Binding, Partial Agonism, and Potentiation of $\alpha_1$-Adrenergic Receptor Function by Benzodiazepines: A Potential Site of Allosteric Modulation

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ABSTRACT

Benzodiazepines, a class of drugs commonly used to induce anesthesia and sedation, can attenuate intracellular calcium oscillations evoked by $\alpha_1$-adrenergic receptor ($\alpha_1$-AR) stimulation in pulmonary artery smooth muscle cells. We postulated a direct action of benzodiazepines in modulating $\alpha_1$-AR function at the receptor level. Benzodiazepines bound to each of the cloned $\alpha_1$-AR subtypes ($\alpha_{1a}$-, $\alpha_{1b}$-, or $\alpha_{1d}$-AR) on COS-1 cell membranes transiently transfected to express a single population of $\alpha_1$-AR subtype. The ability of benzodiazepines to alter $\alpha_1$-AR signal transduction was investigated by measuring total inositol phosphate generation in rat-1 fibroblast cells, stably transfected to express a single $\alpha_1$-AR subtype. By themselves, benzodiazepines displayed partial agonism. At $\alpha_{1b}$-ARs and $\alpha_{1d}$-ARs, the maximal inositol phosphate response to phenylephrine was potentiated almost 2-fold by either midazolam or lorazepam (100 $\mu$M). At $\alpha_{1a}$-ARs, diazepam, lorazepam, and midazolam all increased the maximal response of the partial agonist phenylephrine was unaltered or inhibited. The potentiating actions of midazolam and its partial agonism at $\alpha_1$-ARs was blocked by the addition of 1 $\mu$M prazosin, an $\alpha_1$-AR antagonist, and not by a $\gamma$-aminobutyric acidA-receptor antagonist. These studies show that benzodiazepines modulate the function of $\alpha_1$-ARs in vitro, and this is the first report of a potential allosteric site on $\alpha_1$-ARs that may be therapeutically useful for drug design.

$\alpha_1$-Adrenergic receptors ($\alpha_1$-AR) are cell-surface, heptahelical receptors of the G protein-coupled receptor superfamily that bind the endogenous catecholamines epinephrine and norepinephrine and mediate the actions of the sympathetic nervous system (Graham et al., 1995). Three $\alpha_1$-AR subtypes, $\alpha_{1a}$-AR, $\alpha_{1b}$-AR, and $\alpha_{1d}$-AR, have been characterized from the cloning of their individual cDNAs (Lomasney et al., 1991; Perez et al., 1991; Cotecchia et al., 1988; Perez et al., 1994). Each of these subtypes signals by coupling to membrane-bound G proteins. The predominant second messenger pathway stimulated by $\alpha_1$-ARs is the activation of phospholipase C, a membrane-bound enzyme that generates the formation of soluble inositol triphosphate ($I_3P$) and diacylglycerol from phosphatidylinositol-4,5-bisphosphate (Hwa et al., 1996). $I_3P$ and diacylglycerol subsequently cause the release of calcium from intracellular stores and stimulate protein kinase C, respectively (Berridge, 1993). $\alpha_1$-ARs are distributed extensively throughout the tissues of the cardiovascular system, regulating the flow of blood through large conduit vessels and controlling the peripheral vascular resistance at the arteriolar level, the latter being an important determinant of the systemic blood pressure (Graham et al., 1995; Piascik et al., 1995; Hwa et al., 1996). $\alpha_1$-ARs also are located on cardiomyocytes; current evidence suggests that the $\alpha_1$-AR subtypes may play key and distinct roles in modulating the force and rate of cardiac contraction, especially during pathology. Specifically, the $\alpha_{1a}$-AR has been implicated in promoting abnormal heart rhythms in ischemia, whereas the activation of $\alpha_{1b}$-ARs is thought to promote normal heart rhythms (Anyukhovsky and Rosen, 1991; Anyukhovsky et al., 1992). Therefore, the activation of $\alpha_1$-ARs by circulating epinephrine or norepinephrine released from sympathetic nerves must be carefully controlled to maintain cardiovascular homeostasis.

