0026-895X/96/010096-08$3.00/0

MOLECULAR PHARMACOLOGY, 50:96–103 (1996).

Genetic Evidence for Involvement of Multiple Effector Systems in α_{2A}-Adrenergic Receptor Inhibition of Stimulus-Secretion Coupling

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Received November 20, 1995; Accepted March 20, 1996

SUMMARY

The α_{2A}-adrenergic receptor (α_{2A}AR), via its interaction with the pertussis toxin-sensitive G_i/G_o class of G proteins, modulates multiple effector systems, including inhibition of adenylyl cyclase and Ca^{2+} channels and activation of K^+ channels. Mutation of a membrane-embedded aspartate residue, highly conserved among G protein-coupled receptors, in the α_{2A}AR to asparagine (D79N α_{2A}AR) results in selective uncoupling of the receptor to K^+ currents but retention of inhibition of cAMP production and of voltage-sensitive Ca^{2+} currents when expressed in AtT20 anterior pituitary cells in culture. It is known that attenuation of cAMP synthesis alone cannot account for α_{2A}AR suppression of stimulus-secretion coupling; thus, the D79N α_{2A}AR provides a unique tool with which to assess the relative contribution of K^+ current activation and Ca^{2+} current suppression in mediating the cellular responses of α_{2A}AR. The wild-type α_{2A}AR suppresses basal and secretagogue-evoked adrenocorticotropic hormone (ACTH) release in a manner indistinguishable from response to the endogenous somatostatin receptor. In contrast, the D79N α_{2A}AR does not attenuate basal ACTH release and is only partially effective in suppressing ACTH secretion evoked by the secretagogue isoproterenol. Regulation of ACTH release evoked by 8-bromo-cAMP, which bypasses receptor regulation of cAMP synthesis, suggests that attenuation of cAMP production, although not sufficient for inhibition of ACTH secretion, nevertheless participates in a functionally relevant manner. Taken together, the present findings indicate that α_{2A}AR-mediated suppression of neuropeptide secretion requires concomitant regulation of K^+ and Ca^{2+} currents in parallel with attenuation of cAMP production.

The α_2AR is a member of the G_i/G_o protein-coupled receptor family. This receptor plays an important role in regulation of many physiological processes, such as heart rate and blood pressure, lipolysis, platelet aggregation, pain perception, and epileptiform activity (1). An essential mechanism by which α_2AR achieves this regulatory influence is the receptor-mediated attenuation of neurotransmitter and hormone secretion from nerve terminals and endocrine organs, a characteristic response shared by other G_i/G_o-coupled receptors, such as somatostatin (2), adenosine (3), and opioid (4) receptors.

It has been known for several years that the α_2AR, similar to other members of the G_i/G_o-coupled receptor family, couples to multiple effector systems such as inhibition of adenylyl cyclase, suppression of voltage-activated Ca^{2+} channels, and activation of receptor-operated K^+ channels (5, 6). Studies demonstrating an inhibitory influence of α_2AR on stimulus-secretion coupling even in the presence of cAMP analogues (7) and lack of correlation between intracellular cAMP levels and α_2AR-mediated attenuation of regulated secretion (8) have indicated that suppression of cAMP formation is unable to completely account for α_2AR inhibition of stimulus/secretion coupling. Given the electrical contributions to exocytosis in both endocrine and neuronal cells, it is very likely that an essential component of the α_2AR inhibitory response is modulation of Ca^{2+} and/or K^+ channels.

Activation of α_2AR results in attenuation of voltage-activated Ca^{2+} currents in neuroendocrine and neuronal cells, such as submucosal (9) and locus ceruleus (10) neurons. This action may limit availability of Ca^{2+} for stimulus-secretion coupling, with a resultant inhibition of the secretion. Alternatively, α_2AR-mediated activation of K^+ currents, observed, for example, in pancreatic β cells (11), locus ceruleus (12), and myenteric plexus (13) neurons, leads to hyperpolarization and reduced excitability of a cell, which also can culminate in inhibition of neurotransmitter and hormone secretion. Attempts to understand the relative importance of these

This work was supported by National Institutes of Health Grants HL25182 (L.E.L.) and NS30470 (D.M.L.).

ABBREVIATIONS: α_2AR, α_2-adrenergic receptor; 8-Br-cAMP, 8-bromo-cAMP; α_{2A}AR, α_{2A}-adrenergic receptor; WT, wild-type; ACTH, adrenocorticotropic hormone; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

two electrical pathways in α_2AR-mediated inhibition of stimulus-secretion coupling have yielded ambiguous results, mainly due to confounding effects of ion channel inhibitors on the intrinsic excitability of neuronal and neuroendocrine cells. In fact, Allgaier *et al.* (14) demonstrated that a K^+ channel blocker, tetraethylammonium, attenuated α_2AR-mediated [^{3}H]norepinephrine release from rabbit hippocampal slices. However, they attributed modulation of α_2AR response in the presence of tetraethylammonium to prolongation of the action potential rather than to direct involvement of K^+ channels in the α_2AR response. Another complication in delineating involvement of K^+ versus Ca^{2+} channels in antisecretory actions of the α_2AR may result from the inability to differentiate between a direct effect on Ca^{2+} conductance by receptor-evoked pathways and an indirect inhibition of Ca^{2+} conductance secondary to K^+ channel-mediated hyperpolarization. For example, Koch *et al.* (15) suggested that in cultured pituitary GH_4C_1 cells, inhibition of Ca^{2+} conductance via activation of the somatostatin receptor, another G_i/G_o-coupled receptor, may not be due to direct coupling between the receptor and Ca^{2+} channels but rather may be the result of cell hyperpolarization induced by activation of K^+ channels. These studies demonstrate that conventional tools may not be sufficient to provide an understanding of the relative importance of these two electrical pathways in inhibition of stimulus-secretion coupling by the α_2AR and other G_i/G_o-coupled receptors.

