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Coupling of opiate receptors to adenylate cyclase: Requirement for Na^+ and GTP

(neuroblastoma–glioma hybrid/[D-Ala[2],Met[5]]enkephalin amide/cyclic AMP/prostaglandin E_1)

ARTHUR J. BLUME, DAVID LICHTSHTEIN*, AND GLORIA BOONE

Department of Physiological Chemistry and Pharmacology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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ABSTRACT Inhibition of the adenylate cyclase activity in homogenates of mouse neuroblastoma-glioma hybrid cells (NG108-15) by the opioid peptide [D-Ala[2],Met[5]]enkephalin amide (AMEA) requires the presence of Na^+ and GTP. In this process, the selectivity for monovalent cations is $Na^+ \geq Li^+ > K^+ >$ choline$^+$; ITP will replace GTP but ATP, UTP, or CTP will not. The apparent K_m for Na^+ is 20 mM and for GTP it is 1 μM. Under saturating Na^+ and GTP conditions, the apparent K_i for AMEA-directed inhibition is 20 nM for basal and 100 nM for prostaglandin E_1-activated adenylate cyclase activity. For both cyclase activities, maximal inhibition is only partial (i.e., ≈55% of control in each case). In intact viable NG108-15 cells, the decrease in basal and prostaglandin E_1-stimulated intracellular cyclic AMP concentrations by AMEA is also dependent upon extracellular Na^+. The enkephalin-directed reductions in cyclic AMP concentrations are at least 75%. The specificity of the monovalent cation requirement for enkephalin action on intact cells is the same as for enkephalin regulation of homogenate adenylate cyclase activity. Based on these data, a model is presented in which the transfer of information from opiate receptors to adenylate cyclase requires active separate membrane components, which correspond to the sites of action of Na^+ and GTP in this process.

Opiates decrease the intracellular cyclic AMP (cAMP) concentrations of intact mouse neuroblastoma–rat glioma hybrid cells (NG108-15) by inhibiting the activity of the adenylate cyclase of these cells (1–3). This regulation of cAMP requires occupation of highly specific receptors by opiate agonists and is blocked by opiate antagonists. Recently, these opiate receptors were found to be sensitive to guanine nucleotides (4, 5) and Na^+ (4–7). The effects of Na^+ and nucleotides on NG108-15 opiate receptors parallel the effects of Na^+ (8–14) and nucleotides (15, 16) on brain opiate receptors. Although nucleotides and Na^+ have nonspecific effects on NG108-15 and brain opiate receptors, the actions of ions and nucleotides can be used to discriminate between agonist and antagonist interactions with opiate receptors.

The selective nucleotide and ion regulation of agonist interactions with opiate receptors is reminiscent of the well-documented guanine nucleotide regulation of agonist interaction with hormonal receptors that mediate activations of adenylate cyclase (17–20). In the latter case, agonist occupation of the receptor can take place without any nucleotides present; however, in order for the agonist–receptor complex to cause an increase in adenylate cyclase activity, the presence of a guanine nucleotide is required. Biochemical (21), genetic (22, 23), cell fusion (24, 25), and reconstitution (26–37) studies have shown that the receptor and catalytic units of adenylate cyclase are separate proteins and that another membrane component(s) (here termed the "coupler") is actually required for receptor-directed activation of the catalytic unit.

The published observations of Na^+ and nucleotide sensitivity of opiate–agonist binding therefore raise the possibility that Na^+ as well as guanine nucleotides may be required for opiate receptor-mediated regulation of NG108-15 adenylate cyclase. We report here our findings of an absolute dependence upon the presence of both Na^+ and guanine nucleotides for opiate inhibition of NG108-15 adenylate cyclase.

