0026-895X/93/020380-05$03.00/0

MOLECULAR PHARMACOLOGY, 44:380-384

Mutation of an Aspartate at Residue 89 in Somatostatin Receptor Subtype 2 Prevents Na^+ Regulation of Agonist Binding but Does not Alter Receptor-G Protein Association

HAEYOUNG KONG, KAREN RAYNOR, KAZUKI YASUDA, GRAEME I. BELL, and TERRY REISINE

Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 (H.K., K.R., T.R.), and Howard Hughes Medical Institute and Departments of Biochemistry and Molecular Biology and Medicine, University of Chicago, Chicago, Illinois 60637 (K.Y., G.I.B.)

Received February 11, 1993; Accepted April 30, 1993

SUMMARY

Sodium ions have been shown to reduce the binding of agonists to a number of G protein-linked receptors. They are believed to do so by interacting with aspartate residues in the second membrane-spanning region of these receptors to cause G protein uncoupling, resulting in a diminished affinity of the receptors for agonists. To investigate Na^+ regulation of agonist binding to somatostatin receptors, Na^+ was tested for its effect on the binding of agonists to cloned somatostatin receptor type 1 (SSTR1) and somatostatin receptor type 2 (SSTR2) stably expressed in Chinese hamster ovary cells. Na^+ reduced agonist binding to SSTR2 but not to SSTR1. Because high affinity agonist binding to SSTR1 does not depend on G protein coupling but agonist binding to SSTR2 is reduced by guanosine-5′-(β,γ-imido)triphosphate and pertussis toxin treatment, the selective Na^+ effect on SSTR2 is consistent with previous findings with other receptors showing that Na^+ uncouples receptors from G proteins, thereby reducing the affinity of the receptors for agonists. Conversion of Asp^{89} to Asn^{89} in SSTR2 resulted in a mutant receptor whose affinity for agonists was not altered by Na^+, indicating that Asp^{89} is involved in mediating the effects of Na^+ on agonist binding to SSTR2. However, the affinities of the mutant and wild-type receptors for somatostatin were the same, and both guanosine-5′-*O*-(γ-thio)triphosphate and pertussis toxin treatment reduced agonist binding to the mutant and wild-type receptors. These findings differ from the results of similar mutagenesis studies on other G protein-linked receptors, in that the mutant and wild-type SSTR2 forms associate with G proteins in similar ways. These results indicate that Asp^{89} acts in a novel manner to regulate agonist binding and G protein interaction with SSTR2.

For a number of G protein-linked receptors, including the α_2-adrenergic (1, 2), β-adrenergic (3), dopamine D_2 (4), μ- and δ-opiate (5, 6), and muscarinic (7) receptors, Na^+ has been reported to reduce the affinity of the receptors for agonists. Many of these receptors have been found to contain a conserved aspartate residue in their second membrane-spanning region, and site-directed mutagenesis of the aspartate to an asparagine results in mutant receptors that are insensitive to the effects of Na^+ (2, 8–10). Agonist binding to the mutant receptors was not regulated by GTP analogs. These findings indicate that the conserved aspartate in the second membrane-spanning region of these receptors is the site of Na^+ regulation of agonist binding and that mutations of this site perturb receptor-G protein interactions (2, 9, 10).

Although Na^+ has been shown to affect the binding of agonists to receptors and receptor/G protein association, the mechanisms by which it regulates the properties of receptors have not been clearly established. The mutagenesis studies to date suggest that Na^+ directly interacts with receptors to induce allosteric modifications of the receptor, reducing the affinity of the receptor for agonists. Na^+ could alter the conformation of receptors to induce G protein uncoupling, thereby reducing the affinity of receptors for agonists. Alternatively, Na^+ could interact with this recognition site in receptors to reduce agonist potency independently of effects on G protein coupling.

SRIF receptors, like α_2-adrenergic receptors, are coupled to pertussis toxin-sensitive G proteins, and Na^+ has been shown to reduce the affinity of these receptors for agonists (11, 12). Recently, a family of SRIF receptors have been cloned (13–16). The first two cloned SRIF receptors, referred to as SSTR1 and SSTR2, both express high affinity for SRIF and SRIF-28 (13). However, they differ in their association with G proteins (17). GTPγS and pertussis toxin treatments uncouple SSTR2 from

This work was supported by National Institute of Mental Health Grants 45533 and 48518.