We used a novel genetic approach to explore the role of K^+ channels in α_2AR-mediated inhibition of stimulus-secretion coupling. Mutation in one of the three α_2AR subtypes, the α_{2A}AR, of an aspartate residue (Asp79), predicted to lie in the second transmembrane domain and topographically conserved among many G protein-coupled receptors, to the corresponding asparagine (D79N) results in nonreciprocal changes in receptor/G protein communication (16) as well as loss of allosteric modulation of receptor binding by monovalent cations (17). Furthermore, mutation of the α_{2A}AR to the D79N α_{2A}AR selectively uncouples the receptor from inwardly rectifying K^+ channels without an appreciable effect on coupling to adenylyl cyclase or Ca^{2+} channels when expressed in mouse anterior pituitary AtT20 cells (18). In the current study, we used this mutant receptor structure as a genetic tool to delineate the role of K^+ channels in linking α_{2A}AR activation to attenuation of stimulus-secretion coupling.

Materials and Methods

ACTH secretion. Permanent clonal AtT20 cell lines expressing the WT α_{2A}AR or the D79N mutant α_{2A}AR were developed as described previously (17). The cells were plated onto poly-D-lysine-coated four-well Nunc dishes at a density of 2–4 × 10^5 cells/well and were allowed to grow to near-confluency in a serum-rich DMEM medium (DMEM containing 10% fetal calf serum, 10% horse serum, and 10% Nu-serum). The cells were serum-deprived for 18–24 hr before each experiment to minimize any influence of catecholamines present in the serum on subsequently evaluated α_{2A}AR-mediated responses. At the time of each experiment, the cells were washed at 15-min intervals for four times with serum-free DMEM and then subjected to two treatment periods of 15 min each, termed T1 and T2, respectively. Care was taken to use the minimal time possible for changing medium; the cells were maintained in a humid cell culture incubator equilibrated with 95% O_2/5% CO_2 at 37° during all incubation intervals. During the first treatment period (T1), all of the wells were treated with medium only, whereas during the second treatment period (T2), the wells were treated with only medium (control) or medium containing modulating agents, such as the β-adrenergic receptor agonist isoproterenol (which acts as a secretagogue), the α_2AR agonist UK 14,304, somatostatin, and their combinations. At the end of the treatment periods, the medium was collected separately from each well and analyzed for ACTH content through the use of radioimmunoassay (19). The ratio of the amount of ACTH secreted during the second treatment period to that secreted in the first treatment period (T2/T1) was calculated to quantify the influence exerted by the modulating agents on ACTH secretion. The ratio of ACTH secretion in control wells exposed only to DMEM medium during both treatment periods was usually ~1, indicating that the basal secretion from AtT20 cells was reasonably constant throughout the course of the experiments. Percentage change in ACTH secretion was calculated by comparing the T2/T1 ACTH secretion ratio under control conditions with the T2/T1 ratio observed in the presence of any modulating agent.

Electrophysiology. AtT20 cells, permanently transfected with either WT or D79N mutant α_{2A}AR, were plated onto 35 mM Falcon dishes and were allowed to grow in a serum-rich DMEM medium. For most experiments, the cells were serum deprived 18–24 hr before the experiment to eliminate modulatory effects that might be due to catecholamines present in the serum. On the day of the experiment, the cells were superfused with a normal external solution (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.35, 320–330 mOsM adjusted with sucrose) at room temperature. Whole-cell patch-clamp recordings were carried out to study either K^+ or Ca^{2+} currents with an Axopatch 1D amplifier and patch pipettes with 2–5 MΩ resistance.

After a GΩ seal was obtained, a whole-cell recording was obtained in the presence of normal external solution to confirm the presence of Na^+ current and high input resistance. The external solution was then switched to either K^+ external or Ba^{2+} external to study K^+ or Ca^{2+} channels, respectively. For K^+ current recording, the external solution contained 150 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, whereas the internal solution contained 150 mM K methyl sulfate, 10 mM HEPES, 10 mM EGTA, 2.5 mM MgATP, and 0.1 mM GTP. For Ca^{2+} current recording, the external solution consisted of 120 mM NaCl, 10 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES, and 1 μM tetrodotoxin (to block Na^+ current, all of which is tetrodotoxin sensitive in these cells), whereas the internal solution contained 120 mM Cs gluconate, 10 mM EGTA, 10 mM HEPES, 2.5 mM MgATP, and 0.1 mM GTP. pH and osmolarity of all of the solutions were maintained at 7.35 and 320–330 mOsM, respectively.

A range of magnitude for both K^+ and Ca^{2+} currents was observed in both WT and D79N α_{2A}AR-expressing cells and did not correlate with cell size. A 125-msec pulse was applied to elicit both currents. The magnitude of the current in the absence and presence of various pharmacological agents was measured at a single point corresponding to 80 msec for K^+ currents and 25 msec for Ca^{2+} current. The Ca^{2+} currents were not leak-subtracted.

Statistical analysis. Statistical comparisons within group and between groups were made with paired or unpaired two-tailed *t* tests, respectively, as well as two-factor analysis of variance, followed by Student-Newman-Keuls test. The level of significance was accepted at $p < 0.05$.

Results

The introduction of the D79N α_{2A}AR into cultured mouse anterior pituitary (AtT20) cells reveals a selective uncoupling of the mutant receptor from receptor-operated, Ba^{2+}-sensitive, inwardly rectifying K^+ currents (Fig. 1, A versus B) without any appreciable effects on modulation of voltage-

