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VANADATE STIMULATES ADENYLATE CYCLASE VIA THE GUANINE NUCLEOTIDE REGULATORY PROTEIN BY A MECHANISM DIFFERING FROM THAT OF FLUORIDE*

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Abstract—Vanadate stimulates adenylate cyclase activity in turkey erythrocyte membranes. The maximal stimulation is 7-fold over basal at 3 mM vanadate; higher concentrations are inhibitory. A suboptimal concentration of fluoride (1 mM) together with vanadate (3 mM) activates adenylate cyclase in a non-additive manner; cyclase activation by optimal fluoride (10 mM) is inhibited by vanadate (3 mM). There is no stimulation by vanadate of adenylate cyclase activity (measured either with Mg^{2+} or Mn^{2+}) in CYC^- S49 lymphoma cell membranes. Vanadate (3 mM) shows no effect on binding of β-adrenergic agonists or antagonists to the [^{3}H](−)-dihydroalprenolol binding site in turkey erythrocyte membranes. These results suggest that the effect of vanadate on adenylate cyclase is mediated through the nucleotide regulatory protein and may act by a mechanism similar to fluoride. However, in cholera toxin-treated membranes as well as in GDP-β-S plus isoproterenol-treated membranes, fluoride-stimulated adenylate cyclase activity is significantly reduced, but vanadate stimulation is not. Our results suggest that although the actions of vanadate and fluoride in adenylate cyclase may each involve the nucleotide regulatory unit, the exact mechanisms of activation by the two anions differ.

Cantley *et al.* [1–3] have recently shown that vanadate is a potent inhibitor of (Na^+–K^+) ATPase. Compounds of the trace element vanadium are found in small amounts (0.1–1.0 μM) [1] in sera and tissues of vertebrates. Vanadate, like ouabain, another inhibitor of (Na^+–K^+)ATPase [4], shows a concentration-dependent positive inotropic effect on cat papillary muscle [5]. Since the inotropic effects of certain substances including catecholamines are mediated through cyclic AMP [6], we tested for activation of adenylate cyclase by vanadate. Vanadate stimulated adenylate cyclase in heart and fat [7–9], thus raising the possibility that some of the effects of this endogenous compound might be mediated by stimulation of adenylate cyclase rather than inhibition of (Na^+–K^+)ATPase.

In the present studies, we compared the effects of vanadate on adenylate cyclase with those of another anion, fluoride. The studies were performed primarily using turkey erythrocyte membranes, a model system for a β-adrenergic receptor-coupled adenylate cyclase in which the effects of fluoride have been studied in detail [10]. Current evidence [10–15] supports a mechanism for fluoride activation of adenylate cyclase that depends upon interaction of the catalytic unit and guanine nucleotide regulatory (G) unit. In this paper, we show that, like fluoride, vanadate stimulation of adenylate cyclase involves the G unit but the mechanism of vanadate stimulation of the enzyme differs from that of fluoride.

MATERIALS AND METHODS

Membrane preparations. Turkey blood collected with heparin was shipped on ice to us by Pel-Freeze Biologicals, Inc. (Rogers, AR). Nucleated ghosts were prepared by hypotonic lysis [10] and homogenized in a Waring Blender. The membranes were centrifuged, and the upper layer of the pellet was washed, resuspended in 0.01 M Tris–HCl, pH 7.5, and 0.25 M sucrose, and stored in liquid nitrogen until use.

Turkey erythrocyte membranes were prepared using deoxyribonuclease [10] for those experiments in which specific GTPase was measured and were prepared according to Stadel *et al.* [16] for β-receptor binding experiments. CYC^- S49 lymphoma cell membranes were prepared as previously described [12].

Enzyme assays. Adenylate cyclase activity was determined as previously described [13] using 5 mM $MgCl_2$ and 0.125 mM ATP as substrate. Results are expressed as cAMP generated in pmole/mg of protein/min and are the means of triplicate determinations.

GTPase assay was performed according to Cassel and Selinger [17]. The reaction mixture (final volume 0.1 ml) contained 0.25 μM [γ-^{32}P]GTP (2–20 mCi/mole), 6 mM $MgCl_2$, 0.5 mM App(NH)p, 0.1 mM ATP, 3 mM creatine phosphate, 3 units of creatine phosphokinase, 2 mM β-mercaptoethanol, 0.1 mM EGTA, and 50 mM imidazole–HCl buffer, pH 6.7 The reaction was initiated by the addition of the

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membranes (20–40 μg protein) and terminated after 10 min at 37° by adding 0.5 ml of 5% charcoal (Norit A) suspension in an ice cold sodium phosphate buffer (20 mM, pH 7.0). After 5 min in the cold, the suspension was centrifuged (1,000 *g* for 5 min) and radioactivity determined in aliquots of supernatant. Basal 'specific' GTPase and catecholamine-stimulated GTPase activities were determined as described by Cassel and Selinger [17]. Assays were performed in triplicate.