ABBREVIATIONS: SRIF, somatostatin; CHO, Chinese hamster ovary; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NMDG, *N*-methyl-D-glucamine; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; GTPγS, guanosine-5′-*O*-(γ-thio)triphosphate; SSTR1, somatostatin receptor type 1; SSTR2, somatostatin receptor type 2.

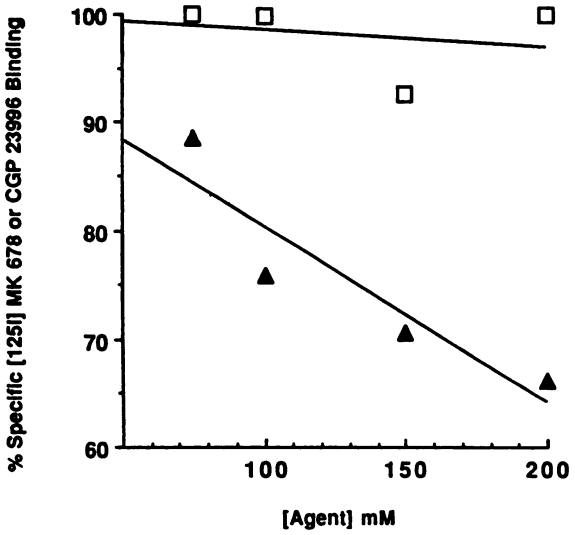


Fig. 1. Effect of Na^+ on agonist binding to SSTR1 and SSTR2. Specific ^{125}I-MK 678 binding to SSTR2 (▲) or specific ^{125}I-CGP 23996 binding to SSTR1 (□) was inhibited by increasing concentrations of NaCl. Alterations in radioligand binding due to changes in ionic strength were determined using similar concentrations of NMDG and were subtracted from the corresponding effects of NaCl to reveal the selective effect of Na^+ on agonist binding. Values are presented as a percentage of specific radioligand binding in the absence of Na^+ or NMDG and are the means of four different experiments, with standard errors of <10% of the means.

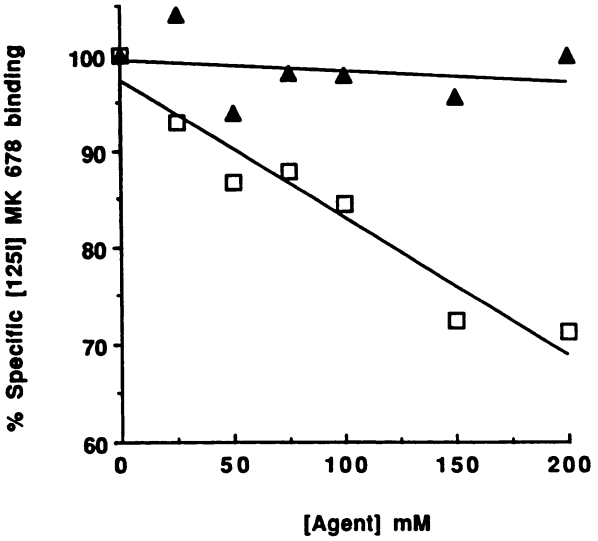


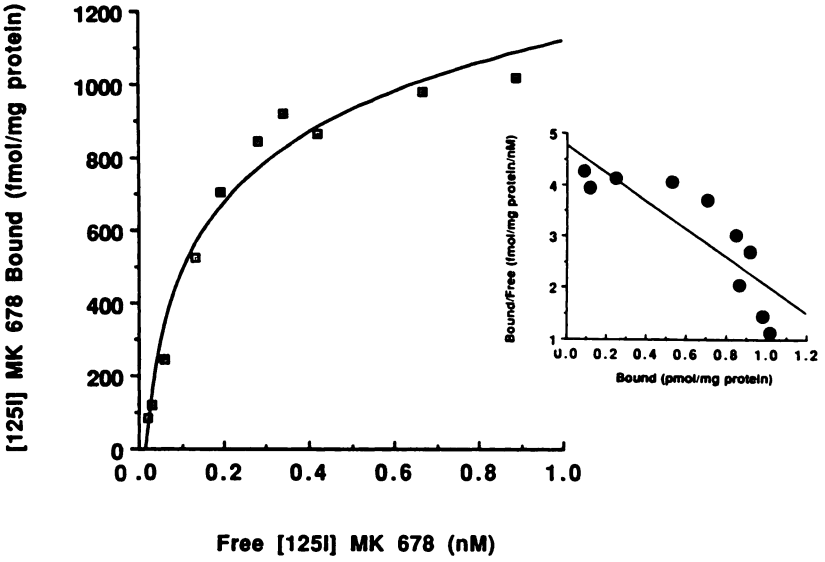
Fig. 2. Effect of Na^+ on specific ^{125}I-MK 678 binding to wild-type SSTR2 and the Asn^{89} mutant SSTR2. Wild-type (□) or mutant (▲) SSTR2 was transiently expressed in COS cells; 2 days after transfection, membranes were prepared and the effect of NaCl on high affinity ^{125}I-MK 678 binding was tested. Values are presented as a percentage of specific binding to control membranes not exposed to Na^+ or NMDG. These are the mean values from four different experiments and the standard errors of these experiments were <10% of the means.

