A point mutation of the alpha-2-adrenoceptor that blocks coupling to potassium but not calcium currents.

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The alpha-2-adrenergic receptor (adrenoceptor) was stably expressed in AtT20 mouse pituitary tumor cells; adrenoceptor agonists inhibited adenyl cyclase, inhibited voltage-dependent calcium currents, and increased inwardly rectifying potassium currents. An aspartic acid residue ([Asp.sup.79]) highly conserved among guanine nucleotide-binding protein (G protein)-coupled receptors was mutated to asparagine; in cells transfected with the mutant [alpha.sub.2-receptor], agonists inhibited adenyl 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 [alpha.sub.2-adrenoceptor] cannot achieve the conformation necessary to activate G proteins that mediate potassium channel activation.

The alpha-2-adrenergic receptors ([alpha.sub.2]ARs) belong to the superfamily of G protein-coupled receptors, specifically to the branch of this family consisting of receptors coupled to the inhibition of adenyl cyclase, the inhibition of [Ca.sup.2+] currents, and the activation of [K.sup.+] 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 [alpha.sub.2]ARs on mammalian neurons[1]. Multiple [alpha.sub.2AR] subtypes have been identified by both pharmacological and molecular biological approaches, and site-directed mutagenesis of [alpha.sub.2]ARs 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 adenyl cyclase[1, 2].

However, it is not known whether cloned [alpha.sub.2]ARs, 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 [alpha.sub.2]AR[3] to [K.sup.+] currents, [Ca.sup.2+] currents, and adenyl cyclase and the consequences of a single amino acid mutation [converting aspartic acid to asparagine at position 79 ([Asn.sup.79] [alpha.sub.2]ARs) on the coupling to these three effector systems.

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

The [alpha.sub.2]AR agonist UK 14304 increased the [K.sup.+] current in cells expressing the WT [alpha.sub.2]AR (Fig. 1); 95% of the current induced by UK 14304 was blocked by 1 mM [Ba.sup.2+] (Fig. 1A), as would be expected if the agonist were opening inwardly rectifying [K.sup.+] channels[9]. Somatostatin (100 nM) increased this current by two- to tenfold in all mock-transfected cells and in cells transfected with the WT [alpha.sub.2]AR or [Asn.sup.79] [alpha.sub.2]AR (Fig. 1, B through E). Maximally effective concentrations of UK 14304 or clonidine produced a 1.5- to 8-fold increase in [K.sup.+] current in cells expressing the WT [alpha.sub.2]AR or [Asn.sup.79] [alpha.sub.2]AR (Fig. 1, B through D). Concentrations of clonidine and UK 14304 that produced half-maximal activation ([EC.sub.50]) of the [K.sup.+] current were 14 and 30 nM, respectively, in cells expressing the WT [alpha.sub.2]AR (Fig. 2A); these are similar to the [EC.sub.50] values for the inwardly rectifying [K.sup.+] conductance activated by pharmacologically characterized [alpha.sub.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 [alpha.sub.2]AR couples to the same set of [K.sup.+] channels as does the endogenous somatostatin receptor. In contrast to the WT [alpha.sub.2]AR, the mutant [Asn.sup.79] [alpha.sub.2]AR did not activate [K.sup.+] currents (Fig. 1, B, C, and E), even in the presence of...
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10,000-fold higher concentrations of clonidine or UK 14304 (Fig. 2A).

