Diazepam is not a direct allosteric modulator of α1-adrenoceptors, but modulates receptor signaling by inhibiting phosphodiesterase-4

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Abstract
α1A- and α1B-adrenoceptors (ARs) are G protein-coupled receptors (GPCRs) that are activated by adrenaline and noradrenaline to modulate smooth muscle contraction in the periphery, and neuronal outputs in the central nervous system (CNS). α1A- and α1B-AR are clinically targeted with antagonists for hypertension and benign prostatic hyperplasia and are emerging CNS targets for treating neurodegenerative diseases. The benzodiazepines midazolam, diazepam, and lorazepam are proposed to be positive allosteric modulators (PAMs) of α1-ARs. Here, using thermostabilized, purified, α1A- and α1B-ARs, we sought to identify the benzodiazepine binding site and modulatory mechanism to inform the design of selective PAMs. However, using a combination of biophysical approaches no evidence was found for direct binding of several benzodiazepines to purified, stabilized α1A- and α1B-ARs. Similarly, in cell-based assays expressing unmodified α1A- and α1B-ARs, benzodiazepine treatment had no effect on fluorescent ligand binding, agonist-stimulated Ca2+ release, or G protein activation. In contrast, several benzodiazepines positively modulated phenylephrine stimulation of a cAMP response element pathway by α1A- and α1B-ARs; however, this was shown to be caused by off-target inhibition of phosphodiesterases, known targets of diazepam. This study highlights how purified, stabilized GPCRs are useful for validating allosteric ligand binding and that care needs to be taken before assigning new targets to benzodiazepines.

KEYWORDS
α1-adrenergic receptors, α1-adrenoceptors, allosteric modulator, benzodiazepines, GPR68, phosphodiesterase, thermostabilized receptor

Abbreviations: AC, adenylate cyclase; AR, adrenoceptor; CaM, calmodulin; CNS, central nervous system; CRE, cAMP response element; DDM, n-dodecyl β-D-maltopyranoside; DMEM, Dulbecco’s modified Eagle medium; GPCR, G protein-coupled receptor; HEAT, [125I]-2-[2-(4-hydroxy-2-iodophenyl)ethylamino]methyl]-3,4-dihydro-2H-naphthalen-1-one; HEK, human embryonic kidney; IP3, inositol 1,4,5-trisphosphate; NMR, nuclear magnetic resonance; PAMs, positive allosteric modulators; PDE, phosphodiesterase; PKC, phosphokinase C; PLCδ, phospholipase C-δ; QAPB, quinazoline piperazine bodipy; SAR, structure–activity relationship; SD, standard deviation; STD, saturation transfer difference; TM, transmembrane; TOCSY, 1H total correlation spectroscopy; WT, wild-type.

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1 INTRODUCTION

Adrenergic receptors, or adrenoceptors (ARs), are a family of G protein-coupled receptors (GPCRs) that bind endogenous adrenaline and noradrenaline and are important for modulating the cardiovascular and nervous systems. GPCRs are seven α-helical transmembrane (TM) proteins that bind ligands on the extracellular face shifting the conformational equilibrium of the GPCR to active states and promoting cytoplasmic interactions with heterotrimeric G proteins and other signaling proteins.1 There are three AR subfamilies, α1, α2, and β-ARs, each comprising three receptor subtypes.2 The α1-AR subtypes, α1A-AR, α1B-AR, and α1D-AR couple to Gα1/11 G proteins to activate phospholipase C-β (PLCβ) that catalyses the formation of the second messenger, inositol 1,4,5-trisphosphate (IP3), thereby stimulating intracellular calcium mobilization. α1-AR signaling stimulates smooth muscle contraction and thus α1-AR antagonists are prescribed for hypertension and benign prostatic hyperplasia.3 α1A-AR and α1B-AR are also highly expressed in the brain, and transgenic rodent models have indicated α1A-AR activation stimulates neurogenesis, while prolonged α1B-AR stimulation promotes apoptotic neurodegeneration.3,4 In the failing rodent heart, α1A-AR stimulation drives adaptive hypertrophy, whereas chronic α1B-AR activation causes maladaptive hypertrophy as a result of hemodynamic overload.3 Thus, selective α1A-AR activation, or α1A-AR blockade, could be useful therapeutic strategies for certain diseases.

While there are some α1A-AR and α1B-AR subtype-selective ligands available, no highly α1A-AR subtype-selective drugs have been approved for use in the clinic. The highly similar ligand binding sites in closely related AR subtypes makes identifying subtype-selective ligands challenging. Allosteric ligands, on the other hand, bind to distinct sites in the receptor and can modulate the activity of the receptor in response to agonist binding. As allosteric sites are less conserved between receptor subtypes it may be possible that allosteric modulators offer scope for achieving subtype selectivity. Several ligands have been reported to act as allosteric modulators of α1A-AR and α1B-AR, including the conotoxin ρ-TIA,5 9-aminoacridine,6 and the benzodiazepines diazepam, lorazepam, and midazolam.7 However, the exact structural mechanisms by which these ligands modulate the receptors are unknown.

