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EHNA as an Inhibitor of PDE2:

Evidences from a Pharmacological and Biochemical study in Cardiac Myocytes

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1. Introduction

1.1. Cardiac Ca^{2+} current is inhibited by cGMP via activation of PDE2

By controling the activity of various cardiac proteins, cyclic nucleotides are well-recognized to contribute to the performances of the normal and of the diseased heart [Hartzell, 1988]. The fine tuning of L-type Ca^{2+} channel activity by cyclic AMP (cAMP) and cyclic GMP (GMP) receives carefull attention since these channels are responsible for the triggering of cardiac contraction. Cyclic AMP activation of cAMP-dependent protein kinase leads to the phosphorylation of cardiac L-type Ca^{2+} channels (or a closely associated protein), resulting in an increase in the mean probability of channel opening and stimulation of macroscopic calcium current (I_{Ca}) [Hartzell, 1988]. Cyclic GMP has often been shown to produce contractile effects opposite to those of cAMP in the heart [Hartzell, 1988; Lohmann et al., 1991]. In isolated cardiomyocytes from different species, exogenous or endogenous cGMP can strongly inhibit I_{ca} (reviewed in [Lohmann et al., 1991]; see also [Levi et al., 1994; Méry et al., 1993; Whaler and Dollinger, 1995]).

In frog ventricular myocytes, the inhibitory effect of cGMP has been attributed to the stimulation of a specific cAMP-phosphodiesterase, the PDE2 [Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987]. This conclusion was suggested by the finding that cAMP level is reduced when cGMP level is increased in the frog heart [Flitney and Singh, 1981]. It is now strengthened by pharmacological and biochemical findings. In the isolated frog myocyte, cGMP inhibited cAMP stimulated I_{Ca} but did not affect I_{Ca} that had been increased by the hydrolysis-resistant analog of cAMP, 8Br-cAMP [Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987]. The sensitivity of the cAMP-stimulated I_{Ca} ($IC_{50} \approx 0.6$ µM) to cGMP correlates well with the ability of cGMP to activate the PDE2 ($K_a \approx 1.1$ µM) [Simmons and Hartzell, 1988]. Moreover, in this preparation, the inhibitory effect of cGMP on cAMP-stimulated I_{Ca} is largely antagonized by isobutyl-methyl-xanthine (IBMX), a non-selective phosphodiesterase inhibitor [Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987]. The effects of cGMP on I_{Ca} and PDE2 are sensitive to the same range of (high) concentrations of IBMX [Simmons and Hartzell, 1988]. Unlike in mammalian myocytes, the cGMP-dependent protein kinase is practicaly excluded from the inhibitory effect of cGMP on

the cAMP-stimulated I_{Ca} in the amphibian cells. Indeed, while being a potent stimulator of this kinase, the weak stimulator of PDE2, 8Br-cGMP, does not mimick the inhibitory effect of cGMP on cAMP-stimulated I_{Ca} [Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987]. Therefore, the inhibition of cardiac I_{Ca} by cGMP in the frog myocyte can be viewed as a sensitive *« in vivo »* assay for PDE2 activity.

1.2. Use of the cardiac I_{Ca} in determining the effects of EHNA on PDE2

Recently, T. Podzuweit and co-workers [1992; 1993] could establish, under *in vitro* conditions, that erythro-9-[2-hydroxyl-3-nonyl]-adenine (EHNA, which they called MEP1) acted as an inhibitor of a cGMP-stimulatable peak of PDE activity purified from both human and porcine hearts. In the micromolar range of concentration, this compound was reported to have no effect on three other purified peaks of cardiac PDE (PDE1, PDE3 and PDE4) from the same preparations [Podzuweit et al., 1992; 1993]. However, EHNA is most commonly used as a specific inhibitor of adenosine deaminase ($K_i = 7$ nM) [Cristalli et al., 1994] and as a potential preservative agent against ischemia-reperfusion injury [Zhu et al., 1994 and Refs. therein]. Therefore it remains to be verified whether EHNA can act as a selective PDE2 inhibitor when applied on intact cells. For this reason, we have studied the effects of EHNA on the regulation of I_{Ca} by exogenously dialysed and endogenously synthetized cGMP. The study was performed in frog ventricular cells because of the number of evidence listed above for the participation of PDE2 in the regulation of I_{Ca} in this preparation.

