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# Selectivity of Agonists for Cloned $\alpha_1$-Adrenergic Receptor Subtypes

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**SUMMARY**

The potencies and intrinsic activities of agonists in activating cloned $\alpha_1$-adrenergic receptor (AR) subtypes were compared. The hamster $\alpha_{1B}$-, bovine $\alpha_{1C}$-, or rat $\alpha_{1A/D}$-ARs were expressed at high levels in human embryonic kidney 293 cells. Catecholamines and phenylethylamines, but not lower efficacy agonists, were more potent in inhibiting radioligand binding to the expressed $\alpha_{1A/D}$ subtype than to the $\alpha_{1B}$ or $\alpha_{1C}$ subtypes; this selectivity remained in the presence of different buffers, nucleotides, and cations. Activation of all three subtypes caused substantial increases in [$^3$H]inositol phosphate formation in cells grown in 96-well plates. Pretreatment with phenoxybenzamine decreased maximal responses to norepinephrine (NE) with only small decreases in apparent potency, suggesting similar small receptor reserves for all three subtypes. The catecholamines NE, epinephrine, and 6-fluoro-NE were full agonists with similar potencies at the three subtypes; $\alpha$-methyl-NE was also a full agonist but was about 20-fold less potent at $\alpha_{1B}$-ARs than at $\alpha_{1C}$- or $\alpha_{1A/D}$-ARs. Phenylephrine had similar potencies at all three subtypes but gave a submaximal response at $\alpha_{1B}$-ARs. Methoxamine was a full agonist at $\alpha_{1C}$- and $\alpha_{1A/D}$-ARs, with about 20-fold greater potency at the $\alpha_{1C}$ subtype, but showed lower intrinsic activity at $\alpha_{1B}$-ARs. A number of imidazolines, amidephrine, and SKF 89748 had substantial intrinsic activity at $\alpha_{1C}$-ARs but little or no intrinsic activity at the other two subtypes. We conclude that the potencies of many agonists in competing for radioligand binding sites are related to their potencies in activating functional responses but that this relationship is not the same for all subtypes. NE and epinephrine activate all three cloned $\alpha_1$-AR subtypes with similar potencies and intrinsic activities, but many widely used agonists show significant selectivity for different $\alpha_1$-AR subtypes.

The $\alpha_1$-ARs are one of the three major families of ARs mediating the actions of NE and EPI. Drugs acting on $\alpha_1$-ARs are useful research tools and have important clinical uses. Selective $\alpha_1$-AR antagonists are used for treatment of diseases such as hypertension, benign prostatic hyperplasia, and congestive heart failure; selective $\alpha_1$-AR agonists are used to treat nasal congestion, for pupillary dilation, to limit absorption of local anesthetics, and for several cardiovascular problems (1).

It is now clear that there are multiple, closely related, $\alpha_1$-AR subtypes (1–7). Two native subtypes ($\alpha_{1A}$ and $\alpha_{1B}$) can be distinguished with selective antagonists (1–3), whereas three subtypes have been cloned (4–7). The cloned subtypes are structurally homologous G protein-linked receptors (referred to here as $\alpha_{1B}$, $\alpha_{1C}$, and $\alpha_{1A/D}$). When expressed in heterologous systems, none of these cloned subtypes results in a receptor with the exact properties of the pharmacologically defined $\alpha_{1A}$ subtype. There may be another subtype yet to be cloned (1, 7–9), although it has been suggested that either the $\alpha_{1A/D}$ (10, 11) or $\alpha_{1C}$ (12) clone encodes the native $\alpha_{1A}$ subtype.

Antagonist specificities of $\alpha_1$-AR 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 (13), whereas radioligand binding studies are influenced by differential affinity states due to G protein coupling (14).

Some studies suggest that agonists may discriminate between $\alpha_1$-AR subtypes. Oxymetazoline shows selectivity in radioligand binding assays, with highest affinities for the native $\alpha_{1A}$ and cloned $\alpha_{1C}$ subtypes (1, 3, 5, 8). Other binding studies suggest that full agonists are more potent at the cloned $\alpha_{1A/D}$ subtype thhn at other subtypes (6–8). Receptor-mediated second messenger responses suggest that methoxamine and oxymetazoline may be selective for the $\alpha_{1A}$, $\alpha_{1A/D}$, and $\alpha_{1C}$ subtypes over the $\alpha_{1B}$ subtype (15, 16). Finally, agonists have different potencies and efficacies in initiating contraction of diverse blood vessels, although this has not yet been clearly correlated with specific subtypes (1, 17).

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**ABBREVIATIONS:** AR, adrenergic receptor; DMEM, Dulbecco's modified Eagle's medium; BE, BE 2254; InsP, inositol phosphate; PBS, phosphate-buffered saline; NE, norepinephrine; PE, phenylephrine; EPI, epinephrine; PBZ, phenoxybenzamine; HEK, human embryonic kidney.

Here we report on the agonist selectivity of cloned $\alpha_1$-AR subtypes, using vectors with strong, constitutively active promoters to drive high levels of expression of each subtype in mammalian cells. Because the three cloned subtypes all activate phospholipase C (4–7, 18) and increase InsP formation, we have used this response as a convenient measure of receptor activation. We also compare the concentration-response relationships for activating InsP formation and competing for radioligand binding sites in membrane preparations from the same cells.

## Experimental Procedures

**Materials.** Materials were obtained from the following sources: expression vectors pREP4, pREP8, and pREP9, Invitrogen; HEK 293 cells, American Type Culture Collection; calf serum and Geneticin, GIBCO; PBZ HCl and SKF 89748, SmithKline Beecham; phentolamine mesylate, Ciba-Geigy; (±)-α-methyl-NE HCl, Sterling Winthrop; 6-fluoro-NE and methoxamine HCl, Research Biochemicals; cirazoline, Synthelabo; oxymetazoline HCl, Schering Corp.; clonidine HCl and St 587, Boehringer Ingelheim; Sgd 101/75, Siegfried Zofingen; amidephrine mesylate, Bristol-Myers Squibb; [$^3$H]inositol (50–80 Ci/mmol), American Radiolabelled Chemicals; carrier-free Na$^{125}$I, Amersham Corp.; DMEM, (−)-NE bitartrate, (−)-EPI bitartrate, PE HCl, xylometazoline, histidinol, hygromycin, penicillin, streptomycin, and other reagents, Sigma.

