Biochemical Pharmacology, Vol. 45, No. 6, pp. 1311–1316, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

INTERACTION OF VANADATE AND IODATE OXYANIONS WITH ADENYLYL CYCLASE OF CILIARY PROCESSES

THOMAS W. MITTAG,*†‡ WEN-BIN GUO*§ and TORU TANIGUCHI*‖
Departments of *Ophthalmology and †Pharmacology, Mount Sinai School of Medicine, CUNY, New York, NY 10029, U.S.A.

(*Received* 11 *June* 1992; *accepted* 17 *November* 1992)

Abstract—Vanadate (VO_3^-) was found to activate adenylyl cyclase (AC) in ocular ciliary process membrane. This response was additive to that of isoproterenol (ISO) and vasoactive intestinal peptide (VIP), but it was potentiative with forskolin (FSK) and also with Ca^{2+}/calmodulin activation of AC activity. The potentiated response of FSK in the presence of VO_3^- was due to an increase in V_{max} without a change in the apparent affinity of FSK or VO_3^-, and therefore differs from the potentiated response of activated G-protein (G_s) and FSK, where the affinity of FSK was increased by 1–2 orders of magnitude. Potentiation occurred at low Mg^{2+} and was not observed at free $[Mg^{2+}] > 3$ mM. Iodate (IO_3^-) inhibited the FSK, ISO, and VO_3^- activations of AC in ciliary process membranes (IC_{50}, 0.3 mM). *In vivo* topical treatment of the rabbit eye with 50 μL of 5% $NaIO_3$ had no effect alone but completely blocked the intraocular pressure response to a 50-μL topical dose of 1% FSK and partially blocked the response to a 50-μL dose of 0.001% ISO. These findings indicate that some AC enzymes may have a binding site for oxyanions which can directly regulate enzyme activity.

The complex biological response to vanadate has been reviewed frequently [1–3] but is poorly understood in terms of physiological or biochemical mechanisms. Cantley *et al.* [4] were the first to demonstrate that vanadate is a potent inhibitor of renal (Na^+,K^+)ATPase and they proposed that this was the basis for its biological effects. These effects include diuresis and natriuresis, a positive inotropic effect on ventricular cardiac muscle, inhibition of active Na^+ and Cl^- transport in the cornea and of water transport in toad bladder, and blockade of Rb^+ uptake by red cells. The ocular effects of vanadate can also be explained by postulating (Na^+,K^+)ATPase inhibition, since intraocular pressure is lowered [5] by a reduction in the rate of aqueous humor secretion [6]. All of these effects are thought to be due to the orthovanadate anion ($H_2VO_4^-$), an analog of the phosphate anion, interacting with the ATPase. However, some additional effects of oxovanadium compounds on tissues such as the insulin-mimetic effect [7] and enhancement of the action of epidermal growth factor [8] suggest that vanadate may also interact with tyrosine kinases or phosphatases.

Vanadate, probably in the form of VO_3^-, is also able to bind to anion transporters, such as the "band 3" HCO_3^-/Cl^- exchanger in erythrocytes [9], and in the corneal epithelium [10]. Vanadate can be reduced *in vivo* to the vanadyl cation by several ubiquitous physiological reductants including ascorbate, glutathione and NADPH. Thus, the vanadyl form may be present inside cells and contribute to biological effects of administered vanadate by competing with other divalent cations such as Ca^{2+} or Mg^{2+}. In fact, vanadyl is thought to be a major intracellular form of vanadium in some cells [11]. Because of the multiple forms of vanadate there is considerable uncertainty as to the mechanism(s) underlying many of the biologic responses to this agent.