MATERIALS AND METHODS

Membrane Preparations of Adenylate Cyclase. The source of adenylate cyclase in all these studies was the mouse neuroblastoma–rat glioma hybrid NG108-15 (passage no. 20-45). Cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum, hypoxanthine, aminopterin, and thymidine as described (1–4). After reaching confluence, the cells were shaken free and washed twice at 4°C in 0.32 M sucrose/5 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$. Subsequently, cell-free homogenates were made (at 4°C) by glass/Teflon homogenization (10–16 strokes with a motor-driven homogenizer) until ≥95% of the nuclei had been released yet still remained intact. Nuclei and any remaining cells were removed from the homogenate by centrifugation at 650 × g for 5 min. This nuclei-free homogenate was then centrifuged at 40,000 × g and the resulting supernatant was discarded. The pelleted membranes were suspended in 0.32 M sucrose/5 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$ at 3–5 mg of protein per ml, frozen, and stored in liquid N_2 until used. The adenylate cyclase activity of this preparation and its susceptibility to opiate inhibition were stable for at least 6 months.

Adenylate Cyclase Assays. Adenylate cyclase activity was determined in a final reaction volume of 100 μl in a standard assay mixture that contained (final concentrations): 50 mM Tris maleate pH 7.4 buffer, 0.2 mM ATP (Na_2 salt), 10 mM $MgCl_2$, 40 μg of creatine kinase, 20 mM creatine phosphate (Tris salt), 2–4 × 10^6 cpm of [α-^{32}P]ATP, 0.5 mM Ro 20-1724 (as inhibitor of cyclic nucleotide phosphodiesterase activity), 0.5 mM cAMP, and homogenate protein (20–60 μg of protein, 20–60 mM sucrose). The various adenylate cyclase preparations were added to the above complete standard reaction mixture plus any other additions (i.e., opiates, Na^+, nucleotides, see text) but lacking [α-^{32}P]ATP and equilibrated at 32°C for 10 min. The [α-^{32}P]ATP was then added and the reaction was continued at 32°C for an additional 20 min. Under all conditions described in this paper, activity was constant for at least 30 min and was directly proportional to protein concentration. Some reaction mixtures (as noted in text) also contained the muscarinic antagonist quinuclidinylbenzylate·HCl (QNB) at 10 μM. These

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Abbreviations: cAMP, cyclic AMP; NG108-15, mouse neuroblastoma–rat glioma hybrid cells; PGE_1, prostaglandin E_1; QNB, quinuclidinylbenzylate·HCl; AMEA, [D-Ala[2], Met[5]]enkephalin amide.

* Present address: Department of Physiology, Hebrew University, Hadassah Medical School, Jerusalem, Israel.

additions were sometimes necessary to ensure the absence of inhibition of activity by acetylcholine (which is made by these cells) and which inhibits NG108-15 adenylate cyclase in a nonadditive fashion with opiates (28). Cyclase reactions were stopped by addition of perchloric acid containing [^{3}H]cAMP as an indicator of recovery, and the [^{32}P]cAMP synthesized was assessed by the method of Solomon *et al.* (29) as amended (30). Values given are the mean of triplicate determinations which varied <5%. Protein was determined by the method of Lowry *et al.* (31).

Determination of Intracellular cAMP. cAMP concentrations in intact viable NG108-15 were determined by the protein binding method of Gilman (32) according the modification of Brostrom and Kon (33). Cells for these experiments were harvested and washed twice with choline buffer [135 mM choline·HCl/50 mM Hepes (Tris), pH 7.4/0.8 mM $MgCl_2$/1.8 mM $CaCl_2$/5.5 mM glucose] and concentrated in this buffer. At the start of each experiment, cells were diluted 1:10 into choline buffer or Na^+ buffer (135 mM NaCl instead of choline·HCl) or sucrose buffer (320 mM sucrose instead of choline·HCl) containing 0.7 mM Ro 20-1724 at 37°C with other additions (see text) and incubated for 10 min. Reactions were terminated with trichloroacetic acid containing [^{14}C]cAMP to monitor recovery. Values given are the mean of quadruplicate assays which varied <5%.

Materials. [α-^{32}P]ATP (10–30 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), [^{14}C]cAMP (40–60 mCi/mmol), and [^{3}H]cAMP (30–50 Ci/mmol) were obtained from New England Nuclear, [D-Ala^2, Met^5]enkephalin amide (AMEA) was from Peninsula Labs, San Carlos, Calif., creatine phosphate (Tris salt) and ATP (Na_2 salt) (prepared from adenosine by phosphorylation) were from Sigma, and Ro 20-1724, prostaglandin E_1 (PGE_1), and QNB were from Hoffmann-LaRoche.