G proteins and greatly reduce the affinity of this receptor for agonists. In contrast, neither treatment affects agonist binding to SSTR1, suggesting that this cloned receptor is inefficiently coupled to G proteins and that high affinity agonist binding to this receptor is not dependent on G protein association. Because SSTR1 and SSTR2 both have the conserved aspartate in their second membrane-spanning regions, we have used these receptors to investigate the mechanisms by which Na^+ allosterically regulates agonist binding to SRIF receptors. We show that Na^+ reduces agonist binding to SSTR2 but not SSTR1, consistent with the hypothesis that Na^+ induces the uncoupling of receptors from G proteins to reduce agonist binding. Furthermore, using site-directed mutagenesis we show that an aspartate at residue 89 in SSTR2 is required for the actions of Na^+. However, agonist binding to the mutant Asn^{89} SSTR2 can be regulated by GTP analogs and pertussis toxin treatment in a manner similar to that of the wild-type receptor. Moreover, the affinities of the mutant and wild-type receptors for agonists are similar. These findings indicate that G protein association with the mutant SSTR2 is not perturbed as it is with other hormone receptors (9, 10) with similar mutations and that the nature of the interaction of SSTR2 with G proteins differs from that of other G protein-linked receptors.

Materials and Methods

Mouse SSTR2 was mutated using the Altered Sites *in vitro* mutagenesis system (Promega Corp., Madison, WI). To convert the aspartate residue at position 89 of SSTR2 to asparagine, the mouse SSTR2 gene was subcloned into the phagemid pSELECT-1 and, with the helper phage R408, single-stranded template was produced. A 21-mer oligonucleotide (GCCATTGCAAATGAACTCTTC) encoding the desired mutation (GAT to AAT) was annealed to the single-stranded template and elongated with T4 DNA polymerase. (Nucleotides underlined were mutated). The heteroduplex DNA was then used to transform the repair-deficient *Escherichla coli* strain BMH 71–18 mut S. Transformants were selected by growth on LB plates containing 125 μg/ml ampicillin. Double-stranded plasmid DNA was sequenced by the Sanger dideoxy chain termination method to confirm the presence of the mutation. No other mutation besides the intended one was detected in the SSTR2 construct. The mutant gene was excised from the pSELECT vector using *Eco*RI and *Sal*I and was subcloned into the corresponding sites in the mammalian expression vector pCMV6c. This construct was then used to transiently transfect COS cells by the calcium phosphate-mediated procedure, as described (18).

