Calmodulin Activated Adenylyl Cyclase in Ciliary Processes: Additivity of Calcium and Cyclic Adenosine Monophosphate Signals on Intraocular Pressure Response of the Rabbit Eye

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Purpose. The regulation of adenylyl cyclase by multiple signal systems was investigated by biochemical studies of the enzyme in ciliary processes and by in vivo intraocular pressure responses in the rabbit eye.

Methods. Adenylyl cyclase enzyme activity was determined by radiometric assay using α-32P-ATP as substrate. Drugs were administered to the rabbit eye by intravitreal injection or topical application, and intraocular pressure was measured by pneumatonography.

Results. Adenylyl cyclase activity in the membrane/particulate fraction of the rabbit ciliary process was activated by calmodulin in the presence of Ca²⁺, Co²⁺, or Mn²⁺, and inhibited by the calmodulin antagonist calmidazolium. The activity was additive to stimulations of adenylyl cyclase by the activating G-protein (Gs) via isoproterenol or vasoactive intestinal peptide receptors, and by forskolin. The biochemical findings were supported by in vivo correlation experiments with intravitreal injection of MnCl₂, and by topical treatment with the Ca²⁺-mobilizing α₁-adrenergic agonist phenylephrine and with agents affecting cyclic adenosine monophosphate levels (forskolin, isobutylmethylxanthine). The intraocular pressure response was augmented by combining threshold doses of phenylephrine with threshold doses of either forskolin or isobutylmethylxanthine. The maximal and most prolonged response (a decrease in intraocular pressure of 4 mm Hg for up to 8 hr) was obtained by combination treatment with all three agents at doses that produced no significant effect by themselves.

Conclusions. These findings provide a biochemical mechanism for ocular hypotensive drugs having both a Ca²⁺-mobilizing activity as well as increasing cyclic adenosine monophosphate levels by receptor-coupled stimulation of adenylyl cyclase. Invest Ophthalmol Vis Sci 1993; 34:2041-2048.

Cyclic adenosine monophosphate (cAMP) is believed to play a major role in the regulation of aqueous humor secretion by ciliary processes¹ and may also be important in controlling outflow mechanisms² in the primate eye. In a previous study we reported that calcium ions stimulated the basal activity but inhibited the forskolin activated adenylyl cyclase activity (AC) in ciliary process membranes.³ The effect of Ca²⁺ to stimulate basal AC activity suggested the possibility of a separate calmodulin (CAM) activated adenylyl cyclase enzyme in rabbit ciliary process membranes. The present study was undertaken to confirm this possibility.

A calcium/CAM activity was found in ciliary processes that was also fully supported by Mn²⁺ ions.⁴ In addition to activating calmodulin, Mn²⁺ significantly...
amplifies cAMP formation induced by other activators of AC, particularly the Gs-protein, in comparison to Mg2+ ions. Thus, Mn2+ increases stimulation of AC by both known physiological activators of the enzyme and we therefore attempted to correlate the biochemical findings with Mn2+ ions by studying the intraocular pressure effects of in vivo administered MnCl2.

The calcium/CAM-dependent adenylyl cyclase activity in ciliary processes was found to be additive to the activation of AC by forskolin. We also attempted an in vivo correlation of this biochemical finding by determining the intraocular pressure response to topical administration of a calcium mobilizing agonist (phenylephrine) in combination with forskolin, compared to their separate effects on intraocular pressure.

MATeRIALS AND METHODS

Chemicals and Reagents
Calmodulin (MWt 18000) from bovine brain (74 x 104 units/mg protein) was purchased from Sigma Chemical Co. (St. Louis, MO).

Synthetic porcine vasoactive intestinal peptide was purchased from Cambridge Research Biochemicals (Atlantic Beach, NY). 3H-cAMP was obtained from New England Nuclear Corp. (Boston, MA) and 32P adenosine triphosphate (ATP) from Amersham (Arlington Heights, IL). Forskolin (FSK), obtained from Calbiochem (La Jolla, CA) or Sigma, was made up as a 10 mM stock in dimethyl sulfoxide. All other reagents, biochemicals, and drugs were obtained from Sigma or from Fisher Scientific (Pittsburgh, PA).

