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Selectivity of the imidazoline α-adrenoceptor agonists (oxymetazoline and cirazoline) for human cloned α_1-adrenoceptor subtypes

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1 To investigate the structure-activity relationships of α-adrenoceptor agonists for the α_1-adrenoceptor subtypes*, we have compared the imidazoline class of compounds, oxymetazoline and cirazoline, with the phenethylamine, noradrenaline, in their affinities and also in their intrinsic activities in Chinese hamster ovary (CHO) cells stably expressing the cloned human α_1-adrenoceptor subtypes (α_{1a}-, α_{1b}-, and α_{1d}-subtypes).

2 Radioligand binding studies with [^{125}I]-HEAT showed that cirazoline and oxymetazoline had higher affinities at α_{1a}-subtype than at α_{1b}- and α_{1d}-subtypes, while noradrenaline had higher affinity at the α_{1d}-subtype than at α_{1a}- and α_{1b}-subtypes.

3 In functional studies, cirazoline caused transients of cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$ response) in a concentration-dependent manner and developed a maximal response similar to that to noradrenaline in CHO cells expressing the α_{1a}-subtype, while it acted as a partial agonist at α_{1b}- and α_{1d}-adrenoceptors. Oxymetazoline, on the other hand, was a weak agonist at α_{1a}-adrenoceptors, and has no intrinsic activity at the other subtypes.

4 Using the phenoxybenzamine inactivation method, the relationships between receptor occupancy and noradrenaline-induced $[Ca^{2+}]_i$ response for α_{1a}- and α_{1d}-subtypes were found to be linear, whereas it was moderately hyperbolic for the α_{1b}-subtype, indicating the absence of receptor reserves in CHO cells expressing α_{1a}- and α_{1d}-subtypes while there exists a small receptor reserve for CHO cells expressing the α_{1b}-subtype.

5 In summary, our data obtained in cells exclusively expressing a single receptor subtype support the idea that the relative role of agonist affinity and intrinsic activity may vary depending on the subtype of α_1-adrenoceptor.

Keywords: α_{1a}-Adrenoceptor; α_{1b}-adrenoceptor; α_{1d}-adrenoceptor; cloned receptors; radioligand binding studies; cytosolic Ca^{2+} concentrations; imidazolines; affinity; intrinsic activity

Introduction

α_1-Adrenoceptors play critical roles in the regulation of a variety of physiological processes. Recently, it was found that α_1-adrenoceptors comprise a heterogeneous family (Minneman & Esbenshade, 1994). Two natively expressed subtypes (α_{1A} and α_{1B}) can be distinguished pharmacologically, while three subtypes (α_{1a}, α_{1b} and α_{1d}) have been cloned (Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Perez *et al.*, 1991; Lomasney *et al.*, 1991). The α_{1b}-adrenoceptor cDNA clone appears to encode the natively expressed, pharmacologically defined α_{1b}-subtype. Earlier studies using heterologous expression systems suggested that neither the α_{1a}- (previously defined as α_{1C}) nor α_{1d}-clones (previously defined as α_{1A} or $\alpha_{1A/D}$) encoded a subtype identical to the native α_{1A}-adrenoceptor. The uncertain relationship between the cloned and native subtypes has been a source of much confusion; however, more recent reports detailing the cloning and expression of the rat homologue of the bovine α_{1a}-(previously α_{1C}) subtype provide strong evidence to support the idea that the α_{1a}-adrenoceptor cDNA encodes the pharmacologically defined α_{1A}-adrenoceptor (Laz *et al.*, 1994; Perez *et al.*, 1994). The functional role of the native α_{1D}-adrenoceptor still remains to be defined.

Antagonist specificities of α_1-adrenoceptor subtypes have been studied extensively, but less information is available on agonists. Evaluation of agonist selectivity is difficult because both binding and functional measurements of agonist/receptor interactions are dependent on the conditions under which they are examined. Agonist-induced functional responses are complicated by differential receptor reserves (Ruffolo, 1982), whereas radioligand binding studies are influenced by differential affinity states due to G protein coupling (Terman *et al.*, 1987). Without reliable information concerning relative receptor reserves for each subtype, comparing the relative intrinsic activity of each agonist may give false conclusions. Since it is known that the subtypes can couple with different efficacies to second messenger systems (e.g., the α_{1a}-adrenoceptor couples to phospholipase C more efficiently than does the α_{1b}-adrenoceptor; Schwinn *et al.*, 1991), simple comparisons of relative receptor density do not address this problem. Therefore, the quantitative relationship between fractional occupation of α_1-adrenoceptor subtype and the receptor-mediated responses needs to be determined when comparing the relative intrinsic activity of agonists.

There are two main classes of α_1-adrenoceptor agonists that have different chemical structures, the phenethylamines, such as noradrenaline, and the imidazolines, such as cirazoline and oxymetazoline (Nichols & Ruffolo, 1991). In general, most phenethylamines are full agonists, whereas imidazolines tend to be partial agonists. Cirazoline, however, is known to be a notable exception to this generalization, in that the drug is an

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* Throughout this paper, we have used the standardized nomenclature system for α_1-adrenoceptor subtypes recently recommended by the IUPHAR Committee on the Classification of Adrenoceptors. In this system, the cloned subtypes are designated by lower case letters as α_{1a}, α_{1b}, and α_{1d} which correspond to the clones previously defined as α_{1c}, α_{1b}, and α_{1a} (or $\alpha_{1a/d}$ and α_{1d}) respectively. The corresponding pharmacological subtypes are designated with upper case letters and are defined as α_{1A}, α_{1B}, and α_{1D}, respectively.

imidazoline but acts as a full α_1-adrenoceptor agonist in several systems (Jim *et al.*, 1985; Ruffolo & Zeid, 1985). The effect of chemical structure on α_1-adrenoceptor agonist activity has been extensively studied in terms of the affinity and efficacy of the drug since agonist potency depends on the two independent properties (Nichols & Ruffolo, 1991). However, there is insufficient information available at present concerning the structure-activity relationships of α-adrenoceptor agonists for the various α_1-adrenoceptor subtypes. As an initial effort to investigate the structure-activity relationships of α-adrenoceptor agonists for α_1-adrenoceptor subtypes, we have compared the affinity and the intrinsic activity of the imidazoline class of compounds, oxymetazoline and cirazoline, with those of the phenethylamine, noradrenaline, in Chinese hamster ovary (CHO) cells stably expressing the cloned α_1-adrenoceptor subtypes (denoted $CHO_{\alpha 1a}$, $CHO_{\alpha 1b}$, and $CHO_{\alpha 1d}$ cells). To avoid potential effects of species difference, we used the α_1-adrenoceptor subtype clones from a single species, (man), in the present study.