1.3. Use of purified cardiac PDE isoforms to determine the selectivity of action

The effect of EHNA on cardiac PDE activity was measured in the particulate fraction of frog ventricle and on the different purified PDEs of the soluble fraction. The crude particulate fraction of frog myocytes is a usefull assay to study the effect of EHNA since it contains a high specific activity of PDE2, sensitive to cGMP [Simmons and Hartzell, 1988]. Moreover, the putative regulatory proteins that modulate PDE2 activity in intact cells are probably kept present in this preparation. For instance, it was found suitable to study the regulation of PDE3 by glucagon involving the GTP-binding protein G_i [Brechler et al., 1992; Méry et al., 1990].

However, the presence of the other PDEs in this type of assay will preclude the study of the precise kinetics of PDE2. Although purification may perturbate the pharmacological sensitivity of cardiac PDEs [Pang, 1992], it is required to determine the site of action of EHNA on PDE2. In addition, there is no better means than the purification of each different PDEs to study the selectivity of EHNA action.

2. Methods

2.1. Electrophysiology

The whole-cell configuration of the patch-clamp technique was used to record L-type Ca^{2+} current (I_{Ca}) on Ca^{2+}-tolerant myocytes isolated from frog cardiac ventricle, as described [Fischmeister and Hartzell, 1986; Fischmeister and Hartzell, 1987].

Several compounds were generously supplied as follows: Milrinone was gift from Sterling-Winthrop, Ro 20-1724 was a gift from Hoffman LaRoche, and SIN-1 was a gift from Dr. J. Winicki, Hoechst Laboratories. EHNA was kindly provided by Dr. Podzuweit or purchased from Sigma Chemical Co. (St Louis, MO), with no change in the results. EHNA was either dissolved immediately before application, or prepared as 10 mM stock solutions in distilled water and stored at -20°C in small aliquots until use.

The results are expressed as mean ± standard error. In the text, the "basal" current refers to the activity of non-phosphorylated Ca^{2+} channels, in the absence of either Iso, or cAMP. In the case of single applications, the effect of a compound is referred to as the percent variation over the basal level. Since EHNA has no effect on basal I_{Ca}, its effects are expressed as the percent variation over the cAMP-dependent stimulation of I_{Ca}, i.e. 100*(test I_{Ca}-reference I_{Ca})/(reference I_{Ca}-basal I_{Ca}).

2.2. PDE assays for cAMP

The preparation of the particulate fraction from frog cardiac ventricle has been described in Brechler et al. [1992] and Méry et al. [1993]. The separation of the soluble PDE isoforms was done according to the method of Bethke et al. [1992]. PDE activity in frog ventricle particulate fraction has been characterized in Brechler et al. [1992] and Méry et al. [1990] and

was determined according to the two-step assay procedure of Thompson et al. [1979]. The assay medium (0.4 ml) consisted in: 20 mM HEPES, pH 7.6, 120 mM CsCl, 5 mM EGTA, 4 mM $MgCl_2$, 2 μM [^{3}H]cAMP (10^5 cpm) with or without 5 μM cGMP, unless indicated. Incubation was initiated by the addition of 50 μg proteins and was terminated after 10 min at 30°C by a 45-s of boiling. Data are the mean of triplicate determinations. Results are expressed as nanomoles of cAMP hydrolyzed /milligram of protein/10 min.

3. Results

3.1. EHNA has no effect on I_{Ca} in the absence of cGMP

Because PDE2 hydrolizes so efficiently cAMP, the effect of EHNA on I_{Ca} was first studied after stimulation of cAMP production with isoprenaline (Iso), a β-adrenergic agonist. In the experiment shown in Fig. 1A, I_{Ca} was stimulated by 1 nM Iso. This concentration of Iso produced a submaximal stimulation of I_{Ca} ($187.2 \pm 32.0\%$, N=21). As reported earlier [Fischmeister and Hartzell, 1990], two inhibitors of PDE3 and PDE4, respectively milrinone (10 μM) and Ro 20-1724 (10 μM), or a large concentration of IBMX (200 μM) produced a substantial further increase in I_{Ca} when not maximally stimulated by cAMP-phosphorylation. Under these conditions, however, EHNA (30 μM) was found to have no effect on I_{Ca} (Fig. 1A). The results of several similar experiments are summarized in Fig. 1B. While milrinone (10 μM), Ro 20-1724 (10 μM) and IBMX (200 μM) produced substantial and significant increases of I_{Ca} on top of a stimulation with 1 nM Iso, the effect of EHNA (30 μM) was not significant.