Receptor binding assays on the cloned SRIF receptors were performed as described previously (14, 17). Membranes were used either from CHO cells stably expressing human SSTR1 or mouse SSTR2 or from COS-1 cells transiently transfected with the wild-type or mutant mouse SSTR2. For the radioligand binding assays, cells were harvested in 50 mM Tris·HCl, pH 7.8, containing 1 mM EGTA, 5 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 200 μg/ml bacitracin, and 0.5 μg/ml aprotinin (buffer 1) and were centrifuged at 24,000 × *g* for 7 min at 4°. The pellet was homogenized in buffer 1 using a Brinkman Polytron (setting 2.5, 30 sec). The homogenate was then centrifuged at 48,000 × *g* for 20 min at 4°. The pellet was homogenized in buffer 1 and used in the binding assay. Cell membranes (20–30 μg protein/tube) were incubated either with ^{125}I-CGP 23996 (0.2 nM; specific activity, 505 Ci/mmol) to label SSTR1 or with ^{125}I-MK 678 (0.05 nM; specific activity, 2200 Ci/mmol) to label SSTR2, in a final volume of 200 μl, for 90 min at 25° in the presence or absence of competing peptides, NaCl, NMDG, choline chloride, or GTP analogs. Nonspecific binding was defined as the radioactivity remaining bound in the presence of 1 μM SRIF. For the saturation studies, increasing concentrations of ^{125}I-MK 678 (0.05–1.5 nM) were incubated in the presence or absence of 1 μM SRIF. The binding reaction was terminated by the addition of ice-cold 50 mM Tris·HCl buffer, pH 7.8, and rapid vacuum filtration over Whatman GF/C glass fiber filters. The filters were then washed with 12 ml of ice-cold Tris·HCl buffer and the bound radioactivity was counted in a γ counter (80% efficiency). Data from saturation studies were used to generate Scatchard curves, from which K_d and B_{max} values were obtained by using the mathematical modeling program NEWFITSITE. For studies investigating the effect of Na^+ on agonist binding to the SRIF receptors, varying concentrations of NaCl or NMDG, as a Na^+



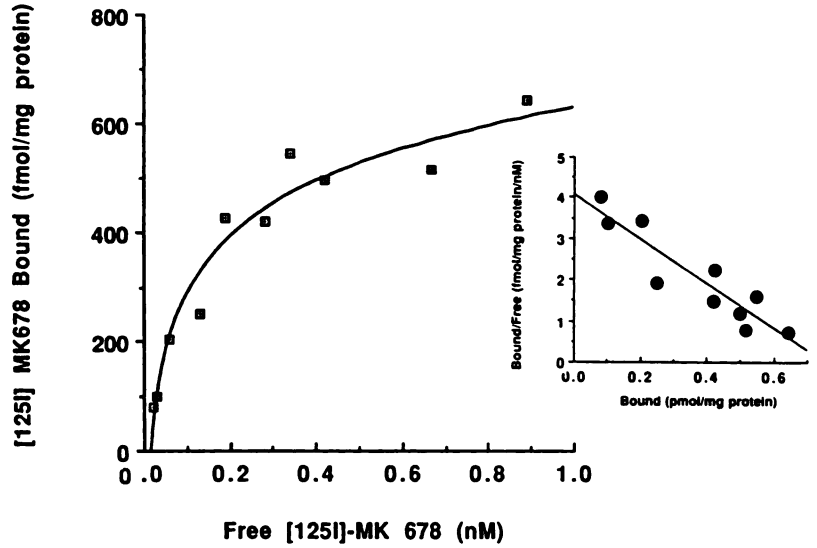


Fig. 3. Saturation analysis of ^{125}I-MK 678 binding to the wild-type (*top*) or Asn^{89} mutant (*bottom*) SSTR2 expressed in COS cells. To determine the relative levels and affinities of the wild-type and mutant SSTR2 expressed in COS cells, saturation analysis of ^{125}I-MK 678 binding was performed. Specific ^{125}I-MK 678 binding is plotted. Linear analysis of the saturation isotherms yielded B_{max} and K_d values for wild-type and mutant SSTR2 of 1262 fmol/mg and 0.18 nM and 727 fmol/mg of protein and 0.17 nM, respectively (see insets).

substitute (choline chloride was also used for this purpose), were tested for their effects on radiolabeled agonist binding. Any effects of NMDG or choline chloride on agonist binding were due to changes in ionic strength of the reaction buffer and were therefore subtracted from the effect of NaCl alone on agonist binding, to reveal the selective effects of Na^+ on radiolabeled agonist binding.