Methods

Cloning of the human α_1-adrenoceptor subtypes

α_{1a} (formerly termed α_{1c}) Human α_{1a}-adrenoceptor clone was isolated from the human prostate cDNA library as described previously (Hirasawa *et al.*, 1993). The 2.1 kb-full length coding region, including 436 bp of 5′ untranslated sequence and 486 bp of 3′ untranslated sequence, was ligated into the EcoRI site of the eukaryotic expression vector pSVK3 containing the neomycin-resistant gene of pMAM-neo (pSVK3neo).

α_{1b} Human α_{1b}-adrenoceptor clone was a cDNA/gene fusion construct from the human prostate cDNA library and PCR products amplified from the human genomic library. A full length cDNA/gene α_{1b}-adrenoceptor fusion construct was obtained by ligation of a partial Pst I - Bss HII cDNA fragment to a PCR product containing 5′ terminal sequence of α_{1b} gene at their common Pst I site and to a PCR product containing 3′ terminal sequence at their common Bss HII site. The deduced amino acid sequence of our α_{1b}-adrenoceptor clone is 100% identical to that recently reported by Weinberg *et al.* (1994). The 1.8 kb-full length coding region, including 26 bp of 5′ untranslated sequence and 203 bp of 3′ untranslated sequence, was ligated into the EcoRI site of the pSVK3neo.

α_{1d} (formerly termed α_{1a} or $\alpha_{1a/d}$) Human α_{1d}-adrenoceptor clone was isolated from the cDNA library prepared from SKNMC cells and also from the human prostate cDNA library (Esbenshade *et al.*, 1995). The 2.1 kb-full length coding region, including 21 bp of 5′ untranslated sequence and 357 bp of 3′ untranslated sequence, was ligated into the EcoRI site of the pSVK3neo.

DNA sequencing

The cloned cDNA, genomic DNA, and constructs were subcloned into pBluescript II KS(+) (Stratagene, La Jolla, U.S.A.). Nucleotide sequence analysis was performed by use of overlapping templates by the ABI 373A DNA Sequencer (Applied Biosystems, Inc., Foster City, U.S.A.) for both complete strands.

Transfection of the human receptor genes

Stable cell lines were obtained by transfection of the pSVK3neo containing the human α_{1a}- or α_{1d}-adrenoceptor cDNA construct, or the human α_{1b}-adrenoceptor cDNA/gene fusion construct into CHO-K1 cells, by use of the Lipofectin technique as described previously (Horie *et al.*, 1994). Cells were grown as monolayers in Ham's F-12 medium (GIBCO, Grand Island, U.S.A.) containing L-glutamine supplemented with 10% foetal bovine serum, penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). Stable clones were then selected for resistance to G418 (600 μg ml^{-1}) as described previously (Horie *et al.*, 1994). Cells were harvested and membrane preparations were assayed for their ability to bind [^{125}I]-HEAT as described below.

Membrane preparation for [^{125}I]-HEAT binding

Subconfluent 150 mm plates of transfected cells were washed twice with 10 ml of phosphate buffered saline (PBS: NaCl 139 mM, KCl 2.7 mM, Na_2HPO_4 8.8 mM, KH_2PO_4 1.48 mM, pH 7.5), and harvested by scraping. Cells were pelleted by centrifugation at 500 *g* for 5 min, washed, and the pellet was homogenized in 2 ml of ice-cold buffer A (sucrose 250 mM Tris HCl 5 mM, $MgCl_2$ 1 mM, pH 7.4) and centrifuged at 1,000 *g* at 4°C for 10 min to remove nuclei. The supernatant was then centrifuged at 35,000 *g* for 20 min at 4°C and the pellet was homogenized and frozen at –80°C until assay. The protein concentration was measured with the bicinchoninic acid protein assay kit (PIERCE, Rockford, U.S.A.).

[^{125}I]-HEAT binding

Radioligand binding studies were performed as described previously (Hirasawa *et al.*, 1993). Briefly, in the radioligand binding assay buffer B (Tris HCl 50 mM, $MgCl_2$ 10 mM, EGTA 10 mM, pH 7.4) was used in the incubation medium. Measurement of specific [^{125}I]-HEAT binding was performed by incubating 0.1 ml of membrane preparation (~10 μg of protein) with [^{125}I]-HEAT (2,200 Ci $mmol^{-1}$) in a final volume of 0.25 ml buffer B for 60 min at 25°C in the presence or absence of competing drugs. The incubation was terminated by adding ice-cold buffer B and immediately filtering through Whatmann GF/C glass-fibre filters with a Brandel cell harvester (Model-30, Gaithersburg, U.S.A.). Each filter was collected and the radioactivity was measured. Binding assays were always performed in duplicate. For competition curve analysis, each assay contained about 70 pM [^{125}I]-HEAT. At this concentration nonspecific binding, defined as binding displaced by 10 μM phentolamine, represented less than 10% of total binding. In some experiments, the incubation was performed in buffer B containing 200 μM Gpp(NH)p to examine the effects of guanine nucleotides on agonist binding affinity.

Measurement of $[Ca^{2+}]_i$

The transfected cells at 50% confluency in 100 mm culture dishes were trypsinized and washed twice with a buffered salt solution (BSS composition mM: NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.25, $NaHPO_4$ 1, HEPES 5, glucose 11 pH 7.4), and incubated in the buffer containing 4 μM fura-2/AM for 30 min at 25°C. The cells were then washed twice and resuspended in BSS buffer without the dye. Mobilization of $[Ca^{2+}]_i$ evoked by various concentrations of agonists was monitored by a JASCO CAF-110 fluorescence spectrophotometer (Nihon Bunkoh, Tokyo, Japan) with dual excitation at 340 nm/380 nm and emission at 500 nm (Horie *et al.*, 1994). For studying the antagonist action of oxymetazoline, various concentrations of oxymetazoline were added 10 min prior to the addition of noradrenaline. Agonists induced an acute $[Ca^{2+}]_i$ increase in the transfected cells that was followed by lower plateau $[Ca^{2+}]_i$ levels. The peak $[Ca^{2+}]_i$ values from the initial transients were used to generate the dose-response curves.