Another mean to elevate intracellular cAMP level is to dialyse exogenous cAMP in the myocyte. On average, perfusion of 10 μM cAMP in frog myocytes induced a $695.1 \pm 43.7\%$ increase of I_{Ca} over its basal amplitude (N=34). EHNA, at concentrations ranging from 0.3 to 30 μM, exerts no significant effects on cAMP-elevated I_{Ca} (Fig. 2B). Thus EHNA does not alter the coupling of the cAMP pathway to the cardiac L-type Ca^{2+} channels, in the absence of cGMP. Furthermore, since EHNA (0.1 to 30 μM) does not affect the basal I_{Ca}, in the absence of cAMP, it is unlikely to bind directly to the Ca^{2+} channels (Fig. 2B).

3.2. EHNA antagonizes the inhibitory effect of cGMP on I_{Ca}

Intracellular perfusion of a frog ventricular cell with cGMP strongly antagonizes the stimulatory action of cAMP [Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987]. Fig. 2A shows such an experiment where I_{Ca} had been first stimulated by 10 μM cAMP and then 20 μM cGMP was added to the intracellular medium containing cAMP. Intracellular perfusion with cGMP antagonized by ≈80% the stimulatory effect of cAMP. When EHNA (30 μM) was superfused on the cell, it fully antagonized the inhibitory effect of cGMP. Thus, unlike the absence of effect on basal and isoprenaline- or cAMP-stimulated I_{Ca}, EHNA produces a strong stimulatory effect on I_{Ca} when cGMP is dialyzing the cell.

Fig. 2B shows the concentration-response curve for the stimulatory effect of EHNA on I_{Ca}. In the presence of 20 μM cGMP, the stimulation of I_{Ca} was on average only 30.9 ± 12.9% (N=27) of its value in cAMP alone (100%). At concentrations above 0.3 μM, EHNA significantly increased I_{Ca}. At 3 μM concentration, EHNA reversed by ≈50% the inhibitory effect of cGMP. Increasing the concentration further induced a larger stimulation of I_{Ca} until the inhibitory effect of cGMP was totally reversed, which occurred at 30 μM concentration. This effect occurs in a voltage-independent manner, since addition of 30 μM EHNA only scales up the current-voltage relationship recorded in the presence of cAMP plus cGMP. Increasing the concentration to 100 μM produced no additional effect on I_{Ca} (not shown). Thus EHNA is a total antagonist of the inhibitory effect of cGMP on I_{Ca} in frog ventricular cells.

Since the non-selective PDE inhibitor, IBMX could antagonize the inhibitory effect of cGMP on I_{Ca} [Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987; 1990; Simmons and Hartzell, 1988], we compared the effects of EHNA and IBMX in the same cells. In 9 cells where 20 μM cGMP decreased cAMP (10 μM)-stimulated I_{Ca} from 777.5 ± 83.6 to 132.0 ± 23.5 % of the basal level, the effects of EHNA (30 μM) and IBMX (500 μM) were similar (74.1 ± 10.1 and 83.7 ± 6.1 % recovery from cGMP inhibition, respectively) and not additive. Although not significant, the effect of IBMX appears somewhat larger than that of EHNA. Since IBMX is likely to antagonize PDE3 and PDE4 [Hartzell and Fischmeister, 1990] together with the effect of cGMP on PDE2, we investigated the effects of Ro 20-1724 and milrinone, in the presence of EHNA. Ro 20-1724 (10 μM) potentiated the effect of 30 μM EHNA (to 98.4 ± 12.5 % recovery from cGMP inhibition, N=5). In contrast, milrinone (10 μM)

did not change the effect of 30 μM EHNA (N=3). Thus, it is likely that PDE3 was inhibited by the dialysis of cGMP. Neither milrinone (N=3) nor Ro 20-1724 (N=5) had any effect on I_{Ca} in the presence of cAMP+cGMP alone, i.e. in the absence of EHNA [Fischmeister and Hartzell, 1990].