Results

To investigate the effect of Na^+ on agonist binding to human SSTR1 and mouse SSTR2 stably expressed in CHO cells (DG44 subclone), the receptors were labeled with the agonists ^{125}I-CGP 23996 and ^{125}I-MK 678, as described previously (14, 17), and the ability of Na^+ to affect specific binding was measured. NMDG was used as a Na^+ substitute, as described previously (2), to account for any effects of changes in ionic strength on agonist binding to SRIF receptors. As shown in Fig. 1, Na^+ reduced ^{125}I-MK 678 binding to SSTR2 in a concentration-dependent manner. NMDG itself reduced specific agonist binding to SSTR2 by approximately 30–40% (data not shown). Similar results were obtained when SSTR2 was labeled with ^{125}I-CGP 23996 and have been obtained with SRIF receptors from rat anterior pituitary and mouse AtT-20 cells, which express SSTR2 (11, 12). In contrast to the effect on agonist binding to SSTR2, Na^+ did not alter the binding of ^{125}I-CGP 23996 to SSTR1 (Fig. 1), indicating that Na^+ does not modulate the affinity of this receptor for agonists (NMDG did not affect agonist binding to SSTR1). This finding is consistent with previous results showing that GTP analogs and pertussis toxin treatment do not affect agonist binding to SSTR1 (17), and it provides further evidence that this receptor subtype is not efficiently coupled to G proteins.

To investigate the site of action of Na^+ in modulating agonist binding to SSTR2, the conserved aspartate (Asp^{89}) in SSTR2 was converted to an asparagine and the mutant and wild-type receptors were expressed in COS cells. As shown in Fig. 2, the specific binding of the SSTR2-selective ligand ^{125}I-MK 678 to wild-type SSTR2 was inhibited by Na^+. Similar results were obtained when wild-type SSTR2 was labeled with ^{125}I-CGP

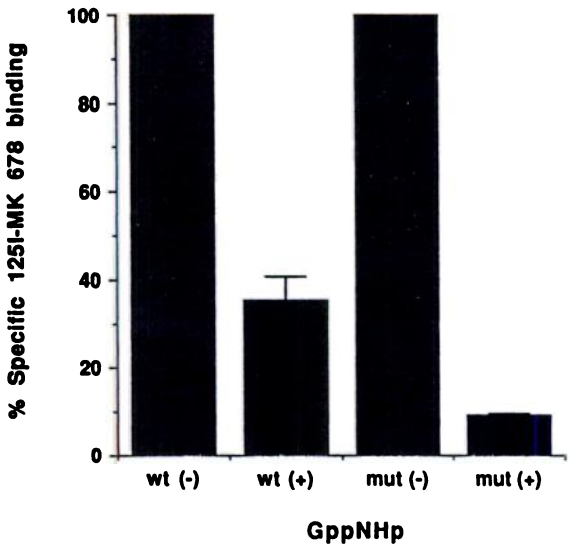


Fig. 4. Effect of Gpp(NH)p on specific ^{125}I-MK 678 binding to the wild-type (*wt*) and Asn^{89} mutant (*mut*) SSTR2. Specific ^{125}I-MK 678 binding to the wild-type and mutant SSTR2 expressed in COS cells was tested in the presence (+) or absence (−) of 100 μM Gpp(NH)p. Values are the means ± standard errors of four different experiments.

23996 (data not shown). In contrast, Na^+ did not selectively affect the binding of ^{125}I-MK 678 (Fig. 2) or ^{125}I-CGP 23996 (data not shown) to the mutant SSTR2 (Fig. 2). These findings indicate that Na^+ reduces the affinity of wild-type SSTR2 for agonists but that the Asn^{89} mutant SSTR2 is insensitive to the effects of Na^+.

Previous studies on adrenergic receptors have shown that mutation of the conserved aspartate in the second membrane-spanning region to an asparagine reduces the affinity of the receptors for agonist and renders the receptors insensitive to GTP (2, 9, 10). The Asn^{89} mutant SSTR2 had high affinity for SRIF and MK 678, similar to that of the wild-type SSTR2 (IC_{50} value for SRIF: wild-type, 1.0 nM; mutant, 0.5 nM; for MK 678: wild-type, 20 pM; mutant, 15 pM). Furthermore, the K_d values for ^{125}I-MK 678 binding to mutant and wild-type SSTR2 were similar (Fig. 3). There were more high affinity wild-type than mutant SSTR2 sites expressed in the COS cell membranes, as indicated from saturation analysis (Fig. 3). Most importantly, Gpp(NH)p was effective in reducing specific ^{125}I-MK 678 binding to both mutant and wild-type receptors (Fig. 4), indicating that both receptors were coupled to G proteins. Additional evidence that high affinity agonist binding to the mutant SSTR2 is dependent on G protein coupling is indicated by the finding that pretreatment of COS cells expressing the mutant SSTR2 with pertussis toxin (100 ng/ml for 24 hr) greatly reduced specific ^{125}I-MK 678 binding to the mutant SSTR2 (binding to control membranes, 261 fmol/mg of protein; binding to pertussis toxin-treated membranes, 18 fmol/mg of protein). These results, taken together, show that the Asn^{89} mutant SSTR2 is insensitive to Na^+ regulation but has agonist affinity and G protein coupling similar to those of the wild-type SSTR2.

