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Erythro-9-(2-Hydroxy-3-nonyl)adenine Inhibits Cyclic GMP-Stimulated Phosphodiesterase in Isolated Cardiac Myocytes

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SUMMARY

Recently, an inhibitor of adenosine deaminase, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), was shown to selectively block the activity of purified cGMP-stimulated phosphodiesterase (PDE) (cGS-PDE, or PDE2) in human and porcine heart [*J. Mol. Cell. Cardiol.* 24 (Suppl. V):102 (1992)]. Because cGS-PDE was found to mediate the cGMP-induced inhibition of L-type Ca^{2+} current (I_{Ca}) in frog ventricular cells, we tested the effects of EHNA in this preparation. I_{Ca} was measured using the whole-cell patch-clamp technique and a perfusing pipette. EHNA (0.3–30 μM) had no significant effect on either basal I_{Ca} or isoprenaline (1 nM)- or cAMP (10 μM)-elevated I_{Ca} . However, EHNA dose-dependently ($\text{IC}_{50} \sim 3 \mu\text{M}$) reversed the inhibitory effect of cGMP on cAMP-stimulated I_{Ca} . EHNA (30 μM) also blocked the inhibitory effect of NO donors, such as sodium nitroprusside (1 mM) and 3-morpholiniosydnonimine (30 μM), on

isoprenaline-stimulated I_{Ca} . In addition, EHNA dose-dependently ($\text{IC}_{50} \sim 4\text{--}5 \mu\text{M}$) inhibited the cGMP-induced stimulation of PDE activity in frog ventricle particulate fraction, as well as purified soluble cGS-PDE. However, EHNA (up to 30 μM) did not modify the activities of three other purified soluble PDE isoforms. Moreover, EHNA did not change the K_a (40 nM) for cGMP activation of cGS-PDE, which suggests that EHNA does not inhibit cGS-PDE by displacing cGMP from the allosteric regulator site. Because adenosine did not mimic the effects of EHNA on I_{Ca} or PDE activity, it is unlikely that the effects of EHNA are due to adenosine deaminase inhibition. We conclude that EHNA acts primarily to inhibit cGS-PDE in intact cardiac myocytes. This compound should be useful in evaluating the physiological role of cGS-PDE in various tissues.

cAMP 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 I_{Ca} (1, 2). cGMP has often been shown to produce effects opposite those of cAMP in the heart (1). For instance, in isolated cells from frog (3, 4), guinea pig (5), rat (6), and embryonic chicken (7, 8) ventricular tissue, cGMP produces an inhibition of I_{Ca} . In frog heart cells, this effect has been attributed to cGMP stimulation of cGS-PDE. Among the evidence supporting this hypothesis are the findings that (i) cGMP inhibited cAMP-stimulated I_{Ca} but did not affect I_{Ca} that had been stimulated by the hydrolysis-resistant cAMP analog 8-Br-cAMP, (ii) 8-Br-cGMP (which is a much better stimulator of cGMP-dependent protein kinase than is cGMP and is a much worse stimulator of cGS-PDE than is cGMP)

had no effect on I_{Ca} , and (iii) the inhibitory effect of cGMP on cAMP-stimulated I_{Ca} was largely antagonized by IBMX, a nonselective PDE inhibitor (3, 4). The hypothesis that I_{Ca} in frog heart is regulated by cGS-PDE is further supported by considerable biochemical evidence (1). This includes (i) the finding that the cAMP level is reduced when the cGMP level is increased (9), (ii) the characterization in frog ventricular cells of a cGS-PDE activity with the same sensitivity to cGMP ($K_a = 1.1 \mu\text{M}$) as I_{Ca} ($\text{IC}_{50} \sim 0.6 \mu\text{M}$) (10), and (iii) similar inhibition of the effects of cGMP on I_{Ca} and cGS-PDE by high concentrations of IBMX (3, 4, 10, 11). The effect of cGMP can be mimicked by NO donors, such as SIN-1 or SNP. In frog ventricle, SIN-1 and SNP stimulate guanylyl cyclase activity and antagonize the stimulatory effects of isoprenaline, forskolin, or cAMP on I_{Ca} (12). This effect was also attributed to cGMP-induced activation of cGS-PDE, because it was prevented by using 8-Br-cAMP instead of cAMP to elevate I_{Ca} (12).