**Stably transfected cell lines.** cDNAs for the hamster $\alpha_{1B}$-AR (4), bovine $\alpha_{1C}$-AR (5), and rat $\alpha_{1A/D}$-AR (7) were kindly provided by Dr. Jon Lomasney (Duke University), Dr. Robert Lefkowitz (Duke University), and Dr. Robert Graham (Cleveland Clinic), respectively. Full-length inserts were subcloned into the Epstein-Barr virus-based vectors pREP4 ($\alpha_{1B}$), pREP8 ($\alpha_{1C}$), and pREP9 ($\alpha_{1A/D}$), each containing a separate resistance marker for selection of mammalian cells. HEK 293 cells were maintained in DMEM containing 10% calf serum, 4.5 g/liter glucose, 100 mg/liter streptomycin, and $10^5$ units/liter penicillin. Cells were transfected by calcium phosphate precipitation with each of the cDNA-containing vectors (20 μg/100-mm plate) individually, allowed to recover for 3 days, and subjected to selection pressure with 0.05 mg/ml hygromycin (pREP4/$\alpha_{1B}$), 2 mg/ml histidinol (pREP8/$\alpha_{1C}$), or 0.15 mg/ml Geneticin (pREP9/$\alpha_{1A/D}$). Resistant cells were propagated and individual subclones were isolated by limiting dilution plating. Subclones were screened for receptor density and propagated in the continued presence of antibiotics.

**Membrane preparation.** Confluent 100-mm plates of cells were washed once with 5 ml of PBS (20 mM sodium phosphate, pH 7.6, 154 mM NaCl). Cells were scraped into 5 ml of PBS, and plates were washed with another 5 ml of buffer. The volume was increased to 30 ml/100-mm plate, and cells were homogenized with a Polytron homogenizer and centrifuged at 17,000 × *g* for 10 min. Pellets were resuspended in 10–300 ml of PBS/100-mm plate. In experiments where effects of buffers on agonist binding affinity were studied, the buffer used for harvesting, washing, and resuspending the membranes was 20 mM Tris·HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride.

**$^{125}$I-BE binding.** $^{125}$I-BE binding was performed in membrane preparations as described previously (3). BE was radioiodinated to theoretical specific activity (19) and stored at −20° in methanol. Specific $^{125}$I-BE binding was usually measured by incubating 0.1 ml of cell membranes with $^{125}$I-BE, in a final volume of 0.25 ml of PBS, for 20 min at 37° in the presence or absence of competing drugs. The incubation was terminated by addition of 10 ml of 10 mM Tris·HCl, pH 7.4, and filtering through a glass fiber filter (Schleicher and Schuell no. 30) under vacuum. The filters were washed once with 10 ml of 10 mM Tris·HCl, pH 7.4, and dried, and radioactivity was measured. Nonreceptor binding was determined in the presence of 10 μM phentolamine.

In some experiments, radioligand binding studies were performed under different incubation conditions, to examine effects on agonist binding affinity. In these experiments, membranes were prepared in 20 mM Tris·HCl containing 1 mM phenylmethylsulfonyl fluoride, and incubations were carried out in 20 mM Tris·HCl, pH 7.4, containing 10 mM $MgCl_2$ or 100 mM NaCl and 0.1 mM GTP. Other details were the same as described above.

Saturation curves were obtained by incubating cell membranes with increasing concentrations of $^{125}$I-BE. Displacement by competitive antagonists was determined by incubating a single concentration of $^{125}$I-BE (40–50 pM) in the presence or absence of 10–16 concentrations of drug. Saturation and displacement curves were analyzed by nonlinear regression analysis, and Hill coefficients were determined from a Hill plot.

**[$^3$H]InsP formation.** [$^3$H]InsP formation was determined on 35-mm plates as described previously (20). For rapid screening of multiple agonist concentration-response curves, most experiments were performed in 96-well plates. Transfected subclones were seeded at low density (1/20 dilution) and two drops (0.1–0.2 ml) of the diluted cells were seeded into each well of a 96-well plate. *myo*-[$^3$H]Inositol was added to the DMEM (1 μCi/ml) at the time of seeding, and cells were allowed to grow to confluence (3–5 days) before experiments were performed. On the day of the experiments, plates were washed by submersion into 1 liter of lithium-containing Kreb's Ringer bicarbonate buffer (110 mM NaCl, 10 mM LiCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 20 mM $NaHCO_3$, 11 mM glucose, 0.029 mM $Na_2EDTA$), where they were gently shaken for 30–60 sec. Plates were removed, and fluid was carefully aspirated from each well and replaced by lithium-containing Kreb's Ringer bicarbonate buffer containing drugs at the appropriate concentrations. Incubations were continued at 37° (in 95% $O_2$/5% $CO_2$) for 1 hr and were then stopped by addition of 0.1 ml of 20 mM formic acid. Wells were sonicated for 10 sec each and [$^3$H]InsPs were isolated by anion exchange chromatography as described (20). Values were normalized to a percentage of the maximal stimulation caused by 10 μM NE. NE caused no stimulation of [$^3$H]InsP formation in nontransfected cells (data not shown).

**PBZ inactivation.** For partial inactivation of the receptor reserve, PBZ was added directly to the culture medium (DMEM with calf serum, antibiotics, and [$^3$H]inositol) in the 96-well plates and cells were incubated for 30 min before washing. Washing was performed by submersion and gentle rocking as described above.

## Results

**Saturation binding of $^{125}$I-BE to expressed clones.** Subclones of HEK 293 cells transfected with the hamster $\alpha_{1B}$-AR, bovine $\alpha_{1C}$-AR, or rat $\alpha_{1A/D}$-AR cDNA-containing vectors were screened for high levels of $\alpha_1$-AR expression, and three subclones with the highest expression of each $\alpha_1$-AR subtype were propagated for further study. $^{125}$I-BE labeled apparently homogeneous populations of binding sites, with similar affinities, in membranes prepared from each of these cell lines (Table 1). The density of $\alpha_1$-AR binding sites in these subclones was highest for the $\alpha_{1B}$-AR and 8–15-fold lower for the $\alpha_{1C}$-AR and $\alpha_{1A/D}$-AR (Table 1).

**Agonist inhibition of specific $^{125}$I-BE binding.** The potencies of a series of agonists in inhibiting specific $^{125}$I-BE

TABLE 1

**Binding of $^{125}$I-BE to cloned $\alpha_1$-AR subtypes expressed in HEK 293 cells**

Saturation analysis of $^{125}$I-BE binding was performed on membrane preparations from subclones of HEK 293 cells expressing high levels of each subtype. Each value is the mean ± standard error of data from three to seven separate experiments performed in duplicate.

| | $K_D$ | $B_{max}$ |
|---|---|---|
| | pM | pmol/mg of protein |
| Hamster $\alpha_{1B}$-AR | 107 ± 28 | 16.0 ± 2.8 |
| Bovine $\alpha_{1C}$-AR | 61 ± 8 | 2.2 ± 0.4 |
| Rat $\alpha_{1A/D}$-AR | 76 ± 23 | 1.1 ± 0.4 |

binding in membrane preparations from each of the transfected cell lines are shown in Table 2. As reported previously (6, 8), catecholamines and phenylethylamines were generally more potent in inhibiting $^{125}$I-BE binding at the expressed $\alpha_{1A/D}$-AR than at the $\alpha_{1B}$- or $\alpha_{1C}$-AR subtypes. This was less true for imidazolines and other agonists (Fig. 1; Table 2).

Most of the agonist inhibition curves were best fit to a single-site model, reflected in Hill coefficients between 0.8 and 1.0. A few curves (six of 42) fit better to a two-site competition curve (Table 2), although this did not appear to correlate with a specific subtype, agonist structure, or degree of intrinsic activity.