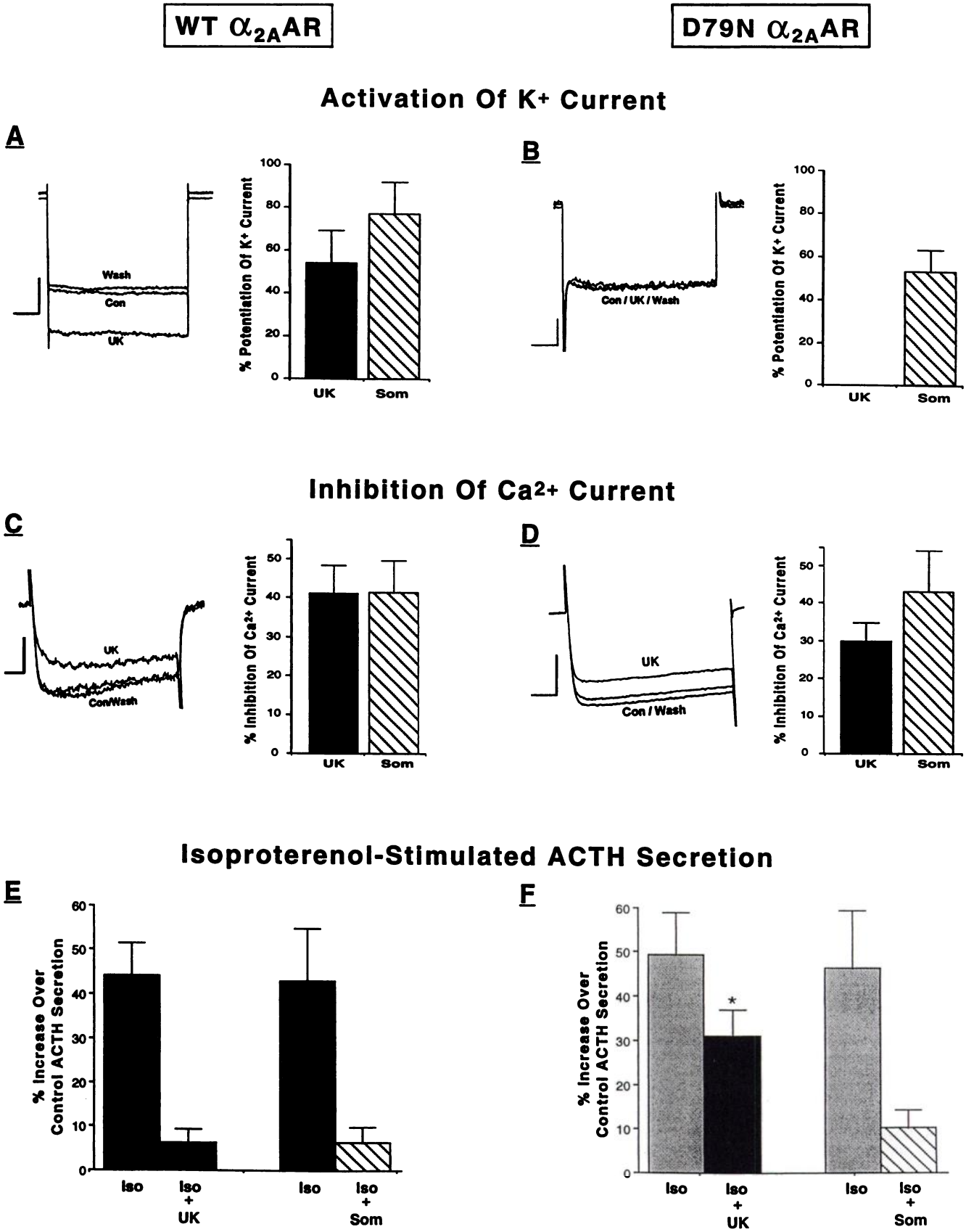

Fig. 1. Regulation of K^+ currents, Ca^{2+} currents, and isoproterenol-stimulated ACTH secretion in AtT20 cells expressing the WT and D79N mutant α_{2A}AR. A and B, Activation of Ba^{2+}-sensitive K^+ currents by 1 μM α_{2A}AR agonist UK 14,304 (*UK*) or 100 nM somatostatin (*Som*) in AtT20 cells expressing endogenous somatostatin receptors and WT (A) or D79N (B) α_{2A}AR. Data are presented as mean ± standard error of 7–12 experiments. After a whole-cell patch was obtained, the cells were held at 0 mV in symmetric K^+ solutions. K^+ currents were evoked in the

sensitive Ca^{2+} currents (Fig. 1, C versus D) or intracellular cAMP levels (18). In contrast, activation of endogenous somatostatin receptors (20, 21), which couple via G_i/G_o to the same effector pathways as does the α_{2A}AR (22–24), elicited comparable activation of K^+ currents and suppression of Ca^{2+} currents in AtT20 cell lines expressing WT or D79N mutant α_{2A}AR (Fig. 1, A–D). These findings eliminate the possibility that the molecular mechanisms linking receptor occupancy to activation of inwardly rectifying K^+ channels have been impaired in AtT20 cells expressing the D79N α_{2A}AR. Furthermore, the failure of the D79N α_{2A}AR to activate K^+ currents, even at supramaximal agonist concentrations (18), cannot be attributed to differences in receptor densities because the receptor density of the D79N α_{2A}AR (10 pmol/mg protein) was higher than that of the WT α_{2A}AR (3 pmol/mg protein). Taken together, these findings suggest that the altered structure of the D79N α_{2A}AR selectively uncouples this mutant receptor from activation of Ba^{2+}-sensitive K^+ currents in AtT20 cells.

The potentiation of K^+ currents by the α_{2A}AR, resulting in hyperpolarization and reduced cell excitability (12, 13), may translate into attenuation of regulated secretion. Earlier attempts to delineate involvement of K^+ channels in α_{2A}AR-mediated inhibition of neurotransmitter release or hormone secretion with the use of K^+ channel blockers have been difficult to interpret due to either possible interaction between K^+ channels and Ca^{2+} conductance (15) or the direct effect of the K^+ channel blockers on the release process itself (14). The D79N α_{2A}AR provided a unique opportunity to unambiguously explore the role of K^+ channel modulation in the α_{2A}AR-mediated attenuation of stimulus-secretion coupling. ACTH secretion in AtT20 cells can be enhanced by endogenous β-adrenergic receptors with the use of isoproterenol as the stimulus. Isoproterenol-stimulated ACTH secretion is exocytotic in nature and represents regulated secretory pathway in these cells (25). Activation of the WT α_{2A}AR by the α_2AR agonist UK 14,304 (1 μM) resulted in a marked inhibition of isoproterenol-stimulated ACTH secretion (Fig. 1E) in a manner analogous to the endogenous somatostatin receptors (inhibition of 77 ± 14% by UK 14,304 and 81 ± 11% by somatostatin). In contrast, the D79N α_{2A}AR was significantly less effective in suppressing isoproterenol-stimulated ACTH secretion (Fig. 1F) compared with the WT α_{2A}AR (inhibition of 39 ± 9% by the D79N α_{2A}AR and 77 ± 14% the WT α_{2A}AR). This attenuated response by the D79N α_{2A}AR represents reduction in the maximal response in the D79N α_{2A}AR compared with the WT receptor rather than a simple dextral shift of the concentration-response curve, as 1 μM UK 14,304 is a supramaximal concentration of the agonist for both the WT and D79N α_{2A}AR (data not shown). Furthermore, we are confident that inhibition of secretion by UK 14,304 was specifically mediated via the α_{2A}AR because UK 14,304 did not inhibit stimulated release in the untransfected AtT20 cells and because yohimbine (1 μM), the α_2AR-selective antagonist, blocked the effects of UK 14,304 on isoproterenol-stimulated ACTH secretion (data not shown). Because stimulation of ACTH secretion by isoproterenol and inhibition by somatostatin (Fig. 1, E and F) were comparable in cell lines expressing the WT or D79N α_{2A}AR, the differential effects of activation of the WT and mutant α_{2A}AR on ACTH release suggest that the failure to activate K^+ channels made the D79N α_{2A}AR less effective in suppressing stimulated ACTH secretion. An attempt to support this conclusion by examining the effect of the WT and D79N α_{2A}AR on stimulated secretion in the presence of the inwardly rectifying K^+ channel blocker Ba^{2+} was confounded due to a substantial effect of Ba^{2+} on basal ACTH secretion itself, probably reflecting depolarization of the cells in the presence of Ba^{2+}. In contrast, the differences in effects of the WT and D79N α_{2A}AR on stimulated ACTH secretion very clearly demonstrate that activation of receptor-operated K^+ channels by the α_{2A}AR is necessary for maximal inhibition of stimulus-secretion coupling in neuroendocrine cells.