Although there is now a large amount of evidence impli-

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ABBREVIATIONS: I_{Ca} , L-type calcium current; PDE, phosphodiesterase; cGS-PDE, cGMP-stimulated phosphodiesterase (phosphodiesterase 2); PDE3, cGMP-inhibited phosphodiesterase; PDE1, Ca^{2+} /calmodulin-activated phosphodiesterase; PDE4, low- K_m , cAMP-specific phosphodiesterase; NO, nitric oxide; SIN-1, 3-morpholiniosydnonimine; SNP, sodium nitroprusside; IBMX, isobutylmethylxanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; I_{400} or I_{200} , leak current after a 400-msec or 200-msec pulse, respectively; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid; 8-Br-cAMP, 8-bromo-cAMP.

cating cGS-PDE in the inhibitory effects of cGMP and NO donors on frog I_{Ca} , it remains to be demonstrated that these effects can be antagonized by a selective inhibitor of cGS-PDE. Until now, no such selective inhibitor of cGS-PDE was available. The only way to fully antagonize the inhibitory effects of cGMP on I_{Ca} in frog ventricular cells was to use massive (500 μ M) concentrations of IBMX (11). However, 5–10 times lower IBMX concentrations are sufficient to block the cGS-PDE activity (10). Therefore, one cannot totally exclude the possibility that cGMP affects I_{Ca} through another mechanism besides cGS-PDE or that the reversal effect of IBMX is due to an additional stimulatory action of this compound, e.g., via inhibition of other PDEs or interaction with adenosine receptors (13). A selective cGS-PDE inhibitor would be useful not only to characterize the role of this PDE after an elevation of cGMP concentration has been elicited, e.g., by intracellular perfusion of cGMP or by administration of exogenous compounds such as natriuretic peptides or NO donors, but also to establish its role under normal conditions, when the cGMP concentration is at its basal level. In that respect, the use of other selective PDE inhibitors, such as milrinone and Ro 20–1724, has contributed to our current understanding of the implications of PDE3 and PDE4 in the control of cardiac I_{Ca} and heart function, respectively (10, 11, 14–18).

In a recent report (19), it was demonstrated that EHNA, initially referred to as MEP1 (19, 20), acts as a selective inhibitor of cGS-PDE in human and porcine heart under *in vitro* conditions. In the micromolar range of concentrations, this compound was reported to have no effect on three other forms of purified cardiac PDE (PDE1, PDE3, and PDE4). However, EHNA is most commonly used as a specific inhibitor of adenosine deaminase ($K_i = 7$ nM) (21) and as a potential preservative agent against ischemia-reperfusion injury (Ref. 22 and references cited therein). Therefore, it remains to be verified whether EHNA can act as a selective cGS-PDE inhibitor when applied to intact cells. For this reason, in the present study we have examined the effects of EHNA on I_{Ca} and its response to cGMP and NO donors in isolated cardiac myocytes. The study was performed in frog ventricular cells because of the number of findings (as listed above) on the participation of cGS-PDE in the regulation of I_{Ca} in this preparation. The effects of EHNA on I_{Ca} were compared with its effects on PDE activity measured in frog heart ventricle particulate fraction and on purified soluble cGS-PDE. A preliminary report of some of these results has appeared elsewhere (23).