Benzodiazepines are positive allosteric modulators (PAMs) of GABA_A receptor ion channels and are widely prescribed as sedatives, anxiolytics, anticonvulsants, and myorelaxants.8 The benzodiazepines, diazepam and lorazepam, inhibit Ca^2+ oscillations in pulmonary artery smooth muscle cells, suggestive of off-target interactions with ARs.9 Waugh et al.7 postulated that benzodiazepines directly bind to α2-ARs. Diazepam, lorazepam, and midazolam also directly competed with the α1-AR antagonist [125I]-2-[2-(4-hydroxy-2-isopropenyl)ethylamino]methyl]-3,4-dihydro-2H-naphthalen-1-one (HEAT) on α1A/B/D-AR expressing COS-1 cells.7 Functional IP₃ assays demonstrated potentiation of agonist responses at α1A/B/D-AR-expressing cells by these benzodiazepines, suggestive of allosteric interaction. However, no further studies have been reported on the mechanism of benzodiazepine modulation of α₂-ARs, or the receptor binding site. We recently engineered thermostabilized mutants of α1A-AR and α1B-AR, which enabled binding epitope determination of orthosteric ligands with nuclear magnetic resonance (NMR) spectroscopy.10 In this work we sought to use these thermostabilized receptors and our NMR approach to further understand how benzodiazepines bind to and modulate α1-ARs so as to apply this information as a starting point for developing more selective modulators targeting this allosteric site. Instead we observed no evidence for the direct binding of several benzodiazepines to purified α1A-AR and α1B-AR using NMR and fluorescent ligand binding assays. Cell-based binding and calcium signaling assays with wild-type (WT) α1-ARs also failed to detect any direct modulatory effects of diazepam on these receptors. While diazepam could positively modulate the stimulation of a CAMP response element (CRE) reporter through α1-AR activation, this was found to be driven through the ability of diazepam to inhibit phosphodiesterases (PDEs), a known target of some benzodiazepines. This study highlights how purified GPCRs can be used to directly investigate allosteric modulator mechanisms that have been proposed from cell-based assays, where off-target actions are difficult to control for.

2 MATERIALS AND METHODS

2.1 Benzodiazepine preparation

Nordiazepam and diazepam were synthesized from 2-amino-5-chlorobenzophenone using the method of Sternbach et al.11 N-hydroxyethyl-nordiazepam was synthesized from nordiazepam according to the method of Archer et al.12 Further details of benzodiazepine preparation and chemical synthesis can be found in Supporting Information.

2.2 Protein expression, purification, and binding assays

α1A-AR variant A4 and α1B-AR variant #15 were expressed in Escherichia coli and purified as described previously.10 Stabilized rat neurotensin receptor 1 (enNTS1) was expressed and purified as described by Bumbak et al.13 BODIPY-FL-prazosin (QAPB [quinazoline-piperazine-bodipy]) competition binding assays were performed as described previously.10 Briefly, 2 nmol of purified receptor, with C-terminal mCherry-Avi tag fusion, were incubated with 100 μL Dynabeads® MyOne Streptavidin T1 paramagnetic beads (Life Technologies, Carlsbad, CA, USA) in 10 mL binding buffer (1× PBS, 0.05% n-dodecyl β-D-maltopyranoside [DDM], 10 mMol/L EDTA), at 4°C for 1.5 hours. The receptor-coated beads were washed in binding buffer, and then aliquoted into a KingFisher 96-DeepWell™ plate (Thermo Fisher Scientific, Waltham, MA, USA) at approximately 20 pmol of receptor per well. A Kingfisher 96 magnetic particle processor was then used to transfer the beads into plates containing QAPB and various competitors, which was incubated for 2 hours at 22°C with gentle mixing. The beads were then washed for 1 minute in...
binding buffer then transferred to 100 μL binding buffer in black Greiner nonbinding 96-well plates. QAPB fluorescence was measured using 485/12 nm excitation and 520/10 nm emission filters, while m-Cherry fluorescence was measured using 544 nm excitation and 590/10 nm emission filters in an Omega POLARstar plate reader (BMG Labtech, Ortenberg, Germany).

2.3 | NMR spectroscopy

Stock solutions of 100 mmol/L diazepam and lorazepam were prepared in deuterated DMSO. Stock solutions of 5 mmol/L prazosin were prepared in deuterated methanol. Saturation transfer difference (STD) NMR samples constituted 5 μmol/L α1A-AR A4, or 5 μmol/L enNATS1, and 500 μmol/L test ligands with and without competition of 10 μmol/L prazosin in 500 μL of DDM buffer (0.05% [or 1 mmol/L] DDM, 50 mmol/L potassium phosphate, 100 mmol/L NaCl, 10% 2H2O, pH 7.4) in 5 mm NMR tubes. STD NMR was performed as described previously on a 700 MHz Bruker Avance III HD spectrometer using a cryogenically cooled triple resonance probe 10 and the resultant spectra were analyzed using M nova NMR 10 (Mestrelab, Santiago de Compostela, Spain).