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 (14) was tested by altering cation and nucleotide concentrations in the radioligand binding assays. The apparent $K_i$ for NE at each of the subtypes was not significantly altered when 10 mM $MgCl_2$ or 0.1 mM GTP was added to the PBS (data not shown). In an attempt to induce high and low affinity agonist binding states, experiments were conducted in 20 mM Tris plus 10 mM $MgCl_2$ to promote high affinity agonist binding or in 20 mM Tris plus 100 mM NaCl plus 0.1 mM GTP to promote low affinity agonist binding. Neither of these conditions significantly increased the affinity of NE for the $\alpha_{1B}$ subtype or decreased the affinity of NE for the $\alpha_{1A/D}$ subtype (Table 3), although $Mg^{2+}$ did cause a 6-fold increase in NE affinity for the $\alpha_{1C}$ subtype. The apparent selectivity of NE for the expressed rat $\alpha_{1A/D}$ subtype remained under all experimental conditions studied (Tables 2 and 3 and data not shown).

**Stimulation of [$^3$H]InsP formation.** The ability of each subtype to activate a response was determined by prelabeling 35-mm plates of cells with *myo*-[$^3$H]inositol (1 $\mu$Ci) for 2 days and measuring agonist-stimulated [$^3$H]InsP formation. Both NE (100 $\mu$M) and PE (100 $\mu$M) caused large increases in [$^3$H]InsP formation in cells transfected with each of the three cloned subtypes (Fig. 2). Stimulation was very large in the $\alpha_{1B}$ and $\alpha_{1C}$ subclones, reaching conversion of 65–70% of total incorporated *myo*-[$^3$H]inositol to [$^3$H]InsPs. Although the response to NE in the $\alpha_{1A/D}$ subclone was smaller, it was still large, reaching 40% hydrolysis of total incorporated label. Concentration-response curves for PE activation of responses in these subclones are also shown in Fig. 2. PE showed similar potencies in activating responses in all three subclones ($EC_{50}$ values of 70–170 nM).

**[$^3$H]InsP responses in 96-well plates.** [$^3$H]InsP responses to NE and PE were examined in cells grown in 96-well plates, to facilitate screening of concentration-response curves for a large number of agonists. Although responses were generally smaller than observed in 35-mm plates, they were still easily measurable. Composite concentration-response curves for NE-stimulated [$^3$H]InsP formation for each of the subclones in 96-well plates are shown in Fig. 3. In 14 experiments, responses to 10 $\mu$M NE averaged 1150 ± 180%, 560 ± 60%, and 254 ± 21% of basal levels for $\alpha_{1B}$-AR-, $\alpha_{1C}$-AR-, and $\alpha_{1A/D}$-AR-expressing subclones, respectively. With maximal agonist stimulation, percentage hydrolysis of total [$^3$H]inositol incorporated into lipid averaged 25%, 12%, and 10% for $\alpha_{1B}$-AR-, $\alpha_{1C}$-AR-, and $\alpha_{1A/D}$-AR-expressing subclones, respectively.

**PBZ inactivation of receptor reserves.** Because the degree of receptor reserve markedly affects the potencies and maximal responses of agonists at the different subtypes, we used the irreversible alkylating agent PBZ to progressively inactivate the receptors in each subclone and examined changes in NE responsiveness. Pretreatment with PBZ (30 or 300 nM for 30 min) caused significant decreases in maximal response (16–40%) for each of the three subclones, with only small effects (<5-fold) on the apparent potency of NE in activating the [$^3$H]InsP response (Fig. 4; Table 4). The method of Furchgott (21) was used to calculate $K_a$ values for NE at each subtype from these data (Table 4). $K_a$ values, like $EC_{50}$ values, showed no significant difference between subtypes.

**Activation by catecholamines.** NE, EPI, $\alpha$-methyl-NE, and 6-fluoro-NE could all elicit similar maximal [$^3$H]InsP responses in cells transfected with each of the three cloned subtypes (Fig. 5), although responses to EPI were routinely 16–30% higher than those to NE (Table 5). NE, EPI, and 6-fluoro-

TABLE 2

**Inhibition by agonists of specific $^{125}$I-BE binding at cloned $\alpha_1$-AR subtypes**

Concentration dependence of agonists in inhibiting specific $^{125}$I-BE binding to membrane preparations of HEK 293 cells expressing high levels of each subtype was determined as described in the text. $K_i$ values were determined by nonlinear regression, and Hill coefficients ($n_H$) were calculated from Hill plots. Each value is the mean ± standard error of data from three to five separate experiments performed in duplicate.

| | −log $K_i$ | | | $n_H$ | | |
|---|---|---|---|---|---|---|
| | Hamster $\alpha_{1B}$-AR | Bovine $\alpha_{1C}$-AR | Rat $\alpha_{1A/D}$-AR | Hamster $\alpha_{1B}$-AR | Bovine $\alpha_{1C}$-AR | Rat $\alpha_{1A/D}$-AR |
| Catecholamines | | | | | | |
| NE | 5.00 ± 0.08 | 4.39 ± 0.03 | 6.30 ± 0.04 | 0.90 ± 0.18 | 0.87 ± 0.04 | 0.88 ± 0.05[a] |
| EPI | 5.01 ± 0.06 | 4.84 ± 0.06 | 6.29 ± 0.05 | 0.85 ± 0.05 | 0.90 ± 0.07 | 0.84 ± 0.05[a] |
| 6-Fluoro-NE | 4.85 ± 0.04 | 4.71 ± 0.14 | 5.98 ± 0.04 | 0.86 ± 0.04 | 0.76 ± 0.03 | 0.92 ± 0.06 |
| $\alpha$-Methyl-NE | 4.10 ± 0.05 | 4.48 ± 0.18 | 5.58 ± 0.04 | 0.89 ± 0.02 | 0.73 ± 0.04 | 0.85 ± 0.08 |
| Phenylethylamines | | | | | | |
| PE | 4.87 ± 0.06 | 4.70 ± 0.07 | 5.86 ± 0.05 | 0.89 ± 0.09 | 0.83 ± 0.07 | 0.84 ± 0.08[a] |
| Methoxamine | 3.38 ± 0.05 | 3.83 ± 0.06 | 4.50 ± 0.05 | 0.90 ± 0.02 | 0.84 ± 0.04[a] | 0.85 ± 0.08 |
| Imidazolines | | | | | | |
| Cirazoline | 6.19 ± 0.04 | 6.30 ± 0.03 | 6.92 ± 0.04 | 0.89 ± 0.03 | 0.99 ± 0.09 | 0.82 ± 0.04[a] |
| Oxymetazoline | 6.76 ± 0.03 | 7.50 ± 0.06 | 6.22 ± 0.09 | 0.90 ± 0.04 | 0.91 ± 0.06 | 0.87 ± 0.04 |
| Clonidine | 6.30 ± 0.05 | 6.00 ± 0.03 | 6.88 ± 0.03 | 0.96 ± 0.14 | 0.81 ± 0.07 | 0.74 ± 0.11 |
| Xylometazoline | 6.67 ± 0.03 | 7.31 ± 0.04 | 6.05 ± 0.04 | 0.94 ± 0.08 | 0.87 ± 0.01 | 0.87 ± 0.06 |
| Sgd 101/75 | 5.21 ± 0.12 | 5.15 ± 0.05 | 5.53 ± 0.06 | 0.86 ± 0.06[a] | 0.88 ± 0.01 | 0.95 ± 0.02 |
| St 587 | 5.38 ± 0.03 | 5.63 ± 0.06 | 6.47 ± 0.06 | 0.91 ± 0.05 | 0.84 ± 0.04 | 0.97 ± 0.06 |
| Other agonists | | | | | | |
| Amidephrine | 4.06 ± 0.11 | 4.91 ± 0.08 | 4.18 ± 0.10 | 0.80 ± 0.04 | 0.84 ± 0.10 | 0.98 ± 0.21 |
| SKF 89748 | 5.32 ± 0.03 | 5.88 ± 0.05 | 6.08 ± 0.03 | 0.86 ± 0.02 | 0.76 ± 0.07 | 0.87 ± 0.12 |