Materials and Methods

Electrophysiology. Ventricular cells were enzymatically dispersed from frog (*Rana esculenta*) heart with a combination of collagenase (Boehringer-Mannheim, Mannheim, Germany, or Yakult, Tokyo, Japan) and trypsin (Sigma Chemical Co.), as described (24). The isolated cells were stored in standard Ringer solution and kept at 4° until use (2–48 hr after dissociation). The whole-cell configuration of the patch-clamp technique was used to record I_{Ca} in Ca^{2+} -tolerant cells. In the routine protocols, the cell was depolarized every 8 or 10 sec from a –80-mV holding potential to 0 mV for 200 or 400 msec. K^+ currents were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+ (24). The fast Na^+ current was blocked by tetrodotoxin. Under these conditions, the only time-dependent currents measured during a depolarization of the membrane poten-

tial could be completely blocked by 100 μ M $CdCl_2$ (24) and were attributed entirely to L-type calcium channels. On the time- and voltage-dependent I_{Ca} is superimposed a small, time-independent, leak current. In frog ventricular cells, this leak current is not modified by activation of cAMP-dependent phosphorylation (3, 24). All experiments were done at room temperature (19–25°).

Solutions. Control external solution contained 107 mM NaCl, 10 mM HEPES, 20 mM CsCl, 4 mM $NaHCO_3$, 0.8 mM NaH_2PO_4 , 1.8 mM $MgCl_2$, 0.8 mM $CaCl_2$, 5 mM D-glucose, 5 mM sodium pyruvate, and 30 nM tetrodotoxin; the pH was adjusted to 7.4 with NaOH. Control or drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of 250- μ m (i.d.) capillary tubing, with a flow rate of ~ 10 μ l/min. Patch electrodes (0.8–2.0 M Ω) were filled with control internal solution, which contained 119.8 mM CsCl, 5 mM EGTA (acid form), 4 mM $MgCl_2$, 5 mM phosphocreatine, 3.1 mM Na_2ATP , 0.42 mM Na_2GTP , 0.062 mM $CaCl_2$ (pCa 8.5), and 10 mM HEPES; the pH was adjusted to 7.1 with KOH or CsOH. Drug-containing solutions were then applied to the interior of the cell by a system that permitted perfusion of the patch electrode with different solutions (3, 4). Perfusion time depended on patch electrode resistance, access to the cell, and the molecular weight of the molecule tested. Typically, with cAMP (M_r 351) the beginning of I_{Ca} stimulation occurred 1.5–3 min after the beginning of intracellular perfusion with this compound (e.g., see Fig. 2).

Drugs. Several compounds used in this study were generously supplied as follows: milrinone was a 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 (Paris, France). Tetrodotoxin was from Latoxan (Rosans, France), and all other drugs were from Sigma Chemical Co. (St. Louis, MO). During the preparation of solutions, SIN-1 and SNP were protected from UV irradiation of natural light. They were solubilized in standard external solution <15 min before being applied to the cell under investigation, i.e., only fresh NO donor-containing solutions were tested. EHNA was kindly provided by Dr. Podzuweit (Max Planck-Institut, Bad Nauheim, Germany) or purchased from Sigma, with no significant differences in the results. In some experiments, EHNA was dissolved immediately before application to the cell studied. In all other experiments, EHNA was prepared as 10 mM stock solutions in distilled water and stored at –20° in small aliquots until use.

Data analysis. The maximal amplitude of whole-cell I_{Ca} was measured as described previously (24). Currents were not compensated for capacitive and leak currents. The leak current (I_{400} or I_{200}) was routinely measured as the current amplitude at the end of the 400-msec or 200-msec pulse. On-line analysis of the recordings was made possible by programming a PC-compatible 486/50 computer in Pascal language (Microsoft) to determine, for each membrane depolarization, peak and steady state current values (24). Current-voltage relationships for I_{Ca} and I_{200} were obtained using voltage-clamp protocols described previously (24).

The results are expressed as mean $\pm$ standard error. Throughout the text, the “basal” condition refers to the absence of either isoprenaline or cAMP, i.e., to the current elicited by nonphosphorylated calcium channels. In the case of single applications, the effect of a compound is referred to as the percentage variation relative to the basal level. Because EHNA has no effect on basal I_{Ca} , its effects are expressed as the percentage variation relative to the cAMP-dependent stimulation of I_{Ca} , i.e., $100 \times (\text{test } I_{Ca} - \text{reference } I_{Ca}) / (\text{reference } I_{Ca} - \text{basal } I_{Ca})$.

Frog heart ventricle particulate fraction. The procedure for preparation of frog heart ventricle particulate fraction has been previously described in detail (12, 25).