RESULTS

Using nuclei-free homogenates of NG108-15, we were able to confirm the observations by Sharma *et al.* (2) on opiate-directed inhibition of adenylate cyclase. In our studies, inhibition was produced by the stable opioid peptide AMEA (Table 1) and was at maximum 50–55%. This partial inhibition had been observed previously for the opiate agonists morphine and etorphine (2, 3). Although not previously noted, with this crude homogenate both Na^+ and GTP were necessary for maximal enkephalin-directed inhibition of adenylate cyclase activity (Table 1). Only about half-maximal inhibition of basal activity was observed in the presence of 135 mM Na^+ without added GTP. With the PGE_1-stimulated activity, addition of Na^+ (but not GTP) allowed 86% of the maximal possible opioid inhibition.

With nuclei- and supernatant-free NG108-15 membranes, a clear requirement for added Na^+ and GTP for maximal oplioid inhibition was observed (Table 2). Basal activity was now ≤10% inhibited by enkephalin when either Na^+ or GTP was added alone; the full 50% inhibition was evident when both Na^+ and GTP were present. In addition, the PGE_1-activated enzyme was only slightly inhibitable (i.e., 27% of maximal inhibition) when Na^+ was added by itself. In these preparations, etorphine and morphine also inhibited basal and PGE_1-stimulated activity to a similar partial extent (i.e., ≈50%) and their action was likewise dependent upon the presence of Na^+ and GTP (data not shown).

As expected from the work of Nirenberg and coworkers (1–3), the opiate antagonists naloxone and naltrexone blocked all opiate and enkephalin inhibition of adenylate cyclase under our assay conditions (see Fig. 4).

It is important to point out that the requirement for "added" Na^+ could be seen only when the Tris salt of creatine phosphate was used as part of the nucleotide triphosphate-regenerating system in the assay mixtures. The commonly purchased creatine phosphate is a Na_2 salt and therefore by itself makes the endogenous contents of these assays 40 mM Na^+. This amount of Na^+ is twice as great as needed to allow for half-maximal enkephalin inhibition (Fig. 1). Furthermore, the ATP used here was made by phosphorylation of adenosine and did not contain enough GTP to obscure adenylate cyclase regulation otherwise dependent upon added GTP (34).

The question arises as to whether or not the requirement for Na^+ is related to the known Na^+ regulation of NG108-15 opiate receptors (4, 5). As reported (5), AMEA binding affinity is selectively decreased by monovalent cations with a potency order of $Na^+ > Li^+ > K > choline^+$ and with an apparent K_m for Na^+ of ≈20 mM. The observed concentration curve for the Na^+ required for opiate-directed inhibition of NG108-15 adenylate cyclase appears narrow (perhaps indicating a cooperative type of action) and has an apparent K_m value of ≈20 mM (Fig. 1). Lithium appears to be slightly less effective, K^+ considerably less effective, and choline$^+$ least effective [only 9% of maximal opiate inhibition was observed with 75 mM choline (Table 3)].

Table 1. Inhibition of adenylate cyclase by 10 μM AMEA in nuclei-free NG108-15 homogenate

	Adenylate cyclase, pmol/min/mg protein		
	No AMEA	With AMEA	% opiate inhibition
Basal:			
—	14.8	13.0	7
Na^+	11.3	8.3	26
GTP	8.2	8.9	−9
Na^+ + GTP	9.7	4.4	55
Activated:			
PGE_1	43.4	36.3	16
PGE_1 + Na^+	82.0	46.3	44
PGE_1 + GTP	41.8	35.9	14
PGE_1 + Na^+ + GTP	65.3	32.4	51

Basal enzyme activity in nuclei-free homogenates was monitored in the standard Na^+-free assay mixture with no further additions. Assays for activated enzyme activity contained PGE_1 (1 μM). Where indicated, Na^+ (0.135 M NaCl) or GTP (0.1 mM) or both also were present.