[a] Significantly better two-site fit (not shown).

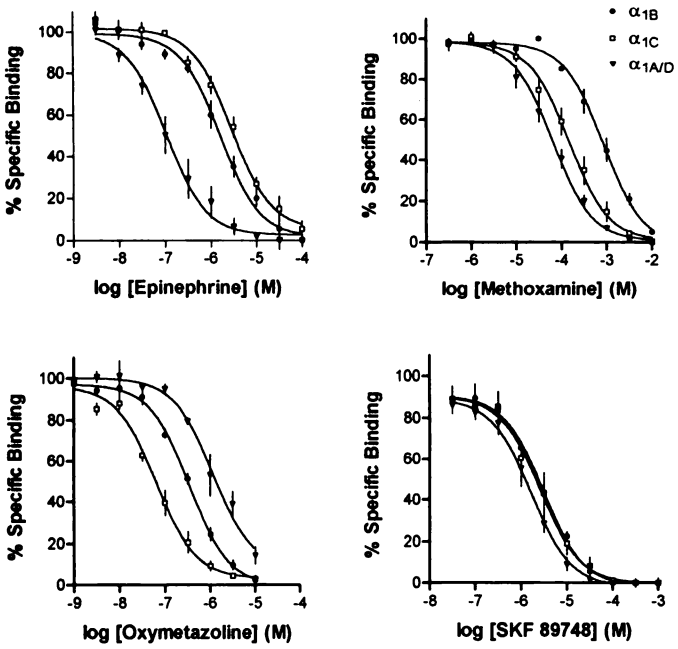



**Fig. 1.** Inhibition by agonists of specific $^{125}$I-BE binding in membrane preparations. Membranes from transfected cells were incubated with $^{125}$I-BE and drugs as described in the text. Each value is the mean ± standard error of data from three or four separate experiments performed in duplicate.

TABLE 3

**Effect of nucleotides and cations on NE inhibition of specific $^{125}$I-BE binding to cloned $\alpha_1$-AR subtypes**

All binding experiments were performed in 20 mM Tris · HCl, pH 7.4, in the presence of 10 mM $MgCl_2$ (Tris/$Mg^{2+}$) or 100 mM NaCl and 100 μM GTP (Tris/$Na^+$/GTP), as described in the text. Membranes were harvested in the presence of 1 mM phenylmethylsulfonyl fluoride. Each value is the mean ± standard error of data from three experiments performed in duplicate.

| | −log $K_i$ | | |
|---|---|---|---|
| | Hamster $\alpha_{1B}$-AR | Bovine $\alpha_{1C}$-AR | Rat $\alpha_{1A/D}$-AR |
| Tris/$Mg^{2+}$ | 5.10 ± 0.095 | 5.38 ± 0.063 | 6.09 ± 0.034 |
| Tris/$Na^+$/GTP | 4.80 ± 0.091 | 4.58 ± 0.068[a] | 5.97 ± 0.012 |

[a] $P < 0.001$, compared with Tris/$Mg^{2+}$.

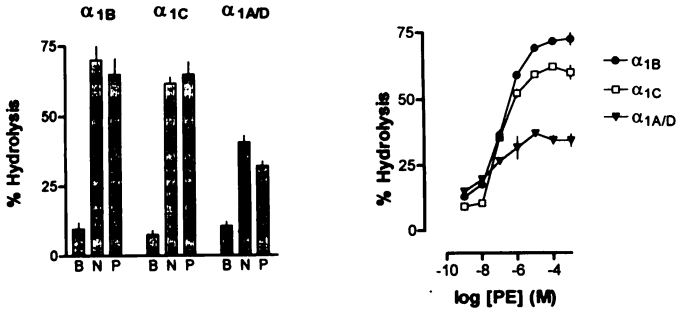



**Fig. 2.** Agonist-stimulated [$^3$H]InsP formation in subclones. *Left*, subclones ($\alpha_{1B}$ clone 5, $\alpha_{1C}$ clone 5, and $\alpha_{1A/D}$ clone 3) were grown in 35-mm dishes and labeled with 1 μCi of [$^3$H]inositol for 2 days. [$^3$H]InsP formation in the absence of drug (basal) (*B*) or in the presence of 100 μM NE (*N*) or PE (*P*) was determined as described in the text. Values are expressed as percentage of total [$^3$H]inositol converted to [$^3$H] inositol-1-phosphate (percentage hydrolysis) and are the mean ± standard error of four to nine separate determinations. *Right*, concentration-response curves for PE in activating [$^3$H]InsP formation in subclones grown in 35-mm dishes are shown. Each value is the mean ± standard error of duplicate values from a single experiment.

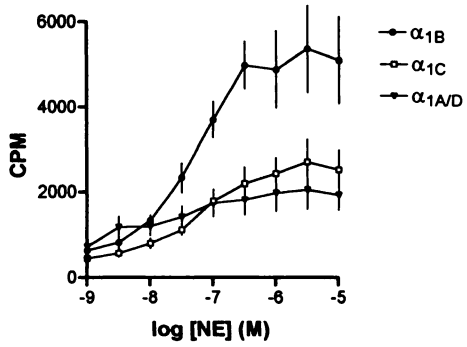



**Fig. 3.** Activation by NE of [$^3$H]InsP formation in subclones grown in 96-well plates. Cells were seeded in 96-well plates and labeled with [$^3$H] inositol, and NE stimulation of [$^3$H]InsP formation was determined as described in the text. Each value is expressed as cpm recovered and is the mean ± standard error of data from six to eight experiments, each performed in duplicate. Total incorporation in each well averaged 23,249 ± 2,613, 21,004 ± 2,164, and 21,414 ± 2,300 cpm for $\alpha_{1B}$, $\alpha_{1C}$, and $\alpha_{1A/D}$ subclones, respectively.