Discussion

Sodium ions have the well known effect of diminishing the affinity of many neurotransmitter receptors for agonists (1–10). They were first reported to affect agonist binding to opiate receptors (5) and were then shown to alter agonist binding to other inhibitory receptors, such as α_2-adrenergic (1, 2) and SRIF (11, 12) receptors. Mutagenesis studies with the catecholamine receptors have shown that a consensus aspartate residue in the second membrane-spanning region is involved in mediating the effects of Na^+ on agonist binding, because mutant receptors with the aspartate converted to an asparagine are insensitive to the effects of Na^+ (2, 10). However, the mutant receptors also have altered interactions with G proteins, because analogs of GTP that induce uncoupling of wild-type receptors from G proteins, reducing the affinity of the receptors for agonist, are unable to induce this effect with the mutant receptors.

We have shown that an aspartate at residue 89 in the SRIF receptor subtype SSTR2 is critical for Na^+ regulation of agonist binding, because mutation of this residue abolished Na^+ inhibition of agonist binding. Other characteristics of the receptor, such as affinity for agonists and regulation by GTP analogs and pertussis toxin, were unaffected by this mutation. These findings differ from those reported with α_2-adrenergic (2, 10) and β_2-adrenergic (9) receptors and indicate that mutations of the conserved aspartate in SSTR2 do not perturb receptor interactions with G proteins. Our results suggest that the aspartate at residue 89 acts as a Na^+ recognition site so that Na^+ can interact with SSTR2 to modulate agonist binding.

Although Na^+ reduced the affinity of SSTR2 for agonists, it did not affect agonist binding to SSTR1, which, like SSTR2, has a conserved aspartate in its second membrane-spanning region. In previous studies, we have found that SSTR1 expressed in CHO cells exhibits high affinity for SRIF and that agonist affinity is not altered by GTPγS or pertussis toxin pretreatment (17). Furthermore, similar results were obtained when SSTR1 was expressed in COS cells, which, unlike CHO cells, express all forms of $G_{i\alpha}$ (17). In addition, a SRIF receptor with characteristics similar to those of SSTR1, expressed in rat brain, exhibits high affinity for agonists, but binding is not affected by GTP analogs or Na^+ (19). These findings indicate that high affinity agonist binding to SSTR1 is not dependent on G protein association, although we cannot exclude the possibility that under some conditions the receptor may be able to interact with G proteins. However, the lack of effect of Na^+ on agonist binding to SSTR1 expressed in CHO cells suggests that this ion may be able to interact with SSTR1 but does not alter agonist binding because the receptor is already uncoupled from G protein. These findings support the hypothesis that Na^+ affects agonist binding to G protein-linked receptor by promoting the dissociation of G proteins from the receptor.

In conclusion, we show that Na^+ can regulate agonist binding to the cloned SRIF receptor SSTR2 but not SSTR1 and that an aspartate in the second membrane-spanning region of SSTR2 is involved in Na^+ regulation of agonist binding. Further structural analysis of SSTR2 may help to identify the mechanisms by which Na^+ regulates agonist binding to this SRIF receptor subtype.

Acknowledgments

The authors wish to thank Dr. Stephanie Rens-Domiano for carrying out initial studies and Ms. Yuan-Jiang Yu for expert technical assistance. We thank Dr. Larry Chasin for providing the CHO DG44 cells.

References

1. Limbird, L., J. Speck, and S. Smith. Sodium ion modulates agonist and antagonist interactions with the human platelet α_2 adrenergic receptor in membrane and solubilized preparations. *Mol. Pharmacol.* **21**:609–617 (1982).