$[Ca^{2+}]_i$ was calculated based on the formula (Grynkiewicz *et al.*, 1985) as follows:

$$[Ca^{2+}]_i = K_D S_{f380/b380} (R - R_{min})/(R_{max}-R)$$

where K_D is 225 nM in the cytosolic environment, $S_{f380/b380}$ is the ratio of the intensities of the free and bound dye forms at 380 nm, R is the fluorescence ratio (340nm/380nm) of the in-

tracellular fura-2, and R_{min} and R_{max} are the minimal and maximal fluorescence ratios, respectively. Calibration of the fluorescence levels was performed for every aliquot by equilibrating intracellular and extracellular Ca^{2+} with 5 μl of 10% Triton-X 100 followed by addition of 5 μl of 300 mM EGTA/3 M Tris buffer (pH 9.0).

Phenoxybenzamine inactivation method

To obtain the quantitative relationship between fractional occupation of α_1-adrenoceptor subtype and the receptor-mediated $[Ca^{2+}]_i$ responses, phenoxybenzamine inactivation analysis was performed as described previously (Tsujimoto *et al.*, 1989). To examine the effect of receptor inactivation by phenoxybenzamine on [^{125}I]-HEAT binding, CHOα_{1a}, CHOα_{1b}, and CHOα_{1d} cells were harvested and suspended into BSS containing specified phenoxybenzamine concentrations (1 pM–10 μM) for 30 min at 37°C. After incubation, cells were washed three times with the buffer A and were homogenized for the membrane binding study. Data were expressed as % B/B_{max}, where B was the amount of [^{125}I]-HEAT specifically bound under the experimental conditions and B_{max} was the amount of specific [^{125}I]-HEAT binding in the absence of any phenoxybenzamine.

To measure phenoxybenzamine inhibition of noradrenaline-induced $[Ca^{2+}]_i$ responses, CHOα_{1a}, CHOα_{1b}, and CHOα_{1d} cells were exposed to BSS containing fura-2/AM and specified phenoxybenzamine concentrations (0.1 nM–10 μM) for 30 min at 37°C. Termination of the incubation was the same as outlined above. Data were expressed as percentages of the $[Ca^{2+}]_i$ response induced by 1 μM noradrenaline alone.

Statistical analysis

Computer analyses with LIGAND (Munson & Rodbard, 1980) were used to evaluate dissociation constant and receptor density. In $[Ca^{2+}]_i$ response experiments, the EC_{50} and IC_{50} values were determined with a sigmoidal function using analytical software SigmaPlot (Jandel Scientific, San Rafael, U.S.A.). Values are expressed as the mean ± s.e.mean. A two-way analysis of variance with 99% confidence limits was performed, followed by Student's t test on individual sets of data.

Materials

Sources of drugs were as follows: [^{125}I]-HEAT (2-[b-(4-hydroxy-3-[^{125}I]-iodophenyl)ethylamino-methyl]tetralone) (Du Pont - New England Nuclear, Boston, U.S.A.); methoxamine, (–)-noradrenaline bitartrate, (–)-adrenaline bitartrate, (+)-adrenaline bitartrate, oxymetazoline, Gpp(NH)p (5'guanylylimidodiphosphate) (Sigma, St. Louis, U.S.A.); WB4101 (2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4benzodioxane), 5-methylurapidil (Research Biochemicals Inc., Natick, U.S.A.); phentolamine HCl (Ciba-Geigy, Summit, U.S.A.); prazosin HCl (Pfizer, Brooklyn, U.S.A.); yohimbine HCl (Wako Pure Chemical Industries, Ltd., Osaka, Japan); cirazoline (Synthelabo Recherche, Bagneux, France); phenoxybenzamine (Smith Kline Beecham Pharmaceuticals, Philadelphia, U.S.A.); fura-2/AM (fura-2 tetrakis(acetoxymethyl)ester) (Dojindo, Kumamoto, Japan); Triton X-100 (polyoxyethylene(10) octhylphenyl ether) (Wako, Osaka, Japan). All other chemicals were of reagent grade. The CHO-K1 cell line was obtained from American Type Culture Collection (Rockville, U.S.A.). The expression vectors pSVK3 and pMAM-neo were from Pharmacia (Uppsala, Sweden) and Clontech (Palo Alto, U.S.A.), respectively.

Results

Stable expression of functional α_1-adrenoceptor subtypes in CHO-K1 cells

Membrane preparations from CHO cells stably transfected with the human cloned α_1-adrenoceptor genes showed saturable binding of [^{125}I]-HEAT; B_{max} and K_D values for the α_{1a}-, α_{1b}-, and α_{1d}-adrenoceptors were 1.3±0.2, 5.5±0.1, and 1.1±0.1 pmol mg^{-1} protein (n=4, each), and 110±20, 60±1.0, and 262±15 pM (n=4, each) respectively. The K_i values of α_1-adrenoceptor agonists and antagonists at the cloned human α_1-adrenoceptors are shown in Table 1. Consistent with their identity as α_1-adrenoceptors, the three cloned α_1-adrenoceptor subtypes showed an extremely low affinity for the α_2-selective antagonist yohimbine. The phenethylamines, noradrenaline and adrenaline, were found to be more than one order of magnitude potent at the human α_{1d}-subtype than at the α_{1a}- or α_{1b}-subtypes. Prazosin showed very small differences in their binding potencies at the three α_1-adrenoceptor subtypes. However, there were several agonists and antagonists that showed marked differences in their potencies to inhibit [^{125}I]-HEAT binding to the three cloned human α_1-adrenoceptor subtypes. Among agonists, methoxamine was 25 fold more potent at the α_{1a}-than at the α_{1b}-subtype, and oxymetazoline was found to be markedly more potent at the α_{1a}-subtype than at α_{1b}- and α_{1d}-subtypes. Moreover, the antagonists WB-4101 and 5-methylurapidil were found to be 17 and 44 fold less potent, respectively, at the α_{1b}- than at the α_{1a}-subtype (Table 1).

Table 1 Pharmacological profile of the cloned human α_1-adrenoceptor subtypes

Drugs	K_i (nM) α_{1a}	α_{1b}	α_{1d}
Agonists			
(–)-Adrenaline	600±250	400±35	56±12
(+)-Adrenaline	8,100±560	7,600±600	920±90
(–)-Noradrenaline	990±100	680±90	42±8.8
Methoxamine	4,400±200	110,000±6,000	11,000±1,300
Cirazoline	120±18	960±130	660±160
Oxymetazoline	6.0±0.60	320±15	390±100
Antagonists			
Prazosin	0.17±0.020	0.26±0.032	0.070±0.0010
Phentolamine	2.5±0.10	30±3.0	7.0±0.83
Yohimbine	400±50	520±5.0	240±25
5-Methylurapidil	0.89±0.081	39±3.1	10±1.4
WB-4101	0.20±0.030	3.4±0.30	0.25±0.012

CHO cell membranes stably expressing α_{1a}-, α_{1b}-, or α_{1d}-adrenoceptor were incubated with [^{125}I]-HEAT, in the absence or presence of increasing concentrations of various agonists and antagonists,. Each value represents the mean ± s.e.mean from at least three individual experiments performed in duplicate. At least ten concentrations of each ligand were tested, and the points were chosen to be the linear portion of the displacement curve. K_i values were generated by the iterative curve-fitting programme LIGAND. For all drugs examined Hill slopes were not significantly different from unity.

