorta might be due to tissue-

specific differences rather than different receptor subtypes. In fact, several factors, including receptor "reserve" and tissue G protein composition, might influence receptor-mediated responses in different tissues (19, 40). Hence, in light of such tissue variability, the study of recombinant receptor subtypes in the same cellular system represents a valuable approach in assessing pharmacological and functional properties of distinct receptor subtypes.

Activation of α_1ARs increases PI hydrolysis in almost every tissue studied so far. However, different sensitivity to extracellular calcium has been reported for α_1AR-mediated responses in a variety of tissues (19, 20). These observations suggested that distinct α_1AR subtypes (specifically the α_{1A}AR subtype) might activate different biochemical pathways, including the regulation of calcium influx (39). Our present data indicate that both the α_{1C}AR and α_{1B}AR expressed in HeLa cells predominantly activate PLC without any direct effect on calcium influx. Activation of PLC is mediated by a pertussis toxin-insensitive G protein for both the α_{1C}- and α_{1B}AR, as shown by the fact that stimulation of both α_1AR subtypes with agonist can activate PLC in a cell-free system only in the presence of guanine nucleotide analogues. In addition, α_{1C}- and α_{1B}AR subtypes seem to activate the same PLC or phospholipases with similar substrate specificity. In fact, the pattern of IPs produced by α_1AR activation is similar between the two α_1AR subtypes when the α_{1C}- and α_{1B}AR-mediated responses are compared in HeLa or COS-7 cells. In addition to coupling to PLC, activation of both the α_{1C}- and α_{1B}AR results in the rapid release of calcium from intracellular stores, resulting in increased cytosolic calcium concentrations. As previously shown for the α_{1B}AR (25), we demonstrate that stimulation of the α_{1C}AR can also increase intracellular cAMP levels (Fig. 4) by an indirect mechanism. Hence, α_{1C}- and α_{1B}AR subtypes appear to couple to similar signal transduction systems.

Although both α_1AR subtypes couple to PLC, the α_{1C}AR couples to PLC with greater efficiency than the α_{1B}AR, because its activation results in 2–3-fold greater increase of IPs, compared with the α_{1B}AR. The greater coupling efficiency to PLC of the α_{1C}AR, compared with the α_{1B}AR, has no obvious explanation. This difference does not seem to be dependent on receptor number or extracellular calcium. Structural differences in the domains of each α_1AR subtype interacting with the G protein-effector system might result in different efficiency of receptor-G protein coupling. Alternatively, other biochemical events in whole cells might influence and differentiate functional responses mediated by the two α_1AR subtypes.

Although α_{1C}- and α_{1B}ARs couple to similar second messenger pathways (albeit with differences in potency and efficacy), these receptor subtypes clearly differ in tissue expression in interesting ways. The presence of α_{1B}ARs in rat liver but not in rabbit liver, the absence of α_{1B}ARs in rat aorta but presence in rabbit aorta, and the absence of α_{1C}ARs in all rat tissues studied but presence in rabbit liver bring into question basic assumptions about receptor distribution between species. Clearly, in the case of α_1AR subtypes, extrapolation of receptor subtype tissue specificity from the rat to other mammalian species may not be valid. This caveat may have important implications for studying cardiovascular disease states and therapeutic drug development in humans. Hence, discovery of species variation in AR tissue specificity further illustrates the power of molecular genetic approaches in discovering and characterizing individual AR subtypes.

