Tris Hcl (pH 7.4), 1 mM EGTA, 2 mM EDTA, 2 mM DTT, 10 mM β-glycerophosphate, Triton X-100 (0.1%), glycerol (10%), before assay of S6 kinase activity.
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23. Cystolic extracts were matched for protein content, diluted with three parts of chromatography buffer [50 mM, β-glycerophosphate (pH 7.2), 1 mM DTT, 1 mM EGTA, 0.1 mM vanadate and applied to a MonoQ HR (515) column]. The column was eluted with a 90-ml gradient of NaCl (0 through 0.4 M final) in chromatography buffer.
24. The cDNA encoding rat p70 S6 kinase αI (*14*), rat p85 S6 kinase (*3*), and rat erk-1 (*21*) were modified by insertion of a nine amino acid–peptide epitope derived from influenza virus hemagglutinin (*22*) at the NH_2-terminus of each polypeptide, immediately after the initiator methionine.
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A Point Mutation of the α_2-Adrenoceptor That Blocks Coupling to Potassium But Not Calcium Currents

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The α_{2A}-adrenergic receptor (adrenoceptor) was stably expressed in AtT20 mouse pituitary tumor cells; adrenoceptor agonists inhibited adenylyl cyclase, inhibited voltage-dependent calcium currents, and increased inwardly rectifying potassium currents. An aspartic acid residue (Asp^{79}) highly conserved among guanine nucleotide–binding protein (G protein)–coupled receptors was mutated to asparagine; in cells transfected with the mutant α_2-receptor, agonists inhibited adenylyl cyclase and calcium currents but did not increase potassium currents. Because distinct G proteins appear to couple adrenoceptors to potassium and calcium currents, the present findings suggest that the mutant α_2-adrenoceptor cannot achieve the conformation necessary to activate G proteins that mediate potassium channel activation.

The α_2-adrenergic receptors (α_2ARs) belong to the superfamily of G protein–coupled receptors, specifically to the branch of this family consisting of receptors coupled to the inhibition of adenylyl cyclase, the inhibition of Ca^{2+} currents, and the activation of K^+ currents by pertussis toxin (PTX)–sensitive G proteins (*1*). One or both of these latter two effects is responsible for the immediate inhibition of neurotransmitter release and neuronal firing produced by activation of presynaptic and postsynaptic α_2ARs on mammalian neurons (*1*). Multiple α_2AR subtypes have been identified by both pharmacological and molecular biological approaches, and site-directed mutagenesis of α_2ARs has identified several amino acids in transmembrane regions II through V as sites of interaction for agonist binding and for receptor coupling to the inhibition of adenylyl cyclase (*1, 2*). However, it is not known whether cloned α_2ARs, when expressed in a heterologous system, can couple to diverse ion channels or whether specific domains can be identified that participate in coupling to specific diverse effector systems. We investigated the coupling of a stably transfected α_{2A}AR (*3*) to K^+ currents, Ca^{2+} currents, and adenylyl cyclase and the consequences of a single amino acid mutation [converting aspartic acid to asparagine at position 79 (Asn^{79} α_2AR)] on the coupling to these three effector systems.

The AtT20 cell does not express endogenous α_2ARs (Table 1) but contains somatostatin receptors that couple to inhibition of adenylyl cyclase (*4*), inhibition of Ca^{2+} currents (*5*), and activation of an inwardly rectifying K^+ current (*6*). Thus, we used AtT20 cells to evaluate the functional properties of wild-type (WT) α_2ARs or Asn^{79} α_2ARs. We compared somatostatin-induced alterations in K^+ and Ca^{2+} currents (*7*) with responses to the α_2AR agonists clonidine and UK 14304 in permanent transformants of AtT20 cells expressing recombinant WT or Asn^{79} α_2ARs (*8*).