NE showed only small differences in potency in activating the three cloned subtypes, although 6-fluoro-NE was about 5–6-fold less potent at $\alpha_{1A/D}$-ARs than $\alpha_{1B}$-ARs or $\alpha_{1C}$-ARs. α-Methyl-NE was also a full agonist at each subtype but was about 20-fold less potent at the $\alpha_{1B}$-ARs than at the other two subtypes (Table 5).

**Activation by phenylethylamines.** PE was a full agonist in stimulating [$^3$H]InsP formation at the $\alpha_{1C}$ and $\alpha_{1A/D}$ subtypes but stimulated a maximum of 73% of the NE response at the $\alpha_{1B}$ subtype (Fig. 6). PE had similar potencies at all three subtypes, with <4-fold differences in $EC_{50}$. Methoxamine gave a maximal response at both the $\alpha_{1C}$ and $\alpha_{1A/D}$ subtypes but only a half-maximal response at the $\alpha_{1B}$ subtype. Methoxamine was 13–20-fold more potent in activating $\alpha_{1C}$-ARs than $\alpha_{1B}$-ARs or $\alpha_{1A/D}$-ARs (Table 5). Ephedrine and phenylpropanolamine gave small responses at each subtype at up to 10 μM (data not shown).

**Activation by imidazolines.** Cirazoline, a widely used imidazoline agonist, was highly selective for the $\alpha_{1C}$ subtype (Fig. 7). Cirazoline gave 74% of the maximal [$^3$H]InsP response to NE at the $\alpha_{1C}$ subtype but much smaller responses at the $\alpha_{1B}$ and $\alpha_{1A/D}$ subtypes. The other imidazoline agonists tested, including oxymetazoline, clonidine, xylometazoline, Sgd 101/75, and St 587, were even more selective for the $\alpha_{1C}$ subtype, with no measurable intrinsic activity at the $\alpha_{1B}$ or $\alpha_{1A/D}$ subtypes (Fig. 7; Table 5). Tramazoline and tolazoline had little measurable activity at any of the subtypes (data not shown).

**Activation by other selective agonists.** Both the substituted phenylethylamine amidephrine and the cyclized compound SKF 89748 were also $\alpha_{1C}$ selective. Amidephrine gave a maximal [$^3$H]InsP response at the $\alpha_{1C}$ subtype but elicited only small responses at the $\alpha_{1B}$ and $\alpha_{1A/D}$ subtypes (Fig. 8). SKF 89748 was a potent agonist with high intrinsic activity at the $\alpha_{1C}$ subtype (Fig. 8). SKF 89748 also caused large responses in cells transfected with the $\alpha_{1A/D}$ subtype, but only at very high agonist concentrations. SKF 89748 gave only a small response at the $\alpha_{1B}$ subtype, although with a fairly high potency (Table 5).

**Comparison of radioligand binding and functional data.** The relationship between the potencies of agonists in competing for radioligand binding in membranes (−log $K_i$) and their potencies in activating responses in intact cells (−log $EC_{50}$) is shown in Fig. 9, using data from Tables 2 and 5.

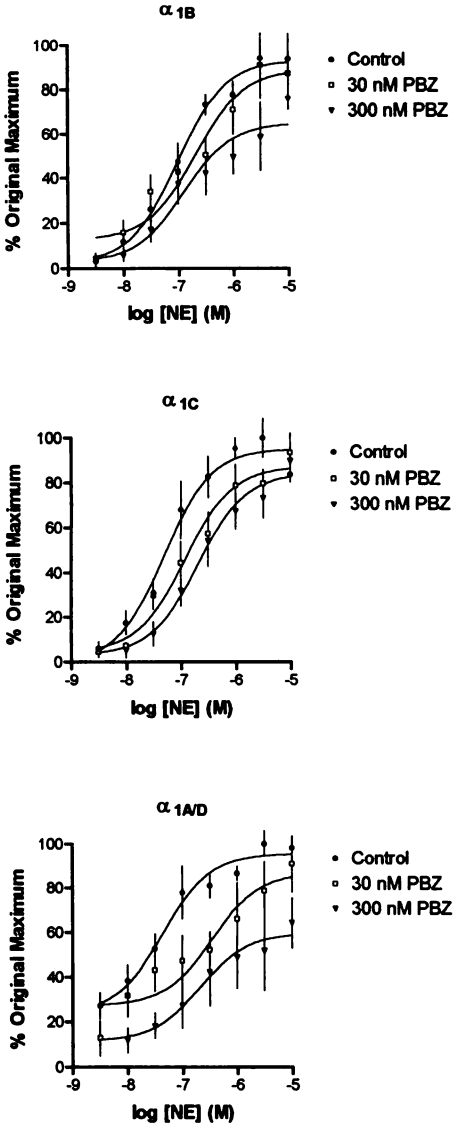



**Fig. 4.** Effect of PBZ pretreatment on NE-stimulated [$^3$H]InsP formation in transfected cell lines. Cells in 96-well plates were prelabeled for 3–5 days with [$^3$H]inositol and pretreated for 30 min with 0 (*Control*), 30 nM, or 300 nM PBZ. NE-stimulated [$^3$H]InsP formation was determined as described in the text. Values are plotted as a percentage of the original maximal response and represent the mean ± standard error of data from four or five separate experiments.

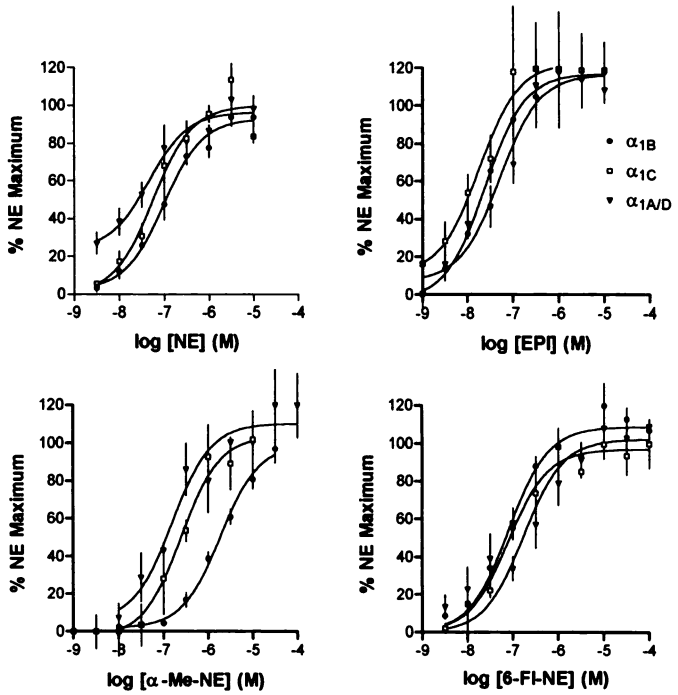



**Fig. 5.** Effects of catecholamines on cloned $\alpha_1$-AR subtypes expressed in HEK 293 cells. Values are expressed as a percentage of the maximal response to NE and represent the mean ± standard error of data from three to five separate experiments performed in duplicate. *α-Me-NE*, α-methyl-NE; *6-Fl-NE*, 6-fluoro-NE.

