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Inhibition and Stimulation of Photoreceptor Phosphodiesterases by Dipyridamole and M&B 22,948

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SUMMARY

Few high affinity inhibitors of the photoreceptor phosphodiesterases have been identified. We show here that dipyridamole and M&B 22,948 (Zaprinast), potent inhibitors of the cGMP-binding, cGMP-specific phosphodiesterase (PDE), also inhibit trypsin- or transducin-activated bovine rod and cone photoreceptor phosphodiesterases at submicromolar concentrations. Dixon plots demonstrated that the inhibition of trypsin-activated rod PDE was competitive, with K_i values of 140 nM for M&B 22,948 and 380 nM for dipyridamole. Both of these drugs were much more potent than other PDE inhibitors, including isobutylmethylxanthine (IBMX). These results reinforce the suggestion that the photoreceptor and the cGMP-binding, cGMP-specific PDE are closely related. In addition, the high affinity and selectivity of these agents should make them useful for probing the regulation and function of PDE in the photoreceptor. At low substrate concentrations, the effects of these drugs on basal unactivated PDE activity were similar to those seen with trypsin- or transducin-activated PDE. At millimolar substrate concentrations, however, the effects of the drugs were biphasic; PDE activity was stimulated at drug concentrations from 1 to 10 μM and inhibited at higher concentrations. Stimulation was not observed with IBMX. This stimulation of activity apparently was not an allosteric effect caused by direct binding of the dipyridamole and M&B 22,948 to the high affinity noncatalytic cGMP binding sites on the PDEs; whereas no cooperativity of cGMP binding to this site has been demonstrated, the drugs actually stimulated the binding of low concentrations of cGMP to this site. In addition, whereas preincubation with cGMP and cGMP analogs blocked the stimulation exerted by the drugs, they did so only at much higher concentrations than those necessary for saturation of the high affinity noncatalytic cGMP site. Because the stimulation can only be seen at higher substrate levels than are thought to exist in the photoreceptor, only the inhibitory effects of the drugs are likely to be pharmacologically relevant. However, the stimulation exerted by these drugs may point to a hitherto unknown allosteric interaction between the catalytic and regulatory sites on the PDE or to a previously unrecognized regulatory site.

The light-activated cGMP-specific PDE of vertebrate rod outer segments plays a pivotal role in visual transduction, hydrolyzing cGMP in response to light (reviewed in Ref. 1). A cone outer segment PDE (2, 3) probably performs a similar function in cone outer segments. Both isozymes preferentially hydrolyze cGMP, with similar kinetics (K_m of about 20 μM and V_{max} of 4000–7000 sec^{-1}; cGMP also binds to noncatalytic binding sites on both enzymes (2, 4, 5). The rod and cone PDEs differ in subunit composition, affinity of noncatalytic cGMP binding, and stimulation by the retinal GTP-binding protein transducin (2, 5–7). It is not clear whether any of these differences are responsible for the distinct electrophysiological properties of rods and cones.

PDE inhibitors have proven instrumental in probing the physiological role of other PDE isozymes (8). Potent selective inhibitors of the photoreceptor PDEs could be useful in examining the role and regulation of these enzymes in the intact photoreceptor or in broken outer segment suspensions. To date, however, no potent and selective inhibitors of the photoreceptor PDEs have been identified. Although a number of studies have examined the effects of common PDE inhibitors like IBMX and papavarine on light responses of photoreceptors (9–12), these drugs are neither potent nor selective; furthermore, because high drug concentrations are required for inhibition, other components of the transduction system also may be affected.

The photoreceptor PDEs share many properties with another cGMP-specific PDE, often called the CGB-PDE. This isozyme has been most thoroughly characterized in lung (13, 14) and platelets (15–17) but may be present in a wide variety of tissues (18). CGB-PDE has been purified to apparent homogeneity from rat lung (14). The photoreceptor PDEs and CGB-PDE

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ABBREVIATIONS: PDE, phosphodiesterase; 8-Br-cGMP, 8-bromoguanosine 3′,5′-cyclic phosphate; CaM, calmodulin; CGB-PDE, cGMP-binding, cGMP-specific PDE; cGMP[S], guanosine 3′,5′-cyclic phosphothioate; IBMX, isobutylmethylxanthine; SDS, sodium dodecyl sulfate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

share substrate specificity, K_m, cGMP-binding properties, subunit molecular weight, and elution position on anion exchange resins (13–17). Many characteristics, however, distinguish the two types of PDE; for example, unlike the photoreceptor PDEs, CGB-PDE has a low turnover (15 sec^{-1}) (14), is phosphorylated by cAMP-dependent and cGMP-dependent protein kinase (19), and is sensitive to fatty acids and oxygen (17).

Although dipyridamole and M&B 22,948 have been reported to inhibit a number of PDEs (16, 20, 21), these drugs are most potent towards the cGMP-hydrolyzing activity that elutes early from anion exchange resins (8, 21). In most tissues, this activity is due to varying amounts of both calcium/CaM-sensitive PDE and CGB-PDE (8). However, the inhibition of this PDE activity by these drugs is always most potent ($IC_{50} < 2$ μM with 1 μM cGMP as substrate) in tissues that contain more CGB-PDE than calcium/CaM-sensitive PDE, such as platelet and lung (21). It has been proposed that most cGMP-specific PDE activity is correlated with this binding activity (8). The evidence thus suggests that dipyridamole and M&B 22,948 are relatively potent and selective inhibitors of the cGMP-specific CGB-PDE.

The physical and kinetic similarities between the CGB-PDE and the photoreceptor PDEs led us to examine the effects of dipyridamole and M&B 22,948 on the photoreceptor PDEs. We show here that these compounds also potently and selectively inhibit rod and cone PDEs that have been activated by tryptic proteolysis. Nonactivated PDEs, by contrast, are stimulated at intermediate concentrations and inhibited by high concentrations of the drugs if millimolar substrate is used.

Experimental Procedures

Materials. M&B 22,948 (Zaprinast), a kind gift from May and Baker Ltd., was dissolved in dimethyl sulfoxide and stored as a stock solution of 50 mM, at −20°. M&B 22,948 was diluted by slowly adding aqueous diluent to the dimethyl sulfoxide-containing stock solution while mixing with a vortex mixer. Dipyridamole and IBMX were purchased from Sigma. Dipyridamole was stored at −20° in 0.05 N HCl at a drug concentration of 5 mM and then was diluted to the appropriate concentration immediately before use. Manipulation and storage of other drugs used in this work are described in the legend to Fig. 2. All other materials were obtained from sources previously described (2) or were of the highest grade available.