Diazepam and lorazepam assignments were confirmed with two-dimensional 1H total correlation spectroscopy (TOCSY) and 1H correlated spectroscopy spectra acquired under the same solution conditions as the STD NMR experiments and in the presence of receptor. Spectra were typically acquired at 25°C with 10 ppm spectral widths and 2K data points in the direct dimension and 256 data points in the indirect dimension. TOCSY spectra were run with a spin lock time of 60 ms.

2.4 | Whole cell QAPB binding and Ca2+ mobilization assays

Saturation binding of QAPB in the absence or presence of 50 μmol/L diazepam was measured using COS-7 cells stably expressing WT human α1A-AR and α1B-AR as previously described.10 Cells were gently suspended with pipetting into 1.4 mL of phenol red-free Dulbecco’s modified Eagle medium (DMEM) at 20°C, resulting in a concentration of approximately 6,000 00 cells/mL. Fifty microliter of the cell suspension was added to each well of a V-bottom 96-well plate (Sarstedt, Nümbrecht, Germany). A further 50 μL of phenol red-free DMEM containing QAPB at varying concentrations was added to make final QAPB concentrations of 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 mmol/L. Separate solutions of these QAPB concentrations were made in the presence of 50 μmol/L diazepam.

To determine nonspecific binding, cells were exposed to QAPB at the same concentrations as above, but in the presence of 100 μmol/L phenotolamine. The cells were incubated with ligands for 1 hour at 20°C prior to detection of bound QAPB with flow cytometry using Cyt oFLE X LX flow cytometer (Beckman Coulter, Brea, CA, USA).

Intracellular Ca2+ mobilization assays were performed on nontransfected COS-7 cells and cells stably expressing either α1A-AR or α1B-AR. Cells were seeded at 25,000 cells per well into 96-well culture plates and allowed to grow overnight at 37°C, 5% CO2. Cells were washed twice with Ca2+ assay buffer (150 mmol/L NaCl, 2.6 mmol/L KCl, 1.2 mmol/L MgCl2, 10 mmol/L D-glucose, 10 mmol/L HEPES, 2.2 mmol/L CaCl2, 0.5% [w/v] BSA, and 4 mmol/L probenecid, pH 7.4) and then incubated in Ca2+ assay buffer containing 1 mmol/L Fluo-4-AM for 1 hour in the dark at 37°C and 5% CO2. After two washes with Ca2+ assay buffer and the addition of phenylephrine solutions (or co-addition of phenylephrine and benzodiazepines) fluorescence was measured for 1.5 minute in a Flexstation plate reader (Perkin Elmer, Waltham, MA, USA) using an excitation wavelength of 485 nm and emission wavelength of 520 nm.

2.5 | CRE reporter assays

cAMP response element (CRE) response assays were performed as previously described by transfecting WT α1A- and α1B-AR stably expressing COS-7 cells, parental COS-7 cells or parental HEK293T cells, with a β-galactosidase expression plasmid under the control of the CRE promoter.10 CRE reporter-transfected cells were seeded into 96-well culture plates for 24 hours before treatment with various compounds for 6 hours at 37°C 5% CO2. Media was aspirated, and the cells frozen at −80°C for at least 24 hours before measurement of cellular β-galactosidase expression using chlorophenol red-β-D-galactopyranoside. Ligands were made up in 0.5% FBS DMEM media. Benidipine (10 μmol/L), bicuculline (30 μmol/L), IBMX (500 μmol/L), or rolipram (10 μmol/L) were co-added with 0.1 μmol/L phenylephrine, or 10 μmol/L forskolin with and without diazepam or lorazepam (50 μmol/L). Inhibitors W-7 hydrochloride (10 μmol/L), 2-APB (40 μmol/L), (R)-(+)-Bay 8644 (4 μmol/L), and KN-93 (20 μmol/L) were preincubated for 30 minute prior to addition of 50 μmol/L diazepam, and 10 μmol/L forskolin or 0.1 μmol/L phenylephrine for 6 hours. HEK293T cells transiently transfected with GPR68 and pCRE were stimulated with sodium bicarbonate-free, low glucose DMEM, supplemented with 20 mmol/L HEPES and 0.5% FBS at pH 6.8, 7.0, 7.2, 7.4, and 7.8. The pH was adjusted at 37°C with NaOH. Cells were incubated for 6 hours at 37°C, in a room atmosphere incubator. Diazepam or lorazepam were made up in media at pH 7.2 or 7.8, co-added with rolipram and stimulated for 6 hours in above conditions.

