

# The Agonism and Synergistic Potentiation of Weak Partial Agonists by Triethylamine in $\alpha_1$ -Adrenergic Receptor Activation: Evidence for a Salt Bridge as the Initiating Process

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## ABSTRACT

$\alpha_1$ -adrenergic receptor (AR) activation is thought to be initiated by disruption of a constraining interhelical salt bridge (Porter *et al.*, 1996). Disruption of this salt bridge is achieved through a competition for the aspartic acid residue in transmembrane domain three by the protonated amine of the endogenous ligand norepinephrine and a lysine residue in transmembrane domain seven. To further test this hypothesis, we investigated the possibility that a simple amine could mimic an important functional group of the endogenous ligand and break this  $\alpha_1$ -AR ionic constraint leading to agonism. Triethylamine (TEA) was able to generate concentration-dependent increases of soluble inositol phosphates in COS-1 cells transiently transfected with the hamster  $\alpha_{1b}$ -AR and in Rat-1 fibroblasts stably transfected with the human  $\alpha_{1a}$ -AR subtype. TEA was also able

to synergistically potentiate the second messenger production by weak partial  $\alpha_1$ -AR agonists and this effect was fully inhibited by the  $\alpha_1$ -AR antagonist prazosin. However, this synergistic potentiation was not observed for full  $\alpha_1$ -AR agonists. Instead, TEA caused a parallel rightward shift of the dose-response curve, consistent with the properties of competitive antagonism. TEA specifically bound to a single population of  $\alpha_1$ -ARs with a  $K_i$  of  $28.7 \pm 4.7$  nM. In addition, the site of binding by TEA to the  $\alpha_1$ -AR is at the conserved aspartic acid residue in transmembrane domain three, which is part of the constraining salt bridge. These results indicate a direct interaction of TEA in the receptor agonist binding pocket that leads to a disruption of the constraining salt bridge, thereby initiating  $\alpha_1$ -AR activation.

$\alpha_1$ -ARs are part of a larger family of ARs comprised of three  $\beta$ -, three  $\alpha_2$ -, and three  $\alpha_1$ -AR subtypes ( $\alpha_{1a}$ -,  $\alpha_{1b}$ -,  $\alpha_{1d}$ -) (Bylund, 1992). ARs are members of a superfamily of G protein-coupled receptors, all of which share the common structural motif of a single polypeptide chain that transverses the cell membrane using seven  $\alpha$ -helical domains. The seven TMDs of the  $\alpha_1$ -AR form a hydrophilic ligand-binding pocket in which the endogenous agonists epinephrine and norepinephrine bind with the receptor. It is postulated that binding of these agonists changes the  $\alpha_1$ -AR tertiary protein structure. Spin-labeling studies of the prototypical G protein-coupled receptor rhodopsin have indicated that during the photoactivation process, rigid body movements of TMD three and six take place (Farahbakhsh *et al.*, 1995; Altenbach *et al.*, 1996). Likewise, changes in TMDs six and seven have been

observed in bacteriorhodopsin, even though this related seven-TMD receptor does not couple to G proteins (Subramaniam *et al.*, 1993). Site-directed mutagenesis studies of rhodopsin have elucidated an activational mechanism involving the disruption of a salt bridge constraint between Glu113 on TMD three and Lys296, which forms a Schiff's base with retinal in TMD seven (Robinson *et al.*, 1992). Light-induced isomerization of *cis*-retinal to the all-*trans* form breaks this salt bridge, which leads to receptor activation. However, for any G protein-coupled receptor other than rhodopsin, little is known about the agonist-dependent molecular mechanisms of receptor stimulation.

Activation of  $\alpha_1$ -ARs is postulated to be conserved in the rhodopsin paradigm by disruption of a similar salt bridge between a conserved aspartic acid in TMD three and a lysine residue in TMD seven (Porter *et al.*, 1996). We have shown previously through mutagenesis that eliminating the charge of the amino acids that form this  $\alpha_{1b}$ -AR salt bridge causes the receptor to become constitutively active. The mechanism of agonist activation is thought to involve a competition for the negative charge of the aspartic acid in TMD three by the

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**ABBREVIATIONS:** AR, adrenergic receptor; TMD, transmembrane domain; IP, inositol phosphates; TEA, triethylamine; [ $^{125}$ I]HEAT, ( $\pm$ )- $\beta$ -([ $^{125}$ I]iodo-4-hydroxyphenyl)-ethyl-aminomethyl-tetralone.

protonated amine of the endogenous ligand and the positively charged lysine in TMD seven. Hence, the basic amine of the catecholamine ligand disrupts the constraining salt bridge, allowing the  $\alpha_{1b}$ -AR to adopt an active conformation.