Binding studies. β-Receptor binding was measured with [^{3}H](−)-dihydroalprenolol ([^{3}H]DHA) according to Stadel *et al.* [16]).

Cholera toxin treatment. Cholera toxin treatment of turkey erythrocyte membranes was performed according to Kaslow *et al.* [18]. Membranes (1.5–5.0 mg/ml) were incubated with 12 mM potassium phosphate, pH 7.5, 20 mM thymidine, 5 mM ADP-ribose, 20 mM arginine–HCl, 100 U/ml Trasylol, 0.1 mM GTP, 100 μg/ml cholera toxin (activated with 20 mM DTT at 30° for 10 min) and 1 mM NAD^+. Control membranes were treated in an identical manner except that cholera toxin was omitted. The reaction was initiated by addition of membranes, and continued for 30 min at 30°. The reaction was terminated by addition of 10 vol. of ice cold 15 mM potassium phosphate, pH 7.5, and washed three times by centrifugation. The final pellet was resuspended in 10 mM Tris–HCl, pH 7.5, buffer containing 0.25 M sucrose and assayed as indicated at 30° for 20 min.

Incubation of turkey erythrocyte membranes with (−)isoproterenol and GDP-β-S. Membranes were thawed and incubated with (−)isoproterenol (50 μM) and GDP-β-S (0.1 mM) at 37° for 10 min in 10 mM Tris–HCl, pH 7.5, buffer containing 0.25 M sucrose, 1 mM $MgCl_2$ and 1 mM dithiothreitol. The membrane protein concentration during incubation was usually 2–3 mg/ml. After incubation, the membranes were centrifuged and resuspended three times in 10 vol. of 10 mM Tris–HCl, pH 7.5, buffer containing 1 mM $MgCl_2$, 1 mM dithiothreitol and 0.25 M sucrose and then resuspended to their original volume prior to enzyme assay. Control membranes were treated similarly except that (−)isoproterenol and GDP-β-S were omitted. Adenylate cyclase assay was performed at 30° for 15 min.

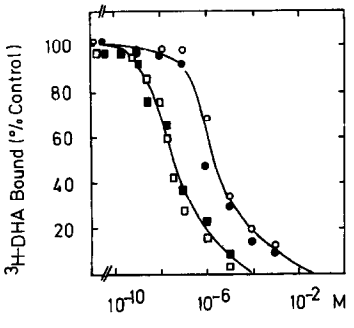

Fig. 1. Effect of vanadate (3 mM) on (−)alprenolol (■——■) and L-isoproterenol (●——●) binding to [^{3}H]DHA binding sites on turkey erythrocyte membranes. Open symbols: control experiments performed without vanadate. Results are means of triplicate determinations.

Protein was measured according to Lowry *et al.* [19].

Materials. Radiolabeled nucleotides were obtained from ICN Chemical and Radioisotope Division (Cleveland, OH). GDP-β-S was from Boehringer (Mannheim, West Germany) and purified as before [10]. Cholera toxin was from Schwarz–Mann (Orangeburg, NY) and L-isoproterenol from Sigma Chemical Co. (London, U.K.). Other chemicals were of the best grade commercially available. Vanadate was used as $Na_3VO_4 \times 14H_2O$ from BDH (Poole, U.K.) and assayed out of a stock solution of 60 mM vanadate, which showed stability in stimulating adenylate cyclase activity over a period of 3 months. Even though vanadium may exist in several oxidation states in solution including orthovanadate VO_4^{3-}, metavanadate VO_3^- and vanadyl VO^{2+}, the term 'vanadate' is used here to describe the activator of the adenylate cyclase, although this may not be the form of the active component.