The assay kit for protein determination by dye-binding was purchased from BioRad (Richmond, CA).

Membrane Preparation
Ciliary processes were obtained by scraping them with a scalpel blade from the iris-ciliary body dissected from albino rabbit eyes within 1 hr of death (sodium pentobarbital overdose). Processes from 6 eyes were hand-homogenized in 3 ml of buffer (0.3 M sucrose, 20 mM Tris pH 7.6, 5 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, 10 µM leupeptin) in a Dounce homogenizer using 25 strokes of the pestle. The centrifuged particulate/membrane fraction (15 min, 27000 g) was washed once in 6 ml of the homogenizing buffer and resuspended in the same buffer (3 ml) containing 0.1 mM indomethacin, 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 7.6, 2 mM MgCl2 or 3 mM MnCl2, 1 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 5 mM creatinine phosphate, 125 µg creatinine phosphokinase, 20 µM GTP, 1 mM cAMP, 4 mM theophylline, 20 µM indomethacin, 0.5 µg leupeptin, 0.2 mM ATP. Fifty-microliter aliquots of the membrane suspension (30-70 µg protein) were added to triplicate tubes in ice containing 1 mM CaCl2, 2 mM CoCl2, or 3 mM MnCl2 (as indicated), and all other incubation ingredients except α-32P-ATP. The tubes were incubated at 30°C to equilibrate. After 3 min the assay was started by adding the α-32P-ATP (1-2 x 106 ciliary process membranes) and terminated at 6 min with SDS stopping solution and placing the tubes in boiling water. The 32P-cAMP with added 3H-cAMP tracer (1-3 x 104 cpm) was isolated by the double-column method (Dowex 50, alumina) of Salomon et al. When FSK was used, the control assay tubes contained dimethyl sulfoxide (<1%) equivalent to the amount used to dissolve the FSK.

Intravitreal Injections, Aqueous Humor Protein Determination, and Intraocular Pressure Measurements
This investigation adhered to the ARVO “Statement for the Use of Animals in Ophthalmic and Vision Research.”

Solutions of MnCl2 or MgCl2 (50 mM) in sterile H2O were injected into the vitreous cavity (20 µl/eye) of one eye in groups of 6 albino rabbits using a microsyringe and a 26-gauge needle under anesthesia with systemic ketamine (12.5 mg/kg) and with topical 0.5% proparacaine. Contralateral eyes received intravitreal injections of 20 µl of the vehicle in all cases. Solutions were made up and coded by one person and the injections and pressure measurements were made by another person on a masked basis. Intraocular pressure measurements were made on rabbits between 3:00 and 4:00 PM just before injection, and for 3 successive days after injection, at 9:00 AM, noon, and 5:00 PM using a manometrically calibrated pneumotonomograph (Digilab Model 30 R) as previously reported. In other groups of rabbits similarly treated, intraocular pressure (IOP) was measured at 40 hr and aqueous humor protein concentration was determined by the Bradford dye-binding method by paracentesis at 42 hr after intravitreal injection of the agents, which is the time giving nearly the maximum IOP effect. In other trials, rabbits were pretreated 4 hr before receiving the intravitreal drug with 20 mg/kg indomethacin (administered intraperitoneally) and 10 mg/kg intraperitoneal indomethacin twice a day thereafter (Table 1). The indomethacin regimen did not itself affect IOP.