The present experiments were initiated as a result of our previous investigation to determine whether the activation of adenylyl cyclase (AC¶) by vanadate played a role in the intraocular pressure (IOP) response [12]. In our earlier study we concluded that the IOP lowering effect of administering topical vanadate to the eye was probably not due to activation of AC because the tissue content at the time of the IOP effect seemed far too low in comparison to that required for *in vitro* activation of the AC enzyme. In view of the complexity of the biological actions of vanadate (briefly reviewed above), a variety of alternative mechanisms can be hypothesized for the reduced secretion of aqueous humor, which is primarily responsible for the lowered IOP response to vanadate [5, 6]. However, the interaction of vanadate with AC is also of biochemical interest and these earlier experiments were not fully in accord with published biochemical studies of vanadate interaction with AC.

Vanadate activation of AC occurs in the membranes of a variety of tissues and this general response has been interpreted as being mediated by

‡ Corresponding author: Dr. Thomas Mittag, Department of Ophthalmology, Box 1183, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029–6574. Tel. (212) 241-7229; FAX (212) 289-5945.

§ W. Guo is a Visiting Associate from the Department of Biochemistry, Sun Yat Sen University of Medical Science, Guang-Zhou, Peoples Republic of China.

‖ T. Taniguchi is a Visiting Associate from the Department of Ophthalmology, Gifu University School of Medicine, Gifu, Japan.

¶ Abbreviations: AC, adenylyl cyclase; IOP, intraocular pressure; FSK, forskolin; VIP, vasoactive intestinal peptide; G_s, stimulatory guanyl nucleotide binding protein; and ISO, isoproterenol.

the activator G-protein (G_s) [13, 14]. Although orthovanadate might behave similarly to fluoroaluminate, both of which have tetrahedral geometry and can mimic phosphate, the activation of G_s by vanadate showed differences from fluoride [14]. In the present study we examined the interaction of vanadate (VO_3^-) and the structurally related iodate anion (IO_3^-) in more detail on AC enzymes in membranes of ciliary processes—the tissue responsible for secretion of aqueous humor and maintenance of the intraocular pressure. We concluded that these agents do not affect AC indirectly by interacting with G-proteins, but instead probably have a direct action on the enzyme.

MATERIALS AND METHODS

Chemicals and reagents. Synthetic porcine VIP was purchased from Cambridge Research Biochemicals (Atlantic Beach, NY) or from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]cAMP was obtained from the New England Nuclear Corp. (Boston, MA) and [^{32}P]ATP from Amersham (Arlington Heights, IL). Forskolin (FSK), from Calbiochem (La Jolla, CA), was made up as a 10 mM stock in dimethyl sulfoxide. All other reagents, biochemicals, and drugs were analytical grade or the best grade available and were obtained from Sigma or from Fisher Scientific (Pittsburgh, PA). The assay kit for protein determination by dye-binding (Bradford) was purchased from Bio-Rad (Richmond, CA).

Membrane preparation. Ciliary processes dissected from albino rabbit eyes within 1 hr of death (sodium pentobarbital overdose) or from slaughterhouse bovine eyes kept on ice less than 6 hr, were frozen quickly at −80° and stored for up to 2 months before use. For a typical concentration–response experiment, thawed processes from six rabbit or three bovine eyes were hand-homogenized in 3 mL of buffer (0.3 mM sucrose, 20 mM Tris, pH 7.4, or 20 mM HEPES, pH 7.8, 5 mM EDTA, 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), 5 mM dithiothreitol (DTT), 10 μg/mL leupeptin, 100 μM indomethacin) in a Dounce homogenizer using 25 strokes of the loose-fitting pestle. Centrifuged membranes (15 min, 27,000 *g*) were washed once in 6 mL of the homogenizing buffer and resuspended in the same buffer (3 mL) containing 0.1 mM indomethacin, 2.5–10 μg/mL leupeptin, at a concentration of 1–2 mg protein/mL.