RESULTS

Vanadate (3 mM) shows no effect on binding of a β-adrenergic antagonist, alprenolol, or agonist, isoproterenol, to specific [^{3}H]DHA binding sites on turkey erythrocyte membranes (Fig. 1). Vanadate stimulates adenylate cyclase in turkey erythrocyte membranes (Fig. 2). A concentration as low as 0.01 mM causes a small but significant increase over basal activity. Stimulation is maximal (7-fold over basal) at 3 mM, with higher concentrations showing significantly less stimulation. The maximal stimulation by fluoride is about 4-fold that of vanadate. Stimulation of adenylate cyclase by a submaximal

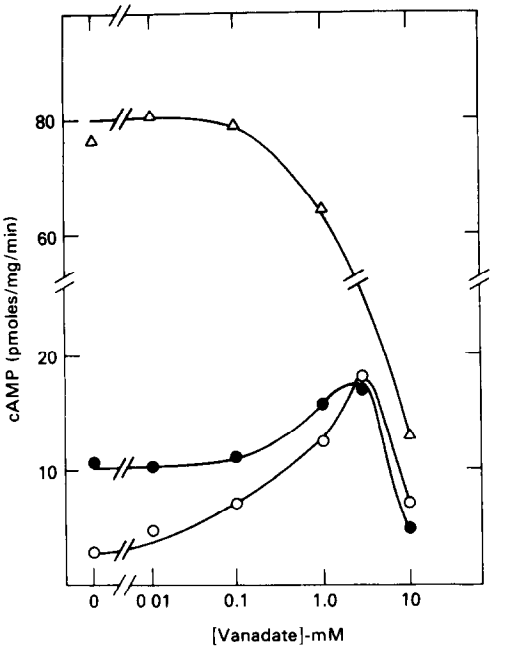

Fig. 2. Effect of increasing concentrations of vanadate on basal (○), 1 mM fluoride-stimulated (●), and 10 mM fluoride-stimulated (△) adenylate cyclase activity in turkey erythrocyte membranes.

concentration of fluoride (1 mM) plus increasing concentrations of vanadate is less than additive. Stimulation by an optimal concentration of fluoride (10 mM) is decreased by 0.1 mM vanadate, with greater inhibition at higher vanadate concentrations (Fig. 2). The magnesium dependency of fluoride and vanadate stimulation are compared in Fig. 3. Maximal stimulation by an optimal concentration of fluoride (10 mM) occurs at 5 mM Mg^{2+}; for vanadate (3 mM) optimal activation of the enzyme occurs at 10 mM Mg^{2+}. Higher concentrations of Mg^{2+} significantly decrease activation by fluoride; the vanadate effect is decreased minimally even at 50 mM Mg^{2+}.

β-Adrenergic catecholamines stimulate GTPase activity in turkey erythrocyte membranes [17]. Fluoride was shown [20] to inhibit both the basal- and the catecholamine-stimulated GTPase activity. Vanadate (3 mM) likewise is capable of inhibiting basal- and isoproterenol-stimulated GTPase activity (Table 1).

Membranes of CYC^- S49 lymphoma cells contain the catalytic unit and the β-adrenergic receptor but no G unit [21]. Minimal adenylate cyclase activity can be measured in CYC^- membranes with 10 mM Mg^{2+} and there is no significant activation with GTP (0.1 mM), fluoride (10 mM) or vanadate (3 mM) (Fig. 4). With 10 mM Mn^{2+}, significant enzyme activity is detected. Addition of GTP (0.1 mM) or fluoride (10 mM) with Mn^{2+} does not activate further and vanadate inhibits activity with Mn^{2+}.

Cholera toxin catalyses the covalent modification (ADP-ribosylation) of the G unit and consequently alters adenylate cyclase activity [22]; inhibition of fluoride-stimulated activity is a prominent feature of cholera toxin-modified enzyme activity [22]. As expected, fluoride stimulation is significantly decreased in toxin-treated membranes (Fig. 5). Vanadate-induced stimulation, by contrast, is increased in cholera toxin-treated turkey erythrocyte membranes compared to untreated membranes (Fig. 5). Slight enhancement of vanadate-stimulated activity in cholera toxin-treated membranes compared with untreated membranes was also observed in cardiac and lung membranes (unpublished observations).

Incubation of turkey erythrocyte membranes with isoproterenol and GDP-β-S also causes a significant loss of fluoride-stimulated activity [10, 14]. Vanadate-stimulated adenylate cyclase activity is not reduced in isoproterenol plus GDP-β-S-treated membranes (Fig. 6). Addition of isoproterenol plus GTP during the assay restores fluoride-stimulated activity to isoproterenol plus GDP-β-S-treated membranes [10, 14] but has no effect on vanadate stimulation (Fig. 6).