3.3. EHNA antagonizes the inhibitory effect of NO-donors on I_{Ca}

The endogenous production of cGMP can be enhanced by nitric-oxide-donors such as SIN-1 and SNP which stimulate guanylyl cyclase activity [Hartzell, 1988; Lohmann et al., 1991]. In frog myocytes, these compounds can mimick the inhibitory effect of exogenous cGMP on Iso- or cAMP-stimulated I_{Ca}, but have no effect on 8Br-cAMP-stimulated I_{Ca} [Méry et al., 1993]. In a first set of experiments, 1 μM Iso increased I_{Ca} by 1326.6 ± 209.1 % (N=5), and the addition of 30 μM SIN-1 inhibited 37.5 ± 3.6 % of the Iso-stimulated I_{Ca} (N=6). The inhibitory effect of SIN-1 was abolished by 30 μM EHNA (to 98.8 ± 2.7 % of the initial Iso-stimulation). In a similar type of experiments, SNP (1 mM) reduced by 77.3 ± 6.5 % the amplitude of the Iso (0.1 μM)-stimulated I_{Ca} (N=5). While further addition of 10 μM EHNA partially antagonized the effect of SNP (to 66.6 ± 6.9 % of the Iso-stimulation, N=4), 30 μM EHNA fully suppressed the effect of the NO-donor on I_{Ca} (to 83.5 ± 5.9 % of the Iso-stimulation, N=4). Again in these experiments, the antagonistic effects of EHNA occured in a voltage-independent manner.

3.4. EHNA inhibits a cGMP-stimulated PDE in the crude particulate fraction

We examined the effects of EHNA on PDE activity in frog ventricle particulate fraction, a condition which approximates those used in electrophysiolgical recordings. In the absence of cGMP, the total PDE activity of the preparation was unaltered by 10 μM EHNA, when measured at concentrations of cAMP ranging from 0.5 to 100 μM (Fig. 3A). In contrast, the PDE activity which increased 4-fold by adding 5 μM cGMP to the preparation, was clearly reduced by 10 μM EHNA (Fig. 3B). A 40% reduction of the apparent cGMP-stimulated PDE was observed at all concentrations of cAMP tested. The antagonistic effect of EHNA was dose-dependent (Fig. 3C) and attributed to a decrease in the maximal velocity (V_{max}). In the

presence of 5 μM cGMP, a maximal 75% reduction in V_{max} was observed with 30 μM EHNA, with half-maximal inhibition occuring at 4 μM EHNA. Overall, these data are in good agreement with the results of patch-clamp experiments.

3.5. EHNA selectively inhibits the purified soluble PDE2

To assess the selectivity of EHNA action, each PDE was purified from the soluble fraction of frog cardiac ventricle. In our conditions, PDE1 activity was twice as high as PDE3 activity, while that of PDE4 was much weaker. In the presence of 5 μM cGMP, PDE2 activity became comparable to that of PDE1. Irrespective of the concentration of cGMP, the activity of the purified PDE2 was suppressed in a dose-dependent manner by EHNA (Fig. 4, left panel). As for the particulate PDE, EHNA effect on the soluble PDE2 resulted from a decrease in V_{max}, with a comparable potency, half maximal inhibition occurring at 5 μM EHNA. Interestingly, inhibition by EHNA of soluble PDE2 occurred in a non-competitive manner with respect to cGMP activation of the enzyme (K_a=40 nM). This suggests that the drug binds at a site other than the allosteric cGMP regulator site. In contrast, the other PDEs were not significantly affected by EHNA (Fig. 4, right panel), even at the highest concentration (30 μM) shown to fully overcome the inhibitory effect of cGMP on cAMP-stimulated I_{Ca}. These experiments demonstrate that EHNA is a selective inhibitor of PDE2, among the different cardiac isoforms of PDE.

3.6. Participation of adenosine deaminase in the effects of EHNA ?

Because EHNA is an inhibitor of adenosine deaminase, an indirect effect of EHNA due to adenosine accumulation was to rule out. Adenosine (10 μM) had no effect on PDE activity in frog ventricle particulate fraction, measured under basal conditions or in the presence of 5μM cGMP to stimulate the PDE2 activity (not shown). At the same concentration, adenosine induced an inhibitory effect on isoprenaline stimulated I_{Ca} in 2 out of 4 cells (not shown; see [Alvarez et al., 1990]). Therefore, it seems unlikely that the effects of EHNA could be due to some contamination by adenosine, because, as shown above, EHNA produces stimulatory, not inhibitory, effects on I_{Ca}.