Catecholamines and phenylethylamines, as "high efficacy" agonists, are grouped together in Fig. 9, *upper*, and imidazolines are shown in Fig. 9, *lower*. There is a highly significant linear correlation between the two parameters, although the values for the $\alpha_{1B}$- and $\alpha_{1C}$-ARs are not close to the line of identity. The $K_i$ and $EC_{50}$ values for catecholamines differed by 43–589-fold for the $\alpha_{1B}$-AR and by 251–631-fold for the $\alpha_{1C}$-AR but by only 2–26-fold for the $\alpha_{1A/D}$-AR. $K_i$ and $EC_{50}$ values were more similar for imidazolines (Fig. 9).

## Discussion

We have systematically examined the selectivities of widely used agonists at the three cloned $\alpha_1$-AR subtypes. Although binding and functional differences observed previously have raised the possibility that some of these drugs might be subtype selective, this has never been quantitatively evaluated. We found that, although the catecholamines are generally full agonists with little subtype selectivity, many synthetic agonists are highly selective for the $\alpha_{1C}$ subtype, having substantially lower relative efficacies at the $\alpha_{1B}$ and $\alpha_{1A/D}$ subtypes.

Subclones of transfected HEK 293 cells that expressed high levels of hamster $\alpha_{1B}$-ARs, bovine $\alpha_{1C}$-ARs, and rat $\alpha_{1A/D}$-ARs were identified. Expression of the $\alpha_{1B}$ subtype was generally higher than that of the other two subtypes, and the subclones with the highest expression levels were used for further analysis. Although this resulted in the use of cells with different expression levels (1–16 pmol/mg of protein), which might complicate pharmacological analyses, we wanted to maximize our ability to evaluate low efficacy agonists. As reported previously (4–7, 18, 22), each subtype activated [$^3$H]InsP formation in transfected cells, although with different maximal stimulation by NE. The $\alpha_{1B}$-ARs, $\alpha_{1C}$-ARs, and $\alpha_{1A/D}$-ARs gave 1050%, 460%, and 150% increases over basal levels, respectively, which

TABLE 4

**Effect of PBZ pretreatment on NE-stimulated [$^3$H]InsP formation in transfected cell lines**

Data are from Fig. 4. $K_a$ values were calculated by the technique of Furchgott (21). Each value is the mean ± standard error of three to five determinations.

| | Hamster $\alpha_{1B}$-AR | Bovine $\alpha_{1C}$-AR | Rat $\alpha_{1A/D}$-AR |
|---|---|---|---|
| Control, –log $EC_{50}$ | 7.0 ± 0.08 | 7.3 ± 0.15 | 7.4 ± 0.15 |
| 30 nM PBZ | | | |
| –log $EC_{50}$ | 6.7 ± 0.21 | 6.9 ± 0.15 | 6.5 ± 0.31[a] |
| Maximum (% of control) | 89 ± 6.7 | 87 ± 4.4[a] | 87 ± 8.7 |
| 300 nM PBZ | | | |
| –log $EC_{50}$ | 6.9 ± 0.24 | 6.7± 0.11 | 6.7 ± 0.14 |
| Maximum (% of control) | 65 ± 5.5[a] | 84 ± 3.7[a] | 60 ± 2.8[a] |
| Calculated, –log $K_a$ | 6.5 ± 0.25 | 6.0 ± 0.13 | 6.3 ± 0.40 |

[a] $P < 0.05$, compared with control.

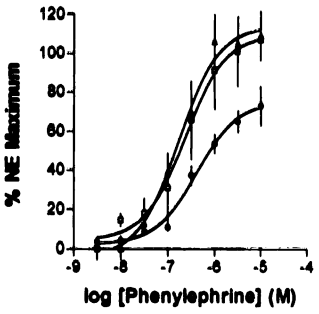
TABLE 5

**Potencies and intrinsic activities of agonists in activating cloned $\alpha_1$-AR subtypes**

Dose-response curves for each compound in activating [$^3$H]InsP formation in HEK 293 cells expressing high levels of each subtype were determined as described in the text. $EC_{50}$ and maximum values were determined by nonlinear regression analysis. Each value is the mean ± standard error of data from three to five separate experiments performed in duplicate.

| | −log $EC_{50}$ | | | Maximum | | |
|---|---|---|---|---|---|---|
| | Hamster $\alpha_{1B}$-AR | Bovine $\alpha_{1C}$-AR | Rat $\alpha_{1A/D}$-AR | Hamster $\alpha_{1B}$-AR | Bovine $\alpha_{1C}$-AR | Rat $\alpha_{1A/D}$-AR |
| | | | | % of NE maximum | | |
| **Catecholamines** | | | | | | |
| NE | 7.32 ± 0.07 | 7.19 ± 0.07 | 7.62 ± 0.20 | 100 | 100 | 100 |
| EPI | 7.62 ± 0.04 | 7.66 ± 0.15 | 7.30 ± 0.15 | 117 ± 1.5 | 130 ± 6.8 | 116 ± 5.6 |
| 6-Fluoro-NE | 7.00 ± 0.12 | 7.11 ± 0.11 | 6.33 ± 0.12 | 109 ± 3.5 | 97 ± 3.0 | 107 ± 3.7 |
| α-Methyl-NE | 5.73 ± 0.06 | 7.24 ± 0.10 | 7.00 ± 0.22 | 100 ± 2.9 | 102 ± 4.5 | 94 ± 1.7 |
| **Phenylethylamines** | | | | | | |
| PE | 6.47 ± 0.05 | 6.77 ± 0.08 | 7.01 ± 0.22 | 73 ± 1.5 | 114 ± 3.7 | 125 ± 10.2 |
| Methoxamine | 4.63 ± 0.10 | 5.76 ± 0.17 | 4.43 ± 0.17 | 52 ± 2.3 | 107 ± 5.6 | 102 ± 7.3 |
| **Imidazolines** | | | | | | |
| Cirazoline | 6.87 ± 0.16 | 7.65 ± 0.17 | 7.32 ± 0.42 | 16 ± 1 | 74 ± 3.7 | 38 ± 4.5 |
| Oxymetazoline | | 8.10 ± 0.19 | | NE[a] | 57 ± 2.0 | NE |
| Clonidine | | 7.09 ± 0.21 | | NE | 64 ± 2.8 | NE |
| Xylometazoline | | 7.39 ± 0.48 | | NE | 41 ± 4.9 | NE |
| Sgd 101/75 | | 5.70 ± 0.10 | | NE | 55 ± 2.2 | NE |
| St 587 | | 6.85 ± 0.06 | | NE | 88 ± 2.3 | NE |
| **Other agonists** | | | | | | |
| Amidephrine | | 6.14 ± 0.32 | | 5 ± 0 | 81 ± 9.4 | 14 ± 4.7 |
| SKF89748 | 6.13 ± 0.33 | 7.09 ± 0.15 | 4.0 ± 0.19 | 27 ± 1.9 | 92 ± 4.3 | 81± 10.1 |

[a] NE, no measurable effects.