The α_2AR agonist UK 14304 increased the K^+ current in cells expressing the WT α_2AR (Fig. 1); 95% of the current induced by UK 14304 was blocked by 1 mM Ba^{2+} (Fig. 1A), as would be expected if the agonist were opening inwardly rectifying K^+ channels (*9*). Somatostatin (100 nM) increased this current by two- to tenfold in all mock-transfected cells and in cells transfected with the WT α_2AR or Asn^{79} α_2AR (Fig. 1, B through E). Maximally effective concentrations of UK 14304 or clonidine produced a 1.5- to 8-fold increase in K^+ current in cells expressing the WT α_2AR (Fig. 1, B through D). Concentrations of clonidine and UK 14304 that produced half-maximal activation (EC_{50}) of the K^+ current were 14 and 30 nM, respectively, in cells expressing the WT α_2AR (Fig. 2A); these are similar to the EC_{50} values for the inwardly rectifying K^+ conductance activated by pharmacologically characterized α_{2A}ARs in autonomic enteric and central locus coeruleus neurons (*10*). The actions of maximally effective concentrations of somatostatin and AR agonists were not additive ($n = 22$), which is evidence that the transfected WT α_2AR couples to the same set of K^+ channels as does the endogenous somatostatin receptor. In contrast to the WT α_2AR, the mutant Asn^{79} α_2AR did not activate K^+ currents (Fig. 1, B, C, and E), even in the presence of 10,000-fold higher concentrations of clonidine or UK 14304 (Fig. 2A).

In contrast to their effects on K^+ currents, α_2AR agonists were effective in inhibiting Ca^{2+} currents in AtT20 cells expressing WT α_2ARs or Asn^{79} α_2ARs (Fig. 3). In either case, the inhibition of Ca^{2+} currents by AR agonists was not quantitatively different from the inhibition of Ca^{2+} currents by somatostatin acting at endogenous receptors (Fig. 3, A and B). Somatostatin inhibits two high-voltage–activated (HVA) Ca^{2+} currents in AtT20 cells, a dihydropyridine-sensitive (HVA/L-type) current and a dihydropyridine-insensitive (HVA/N-type) current (*5*); α_2AR agonists similarly inhibited HVA/L- and HVA/N-type Ca^{2+} currents in cells expressing either WT or Asn^{79} α_2ARs (Fig. 3, C and D). Somatostatin inhibited the Ca^{2+} current in 92% of mock-transfected cells examined but inhibited Ca^{2+} currents in only 50% of cells expressing WT α_2ARs or Asn^{79} α_2ARs (Fig. 3B). The percentage of cells in which somatostatin inhibited the Ca^{2+} current was not correlated with the cell cycle nor the time after cell passage. The explanation for this observation is unclear because the percentage of cells responding to somatostatin with an increase in K^+ current was similar in all cells (Fig. 1C).

There were no apparent differences in the concentration-response curves for clonidine-induced inhibition of the Ca^{2+} current in cells expressing WT or Asn^{79} α_2ARs (Fig. 2B) or the norepinephrine-mediated responses (measured in the presence of pro-

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pranolol to block endogenous β-ARs). Cells expressing WT or Asn^{79} α_2ARs showed no obvious differences in their responses to the α_2AR antagonist idazoxan; idazoxan (100 nM) inhibited the maximum response to UK 14304 by 49 ± 8% (n = 8) in WT α_2AR cells and by 54 ± 6% (n = 10) in Asn^{79} α_2AR cells, and 1 μM idazoxan inhibited responses to agonists by 95 to 100% in both cell types. However, cells expressing the Asn^{79} α_2AR were one-sixth as sensitive to inhibition of the Ca^{2+} current by UK 14304 as cells expressing the WT α_2AR (Fig. 2C). These data suggest that the clonidine analog UK 14304 may behave as a partial agonist for Ca^{2+} current inhibition in comparison to clonidine or norepinephrine in these cells.