4. Discussion

4.1. EHNA acts as a selective inhibitor of PDE2 in cardiac myocytes

We examined the effects of EHNA on Ca^{2+} current and PDE activity in frog ventricular cardiomyocytes. We conclude that EHNA acts primarily to inhibit PDE2 in this preparation.

An important feature of the present study is the correspondence between biochemical and electrophysiological data. For example, EHNA had no effect on Iso- or cAMP-elevated I_{Ca} in contrast to other selective PDE inhibitors, such as milrinone or Ro 20-1724, or the non selective PDE inhibitor IBMX. This suggests that, while different PDEs were active in our experimental conditions, more specifically PDE3 and PDE4 isoforms [Fischmeister and Hartzell, 1990], EHNA did not modify their activity. The data obtained with purified soluble PDE isoforms supported this observation, since EHNA (up to 30 µM) was found to have no or little effect on PDE1, PDE3 and PDE4. Moreover, both in intact myocytes and in particulate fractions, the effect of EHNA requires prior elevation of cGMP level. Also, the concentration-response curve for the effects of EHNA on cGMP-inhibited I_{Ca} was superimposable with that obtained for the effects of EHNA on cGMP-stimulated PDE activity in particulate fraction or on purified soluble cGS-PDE. Finally, both biochemical (this report) and electrophysiological data [Méry et al., 1995] support the hypothesis that EHNA inhibits PDE2 in a non-competitive manner with respect to the effect of cGMP on the enzyme. This may indicate that EHNA does not bind to the cGMP allosteric regulator site of the PDE2.

4.2. EHNA should be useful in evaluating the role of PDE2 in various tissues

Although PDE2 is expressed in numerous tissues, relatively little is known on its function. One reason for this is that no agent has been described as a truly selective inhibitor of PDE2 [Nicholson et al., 1991; Weishaar et al., 1985]. Various compounds such as dipyridamole, or the isoquinoline derivatives HL-725 (trequinsin) and papaverine have been shown to exert a somewhat greater inhibition on PDE2 than on PDE1 and PDE3 [Nicholson et al., 1991; Whalin et al., 1991; Weishaar et al., 1985]. However, in platelets, dipyridamole

inhibits PDE1 and PDE2 activities to a comparable degree [Weishaar et al., 1985], and, in frog heart, dipyridamole and papaverine were shown to inhibit PDE4 with a lower K_i than PDE2 [Lugnier et al., 1992]. For these reasons, the function of PDE2 in various cell types, such as human fibroblasts [Lee et al., 1988], bovine adrenal glomerulosa cells [MacFarland et al., 1991], rat pheochromocytoma cells (PC12) [Whalin et al., 1991], and frog cardiomyocytes [Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987; 1990; Simmons and Hartzell, 1988] was identified by means other than direct and selective inhibition of PDE2.

We suggest EHNA to be a suitable pharmacological agent to identify PDE2 among other PDE activities. As such, the effect of EHNA should be opposite to that of reasonable concentrations of cGMP on cAMP hydrolysis. A major drawback in the use of EHNA to inhibit PDE2 is that EHNA is a potent inhibitor of adenosine deaminase [Cristalli et al., 1994]. An accumulation of adenosine, in the extra- or the intra-cellular compartment, may therefore participate in the effects of EHNA, particularly in complex preparations. For instance, binding of adenosine to purinergic receptors can strongly modify the activity of second messengers pathway [Tucker and Linden, 1993]. In addition, binding of adenosine to the intracellular P-site of adenylyl cyclase can reduce cAMP production. However, adenosine deaminase is not expressed in every cell types. For instance, it could not be detected in cardiac myocytes while present in other cardiac cell types [Schrader and West, 1991]. Besides, the K_m of adenosine deaminase for adenosine is in the range of 20-50 μM [Schrader and West, 1991]. Thus, unless adenosine deaminase activity takes place in a compartment not readily available for internal perfusion, e.g. in the close vicinity of the membrane, the continuous dialysis of the cell in whole-cell patch-clamp experiments will prevent such a high accumulation of adenosine.