Table 2. Inhibition of adenylate cyclase by 10 μM AMEA in nuclei- and supernatant-free NG108-15 membranes

	Adenylate cyclase, pmol/min/mg protein		
	No AMEA	With AMEA	% opiate inhibition
Basal:			
—	30.6	30.1	2
Na^+	21.9	19.8	10
GTP	22.3	20.9	7
Na^+ + GTP	18.9	9.6	50
Activated:			
PGE_1	35.5	36.7	−3
PGE_1 + Na^+	48.9	43.5	11
PGE_1 + GTP	59.8	48.0	20
PGE_1 + Na^+ + GTP	70.5	42.4	40

Assays were conducted on the resuspended membranes that had been pelleted at 40,000 × *g* after a 30-min centrifugation of the nuclei-free NG108-15 homogenates described in Table 1. Where indicated, NaCl (0.135 M), GTP (0.1 mM), and PGE_1 (1 μM) were added to the standard Na^+-free assay mixture.

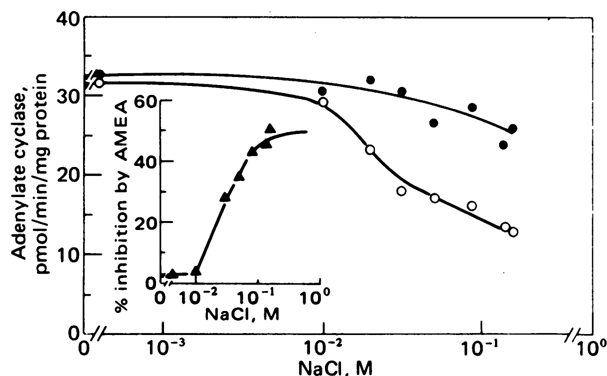

FIG. 1. Na^+ dependence of AMEA-directed inhibition of basal activity of adenylate cyclase. Assays were performed on nuclei- and supernatant-free NG108-15 membranes in the standard Na^+-free assay mixture plus 0.1 mM GTP with (○) or without (●) AMEA (10 μM) at increasing concentrations of NaCl. (*Inset*) Plot of the difference in basal enzyme activity with and without AMEA at increasing Na^+ concentrations, taking the activity observed at each Na^+ concentration without AMEA to be 100%.

The demonstration of this requirement for Na^+ with cell membranes led us to investigate if there is a requirement for Na^+ for opiate action on intact cells. Basal and PGE_1-stimulated cAMP concentrations are decreased by opiates in viable NG108-15 (2). We therefore investigated what would be the effect of receptor-saturating concentrations of AMEA on cells suspended in isotonic buffers containing high Na^+ concentration or devoid of Na^+ (Fig. 2). Removal of extracellular Na^+ ($[Na^+]_{out}$) induced a loss in the ability of enkephalin to regulate cAMP concentration. Neither choline$^+$ nor sucrose was able to replace $[Na^+]_{out}$. Recent experiments indicate that $[Li^+]_{out}$ can replace $[Na]_{out}$, and the apparent K_m for $[Na^+]_{out}$ and $[Li^+]_{out}$ is 20–40 mM (35). However, $[Na^+]_{out}$ influenced other aspects of adenylate cyclase activity. Basal and PGE_1-stimulated cAMP levels decreased from 230 and 2400 pmol/10^6 cells to 45 and 350, respectively, when $[Na^+]_{out}$ was removed. Choline$^+$ or sucrose could not substitute for $[Na^+]_{out}$ here either. This indicates that, although PGE_1 can still activate adenylate cyclase in the absence of $[Na^+]_{out}$, this cation appears to control the amount of adenylate cyclase that can be activated. Regulation of cyclic nucleotide phosphodiesterase activity in the intact cell by Na^+ does not appear to be a likely explanation of these phenomena because the experiments were performed in the presence of Ro 20-1724, a potent inhibitor of this phosphodiesterase activity. However, because $[Na^+]_{out}$ may control $[Ca^{2+}]_{in}$, as well as other intracellular processes and ion concentrations, additional data will be required to prove that the

Table 3. Specificity of monovalent cation requirement for opiate inhibition of adenylate cyclase

	Adenylate cyclase, pmol/min/mg protein		
Ion*	No AMEA	With 10 μM AMEA	% opiate inhibition
None	32.6	31.4	4
Na^+	28.4	16.2	39
Li^+	28.9	20.5	26
K^+	23.5	18.0	19
Choline$^+$	30.6	26.5	9

All assays were of basal enzyme activity in a nuclei- and supernatant-free NG108-15 membrane preparation. Assays were conducted in the standard Na^+-free assay mixture plus 0.1 mM GTP.