For topical drug treatments (Figs. 7-9), groups of 6, 8, or 10 eyes of pigmented Dutch Belt rabbits were treated with a 50 µl dose of vehicle V1, or with freshly prepared 0.2% phenylephrine (PE) or 1% isobutylmethylxanthine (IBMX) in vehicle V1, alone or in com-
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TABLE 1. Intraocular Pressure (IOP) Change and Aqueous Humor Protein

<table>
<thead>
<tr>
<th>Injected Drug</th>
<th>Pretreatment</th>
<th>Change of IOP at 24 hr (%)</th>
<th>Change of IOP at 40 hr (%)</th>
<th>Aqueous Humor Prote (42 hr) (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{MnCl}_2 ), 1 ( \mu \text{mol} ) (n = 3)</td>
<td>None</td>
<td>-40.0 ± 8.2</td>
<td>-38.4 ± 5.0</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>( \text{MnCl}_2 ), 1 ( \mu \text{mol} ) (n = 4)</td>
<td>Indomethacin*</td>
<td>-48.2 ± 4.2</td>
<td>15.0 ± 5.5</td>
<td>5.5 ± 0.5</td>
</tr>
</tbody>
</table>

* Indomethacin pretreatment: initial dose (20 mg/kg, IP) 4 hr before intravitreal \( \text{MnCl}_2 \), thereafter 10 mg/kg IP twice daily.

bination, and additionally with a 50 \( \mu \)l dose of vehicle \( V_1 \), or with 0.2% FSK in vehicle \( V_2 \). The composition of \( V_1 \) was 0.5% hydroxypropylmethyl-cellulose and \( V_2 \), 0.45% hydroxypropylmethylcellulose + 10% dimethylsulfoxide in H\(_2\)O. A two-dose treatment schedule was followed; at time minus 0.5 hr the baseline IOP was determined and immediately thereafter either \( V_1 \), PE, IBMX, or PE+ IBMX administered to the experimental eye. IOP was again measured at time zero immediately prior to administration of \( V_2 \) or FSK to the experimental eye. IOP was measured at intervals for 8 more hours. For each group of treated eyes the difference in IOP between the baseline IOP at -0.5 hr and the IOP at each time point was plotted (\( \Delta \)IOP) as in Figures 7–9. A control group of eyes receiving only the \( V_1 \) and \( V_2 \) vehicles at the same times as the drug-treated eyes showed no significant change of IOP during 8 hrs (data not shown). Groups of rabbits were reused after a 7–10 day drug washout period.

Data Analysis

Adenylyl cyclase specific activities were calculated from triplicate 3 min assays and are based on membrane protein solubilized with NaOH determined by the Bradford dye-binding method with bovine gamma globulin as the reference protein. Error bars representing SEM are omitted from the biochemical response curves (Figs. 1, 2, and 5) for clarity, but similar (less than 5% of the measurement) to those shown in bar graphs (Figs. 3, 4). Complete dose response experiments were done at least twice. IOP responses were evaluated as either the percentage change relative to the control eye’s IOP at the time (Fig. 6) or the difference in millimeters of mercury (\( \Delta \)IOP) at each time-point relative to the baseline measurements before drug treatment (indicated by dotted line in Figs. 7–9). Error bars on Figure 6 represent SD, and on Figures 7–9 represent SEM. Significance (P values) for \( \Delta \)IOP were determined by Students’ t test.

RESULTS

All biochemical experiments were done on a membrane/particulate preparation of ciliary process membranes freshly isolated from albino rabbit eyes. AC activity was routinely measured in the presence of 2 added Mg\(^{2+}\), which is calculated to be approxim 0.5–0.6 mM free Mg ions.  Figure 1 shows the concentration-response of added Ca\(^{2+}\) ions on the basal activity of ciliary process membranes in the presence (+) or absence (−) of 5 \( \mu \)M CAM. The optima

FIGURE 1. Dose response for addition of Ca\(^{2+}\) on basal adenylyl cyclase (AC) activity (+CAM) in rabbit ciliary process membranes and in presence of 5 \( \mu \)M added calmodulin (+CAM).