The structure of norepinephrine can be divided into its basic organic components of a phenol ring attached to an ethylamine moiety. Ethylamine contains the positively charged nitrogen of  $\alpha_1$ -AR agonists known to interact with the conserved aspartic acid in TMD three (Porter *et al.*, 1996). If activation of the  $\alpha_1$ -AR is initiated through disruption of the salt bridge by the protonated amine of the catecholamine, then simple basic amines may also be able to directly activate the  $\alpha_1$ -AR. To test this hypothesis, we used TEA as a mimic of norepinephrine to see if this simple amine could initiate a response mediated by  $\alpha_1$ -AR activation. We found that TEA behaves as an agonist by specifically binding and activating the  $\alpha_1$ -AR. A synergistic potentiation of weak partial receptor agonists by TEA is consistent with the salt bridge being an initial component for receptor activation. These functional responses of TEA are the result of a direct and competitive effect at the site of the receptor salt bridge that causes  $\alpha_1$ -AR activation.

## Experimental Procedures

**Site-directed mutagenesis.** Site-directed mutagenesis was performed on a M13mp19 hamster  $\alpha_{1b}$ -AR construct using the oligonucleotide-mediated double primer method (Sambrook *et al.*, 1989) as described previously (Porter *et al.*, 1996). DNA was purified and sequenced by the dideoxy method to verify the mutation. The mutated  $\alpha_{1b}$ -AR insert was removed from the phage M13mp19 vector, then subcloned into the eukaryotic expression vector, pMT2'. The full length plasmid DNA was again sequenced to verify the mutation.

**Cell culture and transfection.** COS-1 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and the transient transfection was performed as previously described using the diethylaminoethyl-dextran method (Porter *et al.*, 1996). Rat-1 fibroblasts stably transfected with the human  $\alpha_{1a}$ -AR were grown in Dulbecco's modified Eagle's medium plus 500  $\mu$ g/ml Geneticin (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum.

**IP hydrolysis.** Total soluble IP production was determined from rat-1 fibroblasts and COS-1 cells that were prelabeled with [ $^3$ H]inositol (1  $\mu$ Ci/ml) for 16–24 hr before the assay. On the assay day, these cultured cells were prepared as described previously (Porter *et al.*, 1996). Stocks of TEA were titrated to pH 7.3 and assays were performed in Dulbecco's modified Eagle's medium without serum to maintain the physiological pH value. The amount of [ $^3$ H]IP was determined by scintillation and a concentration-response curve was generated using iterative nonlinear regression analysis (Multulsky and Ransnas, 1987).

**Radioligand binding.** Transfected COS-1 cell membranes were prepared as described previously (Perez *et al.*, 1991). The pharmacological profile of expressed  $\alpha_{1b}$ -ARs was determined by saturation and/or competition binding experiments using the selective  $\alpha_1$ -AR antagonist [ $^{125}$ I]HEAT as the radiolabel. All binding experiments were performed as previously described (Porter *et al.*, 1996). Binding curves were generated using iterative nonlinear regression analysis (Multulsky and Ransnas, 1987). Protein concentrations were measured using the method of Bradford (1976).

**Statistical analysis.** For each individual experiment, the fitted iterative nonlinear regression curve that best represented the data was determined using a partial *f*-test ( $p < 0.05$ ). Significance between groups was tested using an unpaired two-tailed Student's *t*

test ( $p < 0.05$ ). All values are reported as the mean  $\pm$  standard error of a certain number of experiments, each performed in duplicate.

**Materials.** Triethylamine, (–)-epinephrine, oxymetazoline, (–)-methoxamine, and prazosin were purchased from Sigma Chemical (St. Louis, MO); clonidine was purchased from RBI (Natick, MA); and [ $^3$ H]inositol and [ $^{125}$ I]HEAT from New England Nuclear Research Products (Boston, MA).