In contrast to their effects on [K.sup.+] currents, [alpha.sub.2]AR agonists were effective in inhibiting [Ca.sup.2+] currents in AtT20 cells expressing WT [alpha.sub.2]ARs or [Asn.sup.79] [alpha.sub.2]ARs (Fig. 3). In either case, the inhibition of [Ca.sup.2+] currents by AR agonists was not quantitatively different from the inhibition of [Ca.sup.2+] currents by somatostatin acting at endogenous receptors (Fig. 3, A and B). Somatostatin inhibits two high-voltage-activated (HVA) [Ca.sup.2+] currents in AtT20 cells, a dihydropyridine-sensitive (HVA/L-type) current and a dihydropyridine-insensitive (HVA/N-type) current (5); [alpha.sub.2]AR agonists similarly inhibited HVA/L- and HVA/N-type [Ca.sup.2+] currents in cells expressing either WT or [Asn.sup.79] [alpha.sub.2]ARs (Fig. 3, C and D). Somatostatin inhibited the [Ca.sup.2+] current in 92% of mock-transfected cells examined but inhibited [Ca.sup.2+] currents in only 50% of cells expressing WT [alpha.sub.2]ARs or [Asn.sup.79] [alpha.sub.2]ARs (Fig. 3B). The percentage of cells in which somatostatin inhibited the [Ca.sup.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.sup.+] 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.sup.2+] current in cells expressing WT or [Asn.sup.79] [alpha.sub.2]ARs (Fig. 2B) or the norepinephrine-mediated responses (measured in the presence of propranolol to block endogenous [beta]-ARs). Cells expressing WT or [Asn.sup.79] [alpha.sub.2]ARs showed no obvious differences in their responses to [alpha.sub.2]AR antagonist idazoxan; idazoxan (100 nM) inhibited the maximum response to UK 14304 by 49 [%] in WT [alpha.sub.2]AR cells and by 54 [%] in [Asn.sup.79] [alpha.sub.2]AR cells, and 1 [mu]M idazoxan inhibited responses to agonists by 95 to 100% in both cell types. However, cells expressing the [Asn.sup.79] [alpha.sub.2]AR were one-sixth as sensitive to inhibition of the [Ca.sup.2+] current by UK 14304 as cells expressing the WT [alpha.sub.2]AR (Fig. 2C). These data suggest that the clonidine analog UK 14304 may behave as a partial agonist for [Ca.sup.2+] current inhibition in comparison to clonidine or norepinephrine in these cells.

Modulation of [K.sup.+] or [Ca.sup.2+] currents by [alpha.sub.2]AR 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.sup.+] and [Ca.sup.2+] currents. In PTX-treated cells, somatostatin (300 nM) increased [K.sup.+] currents in only 1 of 10 mock-transfected cells, 1 of 18 WT [alpha.sub.2]AR cells, and 2 of 36 [Asn.sup.79] [alpha.sub.2]AR cells. Neither clonidine nor UK 14304 (1 to 10 [mu]M) altered [K.sup.+] currents in any of these cells. Clonidine inhibited the [Ca.sup.2+] current in only 1 of 35 WT [alpha.sub.2]AR cells and 2 of 29 [Asn.sup.79] [alpha.sub.2]AR cells that had been treated with PTX.

Cells bearing either the WT or mutant [Asn.sup.79] [alpha.sub.2]AR coupled to inhibition of adenosine 3',5'-monophosphate (cAMP) accumulation (11) through a PTX-sensitive pathway in AtT20 cells. Unlike the inhibition of [Ca.sup.2+] currents, the potency of UK 14304 inhibition of cAMP accumulation was not reduced in cells with mutant [alpha.sub.2]ARs (Table 1). Our observation that WT and [Asn.sup.79] [alpha.sub.2]ARs couple to inhibition of cAMP accumulation and suppression of [Ca.sup.2+] currents through a PTX-sensitive pathway implies that these receptors mediating these two 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.sup.79] [alpha.sub.2]ARs than for WT [alpha.sub.2]ARs (Table 1).

These data are consistent with observations for the [alpha.sub.2]AR(2, 3) that suggest that mutation of [Asp.sup.79] perturbs G protein-dependent agonist interactions but not G protein-independent antagonist interactions with the [alpha.sub.2]AR. Perturbation of G protein-dependent agonist binding has also been reported for the [beta]-AR(13, 14) and the muscarinic [M.sub.1] receptor (15) after mutation of the topologically shared aspartate residue. Our results suggest either that receptor coupling to [Ca.sup.2+] channel- and adenylyl cyclase-associated G proteins, but not to [K.sup.+] channel-associated G proteins, is preferentially retained by the [Asn.sup.79] [alpha.sub.2]AR mutant or that the apparently reduced receptor-G protein coupling manifest in binding studies of the [Asn.sup.79] [alpha.sub.2]AR mutant is still sufficient to inhibit [Ca.sup.2+] and adenylyl cyclase but not to activate [K.sup.+] currents.