* Added as chloride salt to a final assay concentration of 75 mM listed.

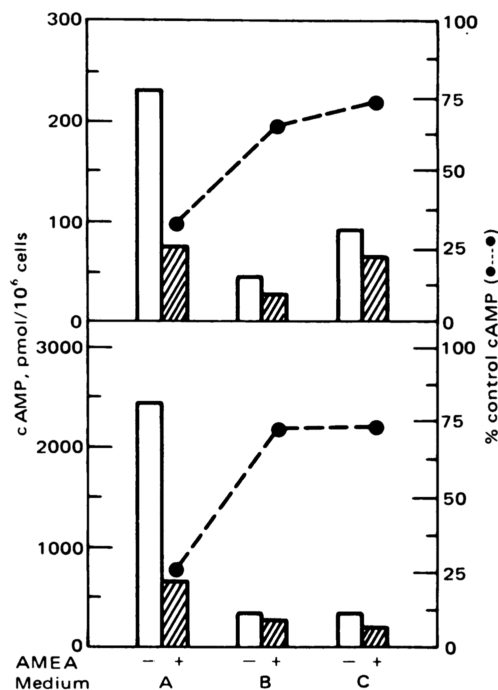

FIG. 2. Influence of extracellular Na^+ on the control of intracellular cAMP concentration in intact NG108-15. NG108-15 washed and concentrated into choline buffer were diluted 1:10 (to 2 × 10^6 cells per ml) into the following media: A, 135 mM NaCl buffer plus 5 mM K^+; B, 135 mM choline·HCl buffer plus 5 mM K^+; or 320 mM sucrose buffer plus 5 mM K^+. When present, AMEA was at 10 μM. (*Upper*) Basal. (*Lower*) With 5 μM PGE_1.

apparent $[Na^+]_{out}$ regulation of adenylate cyclase is direct, not indirect.

As noted in the introduction, guanine nucleotides, acting through a separate membrane component (i.e., the coupler), are necessary for hormonal activation of adenylate cyclase. In many of these systems, nucleotides (by apparently acting through this coupler) also regulate the binding of agonists to these hormone receptors. We have reported (4, 5) that NG108-15 opiate receptor binding is regulated by purine nucleotides, including GTP, ITP, and GDP. Effective coupling of the opiate receptors and adenylate cyclase requires GTP (apparent K_m for GTP, ≈1 μM) and occurs with up to 100 μM GTP without any significant loss of opiate-directed inhibition (Fig. 3). Investigation of the specificity of action of nucleotide triphosphates in this phenomenon revealed that ITP, but not UTP, CTP, or ATP, could replace GTP (Table 4). Our earlier studies on opiate binding to NG108-15 receptors showed that GDP also regulates these opiate receptors (4, 5). The possible effectiveness of GDP as a coupler could not be ascertained because a nucleotide triphosphate-regenerating system is present in the adenylate cyclase assay mixtures in order to maintain constant substrate levels of ATP. Nevertheless, the question of whether GDP can replace GTP for opiate coupling as it does for regulating opiate binding is certainly important.

Although both basal and activated adenylate cyclase were inhibited to a similar partial extent (Table 2; Fig. 4), we wondered whether or not these two enzyme states were equally susceptible to opiate inhibition. Analysis of AMEA inhibition of basal and PGE_1-stimulated enzyme (Fig. 4) showed that less opiate was required to inhibit 50% of basal (apparent K_i ≃20 nM) than 50% of PGE_1-stimulated (apparent K_i = 100 nM) activity. Both inhibitory events, however, appear to be non-