## Results

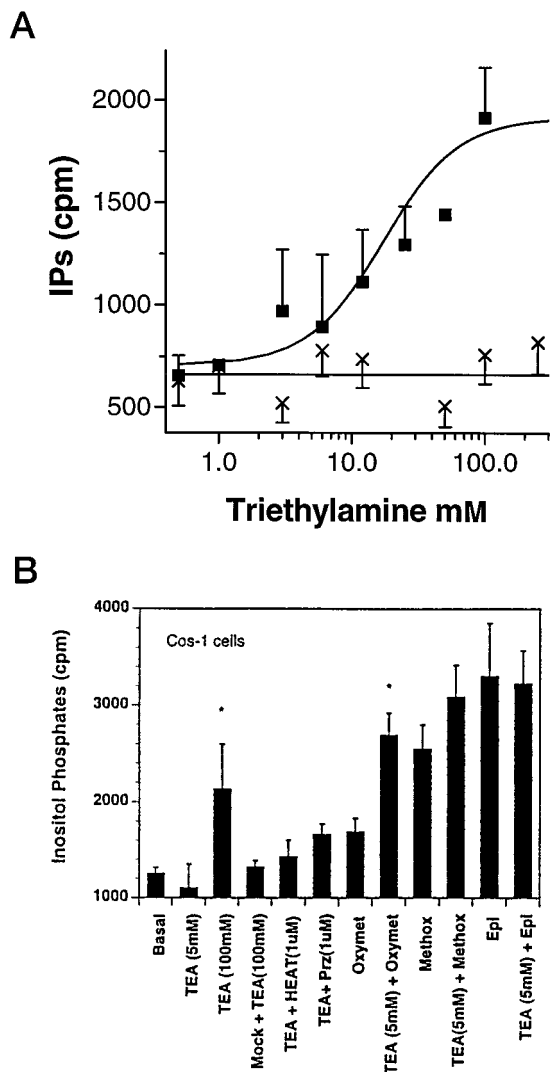
**$\alpha_1$ -AR-mediated increases of soluble IP in cultured cells by TEA.** TEA induced a concentration-dependent increase of soluble IP in AR negative COS-1 cells transiently transfected with the  $\alpha_{1b}$ -AR subtype (Fig. 1A). The EC<sub>50</sub> value for this response was  $17.2 \pm 3.7$  mM. The effect was mediated through activation of  $\alpha_{1b}$ -ARs, as indicated by no significant increase of soluble IP production in mock-transfected COS-1 cells stimulated over the same concentrations of TEA (Fig. 1A). In addition, the increased soluble IP production generated by 100 mM TEA was blocked by 1  $\mu$ M of the AR antagonists HEAT or prazosin (Fig. 1B). Various full and partial receptor agonists were also used at their maximal concentrations in these transfected COS-1 cells (Fig. 1B). When a weak partial agonist, oxymetazoline, was coincubated with a threshold amount (5 mM) of TEA, a synergistic potentiation of the response was observed (Fig. 1B). This potentiating effect of TEA was not reproduced in the presence of the full AR agonist (–)-epinephrine (Fig. 1B).

TEA also generated a concentration-dependent increase of soluble IP production in rat-1 fibroblasts stably transfected with the human  $\alpha_{1a}$ -AR subtype (Fig. 2A). The EC<sub>50</sub> value for this response was  $13.0 \pm 0.4$  mM, which is similar to the value calculated for the  $\alpha_{1b}$ -AR expressed on COS-1 cells. The increase of soluble IP production in rat-1 fibroblasts was blocked by the  $\alpha_1$ -AR antagonist prazosin, suggesting that this response is mediated by  $\alpha_{1a}$ -AR activation (Fig. 2B). Conversely, no significant response over basal was observed when 50 mM KCl was used to generate soluble IP in the absence or presence of prazosin (Fig. 2B). Oxymetazoline and (–)-methoxamine gave large increases of soluble IP production, comparable with (–)-epinephrine (Fig. 2B). When a threshold amount (5 mM) of TEA was included with a maximal concentration (10–100  $\mu$ M) of the same AR agonists, no potentiation in soluble IP was observed (Fig. 2B). Instead, 5 mM TEA caused a significant parallel rightward shift of the dose-response for the full AR agonist, (–)-epinephrine (Fig. 2C). The EC<sub>50</sub> value of (–)-epinephrine for the control response was  $1.2 \pm 0.1$   $\mu$ M and  $3.5 \pm 0.4$   $\mu$ M in the presence of TEA. Conversely, a maximal concentration (1 mM) of clonidine performed as a weak partial  $\alpha_1$ -AR agonist (Fig. 2B). When a threshold dose (5 mM) of TEA was added to increasing amounts of clonidine, there was a synergistic potentiation of the concentration-response curve that was totally blocked by the AR antagonist prazosin (Fig. 2D). In addition, the EC<sub>50</sub> value for clonidine in the presence of TEA ( $3.0 \pm 0.7$   $\mu$ M) was significantly different from control ( $0.7 \pm 0.1$   $\mu$ M).

**Binding of TEA to the  $\alpha_{1b}$ -AR.** To understand whether TEA was activating the  $\alpha_1$ -AR through disruption of the salt bridge, radioligand binding studies were performed. Preliminary time course experiments were performed to ensure that a binding equilibrium was obtained and not affected by TEA under the normal conditions of 1hr (data not shown). In-

ing concentrations of TEA were able to compete for specific [ $^{125}$ I]HEAT binding sites from isolated COS-1 cell membranes expressing the hamster  $\alpha_{1b}$ -AR subtype (Fig. 3). The affinity of TEA for the  $\alpha_{1b}$ -AR was  $28.7 \pm 4.7$  mM. This number is similar to the potency of TEA for increasing soluble IP in cultured cells. Similar TEA competition binding experiments were performed on previously characterized and constitutively active  $\alpha_{1b}$ -AR salt bridge mutants (Porter et al., 1996). The affinity of TEA for the  $\alpha_{1b}$ -AR K331A mutant was, significantly, 7-fold higher ( $4.0 \pm 0.2$  mM) than the

