Potency and Mechanism of Action of E4021, a Type 5 Phosphodiesterase Isozyme-Selective Inhibitor, on the Photoreceptor Phosphodiesterase Depend on the State of Activation of the Enzyme

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This paper is available online at http://www.molpharm.org

ABSTRACT

The ability of inhibitors selective for the type 5 phosphodiesterase isozyme (PDE5) to act on the photoreceptor PDE isozyme (PDE6, the central effector enzyme for visual transduction) is poorly understood. Because PDE5 inhibitors are currently used as therapeutic agents, it is important to assess the potency and mechanism of action of this class of PDE inhibitor on PDE6. We show that E4021 (sodium 1-[6-chloro-4-(3,4-methylenedioxybenzyl)-aminoquinazolin-2-yl]piperidine-4-carboxylate sesquihydrate) inhibits activated PDE6 (K_I = 1.7 nM) as potently as PDE5. This makes E4021 the most potent inhibitor of PDE6 discovered to date. The effectiveness of E4021 to inhibit nonactivated PDE6 (with bound inhibitory γ subunits) is reduced 40-fold compared with the activated enzyme. Furthermore, at intermediate E4021 concentrations and high cGMP concentrations, nonactivated PDE undergoes activation of cGMP hydrolysis rather than inhibition. We demonstrate direct competition of E4021 and the γ subunits for binding to the catalytic site. Measurements of cGMP binding to noncatalytic regulatory sites on the catalytic subunits of PDE6 rule out an allosteric effect of E4021 by direct binding to these noncatalytic sites. We conclude that E4021 is a competitive inhibitor of cGMP hydrolysis and that the γ subunit also competes with both E4021 and substrate for catalytic site binding. An understanding of the effects of PDE5-targeted drugs on retinal PDE6 requires a knowledge of the complex interactions among substrate, drug, and inhibitory γ subunit at the catalytic site of both nonactivated and activated forms of PDE6.

The phosphodiesterase (PDE) isozymes found in rod and cone photoreceptor cells of the retina belong to a large family of cyclic nucleotide PDEs that catalyze cAMP and cGMP hydrolysis. The photoreceptor PDE [constituting the photoreceptor phosphodiesterase (PDE6) subfamily] is the central effector enzyme involved in the pathway of visual excitation in vertebrate photoreceptors (Pfister et al., 1993; Pugh and Lamb, 1993; Takemoto et al., 1993; Helmreich and Hofmann, 1996). In the dark-adapted state, the rod PDE holoenzyme consists of a catalytic heterodimer (P$\alpha\beta$) tightly associated with two inhibitory γ subunits (Pγ); this $\alpha\beta\gamma_2$ holoenzyme is the nonactivated state of the enzyme. The carboxyl terminus of the Pγ subunit is thought to directly bind to the active site and inhibit cGMP hydrolysis in a competitive manner

(Granovsky et al., 1997). Activation of the visual pigment rhodopsin by illumination results in activation of the retinal G protein transducin. The activated form of transducin relieves the inhibitory constraint of the Pγ subunit and activates PDE >300-fold in vitro (Arshavsky et al., 1992). Subsecond decreases in cGMP levels resulting from light-activated PDE6 directly regulate the cyclic nucleotide-gated ion channel, leading to the photoreceptor receptor potential (Koutalos and Yau, 1993; Yarfitz and Hurley, 1994; Palczewski and Saari, 1997). In addition to the catalytic sites, the α and β subunits of PDE6 contain noncatalytic cGMP binding sites, which can bind 2 mol of cGMP/mol of PDE holoenzyme with high affinity (Yamazaki et al., 1980; Gillespie and Beavo, 1989b; Cote and Brunnock, 1993). Activation of PDE by transducin reduces the binding affinity of cGMP at the noncatalytic sites (Yamazaki et al., 1982; Cote et al., 1994).

The PDE6 subfamily is most closely related to the PDE5

ABBREVIATIONS: E4021, sodium 1-[6-chloro-4-(3,4-methylenedioxybenzyl)-aminoquinazolin-2-yl]piperidine-4-carboxylate sesquihydrate; PDE, phosphodiesterase; Pγ, inhibitory γ subunit of type 6 phosphodiesterase; P$\alpha\beta$, catalytic heterodimer of type 6 phosphodiesterase; PDE5, cGMP-binding cGMP phosphodiesterase; PDE6, photoreceptor phosphodiesterase; ROS, rod outer segments; zaprinast, 2-o-propoxyphenyl-8-azapurin-6-one; IBMX, 3-isobutyl-1-methylxanthine; K_D, dissociation constant; K_I, drug inhibition constant; Pγ-1–83BC, recombinant Pγ mutant in which residues 83–87 are deleted and replaced with a cysteine, followed by labeling with 3-(bromoacetyl)-7-diethylaminocoumarin.

(cGMP binding, cGMP-specific PDE) isozyme, as judged by several criteria: 1) similarity of the amino acid sequence of the catalytic subunits (McAllister-Lucas et al., 1993); 2) strong preference for cGMP over cAMP as a substrate (Baehr et al., 1979; Thomas et al., 1990); 3) presence of a putative PDE5-associated, low-molecular-weight protein immunologically related to the PDE6 Pγ subunit (Lochhead et al., 1997); and 4) pharmacological properties (Gillespie and Beavo, 1989a).