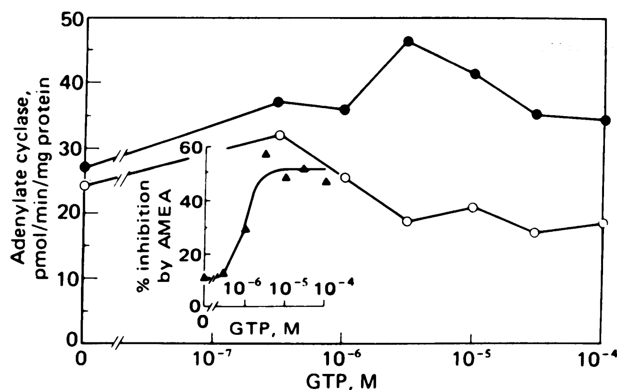

FIG. 3. GTP dependence of AMEA-directed inhibition of basal activity of adenylate cyclase. Assays were performed on the same nuclei- and supernatant-free NG108-15 membranes described in Fig. 1 in the standard Na^+-free assay mixture plus 0.135 M NaCl with (○) or without (●) AMEA (10 μM) at increasing concentrations of GTP. (*Inset*) Plot of the difference in basal enzyme activities with and without AMEA at increasing GTP concentrations, taking the activity observed at each GTP concentration with AMEA to be 100%.

cooperative because their Hill coefficients are ≈1.0. This latter finding is not in agreement with the published data of Sharma *et al.* (2) which showed basal activity to be inhibitable in a positively cooperative manner by a number of opiate agonists. The differences observed might be due to the fact that our assays were done at 32°C on membranes preequilibrated with opiates and in the presence of saturating Na^+ and GTP. The studies by Sharma *et al.* were done at 37°C on total crude homogenates at less than saturating Na^+ with no added GTP and without preequilibration with opiates before initiation of the cyclase assays.

After observing that both Na^+ and guanine nucleotide regulation of opiate binding are apparently related to the coupling of opiate receptors and adenylate cyclase, we decided to investigate the influence of the divalent cations on coupling. We proceeded in this direction because both Mg^{2+} and Mn^{2+} had been shown to reverse Na^+-induced alterations in opiate binding [with both rat brain (36–38) and NG108-15 opiate receptors (4–7)]. In our studies, increasing Mg^{2+} above 12 mM or addition of ≥1.0 mM Mn^{2+} in the presence of 10 mM Mg^{2+} eliminated the ability of opiates to inhibit basal or PGE_1-stimulated adenylate cyclase (data not shown). Increased Mg^{2+} or Mn^{2+} therefore produces "states" of the cyclase which, along with the NaF and guanyl-5′-ylimidodiphosphate, "states" of the enzyme (2), are not significantly susceptible to opiate inhibition. However, it remains to be proven if these effects of

Table 4. Specificity of nucleotide requirement for opiate inhibition of adenylate cyclase

Nucleotide	Adenylate cyclase, pmol/min/mg protein: No AMEA	Adenylate cyclase, pmol/min/mg protein: With 10 μM AMEA	% opiate inhibition
None	27.0	24.0	11
GTP at 100 μM	34.6	18.6	47
ITP at 10 μM	49.4	31.5	37
ITP at 100 μM	52.6	32.1	39
UTP at 100 μM	37.4	34.5	8
CTP at 100 μM	30.1	27.5	9
ATP at 1100 μM	43.6	36.1	7

Assays of the basal enzyme activity in a nuclei- and supernatant-free NG108-15 membrane preparation. Assays were conducted in the standard assay mixture plus 135 mM NaCl. All assays contained 0.2 mM ATP as substrate for the adenylate cyclase.