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References

1. Ahlquist, R. P. A study of the adrenotropic receptors. *Am. J. Physiol.* **153**:586–600 (1948).
2. Lefkowitz, R. J., and M. G. Caron. Adrenergic receptors: models for the study of receptors coupled to guanine nucleotide regulatory proteins. *J. Biol. Chem.* **263**:4993–4996 (1988).
3. Gilman, A. G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–649 (1987).
4. Dixon, R. A. F., B. K. Kobilka, D. J. Strader, J. L. Benovic, H. G. Dohlman, T. Frielle, M. A. Bolanowski, C. D. Bennett, E. Rands, R. E. Diehl, R. A. Mumford, E. E. Slater, I. S. Sigal, M. G. Caron, R. J. Lefkowitz, and C. D. Strader. Cloning of the gene and cDNA for mammalian β-adrenergic receptor and homology with rhodopsin. *Nature* (*Lond.*) **321**:75–79 (1986).
5. Kobilka, B. K., R. A. F. Dixon, T. Frielle, H. G. Dohlman, M. A. Bolanowski, I. S. Sigal, T. L. Yang-Feng, U. Francke, M. G. Caron, and R. J. Lefkowitz. cDNA for the human β_2-adrenergic receptor: a protein with multiple membrane spanning domains and a chromosomal location shared with the PDGF receptor gene. *Proc. Natl. Acad. Sci. USA* **84**:46–50 (1987).
6. Frielle, T., S. Collins, K. W. Daniel, M. G. Caron, R. J. Lefkowitz, and B. K. Kobilka. Cloning of the cDNA for the human β_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**:7920–7924 (1987).
7. Emorine, L. J., S. Marullo, M. M. Briend-Sutren, G. Patey, K. Tate, C. Delavier-Klutchko, and A. D. Strosberg. Molecular characterization of the human *beta*3-adrenergic receptor. *Science* (*Washington D. C.*) **245**:1118–1121 (1989).
8. Kobilka, B. K., H. Matsui, T. S. Kobilka, T. L. Yang-Feng, U. Francke, M. G. Caron, R. J. Lefkowitz, and J. W. Regan. Cloning, sequencing, and expression of the gene coding for human platelet α_2-adrenergic receptor. *Science* (*Washington D. C.*) **238**:650–656 (1987).
9. Regan, J. W., T. S. Kobilka, T. L. Yang-Feng, M. G. Caron, R. J. Lefkowitz, and B. K. Kobilka. Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype. *Proc. Natl. Acad. Sci. USA* **85**:6301–6305 (1988).
10. Zeng, D., J. K. Harrison, D. D. D'Angelo, C. M. Barber, A. L. Tucker, L. Zhihong, and K. R. Lynch. Molecular characterization of a rat α_{2B}-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **87**:3102–3106 (1990).
11. Lomasney, J. W., W. Lorenz, L. F. Allen, K. King, J. W. Regan, T. L. Yang-Feng, M. G. Caron, and R. J. Lefkowitz. Expansion of the α_2-adrenergic receptor family: cloning and characterization of a human α_2-adrenergic receptor, the gene for which is located on chromosome 2. *Proc. Natl. Acad. Sci. USA* **87**:5094–5098 (1990).
12. Cotecchia, S., D. A. Schwinn, R. R. Randall, R. J. Lefkowitz, M. G. Caron, and B. K. Kobilka. Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **85**:7159–7163 (1988).
13. Schwinn, D. A., J. W. Lomasney, W. Lorenz, P. J. Szklut, R. T. Fremeau, Jr., T. L. Yang-Feng, M. G. Caron, R. J. Lefkowitz, and S. Cotecchia. Molecular cloning and expression of the cDNA for a novel α_1-adrenergic receptor subtype. *J. Biol. Chem.* **265**:8183–8189 (1990).
14. Lomasney, J. W., S. Cotecchia, W. Lorenz, W.-Y. Leung, D. A. Schwinn, T. L. Yang-Feng, M. Brownstein, R. J. Lefkowitz, and M. G. Caron. Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor, the gene for which is located on human chromosome 5. *J. Biol. Chem.* **266**:6365–6369 (1991).
15. Morrow, A. L., and I. Creese. Characterization of α_1-adrenergic receptor subtypes in rat brain: a reevaluation of [^{3}H]WB4101 and [^{3}H]prazosin binding. *Mol. Pharmacol.* **29**:321–330 (1986).
16. Han, C., P. W. Abel, and K. P. Minneman. Heterogeneity of α_1-adrenergic receptors revealed by chlorethylclonidine. *Mol. Pharmacol.* **32**:505–510 (1987).
17. Minneman, K. P., C. Han, and P. W. Abel. Comparison of α_1-adrenergic receptor subtypes distinguished by chlorethylclonidine and WB4101. *Mol. Pharmacol.* **33**:509–514 (1988).
18. Hieble, J. P., W. D. Matthers, R. M. DeMarinis, and R. R. Ruffolo. Heterogeneity of *alpha*-1 adrenergic receptors, in *The Alpha-1 Adrenergic Receptors* (R. R. Ruffolo, ed.). Humana Press, Clifton, NJ, 325–343 (1987).
19. Minneman, K. P. α_1-Adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca^{2+}. *Pharmacol. Rev.* **40**:87–119 (1988).
20. Timmermans, P. B. M. W. M., and M. J. M. C. Thoolen. Ca^{2+} utilization in signal transformation of *alpha*-1 adrenergic receptors, in *The Alpha-1 Adrenergic Receptors* (R. R. Ruffolo, ed.). Humana Press, Clifton, NJ, 113–187 (1987).
21. Han, C., P. W. Abel, and K. P. Minneman. α_1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature* (*Lond.*) **329**:333–335 (1987).