Modulation of K^+ or Ca^{2+} currents by α_2AR agonists or by somatostatin was mediated by means of PTX-sensitive G proteins. Incubation of AtT20 cells with PTX (100 ng/ml for 12 to 24 hours before recording) blocked agonist actions on both K^+ and Ca^{2+} currents. In PTX-treated cells, somatostatin (300 nM) increased K^+ currents in only 1 of 10 mock-transfected cells, 1 of 18 WT α_2AR cells, and 2 of 36 Asn^{79} α_2AR cells. Neither clonidine nor UK 14304 (1 to 10 μM) altered K^+ currents in any of these cells. Clonidine inhibited the Ca^{2+} current in only 1 of 35 WT α_2AR cells and 2 of 29 Asn^{79} α_2AR cells that had been treated with PTX.

Cells bearing either the WT or mutant Asn^{79} α_2AR coupled to inhibition of adenosine 3′,5′-monophosphate (cAMP) accumulation (*11*) through a PTX-sensitive pathway in AtT20 cells. Unlike the inhibition of Ca^{2+} currents, the potency of UK 14304 inhibition of cAMP accumulation was not reduced in cells with mutant α_2ARs (Table 1). Our observation that WT and Asn^{79} α_2ARs couple to inhibition of cAMP accumulation and suppression of Ca^{2+} currents through a PTX-sensitive pathway implies that these receptors can productively interact with G proteins mediating these two effector responses. However, when receptor coupling to G proteins was evaluated by guanine nucleotide modulation of agonist binding (*12*), this coupling was less for Asn^{79} α_2ARs than for WT α_2ARs (Table 1).

These data are consistent with observations for the α_2AR (*2*, *3*) that suggest that mutation of Asp^{79} perturbs G protein–dependent agonist interactions but not G protein–independent antagonist interactions with the α_2AR. Perturbation of G protein–