Benzodiazepines are widely used in clinical practice as a premedicant in surgery or a sedative-ammesic. After i.v. ad-
ministration, benzodiazepines are rapidly distributed to the brain. Their principal molecular target is the γ-aminobutyric acid (GABA) receptor, a pentameric integral membrane ion channel. By themselves, benzodiazepines do not activate the GABA receptor, but instead act as allosteric modulators increasing the affinity and efficacy of the endogenous ligand, GABA, to bind and activate the receptor (Costa et al., 1975; Haeffely et al., 1975). Activation of the GABA receptor causes a chloride ion influx that hyperpolarizes the cell, facilitating the inhibitory or sedative actions of benzodiazepines in the central nervous system. Additional studies have suggested that the anxiolytic actions of benzodiazepines use other independent molecular pathways distinct from those by which the sedative actions of benzodiazepines are manifested. Indeed, the triazolobenzodiazepine alprazolam activates brain α2-ARs in reserpine-treated rats and antagonizes the anxiogenic effects of yohimbine at these receptors (Erickson et al., 1986). Other clinical studies to assess the effects of alprazolam on brain noradrenergic function have suggested that the antianxiety mechanism of alprazolam may be due to an interaction between benzodiazepine-sensitive and noradrenergic neural systems (Charny and Heninger, 1985).

Significant hemodynamic alterations have been observed in vivo following the administration of benzodiazepines (Kotryl et al., 1984; Marty et al., 1986; Taneyama et al., 1993). These effects include decreases in the systemic blood pressure and variations ranging from mild decreases to modest increases in heart rate. These effects are mediated in part by the inhibitory actions of benzodiazepines on the sympathetic nervous system. In recent studies, we have demonstrated that individual benzodiazepines differentially inhibit intracellular calcium oscillations generated in response to the selective α1-AR agonist phenylephrine in individual pulmonary artery smooth muscle cells (Hong et al., 1998). Although the addition of lorazepam produced a concentration-dependent decrease in the amplitude of the calcium oscillation in these cells, diazepam resulted in concentration-dependent decreases in the frequency of the oscillations. These inhibitory actions on calcium oscillations in an arterial smooth muscle cell in vitro may conceivably explain the in vivo observations of blood vessel dilation following benzodiazepine administration (Chang et al., 1994). In the present study, we have performed experiments to test our hypothesis that benzodiazepines modulate the signaling responses of α1-AR agonists via a direct interaction with the α1-AR. We have used radioligand binding experiments to determine the affinities with which benzodiazepines occupy binding sites on each of the α1-AR subtypes. In addition, we have measured total inositol phosphate (IP) levels in rat fibroblasts expressing single α1-AR populations to characterize the inherent signaling properties of benzodiazepines and to assess their ability to modify the signaling properties of agonists at each of the α1-AR subtypes. In summary, we report our findings of a novel sympathomimetic property of benzodiazepines and the potential role of allosterism in modulation of α1-AR function.

Experimental Procedures

Materials. Drugs were obtained from the following manufacturers: (-)-epinephrine, phenylephrine, and geneticon, Sigma Chemical Co., St. Louis, MO; [125I]2-β-(4-hydroxy-3-[125I]iodophenyl)ethylaminemethyl][tetralone (1[125I]IHEAT), [3]H]myo-inositol, [3H]muscimol, and [3H]flunitrazepam, DuPont NEN, Boston, MA; PK11195 and clonidine, Research Biochemicals Inc., Natick, MA; lorazepam (Ativan), Wyeth Laboratories, Andover, PA; midazolam (Versed), Roche Laboratories, Nutley, NJ; and diazepam (Elkins-Sinn, Inc., Cherry Hill, NJ).

Cell Culture and Transfection. COS-1 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin and streptomycin. Cells were maintained and passaged upon reaching confluency by standard cell culture techniques. Experiments were conducted on cells between passages 10 and 25. Cells were transiently transfected with the DEAE-dextran method previously described with the cDNA of a single subtype of α1-AR, subcloned into the eukaryotic expression plasmid pMT2 (Perez et al., 1991). Stably transfected rat-1 fibroblasts that express a single human α1-AR subtype were maintained in continuous culture in DMEM supplemented with 10% (v/v) FBS and 500 μg/ml geneticin. The expression level of receptors on the rat-1 fibroblasts ranged from 5.5 to 9 pmol/mg membrane protein.