2. Horstman, D., S. Brandon, A. Wilson, C. Guyer, E. Cragoe, and L. Limbird. An aspartate conserved among G-protein receptors confers allosteric regulation of α_2-adrenergic receptors by sodium. *J. Biol. Chem.* **265**:21590–21595 (1990).
3. Minuth, M., and K. Jakobs. Sodium regulation of agonist and antagonist binding to *beta*-adrenoceptors in intact and N_s-deficient membranes. *Naunyn-Schmeidebergs Arch. Pharmacol.* **333**:124–129 (1986).
4. Neve, K., R. Henningsen, J. Kinzie, T. DePaulis, D. Schmidt, R. Kessler, and A. Janowsky. Sodium-dependent isomerization of dopamine D-2 receptors characterized using ^{125}I-epidepride, a high affinity substituted benzamide ligand. *J. Pharmacol. Exp. Ther.* **252**:1108–1116 (1990).
5. Pert, C., and S. H. Synder. Opiate receptor binding of agonists and antagonists affected differentially by sodium. *Mol. Pharmacol.* **10**:868–879 (1974).
6. Kosterlitz, H., S. Paterson, and L. Robson. Modulation of binding at opioid receptors by mono- and divalent cations and by cholinergic compounds. *J. Recept. Res.* **8**:363–373 (1988).
7. Jakobs, K. Inhibition of adenylate cyclase by hormones and neurotransmitters. *Mol. Cell. Endocrinol.* **16**:147–156 (1979).
8. Strader, C., I. Sigal, R. Register, M. Candelore, E. Rands, and R. Dixon. Identification of residues required for ligand binding to the *beta*-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**:4384–4388 (1987).
9. Chung, F., C. Wang, P. Potter, J. Venter, and C. Fraser. Site-directed mutagenesis and continuous expression of human β-adrenergic receptors. *J. Biol. Chem.* **263**:4052–4055 (1988).
10. Surprenant, A., D. Horstman, H. Akbarali, and L. Limbird. A point mutation of the *alpha*$_2$-adrenoceptor that blocks coupling to potassium but not calcium currents. *Science (Washington D. C.)* **257**:977–980 (1992).
11. Enjalbert, A., R. Rasolonjanahary, E. Moyse, C. Kordon, and J. Epelbaum. Guanine nucleotide sensitivity of ^{125}I-Tyr-somatostatin binding in rat adenohypophysis and cerebral cortex. *Endocrinology* **113**:822–824 (1983).
12. Mahan, L., and T. Reisine. Molecular mechanisms of somatostatin inhibition of hormone release from AtT-20 cells, in *Somatostatin: Basic and Clinical Status* (S. Reichlin, ed.). Plenum Press, New York, 137–145 (1987).
13. Yamada, Y., S. Post, K. Wang, H. Tager, G. Bell, and S. Seino. Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract and kidney. *Proc. Natl. Acad. Sci. USA* **89**:251–255 (1992).
14. Yasuda, K., S. Rens-Domiano, C. Breder, S. Law, C. Saper, T. Reisine, and G. Bell. Cloning of a novel somatostatin receptor, SSTR3, coupled to adenylyl cyclase. *J. Biol. Chem.* **267**:20422–20428 (1992).
15. O'Carroll, A.-M., S. Lolait, M. Konig, and L. Mahan. Molecular cloning and expression of a pituitary somatostatin receptor with preferential affinity for somatostatin-28. *Mol. Pharmacol.* **42**:939–946 (1992).
16. Bruno, J. F., Y. Xu, J. Song, and M. Berelowitz. Molecular cloning and functional expression of a brain-specific somatostatin receptor. *Proc. Natl. Acad. Sci. USA* **89**:11151–11155 (1992).
17. Rens-Domiano, S., S. Law, Y. Yamada, S. Seino, G. Bell, and T. Reisine. Pharmacological properties of two cloned somatostatin receptors. *Mol. Pharmacol.* **42**:28–34 (1992).
18. Sambrook, J., E. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
19. Raynor, K., D. Coy, and T. Reisine. Analogues of somatostatin bind selectively to brain somatostatin receptor subtypes. *J. Neurochem.* **59**:1241–1250 (1992).

Send reprint requests to: Terry Reisine, Department of Pharmacology, University of Pennsylvania School of Medicine, 36th Street and Hamilton Walk, Philadelphia, PA 19104.