Adenylyl cyclase assay. Enzyme activity was determined in glass test tubes in a total volume of 250 μL containing 60 mM sucrose, 80 mM Tris, pH 7.4 (rabbit tissue), or 20 mM HEPES, pH 7.8 (bovine tissue), 2–9 mM $MgCl_2$ as indicated, 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 5 mM creatinine phosphate, 125 μg creatinine phosphokinase, 20 μM GTP, 1 mM cAMP, 4 mM theophylline, 20 μM indomethacin, 0.5 to 2 μg/mL leupeptin, and 0.2 mM ATP. Aliquots (50 μL) of the membrane suspension (30–70 μg protein) were added to preincubated (3 min) triplicate tubes containing drugs (as indicated), all other incubation ingredients, and [α-^{32}P]ATP (1–2 × 10^6 cpm). The tubes were incubated at 30° and after 3 or 5 min the assay was terminated with stopping solution [15] (zinc acetate followed by Na_2CO_3). The [^{32}P]cAMP with added [^{3}H]cAMP tracer (1–3 × 10^4 cpm) was isolated by the double column method (Dowex 50, alumina) of Salomon [15]. When FSK was used, the control assay tubes contained the equivalent amount of dimethyl sulfoxide (<1%) as was used to dissolve the FSK.

Data analysis. Adenylyl cyclase specific activities were calculated from quadruplicate or triplicate assays and are based on membrane protein solubilized with NaOH determined by the Bradford dye-binding method [16] with bovine serum albumin as the reference protein. Error bars representing SEMs (omitted from some concentration–response curves) were all less than 5% of the measurement.

In vivo *experiments.* For topical ocular drug treatments, two or three groups each of two or three pigmented Dutch Belt rabbits were treated with 50-μL doses of freshly prepared isoproterenol (ISO) or FSK in vehicle V_1 and vehicle V_2, respectively. The composition of V_1 was 0.5% hydroxypropylmethyl cellulose and V_2, 0.45% hydroxypropylmethyl cellulose + 10% dimethyl sulfoxide in H_2O. Effective 50-μL doses for ISO and FSK were established in preliminary experiments at 0.001% ISO (0.5 μg/eye) and 1% FSK (0.5 mg/eye). A two-dose treatment schedule was followed for all eyes. For the experimental eyes at time minus 0.5 hr the baseline IOPs were determined with a Digilab manometrically calibrated pneumatonograph (model 30 R), and immediately thereafter either V_1 or 5% $NaIO_3$ in V_1 was administered. IOP was again measured at time zero, and immediately thereafter the second drug was administered. Contralateral (control) eyes were treated similarly with V_1 and V_2 vehicles, and IOP was thereafter measured in both eyes at intervals over an 8-hr period. Thus, groups of experimental eyes (N = 6 or 9) received one of the following six treatments: ISO followed by V_2; V_1 followed by FSK; $NaIO_3$ followed by V_1, or by V_2, or by ISO, or by FSK, while the contralateral control eyes all received V_1 followed either by V_1 or V_2 as appropriate. For each time point the mean difference in IOP between groups of eyes (experimental minus control) is plotted as Δ IOP (mm Hg) in Figs. 7 and 8. At each time point the Δ IOP values were evaluated statistically as to whether Δ IO_3 + FSK was significantly less than Δ FSK (Fig. 7), and whether Δ IO_3 + ISO was significantly less than Δ ISO (Fig. 8).

RESULTS

In albino rabbit ciliary process particulate fraction, vanadate (1 mM $NaVO_3$) activated the basal AC activity. Net responses to ISO or to vasoactive intestinal peptide (VIP) were additive to that of vanadate (Fig. 1). However, when combined with FSK the net response to FSK was increased markedly (Fig. 1). This phenomenon has been reported by other workers and has been ascribed to activation of G_s [14]. We previously determined the effect of activated G_s (by using ISO, VIP or fluoroaluminate) on FSK-activated adenylyl cyclase in rabbit ciliary processes under the same assay conditions as used here [17, 18]. We found that the V_{max} for FSK stimulation was approximately doubled in the