PDE assays. PDE activity was assayed by one of three methods: (i) as previously described with [^{3}H]cGMP (2), (ii) with a modification of the phosphate-release assay that is performed in microtiter plates (described briefly in Ref. 2), or (iii) with the pH assay (22). The [^{3}H]-cGMP assay was used for all experiments that required low concentrations of cGMP (<10 μM), whereas the phosphate-release assay was used for those with higher substrate concentrations. The pH assay was used to confirm that the stimulation was not an artifact of the other assays.

The phosphate-release assay was performed by mixing 10 μl of concentrated assay buffer (final concentrations: 40 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin), water, and drug in the wells of a microtiter plate that was resting in a 30° water bath. The plate was incubated for at least 5 min to equilibrate temperature. An aliquot of a PDE solution (typically 10 μl of a 1–10 nM stock solution) was added to each well, bringing the volume at this point of the assay to 80 μl; where appropriate, samples were then further incubated for 1–10 min to allow drug activation to take place. Snake venom, which contains a 5′-nucleotidase activity, was then added to allow production of phosphate and guanosine from 5-GMP (10 μl of a stock solution, prepared as described below). Finally, the reaction was initiated by addition of 10 μl of a concentrated cGMP solution and the samples were incubated for the desired time (at least 1 min). The reactions were stopped at the end of the assay by adding 50 μl of 6% SDS. Phosphate reagent (150 μl) (23) containing ammonium molybdate (0.1% final), sulfuric acid (0.2 N final), and ascorbic acid (1% final) was added, and the color was allowed to develop at 37° for 30 min. The absorbances of the wells were determined at 700 nm in a Dynatech plate reader controlled by a Macintosh computer. Potassium phosphate was used as a standard. Controls included identical samples that had SDS added before cGMP and samples that had no PDE added, to control for hydrolysis of cGMP by the snake venom.

Although the dependence of absorption on phosphate concentration was only linear to an absorbance of 0.5 (with a slope of 0.04 to 0.05 absorbance units/nmol), it could be readily fit up to 2.0 absorbance units with a second-order polynomial. Thus, the assay reliably detected phosphate from 1 to 50 nmol. The lower limit meant that the initial concentration of cyclic nucleotide had to be at least 10 μM for accurate results. This requirement limited the utility of the assay, requiring relatively high substrate concentrations (best at 500 μM or greater). Replicate samples were routinely within 5%; assays of identical samples by this assay and the [^{3}H]cGMP assay agreed to within 10%.

The snake venom stock from the phosphate-release assay was prepared by dissolving 50 mg of *Crotalus atrox* venom into 2 ml of 10 mM Tris, pH 7.5. The venom was loaded onto a prepacked Pharmacia PD-10 gel filtration column. The column was then washed with 0.5 ml of 10 mM Tris and was eluted with 3 ml of 10 mM Tris. This solution was diluted with 3 ml of 10 mM Tris and stored at 4°. Ten microliters of this snake venom stock could hydrolyze over 50 nmol of 5′-GMP/min. Because samples with hydrolysis of more than 50 nmol of cGMP were not used (they saturated the assay), it was not necessary to stop the PDE assay with IBMX as was done previously (2). During assays of greater than 5 min, the snake venom was added after the cGMP, 2–3 min before the end of the assay, to minimize blank values. The snake venom contains a cGMP PDE activity with a V_{max} of 0.02–0.04 nmol·sec^{-1}/10 μl and a K_m of 33 mM.[2] None of the drugs inhibited the 5′-nucleotidase activity of the snake venom.

Substrate hydrolysis was less than 20% in all of the reported experiments. Velocities are reported in units of sec^{-1} (mol of substrate hydrolyzed/mol of PDE/sec); molecular masses of 210 kDa for the rod PDE and 226 kDa for the cone PDE (2) were assumed.

Enzyme preparations. The membrane-associated rod PDE and the cone PDE were isolated by an antibody affinity chromatography method (24). The rod and cone PDEs bound to the antibody (ROS-1a) used in this method with high affinity at pH 7.5 ($K_d \approx 5$ nM) but were readily eluted at pH 10.7. Control experiments showed that the short exposure to high pH used in the chromatographic purification (<30 min) reduced PDE activity by less than 20%. This method allowed the purification of the rod PDE 1400-fold and the cone PDE 15,000-fold, to apparent homogeneity. Trypsin typically activated the immunoaffinity-purified PDEs from 350 to 7000 sec^{-1} (rod PDE) and from 600 to 4500 sec^{-1} (cone PDE).

Crude rod outer segments were isolated as described previously (2) and were further purified on a discontinuous sucrose density gradient (25). The rhodopsin concentration of the outer segments was measured in 1.2% Emulphogene BC-720, 100 mM Tris, pH 7.5, with an assumed molar absorption coefficient of 40,000 at 500 nm. Rod PDE γ-subunit was purified by elution of washed outer segment membranes with GTPγS, followed by ion exchange chromatography (2). The bovine heart Ca^{2+}/CaM-stimulated PDE, cGMP-stimulated PDE, and cGMP-inhibited PDE were purified as described elsewhere (26, 27). These enzymes were judged pure by SDS-polyacrylamide gel electrophoresis and by specific activity.

The cGMP-binding, cGMP-specific PDE of rat lung was partially purified using a modification of a published procedure (15). Frozen rat lung tissue (stored at −70° after dissection) was thawed, weighed (20–25 g wet weight), and homogenized with a Polytron in 200 ml of 20 mM

[2] P. G. Gillespie and J. A. Beavo, unpublished data.

Tris, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol (homogenization buffer). The volume was adjusted to 250 ml and the extract was centrifuged in a GSA rotor at 27,500 × *g* for 40 min. The supernatant was filtered through a 100-μm nylon mesh and was adjusted to 65% ammonium sulfate by slowly adding the dry powder. The mixture was stirred for 3 hr at 4° and then was centrifuged in a GSA rotor at 27,500 × *g* for 40 min. The pellet was dissolved with homogenization buffer to a final volume of about 70 ml. The sample was then dialyzed against two changes of homogenization buffer (2 liters each) over 10 hr. The sample was centrifuged at 100,000 × *g* in a Ti45 rotor and then was filtered through a 0.2-μm filter. Aliquots of the filtrate were injected onto a TSK DEAE 5-PW high pressure liquid chromatography column. A gradient of 0 to 500 mM NaCl in homogenization buffer over 20 min (1 ml/min) was used to separate CGB-PDE from other PDE activities. When assayed with 30 μM [^{3}H]cGMP, two large peaks of activity were observed. The first peak of activity was not stimulated by calcium and CaM and was coincident with a large peak of IBMX-stimulated cGMP binding. This activity peak was identified as primarily CGB-PDE by these criteria. It is possible, however, that some proteolytically activated CaM-PDE was present in this peak. The second large peak was probably cyclic GMP-stimulated PDE (28).