affinity for the wild-type  $\alpha_{1b}$ -AR, implying that TEA is an AR agonist (Fig. 3). Previous work has also documented the 5-fold lower affinity of (-)-epinephrine for the  $\alpha_1$ -AR D125A mutant compared with the wild-type  $\alpha_{1b}$ -AR, which is consistent with D125 interacting with the protonated amine of catecholamines (Porter et al., 1996). However, increasing concentrations of TEA were not able to compete for specific [ $^{125}$ I]HEAT binding sites from COS-1 cell membranes expressing the mutant  $\alpha_{1b}$ -AR D125A (Fig. 3). In addition, when the  $\alpha_{1b}$ -AR D125A mutant expressed on COS-1 cells was incubated with 50 mM TEA, no detectable IP production was measured (data not shown).



**Fig. 1.** Agonist properties of TEA for generating soluble IP in COS-1 cells transfected with the hamster  $\alpha_{1b}$ -AR subtype. A, COS-1 cells transfected with the wild-type  $\alpha_{1b}$ -AR (■) or mock (×) were stimulated with increasing concentrations of TEA to generate soluble IP. The  $EC_{50}$  value of this TEA response was  $17.2 \pm 3.7$  mM. Measurements are presented as the mean  $\pm$  standard error for three experiments performed in duplicate. B, COS-1 cells transfected with the  $\alpha_{1b}$ -AR were stimulated to generate [ $^3$ H]-IP with threshold (5 mM) and maximal (100 mM) concentrations of TEA. In separate experiments the  $\alpha_1$ -AR antagonists HEAT (HEAT; 1  $\mu$ M) and prazosin (Prz; 1  $\mu$ M) were used to inhibit the 100 mM TEA response. Maximal amounts of the  $\alpha_1$ -AR agonists oxymetazoline (Oxymet; 1 mM), (-)-methoxamine (Methox; 1 mM), and (-)-epinephrine (Epi; 10  $\mu$ M) were used in the presence or absence of a threshold concentration (5 mM) of TEA. \*, TEA (100 mM) is significantly different than basal ( $p < 0.05$ ) and oxymetazoline plus TEA is significantly different than oxymetazoline alone ( $p < 0.01$ ). The density of the  $\alpha_{1b}$ -AR expressed on COS-1 cells was approximately 1 pmol/mg membrane protein. Points, mean  $\pm$  standard error for three experiments performed in duplicate.