In the only pharmacological comparison of the efficacy of different PDE inhibitors to block PDE6 catalysis, Gillespie and Beavo (1989a) reported that two inhibitors that preferentially act on PDE5 (i.e., zaprinast and dipyridamole) were also good inhibitors of PDE6. Surprisingly, these two drugs could also activate PDE6 catalysis under certain conditions (Gillespie and Beavo, 1989a). However, this study was unable to determine the molecular basis by which these drugs exerted both inhibitory and stimulatory effects on PDE6.

The interest in PDEs as molecular targets of drug action has grown with the development of isozyme-selective PDE inhibitors that offer potent inhibition of selected isozymes without the side effects attributed to nonselective inhibitors such as theophylline. PDE5-selective inhibitors show promise as therapeutic agents in several areas, including pulmonary and cardiovascular diseases (Sybertz et al., 1995). However, little is known about the potency, selectivity, and mechanism of action of PDE5-selective inhibitors with PDE6. We have chosen to examine E4021 (Takase et al., 1993, 1994; Saeki et al., 1995), a PDE5 inhibitor that is significantly more potent and selective than zaprinast (an early PDE5 inhibitor) and comparable in its PDE inhibitory properties to sildenafil (Viagra; Ballard et al., 1998), a drug recently approved for the treatment of male erectile dysfunction.

In this article, we report on the mechanism of action of E4021 on both the nonactivated and activated forms of rod PDE6 because both states are relevant to understanding how PDE5-selective inhibitors may alter signal transduction pathways in photoreceptor cells. Our results are consistent with a single site of action of E4021 at the catalytic site of the enzyme. However, the effects of E4021 on PDE6 depend on whether the Pγ subunits are associated with the catalytic Pαβ dimer. We conclude that the mutually exclusive binding of drug, Pγ, and cGMP at the catalytic site must be accounted for when evaluating the potency and selectivity of PDE5-targeted drugs to alter PDE6 activity in retinal photoreceptor cells.

Experimental Procedures

Materials. E4021 was a gift of Eisai Co., Ltd. (Tokyo, Japan), and zaprinast was a gift from Rhone-Poulenc Rorer (Dagenham, UK). Stock solutions of the PDE inhibitors were prepared as follows: 100 mM E4021 was prepared fresh daily in 1-methyl-2-pyrrolidinone; 400 mM zaprinast was dissolved in 1-methyl-2-pyrrolidinone and stored for up to 3 months at −20°C; and 600 mM 3-isobutyl-1-methylxanthine (IBMX) was prepared in 1-methyl-2-pyrrolidinone and stored at −20°C.

The Ringer's solution used to isolate the rod photoreceptors contained 105 mM NaCl, 2 mM KCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$, and 10 mM HEPES, pH 7.5. The PDE activity assay medium contained 100 mM Tris, pH 7.5, 10 mM MgCl$_2$, 0.5 mM EDTA, 2 mM dithiothreitol, 0.5 mg/ml BSA, 5 μM leupeptin, and 50 kallikrein-inactivating unit (KIU)/ml aprotinin. The binding assay contained 100 mM

Tris, pH 7.5, 10 mM EDTA, 0.5 mg/ml BSA, 5 μM leupeptin, and 50 KIU/ml aprotinin.

Radiochemicals were purchased from New England Nuclear (Boston, MA). Unless noted above, all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Preparation of PDE. Purified frog rod outer segments (ROS) were isolated in the dark (using infrared illumination) using modifications of a method described previously (Cote and Brunnock, 1993). Briefly, frog retinas were removed from enucleated eyes, and ROS were detached from the retinas by gentle shaking into a Ringer's solution containing 5% Percoll. The ROS were then purified by centrifugation in a discontinuous Percoll gradient consisting of 5%, 30%, and 60% Percoll. After dilution of the Percoll with Ringer's and brief centrifugation of the ROS (1 min at 3000g), the ROS were resuspended in the appropriate assay medium and homogenized at 4°C using a nylon pestle and glass mortar. No structure was detectable by phase-contrast microscopy after homogenization. Endogenous nucleotides were depleted by incubating the homogenized ROS at room temperature for ≥30 min; this treatment has been shown to remove >95% of the endogenous, bound cGMP (Cote and Brunnock, 1993).

The PDE concentration was routinely determined by measuring the rhodopsin concentration spectrophotometrically (Bownds et al., 1971), along with a knowledge of the molar ratio of rhodopsin/PDE (330:1) in frog ROS (Cote and Brunnock, 1993). In some experiments, we checked this estimate of PDE concentration by also measuring the maximum extent of binding of saturating (1 μM) amounts of [^{3}H]cGMP to the high-affinity sites on nonactivated PDE, assuming a binding stoichiometry of two high-affinity noncatalytic sites per holoenzyme (Gillespie and Beavo, 1989b; Cote et al., 1994). [Note that PDE is the only high-affinity cGMP binding protein in Percoll-purified ROS (Cote and Brunnock, 1993; Cote et al., 1994).] In some instances, we also measured the rate of cGMP hydrolysis by transducin-activated PDE at saturating concentrations of cGMP (10 mM) to obtain the V_{max} for our sample, and we calculated the enzyme concentration with a knowledge of the k_{cat} (4400 cGMP hydrolyzed/s/Pαβ; Dumke et al., 1994).