Trypsin activation. Rod or cone PDE (10 nM) was activated with 3 μM trypsin at 4° in a buffer containing 20 mM Tris, pH 7.5, 1 mM $MgCl_2$, and 0.5 mg/ml bovine serum albumin. The time of activation was determined for each batch of PDE and trypsin and was typically 30–45 min. The activation was stopped by the addition of soybean trypsin inhibitor to 15 μM. Although trypsin-activated PDE activity was stable for over 1 week, each particular sample was always used within 36 hr of activation.

Other methods. Cyclic GMP binding was performed as described previously (2). Protein was estimated by the Coomassie dye-binding method (29).

Modeling. Curves for the data shown in Fig. 5 were generated from Eq. 1, shown below, using the program Mathematical (Macintosh II version; Wolfram Research Inc., Champaign, IL). This equation describes the velocity of cGMP hydrolysis in terms of a mixture of two components of PDE activity. The first component is considered constitutively active, as for example might occur as a result of basal unstimulated activity or as a result of limited proteolysis. The activity of the second component, however, is expressed as dependent on drug binding to an activation site on the enzyme, with the extent of activation directly proportional to the extent of drug binding to the activation site. Each component is presumed to hydrolyze cGMP with Michaelis-Menten kinetics, with identical K_m and differing V_{max} values. The model of Eq. 1 further assumes that the catalytic sites on both forms can be inhibited by the drug with the same K_i and that there is no competition for cGMP at the second drug binding site. This condition is met in the experiment of Fig. 5 because the drug was added before cGMP, with the further assumption that the dissociation of drug from the activation site is slow relative to the time of the assay. The velocity equation is:

$$\text{Velocity} = \frac{V_{max_1}[\text{cGMP}]}{K_m\left(1 + \frac{[D]}{K_i}\right) + [\text{cGMP}]} + \frac{[D]}{K_a + [D]}\left[\frac{V_{max_2}[\text{cGMP}]}{K_m\left(1 + \frac{[D]}{K_i}\right) + [\text{cGMP}]}\right] \quad (1)$$

in which V_{max_1} is the V_{max} in the absence of drug, V_{max_2} is the V_{max} of the activated fraction of the PDE, [cGMP] is the cGMP concentration, [*D*] is drug concentration, and K_m, K_i, and K_a take on their usual meanings (for cGMP, drug, and drug, respectively). The data can also be fit well by more complete equations such as Eq. 2:

$$\text{Velocity} = \frac{V_{max_1}[\text{cGMP}]}{K_m\left(1 + \frac{[D]}{K_i}\right) + [\text{cGMP}]} + \left[\frac{[D]}{K_a\left(1 + \frac{[\text{cGMP}]}{K_{i_2}}\right) + [D]}\right]\left[\frac{V_{max_2}[\text{cGMP}]}{K_m\left(1 + \frac{[D]}{K_i}\right) + [\text{cGMP}]}\right] \quad (2)$$

In this case, the assumption is made that cGMP and drug do compete at the second drug binding site (activation site) and that cGMP acts as a competitive inhibitor of the drug's activation at this site. K_{i_2} is the K_i for this inhibition. If K_{i_2} is very large or the drug is added first (and its off-rate is slow), then Eq. 2 reduces to Eq. 1.

Results

Inhibition of trypsin-activated photoreceptor phosphodiesterases. We first examined the potency and selectivity of dipyridamole and M&B 22,948 with trypsin-activated rod and cone PDEs. Both drugs potently inhibited photoreceptor PDEs. Fig. 1 shows the effects of M&B 22,948 on several purified or partially purified bovine PDE isozymes that were assayed at 35% of their reported K_m values for cGMP. M&B 22,948 was 10-fold more potent towards trypsin-activated rod PDE than towards rat lung CGB-PDE (partially purified as described in Experimental Procedures). In agreement with a previous report (21), M&B 22,948 was a poor inhibitor of the cGMP-stimulated PDE, the Ca^{2+}/CaM-stimulated PDE, and the cGMP-inhibited PDE (each purified from bovine cardiac muscle). Both dipyridamole and M&B 22,948 inhibited the rod or cone PDEs at much lower concentrations than several other PDE inhibitors, including the commonly used inhibitor IBMX (Fig. 2). Milrinone and RO 20-1724, selective inhibitors of the

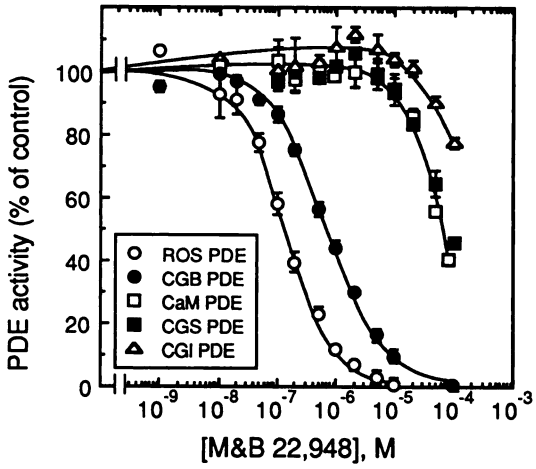

Fig. 1. M&B 22,948 is a highly potent selective inhibitor of cyclic GMP-specific PDEs. Catalytic activities of the PDEs were measured with the [^{3}H]cGMP assay at cGMP concentrations that were 35% of the reported K_m value for the particular isozyme (8). Other components of the assay mixture included 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 0.5 mg/ml bovine serum albumin. The cGMP concentrations used were: trypsin-activated rod PDE, 6 μM; CGB-PDE, 2 μM; CaM-stimulated PDE, 1.8 μM; cGMP-stimulated PDE, 4 μM; and cGMP-inhibited PDE, 0.35 μM. *ROS PDE*, trypsin-activated purified bovine rod outer segment PDE; *CGB-PDE*, PDE partially purified from rat lung (see Experimental Procedures); *CaM PDE*, CaM-stimulated PDE; *CGS PDE*, cGMP-stimulated PDE; *CGI PDE*, cGMP-inhibited PDE. CaM, CGS, and CGI PDEs were all purified from bovine heart. IC_{50} values (determined from Hill plots) were ROS PDE, 160 nM; CGB PDE, 760 nM; CaM PDE, 71 μM; CGS PDE, 78 μM; CGI PDE, 224 μM. *Error bars* represent ± 1 SD (three determinations for each point).