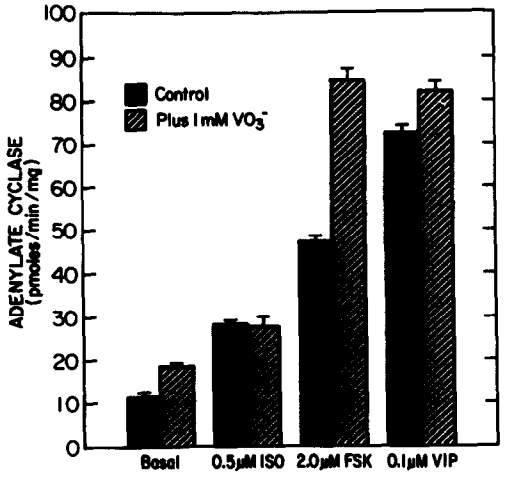

Fig. 1. Effect of $NaVO_3$ on net adenylyl cyclase responses to ISO, FSK and VIP in rabbit ciliary process membrane fraction (▨) compared to responses in the absence of VO_3^- (control, ■). Values are means ± SEM (N = 3).

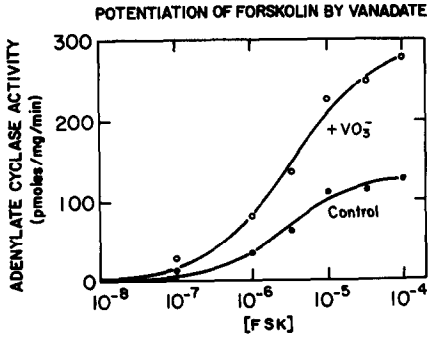

Fig. 2. Representative concentration–response for FSK activation of rabbit ciliary process adenylyl cyclase in the presence of absence of 3.3 mM $NaVO_3$ in pH 7.4 Tris buffer with 2 mM Mg^{2+}. The V_{max} was increased (+120%) but the EC_{50} for FSK was not significantly different (3.5 and 2.9 μM, respectively). Points are mean values for N = 3, and SEM for each point was less than 5% of the measurement. Both curves were generated from data from a single experiment.

presence of maximally activated G_s, and the apparent affinity of FSK was increased by about 80-fold [19]. In the present study the concentration–response to FSK in the presence of 3.3 mM vanadate (Fig. 2) also showed a doubling of the V_{max}, but the EC_{50} for FSK was not altered significantly when vanadate was present. These data in comparison with results previously obtained with fluoroaluminate [19] do not support a G_s-activation mechanism for the stimulation of AC by vanadate.

We also tested vanadate on another regulator of AC in ciliary processes of the rabbit eye, namely calcium/calmodulin-stimulated activity [20]. This activity was also potentiated significantly by vanadate

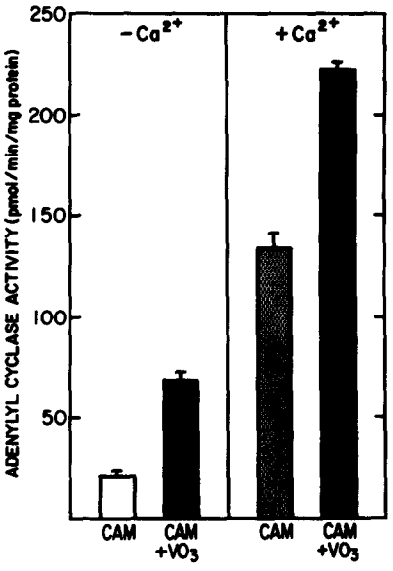

Fig. 3. Responses of rabbit ciliary process adenylyl cyclase to calmodulin (CAM) and to calmodulin + $NaVO_3$ (2 μM + 3.3 mM) with 2 mM Mg^{2+} in the presence or absence of added Ca^{2+} (1 mM). The net AC response to Ca^{2+}/calmodulin was increased 37% ($P < 0.01$) in the presence of VO_3 anion relative to the sum of their separate activities. Values are means ± SEM (N = 4).