To prepare activated PDE lacking bound Pγ subunits, we performed limited proteolysis to degrade the inhibitory Pγ subunits (Hurley and Stryer, 1982). ROS homogenates (40 μM rhodopsin) were incubated with 100 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin for 10 min at 4°C and then mixed with a 6-fold excess of soybean trypsin inhibitor; the PDE activity assay medium lacked leupeptin and aprotinin during the trypsinization step. The proteolysis conditions were optimized to minimize exposure to the protease while achieving maximal hydrolytic rates. The addition of 2 mol of recombinant Pγ/mol of Pαβ inhibited >90% of the enzyme activity and restored its ability to undergo stimulation by E4021 (under the conditions described in Fig. 4), demonstrating that the activation by trypsin was directed at the Pγ subunits and did not significantly affect the catalytic sites of the enzyme.

PDE Activity Assay. Nucleotide-depleted PDE was diluted to 45 μl with PDE activity assay medium. Unless indicated, PDE was preincubated with the inhibitor for 15 min at 21°C before initiating the PDE activity assay with the addition of 5 μl of the cGMP substrate. The reaction was stopped by quenching 10-μl samples in 50 μl of 100 mM HCl at various times. To quantify the amount of cyclic nucleotide that was hydrolyzed, each sample was neutralized with 50 μl of 100 mM Trizma base, treated with 10 μl of 12.5 mg/ml snake venom (to convert the 5′-nucleoside monophosphate to the nucleoside plus inorganic phosphate), and analyzed using either a radiotracer assay followed by separation of nucleotides by diethylaminoethyl anion exchange chromatography (Kincaid and Manganiello, 1988) or a colorimetric assay to quantify the amount of inorganic phosphate (Artemyev et al., 1998). Total substrate hydrolysis was <30% in all experiments. The calculation of the rate of cyclic nucleotide hydrolysis was based on three to six individual time points during which the time course remained linear.

Filter Binding Assay of cGMP Binding to Noncatalytic Sites of PDE. Nucleotide-depleted PDE holoenzyme was diluted to 7.5 nM with binding assay medium and incubated overnight at 4°C with 10 mM EDTA to prevent hydrolysis of [^{3}H]cGMP used in the binding assay. [EDTA inhibits hydrolytic activity by chelating divalent cations, which are a required cofactor for catalysis (Srivastava et al., 1995). The long incubation period for effective inhibition probably reflects slow dissociation of divalent cations from the active site due to high-affinity binding of Pγ to this region (Granovsky et al., 1997). The effects of EDTA on PDE function were limited to inhibition of the catalytic site and were fully reversible on the addition of excess Mg^{2+}.] The PDE was then mixed with the indicated concentration of PDE inhibitor, and preincubated for 15 min at 21°C. The sample was cooled to 4°C, and the binding assay was initiated with [^{3}H]cGMP (final PDE concentration, 3 nM). After incubating for 1 h at 4°C, 50-μl samples were filtered in duplicate on prewet nitrocellulose filters (HAWP 025; Millipore) using a vacuum manifold system. PDE activity measurements showed that $\leq$2% of the cGMP was hydrolyzed during the filter binding assay in the absence of PDE inhibitor.

The filters were then rinsed with three 1-ml portions of the ice-cold binding assay medium. Sample application and filter washing were completed within 4 s, during which <5% of the bound cGMP dissociates. Nonspecific binding was determined as described previously (Cote and Brunnock, 1993).

Fluorescent Assay of Pγ-1–83BC Binding to PDE. The ability of E4021 to displace Pγ bound to P$\alpha\beta$ was measured with a fluorescence assay essentially as described previously (Granovsky et al., 1997; Artemyev et al., 1998). Briefly, a mutant of Pγ was constructed in which the four C-terminal residues were replaced with a cysteine; this mutant was then labeled with 3-(bromoacetyl)-7-diethylaminocoumarin to form Pγ-1–83BC. Pγ-1–83BC (10 nM) was mixed with 3.6 nM trypsin-activated PDE. To perform the fluorescence measurements, the trypsin-activated PDE was separated from the ROS membranes by centrifugation (180,000g for 5 min at 21°C) before addition to the cuvette.

The fluorescence change on binding and dissociation of Pγ-1–83BC with P$\alpha\beta$ was monitored in an Aminco-Bowman Series 2 luminescence spectrometer in 600 μl of modified PDE assay buffer (lacking protease inhibitors and with BSA reduced to 0.1 mg/ml). Fluorescence was monitored with excitation at 445 nm and emission at 495 nm. The displacement of bound Pγ-1–83BC by E4021 was determined by adding increasing concentrations of drug to the cuvette (total volume change, <3%). Control experiments indicated that E4021 addition alone caused no fluorescence change to γ-1–83BC solutions. Assays were conducted at equilibrium.

Data Analysis. Dose-response curves at a single substrate concentration and in the absence of drug-induced stimulation were fit to the Hill equation using nonlinear least-squares analysis. The IC$_{50}$ value determined in this way was related to the drug inhibition constant (K_I) by the following equation (Cheng and Prusoff, 1973): K_I = IC$_{50}$/(1 + [S]/K_M), where [S] is the substrate concentration, K_M is the Michaelis constant, and the mechanism of inhibition is assumed to be competitive. For nonactivated PDE holoenzyme, a K_M value for cGMP (95 μM) was verified in our laboratory to be the same as that reported earlier (Dumke et al., 1994); for trypsin-activated PDE, the K_M for cGMP hydrolysis was experimentally determined to be 21.8 ± 2.2 μM (mean ± S.E.M., n = 7). Kinetic analyses over a range of cGMP and E4021 concentrations were used to more accurately determine the K_I value using a Dixon plot (Segel, 1975). Analysis of fluorescence data was performed as described previously (Granovsky et al., 1997). All experiments were repeated at least three times, and average values are reported as the mean ± S.E.M.