FIGURE 2. Dose dependency for CAM activation of process membrane adenylyl cyclase in presence (+Ca\(^{2+}\)) absence (−Ca\(^{2+}\)) of 1 mM added CaCl\(_2\).
FIGURE 3. Comparison of net responses of ciliary process adenylyl cyclase to added 2mM CoCl₂, 1mM CaCl₂, 3 mM MnCl₂, and to 3 mM MnCl₂ + 1 mM CaCl₂ in absence and presence of 2 μM calmodulin. Activities were determined with 2 mM Mg²⁺ present and are corrected for the basal response ± in presence (8.8 ± 1.0) and absence (12.0 ± 0.6) of calmodulin without any other divalent cations.

tion of 1 mM added Ca²⁺. There is a clear dose dependency, but the maximum response requires CAM in excess of 6 μM, the highest dose tested. (To conserve CAM we routinely used a low submaximal dose of 2 μM for most subsequent experiments.) Figure 3 shows that the CAM-dependent activity was supported by addition of either 1 mM Ca²⁺, 2 mM Co²⁺, or 3 mM Mn²⁺. The data show that Mn²⁺ can fully substitute for Ca²⁺. Manganese ions can also substitute for magnesium in AC assays in both the substrate (as MnATP), and as the divalent ion regulator of AC catalytic activity induced by FSK as well as for receptor-mediated activations of AC.⁸ Adenylyl cyclase assays are simplified by these properties of Mn²⁺ because only one divalent ion is required in the reaction mix. This allowed us to avoid using 1 mM Ca²⁺ to determine the CAM-dependent component in the presence of other AC.

FIGURE 4. Comparison of net responses of ciliary process adenylyl cyclase to maximal doses of isoproterenol (ISO, 10 μM), vasoactive intestinal peptide (VIP, 1 μM) and forskolin (FSK, 60 μM) in absence or presence of 2 μM calmodulin assayed with 3 mM Mn²⁺ as the only divalent cation. Net activities were obtained by subtraction of the basal response (278 ± 11) in absence (left) or the response (629 ± 7) in presence of calmodulin (right), respectively. The net response to calmodulin in this experiment is the difference between these two values; 351 pmoles/min/mg protein.

FIGURE 5. Dose response for inhibition by calmidazolium of net Ca²⁺/CAM (1nM/2μM) adenylyl cyclase activity [open symbols, left activity scale (y axis)] compared to forskolin (100 μM) activated adenylyl cyclase activity [closed symbols, right activity scale (y axis)]. Activities are corrected for the basal responses determined at each dose of calmidazolium.

FIGURE 6. Effect of intravitreal injection of MnCl₂ (1 μmole per eye, solid line) on IOP of albino rabbits (n = 6) relative to contralateral eyes injected with 1 μmole MgCl₂. Data are plotted as the IOP (mm Hg) of MnCl₂-injected eyes as a percentage of the IOP of their contralateral MgCl₂-injected eyes measured at the same time. Error bars indicate SD of the measurement.
Figure 7. Effect of threshold doses of FSK (0.2%, panel A), phenylephrine (0.2% PE, panel B) and IBMX (1%, panel C) on IOP of groups of pigmented rabbits (n = 6 per group) over an 8 hour period. Each eye was dosed twice, at -0.5 hr with vehicle V1, with or without the indicated drug, and again at time 0 hr with vehicle V2, with or without FSK. Data at each time-point are plotted as the differences (ΔIOP, mm Hg) between the baseline IOP measured prior to drug treatment at -0.5 hrs. Significance symbols, * = P < 0.05, no symbol = P > 0.05 (not significant).

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The distinction between CAM activation and FSK activation of AC was further illustrated by inhibition curves to calmidazolium, a specific CAM antagonist. Inhibition of net activity due to Ca2+/CAM by calmidazolium is compared to its effect on FSK-activated AC by the data plotted in Figure 5 (left and right activity axis, respectively). There is no effect up to 50 μM on FSK activity, but for the Ca2+/CAM stimulated activity the IC50 of calmidazolium was approximately 20 μM. Similar results were obtained with another structurally different CAM-antagonist, W-7 (data not shown).