Table 1. Effect of vanadate on high-affinity, specific GTPase activity in turkey erythrocyte membranes

Additions	Experiment 1	Experiment 2
None	7.4	5.9
0.1 mM isoproterenol	11.7	13.2
0.1 mM isoproterenol + 0.1 mM propranolol	8.5	8.1
10 mM F^-	4.0	4.2
3 mM vanadate	3.7	4.6
3 mM vanadate + 0.1 mM isoproterenol	5.9	7.5

Values in pmole P_i/mg/min are the means of triplicate determinations.

GTPase assay was performed as described in Materials and Methods.

Results from two different experiments are shown.

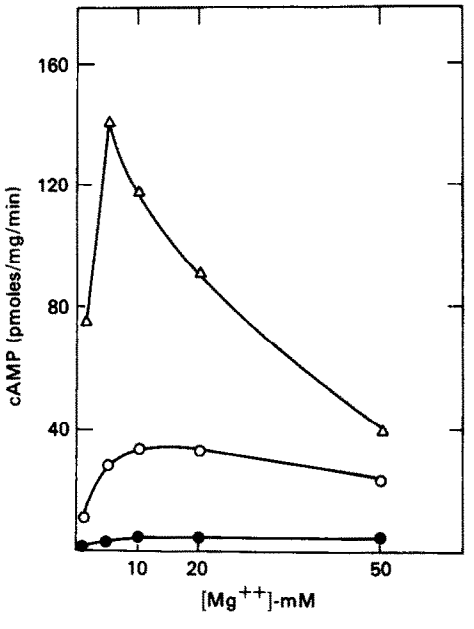

Fig. 3. Effect of increasing magnesium concentration (from 1 to 50 mM) in the assay mix on basal (●), 3 mM vanadate-stimulated (○), and 10 mM fluoride-stimulated (△) adenylate cyclase activity in turkey erythrocyte membranes.

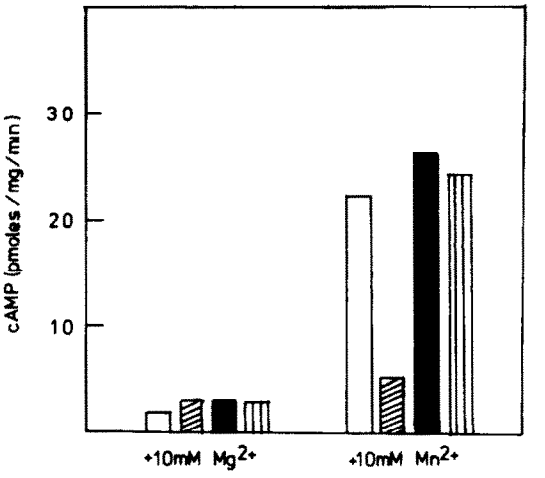

Fig. 4. Effect of vanadate on CYC^- S49 lymphoma cell membrane adenylate cyclase activity. Enzyme activity was assayed with either 10 mM Mg^{2+} or 10 mM Mn^{2+} in the reaction mixture with no further addition (□), 3 mM vanadate (▨), 0.1 mM GTP (■) or 10 mM fluoride (▥).

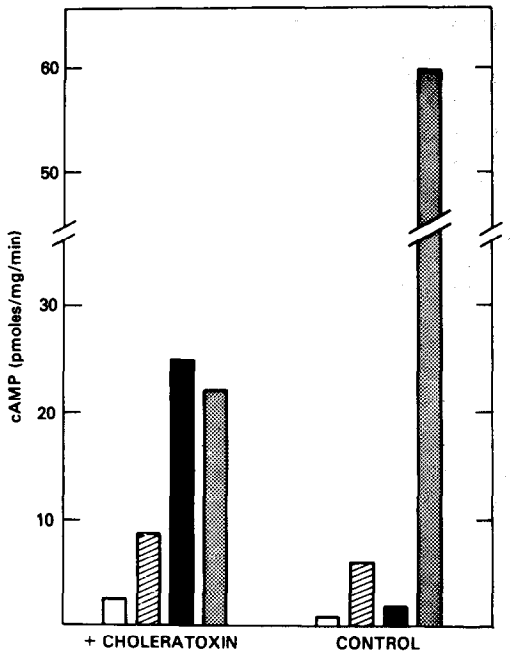

Fig. 5. Effect of cholera toxin treatment on basal (□), 3 mM vanadate-stimulated (▨), isoproterenol (0.1 mM) + GTP- (0.1 mM) stimulated (■), and 10 mM fluoride-stimulated (▩) adenylate cyclase activity. Turkey erythrocyte membranes were treated with cholera toxin, as described in Materials and Methods, before enzyme assay.