Results

E4021 Is a Simple Competitive Inhibitor of Activated PDE6. We first examined the potency of E4021 to inhibit the activated form of frog rod PDE6 in which the Pγ subunits

have been removed by limited proteolysis. We found that E4021 is able to effectively inhibit catalysis at an IC$_{50}$ value of 70 nM (Fig. 1). This value is 20- and 3000-fold lower than the IC$_{50}$ values for zaprinast (a prototype PDE5 inhibitor) and IBMX (a nonselective PDE inhibitor), respectively (Fig. 1). With the IC$_{50}$ data in Fig.1 and knowledge of the K_M value for trypsin-activated PDE (21.8 ± 2.1 μM), we can calculate K_I values of 1.6 nM for E4021, 32 nM for zaprinast, and 4.7 μM for IBMX. These results indicate that E4021 is the most potent inhibitor of PDE6 identified to date.

To verify the mechanism of inhibition of E4021 with activated PDE, the ability of various concentrations of the drug to inhibit cGMP hydrolysis was tested at several fixed substrate concentrations. As shown in Fig. 2, a Dixon plot of the reciprocal hydrolytic velocity as a function of inhibitor concentration at several cGMP concentrations resulted in a series of lines that intersect above the x-axis. From the point of intersection, a K_I value of 2.0 nM can be inferred. To distinguish between several inhibition mechanisms that can yield similar Dixon plots, the slopes of each line were replotted as a function of the reciprocal of the substrate concentration (Fig. 2, inset). The intersection of the slope replot at the origin is consistent with a mechanism of simple competitive inhibition. The average K_I value obtained from five experiments is 1.7 ± 0.3 nM, which is in excellent agreement with the K_I value calculated from the dose-response curve in Fig. 1. We conclude that activated PDE is potently inhibited by E4021 through direct competition between the substrate and drug at the active site of the enzyme.

PDE Holoenzyme Is Inhibited by E4021 in a Complex Manner. Because PDE is normally associated with its Pγ subunits in vivo, we next tested the relative potency of E4021 to inhibit PDE holoenzyme ($\alpha\beta\gamma_2$) that was not activated. The hydrolytic activity of this PDE preparation is low compared with the maximum rate attainable for fully activated PDE; the basal rate reflects the equilibrium binding of the Pγ subunits to the catalytic P$\alpha\beta$ dimer (Hurley and Stryer, 1982;
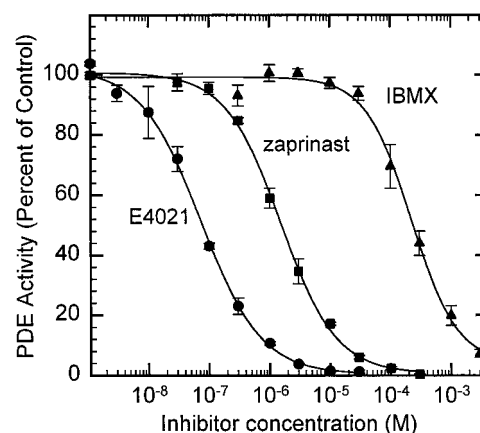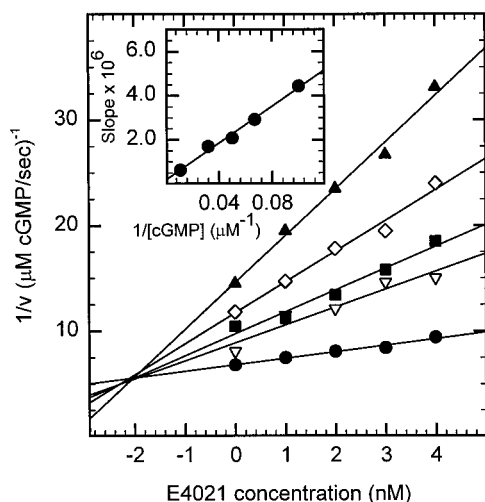

Fig. 1. E4021 is a potent inhibitor of the photoreceptor PDE. Activated PDE (0.1 nM final concentration) was prepared as described in *Experimental Procedures* and mixed with the indicated concentrations of E4021 (●), zaprinast (■), or IBMX (▲) for 15 min before the addition of 1.0 mM cGMP. Samples were quenched, and PDE activity was determined with a colorimetric assay. Results were normalized to the rate in the absence of drug (34 ± 15 pmol of cGMP hydrolyzed/s) for three separate experiments. Lines are a fit of data to the Hill equation with the following coefficients: E4021: IC$_{50}$ = 73 ± 5.0 nM, n = 0.9 ± 0.1, r^2 = 0.998; zaprinast: IC$_{50}$ = 1.5 ± 0.1 μM, n = 1.0 ± 0.1, r^2 = 0.999; and IBMX: IC$_{50}$ = 220 ± 30 μM, n = 1.2 ± 0.2, r^2 = 0.994.