2.6 | Data analysis

Nuclear magnetic resonance (NMR) data were processed in Topspin 3.5 using squared cosine-bells in both dimensions and zero-filled once, prior to Fourier-transformation. All other data was analyzed with Graphpad prism 7 (San Diego, CA, USA). All error bars are standard deviation (SD) from three independent experiments. Competition binding assays were performed in duplicate wells. Curves were fit with one site nonlinear regression. For whole cell QAPB binding, the mean QAPB fluorescence intensity (MFI) of at least 5000 cells was measured, and plotted against QAPB concentration. Values reported represent the mean and SD of at least three Kd values calculated from separate measurements. Ca2+ mobilization data were
performed in triplicate wells and normalized to the peak response elicited by 3 μm/L ionomycin. CRE assay was conducted in triplicate, and data were normalized to the response elicited by the vehicle. Curves were fitted with three variable nonlinear regressions. For GPR68 assays, responses were normalized to CRE response at pH 6.8 (100%) and pH 7.8 (0%).

3 RESULTS

Previously the benzodiazepines lorazepam, diazepam, and midazolam were shown to behave as positive modulators of α1-AR agonists in cell lines overexpressing α1-ARs, with low micromolar potencies. However, direct binding of benzodiazepines to purified α1-AR proteins has never been demonstrated. Thermostabilized GPCRs are receptors containing mutations that improve the protein stability upon solubilization and purification using detergents. The retention of natural receptor pharmacology enables thermostabilized receptors to be used for structural biology and for probing the mechanisms and kinetics of ligand binding in a purified system. The thermostabilized variants α1A-AR A4 and α1B-AR #15 were recently described and exhibit the stability required to probe the binding of benzodiazepines to the purified receptors. We hypothesized that if benzodiazepines are direct allosteric modulators of α1-AR, then they should influence the binding of orthosteric antagonists and/or agonists to α1-AR. Biotinylated α1A-AR A4 or α1B-AR #15 was immobilized onto streptavidin-coated paramagnetic beads and placed in a 10 nmol/L solution of fluorescent-labeled BODIPY-FL prazosin (QAPB), an approximately Kd concentration, with serial dilutions of validated competitors, diazepam or lorazepam, for 2 hours. The Kd of QAPB at α1A-AR A4 and α1B-AR #15 has been previously reported to be 11.6 and 8.5 nmol/L respectively. The agonist phenylephrine displaced QAPB at both receptor subtypes in an expected dose-dependent manner (Figure 1A,B). Conversely, neither diazepam nor lorazepam displaced QAPB at either receptor, even at concentrations of up to 200 μmol/L (Figure 1A,B). As diazepam is predicted to bind to an allosteric site distinct from the orthosteric site, where QAPB binds, this discrepancy could be due to noncompetitive binding of the two ligands. However, the ability of diazepam to increase the potency and efficacy of the α1-AR agonist phenylephrine suggests that allosteric binding of diazepam influences the affinity of phenylephrine for the receptors. Thus, QAPB competition binding assays were performed at α1A-AR A4 and α1B-AR #15 where a sub-IC50 concentration of phenylephrine (2.5 mmol/L) was included and the ability of increasing concentrations of diazepam to positively modulate QAPB displacement by phenylephrine was monitored. No cooperativity between diazepam and phenylephrine was observed at either receptor subtype. These data suggest that diazepam is either not binding to the thermostabilized α1-ARs, has a very low affinity interaction, or is allosterically binding, but not able to modulate phenylephrine binding in this purified system.

Saturation transfer difference (STD) NMR is a ligand-observed experiment that is especially sensitive at monitoring ligands that bind weakly to proteins (Kd > 1 μmol/L) and does not require labeled...
ligands or proteins.\textsuperscript{17} We recently applied STD NMR to the study of orthosteric agonist binding at $\alpha_{1A}$-AR A4 and $\alpha_{1B}$-AR #15.\textsuperscript{10} Here, STD NMR was applied to determine if diazepam and lorazepam bind to purified $\alpha_{1A}$-AR A4. STD NMR signals were observed for diazepam and lorazepam (Figure 2A,E) when incubated with DDM micelles that were not loaded with protein (Figure 2B,F), suggesting significant nonspecific interactions with detergent. Stronger STD NMR signals were observed for diazepam and lorazepam when incubated with purified $\alpha_{1A}$-AR A4, solubilized in DDM (Figure 2C,G) and this signal was not reduced upon the addition of 10 $\mu$mol/L prazosin as a competitor, suggesting either the detection of nonspecific or allosteric binding of the benzodiazepines to $\alpha_{1A}$-AR A4. However, strong STD NMR signals were also observed when diazepam and lorazepam were studied in the presence of an unrelated receptor, stabilized neurotensin receptor 1 (enNTS1)\textsuperscript{13} (Figure 2D,H), suggesting that the STD NMR signals of diazepam and lorazepam in all these experiments were due to nonspecific interactions with detergent micelles.