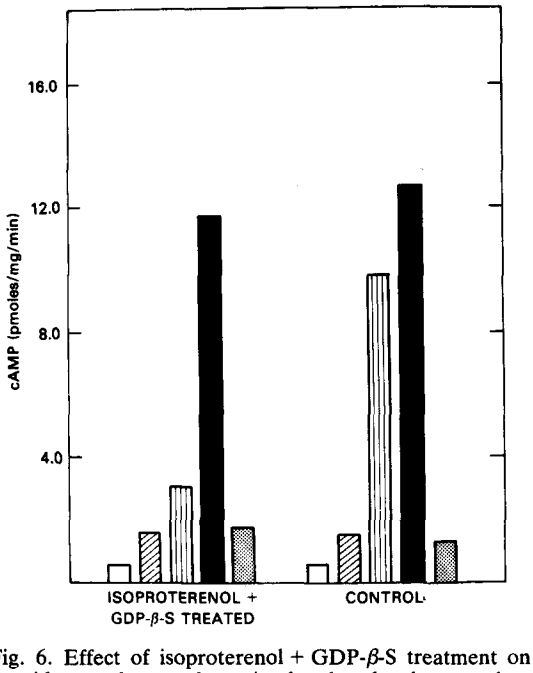

Fig. 6. Effect of isoproterenol + GDP-β-S treatment on fluoride- and vanadate-stimulated adenylate cyclase activity. Turkey erythrocyte membranes were treated with isoproterenol + GDP-β-S, as described in Materials and Methods, and adenylate cyclase was then assayed with no addition (□), 3 mM vanadate (▨), 10 mM fluoride (▥), fluoride + isoproterenol + GTP (■), and vanadate + isoproterenol + GTP (▩).

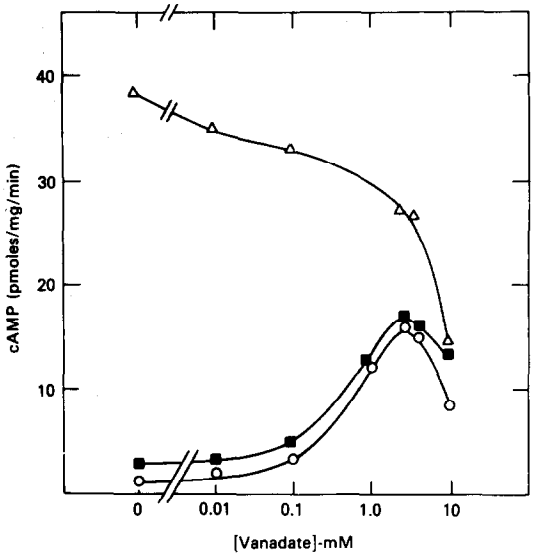

Fig. 7. Effect of increasing concentrations of vanadate on cholera toxin-treated turkey erythrocyte membrane adenylate cyclase activity. Membranes were treated with cholera toxin, as described in Materials and Methods, and assayed with increasing concentrations of vanadate and no further addition (○), GTP (0.1 mM) (■), and isoproterenol (0.1 mM) + GTP (0.1 mM) (△).

In cholera toxin-treated turkey erythrocyte membranes, isoproterenol plus GTP stimulation of adenylate cyclase is substantially enhanced [22]. The effect of vanadate, unlike that of β-adrenergic agonists in cholera toxin-treated membranes, is not enhanced by addition of GTP. Vanadate in fact causes a concentration-dependent decline in isoproterenol plus GTP-stimulated activity (Fig. 7).

DISCUSSION

The mechanism whereby vanadate acts on different enzymes is under active investigation [23]. The inhibitory effect of vanadate on $(Na^+–K^+)$ATPase has been well characterized. Cantley *et al.* have suggested that vanadate acts as a phosphate transition analog [3]. Contrary to the inhibitory effect on $(Na^+–K^+)$ATPase, vanadate stimulates adenylate cyclase in rat fat cells [9] and in cardiac tissue [8, 24]. In this study, we show that vanadate also stimulates turkey erythrocyte adenylate cyclase. Maximal stimulation occurred at 3 mM vanadate; higher concentrations caused significantly less stimulation. In cardiac tissue, a decline in stimulation at higher vanadate concentrations was not observed [8]. The reason for this difference is unclear but it is presumably related to differences in the adenylate cyclase complex or associated membrane components in turkey erythrocyte vs other tissues.