22. Cullen, B. R. Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* **152**:684–704 (1987).
23. Gey, G. O., W. D. Coffman, and M. T. Kubicek. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.* **12**:264–265 (1952).
24. Gold, M. *A Conspiracy of Cells: One Woman's Immortal Legacy and the Medical Scandal It Caused.* State University of New York Press, Albany, NY (1986).
25. Cotecchia, S., B. K. Kobilka, K. W. Daniel, R. D. Nolan, E. Y. Lapetina, M. G. Caron, R. J. Lefkowitz, and J. W. Regan. Multiple second messenger pathways of α-adrenergic receptor subtypes expressed in eukaryotic cells. *J. Biol. Chem.* **265**:63–69 (1990).
26. DeLean, A., A. A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* **21**:5–16 (1982).
27. Martin, T. F. J. Thyrotropin-releasing hormone rapidly activates the phosphodiester hydrolysis of polyphosphoinositides in GH3 pituitary cells: evidence for the role of a polyphosphoinositide-specific phospholipase C in hormone action. *J. Biol. Chem.* **258**:14816–14822 (1983).
28. Berridge, M. T., R. M. C. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212**:473–482 (1983).
29. Molina, Y. V. L., R. D. Nolan, and E. G. Lapetina. Subcellular localization of the enzymes that dephosphorylate *myo*-inositol polyphosphates in human platelets. *Biochem. J.* **255**:795–800 (1988).
30. Okajima, F., K. Sato, and Y. Kondo. P_2-purinergic agonists activate phospholipase C in a guanine nucleotide- and Ca^{2+}-dependent manner in FTRL-5 thyroid cell membranes. *FEBS Lett.* **253**:132–136 (1989).
31. Middleton, J. P., J. R. Raymond, A. R. Whorton, and V. W. Dennis. Short-term regulation of Na^+/K^+ ATPase by recombinant human serotonin (5-HT_{1A}) receptor expressed in HeLa cells. *J. Clin. Invest.* **86**:1799–1805 (1990).
32. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
33. Schild, H. O. p*A*, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol.* **2**:189–206 (1947).
34. Schild, H. O. Drug antagonism and pA_X. *Pharm. Rev.* **9**:242–245 (1957).
35. Eberhard, D. A., and R. W. Holz. Intracellular Ca^{2+} activates phospholipase C. *Trends Neurosci.* **11**:517–520 (1988).
36. Berridge, M. J., and R. F. Irvine. Inositol phosphates and cell signalling. *Nature* (*Lond.*) **341**:197–205 (1989).
37. Benham, C. D., and R. W. Tsien. A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature* (*Lond.*) **328**:275–278 (1987).
38. Berridge, M. J. Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.* **56**:159–193 (1987).
39. Tsujimoto, G., A. Tsujimoto, E. Suzuki, and K. Hashimoto. Glycogen phosphorylase activation by two different α_1-adrenergic receptor subtypes: methoxamine selectively stimulates a putative α_1-adrenergic receptor subtype (α_{1a}) that couples with Ca^{2+} influx. *Mol. Pharmacol.* **36**:166–176 (1989).
40. Minneman, K. P., and P. W. Abel. Relationship of *alpha*-1 adrenergic receptor occupancy to tissue response, in *The Alpha-1 Adrenergic Receptors* (R. R. Ruffolo, ed.). Humana Press, Clifton, NJ, 267–324 (1987).

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