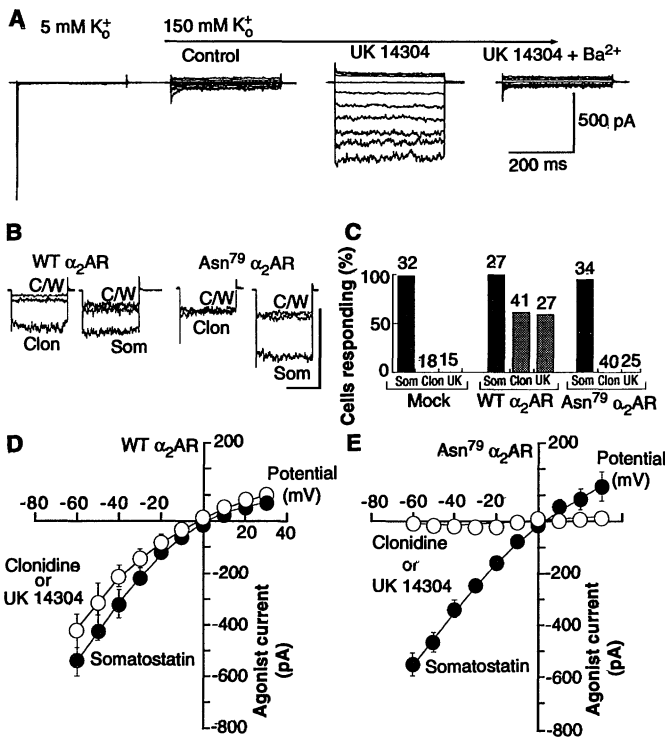

Fig. 1. Activation of K^+ currents by transfected α_2ARs and endogenous somatostatin receptors in AtT20 cells. (**A**) Depiction of superimposed traces of K^+ currents elicited by four sequentially increasing depolarizing pulses, six similar hyperpolarizing pulses applied in 5 mM external K^+ (K_o^+) and after switching from 5 mM K_o^+ to 150 mM K_o^+ (control) after addition of UK 14304 (1 μM) and after Ba^{2+} (1 mM) was added to the UK 14304–containing solution. Holding potential in 5 mM K_o^+ was −70 mV, and in 150 mM K_o^+ it was 0 mV (the K^+ equilibrium potential); large downward transient seen in 5 mM K_o^+ is the fast Na^+ current. (**B**) K^+ currents evoked by a 40-mV hyperpolarizing pulse from 0 mV in 150 mM K_o^+ solution in cells expressing WT α_2ARs and Asn^{79} α_2ARs; each set of traces consists of superimposed currents recorded in control (C) solution in the presence of agonist and after washout (W). Somatostatin (Som, 100 nM) and clonidine (Clon, 100 nM) were added to WT α_2AR cells; these agonists were added at a final concentration of 100 nM and 10 μM in Asn^{79} α_2AR cells. Calibrations are as in (A). (**C**) Percent of cells that exhibited an increased K^+ conductance in response to application of somatostatin (Som), clonidine (Clon), or UK 14304 (UK) in mock-transfected, WT α_2AR–expressing, and Asn^{79} α_2AR–expressing cells; numbers above each bar are numbers of cells examined. (**D** and **E**) Summary of agonist-induced K^+ current recorded in AtT20 cells transfected with the WT α_2AR (D) and the Asn^{79} α_2AR (E) mutant. Currents recorded in high-concentration K_o^+ solution (control) were subtracted from currents recorded in 100 nM somatostatin (closed circles) and 1 μM clonidine or UK 14304 (open circles). Each point in (D) is mean ± SEM from 14 experiments; points in (E) are from nine experiments.

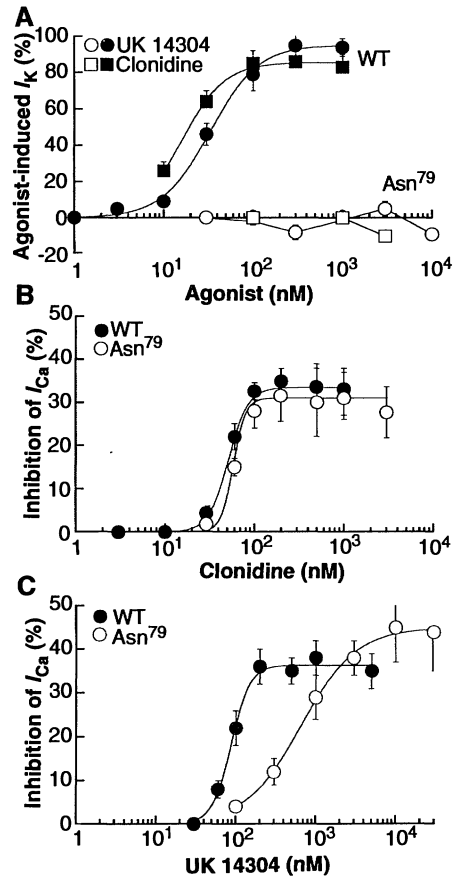

Fig. 2. Actions of AR agonists on K^+ currents (I_K) and Ca^{2+} currents (I_{Ca}) in AtT20 cells transfected with WT α_2ARs (filled symbols) or Asn^{79} α_2ARs (open symbols). (**A**) UK 14304 (circles)– and clonidine (squares)–induced increase in K^+ current in response to a 60-mV hyperpolarizing command (Fig. 1B); we normalized results by expressing them as a percentage of response produced by somatostatin (100 nM). Each point is mean ± SEM from six to eight cells for those expressing WT α_2ARs and four to five cells for those expressing Asn^{79} α_2ARs. Somatostatin EC_{50} values were 15 nM and 22 nM in cells expressing WT and Asn^{79} α_2ARs, respectively, and these values were not significantly different (student's t test). (**B** and **C**) Concentration-response curves for inhibition of I_{Ca} by clonidine (B) and UK 14304 (C); inhibition was measured as percent inhibition of the current elicited by depolarizing the cell from −70 mV to +20 mV. Each point is mean ± SEM [n = 15 for each point in (B); in (C), n = 12 for each point from WT α_2AR cells, and n = 8 to 10 for each point from Asn^{79} α_2AR cells]. EC_{50} values for somatostatin inhibition of I_{Ca} were 30 and 52 nM in WT α_2AR and Asn^{79} α_2AR cells, respectively, and these values were not significantly different (student's t test).