Membrane Preparation. Transiently transfected COS-1 cells were scraped 72 h post-transfection, collected, and washed in Hanks’ balanced salt solution, then pelleted under low-speed centrifugation (1260g for 5 min). The cell pellet was resuspended in a 0.25 M sucrose solution and after another low-speed centrifugation step, the pellet was resuspended in a 10-ml volume of water containing a cocktail of protease inhibitors (40 μg of leupeptin,68 μg of phenylmethylsulfonyl fluoride, 400 μg of bacitracin, and 400 μg of benzamidine) and frozen at −70°C for 30 min. Membranes were prepared from the cell suspension by 20 strokes of a “B” glass Dounce homogenizer. Nuclear debris was removed by a low-speed centrifugation step. Membranes in the supernatant were washed with HEM buffer [20 mM HEPES, pH 7.4, 1.4 mM ethylene glycol bis(β-aminoethyl ether)N,N,N′,N″-tetraacetic acid, and 12.5 mM MgCl2] and pelleted by high-speed centrifugation (30,000g for 15 min). Two additional washes of the membrane pellet with HEM buffer (20 ml) were performed, and the final pellet was reconstituted in a known volume of HEM buffer containing 10% (v/v) glycerol and stored at −70°C until use. The protein concentration of the membrane preparation was determined by performing a Bradford assay (Bradford, 1976), with bovine serum albumin as the known standard.

Measurement of Ligand-Binding Affinities. The binding affinities of benzodiazepines were determined in a series of competition-binding experiments with the α1-AR-selective antagonist [125I]IHEAT as the radioligand. Assays were performed in duplicate in HEM buffer, in a total assay volume of 250 μl. A fixed amount of COS-1 cell membranes expressing a single α1-AR subtype was incubated with 100 pM [125I]IHEAT and a range of 10 different concentrations of the competing benzodiazepine. Nonspecific binding was determined experimentally in the presence of 10−4 M phenolamine. After incubation in a shaking water bath at 22°C for 60 min, unbound radioactivity was separated from membrane-bound radioactivity by filtration through Whatman GF/C filter paper with a Brandel cell harvester (Brandel, Gaithersburg, MD). Filters were washed with 20 ml of ice-cold HEM buffer to remove further nonspecifically bound radioactivity. Bound radioactivity remaining on the filters was counted on an ICN gamma counter operating at 79.8% efficiency.

Quantitation of Intracellular IP. Rat-1 fibroblasts plated on 60-mm culture plates were grown in DMEM supplemented with 5% FBS. Upon reaching 90% confluency, 3 μl of [3H]myo-inositol was added 16 h before experimentation to permit uptake by the fibroblasts. Measurement of intracellular IP was performed under serum-free conditions by washing fibroblasts with 10 ml of serum-free DMEM. To prevent complete hydrolysis of IP moieties, assays were conducted in the presence of the phosphatase inhibitor LiCl (10 mM) in a total assay volume of 5 ml. Agonists were added directly to the media and incubated at 37°C for 45 min in a 5% CO2 atmosphere. In
certain studies, antagonists were added 30 min before the addition of the agonist. Complete concentration-response curves for agonists were constructed over a suitable range of concentration, with at least two concentrations per order of magnitude and performing each data point in duplicate. Incubations were terminated by removal of the media containing the agonist and by adding a 1-ml volume of a 0.4 M perchloric acid solution. The cell lysate was scraped, collected, and neutralized by the addition of a 0.5-ml volume of a 0.72 N KOH/0.6 M KHCO₃ solution. Soluble IPs in the lysate were isolated by passage through a Bio-Rad AG 1X-8 resin column that was buffered with a 0.1 M formic acid solution. After washing the column with 0.1 M formic acid, bound ³H-IPs were displaced from the column by eluting the column with a 0.1 M formic acid solution containing 1 M ammonium formate. The eluant was collected directly in scintillation vials, scintillant was added, and the radioactivity was detected with a beta-counter (Beckman Instruments, Berkeley, CA).