Wensel and Stryer, 1986). The PDE holoenzyme preparation was incubated with inhibitor 15 min to permit the inhibitor to attain binding equilibrium at the catalytic site. After pre-incubation with the drug, the hydrolytic activity of PDE was determined by the addition of 200 μM cGMP. Figure 3 shows that even though the cGMP concentration (200 μM) is 5-fold less than that shown in Fig. 1, higher concentrations of PDE5-selective drugs were needed to inhibit 50% of the PDE activity of the nonactivated holoenzyme compared with the activated catalytic dimer. In contrast, IBMX displayed a similar potency of inhibition for nonactivated holoenzyme and activated PDE. To quantify the difference in the ability of E4021 to inhibit nonactivated and activated PDE, a Dixon plot was also constructed for PDE holoenzyme at several cGMP and E4021 concentrations. The lines intersected at a single point above the x-axis with a K_I value of 70 nM; the slope replot was linear and intersected the y-axis at the origin (data not shown). These results are consistent with E4021 acting as a pure competitive inhibitor of cGMP hydrolysis at the active site. However, the presence of the Pγ subunits associated with the P$\alpha\beta$ catalytic dimer reduce the inhibitory potency of the drug $\sim$40-fold compared with the activated P$\alpha\beta$ dimer.
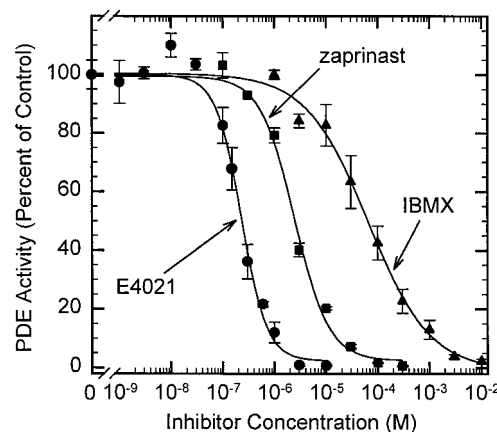
Further evidence that E4021 behaves differently when the Pγ subunits are associated with P$\alpha\beta$ was obtained by examining the behavior of the drug at high cGMP concentrations. Previously, it has been reported that at $\geq$1 mM cGMP concentrations, zaprinast can stimulate cGMP hydrolysis of bovine rod PDE holoenzyme; this stimulatory effect was not seen for activated PDE (Gillespie and Beavo, 1989a). Figure 4 shows that when the cGMP concentration is raised from 200 μM to 1 or 10 mM, submicromolar concentrations of E4021 can stimulate the rate of cGMP hydrolysis by as much

as 60%. In contrast, activated PDE is unable to be stimulated by E4021, either at 1 mM cGMP (Fig. 4, dashed line) or at 10 mM cGMP (not shown). Only the inhibitory action of the drug is apparent for the activated enzyme. It is clear from Fig. 4 that E4021 is acting on PDE holoenzyme in a complex manner, such that under certain conditions, the drug can activate the enzyme rather than simply serve as a catalytic site inhibitor.
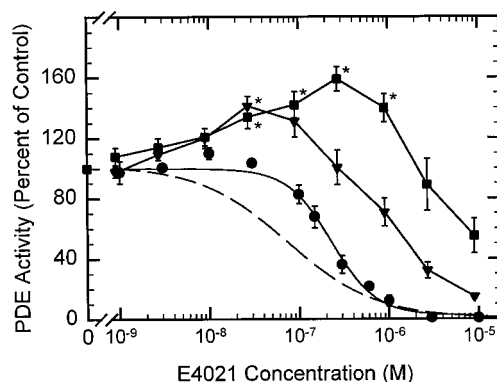
E4021 Interacts Very Weakly with Noncatalytic cGMP Binding Sites on PDE Holoenzyme. Previous work has shown that high-affinity binding of cGMP to the noncatalytic binding sites on PDE requires that Pγ be asso-

Fig. 3. Complex inhibition of nonactivated PDE by inhibitors. PDE holoenzyme (12 nM) was incubated for 15 min with the indicated concentrations of E4021 ($\bullet$), zaprinast ($\blacksquare$), or IBMX ($\blacktriangle$) as described in *Experimental Procedures*. Hydrolytic activity of PDE was determined after addition of 200 μM cGMP. PDE activity is reported as the percent of the rate observed in the absence of inhibitor: E4021, 90 $\pm$ 18 cGMP hydrolyzed/PDE holoenzyme/s ($n = 5$); zaprinast, 90 $\pm$ 17 cGMP hydrolyzed/PDE holoenzyme/s ($n = 3$); and IBMX, 120 $\pm$ 46 cGMP hydrolyzed/PDE holoenzyme/s ($n = 3$). The lines are fits to the Hill equation, with the following parameters: E4021: $IC_{50} = 230 \pm 30$ nM, $n = 1.8 \pm 0.4$, $r^2 = 0.992$; zaprinast: $IC_{50} = 2.4 \pm 0.4$ μM, $n = 1.3 \pm 0.2$, $r^2 = 0.996$; and IBMX: $IC_{50} = 65 \pm 12$ μM, $n = 0.7 \pm 0.1$, $r^2 = 0.993$.