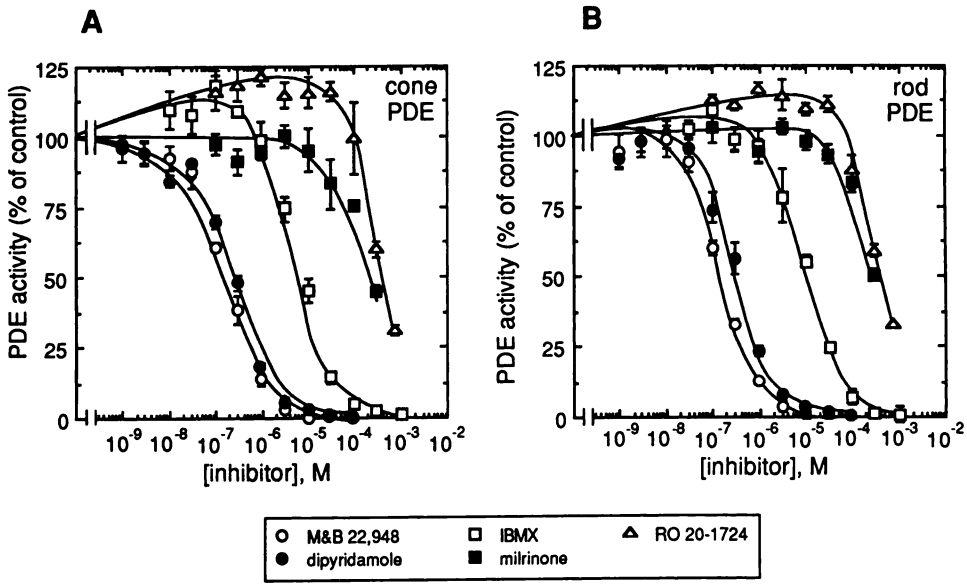

Fig. 2. Dipyridamole and M&B 22,948 are potent inhibitors of purified, trypsin-activated, rod and cone photoreceptor PDEs. Purified cone (A) or rod (B) PDEs (1 pM) were activated by trypsin and were assayed using 6 μM [^{3}H]cGMP in the presence or absence of various inhibitors. All drugs were diluted in water from stock solutions. M&B 22,948 was dissolved in dimethyl sulfoxide at 50 mM and was stored at −20°. Dipyridamole was dissolved in 50 mM HCl at a concentration of 10 mM and was stored at −20°. IBMX was dissolved in 0.5 N NaOH at a concentration of 500 mM, diluted to 50 mM, and stored at room temperature. Milrinone was dissolved in 0.5 N HCl at a concentration of 500 mM, diluted to 2.5 mM, and stored at 4°. RO 20-1724 was dissolved in 40% propylene glycol, 10% ethanol, 0.1 N NaOH at a concentration of 25 mM, diluted to 2.5 mM, and stored at 4°. IC_{50} values (determined from Hill plots) for trypsin-activated cone PDE: M&B 22,948, 127 nM; dipyridamole, 125 nM; IBMX, 11.9 μM; milrinone, 270 μM; 20-1724, 402 μM. IC_{50} values for trypsin-activated rod PDE: M&B 22,948, 154 nM; dipyridamole, 376 nM; IBMX, 11.5 μM; milrinone, 336 μM; 20-1724, 436 μM. *Error bars* represent ± 1 SD (three determinations).

cGMP-inhibited PDE and the cAMP-selective low K_m PDEs, respectively (8), were very poor inhibitors of photoreceptor PDE activity. The order of potency of inhibition of the photoreceptor PDEs by these drugs matches that of CGB-PDE (8), adding to the similarities between these isozymes. Dipyridamole also inhibited rod PDE that had been activated with purified transducin α-subunit-GTPγS complex, with dose-response characteristics that were similar to those of the trypsin-activated rod PDE (data not shown).

The mechanism of inhibition of trypsin-activated rod PDE by these drugs was examined with Dixon plots (Fig. 3). The intersection above zero of the lines representing different cGMP concentrations indicated that both drugs may be competitive inhibitors (30). Because several complex inhibition mechanisms can also produce similar Dixon plots (30), the slopes of the lines were plotted as a function of the reciprocal of the cGMP concentrations (Fig. 3, *insets*). The straight lines intersecting the origins indicated that inhibition of trypsin-activated rod PDE by both dipyridamole and M&B 22,948 was simple competitive. K_i values for inhibition of cGMP hydrolysis [140 ± 20 nM for M&B 22,948 (five experiments) and 380 ± 30 nM for dipyridamole (five experiments)] were determined from the absolute value of the x-axis coordinate the point of intersection (30). Thus, these drugs are potent competitive inhibitors of photoreceptor PDE activity.

Biphasic effects on purified nonactivated PDEs. Tryptic activation of the photoreceptor PDEs can affect their regulatory and kinetic properties (2, 4, 7, 31, 32). We, therefore, examined the effects of dipyridamole and M&B 22,948 on nonactivated PDEs. Although dipyridamole inhibited trypsin-activated rod and cone PDEs, when analyzed at millimolar substrate levels it had complex effects on purified nonactivated rod or cone PDE (Fig. 4). Whereas low drug concentrations had no effect, intermediate concentrations (1–10 μM) of the drug stimulated both rod and cone PDE activity; higher concentrations inhibited the activity. The stimulation exerted by

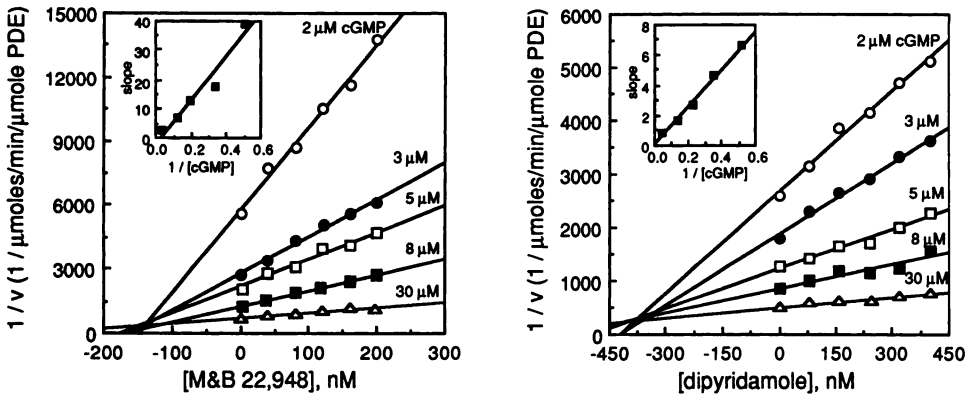

Fig. 3. Dipyridamole and M&B 22,948 are competitive inhibitors of trypsin-activated rod PDE. Assays were conducted as described in Experimental Procedures except that [^{3}H]cGMP was present at the indicated concentrations. Hydrolysis of cGMP was less than 15% of total at each point. *Inset*, replot of the slopes of the Dixon plots versus the reciprocal of the corresponding cGMP concentration. The intersection of the Dixon plot lines above the x-axis and the linear replot that goes through zero both indicate that the inhibition is simple competitive (30).