Having failed to detect any evidence for specific interactions between diazepam and lorazepam and $\alpha_{1A}$-AR A4 and $\alpha_{1B}$-AR #15, we sought to validate the work of Waugh et al.\textsuperscript{7} by measuring competitive binding and positive agonist modulation of benzodiazepines using cells overexpressing WT human $\alpha_{1A}$-AR and $\alpha_{1B}$-AR. A benzodiazepine related to diazepam and midazolam, was previously shown to compete with iodinated HEAT, a selective $\alpha_1$-AR antagonist, at cells expressing $\alpha_{1A}$-AR, $\alpha_{1D}$-AR or $\alpha_{1D}$-AR.\textsuperscript{7} HEAT is thought to bind in the orthosteric binding site, thus we sought to validate this competitive binding behavior of benzodiazepines using QAPB on COS-7 cells stably expressing unmodified $\alpha_{1B}$-AR. Flow cytometry-based QAPB saturation binding assays were performed in the absence or presence of 50 $\mu$mol/L diazepam. QAPB exhibited the expected affinity for WT $\alpha_{1B}$-AR ($K_d = 12 \pm 3$ nmol/L), which was not significantly different in the presence of 50 $\mu$mol/L diazepam ($K_d = 20 \pm 13$ nmol/L) (Figure 3A,B).

To probe whether allosteric binding of diazepam could modulate $\alpha_1$-AR signaling, COS-7 cells stably expressing human $\alpha_{1A}$-AR and

![Figure 2](image-url)
α1B-AR were used for calcium mobilization assays. The agonist, phenylephrine, stimulated calcium mobilization in α1-AR expressing cells, but not untransfected COS-7 cells, with expected potencies (Figure 3C). To probe positive modulation of this response, α1A-AR and α1B-AR expressing cells were treated with an EC50 concentration of phenylephrine (10 nmol/L) and increasing concentrations of diazepam or lorazepam before measuring the mobilization of intracellular calcium 5 minute after treatment. Treatment with these benzodiazepines (0 to 50 μmol/L), had no effect in the absence (Figure 3D), or presence of phenylephrine on both receptor expressing cell lines (Figure 3E,F). To gain a more complete measure of phenylephrine-induced α1-AR signaling over a 6-h stimulation period, a CRE-reporter gene assay was employed. In this assay, diazepam positively modulated the potency of phenylephrine at α1A-AR and α1B-AR expressing cells and also increased the E_{max} of phenylephrine at α1B-AR expressing cells (Figure 4A,B and Table 1). Diazepam modulated the phenylephrine CRE response with potencies of 6.5 ± 4.8 μmol/L on α1A-AR and 7.8 ± 1.9 μmol/L on α1B-AR (mean EC50 ± SD from three experiments, Figure 4C). Critically, diazepam treatment in the absence of phenylephrine did not induce a CRE response (Figure 4C). However, diazepam positively modulated the phenylephrine-induced CRE response on COS-7 cells that do not express α1A-AR and α1B-AR (Figure 4D), suggesting that the action of diazepam on CRE signaling is independent of α1-AR stimulation.

**FIGURE 3** Cell-based assays of benzodiazepine action at WT α1-ARs. (A) Pooled quinazoline piperazine bodipy (QAPB) saturation binding curves in the absence (+ vehicle) and presence of 50 μmol/L diazepam and (B) the resultant K_d values from each experimental replicate. (C) Phenylephrine-induced intracellular Ca^{2+} mobilization in COS-7 cells stably expressing either WT α1A-AR (blue circles), WT α1B-AR (red squares) or no receptor (green circles). (D) Diazepam and lorazepam did not stimulate Ca^{2+} mobilization in any of the cell lines, nor did they potentiate phenylephrine stimulation of Ca^{2+} mobilization in (E) WT α1A-AR stable cells or (F) WT α1B-AR stably expressing cells.
All α1-ARs signal primarily through Gαq/11 G proteins, which activate the effector protein phospholipase C (PLC). PLC catalyses the formation of diacylglycerol (DAG), which then activates phosphokinase C (PKC), and IP3 causing Ca2+ release from the endoplasmic reticulum via InsP3R Ca2+ channels. α1-AR activation also has a secondary effect of stimulating cAMP production via calmodulin (CaM)-stimulated adenylate cyclase (AC). PKC and secondary G protein coupling may also play a role in AC activation. CaM family kinases I and IV are activated by CaM which phosphorylate the transcription factor cAMP response binding protein, leading to its activation, and upregulation of CRE genes. Thus, there are many molecular targets in the CRE pathway that benzodiazepines may be interacting with to positively modulate the α1-AR CRE response. To assess the specificity of this action of diazepam, CRE assays were performed on human embryonic kidney (HEK) 293-T cells, which endogenously express β-ARs, treated with the β2-AR agonist isoprenaline. Treatment with diazepam at 50 μmol/L significantly increased the CRE efficacy of isoprenaline at HEK293-T cells (Figure 4E), suggesting that diazepam modulation of the CRE response is not specific to α1-AR stimulation. Furthermore, 50 μmol/L diazepam positively modulated the potency of the AC activator forskolin in HEK293-T cells (Figure 4F), indicating that diazepam is not modulating CRE activity at the receptor level, but at some other step of the signaling pathway.