Data Analysis. Competition-binding data and functional data from intracellular IP measurements were analyzed with the nonlinear regression functions of the noniterative curve-fitting program GraphPad Prism. Binding affinities (Kᵢ) were determined by transformation of the program-calculated IC₅₀ value with the Cheng-Prusoff equation. The binding data for benzodiazepine binding was modeled to one- or two-site binding. The most suitable model was determined by performing an F test comparison of the least sum-of-squares fit of the data to these equations. Functional data for IP stimulation in fibroblasts were analyzed by nonlinear regression analysis, with the sigmoidal curve-fitting equation of GraphPad Prism. In these studies, potency refers to the concentration of the agonist that stimulates the half-maximal IP response and was calculated directly from nonlinear regression analysis. Statistically significant differences in the potency of responses relative to control were determined by t test analysis. Statistically significant differences in the maximal responses from control were determined with a repeated measures two-tailed analysis of variance test followed by a post hoc Dunnett’s multiple comparison test.

Results

Binding of Benzodiazepines to α₁-ARs

Binding studies were performed on COS-1 cell membranes that had been transiently transfected to singly express one of the three α₁-AR subtypes. The affinities of diazepam, lorazepam, and midazolam at each of the α₁-AR subtypes were determined in heterologous competition-binding assays, measuring the ability of each of these drugs to compete for binding sites on the receptors that were labeled with the selective α₁-AR antagonist [¹²⁵I]HEAT. Increasing concentrations of diazepam, lorazepam, and midazolam resulted in concomitant decreases in the specific binding of [¹²⁵I]HEAT at each of the α₁-AR subtypes. Complete displacement of [¹²⁵I]HEAT binding was observed with each of these benzodiazepines at each α₁-AR subtype. Analysis of the data with nonlinear regression indicated that a single-site model was the most appropriate fit of the inhibition curves generated for benzodiazepine binding at each of the α₁-ARs. A composite displacement curve for midazolam is shown in Fig. 1 and the affinities of individual benzodiazepines at the α₁-AR subtypes are listed in Table 1.

Effects of Benzodiazepines on Signaling Properties of α₁-ARs

Because these benzodiazepines demonstrated the ability to occupy binding sites on the α₁-AR subtypes, additional experiments were conducted to examine the signaling properties of benzodiazepines at these receptors. Because the predominant signaling pathway for α₁-AR is activation of phospholipase C via Gₛₐ coupling, we measured the intracellular levels of IPs in rat-1 fibroblasts that are stably transfected to express a single α₁-AR subtype. These cell lines have two distinct advantages over the use of transient transfection of COS-1 cells in signaling studies. These fibroblasts maintain a uniform and high level of α₁-AR expression, limiting the variability between experiments that could result from variations in transfection efficiency. In addition, the stimulation resulting from the challenge with agonists produces large increases over the basal levels measured in the absence of agonist, thereby enhancing the ability to detect small changes in either the potency (EC₅₀) or maximal responses. Indeed, a full agonist at the α₁a-AR in these rat-1 fibroblasts can produce specific increases in IP release up to 40,000 cpm (~100-fold). Experiments were conducted to observe the effects of benzodiazepines on α₁-AR-mediated IP accumulation in the presence and absence of α₁-AR agonists.

To test whether the benzodiazepines had any intrinsic agonist properties by themselves, the stimulation of IPs by diazepam, lorazepam, or midazolam at the α₁a-AR subtype is shown in Fig. 2A. Each of these compounds induced a dose-dependent increase over the basal levels of IPs at concentrations that approximate the affinity with which they occupy the α₁-AR. Each of these benzodiazepines also induced increases over basal levels in similar experiments conducted on the α₁b-AR (data not shown). The increases in IP, although significant, are weak compared with the full α₁b-AR agonist phenylephrine (Fig. 2A). The increases in total IPs observed in response to 1 mM midazolam were reversed to basal levels in the presence of a saturating concentration of the α₁-AR antagonist prazosin (Fig. 2B). The GABA receptor nonbenzodiazepine antagonist PK11195 did not diminish the IP response to midazolam, even at a concentration of 1 μM, which would saturate GABA receptors (Doble et al., 1985).