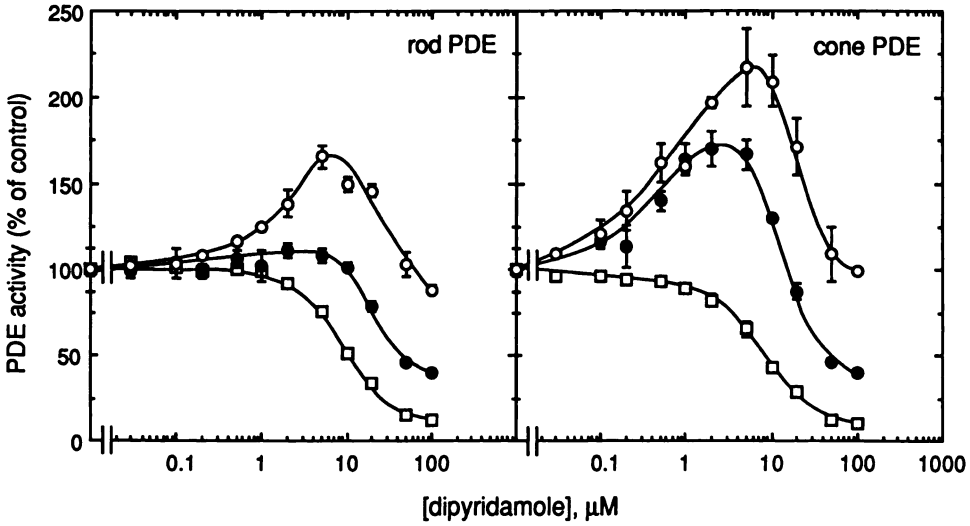

Fig. 4. Dipyridamole has stimulatory and inhibitory effects on nonactivated, but not trypsin-activated, purified rod and cone PDEs. Purified rod or cone PDEs were preincubated at 30° with the indicated concentrations of dipyridamole for 10 min using standard phosphate-release assay conditions (see Experimental Procedures). Cyclic GMP was added and the samples were assayed for 3 min. □, Rod (0.25 nM) or cone (1 nM) PDE preactivated with trypsin and assayed with 1 mM cGMP; ●, nonactivated rod or cone (1 nM) PDE assayed with 1 mM cGMP; ○, nonactivated rod or cone (1 nM) PDE assayed with 5 mM cGMP. Control (no dipyridamole) activities: trypsin-activated rod PDE, 5390 sec^{-1}; nonactivated rod PDE, 560 sec^{-1} at 1 mM cGMP and 580 sec^{-1} at 5 mM cGMP; trypsin-activated cone PDE, 3110 sec^{-1}; nonactivated cone PDE, 330 sec^{-1} at 1 mM cGMP and 330 sec^{-1} at 5 mM cGMP. *Error bars* represent ± 1 SD (three determinations).

dipyridamole was greatest when PDE activity was assayed at high cGMP concentrations and was not seen at lower substrate levels (Fig. 4); in addition, the stimulation of the cone PDE exceeded that of the rod PDE. No stimulation of transducin-activated rod PDE was observed, even when assayed with 10 mM cGMP. M&B 22,948 also had biphasic effects on purified unactivated rod and cone PDEs (data not shown). The stimulatory effects of dipyridamole or M&B 22,948 were maximal only if the PDE was exposed to the drug, in the presence of Mg^{2+}, for at least 2 min before cGMP was added for the assay (data not shown). Finally, the stimulation exerted by these compounds was observed with three separate PDE assays, the [^{3}H]cGMP assay, the phosphate-release assay, and the pH assay (data not shown), eliminating assay artifact as the source of the observed effects.

Biphasic effects on PDE activity of purified rod outer segments. In the intact photoreceptor, rod PDE is bound to outer segment membranes (6). Because the effects of the drugs might be different when PDE was bound to a membrane, we examined the effects of dipyridamole and M&B 22,948 on the basal PDE activity of purified broken rod outer segments. The stimulatory effect of dipyridamole was also seen when this PDE activity is measured at varying cGMP concentrations (Fig. 5). As with the purified PDE, the stimulation was much greater at very high cGMP concentrations. Furthermore, whereas M&B 22,948 exhibited the same biphasic effects on basal rod outer segment activity, IBMX did not appear to stimulate PDE activity (Fig. 6). Again, stimulation was only seen at millimolar substrate levels.

The data in Fig. 5 were fit with a simple model that assumes dipyridamole binds to an activator site and, under the conditions of the experiment, does not interact with cGMP at this side. The model makes no assumptions, however, about the nature of the activator site or mechanism of activation. Dipyridamole also is assumed to inhibit both stimulated and unstimulated PDE activity with the same K_i that was determined for the trypsin-activated enzyme (380 nM). Using this model, the data are fit reasonably well assuming that the K_a for dipyridamole activation of rod PDE is 10 μM and that the V_{max} in the presence of dipyridamole is 225% of the V_{max} determined without the drug. The qualitative features of the stimulation (particularly the requirement for high substrate concentration) are mirrored in the curves generated by the model. Curves generated by an alternative model, wherein the K_m of the stimulated enzyme increases by 10-fold, did not fit the data as well as the model that did not change K_m (not shown), suggesting that the drug-stimulated activity is unlike the stimulated PDE activity in broken rod outer segment suspensions stimulated by light, where K_m rises 10-fold or greater (31).

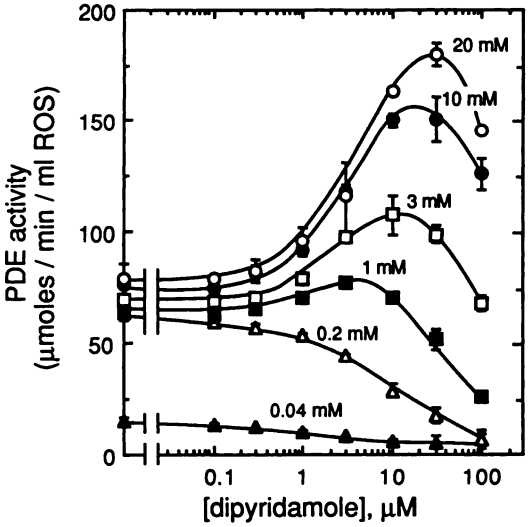

Fig. 5. Dipyridamole has stimulatory and inhibitory effects on nonactivated rod outer segment PDE activity. Rod outer segments (*ROS*) (0.3 μM rhodopsin) were preincubated at 30° for 3 min in the presence of various concentrations of dipyridamole in a buffer containing 150 mM NaCl, 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin, and *C. atrox* snake venom in a microtiter plate. No triphosphates or analogs (i.e., GTP or GTPγS) were present. Cyclic GMP was added to the samples at the indicated concentrations and the samples were assayed for 2 min. After the reaction was stopped, the samples were spectrophotometrically assayed for phosphate. △, 20 mM cGMP; ■, 10 mM cGMP; □, 3 mM cGMP; ●, 1 mM cGMP; ○, 0.2 mM cGMP. *Curves* drawn use the model described in Experimental Procedures, with the following parameters: K_a = 10 μM, K_m = 20 μM, K_i = 380 nM, V_{max} in the presence of drug = 225% of control. *Error bars* represent ± 1 SD (three determinations).