The observation that the mutant D79N α_{2A}AR was able to inhibit at least a part of the isoproterenol-stimulated ACTH secretion (Fig. 1F) implies that additional effector systems are involved in the inhibitory actions of this receptor. The effects of isoproterenol on ACTH release have been shown to result from β-adrenergic receptor-mediated activation of adenylyl cyclase and cAMP-dependent protein kinase (26, 27). To bypass regulation of cAMP synthesis by the α_{2A}AR and thereby evaluate the importance of inhibition of voltage-sensitive Ca^{2+} currents and/or other effector pathways by the α_{2A}AR in suppressing ACTH release, we added the membrane permeant analogue of cAMP, 8-Br-cAMP, to directly activate cAMP-dependent protein kinase and ACTH secretion (28). One limitation of this approach is that membrane-permeant analogues cannot faithfully replicate the kinetics or cellular distribution of cAMP synthesis and turnover evoked by an extracellular secretagogue such as isoproterenol. In addition, a cAMP analogue could induce a physiological response independent of cAMP-dependent protein kinase. Nevertheless, we are reasonably confident that the secretory action of 8-Br-cAMP we observed was mediated via a cAMP-dependent protein kinase pathway because concomitant treatment of AtT20 cells with 8-Br-cAMP and N^6-benzyl-cAMP, another cAMP analogue, produced a synergistic effect on ACTH secretion (34% increase) compared with the effects of 3 mM 8-Br-cAMP (15% increase) or 300 μM N^6-benzyl-cAMP (0% increase) alone. This synergism is a prop-

absence and presence of UK 14,304 or somatostatin by deliverance of a hyperpolarizing pulse from 0 mV to −40 mV. *Vertical scale bar*, 100 pA; *horizontal scale bar*, 25 msec. K^+ currents were significantly greater in magnitude in the presence of UK 14,304 or somatostatin compared with their respective controls, except in the case of the mutant D79N α_{2A}AR, which failed to potentiate K^+ currents. C and D, Inhibition of voltage-activated Ca^{2+} currents by 1 μM α_{2A}AR agonist UK 14,304 (*UK*) or 100 nM somatostatin (*Som*) in AtT20 cells expressing endogenous somatostatin receptors and WT (C) or D79N (D) α_{2A}AR. Data are presented as mean ± standard error of four to seven experiments. After a whole-cell patch was obtained, the cells were held at −70 mV. Ca^{2+} currents were evoked in the absence and presence of UK 14,304 or somatostatin by deliverance of a depolarizing pulse from the holding potential of −70 mV to +10 mV. *Vertical scale bar*, 125 and 500 pA for the WT and D79N α_{2A}AR, respectively; *horizontal scale bar*, 25 msec for both. Ca^{2+} currents were significantly inhibited in the presence of UK 14304 or somatostatin compared with their respective controls in all AtT20 cell lines tested. E and F, Inhibition of 100 nM isoproterenol (*Iso*)-stimulated secretion of ACTH by 1 μM α_{2A}AR agonist UK 14,304 (*UK*) or 100 nM somatostatin (*Som*) in AtT20 cells expressing endogenous somatostatin receptors and WT (E) or D79N (F) α_{2A}AR. Data are presented as mean ± standard error for seven experiments. Isoproterenol-stimulated ACTH secretion was significantly inhibited in the presence of UK 14304 or somatostatin in all AtT20 cell lines tested. *, Significant difference between the inhibitory effect of UK 14,304 in AtT20 cells expressing the WT α_{2A}AR (E) and AtT20 cells expressing the D79N α_{2A}AR (F).