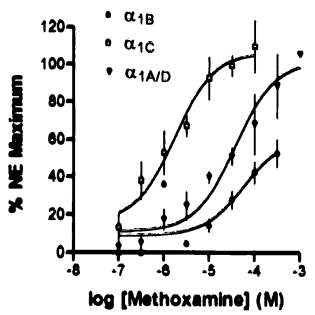
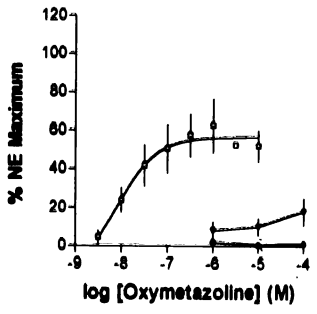
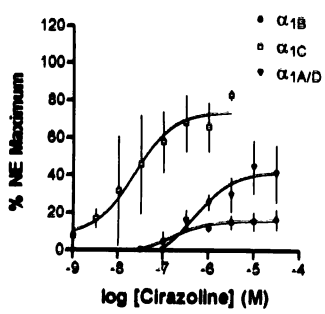
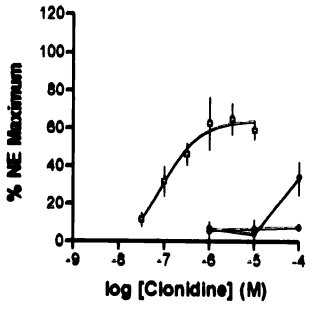




Fig. 6. Effects of phenethylamines on cloned $\alpha_1$-AR subtypes expressed in HEK 293 cells. Values are expressed as a percentage of the maximal response to NE and represent the mean ± standard error of data from four or five separate experiments performed in duplicate.



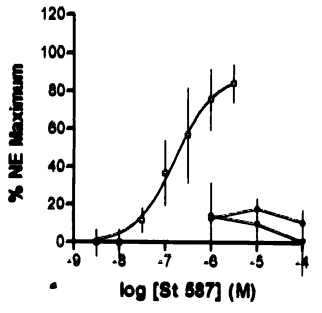




Fig. 7. Effects of imidazolines on cloned $\alpha_1$-AR subtypes expressed in HEK 293 cells. Values are expressed as a percentage of the maximal response to NE and represent the mean ± standard error of data from three to five separate experiments performed in duplicate.

generally correlates with their expression levels (16, 2.2, and 1.1 pmol/mg of protein, respectively). An exact correlation is not expected, because the $\alpha_{1C}$ subtype has been suggested to couple more efficiently to [$^3$H]InsP formation than do the $\alpha_{1B}$ or $\alpha_{1A/D}$ subtypes (18, 22), consistent with our observations.

Evaluation of agonist selectivity is complicated by the possibility of nonlinear relationships between receptor occupancy and response (13). 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. We studied concentration-response curves for agonist-stimulated [$^3$H]InsP formation after partial receptor inactivation (21) with the site-directed alkylating agent PBZ. We could not wash the cells multiple times because they tended to detach from the wells, so we performed PBZ pretreatment in serum-containing medium. High concentrations of PBZ (30–300 nM) were required to produce observable effects, probably because of the serum. However, in cells expressing each of the three subtypes, PBZ pretreatment decreased the maximal response to NE with only small effects on $EC_{50}$. The ratio between calculated $K_a$ and $EC_{50}$ suggested only small receptor reserves for any of the subtypes in these cells. Although this ratio appeared smaller for the $\alpha_{1B}$ subtype than for the $\alpha_{1C}$ or $\alpha_{1A/D}$ subtype, because the $\alpha_{1B}$ subtype showed by far the highest expression levels and neither $EC_{50}$ nor $K_a$ values differed significantly from those for the other subtypes this difference is probably not significant. Overall, although a moderate receptor reserve cannot be ruled out for any of the three subtypes, potency and observed intrinsic activity appear to be first approximations of $K_a$ and relative intrinsic efficacy, respectively.

The four catecholamines tested were full agonists at all three

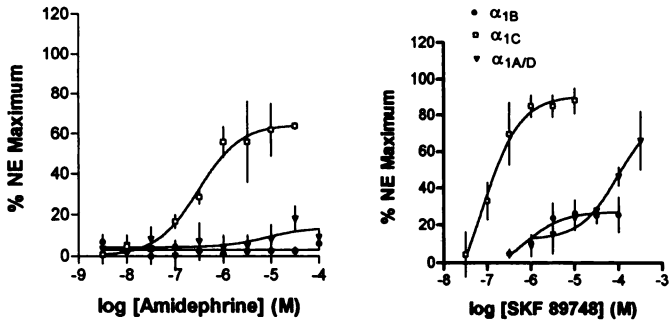



**Fig. 8.** Effects of other agonists on cloned $\alpha_1$-AR subtypes expressed in HEK 293 cells. Values are expressed as a percentage of the maximal response to NE and represent the mean ± standard error of data from three or four separate experiments performed in duplicate.

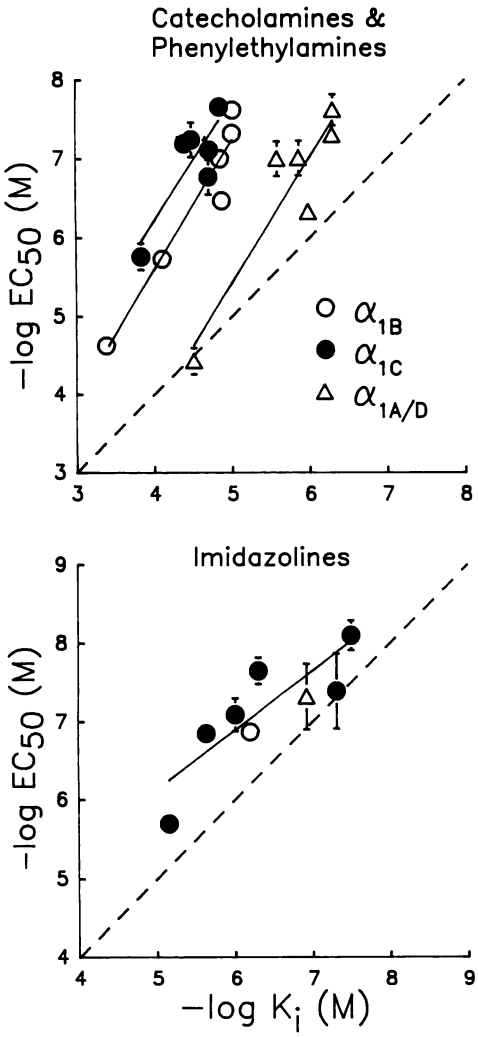



**Fig. 9.** Comparison of $EC_{50}$ and $K_i$ values for catecholamines and phenylethylamines (*upper*) and imidazolines (*lower*). Data are from Tables 2 and 5. *Dashed line*, line of identity. Linear regression gave the following results: *upper*, $\alpha_{1B}$, $r^2 = 0.96$, slope = 1.63; $\alpha_{1C}$, $r^2 = 0.85$, slope = 1.52; $\alpha_{1A/D}$, $r^2 = 0.92$, slope = 1.58; *lower*, $\alpha_{1C}$, $r^2 = 0.75$, slope = 0.85.

subtypes. NE, EPI, and 6-fluoro-NE had similar potencies in activating each subtype, but α-methyl-NE was 20-fold less potent at the $\alpha_{1B}$ subtype. PE and methoxamine, phenylethylamines that are widely used as selective $\alpha_1$-AR agonists, were full agonists at $\alpha_{1C}$- and $\alpha_{1A/D}$-ARs but had submaximal intrinsic activity at the $\alpha_{1B}$ subtype. PE and methoxamine are usually assumed to be full agonists; however, there are reports of submaximal intrinsic activity in the literature (15–17, 23). PE showed similar potencies at all three subtypes, but methoxamine was 20-fold more potent at the $\alpha_{1C}$ subtype than at the other two subtypes.