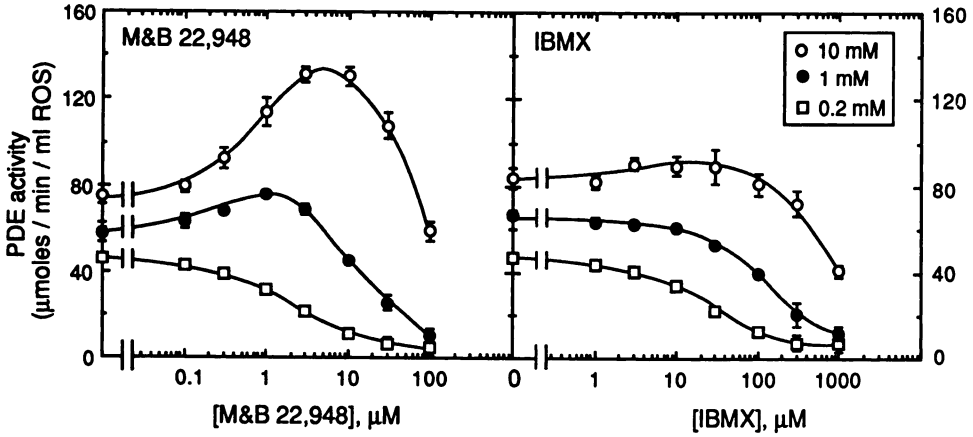

Fig. 6. M&B 22,948 but not IBMX has stimulatory effects on nonactivated broken rod outer segment PDE activity. Rod outer segments (*ROS*) were broken open by freeze/thaw. They were then assayed for PDE activity wit the phosphate-release assay, using the conditions described in Fig. 5. *Error bars* represent ± 1 SD (three determinations).

Examination of mechanism of action of stimulation. Dipyridamole or M&B 22,948 must bind to one of three classes of sites on the rod or cone PDE: (i) the noncatalytic cGMP binding site (2, 4, 5); (ii) the catalytic site, perhaps in an inactive state; or (iii) a third class of site, as yet undescribed. In order to address the mechanism of action of the drugs, we examined the effects of the drugs on [^{3}H]cGMP binding to the cone PDE (Fig. 4). M&B 22,948 (Fig. 7) and dipyridamole (data not shown) stimulated [^{3}H]cGMP binding to cone PDE at drug concentrations that inhibited PDE activity. No inhibition was seen. This observation suggests that the stimulatory effects of this drug on PDE activity were not due to binding of the drug to the noncatalytic cGMP binding site. However, the conditions required for measurement of cGMP binding (nanomolar cGMP and the presence of purified γ-subunit) are different from those used for the demonstration of the stimulatory effects of the drugs. The presence of γ-subunit was necessary to prevent hydrolysis of the [^{3}H]cGMP used for measuring cGMP binding, particularly at low concentrations of the drug. Dipyridamole also stimulates [^{3}H]cGMP binding to CGB-PDE (16).

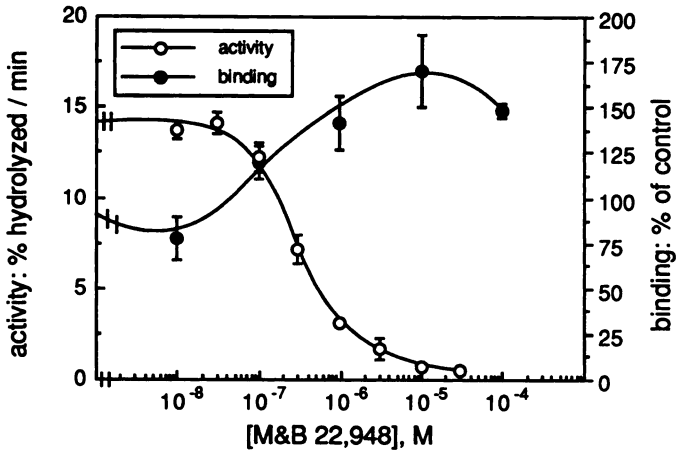

Fig. 7. M&B 22,948 increases [^{3}H]cGMP binding to cone PDE. *Binding*, purified cone PDE (2.6 nM) was preincubated at 4° for 1 hr with 24 nM purified rod PDEγ-subunit, 10 mM EDTA, 1 mM dithiothreitol, 50 mM Tris at pH 7.5, and various concentrations of M&B 22,948. The excess γ-subunit minimized hydrolysis of cGMP used in the binding reaction. After the preincubation, [^{3}H]cGMP (10 nM) was added and the binding reaction was allowed to proceed for 1 hr. Samples were then filtered on nitrocellulose filters, which were dried at 80°, solubilized with methoxyethanol, and counted. *Activity*, the PDE activity was measured under exactly the same conditions as the binding except that no rod γ-subunit was present. Error bars represent ± 1 SD (three determinations).

Further evidence against the noncatalytic site being responsible for stimulation is shown in Fig. 8. When preincubated with cone PDE before adding dipyridamole, 8-Br-cGMP [and cGMP and (Sp)cGMP[S], not shown] blocked the stimulation of PDE activity under conditions where 8-Br-cGMP did not inhibit catalysis. The half-maximally effective concentrations of (Sp)cGMP[S] (17 μM; two experiments) and 8-Br-cGMP (2.3 mM; two experiments) that were required to block dipyridamole stimulation are, however, several orders of magnitude greater than the corresponding K_i values for the high affinity noncatalytic cGMP binding site (4 and 62 nM) (2). Thus, at micromolar cGMP analog concentrations, the high affinity noncatalytic cGMP binding site should be fully saturated by the analog, yet the stimulation by dipyridamole remained maximal. Because it was rapidly hydrolyzed during the preincubation, the half-maximally effective concentration of cGMP was