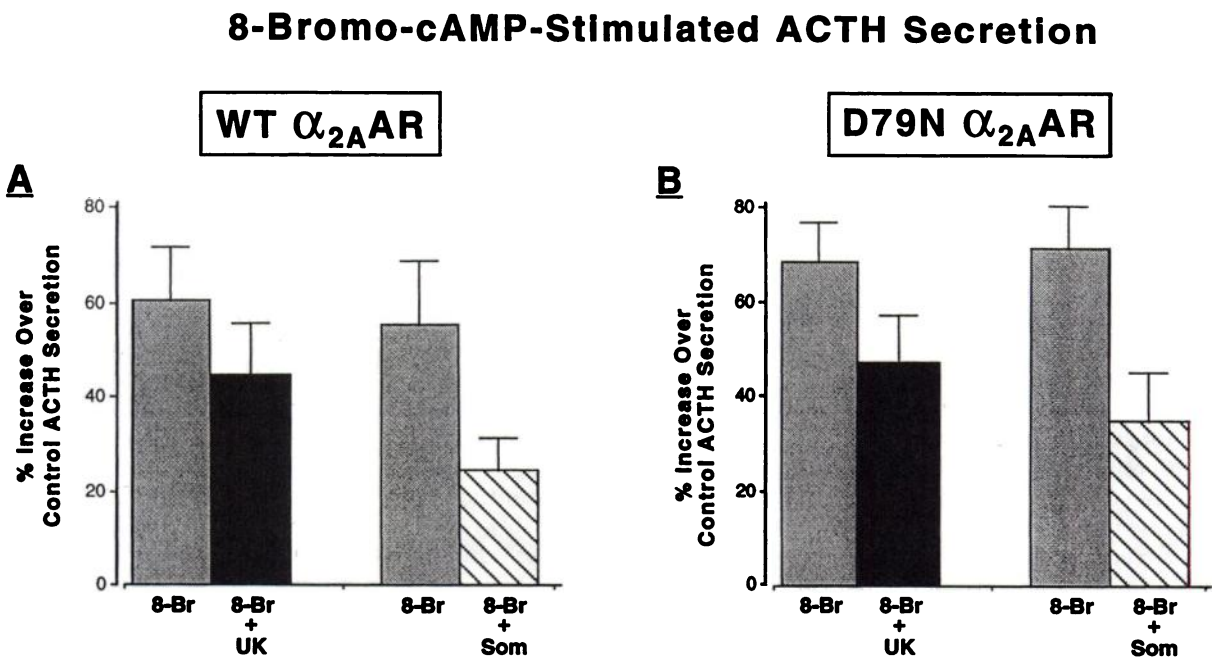

Fig. 2. Comparison of regulation of 8-Br-cAMP-stimulated ACTH secretion in AtT20 cells expressing the WT and D79N mutant α_{2A}AR. Inhibition of 3 mM 8-Br-cAMP (*8-Br*)-stimulated secretion of ACTH by 3 μM α_{2A}AR agonist UK 14,304 (*UK*) or 100 nM somatostatin (*Som*) was examined in AtT20 cells expressing endogenous somatostatin receptors and WT (A) or D79N (B) α_{2A}AR. Data are presented as mean ± standard error for eight or nine experiments. ACTH secretion evoked by 8-Br-cAMP was significantly inhibited by UK 14,304 or somatostatin in the D79N α_{2A}AR-expressing AtT20 cells and by somatostatin in the WT α_{2A}AR-expressing AtT20 cells. UK 14,304 clearly showed a trend towards inhibition in WT α_{2A}AR-expressing cells (seven of nine experiments exhibited inhibition), but overall, the inhibition was not statistically significant.

erty characteristic of allosteric regulation of the cAMP-dependent protein kinase regulatory subunit (29).

As shown in Fig. 2A, the WT α_{2A}AR showed a clear trend toward suppressing the response to 8-Br-cAMP in that the suppression was observed in seven of nine experiments, although this effect was not statistically significant (paired two-tailed *t* test). The WT α_{2A}AR was approximately half as effective in inhibiting the response to 8-Br-cAMP (34 ± 10% inhibition) (Fig. 2A) compared with WT α_{2A}AR inhibition of isoproterenol-evoked secretion (77 ± 14% inhibition) (Fig. 1E). Somatostatin also was less effective in suppressing secretion in response to 8-Br-cAMP than in response to isoproterenol as a secretagogue (Fig. 2, A and B, versus 1, E and F) in a manner analogous to previous observations for the somatostatin receptors in GH cells (30) and AtT20 cells (31). These results suggest that both the α_{2A}AR and the somatostatin receptor mediate at least a part of their inhibitory responses by attenuating cAMP synthesis because suppression of ACTH secretion is attenuated when this signaling pathway is bypassed. Surprisingly, the extent of inhibition elicited by the D79N α_{2A}AR of 8-Br-cAMP-stimulated ACTH secretion was comparable to that observed when isoproterenol was the secretagogue (Fig. 2B versus Fig. 1F), implying that for the D79N α_{2A}AR (unlike for the WT α_{2A}AR), adenylyl cyclase modulation was not involved in the inhibitory response. At present, our data do not clarify the molecular basis for this difference in response to 8-Br-cAMP in cells expressing the WT versus D79N α_{2A}AR. However, the important and readily interpretable observation is that the D79N α_{2A}AR still significantly inhibits 8-Br-cAMP-stimulated ACTH secretion, implying that an effector pathway in addition to attenuation of cAMP production and activation of K^+ currents is involved in transducing inhibition of stimulus-secretion coupling. This additional pathway required by the α_{2A}AR may involve inhibition of Ca^{2+} channels (33) or other downstream mechanisms such as stimulation of phospholipase A_2 (32) or direct inhibition of release mechanism (37) or other, not-yet-revealed effector systems.

In addition to examining α_{2A}AR-mediated inhibition of stimulated ACTH secretion, we evaluated α_{2A}AR modulation of basal ACTH secretion from AtT20 cells. Basal ACTH secretion, which occurs in the absence of any external stimulus, consists of ACTH secreted by a constitutive pathway as well as ACTH released by a Ca^{2+}-dependent pathway (34). Activation of the WT α_{2A}AR by UK 14,304 resulted in small but consistent and statistically significant inhibition of basal ACTH secretion (Table 1). The D79N mutant α_{2A}AR was significantly less effective in attenuating basal ACTH secretion than the WT α_{2A}AR, although inhibition by somatostatin was comparable in both cell lines (Table 1). Although the role of K^+ channels in ACTH secretion under basal conditions has not been delineated in AtT20 cells, the lack of D79N α_{2A}AR

TABLE 1

Inhibition of basal ACTH secretion in AtT20 cells expressing the WT and D79N mutant α_{2A}AR

The α_{2A}AR agonist UK 14,304 (1 μM) and the somatostatin receptor agonist somatostatin (100 nM) were used to assess inhibition of basal secretion. Data are presented as mean ± standard error. Each experiment was performed in triplicate or quadruplicate.

Cell line	UK 14,304	No. of experiments	Somatostatin	No. of experiments
	% Inhibition		*% Inhibition*	
WT	15.95 ± 4.16	6	33.45 ± 6.96	7
D79N	2.67 ± 1.12[a]	6	23.42 ± 3.76	7

[a] Significant difference between UK 14,304-mediated inhibition of basal secretion in WT and D79N α_{2A}AR-expressing AtT20 cells.

influence on basal ACTH secretion implies an important involvement of K^+ channels in regulation of ACTH secretion in the absence of any external excitatory stimulus.