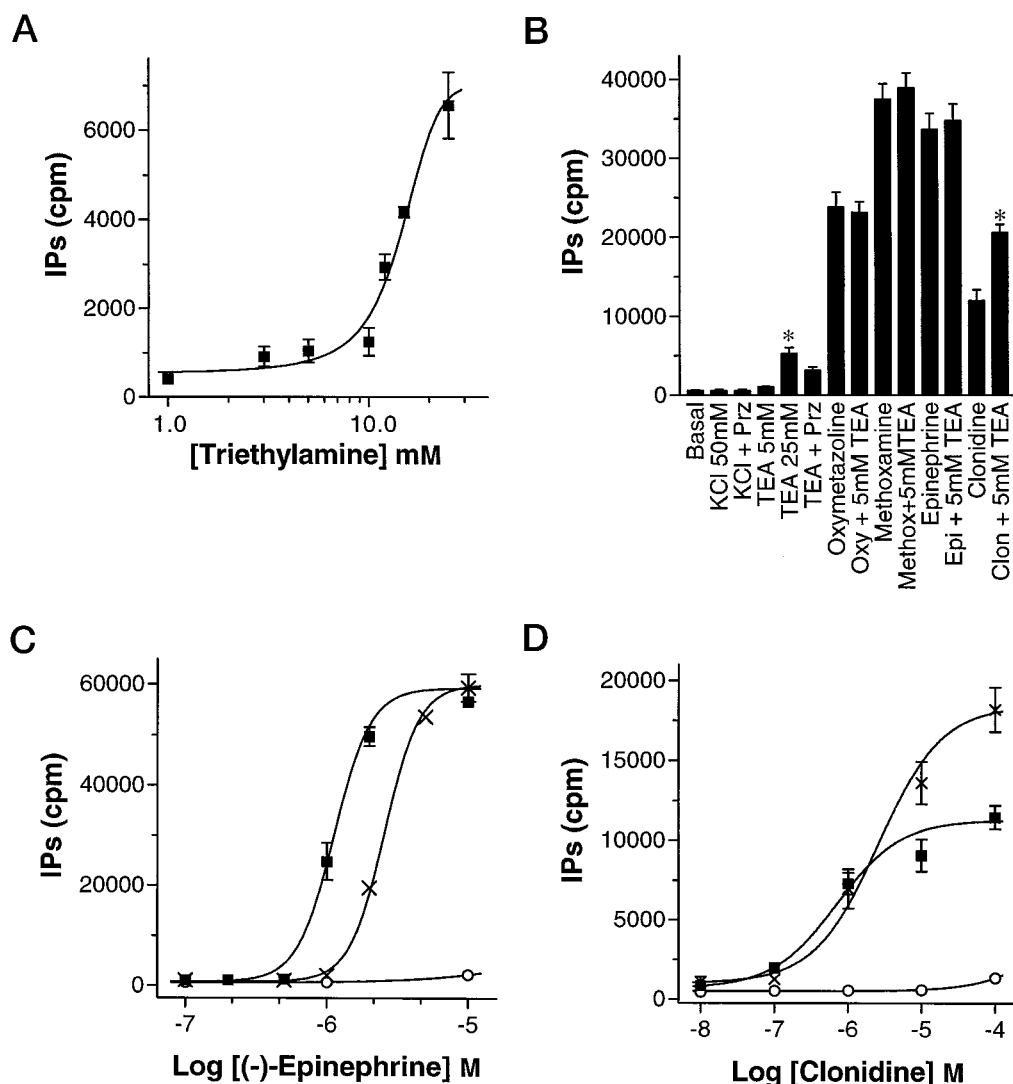
## Discussion

In previous studies, we have postulated that the initial event of agonist-induced  $\alpha_{1b}$ -AR activation involves the disruption of an interhelical salt bridge (Porter et al., 1996). In addition, the amino acids that form the salt bridge are conserved in all other  $\alpha_1$ -AR subtypes. For this investigation, we hypothesized that if an ionic constraint were part of the  $\alpha_1$ -AR activation process, a basic amine such as TEA could act as a receptor agonist. In this study, we have shown that TEA is indeed an agonist for  $\alpha_1$ -AR subtypes in various cell lines (Fig. 1–2). The agonistic properties of TEA are not due solely to a charge effect because no response was observed for 50 mM KCl (Fig. 2B). Instead, it is the strength of the charge or the high basicity that enables TEA to compete for the negative D125, thereby initiating the  $\alpha_1$ -AR activation process. However, the intrinsic activity of TEA, measured as a fraction of the full receptor agonist response in transfected cell lines, is rather weak. This suggests that salt bridge disruption is not the only process required in  $\alpha_1$ -AR activation. However, this inference is consistent with the salt bridge mechanism being an initiating event of receptor stimulation. These results are similar to the rhodopsin system, in which neither mutations of the salt bridge nor addition of salts produced full receptor activation (Sakmar et al., 1991).

Certain quaternary ammonium compounds such as galamine and tetramethylammonium have been shown to bind at the same time as the radioligand in muscarinic receptors; therefore, these allosteric ligands bind at a site distinct from the radiolabel. Interactions of these ammonium compounds with the muscarinic receptor have been localized to extracellular residues and not to the agonist binding pocket (Ellis et al., 1993; Leppick et al., 1994). In addition, quaternary ammonium compounds have been shown to either block or activate responses, depending upon the chemical structure, mediated by muscarinic receptors and/or acetylcholine channels (Volle and Koelle, 1969). Because TEA is also an ammonium compound, albeit a tertiary amine, it is possible that this drug could also bind to the  $\alpha_1$ -AR in an allosteric fashion analogous to the type of binding reported for the muscarinic receptor subtype. This would contradict a competitive mechanism occurring at the  $\alpha_1$ -AR salt bridge. To explore this possibility, we studied extensively the binding properties of TEA for the  $\alpha_1$ -AR. TEA can compete for the specific [ $^{125}$ I]HEAT binding to  $\alpha_{1b}$ -ARs (Fig. 3) with an affinity similar to its functional  $EC_{50}$  value, a common property of partial receptor agonists. The curves generated from these binding assays fit best to a one-site model, indicating that TEA was interacting at a single location on the  $\alpha_{1b}$ -AR.

We (Porter *et al.*, 1996) and others (Strader *et al.*, 1994) have previously demonstrated that the protonated amine of norepinephrine binds to the conserved Asp125 in TMD three of the  $\alpha_{1b}$ -AR as well as other ARs. Because TEA is a chemical mimic for the amine side chain of norepinephrine, we postulated that this simple amine should also bind to the negatively charged Asp125. To test this hypothesis, TEA competition binding was performed on a mutant  $\alpha_{1b}$ -AR, where this conserved aspartic acid was changed to a neutral alanine. This  $\alpha_{1b}$ -AR D125A mutation can still specifically