In previous studies, benzodiazepines were able to exert profound changes in the calcium signaling attributed to the
stimulation of \( \alpha_1 \)-ARs by phenylephrine (Hong et al., 1998). Therefore, experiments were conducted to observe the IP responses in rat fibroblasts to \( \alpha_1 \)-AR agonists in the presence or absence of benzodiazepines. At the \( \alpha_{1a} \)-AR subtype, phenylephrine alone produced a 100-fold increase over the basal levels of intracellular IPs with a potency of 0.99 ± 0.18 \( \mu \text{M} \) (\( n = 6 \)). When the phenylephrine concentration-response curve was repeated in the presence of 100 \( \mu \text{M} \) diazepam (Fig. 3A), a 3-fold and statistically significant decrease in the potency of phenylephrine in stimulating IPs was observed (\( p < .05 \)). Neither midazolam nor lorazepam produced any statistically significant change in the maximal signal output to phenylephrine in the presence of any of these benzodiazepines.

Phenylephrine concentration-response curves also were generated on rat fibroblasts expressing the \( \alpha_{1b} \)-AR (Fig. 3B) or \( \alpha_{1d} \)-AR subtypes (Fig. 3C). Phenylephrine produced a 20-fold increase in the IP levels compared with basal levels at the \( \alpha_{1b} \)-AR subtype with a potency of 1.14 ± 0.26 \( \mu \text{M} \) (\( n = 6 \)). In the presence of 100 \( \mu \text{M} \) of either lorazepam or midazolam, no significant decreases in the potency of the response were observed but the maximal response was significantly increased being 1.75% of control in both instances (\( p < .05 \)). Coincubation with diazepam (100 \( \mu \text{M} \)) reduced the potency of phenylephrine at the \( \alpha_{1b} \)-AR (Fig. 3B). A similar potentiation of phenylephrine signaling was observed in the presence of benzodiazepines at the \( \alpha_{1d} \)-AR subtype (Fig. 3C), whereas lorazepam (\( p < .01 \)) and midazolam (\( p < .05 \)) both caused statistically significant increases of the signal to 175% of the control. The potentiation seen at either subtype is synergistic with respect to that of a benzodiazepine response alone. The \( \alpha_1 \)-AR-selective antagonist prazosin blocked the IP response to phenylephrine in the presence of midazolam at the \( \alpha_{1b} \)-AR (Fig. 4). The nonbenzodiazepine GABA receptor antagonist PK11195, used at a saturating concentration of 1 \( \mu \text{M} \), also did not have any effect on the signal potentiation of the phenylephrine response by midazolam at the \( \alpha_{1b} \)-AR (Fig. 4), again indicating that the effect of the benzodiazepine is being mediated through its interaction with the \( \alpha_1 \)-AR.

Additional signaling experiments were performed to examine...
ine the effects of benzodiazepines on the signaling properties of a partial agonist clonidine at the \( \alpha_{1\alpha}-AR \) and a full agonist epinephrine at the \( \alpha_{1b}-AR \). Each of the three benzodiazepines significantly potentiated the clonidine stimulation of IPs (Fig. 5). Again, the most dramatic increases in signaling were observed with lorazepam (\( p < .01 \)) and midazolam (\( p < .05 \)), producing 250 and 200% increases in the maximal response relative to that of clonidine alone, respectively. At the \( \alpha_{1b}-AR \), both lorazepam and midazolam significantly increased the maximal IP levels measured in response to challenge with epinephrine (\( p < .05 \)) (Fig. 6).