dependent agonist binding has also been reported for the β-AR (*13, 14*) and the muscarinic M_1 receptor (*15*) after mutation of the topologically shared aspartate residue. Our results suggest either that receptor coupling to Ca^{2+} channel– and adenylyl cyclase–associated G proteins, but not to K^+ channel–associated G proteins, is preferentially retained by the Asn^{79} α_2AR mutant or that the apparently reduced receptor–G protein coupling manifest in binding studies of the Asn^{79} α_2AR mutant is still sufficient to inhibit Ca^{2+} currents and adenylyl cyclase but not to activate K^+ currents.

Inhibition of adenylyl cyclase, inhibition of Ca^{2+} currents, and increase of K^+ currents are three consequences of activating α_2ARs and other related receptors (*1, 16*). All three effects were produced in the same cell by a single molecular species of the α_2AR. Although each effect involves a PTX-sensitive G protein, each appears to represent an independent signal transduction pathway (*17*). Distinct G protein subunits appear to specify the coupling of receptor to different effectors (*18*); α_2ARs, dopamine D_2, muscarine M_2, and somatostatin receptors couple to inhibition of Ca^{2+} currents through G_o proteins and can activate K^+ currents through one or more of the G_i subunits (*19*). AtT20 cells contain three $G_{i\alpha}$ and at least one $G_{o\alpha}$ subunits (*20*). The endogenous somatostatin receptor appears to be linked to $G_{i\alpha1}$, $G_{i\alpha3}$, and $G_{o\alpha}$, and inhibition of adenylyl cyclase is mediated by $G_{i\alpha1}$ (*20*); the relative abundance of these G proteins in AtT20 cells is not known. In other cells, inhibition of adenylyl cyclase by endogenous α_2-adrenergic and opiate receptors is transduced by the $G_{i\alpha2}$ protein (*21*). It is not known which G protein subunits are coupled to the transfected WT or mutant α_2ARs in AtT20 cells. However, if K^+ currents couple to a G protein with low concentrations in AtT20 cells, one interpretation of our results may be that the Asn^{79} α_2AR mutation reduces coupling efficiency to all G proteins to the same extent, with the result that the blockade of K^+ current transduction would be most apparent.

An aspartate in the topological position of Asp^{79} is conserved among almost all G protein–coupled receptors cloned to date; mutation of this aspartate to asparagine reduces agonist affinity (*2, 13–15*) and prevents the modulation of agonist binding by cations (*22*) and nonhydrolyzable guanosine triphosphate analogs (Table 1) (*14*). This implies that the carboxylic acid side chain can bind cations and contribute to a conformational state of the receptor that functions in receptor–G protein interactions, either by influencing efficacy of interaction with a given G protein or by allowing preferential interaction with particular G protein subunits. If distinct G protein α subunits couple the α_2AR to these three effectors (*23*), our results suggest that these α subunits may bind in different ways to the receptor and that the requirements for conferring G protein specificity may be subtle.