Inhibition of adenylyl cyclase, inhibition of [Ca.sup.2+] currents, and increase if [K.sup.+] currents are three consequences of activating [alpha.sub.2]ARs and other related receptors (1, 16). All three effects were produced in the same cell by a single molecular species of the [alpha.sub.2]AR. Although each effect involves a PTX-sensitive G protein, each appears to represent an
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Independent signal transduction pathway[17]. Distinct G protein sub-units appear to specify the coupling of receptor to different effectors[18]; [alpha.sub.2]ARs, dopamine [D.sub.2], muscarine [M.sub.2], and somatostatin receptors couple to inhibition of [Ca.sup.2+] currents through [G.sub.o] proteins and can activate [K.sup.+.] currents through one or more of the [G.sub.i] subunits[19]. AtT20 cells contain three [G.sub.o] and at least one [G.sub.i] subunits[20]. The endogenous somatostatin receptor appears to be linked to [G.sub.i] and [G.sub.o] proteins; and inhibition of adenyl cyclase is mediated by [G.sub.o] [12]. The relative abundance of these G proteins in AtT20 cells is not known. In other cells, inhibition of adenyl cyclase by endogenous [alpha.sub.2]-adrenergic and opiate receptor is transduced by the [G.sub.o][alpha] protein[21]. It is not known which G protein subunits are coupled to the transfected WT or mutant [alpha.sub.2]ARs in AtT20 cells. However, if [K.sup.+.] currents couple to a G protein with low concentrations in AtT20 cells, one interpretation of our results may be that the [Asn.sup.79] [alpha.sub.2]AR mutation reduces coupling efficiency to all G proteins to the same extent, with the result that the blockade of [K.sup.+.] current transduction would be most apparent.

An aspartate in the topological position of [Asp.sup.79] is conserved among almost all G protein-coupled receptors cloned to date; mutation of this aspartate to asparagine reduce 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 [alpha] subunits couple the [alpha.sub.2]AR to these three effectors[23], our results suggest that these [alpha] subunits may bind in different ways to the receptor and that the requirements for conferring G protein specificity may be subtle.

REFERENCES AND NOTES

[1.] R. J. Lefkowitz and M. G. Caron, Rec. Prog. Hormone Res. 43, 469 (1987); A. Surprenant, Semin. Neurosci. 1, 126 (1989); K. Starke, Rev. Physiol. Biochem. Pharmacol. 107, 73 (1987). [2.] C. D. Wang, M. A. Buck, C. M. Fraser, Mol. Pharmacol. 40, 168 (1991). [3.] D. A. Horstman et al., J. Biol. Chem. 265, 21590 (1990). [4.] T. Reisine, Endocrinology 116, 2259 (1985). [5.] A. Luini et al., J. Neurosci. 6, 3128 (1986); J. Stack, thesis, Oregon Health Sciences University, Portland (1990). [6.] P. S. Pennefather, S. Heisler, J. F. MacDonald, Brain Res. 444, 346 (1988). [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.sup.+.] 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.sup.+.] currents were recorded with an internal solution of 150 mM potassium gluconate, 10 mM EGTA, 10 mM Hepes, 2.5 mM [Mg.sup.2+]-adenosine triphosphate (MgATP), and 0.1 mM GTP and with an external solution of 150 mM KCl, 10 mM Hepes, 10 mM glucose, and 2.5 mM [MgCl.sub.2]. Whole-cell recordings were established in an external solution of 120 mM NaCl, 5 mM KCl, 2.5 mM [CaCl.sub.2], 2.5 mM [MgCl.sub.2], 10 mM glucose, and 10 mM Hepes, and then cells were switched to the high-concentration [K.sup.+.] solution after the adequacy of the recording was verified by the presence of a large [Na.sup.+.] current, high input resistance, and minimal leak current (Fig. 1 A). [Ca.sup.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, 3M [CaCl.sub.2], 1 mM [MgCl.sub.2], 10 mM Hepes, 10 mM glucose, and 0.5 to 5 [mu]M tetrodotoxin. The pH of all solutions was maintained at 7.35. We obtained the traces of [Ca.sup.2+] currents by subtracting currents recorded in the presence of [Cd.sup.2+] (300 [mu]M) from currents recorded in control and agonist solutions; no subtraction protocols were carried out on traces of [K.sup.+.] currents. [8.] AtT20 cells were cotransfected by the lipofectin method[24] with the pCMV4 [alpha.sub.2]AR plasmid[22], which encodes either the WT or [Asn.sup.79] AR of the [alpha.sub.2A] subtype, and with pRSVneo, which codes for neomycin resistance. Neomycin-resistant colonies were screened for receptor expression by [l.sup.125]I-labeled p-iodo-clonidine (PIC) binding. Receptor density, calculated from Scatchard transformations of [H.sup.3]yohimbine binding data (3,22), was 3.1 pmol of [alpha.sub.2]AR per milligram of protein WT and 10 pmol [alpha.sub.2]AR per milligram of protein for [Asn.sup.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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