**Mechanism of Diazepam Inhibition at \( \alpha_{1\alpha}-AR \).** In our previous experiments at the \( \alpha_{1\alpha}-AR \), a single concentration of diazepam inhibited the potency of phenylephrine without reducing the maximal response (Fig. 3A). To determine whether diazepam inhibits \( \alpha_{1\alpha}-AR \) signaling via a competitive or noncompetitive interaction, experiments were con-
Fig. 6. Synergistic potentiation of a full agonist response by benzodiazepines at the α₁b-AR. Generation of epinephrine-induced increases in IPs in the absence (■, thick line) or presence of 100 μM diazepam (▲), 100 μM lorazepam (▼), or 100 μM midazolam (●) in rat-1 fibroblasts expressing the α₁b-AR. Data are shown as means ± S.E. from three individual experiments performed in duplicate.

Fig. 7. The synergistic action of benzodiazepines in modulating α₁-AR function is due to a noncompetitive mechanism. Phenylephrine-induced increases in IPs in the absence (■, thick line) or presence of 100 μM diazepam (▲), 300 μM diazepam (▼), or 1000 μM diazepam (●) in rat-1 fibroblasts expressing the α₁a-AR. Data are shown as means ± S.E. from three individual experiments performed in duplicate.

Discussion

Intravenous anesthetics can differentially modulate the intracellular calcium oscillations in pulmonary artery smooth muscle cells stimulated by the α₁-AR-selective agonist phenylephrine (Hong et al., 1998). α₁-AR evoked intracellular calcium oscillations arise following phospholipase C activation that generates IP₃, which promotes calcium release from the sarcoplasmic reticulum (Berridge, 1993; Hwa et al., 1996). Because benzodiazepines are lipophilic, they may cross the plasma membrane and interact with numerous intracellular molecular targets along this signaling pathway. Alternatively, the benzodiazepines may alter the interaction of phenylephrine at the agonist binding pocket of the α₁-AR itself or modulate the α₁-AR function via the signaling cascade of the GABA receptor. Benzodiazepines, like α₁-AR ligands, possess a protonated amine functionality and retain a high degree of aromatic character that is found in many α₁-AR agonists and antagonists. Therefore, we rationalized that the similarity of their size and the conservation of these key sympathomimetic pharmacophores made an interaction at the α₁-AR the most likely site of action for these benzodiazepines. Accordingly, our experiments were conducted to investigate such an interaction of benzodiazepines with the α₁-AR, quantitating their affinity for the receptor and their effects on the signaling properties of the α₁-AR.

All three benzodiazepines used in our study inhibited the binding of the selective α₁-AR antagonist [125I]HEAT from each of the α₁-AR subtypes. The affinities of the benzodiazepine at the α₁-ARs are listed together with our previously determined binding affinities of the full agonist epinephrine and the partial agonist methoxamine at α₁-ARs in Table 1. Although the benzodiazepine affinities are considerably lower than the affinity of epinephrine at all three subtypes, the affinities of diazepam and midazolam are only 2-fold lower than that of methoxamine at the α₁a-AR and are actually 4-fold higher at the α₁b-AR. Therefore, these benzodiazepines bind to and occupy sites on each of the three α₁-AR subtypes and with affinities that are comparable to other sympathomimetic drugs. Although this experimental method is designed to measure the competition between a drug and a radiolabel for a similar site on the receptor, this assay does not provide definitive proof of a competitive interaction. For example, allosteric modulators of muscarinic receptors can displace specific radioligand binding from these receptors in similar assays (Tucek and Prosek, 1995). The determination of whether the interaction between benzodiazepines and [125I]HEAT at α₁-ARs is a truly competitive or an allosteric interaction can only be determined from further detailed kinetic studies.

In signaling studies, each of the benzodiazepines demonstrated a concentration-dependent stimulation of IPs over basal levels in fibroblast cells expressing either the α₁a-AR (Fig. 2A) or the α₁b-AR (data not shown). Because these benzodiazepine-mediated increases in IPs could be reversed by the addition of prazosin, an α₁-AR antagonist, but not by PK11195, we conclude that the IP stimulation at the α₁a-AR is due to an interaction of these benzodiazepines with the α₁-ARs and not with GABA receptors on the rat-1 fibroblast. Subsequent saturation-binding experiments with the GABA receptor ligands [3H]flunitrazepam or competitive-binding studies with [3H]muscimol failed to detect the presence of any GABA receptors on these fibroblasts (data not shown), confirming our conclusion that the weak partial agonist activity is due to their interaction with the α₁-AR.