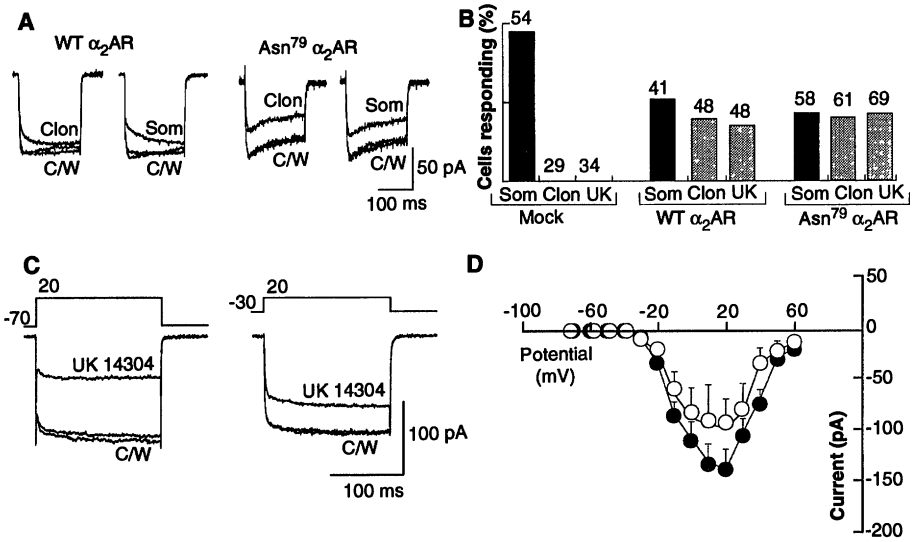

Fig. 3. Inhibition of Ca^{2+} currents by transfected α_2ARs and endogenous somatostatin receptors in AtT20 cells. Abbreviations are as in Fig. 1. (**A**) Whole-cell recordings of Ca^{2+} currents evoked by depolarizations from a holding potential of −70 mV to +20 mV; each set of superimposed traces is as in Fig. 1B [before, during, and after application of agonist, somatostatin (100 nM), and clonidine (1 μM)]. These agonist concentrations elicited maximum inhibitions in each cell shown. (**B**) Percent of cells in which agonists inhibited Ca^{2+} current; numbers above each bar are numbers of cells examined. (**C**) Ca^{2+} currents evoked from a holding potential of −70 mV or −30 mV in one cell transfected with the Asn^{79} α_2AR mutant. In this cell, the dihydropyridine antagonist nifedipine (1 μM) inhibited the current evoked from −70 mV and −30 mV by 41% and 85%, respectively, and ω-conotoxin (3 μM) inhibited these currents by 58% and 22%, respectively. This indicates that the current recorded from holding potential of −30 mV is predominantly HVA/L-type, whereas that evoked from −70 mV is both HVA/L- and HVA/N-type. (**D**) Summary of current-voltage relation of Ca^{2+} current evoked from −70 mV in control (open circles) and in 1 μM clonidine or UK 14304 (closed circles) in AtT20 cells transfected with the WT α_2AR; each point is mean ± SEM of six experiments. Similar inhibition was observed when the holding potential was −30 mV (n = 4).

Table 1. Biochemical properties of WT and Asn^{79} α_2ARs in permanent transformants of AtT20 cells. Modulation of [^{3}H]cAMP production by UK 14304 was measured in the WT α_2AR-expressing clone 66N or the mutant Asn^{79} α_2AR clone 32-8. The ability of maximally effective concentrations of UK 14304 to decrease [^{3}H]cAMP production in response to 40 nM isoprenaline was measured as described (*25*). The EC_{50} values for UK 14304 inhibition of adenylyl cyclase cannot be compared directly with those for activating K^+ currents or inhibiting Ca^{2+} currents because the position of the α_2AR agonist curve for inhibition of isoprenaline-stimulated [^{3}H]cAMP accumulation is dependent on the concentration of isoprenaline added to activate adenylyl cyclase. The effect of UK 14304 was eliminated when cells were treated first with PTX (100 ng/ml for 18 hours, n = 2). We evaluated the interactions of α_2ARs with G proteins by testing the ability of Gpp(NH)p to suppress binding of the partial agonist [^{125}I]PIC (*26*). ND, not detected.