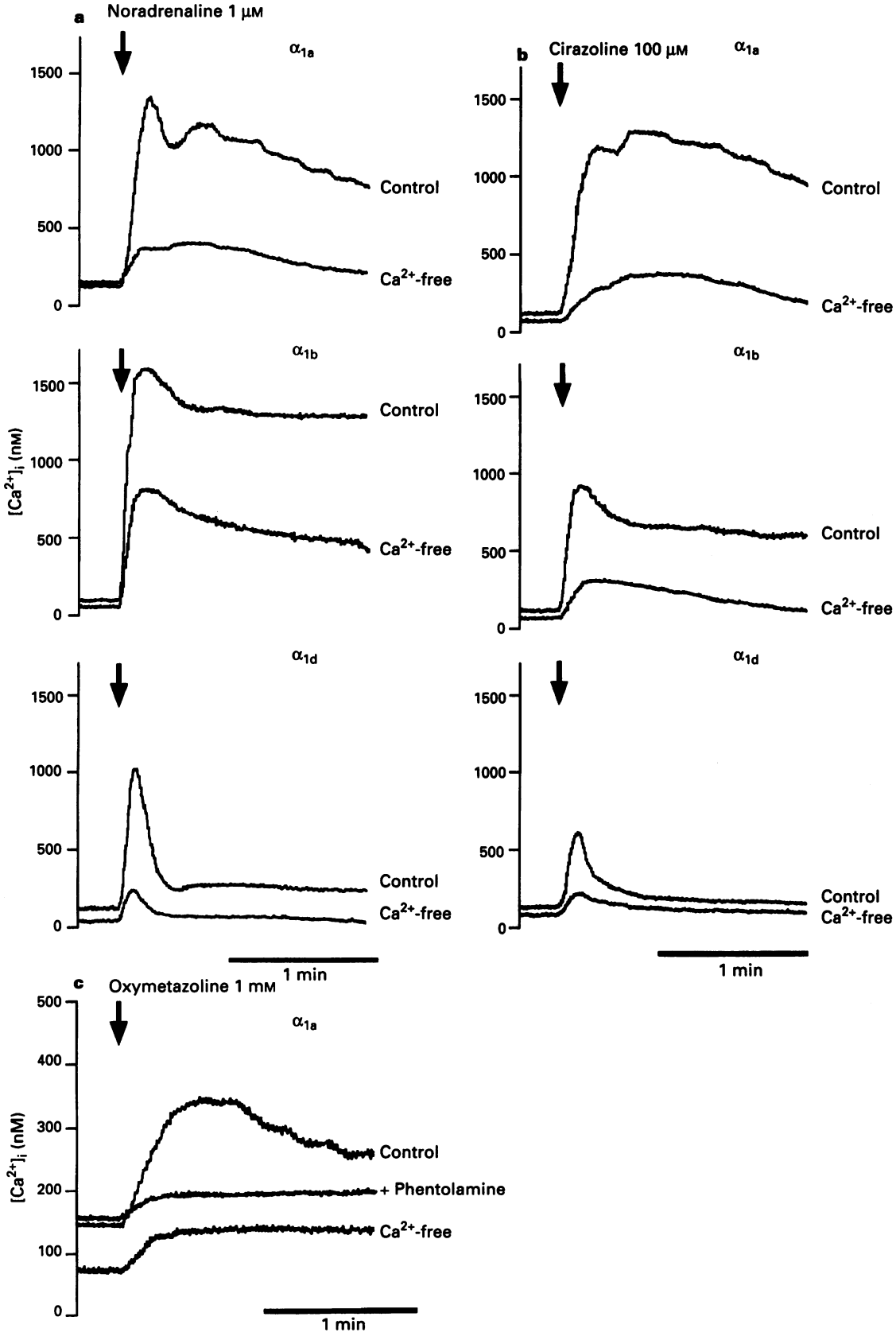

Figure 1 Effects of removal of extracellular Ca^{2+} on $[Ca^{2+}]_i$ responses induced by (a) noradrenaline and (b) cirazoline in $CHO\alpha_{1a}$, $CHO\alpha_{1b}$, and $CHO\alpha_{1d}$ cells, and effects of removal of extracellular Ca^{2+} or pretreatment with phentolamine (10 μM, 10 min) on $[Ca^{2+}]_i$ responses induced by (c) oxymetazoline in $CHO\alpha_{1a}$ cells. Each cell line was loaded with fura-2/AM, and $[Ca^{2+}]_i$ was determined by a fluorescence spectrophotometer with dual excitation at 340 nm/380 nm and emission at 500 nm, as described in Methods. Cells were stimulated with agonists in the presence or absence of 1.25 mM extracellular Ca^{2+}. The results presented are representative experiments of at least three experiments. The points indicating added agonist stimulation were arranged in line to help in comparison of two or three waves in each figure.

The possibility that differences in apparent agonist affinity might be caused by the existence of multiple affinity states related to ternary complex formation with G proteins (Terman *et al.*, 1987) was tested by addition of guanine nucleotides in the radioligand binding assays. The apparent K_i for noradrenaline at each subtype was slightly increased but not significantly altered when 200 μM Gpp(NH)p was added to the incubation buffer (data not shown).