Fig. 2. Dixon plot shows that E4021 is a simple competitive inhibitor of activated PDE. PDE activity assays were conducted with 50 pM activated PDE and at cGMP concentrations of 89 ($\bullet$), 31 ($\triangledown$), 21 ($\blacksquare$), 15 ($\diamond$), and 10 μM ($\blacktriangle$) and with the indicated amounts of E4021 added (see *Experimental Procedures*). The reciprocal of the initial rate of cGMP hydrolysis is plotted versus the final E4021 concentration. Point of intersection in this individual experiment was K_I = 2.0 nM; the average K_I value for five separate determinations was 1.7 $\pm$ 0.3 nM. Inset, slope of each line at a fixed cGMP concentration was determined by regression analysis and plotted as a function of the reciprocal substrate concentration. Intersection of the line on the y-intercept (0.16 $\pm$ 0.14) was not statistically different from zero at the 95% level of confidence, consistent with a simple competitive inhibition mechanism.
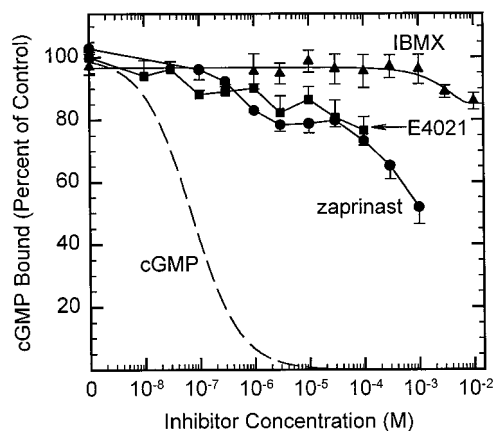

Fig. 4. E4021 stimulates the activity of PDE holoenzyme when high levels of cGMP are used. The indicated concentrations of E4021 were preincubated for 15 min with 12 nM PDE; then 0.2 mM ($\bullet$), 1.0 mM ($\blacktriangledown$), or 10 mM ($\blacksquare$), cGMP was added, and hydrolytic activity was determined. The y-axis is normalized to the rate of hydrolysis in the absence of E4021 and corresponds to 90 $\pm$ 18 (0.2 mM cGMP; $n = 5$), 122 $\pm$ 20 (1 mM cGMP; $n = 3$), or 150 $\pm$ 15 (10 mM cGMP; $n = 3$) cGMP hydrolyzed/PDE/s. The 0.2 mM cGMP line was fit using the Hill equation ($IC_{50} = 230 \pm 30$ nM, $n = 1.8 \pm 0.4$, $r^2 = 0.992$); the other curves did not fit a simple competitive displacement model. The dashed line is the dose-response function of activated PDE with 1 mM cGMP as substrate, taken from Fig. 1. *PDE activities that were significantly greater than 100% at the 95% level of confidence.

ciated with the catalytic P$\alpha\beta$ dimer (Yamazaki et al., 1980; Cote et al., 1994); we verified for this study that trypsin-activated PDE (in which the Pγ subunits have been proteolyzed) was unable to bind [^{3}H]cGMP (<0.2 mol of cGMP/mol of P$\alpha\beta$; M.R. D'Amours and R.H. Cote, unpublished observations). We then reasoned that the differences in how E4021 acts on the activated P$\alpha\beta$ dimer (Fig. 1) versus the nonactivated PDE holoenzyme (Figs. 3 and 4) might be related to the occupancy of the noncatalytic sites by cGMP in the latter case.

To test the hypothesis that E4021 exerts its complex effects by displacing bound cGMP at the noncatalytic sites, we measured the extent of [^{3}H]cGMP binding as a function of E4021 concentration. To perform these experiments, we first needed a method to study cGMP binding without significant cGMP hydrolysis. Furthermore, because of the potential involvement of the Pγ subunit in the complex effects of E4021 on PDE holoenzyme, we avoided the use of Pγ to suppress cGMP hydrolysis, as had been done previously (Gillespie and Beavo, 1989a). Instead, we found that extended incubation of PDE holoenzyme with 10 mM EDTA (to remove free divalent cations, which are required cofactors for catalysis; Srivastava et al., 1995) served to reduce cGMP hydrolysis to ≤2% of the total added [^{3}H]cGMP used for the binding assays.

Figure 5 shows that both E4021 and zaprinast are only weakly able to displace [^{3}H]cGMP from the noncatalytic binding sites on PDE, whereas IBMX is even less effective in competing at these sites. In contrast, unlabeled cGMP is ≥1000-fold more potent than these drugs in competing with radiolabeled cGMP at the noncatalytic sites (Fig. 5, dashed line). Note that E4021 and zaprinast do not substantially differ in their ability to displace cGMP at the noncatalytic sites (Fig. 5), whereas E4021 is 20-fold more potent than zaprinast as a catalytic site inhibitor (Fig. 1). This suggests that the weak effect of these PDE5-selective drugs on [^{3}H]cGMP binding is being exerted directly at the noncata-

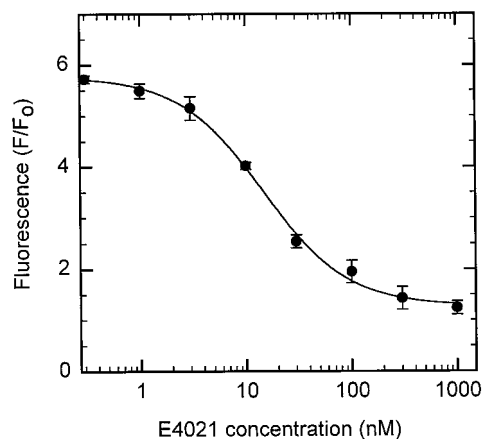lytic sites and is not an allosteric effect of drug binding to the catalytic sites. These results are consistent with previous work demonstrating the high specificity for cGMP binding exhibited by noncatalytic cGMP binding sites (Hebert et al., 1998). In summary, the anomalous effects of E4021 (described in the previous section) or zaprinast (Gillespie and Beavo, 1989a) cannot be attributed to direct binding of the drug to the noncatalytic sites of PDE6.