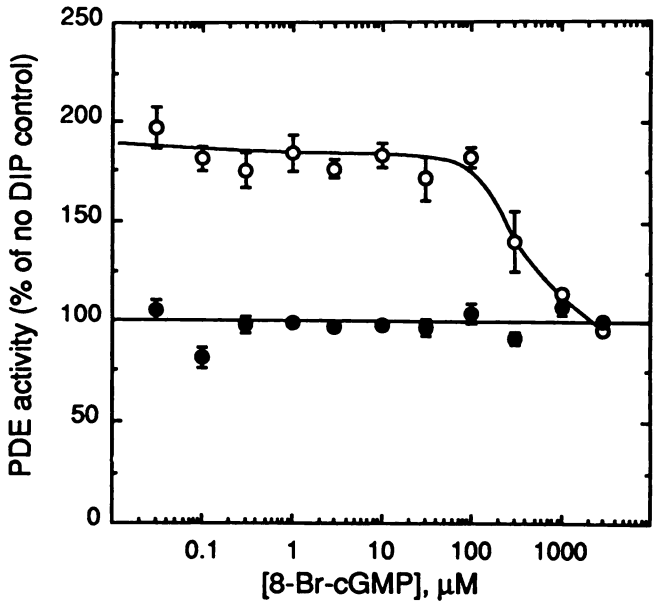

Fig. 8. 8-Br-cGMP blocks the stimulation of cone PDE activity by dipyridamole. PDE (0.5 nM) was incubated for 15 min at 30° in a microtiter plate with 40 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin, indicated concentrations of 8-Br-cGMP, and 0 or 10 μM dipyridamole. Cyclic GMP (10 mM final) was added and the samples were assayed for 2 min (phosphate assay). ○, 10 μM dipyridamole during preincubation and assay; ●, control with no dipyridamole. *Error bars* represent ± 1 SD (three determinations). PDE activity is plotted relative to that activity in the absence of dipyridamole and 8-Br-cGMP. Qualitatively similar results were obtained with both cGMP and (Sp)cGMP[S].

more difficult to measure. If the preincubation was done in the presence of EDTA, and then Mg^{2+} and 5 mM cGMP were added for the assay, however, the half-maximally effective concentration of cGMP was about 10 μM. Interestingly, whereas cGMP, (Sp)cGMP[S], and 8-Br-cGMP antagonized dipyridamole stimulation, preincubation with either $O^{2'}$-monobutyryl-cGMP or N^6,$O^{2'}$-dibutyryl-cGMP led to stimulation of unactivated cone PDE, when assayed with 10 mM cGMP (data not shown).

Dipyridamole could also exert its action by increasing the dissociation of cGMP from the high affinity noncatalytic binding site. This possibility was examined by saturating cone PDE with [^{3}H]cGMP under conditions that promote cGMP binding (1 mM IBMX, 10 mM EDTA) and then removing free [^{3}H]-cGMP, IBMX, and EDTA by gel filtration at 4°. The dissociation of [^{3}H]cGMP from the cone PDE was then examined under conditions identical to those used for the demonstration of dipyridamole stimulation of PDE activity. Dipyridamole (10 μM) had no effect on the dissociation of [^{3}H]cGMP at 30°, in the presence or absence of 10 mM cGMP (data not shown). These results (and those above) indicate that it is unlikely that the stimulation of PDE activity by dipyridamole or M&B 22,948 involves direct binding of the drugs to the high affinity noncatalytic cGMP binding site.

The block of stimulation by preincubation with cGMP and analogs raised the possibility that cGMP normally inhibits rod and cone PDE activity and that the stimulation exerted by the drugs resulted from occupation of the inhibitory site. Furthermore, whereas most reports of unactivated photoreceptor PDE activity have indicated Michaelis-Menten kinetics, one report (33) suggested that unactivated rod PDE displayed complex kinetics at cGMP concentrations of 1–10 μM. The unactivated cone PDE, however, displays normal Michaelis-Menten kinetics between 0.15 and 20 μM cGMP (Fig. 9), with a K_m (16 μM) that is nearly identical to that obtained for the trypsin-activated enzyme (2).

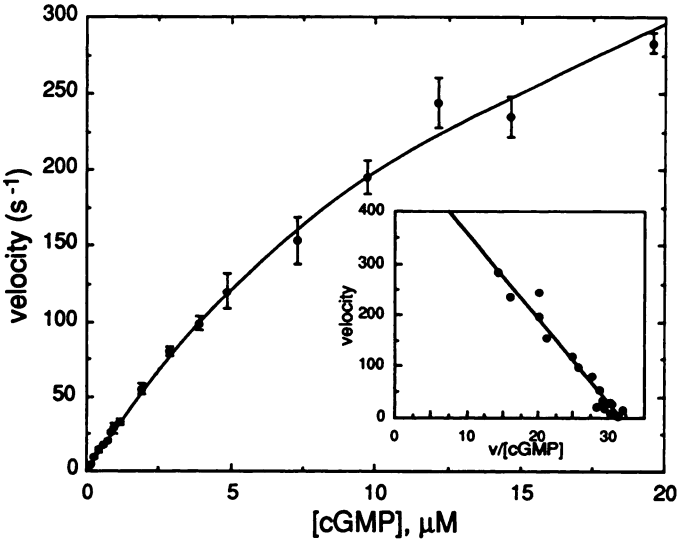

Fig. 9. Kinetics of hydrolysis of low concentrations of cGMP by cone PDE. Cone PDE (50 pM final) was mixed with 40 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin, and 0.15–20 μM [^{3}H]cGMP for 2 min at 30°. The velocity (mol of cGMP hydrolyzed/mol of PDE/sec) is plotted against the average cGMP concentration during the assay (corrected for substrate hydrolysis of 11% or less at all points). *Error bars* represent ± 1 SD (three determinations). *Inset*, a Woolf-Augustinsson-Hofstee plot (30); from these data, K_m = 16 μM and V_{max} = 520 sec^{-1}.

Discussion

Both dipyridamole and M&B 22,948 have been reported to be selective inhibitors of "cGMP-PDE" activity in several tissues (21). Several different PDE isozymes will hydrolyze cGMP, however, and our current view is that these inhibitors most potently inhibit members of the cGMP-binding, cGMP-specific PDE family of isozymes (8). The data presented here demonstrate that both dipyridamole and M&B 22,948 are potent inhibitors of the trypsin-activated photoreceptor PDE. Thus, these data add to the structural and functional similarities between the cGMP-binding, cGMP-specific PDE and the photoreceptor PDEs and suggest that both types of PDE can be considered as members of one larger isozyme family. It will be interesting to see whether amino acid sequence homology underlies these similarites.

The effects of these drugs on trypsin-activated photoreceptor PDEs are relatively uncomplicated. Trypsin activates rod and cone PDEs at least in part by proteolyzing the inhibitory subunits (2, 7). The results described here suggest that, when the inhibitory constraints are removed, dipyridamole and M&B 22,948 inhibit rod and cone PDE catalytic activity with simple competitive kinetics. This suggestion is confirmed by the Dixon plots and the corresponding replots (Fig. 3).