$[Ca^{2+}]_i$ measurements

Effects of α-adrenoceptor agonists on the elevation of $[Ca^{2+}]_i$ were examined in $CHO\alpha_{1a}$, $CHO\alpha_{1b}$, and $CHO\alpha_{1d}$ cells preloaded with fura-2. As shown in Figure 1a and b, noradrenaline (1 μM) and cirazoline (100 μM) caused rapid increases in $[Ca^{2+}]_i$, consisting of quick transient peaks and more sustained components in $CHO\alpha_{1a}$ and $CHO\alpha_{1b}$ cells. In $CHO\alpha_{1d}$ cells, however, the increases in $[Ca^{2+}]_i$ induced by noradrenaline and cirazoline were transient, with small sustained components. The peak $[Ca^{2+}]_i$ values induced by noradrenaline (1 μM) and cirazoline (100 μM) were 1,310±205 nM and 1,297±183 nM in $CHO\alpha_{1a}$ cells, 1,550±250 nM and 822±108 nM in $CHO\alpha_{1b}$ cells, 960±130 nM and 451±49 nM in $CHO\alpha_{1d}$ cells, respectively ($n=4$, each). The $[Ca^{2+}]_i$ responses induced by noradrenaline and cirazoline were α_1-adrenoceptor-mediated in all of the transfected cells, as shown by the fact that those responses were abolished by pretreatment of the cells with 10 μM phentolamine or 0.3 μM prazosin but not 1 μM rauwolscine (data not shown). Although exposure to medium containing no extracellular Ca^{2+} and 1 mM EGTA caused a reduction in basal levels of $[Ca^{2+}]_i$, noradrenaline and cirazoline still gave substantial increases in $[Ca^{2+}]_i$ in all transfected cells; however, these increases in $[Ca^{2+}]_i$ were transient, with no evidence for sustained components. Oxymetazoline (1 mM) caused a rapid increase in $[Ca^{2+}]_i$ only in $CHO\alpha_{1a}$ cells in the presence of extracellular Ca^{2+} (Figure 1c); however, the peak $[Ca^{2+}]_i$ values were significantly lower ($P<0.01$) than those of noradrenaline and cirazoline in $CHO\alpha_{1a}$ cells (the peak $[Ca^{2+}]_i$ responses of oxymetazoline were 328±39 nM, $n=4$, in $CHO\alpha_{1a}$ cells). In $CHO\alpha_{1b}$ and $CHO\alpha_{1d}$ cells, on the other hand, oxymetazoline produced no or little measurable $[Ca^{2+}]_i$ response (Table 2). Furthermore, the peak $[Ca^{2+}]_i$ responses to oxymetazoline in $CHO\alpha_{1a}$ cells were significantly reduced in Ca^{2+}-free medium, and the sustained components were maintained for more than 10 min. The oxymetazoline-induced $[Ca^{2+}]_i$ response was, however, partly mediated by a mechanism other than the α_1-adrenoceptor, because the oxymetazoline-induced $[Ca^{2+}]_i$ response was not completely inhibited with pretreatment of the cells with 10 μM phentolamine (26.6±3.3% of the oxymetazoline-induced $[Ca^{2+}]_i$ response remained, $n=3$, Figure 1c).

α_1-Adrenoceptor occupancy-$[Ca^{2+}]_i$ response relationships defined by phenoxybenzamine inactivation

The quantitative relationship between fractional occupation of α_1-adrenoceptor and noradrenaline-induced $[Ca^{2+}]_i$ responses was examined in each subtype by use of the phenoxybenzamine

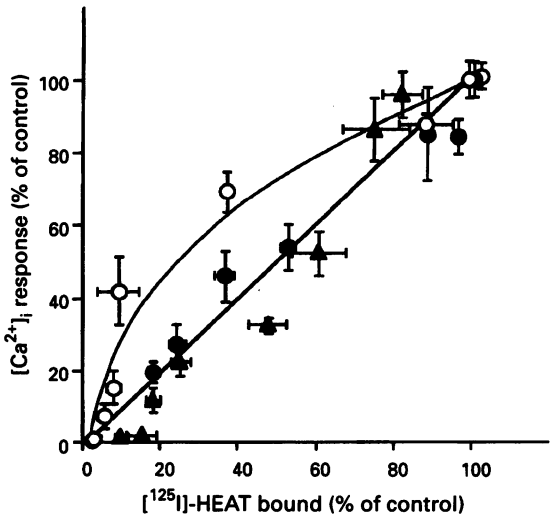

Figure 2 Relationships between maximal [^{125}I]-HEAT binding capacities and maximal noradrenaline (1 μM)-induced $[Ca^{2+}]_i$ responses in $CHO\alpha_{1a}$ (●), $CHO\alpha_{1b}$ (○) and $CHO\alpha_{1d}$ (▲) cells after incubation with phenoxybenzamine. Maximal specific [^{125}I]-HEAT binding capacities were 1.4±0.3, 5.4±0.2, and 1.0±0.2 pmol mg^{-1} protein for the α_{1a}, α_{1b} and α_{1d}-adrenoceptors, respectively ($n=3$, each). Maximal $[Ca^{2+}]_i$ responses induced by noradrenaline (1 μM) were 1,250±210 nM, 1,650±270 nM, and 1,010±170 nM in $CHO\alpha_{1a}$, $CHO\alpha_{1b}$, and $CHO\alpha_{1d}$ cells, respectively ($n=4$, each). Values represent the mean±s.e.mean from three to four individual experiments performed in duplicate.

Table 2 Potencies and intrinsic activities of noradrenaline, cirazoline and oxymetazoline in the $[Ca^{2+}]_i$ responses in CHO cells stably expressing the cloned human α_1-adrenoceptor subtypes

Drugs	n	*-log EC_{50}*	E_{max} (% of NA maximum)
α_{1a}			
Noradrenaline	4	7.37±0.01	100
Cirazoline	4	7.15±0.14	99±14
Oxymetazoline	4	7.39±0.06	25±3.0[a]
α_{1b}			
Noradrenaline	4	7.59±0.11	100
Cirazoline	4	7.10±0.04	53±7.0[a]
Oxymetazoline	4		NE[b]
α_{1d}			
Noradrenaline	4	8.49±0.18	100
Cirazoline	4	6.62±0.17	47±5.1[a]
Oxymetazoline	4		NE[b]

-log EC_{50} values were determined from agonist dose-response curves. E_{max} for each agonist was calculated as the maximum response produced, divided by the maximal response produced by noradrenaline (the maximal responses produced by noradrenaline were 1,310±205 nM, 1,550±250 nM and 960±130 nM in $CHO\alpha_{1a}$, $CHO\alpha_{1b}$, and $CHO\alpha_{1d}$ cells, respectively, $n=4$ each). Each value is the mean±s.e.mean from four individual experiments performed in duplicate.
[a] Significantly different from E_{max} for noradrenaline at each subtype ($P<0.01$).
[b] NE, no measurable effects.