FIGURE 4  Modulation cAMP response element (CRE) activation by diazepam. Phenylephrine-induced CRE response in the absence, or presence of 50 μmol/L diazepam or lorazepam at (A) COS-7 cells stably expressing wild-type (WT) α1A-AR or (B) COS-7 cells stably expressing WT α1B-AR. (C) Dose–response curves of diazepam modulating the phenylephrine-induced CRE response in WT α1A-AR (200 nmol/L phenylephrine used) and WT α1B-AR (800 nmol/L phenylephrine used) expressing COS-7 cells. (D) Phenylephrine- and forskolin-induced CRE response in the absence, or presence of 50 μmol/L diazepam at untransfected COS-7 cells. (E) Isoprenaline-induced CRE response in the absence, or presence of 50 μmol/L diazepam at untransfected human embryonic kidney (HEK) 293T cells. (F) Forskolin-induced CRE response in the absence, or presence of 50 μmol/L diazepam at untransfected HEK293T cells
TABLE 1 Structure activity relationship governing benzodiazepine modulation of the CRE response

| Name   | R1= | R2= | R3= | R4= | phe pEC50 | phe Emax (% veh) | fsk Emax (% veh) |
|--------|-----|-----|-----|-----|-----------|-----------------|-----------------|
| DMSO   | N.A.| N.A.| N.A.| N.A.| 6.07 ± 0.07| 100             | 100             |
| nordiaz | H   | H2  | Cl  | H   | 6.27 ± 0.11| 114 ± 3         | 107 ± 11        |
| diaz   | CH3 | H2  | Cl  | H   | 6.99 ± 0.08*| 146 ± 3*        | 167 ± 3*        |
| loraz  | H   | OH  | Cl  | Cl  | 6.05 ± 0.12| 89 ± 5          | 112 ± 16        |
| temaz  | CH3 | H   | OH  | Cl  | 6.49 ± 0.1*| 127 ± 2*        | N.D.            |
| oxaz   | H   | OH  | Cl  | H   | 6.44 ± 0.10*| 121 ± 8*        | N.D.            |
| OH-diaz| C2H6OH| H2  | Cl  | H   | 6.37 ± 0.09*| 115 ± 9         | N.D.            |
| 7-Br   | CH3 | H2  | Br  | H   | 7.10 ± 0.08*| 172 ± 11*       | 146 ± 31        |
| 7-Ph   | CH3 | H2  | Ph  | H   | 6.56 ± 0.06*| 118 ± 6*        | 138 ± 16        |

The potency of each benzodiazepine at modulating the phenylephrine-stimulated cAMP response element (CRE) response at α1B-AR expressing COS-7 cells (phe pEC50) are expressed as the mean ± SD from three independent experiments. The maximum CRE response induced by either phenylephrine or forskolin at untransfected human embryonic kidney 293T cells (fsk Emax), in the presence of each benzodiazepine are expressed as percentages compared to the vehicle (DMSO) from three independent experiments. nordiaz, nordiazepam; diaz, diazepam; loraz, lorazepam; temaz, temazepam; oxaz, oxazepam; OH-nor, N-hydroxyethyl-nordiazepam; 7-Br, 7-Bromo-diazepam; 7-Ph, 7-phenyl-diazepam; N.D., indicates not determined.

*Statistically significant difference (P < 0.05), evaluated using one-way ANOVA and Tukey multiple comparisons tests.

The structure–activity relationship (SAR) of benzodiazepines at their clinical target, GABAAR is well understood, and thus the SAR of CRE modulation was investigated to gain insight into what the underlying molecular target may be. The benzodiazepines tested, and their modifications compared to diazepam, are listed in Table 1. Interestingly, the N-methyl group of diazepam, which is lacking in nordiazepam, was found to be critical for positive modulation of the phenotype-induced CRE activation in α1B-AR expressing COS-7 cells and forskolin stimulation of CRE in untransfected HEK cells (Figure 5 and Table 1). Furthermore, the GABAAR-inactive benzodiazepine, 7-phenyl-diazepam, was able to modulate the phenotype-induced CRE activation, although its actions on forskolin failed to reach statistical significance (Figure 5 and Table 1). This defined SAR indicated that the benzodiazepine mechanism driving this positive modulation of phenylephrine was likely being driven by a specific binding interaction with a target other than GABAAR in the cells rather than nonspecific interactions, for example, with the cell membrane.