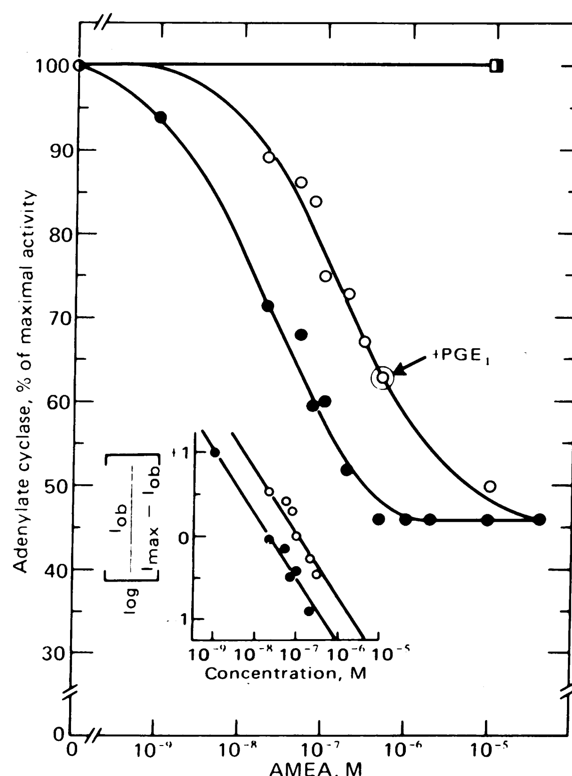

FIG. 4. Inhibition of basal (●) and PGE_1-stimulated (○) adenylate cyclase by AMEA, assayed in a nuclei- and supernatant-free membrane preparation preincubated with or without PGE_1 (5 μM) and varying concentrations of AMEA for 10 min at 32°C in complete reaction mixtures lacking [α-^{32}P]ATP. The assays were subsequently initiated by addition of [α-^{32}P]ATP. Basal (■) and PGE_1-stimulated activities (□) also were assayed with both 100 μM naloxone and AMEA present. (*Inset*) Hill plot of AMEA-directed inhibition. Maximal basal and PGE_1-stimulated activities were 28 and 75 pmol/min per mg protein, respectively.

Mg^{2+} and Mn^{2+} are directly related to their ability to reverse a "Na^+ state" of the opiate receptors.

DISCUSSION

The results presented here conclusively show that Na^+ and GTP are required for opiate inhibition of NG108-15 adenylate cyclase. A guanine nucleotide requirement for receptor-directed inhibition of adenylate cyclase appears to be a general phenomenon. It is also present for muscarinic cholinergic (unpublished data) and α-adrenergic receptor-directed (28) inhibitions of NG108-15 adenylate cyclase and adenosine receptor-directed inhibitions of the fat cell adenylate cyclase (39). In agreement with the idea that all these inhibitions are produced through a similar regulatory process is the observation that opiate, α-adrenergic, and muscarinic inhibitions of NG108-15 adenylate cyclase are not additive (28) and that Na^+ is required for opiate as well as muscarinic and adenosine inhibition of adenylate cyclase (see later in *Discussion*).

The discovery of the need for a guanine nucleotide for receptor-directed inhibition of adenylate cyclase does not come as a surprise, because all known hormonal receptor-mediated activations of adenylate cyclase require the presence of guanine nucleotides. In both cases: (*i*) GTP and ITP allow functional coupling, whereas ATP, UTP, and CTP do not; (*ii*) GTP and ITP decrease the affinity of agonist ligands for their specific receptors; and (*iii*) once fully activated by guanyl-5′-yl imidodiphosphate, the cyclase is no longer "regulatable" by the receptor. However, the apparent K_m for GTP in the activation

process appears to be much lower than it is in the inhibitory process. Work by many investigators (26, 27, 40–44) indicates that the site of action of guanine nucleotides in the activation process is on a separate membrane unit (coupler) that confers not only receptor but also NaF, divalent cation, and cholera toxin sensitivity to the catalytic unit of adenylate cyclase. It appears likely that some type of coupler is also involved in transferring inhibitory information from plasma membrane receptors to the adenylate cyclase.

The processes of transferring inhibitory and excitatory information from receptors to adenylate cyclase are clearly differentiated because Na^+ is apparently only involved in the inhibitory process. Because Na^+ is also required for muscarinic inhibition of NG108-15 (unpublished data) and adenosine inhibition of the fat cell adenylate cyclase (C. Londos, personal communication), we propose that there is another coupler function (or another coupler) within plasma membranes. This entity would be not only the site through which Na^+ regulates receptors but also the site through which Na^+ mediates the receptor-directed inhibitions of the catalytic unit of adenylate cyclase. At present, we cannot distinguish whether Na^+ and guanine nucleotides interact with the same or different couplers.

In conclusion, the fact that such a requirement is exhibited by intact cells indicates that Na^+ regulation is not an artifact of membrane preparations but is a physiologically important phenomenon. Finally, whether or not all of the central nervous system receptors that recently have been shown to exhibit a similar agonist-specific Na^+ (and GTP) sensitivity can initiate a response via a coupling to adenylate cyclase remains to be determined.

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