inactivation method. The pretreatment with phenoxybenzamine was performed for 30 min at 37°C before the assays for [^{125}I]-HEAT binding and $[Ca^{2+}]_i$ measurements. Maximal [^{125}I]-HEAT binding capacity was progressively decreased by exposure to increasing concentrations of phenoxybenzamine in all of the transfected cells, reaching a minimal level of 1.1±0.3%, 2.7±0.5%, and 1.5±0.3% at a phenoxybenzamine concentration of 10 μM for CHOα_{1a}, CHOα_{1b}, and CHOα_{1d} cells, respectively ($n=3$ each). The phenoxybenzamine concentrations necessary to block half of the [^{125}I]-HEAT sites were 0.29±0.05 nM, 22.8±1.1 nM, and 11.2±1.8 nM in CHOα_{1a} cells, CHOα_{1b} cells, and CHOα_{1d} cells, respectively ($n=3$ each). The half-maximal inhibitory concentrations (IC_{50}) for noradrenaline-induced $[Ca^{2+}]_i$ responses were 0.19±0.04 nM, 65.7±5.3 nM, and 19.6±8.7 nM phenoxybenzamine for CHOα_{1a}, CHOα_{1b}, and CHOα_{1d} cells, respectively ($n=4$ each). Figure 2 shows the relationships between fractional occupation of α_1-adrenoceptors and noradrenaline-induced $[Ca^{2+}]_i$ responses in each transfected cell, determined by the phenoxybenzamine inactivation method. The relationships were linear in CHOα_{1a} and CHOα_{1d} cells, while the relationship was moderately nonlinear hyperbolic in CHOα_{1b} cells.

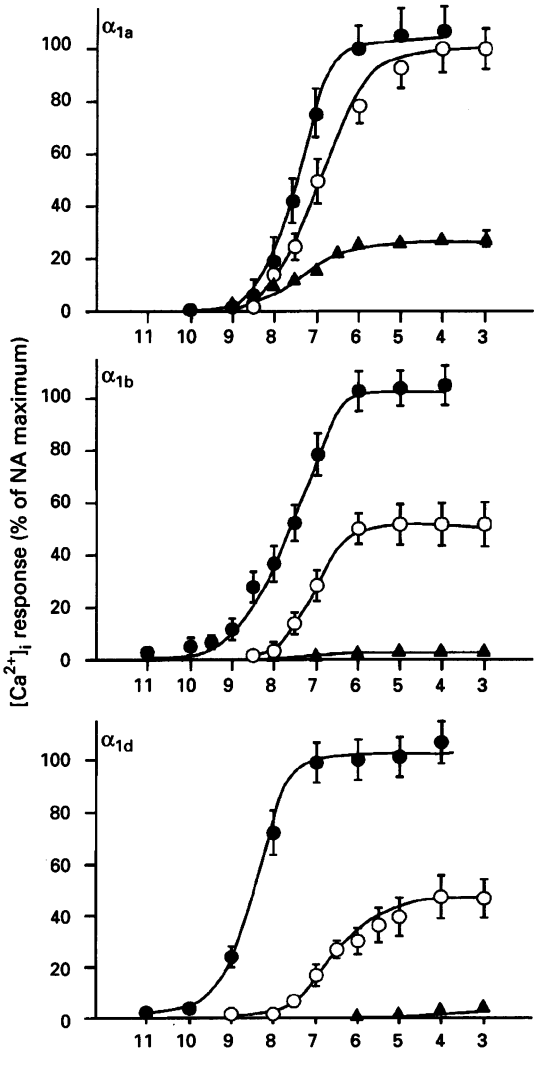

Figure 3 Concentration-response curves for $[Ca^{2+}]_i$ responses induced by noradrenaline (●), cirazoline (○) and oxymetazoline (▲) in CHO cells stably expressing the cloned human α_1-adrenoceptor subtypes. All responses are expressed as percentages of the maximum response induced by noradrenaline (1 μM) in each cell line, as described in Table 2. Values represent the mean± s.e.mean from four individual experiments performed in duplicate.

Agonistic effects of noradrenaline and imidazolines on α_1-adrenoceptor subtypes

The potencies of agonists in the $[Ca^{2+}]_i$ responses were compared by constructing concentration-$[Ca^{2+}]_i$ response curves for noradrenaline, cirazoline, and oxymetazoline in CHOα_{1a}, CHOα_{1b}, and CHOα_{1d} cells (Figure 3 and Table 2). Cirazoline exerted a maximal response that was not different from that of noradrenaline in CHOα_{1a} cells, while it produced a significantly ($P<0.01$) lower maximal response than noradrenaline in CHOα_{1b} or CHOα_{1d} cells, indicating that cirazoline worked as a partial agonist on α_{1b}- and α_{1d}-subtypes. Oxymetazoline was found to elicit $[Ca^{2+}]_i$ responses only in CHOα_{1a} cells, whereas it produced no noticeable responses in CHOα_{1b} and CHOα_{1d} cells (Figure 3 and Table 2). EC_{50} values of concentration-$[Ca^{2+}]_i$ response curves for oxymetazoline were thus determined only for the α_{1a}-adrenoceptor subtype.

Antagonistic effects of oxymetazoline on α_1-adrenoceptor subtypes

As oxymetazoline was found to be a weak agonist in producing a $[Ca^{2+}]_i$ response only for the α_{1a}-adrenoceptor subtype and to have little or no intrinsic activities for α_{1b}- or α_{1d}-subtypes, we attempted to determine whether the compound might possess a blocking effect on noradrenaline-induced $[Ca^{2+}]_i$ responses in each transfected cell. Fura-2 loaded cells were incubated at variable concentrations (0.1 nM–1 mM) for 10 min with oxymetazoline prior to the stimulation with 1 μM noradrenaline. As shown in Figure 4, oxymetazoline inhibited noradrenaline-induced $[Ca^{2+}]_i$ responses in all of the three transfected cells; however, oxymetazoline showed marked differences in its inhibitory abilities in the three transfected cells. The inhibitory ability of oxymetazoline was markedly potent in CHOα_{1a} cells (IC_{50} values in CHOα_{1a}, CHOα_{1b}, and CHOα_{1d} cells were 104±16 nM, 7.0±1.8 μM, and 44±18 μM, respectively, $n=4$ each).