The effects of the benzodiazepines on the signaling prop-
erties of the α1-AR-selective agonist phenylephrine were subtype-dependent. At the α1a-AR, diazepam inhibited the potency of phenylephrine in stimulating IP; however, no statistically significant inhibition of the phenylephrine response was observed with midazolam or lorazepam. In fibroblasts expressing either the α1b-AR or α1d-AR subtype, the maximal response to phenylephrine in the presence of lorazepam and midazolam was markedly enhanced. We interpreted the differential benzodiazepine-mediated signaling inhibition or potentiation as being due to the intrinsic activity of phenylephrine, which is lower at the α1b-AR and α1d-AR subtypes compared with its full agonism at the α1a-AR. We confirmed our hypothesis by observing significant potentiation of the IP response to clonidine, a weak partial agonist at the α1a-AR subtype, in the presence of lorazepam and midazolam (Fig. 5). Therefore, we hypothesize that the intrinsic activity of the α1-AR agonist is one determinant of whether potentiation of the signal is observed.

However, contrary to our expectations, both lorazepam and midazolam significantly increased the IP levels measured in response to the full agonists epinephrine (Fig. 6) and norepinephrine (data not shown) at the α1a-AR. The maximal stimulation by a full agonist at the α1a-AR produces levels of [3H]IP exceeding 40,000 cpm; however, under similar conditions, the full agonist epinephrine produced maximal responses of only 9,000 cpm at the α1a-AR. Because the receptors are expressed at similar receptor densities on the fibroblasts, it can be argued that the relative differences in [3H]IP levels reflect the greater signaling efficiency of the α1a-AR over the α1b-AR. Indeed, previous studies that titrate receptor density have shown that the α1b-AR subtype is always more efficacious than the α1b-AR or α1d-AR at any receptor number (Esbenshade et al., 1993; Theroux et al., 1996). It is conceivable that the binding of the benzodiazepine at the α1b-AR potentiates the response of the full agonist epinephrine by altering the receptor conformation to one that enhances the interaction of the receptor with the G protein, consistent with an allosteric effect. Therefore, our experiments have illustrated that the potentiating effects of benzodiazepines on α1-AR signaling are not only dependent on the intrinsic activity of the agonist but also may be dependent on the efficacy with which the receptor is coupled to the signaling pathway.

The inhibitory profile of diazepam on phenylephrine-induced stimulation of IP at the α1a-AR illustrates an effect consistent with that of a noncompetitive interaction between phenylephrine and diazepam at this receptor (Fig. 7). The unequivocal demonstration of a noncompetitive interaction in these signaling studies is more definitive of an allosteric effect than by using the simple displacement of radiolabel binding at the receptor to determine the mechanism of the interaction. In addition, our other signaling studies demonstrated that lorazepam and midazolam potentiate the responses of full and partial agonists at the α1b-AR (Figs. 3B and 6). We argue that to observe signal potentiation of agonists requires the simultaneous occupation of the receptor by the benzodiazepine and the agonist, and thus separate binding sites exist for each compound on the receptor. Our observations allow us to speculate that the benzodiazepine is behaving as an allosteric modulator of α1-AR function, in a similar fashion to their documented allosteric modulation of GABA binding and channel activation at the GABA_A receptor.

Recent studies have shown that aromatic and hydrophobic residues on the Y1 and Y2 subunits of the GABA_A receptor mediate benzodiazepine binding at the allosteric binding sites on these receptors (Wieland et al., 1992; Sigel and Buhr, 1997; Sigel et al., 1998). Likewise, the juxtamembrane regions of the transmembrane helices and the extracellular loops of the α1-ARs contain numerous aromatic and hydrophobic amino acids that may facilitate binding of the benzodiazepines to the α1-AR. Therefore, we speculate that the benzodiazepine binding site on α1-ARs lies above the catecholamine binding pocket, previously mapped in the hydrophilic core within the circular array of transmembrane spanning helices of the α1b-AR (Hwa et al., 1995; Hwa and Perez, 1996). Alternatively, the lipophilic characteristics of benzodiazepines may facilitate their interaction with the lipophilic core of the α1-AR, or the benzodiazepine may bind in a pocket formed within the circular array of transmembrane helices but on the opposite side from that of the agonists.