More interestingly, the drugs have biphasic effects on PDE that has not been activated by trypsin. At low substrate concentrations (i.e., below 1 mM), PDE activity is inhibited at all drug concentrations examined (Fig. 5). At higher substrate concentrations (i.e., 1 mM and above), low concentrations of either drug stimulate PDE activity. Moreover, another commonly used nonspecific PDE inhibitor, IBMX, does not stimulate photoreceptor PDE activity. IBMX does have biphasic effects on the activity of another PDE isozyme, the cGMP-stimulated PDE, but only at low substrate concentrations (34). Thus, the mechanism of stimulation of the photoreceptor PDE by dipyridamole and M&B 22,948 differs from that of the cGMP-stimulated PDE by IBMX.

Because the stimulatory effect is seen with three separate PDE assays, it is unlikely to be the result of an assay artifact. Instead, it is probable that dipyridamole and M&B 22,948 directly bind to a site on the PDE that leads to stimulation of activity. This stimulation site for the drugs may be one of three possible types, the high affinity noncatalytic cGMP binding site, the catalytic site, or a third, as yet undescribed, site. The stimulation has four characteristics that restrict the possible mechanisms. First, because cGMP and cGMP analogs can block the stimulation, they can influence and presumably bind to the drugs' stimulatory site. Second, an incubation of several minutes is necessary, in the absence of cGMP and the presence of Mg^{2+}, before the maximal stimulation is exerted by these drugs. If the apparent K_a determined from the experiment in Fig. 5 (10 μM) is accurate, it is unlikely that this time reflects a slow on-rate of the drug. Instead, a slow conformational change may be required before stimulation (and perhaps the release of a γ-subunit) can take place. Third, in the absence of the drugs, there is no indication for any allosteric behavior by the PDE. Finally, the stimulation is seen only at high substrate concentration.

Upon first examination, the requirement for high cGMP concentrations for the stimulatory effect appears to argue against any physiological significance for the stimulation exerted by these drugs. The data can be fit well by at least one

model (Eq. 1 in Experimental Procedures), however, wherein the high substrate concentrations are necessary simply because of competitive interaction between the drugs and cGMP at the catalytic site. Although this model explicitly disallows any interaction between cGMP and the drugs at the site responsible for stimulation, the experiments are conducted with drug added first. Thus, the block of drug stimulation by cGMP is not inconsistent with the model; if the drug were to dissociate during the assay period, however, the extent of maximal activation would be underestimated due to binding of cGMP to the drug site. This possible inaccuracy and others (e.g., the K_i value used in the model was determined from the trypsin-activated rod PDE) currently prevent application of the model to the intact cell. Nevertheless, an interesting implication of this model is that, if a compound is a pure activator, i.e., with no interaction with the catalytic site, it would stimulate PDE activity at any cGMP concentration. Whether any endogenous compounds exist that might utilize this mechanism of stimulation of the PDE is unknown.

Several experiments demonstrate that the drugs do not exert their stimulatory effects either by binding to the high affinity, noncatalytic cGMP binding site or by decreasing cGMP occupancy at this site. Because the concentrations of cGMP used to assay the PDE are many orders of magnitude above the K_d for these sites, if the drugs do not affect binding the sites should be saturated during the stimulation of PDE activity. At drug concentrations where PDE activity is stimulated, dipyridamole and M&B 22,948 do not decrease [^{3}H]cGMP binding to the cone PDE noncatalytic cGMP binding site (Fig. 7). In fact, binding is increased nearly 2-fold. Furthermore, although cGMP and other analogs block the stimulation exerted by dipyridamole, the concentrations required are several orders of magnitude greater than would be expected if this effect was due to occupation of the high affinity binding site. Finally, although it is possible that the cGMP binding site allosterically regulates the catalytic site, no such regulation has been demonstrated. For these reasons, it seems unlikely that the site to which these drugs bind (and that is responsible for the stimulation of PDE activity) is the noncatalytic binding site. In addition, the lack of effect of dipyridamole on the off-rate of [^{3}H]cGMP from the high affinity binding site indicates that dissociation of cGMP is not involved in the dipyridamole stimulation.

The catalytic site is the other known nucleotide binding site that could be responsible for the drugs' stimulation; because both agents competitively inhibit trypsin-activated PDE, they apparently are capable of binding to this site. If the drugs bound to an inhibited catalytic site, they might stimulate activity at the other catalytic site of the PDE dimer through an allosteric mechanism. No allosteric catalytic behavior, however, has been shown for these PDEs. Furthermore, whereas the half-maximally effective concentration of (Sp)cGMP[S] for block of dipyridamole stimulation is similar to its K_i value for inhibition of trypsin-activated cone PDE (17 versus 21 μM) (2), 8-Br-cGMP is a much poorer blocker of stimulation (IC_{50} = 2.3 mM) than inhibitor of hydrolysis (K_i = 32 μM). This result indicates that the site with which the analogs interact, presumably the same site as the drug stimulatory site, has different characteristics than the trypsin-activated catalytic site. Although these results do not rule out the catalytic site as the stimulatory site, they make this explanation less likely. The drugs might also exert their stimulation by disrupting the $\alpha\beta$-γ interaction at a single catalytic site, although it is difficult to see how they could activate a single site without concomitantly inhibiting activity.

The experimental data are also consistent with the hypothesis that dipyridamole and M&B 22,948 stimulate PDE activity by binding to a previously undescribed regulatory site. The block of drug stimulation by cGMP suggests that it would be capable of binding to or interacting with this site. Equilibrium binding experiments with cone PDE do not show an additional class of [^{3}H]cGMP binding sites (2); if, however, the dissociation of cGMP is relatively fast (k_{off} less than 5–10 sec^{-1}), the filter binding assay used in those experiments would not be capable of detecting the site. Furthermore, there is evidence for a relatively low affinity class of cGMP binding sites on the rod PDE (2, 4, 5, 32). If there is an additional regulatory site, it will be interesting to investigate whether cGMP acts as an antagonist to the stimulation by the drugs (or another cellular factor) or whether it acts as an inhibitory agonist (so that the drugs prevent access of cGMP to this site).

One goal of these experiments was to find new high affinity inhibitors of the photoreceptor PDEs for pharmacological studies of the light response in intact photoreceptors. Because the free concentration of cGMP in the intact rod outer segment is low (≈5 μM), it is unlikely that the stimulatory effects of the drugs will overcome their inhibitory effects. Furthermore, their effects on transducin-activated PDE activity should be strictly inhibitory. In fact, studies of the effects of these drugs on lizard photoreceptors voltage-clamped with the tight-seal, whole-cell, recording method have indicated that they potently inhibit the photocurrent sensitivity (35). The details of their effects on dark- and photo-currents are, however, unlike those of IBMX. Thus, these inhibitors may be useful for exploring regulation of the PDE and other components of the photoreceptor transduction system.

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