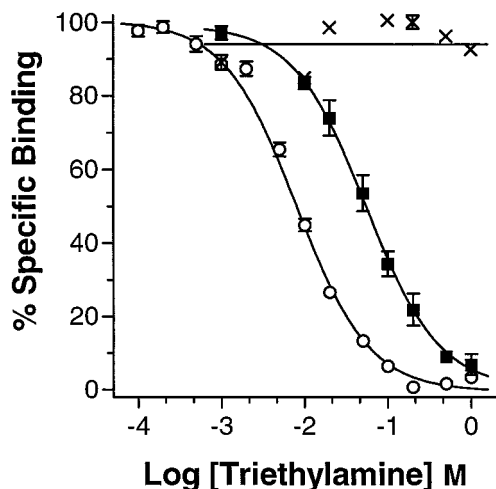
bind [ $^{125}$ I]HEAT with near wild-type affinity and has been characterized previously to be constitutively active (Porter *et al.*, 1996). This mutant receptor also specifically binds other  $\alpha_1$ -AR ligands, although receptor agonists bind at a lower affinity because the altered D125 is a direct agonist binding contact site (Porter *et al.*, 1996). However, with increasing concentrations of TEA, no change in the percent of specific [ $^{125}$ I]HEAT binding was observed for the  $\alpha_{1b}$ -AR D125A mutant (Fig. 3). Moreover, when the  $\alpha_{1b}$ -AR D125A mutant was used in signal transduction studies, TEA was unable to in-



**Fig. 2.** Agonist properties of TEA for generating soluble IP in rat-1 fibroblasts transfected with the human  $\alpha_{1a}$ -AR subtype. A, Rat-1 fibroblasts that had been stably transfected with the human  $\alpha_{1a}$ -AR (■) were stimulated with increasing concentrations of TEA to generate soluble IP. The  $EC_{50}$  value of this TEA response was  $13.0 \pm 0.4$  mM. Measurements are presented as the mean  $\pm$  standard error for three experiments performed in duplicate. B, Rat-1 fibroblasts transfected with the  $\alpha_{1a}$ -AR were stimulated to generate [ $^3$ H]-IP with threshold (5 mM) and maximal (25 mM) concentrations of TEA. The  $\alpha_1$ -AR antagonist prazosin (Prz; 1  $\mu$ M) was used to inhibit the 25 mM TEA response. KCl (50 mM) was also used in the absence and presence of 1  $\mu$ M prazosin. Maximal amounts of the  $\alpha_1$ -AR agonists oxymetazoline (Oxymet; 100  $\mu$ M), (-)-methoxamine (Methox; 100  $\mu$ M), clonidine (Clon; 1 mM), and (-)-epinephrine (Epi; 10  $\mu$ M) were used in the presence or absence of 5 mM TEA. \*, TEA (25 mM) is significantly different than basal ( $p < 0.001$ ) and clonidine plus TEA is significantly different than clonidine alone ( $p < 0.02$ ). Values are presented as the mean  $\pm$  standard error for three experiments performed in duplicate. C, Rat-1 fibroblasts expressing the  $\alpha_{1a}$ -AR were exposed to increasing concentrations of the AR agonist (-)-epinephrine, in the absence (■) or presence (×) of 5 mM TEA or (○) 5 mM TEA and 1  $\mu$ M prazosin to generate soluble IP. The  $EC_{50}$  value of this (-)-epinephrine response was  $1.2 \pm 0.1$   $\mu$ M for control and  $3.5 \pm 0.4$   $\mu$ M in the presence of TEA. The  $EC_{50}$  value for (-)-epinephrine plus TEA is significantly different than (-)-epinephrine alone ( $p < 0.0001$ ). Points, mean  $\pm$  standard error for four to six experiments performed in duplicate. D, Rat-1 fibroblasts expressing the  $\alpha_{1a}$ -AR were exposed to increasing concentrations of the AR agonist clonidine, in the absence (■) or presence (×) of 5 mM TEA or (○) 5 mM TEA and 1  $\mu$ M prazosin to generate soluble IP. The  $EC_{50}$  value of this clonidine response was  $0.7 \pm 0.1$   $\mu$ M for control and  $3.0 \pm 0.7$   $\mu$ M in the presence of TEA. The  $EC_{50}$  value for clonidine plus TEA is significantly different than clonidine alone ( $p < 0.0003$ ). Points, mean  $\pm$  standard error for two to six experiments performed in duplicate. The density of the  $\alpha_{1a}$ -AR expressed on rat-1 fibroblasts was 8.3 pmol/mg membrane protein.