Discussion

Utilizing Chinese hamster ovary (CHO) cells stably expressing the human α_1-adrenoceptor subtype, we have compared the imidazoline class of compounds, oxymetazoline and cirazoline,

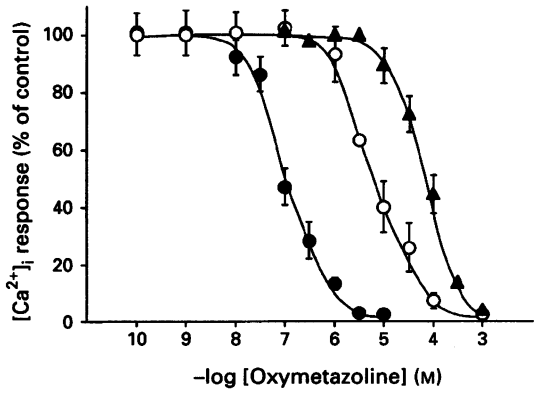

Figure 4 Concentration-response curves for the inhibitory effects of oxymetazoline on noradrenaline-induced $[Ca^{2+}]_i$ responses in CHOα_{1a} (●), CHOα_{1b} (○), and CHOα_{1d} (▲) cells. Various concentrations of oxymetazoline were added 10 min prior to stimulation with 1 μM noradrenaline. All responses are expressed as percentages of the control response induced by noradrenaline (1 μM) in each cell line (the maximal responses produced by noradrenaline were 1,420±220 nM, 1,610±270 nM, and 980±150 nM in CHOα_{1a}, CHOα_{1b}, and CHOα_{1d} cells, respectively, $n=4$, each). Values represent the mean±s.e.mean from four individual experiments performed in duplicate.

with the phenethylamine noradrenaline in their affinities and also in their intrinsic activities. Radioligand binding studies showed that cirazoline and oxymetazoline had higher affinities at the α_{1a}-subtype than at α_{1b}- and α_{1d}-adrenoceptor subtypes, while noradrenaline had higher affinity at α_{1d}-subtype than at α_{1a}- and α_{1b}-subtypes. Using the phenoxybenzamine inactivation method, we found the absence of receptor reserve in CHOα_{1a} and CHOα_{1d} cells, whereas there exists a small receptor reserve in CHOα_{1b} cells. In functional studies with $[Ca^{2+}]_i$ monitoring, we found that imidazolines were highly selective for the α_{1a}-subtype, having substantially lower intrinsic activities at the α_{1b}- and α_{1d}-adrenoceptor subtypes. Oxymetazoline was a weak agonist for the α_{1a}-subtype, and had little or no intrinsic activities at the α_{1b}- and α_{1d}-subtypes. Cirazoline behaved as a full agonist at the α_{1a}-subtype, whereas it worked as a partial agonist at the α_{1b}- and α_{1d}-subtypes. Taken together, our present study performed in CHO cells which exclusively express a single receptor subtype supports the idea that the relative role of agonist affinity and intrinsic activity may vary depending on the subtype.

As indicated in the introduction, evaluation of agonist selectivity can be complicated by the possibility of nonlinear relationships between receptor occupancy and response (Ruffolo, 1982). In the presence of receptor reserves, only a fraction of receptors must be occupied to generate a maximal response, making it impossible to determine the affinities and relative efficacies of agonists from simple concentration-response relationships. For example, if there were a much larger receptor reserve in CHOα_{1b} cells than in CHOα_{1a} cells, cirazoline could have similar intrinsic activities at both subtypes. In fact, as shown by the phenoxybenzamine inactivation analysis, CHOα_{1a} and CHO$_{1d}$ cells have little, if any, receptor reserve, and CHOα_{1b} cells have a small receptor reserve. An almost five fold greater expression of the α_{1b}-adrenoceptor (1.3, 5.5 and 1.1 pmol mg^{-1} protein for CHOα_{1a}, CHOα_{1b} and CHOα_{1d}, respectively) might influence the receptor reserve, and thereby the intrinsic activity. Thus, the presence of receptor reserve cannot be ruled out for any of the three subtypes, and the observed intrinsic activity hence appears to be a first approximation of relative intrinsic efficacy, and the intrinsic activity in CHOα_{1b} cells may be overestimated.

The different receptor occupancy-response relationships for oxymetazoline and cirazoline observed at different subtypes may partly explain the markedly differing efficacies of these agonists in various native tissues. Oxymetazoline is known to have moderately high intrinsic activity in causing the contraction of a variety of tissues including rat caudal artery (Abel & Minneman, 1986) and rabbit iris dilator (Konno & Takayanagi, 1989), while it did not elicit any positive inotropic effect in rabbit ventricular myocardium (Hiramoto *et al.*, 1988) or rat myocardium (Gross *et al.*, 1988). Cirazoline is generally regarded as a full α_1-adrenoceptor agonist in various tissues (Jim *et al.*, 1985; Ruffolo & Zeid, 1985); however, the present data suggested that cirazoline may have different intrinsic activities depending on the subtype. Therefore, the differing efficacy of these agonists in different tissues could partly be explained by the existence of distinct receptor subtypes involved, although several other factors including receptor density and tissue G protein composition might influence receptor-mediated responses in different tissues.

Lack of correlation between agonist affinity and efficacy has been described in a variety of tissues where receptor subtypes co-exist (Abel & Minneman, 1988). In the present study, we also observed the independence of affinity and intrinsic activity even in the cell expressing exclusively single receptor subtypes (Tables 1 and 2); thus, typically oxymetazoline has a high affinity but a low intrinsic activity at the α_{1a}-receptor (Table 2). Oxymetazoline was found to be partial agonist in causing $[Ca^{2+}]_i$ response only at the α_{1a}-subtype. The observation is in good agreement with the recent observation by Minneman *et al.* (1994), although they examined [^{3}H]-inositol phosphate formation in human embryonic kidney 293 cells in their study. Since oxymetazoline has a high affinity and a very low intrinsic activity for the α_{1a}-subtype, oxymetazoline can be expected to behave as a competitive antagonist. In fact, oxymetazoline was found to antagonize the full agonist noradrenaline-induced $[Ca^{2+}]_i$ responses in a dose-dependent manner at all subtypes (Figure 4). The inhibitory effect, however, was markedly selective for the α_{1a}-subtype, possibly due to its higher affinity for the subtype. Interestingly, oxymetazoline was previously reported to act as a competitive antagonist in the α_1-adrenoceptor-mediated positive inotropic effect in rabbit ventricular myocardium (Hiramoto *et al.*, 1988).

In summary, our data obtained in cells exclusively expressing the single receptor subtype support the idea that the relative role of agonist affinity and efficacy may vary depending on the subtype of α-adrenoceptor. Since very few native tissues exclusively expressing a single receptor subtype have been identified, accurate information regarding agonist affinity and efficacy at the distinct receptor subtypes cannot be obtained. As exemplified in the present study, the approach using classical pharmacological methods to analyze the properties of cloned receptors in transfected cells may have important implications for the validation and improvement of receptor theory.