In turkey erythrocyte membrane (and in other tissues [7, 9]), vanadate stimulates adenylate cyclase at lower concentrations than fluoride. This observation and the occurrence of vanadate at micromolar concentrations [1] in cells raise the possibility that vanadate is a physiologic modulator of adenylate cyclase activity. Such a possibility must take into

account the relative effectiveness of various oxidation states of vanadate in stimulating adenylate cyclase. Cantley and Aisen [2] have shown that most vanadate taken up into human red cells is converted to the +4 oxidation state and that this state is less effective than the +5 oxidation state in inhibiting $(Na^+–K^+)$ATPase. The relative effectiveness of various oxidation states of vanadate in stimulating adenylate cyclase has not yet been defined.

The mechanism of vanadate stimulation of adenylate cyclase has not been characterized. The stimulatory effect of vanadate on the adenylate cyclase complex may, in theory, be mediated by any of the three components of a receptor-coupled adenylate cyclase. Vanadate may: (1) interact with hormone receptors coupled to the adenylate cyclase system: by changing receptor-characteristics, vanadate could alter enzyme activity; (2) directly bind at the catalytic unit and thereby stimulate the enzyme activity; and (c) activate the adenylate cyclase via the guanine nucleotide regulatory protein (G unit). Also possible is an interaction with other, as yet uncharacterized, components of the adenylate cyclase complex.

Our data are not compatible with an action of vanadate involving the hormone receptor. Vanadate, like fluoride, stimulates adenylate cyclase in tissues with different receptors and also in detergent-solubilized membrane extracts (unpublished observations) in which receptors are functionally uncoupled. Also, vanadate did not enhance GTP-stimulated activity in cholera toxin-treated turkey erythrocyte membrane in a manner analogous to isoproterenol. Vanadate, moreover, had no effect on agonist or antogonist binding to turkey erythrocyte β-adrenergic receptors as measured directly with [^{3}H]DHA.

The hypothesis of a direct stimulatory effect by vanadate on the catalytic unit was tested in CYC^- S49 lymphoma cell membranes. Neither GTP, fluoride, nor vanadate shows any stimulation with Mg^{2+} in the assay. Significant activity is detected with Mn^{2+} in the assay but this is not increased by vanadate. These results suggest that vanadate does not stimulate adenylate cyclase directly via the catalytic unit. Vanadate, in fact, inhibited Mn^{2+}-dependent activity in CYC^- membranes. The decline in turkey erythrocyte adenylate cyclase activity at high vanadate concentration may reflect a similar inhibitory effect. The basis for this inhibition is unknown. It may involve interaction with the substrate (ATP) binding site of the catalytic unit or some less specific effect of vanadate. Further studies are necessary to define the mechanism of inhibition at high vanadate concentrations.

The third possibility for vanadate stimulation is an action at the guanine nucleotide regulatory protein. A comparison of the effects of vanadate and fluoride in turkey erythrocyte membranes initially suggested that both activate adenylate cyclase by a similar mechanism involving the G unit. Thus, both agents are ineffective in CYC^- membranes which lack the G unit. Both fluoride [20] and vanadate inhibit high affinity-specific GTPase activity in turkey erythrocyte membranes. The inhibitory effect is, however, not limited to the isoproterenol-stimulated GTPase but is also observed for 'basal' specific GTPase which Cassel and Selinger [20] concluded was unrelated to adenylate cyclase activity. Thus, as pointed out by Cassel and Selinger, the relationship, if any, between the inhibitory effect of fluoride (or vanadate) on GTPase and stimulation of adenylate cyclase is not clear.

Further studies have shown that fluoride and vanadate do not act in an identical manner, since modification of the G unit by two separate types of agents results in different consequences for fluoride and vanadate stimulation. Cholera toxin-catalyzed ADP-ribosylation of the G unit profoundly inhibits fluoride action but slightly enhances vanadate stimulation; similarly, hormone dependent introduction of the analog GDP-β-S onto the G unit inhibits fluoride activation without affecting vanadate stimulation. Our data are compatible with a model in which both fluoride and vanadate act by promoting association of an otherwise inactive G unit with the catalytic unit to cause increased activity, but in which the two anions differ in effecting this association with certain states (e.g. cholera toxin modification) of the G unit. These qualitative differences in vanadate- and fluoride-stimulated activities after cholera toxin treatment and incubation with isoproterenol plus GDP-β-S are also compatible with differences in the interaction of these anions with other, as yet uncharacterized, components of the adenylate cyclase system. Further studies with resolved components of the enzyme will be necessary to define the mechanism of vanadate stimulation.

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