E4021 Is a Competitive Inhibitor of Pγ Binding to Catalytic Site on PDE. Having ruled out the noncatalytic cGMP binding sites on PDE as the site by which E4021 can activate PDE holoenzyme, we considered other potential drug binding sites that might account for the anomalous behavior of E4021. Although all of the unusual effects of E4021 are exhibited only when Pγ is present, we consider it very unlikely that E4021 can bind directly to Pγ because the Pγ subunit lacks sufficient secondary and tertiary structure (Berger et al., 1997) to form a high-affinity binding pocket for E4021 or other PDE5-selective drugs. Rather, it seems most likely that E4021 occupies a high-affinity binding site on P$\alpha\beta$ that also is a site of interaction with the Pγ subunit. Although a novel drug-binding site on PDE cannot be excluded, we first considered whether the effects of E4021 on PDE could be ascribed to high-affinity binding at the active site of the enzyme in direct competition with Pγ binding there.

To test this possibility, we used a fluorescently labeled mutant of the Pγ subunit (Pγ-1–83BC) that retains its ability to bind to and inhibit cGMP hydrolysis of bovine rod P$\alpha\beta$ dimers; the fluorescent probe attached at the truncated C-terminal end of Pγ undergoes a fluorescence change on binding to the active site of the enzyme (Granovsky et al., 1997). On incubation of activated frog PDE with Pγ-1–83BC, a 5-fold maximal increase in fluorescence occurred in a dose-dependent fashion. This fluorescence change was reversed on addition of recombinant, unlabeled Pγ subunits, demonstrating the specificity of the fluorescence change. Figure 6 shows that the addition of increasing amounts of E4021 to the P$\alpha\beta$/Pγ-1–83BC complex resulted in a progressive displacement of the fluorescent Pγ mutant with an IC$_{50}$ value of 15

Fig. 5. PDE inhibitors weakly displace [^{3}H]cGMP from noncatalytic binding sites on PDE holoenzyme. EDTA-treated PDE (final concentration 3.0 nM) was mixed with the indicated concentration of inhibitor for 15 min at 21°C before cooling to 4°C and the subsequent addition of 16 nM [^{3}H]cGMP. After a 60-min incubation period at 4°C, the amount of bound [^{3}H]cGMP was determined with a filter binding assay (see *Experimental Procedures*). The data from three separate experiments was first normalized to the extent of cGMP binding in the absence of drug (0.8 ± 0.1 cGMP bound/PDE holoenzyme). The dashed line represents the predicted displacement curve on the addition of unlabeled cGMP, assuming a K_D value of 10 nM for cGMP binding to EDTA-treated PDE holoenzyme (M. R. D'Amours and R. H. Cote, unpublished observations) and simple competitive inhibition.

Fig. 6. E4021 displaces a fluorescently labeled mutant of Pγ at the active site of PDE. Activated PDE (3.6 nM) lacking endogenous Pγ subunits was mixed with 10 nM Pγ-1–83BC (intrinsic fluorescence before adding PDE: F/F_0 = 1.2), and the fluorescence enhancement was allowed to reach equilibrium. The indicated concentrations of E4021 were added to the cuvette, and the change in fluorescence was recorded. Data points represent the mean ± S.D. of three experiments. The line is the fit to a Hill equation (IC$_{50}$ = 14.6 ± 1.6 nM, n = 1.1 ± 0.1; r^2 = 0.99).

nM and a Hill coefficient of 1.1. Although the actual K_I value for E4021 competition with Pγ-1–83BC cannot be determined from this data, it must be less than the IC_{50} value. This high-affinity competition of E4021 with Pγ at the active site is consistent with the high-affinity competition of E4021 with cGMP (K_I = 1.7 nM) to block hydrolysis. We conclude from this result that E4021, the fluorescent probe portion of Pγ-1–83BC, and cGMP all bind in a mutually exclusive fashion to the catalytic site on Pαβ. This ability of PDE5-selective inhibitors to compete not only with cyclic nucleotides but also with the binding of endogenous Pγ inhibitory subunits provides the most reasonable explanation for the complex behavior these drugs have on the PDE6 holoenzyme (see *Discussion*).

Discussion

Our results demonstrate that E4021 is the most potent inhibitor of PDE6 studied to date. However, it appears that although PDE5-selective inhibitors may show good discrimination of PDE5 from most other PDE isoforms, there is likely to be poor discrimination of these drugs between PDE5 and PDE6 isozymes. Our results also demonstrate that the efficacy and mechanism of drug inhibition depend on the subunit composition and state of activation of PDE6. Activated PDE (lacking bound Pγ) is inhibited by E4021 (Fig. 1) or zaprinast (Gillespie and Beavo, 1989a) (Fig. 1) in a simple competitive manner, whereas the nonactivated PDE holoenzyme (containing bound Pγ) is affected by PDE5-selective inhibitors in a complex manner that depends on several factors. Our results rule out a major effect of E4021 on the noncatalytic sites on PDE and support a mechanism of action in which E4021, Pγ, and cGMP all compete in a mutually exclusive manner at the active site.

Comparison of Potency and Selectivity of E4021 with Other PDE5-Selective Inhibitors. With a K_I value of 1.7 nM for the activated enzyme, E4021 is the most effective pharmacological inhibitor of PDE6 thus far reported. By comparison, older PDE5-selective drugs such as zaprinast and dipyridamole are >10-fold less potent in inhibiting the activated PDE6. Recently, another PDE5-selective drug, sildenafil, was reported to inhibit nonactivated bovine PDE6 with a K_I value of 30 nM (Ballard et al., 1998), ~2-fold lower than the K_I value for E4021 inhibition of nonactivated frog PDE6 (see *Results*). Based on the 40-fold increase in potency of E4021 with activated PDE, we predict that sildenafil may also act much more potently ($K_I \sim 1$ nM) on the activated form of PDE6.