Next, we sought to define the mechanism by which diazepam was modulating CRE activation by using inhibitors of various potential targets. Co-addition of the GABAAR antagonist bicuculline had no effect on the positive modulation of phenylephrine or forskolin by diazepam (Figure 6A,B). Voltage-gated calcium channels, through a CaM-dependent mechanism, are known to play a role in α1-AR-induced cAMP production and diazepam is thought to bind to some Ca2+ channels.23 Cotreatment of cells with the broad spectrum Ca2+ channel inhibitor benidipine significantly reduced both the phenylephrine response and the diazepam modulation of the CRE response in α1A-AR expressing cells but had no effect on the positive modulation of forskolin-stimulated CRE response in the same cells (Figure 6A,B). Similarly, the CaM inhibitor W-7 hydrochloride significantly reduced both the phenylephrine response and the diazepam modulation of the CRE response in α1A-AR expressing cells, probably by blocking the same pathway as the calcium channel inhibitor but had no significant effect on forskolin stimulation (Figure 6C,D). These results implicate Ca2+ channels in α1-AR-induced CRE activation, however are unlikely to be driving diazepam mediated positive modulation as diazepam modulation of forskolin-induced CRE was unaffected by benidipine or W-7 hydrochloride.

Other inhibitors such as the InsP3R antagonist 2-APB, the L-type calcium channel inhibitor (R)-(+)-Bay K 8644, and the Ca2+/CaM-dependent protein kinase II inhibitor KN-93, had no significant effect on the ability of diazepam to positively modulate the phenylephrine-or forskolin-stimulated CRE responses (Figure 6C,D).

Diazepam is an inhibitor of PDEs, especially PDE-4,24 and thus should increase the levels of cAMP in cells during the CRE assay, potentially explaining our observations. In this case, we would expect co-addition of the broad-spectrum PDE inhibitor IBMX, or the PDE-4 inhibitor rolipram, to have no additional modulatory effect on top of diazepam treatment in our CRE assays. Indeed, in α1A-AR expressing COS-7 cells, IBMX and rolipram positively modulated the CRE response of phenylephrine to a similar level as diazepam (Figure 6E); however, cotreatment using IBMX or rolipram with diazepam had no additional positive modulatory effect on the phenylephrine response (Figure 6E). Similarly, IBMX and rolipram positively modulated the CRE response of forskolin on these same cells, but no additive effect
was observed for either IMBX plus diazepam or rolipram plus diazepam (Figure 6F). In fact, cotreatment of rolipram and diazepam significantly decreased the forskolin‐induced CRE response compared to rolipram alone (Figure 6F), possibly indicating competition between diazepam and rolipram at the same binding site on PDE4. These data strongly suggest that the positive modulation of the phenylephrine‐induced α1‐AR CRE response by diazepam, and other benzodiazepines, is caused through inhibition of PDEs.

Lorazepam was recently reported to be an allosteric modulator of the pH-sensitive GPCR, GPR68.25 GPR68 has been shown to couple to Gq, Gs, G12/13, and Gi/o proteins25 and thus should activate the CRE reporter assay. We thus sought to use lorazepam modulation of GPR68 as a positive control for direct receptor allosterism in our CRE assay. A pH‐dependent CRE response was observed in HEK cells transfected with GPR68, with the observed EC50 of pH 7.2 reflective of other reports25 (Figure 7A). Critically lorazepam, but not diazepam, positively modulated the CRE response driven by pH 7.2 stimulation of GPR68 (Figure 7B). Treatment with both lorazepam and rolipram resulted in a CRE response similar to that of lorazepam alone. This contrasts with the competitive effect observed upon diazepam and rolipram cotreatment of phenylephrine‐stimulated α1‐AR expressing COS‐7 cells (Figure 6) and suggests that lorazepam modulates GPR68 through a PDE‐independent, possibly direct, mechanism.