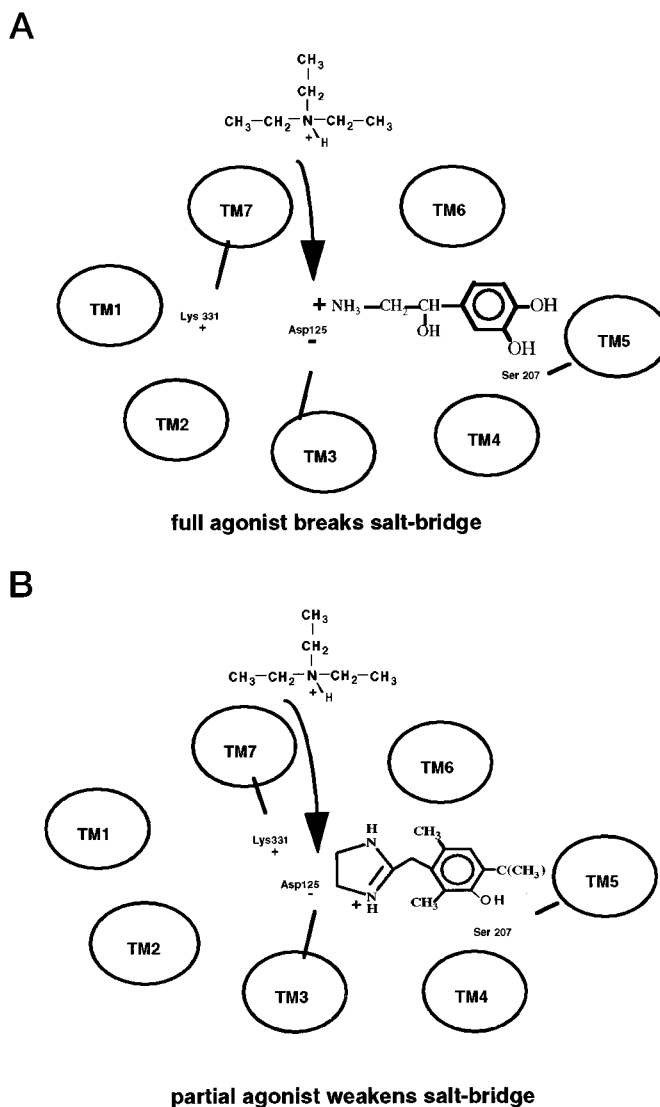
voke an IP response. Supporting these results, the  $\alpha_{1b}$ -AR K331A salt bridge mutant is also constitutively active but leads to a higher binding affinity for all receptor agonists tested with no changes in receptor antagonist affinities (Porter et al., 1996). It is thought that these constitutively active mutations mimic the "activated state" of the receptor, adopting a conformation that shows a phenotype of high affinity binding for agonists, thus conforming to the revised ternary complex model (Samama et al., 1993). Here, TEA indeed shows a significantly higher binding affinity for the  $\alpha_{1b}$ -AR K331A mutant than the wild-type receptor, also suggesting an agonist phenotype (Fig. 3). These studies indicate that TEA is indeed docking in the agonist binding pocket with the conserved Asp125 in TMD three of the  $\alpha_{1b}$ -AR and that this is the site of its activational properties. Taken together, these results support the assumption of a competitive interaction by TEA in the  $\alpha_1$ -AR ligand binding pocket.

A reproducible effect of TEA was its ability to synergistically potentiate the activation of  $\alpha_1$ -ARs by weak partial agonists (Figs. 1B and 2D). This potentiation was specific for only weak partial agonists because use of fuller receptor agonists showed no intrinsic activity differences in the presence of TEA (Fig. 1B, 2B). However, TEA demonstrated a competitive antagonistic property for the (-)-epinephrine dose-response in rat-1 fibroblasts (Fig. 2C). The competitive property of partial receptor agonists to inhibit the response induced by full receptor agonists with higher relative efficacies has been documented previously (Kenakin, 1993). Therefore, it is not unexpected to observe a competitive inhibition of the (-)-epinephrine-induced IP release by TEA. Oxymetazoline and TEA showed a synergistic potentiation of the IP production in COS-1 cells expressing the  $\alpha_{1b}$ -AR subtype, although this agonist combination did not produce these same effects in rat-1 fibroblasts expressing the human  $\alpha_{1a}$ -AR subtype. Intrinsic efficacy of a drug is not only dependent



**Fig. 3.** Competitive binding properties of TEA for the  $\alpha_{1b}$ -AR. Increasing concentrations of TEA were used to compete for specific [ $^{125}$ I]HEAT binding on COS-1 cell membranes expressing the wild-type  $\alpha_{1b}$ -AR (■), the  $\alpha_{1b}$ -AR D125A (×), or the  $\alpha_{1b}$ -AR K331A mutants (○). The affinity of TEA for the  $\alpha_{1b}$ -AR or the  $\alpha_{1b}$ -AR K331A mutant is  $28.7 \pm 4.7$  mM and  $4.0 \pm 0.2$  mM, respectively. The  $K_i$  of TEA for the  $\alpha_{1b}$ -AR K331A mutant was significantly different ( $p < 0.0001$ ) from wild-type receptor. No changes in the specific binding of the radioligand were calculated for the mutant  $\alpha_{1b}$ -AR D125A over the range of TEA concentrations used. Points, mean  $\pm$  standard error for two to five experiments performed in duplicate.