Discussion

We demonstrated that a point mutation in the α_{2A}AR (D79N α_{2A}AR) results in selective uncoupling of the receptor from inwardly rectifying K^+ channels; in contrast, no appreciable influence on coupling to other signaling pathways, such as adenylyl cyclase and voltage-activated Ca^{2+} channels, has been observed because the WT and D79N α_{2A}AR are equally effective in suppressing cAMP accumulation (18) and in inhibiting voltage-activated Ca^{2+} current (current study and Ref. 18). We took advantage of this phenomenon to explore the involvement of inwardly rectifying K^+ channels in the antisecretory effects of the α_{2A}AR.

The most important observation of the current study was that the WT α_{2A}AR and the mutant D79N α_{2A}AR differ significantly in their ability to suppress isoproterenol-enhanced ACTH secretion from AtT20 cells. Thus, the D79N α_{2A}AR is essentially half as efficacious as the WT α_{2A}AR in inhibiting isoproterenol-evoked secretion (Fig. 1, E and F). Because the major difference between the WT and D79N α_{2A}AR is their ability (or inability) to couple to inwardly rectifying K^+ channels, this result indicates that the WT α_{2A}AR coupling to the K^+ channels must play a significant role in antisecretory action of this receptor. The α_{2A}AR activation of K^+ channels may contribute to the antisecretory action of the receptor by changing electrical properties of the cells. It has been demonstrated that AtT20 cells exhibit spontaneous action potentials accompanied by Ca^{2+} transients (35). Stimulation of the β-adrenergic receptors increases spontaneous activity and intracellular Ca^{2+} levels in these cells, with a resultant increase in ACTH secretion (25). Activation of inwardly rectifying K^+ channels by the WT α_{2A}AR would hyperpolarize the cell, thus probably limiting Ca^{2+} influx in the cell and suppressing ACTH secretion. Because the mutant receptor is unable to activate the K^+ channels, it would be less effective in inhibiting the isoproterenol-regulated ACTH secretion. A similar conclusion was reached regarding the action of another G_i/G_o-coupled receptor endogenous to AtT20 cells, somatostatin receptor; Pennfather *et al.* (36) demonstrated that somatostatin produced hyperpolarization of AtT20 cells and reduced frequency of spontaneous action potentials. In addition, in the presence of cesium, which blocks inwardly rectifying K^+ channels, somatostatin was less effective at suppressing secretagogue-enhanced ACTH secretion from these cells. Taken together, these findings suggest that recruitment of inwardly rectifying K^+ channels may be a generalized mechanism for the α_{2A}AR, somatostatin receptor, and other G_i/G_o-coupled receptors to attenuate stimulus-secretion coupling, as these receptors hyperpolarize a wide variety of neuroendocrine and neuronal cells, such as pancreatic islet β cells (11), GH_3 pituitary cells (15), and locus ceruleus neurons (12) with concomitant attenuation of either hormonal secretion or neurotransmitter release in all of these systems.

Because the α_{2A}AR and other G_i/G_o-coupled receptors are known to inhibit adenylyl cyclase and lower intracellular cAMP levels, we examined the possible involvement of this pathway in α_{2A}AR-mediated inhibition of stimulus-secretion coupling. When we used 8-Br-cAMP as a secretagogue, there was a clear reduction in the inhibitory response of the WT α_{2A}AR, suggesting that suppression of cAMP-mediated pathway contributes to α_{2A}AR-induced attenuation of regulated secretion. Other G_i/G_o-coupled receptors also exhibit the same dependency on cAMP modulation to exert their full inhibitory response on hormonal secretion (30, 31). In contrast, this modulatory pathway seems to be of less importance in mediating the suppressive actions of these receptors in neuronal cells, such as hippocampal neurons (8).

When 8-Br-cAMP was used as the secretagogue, the D79N α_{2A}AR was still able to suppress secretion. Because this receptor structure cannot activate K^+ channels and because 8-Br-cAMP bypasses the role of this mutant receptor to attenuate cAMP accumulation, this finding provides strong evidence that an additional pathway, such as direct suppression of Ca^{2+} currents (33) and/or activation of phospholipase A_2 (32), plays an important role in α_{2A}AR-mediated inhibition of peptide hormone release. It was somewhat unexpected that the WT and D79N mutant α_{2A}AR were equieffective in attenuating stimulus-secretion coupling when 8-Br-cAMP was the secretagogue, as if activation of K^+ current were not a contributing factor to the inhibitory effect of the WT α_{2A}AR under these conditions. Furthermore, the D79N α_{2A}AR-mediated inhibition of ACTH secretion was similar regardless of whether the receptor could (with isoproterenol as a secretagogue) or could not modulate intracellular cAMP levels (with 8-Br-cAMP as a secretagogue), which is in contrast to the observation with the WT α_{2A}AR, which had an attenuated response to suppression of 8-Br-cAMP-evoked secretion compared with suppression of isoproterenol-evoked response (Fig. 1E versus Fig. 2A). We are unable to account for these unexpected observations, although a number of molecular possibilities exist. For example, it can be postulated that cAMP inhibits the α_{2A}AR-K^+ channel coupling by channel phosphorylation, and removal of such an inhibition (by reducing cAMP levels) is required for the α_{2A}AR-K^+ channel coupling to occur. In such a scenario, the presence of 8-Br-cAMP, which bypasses attenuation of cAMP production by the α_{2A}AR, may result in noncontribution of inwardly rectifying K^+ currents to the receptor-mediated attenuation of ACTH secretion; for the mutant α_{2A}AR, cAMP pathway would not play a significant role because receptor-dependent K^+ channel activation is already nonexistent. Such a hypothesis is supported by earlier reports that phosphorylation or dephosphorylation of inwardly rectifying K^+ channels influences the channel activity (38, 39). An alternative possibility is that 8-Br-cAMP evokes inhibition of the K^+ channels by acting at an extracellular site, similar to that reported for 8-Br-cAMP action on $GABA_A$ channels (40). In the context of the goal of the current study, however, the experiments with isoproterenol as a secretagogue are more relevant to our efforts to define whether one or more signaling pathways is involved in α_{2A}AR-mediated inhibition of stimulus-secretion coupling. The findings in the current study suggest that in response to a secretagogue acting at cell surface receptors, such as isoproterenol at β-adrenergic receptors, the α_{2A}AR recruits multiple pathways to evoke maximal inhibition of the stimulated secretion.