as shown in Fig. 3, but not as much as the forskolin AC response. However, we do not know whether the calcium/calmodulin AC in this tissue represents a separate fraction of the AC enzymes activated by FSK, or whether it is the type of enzyme controlled by both calmodulin and G_s [21]. The above effects of vanadate were not prevented, but actually increased somewhat, by omitting GTP from the assay medium and substituting 0.1 mM GDPβS, which blocks activation of G_s. In three separate experiments, GDPβS decreased the basal value to 49.0 ± 2.9% and the ISO (10 μM) response to 50.6 ± 5.6%, but increased the vanadate (1 mM) response to 128.8 ± 3.6%, relative to control AC activities with only GTP present determined in the same experiment.

The role of Mg^{2+} in the potentiated effect of vanadate with FSK was examined over the range of 2–9 mM added $MgCl_2$ (approximate free Mg^{2+} from 0.8 to 6.8 mM) in bovine ciliary process membranes at pH 7.8 (HEPES buffer). Per cent potentiation was calculated from the ratio:

$$\frac{(VO_3^- + \text{FSK})\text{ response} \times 100}{VO_3^-\text{ response} + \text{FSK response} - \text{basal response}}$$

Figure 4 shows that potentiation occurred only when free [Mg^{2+}] was <3 mM.

These findings indicated that vanadate may interact directly with AC and that its activating effect was independent of GTP and thus different from G_s activation. We therefore tested the related anions iodate (IO_3^-) and nitrate (NO_3^-). Nitrate was without

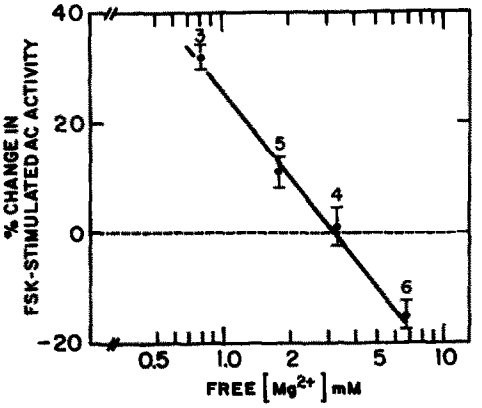

Fig. 4. Dependence of vanadate potentiation as per cent of forskolin-stimulated adenylyl cyclase on the free Mg^{2+} concentration. Experiments were conducted with 3.3 mM $NaVO_3$ at pH 7.8, 60 μM forskolin, and various concentrations of added $MgCl_2$ (2–9 mM). Free Mg^{2+} was estimated by subtracting the concentration of chelators (EDTA + EGTA) in the assay. The absolute response of AC to 60 μM FSK alone ranged from 290 to 410 pmol/min/mg protein in different experiments. Numbers above each point refer to the number of separate determinations and error bars are 2 × SEM of the measurement.

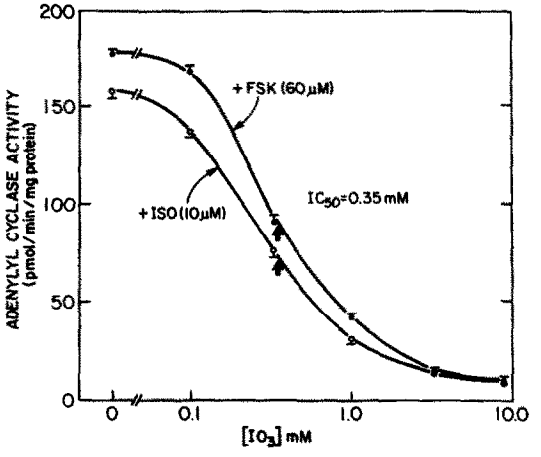

Fig. 6. Inhibition curve for IO_3 anion against ISO- and FSK-stimulated adenylyl cyclase (with 9 mM Mg^{2+} present) in bovine ciliary process particulate fraction (IC_{50}, 0.35 mM). Values are means ± SEM (N = 4).