Because Mn2+ supports and amplifies both physiologic signals for stimulation of AC, (via Gs and CAM) we studied the IOP effects of intravitreal MnCl2 (1 μmole per eye), compared to 1 μmole of intravitreal MgCl2 (Fig. 6). MnCl2 produced a long-lasting fall in IOP. However at 40 hours postinjection, when there was a maximal IOP response, MnCl2 also caused a significant increase in aqueous humor protein content to about 18 mg/ml (Table 1). This AH protein response could be largely eliminated by using animals pretreated with indomethacin, without significantly affecting the IOP response to MnCl2 (Table 1).

Additionally, we attempted a biological correlation of the additivity experiments between Ca2+/CAM and forskolin on AC shown in Figure 4. In vivo treatment with a Ca2+-mobilizing agent (phenylephrine) can be expected to stimulate Ca2+/CAM cyclase in cells where PE-binding α1-adrenergic receptors, endogenous CAM levels, and CAM-regulated AC activity are present. In vivo treatment with forskolin would also cause stimulation of cAMP levels in cells containing the FSK-activated AC activity. Therefore, the combination of these two agents in vivo could be predicted to give a greater cAMP response, with the response further amplified by an agent blocking cAMP metabolism, namely the phosphodiesterase inhibitor isobutylmethyl xanthine (IBMX). The experimental design for this three-drug study was based on using combinations...
of threshold doses of the agents, which by themselves produce no or a barely significant response.

Because two different vehicles were used, (Vj for PE and IBMX, V2 for forskolin), a two-dose protocol for all eyes was used in the experiments as described in Methods (Figs. 7–9). Figure 7 shows that the threshold doses selected (0.2% FSK shown in panel A, 0.2% phenylephrine in panel B, and 1% IBMX in panel C) did not significantly change IOP during the 8-hour period of measurements (except for IBMX in the first hour). Figure 8 shows responses to combinations of the drugs at the same threshold dose levels used in Figure 7; in panel A—PE with FSK; in panel B—PE with IBMX. Each combination gave an IOP response at a significant level. Similarly, when FSK was combined with IBMX, a much greater and more prolonged potentiation of response was seen than with either drug alone. (Compare panels A and C, Fig. 7 to panel A in Fig. 9). When all three agents were given (Fig. 9, panel B) the response was greatest, and the combined response approximated the sum of the responses seen with FSK + PE (Fig. 8, panel A), and FSK + IBMX (Fig. 9, panel A).

DISCUSSION

Our results demonstrate the presence of a CAM-stimulated AC activity in ciliary processes of the rabbit eye. The observation that the basal AC was stimulated by the addition of Ca2+ alone indicates the presence of some endogenous CAM in membrane/particulate preparations. This response was further increased by addition of exogenous CAM and appropriately stimulated by CO2+ and by Mn2+ ions, both of which can substitute for Ca2+ to form an active complex with CAM.11 The specific CAM antagonists, W7 and calmidazolium, blocked the response. At the maximal level of CAM used in these studies (6 µM) the response has the same magnitude as that elicited by a maximal dose of isoproterenol (10 µM) in this tissue, but was much less than the maximal response to forskolin. The CAM-sensitive response was additive to receptor-mediated stimulation of AC via the Gs protein (with isoproterenol or with vasoactive intestinal peptide),12 and also to the direct activator of the AC enzyme, forskolin. The additivity of these responses might be interpreted as evidence that the CAM sensitive activity is a different enzyme type from that stimulated by Gs and by forskolin. However, it is unclear whether this is a valid assumption, because two different regulators binding simultaneously to the same enzyme can give additive or even potentiative responses, (for example, when the AC enzyme is activated by both Gs and FSK).13 Recent biochemical and cloning studies of AC enzymes from brain14 and from olfactory epithelium15 have identified several major types of AC. The most abundant brain enzyme is activated by Ca2+/CAM and also activated by Gs and FSK, showing that all three regulators can function on a single type of AC enzyme molecule.16 Thus, the present findings could be attributable to a single molecular form of AC enzyme.