In addition to examining the WT and D79N α_{2A}AR effects on regulated secretion, we studied their influence on basal secretion, which occurs in the absence of any exter-

nal stimulus. In AtT20 cells, basal ACTH secretion consists of constitutive secretion, which is Ca^{2+} independent, and regulated secretion, which is Ca^{2+} dependent (34). However, molecular mechanisms that may underlie regulation of these secretory pathways remain in large part undefined. As mentioned, AtT20 cells exhibit spontaneous electrical activity, resulting in Ca^{2+} influx, which may contribute to ACTH secretion in the absence of any external stimulus. Somatostatin receptor, by attenuating Ca^{2+} influx, lowers basal Ca^{2+} levels (24), which may lead to inhibition of basal secretion. On examination of basal ACTH secretion, we observed that although the WT α_{2A}AR was effective in reducing basal secretion, the D79N mutant α_{2A}AR failed to influence basal secretion significantly. The WT α_{2A}AR may have reduced the basal secretion, especially the Ca^{2+}-dependent component of basal secretion, by hyperpolarizing the cells and reducing Ca^{2+} influx and basal Ca^{2+} levels, similar to that reported for somatostatin receptors (24). Although the WT α_{2A}AR-mediated inhibition of basal secretion is small (but consistent), reflecting a possibility that contribution of a Ca^{2+}-dependent component to basal secretion is less than that of a Ca^{2+}-independent component, these results again underscore the importance of K^+ channel modulation in the antisecretory effects of the α_{2A}AR, even under basal conditions.

In summary, in the current study, we used a novel genetic approach to reveal an important role for inwardly rectifying K^+ channel activation in maximal inhibition of stimulus-secretion coupling by the α_{2A}AR. In addition, the data suggest that concomitant modulation of cAMP production and of voltage-sensitive Ca^{2+} currents or other effector pathways is necessary for the α_{2A}AR to optimally suppress regulated secretion. Because many G_i/G_o-coupled receptors, such as somatostatin (2), A_1 adenosine (3), and δ- and μ-opioid (4), inhibit neuronal and neuroendocrine release and couple to the same effector systems as the α_{2A}AR, it is likely that the interpretations derived from this study are not limited to the α_{2A}AR but may reflect the need of these multiple receptor populations to recruit all of their effector pathways to elicit maximal physiological responses.