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References

ABEL, P.W. & MINNEMAN, K.P. (1986). α_1-Adrenergic receptor binding and contraction of rat caudal artery. *J. Pharmacol. Exp. Ther.*, **239**, 678–686.

COTECCHIA, S., SCHWINN, D.A., RANDALL, R.R., LEFKOWITZ, R.J., CARON, M.G. & KOBILKA, B.K. (1988). Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 7159–7163.

ESBENSHADE, T.A., HIRASAWA, A., TSUJIMOTO, G., TANAKA, T., YANO, J., MINNEMAN, K.P. & MURPHY, T.J. (1995). Cloning of the human α_{1d}-adrenergic receptor and inducible expression of three human subtypes in SKNMC cells. *Mol. Pharmacol.*, (in press).

GROSS, G., HANFT, G. & RUGEVICS, C.U. (1988). α_1-Adrenoceptors of rat myocardium: comparison of agonist binding and positive inotropic response. *Naunyn Schmied. Arch. Pharmacol.*, **338**, 582–588.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HIRAMOTO, T., KUSHIDA, H. & ENDOH, M. (1988). Further characterization of the myocardial α-adrenoceptors mediating positive inotropic effects in the rabbit myocardium. *Eur. J. Pharmacol.*, **152**, 301–310.

HIRASAWA, A., HORIE, K., TANAKA, T., TAKAGAKI, K., MURAI, M., YANO, J. & TSUJIMOTO, G. (1993). Cloning, functional expression and tissue distribution of human cDNA for the α_{1C}-adrenergic receptor. *Biochem. Biophys. Res. Commun.*, **195**, 902–909.

HORIE, K., HIRASAWA, A. & TSUJIMOTO, G. (1994). The pharmacological profile of cloned and stably expressed α_{1B}-adrenoceptors in CHO cells. *Eur. J. Pharmacol. (Mol. Pharmacol. Section)*, **268**, 399–407.

JIM, K.F., MACIA, R.A. & MATTHEWS, W.D. (1985). An evaluation of the ability of a series of full α_1-adrenoceptor agonists to release internal calcium in venous smooth muscle. *J. Pharmacol. Exp. Ther.*, **235**, 377–381.

KONNO, F. & TAKAYANAGI, I. (1989). Involvement of threshold level in the contractile responses for some α_1-adrenoceptor agonists in the rabbit iris dilators. *Gen. Pharmacol.*, **20**, 71–76.

LAZ, T.M., FORRAY, C., SMITH, K., BARD, J.A., VAYSSE, P.J.-J., BRANCHEK, T.A. & WEINSHANK, R.L. (1994). The rat homologue of the bovine α_{1c}-adrenergic receptor shows the pharmacological properties of the classical α_{1A} subtype. *Mol. Pharmacol.*, **46**, 414–422.

LOMASNEY, J.W., COTECCHIA, S., LORENZ, W., LEUNG, W.Y., SCHWINN, D.A., YANG, F.T., BROWNSTEIN, M., LEFKOWITZ, R.J. & CARON, M.G. (1991). 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.

MINNEMAN, K.P. & ESBENSHADE, T.A. (1994). α_1-Adrenergic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **34**, 117–133.

MINNEMAN, K.P., THEROUX, T.L., HOLLINGER, S., HAN, C. & ESBENSHADE, T.A. (1994). Selectivity of agonists for cloned α_1-adrenergic receptor subtypes. *Mol. Pharmacol.*, **46**, 929–936.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107**, 220–239.

NICHOLS, A.J. & RUFFOLO, R.R. Jr. (1991). Structure-activity and relationship for α-adrenoceptor agonists and antagonists. In *α-Adrenoceptors: Molecular Biology, Biochemistry and Pharmacology*. ed. Ruffolo, R.R.Jr., pp. 75–114. Basel: Karger.

PEREZ, D.M., PIASCIK, M.T. & GRAHAM, R.M. (1991). Solution-phase library screening for the identification of rare clones: isolation of an α_{1D}-adrenergic receptor cDNA. *Mol. Pharmacol.*, **40**, 876–883.

PEREZ, D.M., PIASCIK, M.T., MALIK, N., GAIVIN, R. & GRAHAM, R.M. (1994). Cloning, expression, and tissue distribution of the rat homolog of the bovine α_{1C}-adrenergic receptor provide evidence for its classification as the α_{1A} subtype. *Mol. Pharmacol.*, **46**, 823–831.

RUFFOLO, R.R.Jr. (1982). Review important concepts of receptor theory. *J. Auton. Pharmacol.*, **2**, 277–295.

RUFFOLO, R.R.Jr. & ZEID, R.L. (1985). Relationship between α-adrenoceptor occupancy and response for the α_1-adrenoceptor agonist, cirazoline, and the α_2-adrenoceptor agonist, B-HT 933, in canine saphenous vein. *J. Pharmacol. Exp. Ther.*, **235**, 636–643.

SCHWINN, D.A., LOMASNEY, J.W., LORENZ, W., SZKLUT, P.J., FREMEAU, R.J., YANG, F.T., CARON, M.G., LEFKOWITZ, R.J. & COTECCHIA, S. (1990). Molecular cloning and expression of the cDNA for a novel α_1-adrenergic receptor subtype. *J. Biol. Chem.*, **265**, 8183–8189.

SCHWINN, D.A., PAGE, S.O., MIDDLETON, J.P., LORENZ, W., LIGGETT, S.B., YAMAMOTO, K., LAPETINA, E.G., CARON, M.G., LEFKOWITZ, R.J. & COTECCHIA, S. (1991). The α_{1C}-adrenergic receptor: characterization of signal transduction pathways and mammalian tissue heterogeneity. *Mol. Pharmacol.*, **40**, 619–626.

TERMAN, B.I., SLIVKA, S.R., HUGHES, R.J. & INSEL, P.A. (1987). α_1-Adrenergic receptor-linked guanine nucleotide-binding protein in muscle and kidney epithelial cells. *Mol. Pharmacol.*, **31**, 12–20.

TSUJIMOTO, G., TSUJIMOTO, A., SUZUKI, E. & HASHIMOTO, K. (1989). 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.

WEINBERG, D.H., TRIVEDI, P., TAN, C.P., MITRA, S., PERKINS, B.A., BORKOWSKI, D., STRADER, C.D. & BAYNE, M. (1994). Cloning, expression and characterization of human α-adrenergic receptors α_{1a}, α_{1b} and α_{1c}. *Biochem. Biophys. Res. Commun.*, **201**, 1296–1304.

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