To correlate the biochemical results with in vivo responses we first focused on the actions of Mn2+. This ion could have a significant stimulatory effect on endogenous cAMP levels because it activates CAM as well as amplifying AC catalytic activity, including FSK- and receptor-mediated (via Gs) stimulations of the enzyme. Although it has been shown that some tissues have a specific high affinity transport of Mn2+ into cells,17 there was no observable ocular response to topical Mn2+ treatment even at very high doses. When administered by intravitreal injection, MnCl2 caused a significant and long-lasting decrease in IOP compared to an equivalent dose of MgCl2, which was dissociated from an apparent inflammatory breakdown of the blood/aqueous barrier by using indomethacin-pre-treated animals (Table 1).

Because there are many possible interpretations of the mechanism of the Mn2+ response, we sought an alternative in vivo correlation of the Ca2+/CAM findings. Therefore, we attempted to show additivity of FSK and CAM stimulation of AC using topical agents applied to the rabbit eye. Phenylephrine, which is a specific agonist at α1-adrenergic receptors acting to...
mobilize Ca\(^{2+}\) via the inositolphosphate system therefore can potentially activate Ca\(^{2+}/\)CAM-dependent systems in cells bearing \(\alpha_1\)-adrenergic receptors. We used combinations of threshold doses of PE,\(^{18}\) FSK,\(^{19}\) and IBMX,\(^{20}\) which have none or a barely significant IOP response by themselves (Fig. 7). PE together with FSK elicited a significant response and PE together with IBMX also elicited a somewhat increased response compared to the drugs given alone. It is to be expected that the response to a low dose of PE would be relatively weak and short-lived, and that potentiation and prolongation by the phosphodiesterase inhibitor IBMX would be modest (Fig. 8). Forskolin is, however, a longer lasting agent than is PE and is also more effective in increasing cAMP levels. FSK was significantly potentiated by IBMX and gave a prolonged IOP response (Fig. 9A). The largest prolonged response was observed when all three agents were administered (Fig. 9B). IOP responses obtained with the drug combinations do not specifically distinguish the separate components of aqueous humor dynamics (secretion, trabecular outflow, and uveoscleral outflow). Although the biochemical results were obtained on a specific tissue, (ciliary processes) which controls aqueous secretion, this may not be the only mechanism for the IOP response because the FSK and CAM responsive AC activity may be a single enzyme that occurs in several intraocular tissues controlling aqueous humor dynamics.

There are other possible interpretations of the PE/FSK potentiation of IOP response that do not involve a Ca\(^{2+}/\)CAM-sensitive AC enzyme. Crossover of the Ca\(^{2+}\) and cAMP signal systems has been reported in many tissues, including ciliary process, which involve Ca\(^{2+}\)-dependent protein kinases. In a previous in vitro study, we demonstrated that phorbol ester activation of protein kinase C significantly increased hormone stimulated responses of rabbit ciliary process adenyl cyclase, but did not alter the forskolin response.\(^{6}\) In another study using cultured human non-pigmented ciliary epithelial cells, it was found that treatment of the cells with phorbol ester decreased responsiveness of AC to hormone signals.\(^{21}\) Thus, the available evidence on crossover effects of the Ca\(^{2+}\)-protein kinase C system does not explain the findings of potentiated in vivo drug responses. When taken together, the present in vivo results with MnCl\(_2\) and PE lend support to a hypothesis involving increased cAMP levels mediated by direct activation of AC by Ca\(^{2+}/\)CAM.

Although we do not know the detailed mechanism(s) responsible for the IOP effects, the in vivo results correlate well with the biochemical experiments. These findings also help to explain previous studies showing that the IOP response to epinephrine in the rabbit eye depends on both \(\alpha_1\)- (Ca\(^{2+}\)) and on \(\beta-(\text{cAMP})\) adrenergic receptor signal systems\(^{22,23}\) and the effects of IBMX on IOP.\(^{20}\) Our studies provide a mechanistic framework for drugs having both Ca\(^{2+}\) mobilizing activity as well as causing G\(_s\) stimulation of AC being particularly effective in lowering IOP.

Key Words
adrenyl cyclase, calmodulin, ciliary processes, intraocular pressure, rabbit eye

References

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