Although E4021, sildenafil, and other recently discovered, highly potent PDE5 inhibitors show strong selectivity for PDE5 relative to the PDE1 through PDE4 isoforms (Coste and Grondin, 1995; Miyahara et al., 1995; Saeki et al., 1995; Ballard et al., 1998), it is not clear whether any of these drugs can effectively target PDE5 over PDE6. For example, E4021 has been reported to have a K_I value of 2 to 4 nM for PDE5 (Miyahara et al., 1995; Saeki et al., 1995), which is comparable to the K_I value of 1.7 nM for activated PDE6 (Fig. 2), indicating a lack of selectivity for the two isozymes. Likewise, although the K_I value of sildenafil (2–4 nM) for PDE5 (Ballard et al., 1998; Moreland et al., 1998) is significantly lower than the K_I value for nonactivated bovine PDE6 (Ballard et al., 1998), sildenafil may act on activated PDE6 with compa-

rable potency to PDE5 (see above). We conclude that E4021, and probably the other known high-potency PDE5-selective inhibitors, lack adequate selectivity for PDE5 over PDE6 to be able to target PDE5 without significantly affecting the activity of PDE6 in vivo.

Mutually Exclusive Competitive Binding of E4021 and Pγ at Active Site Can Account for Complex Effects of E4021 on PDE Holoenzyme. The high affinity with which E4021 can both inhibit cGMP hydrolysis at the active site of PDE and displace a fluorescently labeled Pγ mutant (Fig. 6) supports the idea that E4021 exerts its complex effects on PDE holoenzyme solely through direct binding of the drug at the active site. We describe how the K_I shift and the apparent stimulation of hydrolysis by E4021 can be explained by a model in which Pγ, E4021, and cyclic nucleotides compete for binding to the active site of PDE.

The fact that the K_I value for E4021 shifts 40-fold when comparing activated PDE (Pαβ) with nonactivated PDE ($\alpha\beta\gamma_2$) is a consequence of the competition between drug and Pγ subunit for mutually exclusive binding at the active site. This system of two pure competitive inhibitors is expected to behave similarly to a single competitive inhibitor (Segel, 1975). However, the K_I value measured for PDE holoenzyme now represents an apparent K_I value for E4021 that reflects the combined effects of E4021 binding and Pγ binding to the active site of the enzyme. Removal of the competing Pγ increases the apparent potency of E4021 by eliminating this competition. The value of 1.7 nM for the K_I value of E4021 with activated PDE represents the intrinsic affinity of the drug to bind to and inhibit hydrolysis at the active site.

The ability of E4021 to stimulate cGMP hydrolysis of nonactivated PDE holoenzyme under the conditions of Fig. 4 can be explained by the action of E4021 in reducing the concentration of PDE containing Pγ bound at the active site. Before incubation with E4021, Pγ bound to PDE holoenzyme is in equilibrium with free Pγ. Because the K_D value for Pγ binding is in the low picomolar range for bovine (Wensel and Stryer, 1986; Hamilton et al., 1993) and frog (M. R. D'Amours and R. H. Cote, unpublished observations) PDE, only a small fraction of the PDE molecules are active; the majority of enzyme molecules retain bound Pγ and are unable to hydrolyze cGMP. When E4021 is added at concentrations that exhibit stimulation of hydrolysis (~100 nM; Fig. 4), the drug displaces some bound Pγ at the active site to give a population of Pαβ/E4021 complexes. With submillimolar levels of cGMP, the substrate cannot be hydrolyzed because it cannot compete effectively with E4021 bound at the catalytic site; this reasoning is supported by the 10^4 difference in relative binding affinities of E4021 (K_I = 1.7 nM) compared with cGMP (K_M = 22 μM). Thus, no apparent activation of cGMP hydrolysis is seen. However, at very high (≥1 mM) substrate concentrations, cGMP can effectively compete with and displace E4021 bound to Pαβ, leading to elevated cGMP hydrolytic rates. The stimulatory effect of E4021 is confined to a limited range of concentrations (30–1000 nM with 10 mM substrate) because lower drug concentrations would not displace bound Pγ from PDE holoenzyme, whereas higher concentrations of E4021 would require proportionately higher cGMP concentrations than those we tested to be effective in competing with the drug.

In conclusion, E4021 is able to inhibit catalysis at the active site of PDE6 better than any other known phosphodi-

esterase inhibitor. The potency of E4021 for inhibiting activated PDE6 is comparable to the effects of the drug on PDE5, making E4021, and probably other PDE5-targeted drugs, essentially nonselective in its inhibitory potency for PDE5 versus PDE6. The presence of an inhibitory Pγ subunit that binds tightly to PDE6 in its nonactivated state has a pronounced effect on the potency of E4021. The simplest model that accounts for our observations is that E4021 binds in a mutually exclusive manner in competition with the Pγ subunit at the active site of the enzyme. Thus, it will be important to carefully evaluate the extent to which PDE5-selective drugs can act on both the nonactivated and activated forms of the retinal PDE6 isozymes. Future efforts should be directed toward identifying the differences that exist in the active sites of PDE5 and PDE6 to design inhibitors that discriminate PDE5 from PDE6 with high selectivity.

Acknowledgments

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