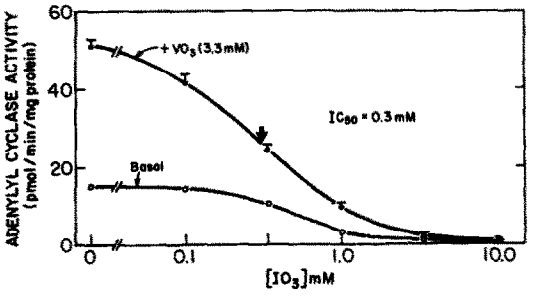

Fig. 5. Inhibition curve for IO_3 anion against basal AC activity and against VO_3 anion (3.3 mM) stimulated AC activity (with 9 mM Mg^{2+} present) in bovine ciliary process particulate fraction (IC_{50} for IO_3^-, 0.3 mM). Values are means ± SEM (N = 4).

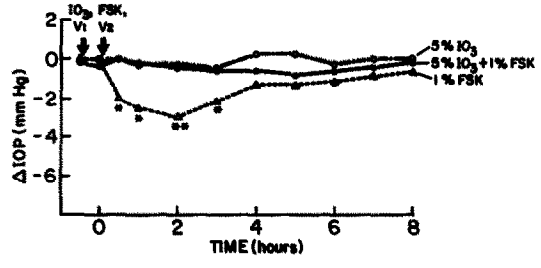

Fig. 7. Effect of topical 5% $NaIO_3$ in vehicle V_1 (50-μL dose) and/or 1% FSK in vehicle V_2 (50-μL dose) on intraocular pressure (IOP) in the rabbit eye when given separately or in combination. The IOP measurements are given as Δ IOP in mm Hg, which represents the difference between the IOP of vehicle-treated contralateral eyes (V_1, V_2) and the experimental eyes measured at the same time. (* = $P < 0.05$, ** = $P < 0.01$, unpaired t-test.)

effect up to 10 mM but iodate inhibited both basal AC activity and the stimulation of AC by 3.3 mM $NaVO_3$ with an IC_{50} of about 0.3 mM (Fig. 5). Iodate was similarly tested against FSK-activated adenylyl cyclase and ISO-activated adenylyl cyclase. Both were inhibited with an IC_{50} of 0.35 mM (Fig. 6). These results suggested that the AC enzyme contains a binding site for oxyanions which can modulate catalytic activity. When vanadate is bound to this site catalytic activity is enhanced, but when iodate is bound catalytic activity is decreased.

We next carried out experiments to determine whether the inhibition of AC by iodate was manifested *in vivo*. The IOP response of the albino rabbit eye to topical FSK [22] and topical ISO [23] was used for *in vivo* evaluation of iodate. Figure 7 shows that topically administered iodate (50 μL of 5%) had no effect on IOP over a period of 8 hr, while 1% FSK caused a significant fall in IOP over the first 3 hr. When 5% iodate treatment preceded the 1% FSK treatment, the response to FSK was blocked. In a similar experiment the IOP response to 0.001% ISO, which decreased intraocular pressure for a period of at least 6 hr, was blocked by prior treatment with 5% iodate, except for the initial response occurring in the first 2 hr (Fig. 8).

DISCUSSION

Meta vanadate anion (VO_3^-) is a known stimulator of AC. Previous studies of this phenomenon concluded that G_s was involved [14, 24]. A possible mechanism is that the hydrated form (orthovanadate)

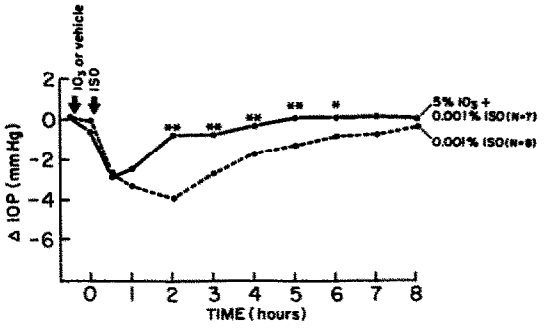

Fig. 8. Effect of topical isoproterenol (ISO, 50 μL of 0.001%) with or without pretreatment with $NaIO_3$ (50 μL of 5.0%) on IOP of the rabbit eye. The IOP measurements are given as Δ IOP in mm Hg, representing the difference between IOP of drug-treated (experimental) eyes and the contralateral vehicle-treated (control) eyes measured at the same time. (* = $P < 0.05$, ** = $P < 0.01$; unpaired *t*-test.)