Cell line	Inhibition of stimulated cAMP production (%)	EC_{50} for UK 14304 inhibition (nM)	Agonist binding modulated by Gpp(NH)p (%)
Untransfected	0	ND	ND
WT α_2AR	28 ± 3.7 (n = 4)	65 ± 15 (n = 4)	80 ± 3 (n = 4)
Asn^{79} α_2AR	30 ± 2.2 (n = 4)	14 ± 5.8 (n = 3)	4 ± 10 (n = 4)

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7. Whole-cell patch-clamp recordings were obtained with an Axopatch 1B amplifier and patch pipettes with a resistance of 4 to 6 megohms; cell input resistance and capacitance measured in 5 mM external K^+ with potassium gluconate as the internal solution were 5 to 25 gigaohms and 8 to

19 pF, respectively, and were not significantly different among subclones (Student's t test). K^+ currents were recorded with an internal solution of 150 mM potassium gluconate, 10 mM EGTA, 10 mM Hepes, 2.5 mM Mg^{2+}–adenosine triphosphate (MgATP), and 0.1 mM GTP and an external solution of 150 mM KCl, 10 mM Hepes, 10 mM glucose, and 2.5 mM $MgCl_2$. Whole-cell recordings were established in an external solution of 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes, and then cells were switched to the high-concentration K^+ solution after the adequacy of the recording was verified by the presence of a large Na^+ current, high input resistance, and minimal leak current (Fig. 1A). Ca^{2+} currents were recorded with an internal solution of 120 mM cesium gluconate, 10 mM EGTA, 10 mM Hepes, 2.5 mM MgATP, and 0.1 mM GTP and an external solution of 120 mM NaCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, and 0.5 to 5 μM tetrodotoxin. The pH of all solutions was maintained at 7.35. We obtained the traces of Ca^{2+} currents by subtracting currents recorded in the presence of Cd^{2+} (300 μM) from currents recorded in control and agonist solutions; no subtraction protocols were carried out on traces of K^+ currents.

8. AtT20 cells were cotransfected by the lipofectin method (*24*) with the pCMV4 α_2AR plasmid (*22*), which encodes either the WT or Asn^{79} AR of the α_{2A} subtype, and with pRSVneo, which codes for neomycin resistance. Neomycin-resistant colonies were screened for receptor expression by ^{125}I-labeled *p*-iodo-clonidine (PIC) binding. Receptor density, calculated from Scatchard transformations of [^{3}H]yohimbine binding data (*3, 22*), was 3.1 pmol of α_2AR per milligram of protein for WT and 10 pmol of α_2AR per milligram of protein for Asn^{79}. Mock-transfected cells were isolated after cotransfection of AtT20 cells with the pCMV4 expression plasmid and an expression plasmid encoding a neomycin resistance gene.
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11. For cAMP assays, AtT20 clones were incubated 12 to 15 hours with 1.5 μCi of [^{3}H]adenine in Dulbecco's minimum essential medium plus fetal calf serum (10%) in 12-well dishes (Costar). For the assay, attached cells were incubated in phosphate-buffered saline with isobutyl methylxanthine (500 μM) for 10 min with or without drugs, and reactions were stopped by addition of trichloroacetic acid (6%). Samples were neutralized, and [^{3}H]cAMP was isolated by sequential Dowex (50W-X4, Bio-Rad AG., Richmond, CA) and alumina chromatography (*25*).
12. For agonist binding experiments, washed AtT20 membranes were prepared by hypotonic lysis of cells and collection by centrifugation at 39,000*g* for 30 min at 4°C. [^{125}I]PIC binding was measured after incubation with 0.7 nM [^{125}I]PIC (180,000 cpm per 100 μl) in buffer containing 50 mM tris HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM EGTA at 25°C for 30 min in the presence or absence of 10 μM 5′-guanylyl imidodi phosphate [Gpp(NH)p], a nonhydrolyzable analog of GTP. Bound ligand was separated from free ligand by filtration over Whatman GF-C glass fiber filters. Specific binding was defined as binding that was displaced by 10 μM yohimbine.
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