Nevertheless, the effect of EHNA can be compared to that of adenosine. In frog myocytes, adenosine does not mimic the effects of EHNA on either PDE or I_{Ca}. Superfusion of adenosine on frog has either no effect on, or induces an inhibition of isoprenaline stimulated I_{Ca}, likely mediated by the activation of A1-adenosine receptors which are negatively coupled to adenylyl cyclase [Alvarez et al., 1990; Tucker and Linden, 1993]. In addition, adenosine deaminase is also inhibited by pentostatin (2'-deoxycoformycin; Park-Davies, Ann Harbor, MI), the chemical structure of which is totally different from that of EHNA. Lacking the adenine

ring, this compound is unlikely to mimick the effect of EHNA on PDE2, and may also be used to discriminate between PDE2 and adenosine deaminase activities.

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FIGURE LEGENDS

Fig. 1. Effects of EHNA and other PDE inhibitors on isoprenaline-stimulated I_{Ca}. *A*: a frog ventricular cell was initially superfused with control Cs Ringer solution. During the periods indicated, the cell was successively exposed to isoprenaline (1 nM) alone and in the presence of either Ro 20-1724 (10 µM), EHNA (30 µM), milrinone (10 µM), or IBMX (200 µM). *B*: summary of the effects of milrinone (10 µM), Ro 20-1724 (10 µM), IBMX (200 µM) and EHNA (30 µM) on isoprenaline (1 nM) stimulated I_{Ca}. The *bars* indicate the means and S.E. of the number of experiments indicated near the *bars*. Significant statistical differences from isoprenaline-stimulated level (100%) are indicated as $*p<0.05$, $**p<0.005$.

Fig. 2. Effects of EHNA on cGMP-inhibited I_{Ca}. *A*: a frog ventricular cell was initially superfused with control Cs Ringer solution and internally dialyzed with control intracellular Cs solution. At the *first arrow*, 10 µM cAMP was added to the intracellular solution which then perfused the cell throughout the rest of the experiment. At the *second arrow*, 20 µM cGMP was added to the cAMP containing intracellular solution. During the period indicated, the cell was exposed to 30 µM EHNA. *B*: summary of the effects of EHNA on I_{Ca} in the presence of 10 µM cAMP (*upper panel, triangles*), or in thepresence of 10 µM cAMP plus 20 µM cGMP (*upper panel, squares*) in the intracellular solution; or in the absence of cyclic nucleotides (*lower panel*),. The *bars* indicate the means and S.E. of the number of experiments indicated near the *bars*. Addition of cGMP reduced the cAMP induced stimulation (100%) to the percentage level indicated by the *dotted line* in *B*. Significant statistical differences from cAMP-stimulated level (# and ##) or cAMP + cGMP level (**) are indicated as # $p<0.05$, and ** or ## $p<0.005$.

Fig. 3. Effect of EHNA on the PDE activity of frog ventricle particulate fraction. The effect of EHNA on basal (*part A*) and cGMP-stimulated (*part B*) PDE activities was measured at varying cAMP concentrations. *A*: basal PDE activity was measured in frog particulate fraction, in the absence (control) and in the presence of 10 µM EHNA, as indicated in « Methods ». *B*: PDE activity was measured in the presence of 5 µM cGMP, either in the absence (control) ot in the presence of 10 µM EHNA. Note that EHNA inhibited cGS-PDE activity to the same degree, irrespective of the concentration of cAMP. *C*: Dose-dependent inhibition of cGMP stimulated PDE activity by EHNA in frog ventricle particulate fraction. PDE activity was measured in the presence of 2 µM cAMP, at different cGMP concentrations, and with varying EHNA concentrations as described in "Methods".

Fig. 4. *A:* Dose-dependent inhibition of purified soluble cGS-PDE activity by EHNA examined under the same conditions as for the particulate fraction. *B:* Specificity of EHNA effect. The effects of 10 and 30 µM EHNA were examined on the purified soluble PDE 1 to 4 isoforms under the same conditions as above. The data are normalized with respect to the PDE activities in the absence of EHNA. These were: PDE1, 14.2 ± 2.5; PDE3, 7.0 ± 0.6; PDE4, 1.0 ± 0.1 nmol cAMP/mg/10 min. The *dotted line* represents the effect of EHNA on cGS-PDE activity in the presence of 0.1 µM cGMP and is taken from A.