forms a complex with GDP bound to G_s and thereby mimics GTP to catalyze activation of G_s. We have previously studied vanadate because topical treatment of the eye with vanadate lowers intraocular pressure and this might be mediated in part by effects on AC [5, 6]. However, the experimental evidence obtained by us [12] and by others [25, 26] makes it unlikely that the IOP effect of vanadate is due to its action on AC. Although the mechanism(s) of the *in vivo* effects of vanadate remains uncertain, its interaction with AC has some biochemical interest. The present studies show that the effect of vanadate on AC in membranes from ciliary processes—the tissue which secretes the aqueous humor of the eye—gave results inconsistent with the proposed mechanism that vanadate activates G_s. However, the ciliary process is not a homogeneous tissue and consists of several cell types—two types of epithelial cells (pigmented and non-pigmented) bounding a stroma containing capillary blood vessels. It is possible that these different cell types may contain different AC enzymes. As shown in Fig. 1, vanadate neither potentiated nor inhibited, but the basal vanadate response was additive to G_s-stimulated AC (via ISO or VIP receptors). It stimulated basal activity dose-dependently [12] but potentiated the FSK activity. Vanadate potentiation of forskolin AC in ciliary processes did not show the expected G_s-mediated large increase in FSK affinity documented in other tissues and in our previous study with ciliary processes on the interaction of FSK and activated G_s [19]. Furthermore, vanadate also potentiated the activation of AC by calcium/calmodulin, though not to the same extent as with FSK. These findings together with an increased rather than a decreased response in the presence of GDPβS suggested that vanadate does not work via G_s but may interact directly with the AC enzyme(s). The structurally related iodate anion was found to be an inhibitor of AC. The IO_3^- inhibition curves against ISO, FSK, fluoroberyllate (data not shown), and VO_3^- activations of AC were all similar and gave an IC_{50} of about 0.3 mM. The influence of Mg^{2+} concentration on the potentiative effect of VO_3^- (Fig. 4) strongly indicates that this ligand increases the binding of Mg^{2+} to the AC enzyme, an effect which appears to be synergistic with the regulation of Mg^{2+} binding and enzyme activation by FSK.

Based on the AC enzyme results, we used the lowering of intraocular pressure in the living eye in response to stimulators of AC to support the biochemical data and test whether IO_3^- inhibition of AC might occur *in vivo*. The cAMP-mediated IOP response can be elicited by local topical treatment of the rabbit eye with FSK [22], the direct activator of AC, or by ISO [23], which acts via the β_2-adrenergic receptors. The mechanism(s) whereby cAMP causes a decrease in IOP in eyes of various mammalian species remains uncertain despite extensive investigation. However, in the rabbit eye there is general agreement that the principal mechanism appears to be a decrease in aqueous humor formation [22].

Pretreatment of the rabbit eye with 5% iodate (250 mM) had no effect alone but completely or significantly blocked the IOP response to FSK and to ISO, respectively (Figs. 7 and 8).

Both the biochemical and biological experiments in this study support the hypothesis that AC enzymes contain a binding site for oxyanions which can modulate enzyme activity by increasing or decreasing the apparent catalytic activity. However, studies on the purified AC will be required to prove that these findings represent an entirely new mode for the direct regulation of the AC enzyme(s).

Acknowledgements—This work was supported in part by USPHS National Institutes of Health Grants EY02619, EY01867 and EY07400 and an unrestricted grant from Research to Prevent Blindness, Inc.

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