upon the subtype and density of receptors present but also is influenced by cell type, because the battery of G proteins expressed can be different. Accordingly, it has been previously demonstrated that many receptor agonists, including oxymetazoline, that activate the  $\alpha_{1a}$ -AR display a higher intrinsic activity and essentially full agonism compared with other  $\alpha_1$ -AR subtypes (Minneman et al., 1994). For this reason, we used the full  $\alpha_2$ -AR agonist clonidine, which is also a weak partial agonist for the  $\alpha_{1a}$ -AR subtype. It was only when we used clonidine in the presence of TEA that we could demonstrate a synergistic potentiation of the



**Fig. 4.** Molecular model for the interaction of TEA in the ligand binding pocket of the  $\alpha_1$ -AR. View from the extracellular surface into the ligand binding pocket of the  $\alpha_{1b}$ -AR subtype. A, Docking of the full  $\alpha_1$ -AR agonist norepinephrine causes disruption of the constraining interhelical salt bridge between the TMD three aspartic acid (Asp125) and the lysine (Lys331) in TMD seven. TEA does not potentiate the response of a full receptor agonist because the salt bridge constraint is already disrupted. Instead, there is a competitive antagonism between TEA and the protonated amine of norepinephrine for D125. B, Interaction of the weak partial  $\alpha_1$ -AR agonist oxymetazoline in the ligand binding pocket only reduces the constraining salt bridge bond strength because of an incomplete association with the TMD three aspartic acid. This allows room for TEA to attack the Asp125 and break the  $\alpha_{1b}$ -AR salt bridge. The synergistic potentiation would be an expected phenotype if the salt bridge were the initial constraining process for  $\alpha_1$ -AR activation.

$\alpha_{1a}$ -AR response. These studies also indicate that it is not a particular structure but a poor intrinsic activity of the receptor agonist that is required for the TEA potentiation response. However, the full intrinsic activity range of weak receptor agonists required by TEA to potentiate a response has not been investigated thoroughly. Theoretically, this range of agonist intrinsic activity and the degree of TEA potentiation could be dependent on the receptor subtype, the strength of the receptor salt bridge, and type of cell or tissue studied.

The synergistic potentiation by TEA was caused solely by an  $\alpha_1$ -AR mediated event, because this response was fully blocked by prazosin (Fig. 2D). We suggest that the data are consistent with a model in which TEA disrupts the  $\alpha_{1b}$ -AR salt bridge by virtue of its own agonism and binding properties for the conserved Asp125. This synergistic potentiation of partial receptor agonists is consistent with TEA mediating a concerted mechanism of  $\alpha_1$ -AR activation by releasing an initial constraint. This mechanism would also account for why fuller receptor agonists are not potentiated in the presence of TEA, because the  $\alpha_{1b}$ -AR salt bridge is already broken. In this scenario, TEA acts as a competitive receptor antagonist because the protonated amine of full receptor agonists optimally binds and interacts with the Asp125 (Fig. 4A). On the other hand, the protonated amine of partial receptor agonists may not be in position to cause disruption of the  $\alpha_{1b}$ -AR salt bridge and instead may only weaken the ionic bond strength. Suboptimal positioning of weak receptor agonists in the ligand binding pocket now allows room for TEA to interact with the Asp125. This TEA interaction breaks the  $\alpha_{1b}$ -AR salt bridge and potentiates the activation mechanism, which in turn synergistically benefits a weak partial receptor agonist (Fig. 4B). We hypothesize that this synergism could be achieved once the  $\alpha_{1b}$ -AR ionic constraint is released, because other helical movements secondary to salt bridge disruption and potentially involved in receptor activation would instantly occur with the partial receptor agonist in place. Alternatively, the weakened bond strength of the  $\alpha_{1b}$ -AR salt bridge caused by the partial receptor agonist positioning in the binding pocket could synergistically potentiate the bond-breaking ability of TEA because the free energy potential of the ionic bond is lowered. In either case, the synergism by TEA of partial but not full receptor agonists suggests disruption of the  $\alpha_{1b}$ -AR salt bridge as the distinguishing process in receptor activation.

Although these studies have used a drug with poor affinity and low intrinsic activity, the results are consistent with a salt bridge mechanism of  $\alpha_1$ -AR activation. In addition, the synergism of weak agonists by TEA suggests it is this initial constraint that needs to be disrupted to cause further  $\alpha_1$ -AR activation. Just as allosteric modification of  $\gamma$ -aminobutyric acid<sub>A</sub> or *N*-methyl-D-aspartate receptors has valuable thera-

peutic effects (Smith and Olsen, 1995), ammonium compounds of higher affinity, potency, and intrinsic activity may be found to act on  $\alpha_1$ -ARs. Currently, there are no advantageously selective agonists that are effective for activating the  $\alpha_{1b}$ - or the  $\alpha_{1d}$ -AR subtypes. Here, a possible therapeutic potential would be to coadminister an  $\alpha_1$ -AR specific ammonium compound to increase the potency and intrinsic activity of weak partial receptor agonists that are selective for particular  $\alpha_1$ -AR subtypes.

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