References

1. Ruffolo, R. R. Jr, A. J. Nichols, J. M. Stadel, and J. P. Hieble. Pharmacologic and therapeutic applications of α_2-adrenoceptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **33**:243–79 (1993).
2. Dorflinger, L. J., and A. Schonbrunn. Somatostatin inhibits basal and vasoactive intestinal peptide-stimulated hormone release by different mechanisms in GH pituitary cells. *Endocrinology* **113**:1551–1558 (1983).
3. Dolphin, A. C., and S. A. Prestwich. Pertussis toxin reverses adenosine inhibition of neuronal glutamate release. *Nature (Lond.)* **316**:148–150 (1985).
4. Schoffelmeer, A. N. M., and A. H. Mulder. Presynaptic opioid receptor and α_2-adrenoceptor-mediated inhibition of noradrenaline release in the rat brain: role of hyperpolarization? *Eur. J. Pharmacol.* **105**:129–135 (1984).
5. Isom, L. L., and L. E. Limbird. What happens next? A hypothesis linking the biochemical and electrophysiological sequelae of α_2 adrenergic receptors, in *The Alpha-2 Adrenergic Receptors* (L. E. Limbird, ed). Humana Press, Clifton, New Jersey, 323–326 (1988).
6. Limbird, L. E. Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB J.* **2**:2686–2695 (1988).
7. Ullrich, S., and C. B. Wollheim. Islet cyclic AMP levels are not lowered during α_2-adrenergic inhibition of insulin release: studies with epinephrine and forskolin. *J. Biol. Chem.* **259**:4111–4115 (1984).
8. Fredholm, B. B., and E. Lindgren. Protein kinase C activation increases noradrenaline release from the rat hippocampus and modifies the inhibitory effect of α_2-adrenoceptor and adenosine A_1-receptor agonists. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **337**:477–483.
9. Surprenant, A., K.-Z, Shen, R. A. North, and H. Tatsumi. Inhibition of calcium currents by noradrenaline, somatostatin and opioids in guinea-pig submucosal neurons. *J. Physiol.* **431**:585–608 (1990).
10. Williams, J. T., and R. A. North. Catecholamine inhibition of calcium action potentials in rat locus coeruleus neurones. *Neuroscience* **14**:103–109 (1985).
11. Cook, D. L., and E. Perara. Islet electrical pacemaker response to *alpha*-adrenergic stimulation. *Diabetes* **31**:985–990 (1982).
12. Aghajanian, G. K., and C. P. VanderMaelen. α_2-adrenoceptor-mediated hyperpolarization of locus coeruleus neurons: intracellular studies *in vivo*. *Science (Washington D. C.)* **215**:1394–1396 (1982).
13. Morita, K., and R. A. North. Clonidine activates membrane potassium conductance in myenteric neurons. *Br. J. Pharmacol.* **74**:419–428 (1981).
14. Allgaier, C., H. Repp, and G. Herttig. Effect of K^+ channel blockers on the α_2-adrenoceptor-coupled regulation of electrically evoked noradrenaline release in hippocampus. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **347**: 14–20 (1993).
15. Koch, B. D., J. B. Blalock, and A. Schonbrunn. Characterization of the cyclic AMP-independent actions of somatostatin in GH cells. I. An increase in potassium conductance is responsible for both the hyperpolarization and the decrease in intracellular free calcium produced by somatostatin. *J. Biol. Chem.* **263**:216–225 (1988).
16. Ceresa, B. P., and L. E. Limbird. Mutation of an aspartate residue highly conserved among G protein-coupled receptors results in non-reciprocal disruption of α_2-adrenergic receptor-G protein interactions. *J. Biol. Chem.* **269**:29557–29564 (1994).
17. Horstman, D. A., S. Brandon, A. L. Wilson, C. A. Guyer, E. J. Cragoe, Jr, and L. E. Limbird. An aspartate conserved among G protein-coupled receptors confers allosteric regulation of α_2-adrenergic receptors by sodium. *J. Biol. Chem.* **265**:21590–21595 (1990).
18. Surprenant, A., D. A. Horstman, H. Akbarali, and L. E. Limbird. A point mutation of the α_2-adrenoceptor that blocks coupling to potassium but not calcium currents. *Science (Washington D. C.)* **257**:977–980 (1992).
19. Nicholson, W. E., D. R. Davis, B. J. Sherrell, and D. N. North. Rapid radioimmunoassay for corticotropin in unextracted human plasma. *Clin. Chem.* **30**:259–265 (1984).
20. Mahy, N., M. Woolkalis, K. Thermos, K. Carlson, D. Manning, and T. Reisine. Pertussis toxin modifies the characteristics of both the inhibitory GTP binding proteins and the somatostatin receptor in anterior pituitary tumor cells. *J. Pharmacol. Exp. Ther.* **246**:779–785 (1988).
21. Richardson, U. I., and A. Schonbrunn. Inhibition of adrenocorticotropin secretion by somatostatin in pituitary cells in culture. *Endocrinology* **108**: 281–290 (1981).
22. Dousmanis, A. G., and P. S. Pennefather. Inwardly rectifying potassium conductances in AtT-20 clonal pituitary cells. *Pfluegers Arch.* **422**:98–104 (1992).
23. Heisler, S., T. D. Reisine, Y. H. V. Hook, and J. Axelrod. Somatostatin inhibits multireceptor stimulation of cyclic AMP formation and corticotropin secretion in mouse pituitary tumor cells. *Proc. Natl. Acad. Sci. USA* **79**:6502–6506 (1982).
24. Luini, A., D. Lewis, S. Guild, G. Schofield, and F. Weight. Somatostatin, an inhibitor of ACTH secretion, decreases cytosolic free calcium and voltage-dependent calcium current in a pituitary cell line. *J. Neurosci.* **6**:3128–3132 (1986).
25. Surprenant, A. Correlation between electrical activity and ACTH/β-endorphin secretion in mouse pituitary tumor cells. *J. Cell. Biol.* **95**:559–566 (1982).
26. Litvin, Y., R. PasMantier, N. Fleischer, and J. Erlichman. Hormonal activation of the cAMP-dependent protein kinases in AtT20 cells: preferential activation of protein kinase 1 by corticotrophin releasing factor, isoproterenol, and forskolin. *J. Biol. Chem.* **259**:10296–10302 (1984).
27. Schecterson, L. C., and G. S. McKnight. Role of cyclic adenosine 3′,5′-monophosphate-dependent protein kinase in hormone-stimulated β-endorphin secretion in AtT20 cells. *Mol. Endocrinol.* **5**:170–178 (1991).
28. Miller, J. P. Cyclic nucleotide analogs, in *Cyclic-3′,5′-Nucleotides: Mechanism of Action*. (J. Schulz, ed.). Wiley and Sons, New York, 77–104 (1977).
29. Beebe, S. J., P. F. Blackmore, T. D. Chrisman, and J. D. Corbin. Use of synergistic pairs of site-selective cAMP analogs in intact cells. *Methods Enzymol.* **159**:118–139 (1988).
30. Koch, B. D., L. J. Dorflinger, and A. Schonbrunn. Pertussis toxin blocks both cyclic AMP-mediated and cyclic AMP-independent actions of somatostatin: evidence for coupling of N_i to decreases in intracellular free calcium. *J. Biol. Chem.* **260**:13138–13145 (1985).
31. Reisine, T. D. Somatostatin inhibition of cyclic AMP accumulation and adrenocorticotropin release from mouse anterior pituitary tumor cells: mode of action and self-regulation. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**:169–177 (1985).
32. Jones, S. B., S. P. Halenda, and D. B. Bylund. α_2-Adrenergic receptor stimulation of phospholipase A_2 and of adenylate cyclase in transfected Chinese hamster ovary cells is mediated by different mechanisms. *Mol. Pharmacol.* **39**:239–245 (1991).
33. Lipscombe, D., S. Kongsamut, and R. W. Tsien. *Alpha*-adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium channel gating. *Nature (Lond.)* **340**:639–642 (1989).
34. Matsuuchi, L., and R. B. Kelly. Constitutive and basal secretion from the endocrine cell line, AtT-20. *J. Cell Biol.* **112**:843–852 (1991).
35. Adler, M., B. S. Wong, S. L. Sabol, N. Busis, M. B. Jackson, and F. F.

Weight. Action potentials and membrane ion channels in clonal anterior pituitary cells. *Proc. Natl. Acad. Sci. USA* **80**:2086–2090 (1983).
36. Pennfather, P. S., S. Heisler, and J. F. MacDonald. A potassium conductance contributes to the action of somatostatin-14 to suppress ACTH secretion. *Brain Res.* **444**:346–350 (1988).
37. Lang, J., I. Nishimoto, T. Okamoto, R. Regazzi, C. Kiraly, U. Weller, and C. B. Wollheim. Direct control of exocytosis by receptor-mediated activation of the heterotrimeric GTPases G_i and G_o or by the expression of their active G_α subunits. *EMBO J.* **14**:3635–3644 (1995).
38. Inoue, M., and I. Imanaga. Phosphatase is responsible for run down, and probably G protein-mediated inhibition of inwardly rectifying K^+ currents in guinea pig chromaffin cells. *J. Gen. Physiol.* **105**:249–266 (1995).
39. Kim, D. Modulation of acetylcholine-activated K^+ channel function in rat atrial cells by phosphorylation. *J. Physiol.* **437**:133–155 (1991).
40. Lambert, N. A., and N. L. Harrison. Analogs of cyclic AMP decrease *gamma*-aminobutyric $acid_A$ receptor-mediated chloride current in cultured rat hippocampal neurons via an extracellular site. *J. Pharmacol. Exp. Ther.* **255**:90–94 (1990).

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