Several imidazolines have been introduced as selective $\alpha_1$-AR agonists (24). Oxymetazoline is one of the most selective of the currently available drugs for distinguishing $\alpha_1$-AR subtypes with radioligand binding techniques (1), and cirazoline is widely used as a selective agonist in isolated tissue studies (24). However, all six of the imidazolines we tested were selective for the $\alpha_{1C}$ subtype and had little or no intrinsic activity at the other two subtypes. Even clonidine, which is usually considered a selective $\alpha_2$-AR agonist, had an intrinsic activity at the $\alpha_{1C}$ subtype that was almost as large as that of cirazoline. Oxymetazoline, which is usually considered a fairly low efficacy agonist, also had substantial intrinsic activity at the $\alpha_{1C}$ subtype.

Amidephrine, a substituted phenylethylamine, and SKF 89748, a cyclized phenylethylamine, are also $\alpha_1$-AR-selective agonists that have been reported to show selectivity between $\alpha_1$-ARs in different tissues (24). We found that amidephrine and SKF 89748, like the imidazolines, were highly selective for the $\alpha_{1C}$ subtype, with much lower intrinsic activity at the $\alpha_{1B}$ and $\alpha_{1A/D}$ subtypes. Overall, it is clear that many widely used agonists show selectivity between $\alpha_1$-AR subtypes. Such selectivity will greatly complicate interpretation of the effects of these drugs in isolated tissues containing mixtures of subtypes but may also be useful, when used in conjunction with subtype-selective antagonists, in separating responses to individual $\alpha_1$-AR subtypes. It is interesting, however, that such widely used agonists as methoxamine, cirazoline, amidephrine, and SKF 89748 are all quite selective for the $\alpha_{1C}$ subtype, suggesting that many of the previously observed effects of these compounds may be due to activation of only a subpopulation of the total $\alpha_1$-ARs in tissues.

In contrast to our functional studies in intact cells, where catecholamines and PE showed similar potencies in activating all three subtypes, radioligand binding studies on membranes have suggested that NE, EPI, and PE are much more potent at the expressed $\alpha_{1A/D}$ subtype than at the $\alpha_{1B}$ or $\alpha_{1C}$ subtypes (6, 8). We have confirmed and extended these data, comparing membrane binding properties of all three subtypes expressed in the same cell line. All drugs we studied that had substantial intrinsic activity at each of the three subtypes were 5–30-fold more potent in competing for radioligand binding to the $\alpha_{1A/D}$ subtype, compared with the $\alpha_{1B}$ and $\alpha_{1C}$ subtypes. Even methoxamine, which was 10-fold less potent in activating the $\alpha_{1A/D}$ subtype, compared with the $\alpha_{1C}$ subtype, was 5-fold more potent in inhibiting binding at the $\alpha_{1A/D}$ subtype than at the $\alpha_{1C}$ subtype. Other drugs that did not significantly activate the $\alpha_{1B}$ or $\alpha_{1A/D}$ subtypes but had intrinsic activity at the $\alpha_{1C}$ subtype did not show such selectivity in radioligand binding assays.

Comparison of functional $EC_{50}$ and binding $K_i$ values reveals a highly significant linear relationship for each subtype (Fig. 9), suggesting that the rank orders of agonist potency are similar in binding and functional experiments. However, for catecholamines and phenylethylamines the values for the $\alpha_{1B}$ and $\alpha_{1C}$ subtypes are grouped together away from the line of identity, whereas the values for the $\alpha_{1A/D}$ subtype are much closer. This suggests that for these agonists the binding and functional measurements differ by a semiquantitative factor that appears to be subtype dependent and independent of receptor reserve. Imidazoline agonists showed fewer differences

between $EC_{50}$ and $K_i$ values, although in most cases $EC_{50}$ values could be obtained only for the $\alpha_{1C}$ subtype.

The differences between the binding and functional data are difficult to explain. It should be noted that NE and EPI do not show biphasic inhibition curves in radioligand binding assays in membranes from animal tissues, and the apparent potencies ($-\log K_i$ of approximately 5.0) and Hill coefficients (0.7–0.8) in rat tissues (vas deferens, spleen, hippocampus, and liver) (25) and cell lines (14) known to contain different subtypes are similar to those observed here for the expressed $\alpha_{1B}$ and $\alpha_{1C}$ subtypes. Affinity values of NE and EPI for native $\alpha_1$-ARs similar to those seen at the expressed $\alpha_{1A/D}$ subtype ($\log K_i$ of approximately 6.3) can be seen only when high affinity agonist binding states are favored (14, 26).

The expressed $\alpha_{1A/D}$-ARs (but not the $\alpha_{1B}$- and $\alpha_{1C}$-ARs) might be "locked" in a state with high affinity for agonists in these cells. However, promoting high affinity agonist binding with $Mg^{2+}$ did not increase NE affinity for the $\alpha_{1B}$ subtype, and promoting low affinity agonist binding with NaCl and GTP did not decrease NE affinity for the $\alpha_{1A/D}$ subtype. This does not support the conclusion that different agonist/receptor/G protein ternary complex formation is responsible for the observed differences in agonist affinity. $Mg^{2+}$ did increase the affinity of NE for the $\alpha_{1C}$ subtype, but this "high affinity" state was still of lower affinity than the "low affinity" state of the $\alpha_{1A/D}$ subtype. Other explanations for the observed differences in agonist binding affinity, possibly related to differences in desensitization or other compensatory processes occurring in intact cells, are currently being explored with whole-cell binding experiments.

In conclusion, NE and EPI activate all three cloned $\alpha_1$-AR subtypes with similar potencies and intrinsic activities. However, many widely used $\alpha_1$-AR agonists show significant selectivity in binding to and/or activating these subtypes. Such selectivity has important implications for the use of these drugs as experimental tools and therapeutic agents. The potencies of agonists in activating functional responses are highly correlated with, but different from, their absolute potencies in competing for radioligand binding sites. This relationship is subtype dependent and does not appear to be explained by known effects of nucleotides and cations. These observations further emphasize the importance of functional experiments in evaluation of agonist potency and selectivity.

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