4 | DISCUSSION

The benzodiazepines diazepam, lorazepam and midazolam have been reported to be PAMs at the α1‐ARs.7 Waugh et al.7 used indirect radioligand binding and signaling assays on receptor‐overexpressing cell lines to demonstrate positive modulation of these benzodiazepines on α1A‐AR, α1B‐AR, and α1D‐AR; however, direct binding of the benzodiazepines to α1‐ARs has never been measured. Here, purified, thermostabilized α1‐AR variants were used as a tool to probe whether diazepam and lorazepam positively modulate α1‐AR signaling via direct binding to the receptors. Interestingly, no evidence was obtained that diazepam or lorazepam could bind to either purified α1‐AR subtype using fluorescent ligand binding assay (Figure 1) or STD NMR (Figure 2). Possibly, this may have been due to the thermostabilizing mutations in the receptors and/or the solubilization state in detergent micelles perturbing the natural benzodiazepine
binding sites. In fact, agonists do exhibit weaker affinities for these thermostabilized receptors in solution and it is not clear whether the receptors can sample active states in detergent, which might preclude binding at allosteric sites. However, diazepam also had no effect on the binding of an orthosteric antagonist (QAPB) to cells stably expressing WT α₁B-AR and did not positively modulate phenylephrine-induced CRE stimulation in WT α₁A-AR and α₁B-AR expressing cells. The ability of diazepam to modulate the CRE response was independent of α₁-AR stimulation, which was shown using β-AR agonists and cells that natively express β-ARs. This suggests that diazepam acts upon signaling elements downstream of the receptor. Interestingly, while diazepam treatment improved the potency of phenylephrine at activating CRE in both α₁A-AR and α₁B-AR expressing cells, it enhanced the efficacy of phenylephrine only at α₁B-AR expressing cells. This likely reflects differences between how phenylephrine activates CRE at α₁A-AR compared to α₁B-AR.

The SAR governing benzodiazepine action at GABA_A receptors is well understood. The substituent at C-7 is of paramount importance; small electron-withdrawing substituents at C-7 generally impart high activity, whereas electron donors or large groups are inactive. Substitutions at N-1 in contrast are generally tolerated, even though...
they can impart significant modulatory effects on GABA<sub>A</sub> activity. By screening various analogues of diazepam, the SAR governing CRE modulation was found to be different to that at GABA<sub>A</sub>R (Table 1), with the methyl group at the N-1 position of diazepam shown to be vital for modulating the CRE response, whereas phenyl substitution at C-7 was tolerated. These chemical differences indicate that the effect observed here is through a target other than GABA<sub>A</sub>R.

Benzodiazepine interactions have been reported against cholecystokinin receptors, HIV-1 reverse transcriptase, opioid receptors, muscarinic receptors, translocator protein, Ca<sup>2+</sup> channels, Ca<sup>2+</sup>/CaM-dependent protein kinases, and PDE. Using inhibitors of several of the other potential target proteins that could be responsible for CRE modulation we were able to conclude that inhibition of PDE, most likely PDE-4, by diazepam causes modulation of the CRE response (Figure 6). Diazepam inhibits the activity of guinea pig PDE-4 with an IC<sub>50</sub> of 9 μmol/L, which is similar to the potency of diazepam for modulating the CRE response (Figure 4). Diazepam also competes with <sup>3</sup>H-rolipram at purified guinea pig PDE-4, which potentially explains the ability of diazepam to compete with rolipram (Figure 6). Collado et al. screened several benzodiazepines for activity at guinea pig PDE-4 and found that clonazepam, nitrazepam, and lorazepam, each of which are unsubstituted at N-1, were less potent than diazepam, which broadly matches the SAR observed here. A similar benzodiazepine, lorazepam, also lacked the ability to modulate the CRE response, but was recently found to positively modulate the pH-sensitive GPCR and GPR68. Here, we confirmed the activity of lorazepam upon GPR68 using the CRE assay and demonstrated that its activity is not due to PDE inhibition. Notably, lorazepam induced a significantly higher GPR68-induced CRE response than diazepam or rolipram treatment. Interestingly, diazepam did not significantly increase the CRE response of GPR68 at pH 7.2, suggesting that GPR68 stimulates CRE in a different, more robust way to α<sub>1</sub>-ARs. This may be related to the fact that GPR68 can couple to Gs proteins to directly activate cAMP accumulation, and thus CRE, whereas α<sub>1</sub>-ARs stimulate CRE indirectly through Gq.

In summary, this work shows the value of stabilized, purified α<sub>1</sub>-ARs to probe direct molecular interactions, allowing us to show that the modulation of α<sub>1</sub>-AR activity by benzodiazepines in cell-based assays is not a result of direct ligand binding. We further demonstrated that significant modulation of cellular CRE responses by diazepam most likely occurred through inhibition of PDE-4. Given the widespread use of benzodiazepines therapeutically, this off-target effect may contribute to their clinical actions and side effects and warrants further study. In contrast to this, lorazepam was validated as a direct-acting, PAM of the pH sensitive receptor GPR68.

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**FIGURE 7** GPR68-induced cAMP response element (CRE) response is modulated by lorazepam but not diazepam. (A) pH-response curve of CRE stimulation in human embryonic kidney (HEK)-293T cells transfected with GPR68. Points are the mean ± SD of three independent experiments. (B) Modulation of pH 7.2 stimulation of the CRE response in HEK-293T cells transfected with GPR68 and treated with lorazepam and diazepam in the presence or absence of rolipram. * Indicates statistically significant difference between the indicated groups as determined using one-way ANOVA and Dunnett multiple comparisons test. Points represent mean values from individual replicate experiments, horizontal lines the mean of the replicates, and error bars the standard deviations.
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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