Our laboratory has proposed that the activation of α1-ARs involves the agonist-mediated disruption of an interhelical salt bridge formed between an aspartic acid (Asp125) in transmembrane 3 and a lysine residue (Lys313) in transmembrane 7 (Porter et al., 1998). Our model predicts that the protonated amine group of the agonist projects toward and forms an ion pair with Asp125 after salt bridge breakage. We speculate that the protonated amine group present on the benzodiazepine is orientated so that it too can project toward Asp125 and break the salt bridge even when the agonist binding pocket is occupied. When the α1-AR is occupied by a partial agonist, the simultaneous interaction of the benzodiazepine’s protonated amine provides additional energy to release the interhelical salt-bridge conformational restraint and potentiates the actions of the agonist. This is consistent with our experimental observations in which the greatest signal potentiation occurs when a weak partial agonist occupies the agonist binding pocket of the α1-AR, e.g., clonidine at the α1b-AR (Fig. 5). In contrast, a full agonist possesses sufficient intrinsic strength to disrupt the constraining salt bridge itself and requires no additional energetic requirement from the benzodiazepine to activate the receptor, e.g., phenylephrine at the α1a-AR (Fig. 3A). Indeed, at high concentrations (1 mM), benzodiazepines actually inhibit the signal of the full agonist (Fig. 7), an effect probably the result of steric hindrance. Because receptor efficacy also determines benzodiazepine potentiation, we predict that the alignment and orientation of epinephrine in the binding pockets of the α1a-AR and α1b-AR are slightly different. A closer projection of the protonated amine group of epinephrine toward the aspartic acid residue in the α1a-AR would be more energetically favorable for salt bridge breakage and explain the minimal effects of the benzodiazepine at the α1a-AR. This hypothesis is based upon our previously reported observations with triethylamine, a chemical mimic of the ethylamine substituent in epinephrine, to also potentiate clonidine but not epinephrine signaling at the α1a-AR (Porter et al., 1998). Indeed it is important to note that the protonated amine group in triethylamine and benzodiazepines is the only commonality between the two potentiators. If our hypothesis is correct, the degree of potentiation depends upon the efficacy of the system, whether it is controlled at the level of the agonist (i.e.,
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In summary, we have reported the findings that three benzodiazepines, diazepam, lorazepam, and midazolam, bind to and modulate the intracellular signaling of all three \(\alpha_1\)-AR subtypes, probably through an allosteric interaction at the receptor level. Benzodiazepines possess low intrinsic activity in stimulating IPs but exert profound effects on the signaling properties of full and partial agonists at \(\alpha_1\)-ARs. Although our observations are made at concentrations 100-fold in excess of the reported 1 \(\mu\)M plasma concentration of benzodiazepines (Gamble et al., 1976; Dundee et al., 1978), the blood chemistry of lipophilic agents makes accurate determination of their concentration difficult, and the possibility remains that the blood levels may be higher than reported. However, it is unlikely that the clinical administration of benzodiazepines will result in their cross-reactivity with adrenergic receptors. Nevertheless, the actions of benzodiazepines at \(\alpha_1\)-ARs may represent part of a general allosteric site on the \(\alpha_1\)-AR, similar to the binding of amiloride analogs at the \(\alpha_2\)-AR (Leppik et al., 1998) and suggest that other higher-affinity modulators of adrenergic receptors may exist. Such a site to modulate \(\alpha_1\)-AR function may be therapeutically useful such as with the allosteric modulators at the muscarinic acetylcholine receptors (Tucek and Prosek, 1995) and GABA\(_A\) receptors (Sigel and Buhr, 1997).

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References


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