Isolation of soluble PDE1–4 from frog heart ventricle. The procedure to separate the soluble PDE isoforms was performed according to the method described in Ref. 26.

cAMP hydrolysis assay for PDE. PDE activity in frog ventricle particulate fraction has been characterized previously (25, 27) and was determined according to the two-step assay procedure described

in Ref. 28. The assay medium was derived from the control internal solution and contained, in a final volume of 0.4 ml, 20 mM HEPES, pH 7.6, 120 mM CsCl, 5 mM EGTA, 4 mM MgCl_2 , and 2 μM [^3H]cAMP (10^5 cpm), with or without cGMP as indicated. Incubation was initiated by the addition of 50 μg of protein and was terminated after 10 min at 30° by 45 sec of boiling. Data are the mean of triplicate determinations. Results are expressed as nanomoles of cAMP hydrolyzed/milligram of protein/10 min.

Results

Effects of EHNA on I_{Ca} in the absence of cGMP. To examine the effects of EHNA on I_{Ca} , we first examined the effects of this compound on basal I_{Ca} , i.e., in the absence of cAMP or cAMP activators, when the current was elicited by nonphosphorylated calcium channels. Fig. 1A shows the absence of effect of an external application of 30 μM EHNA on basal I_{Ca} recorded from a frog ventricular cell. The results of several similar experiments using different concentrations of EHNA are summarized in Fig. 1B. In the range of 0.1–30 μM concentrations, EHNA produced no significant effect on the amplitude of basal I_{Ca} , suggesting that this compound does not directly modulate Ca^{2+} channel activity or activate cAMP pathways.

We then examined the effects of EHNA on I_{Ca} after various manipulations to elevate intracellular cAMP. First, I_{Ca} was strongly enhanced by switching the intracellular solution to one containing 5 μM cAMP (Fig. 2A). The cell was then challenged with a 30 μM concentration of extracellularly applied EHNA. As shown in Fig. 2A, EHNA did not modify I_{Ca} .

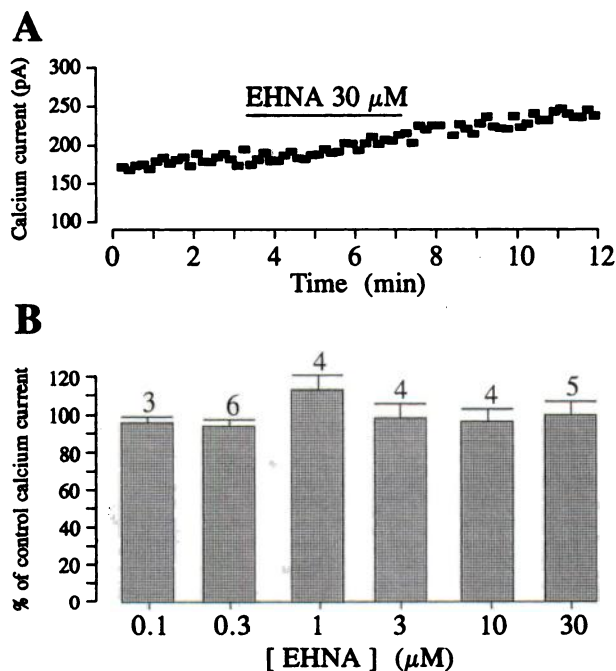


Fig. 1. Effects of EHNA on basal I_{Ca} . A, Time course of the effect of EHNA on basal I_{Ca} in an isolated frog ventricular cell. Each symbol corresponds to a measure of I_{Ca} at 0 mV, obtained every 8 sec (see Materials and Methods). The cell was initially superfused with control cesium-containing Ringer solution. During the period indicated, the cell was exposed to 30 μM EHNA. B, Summary of the effects of EHNA on basal I_{Ca} . Bars, means and standard errors of the number of experiments indicated (numbers above the bars). In these experiments, the mean I_{Ca} amplitude in the absence of EHNA was 215 ± 56 pA (mean $\pm$ standard error, $n = 9$).

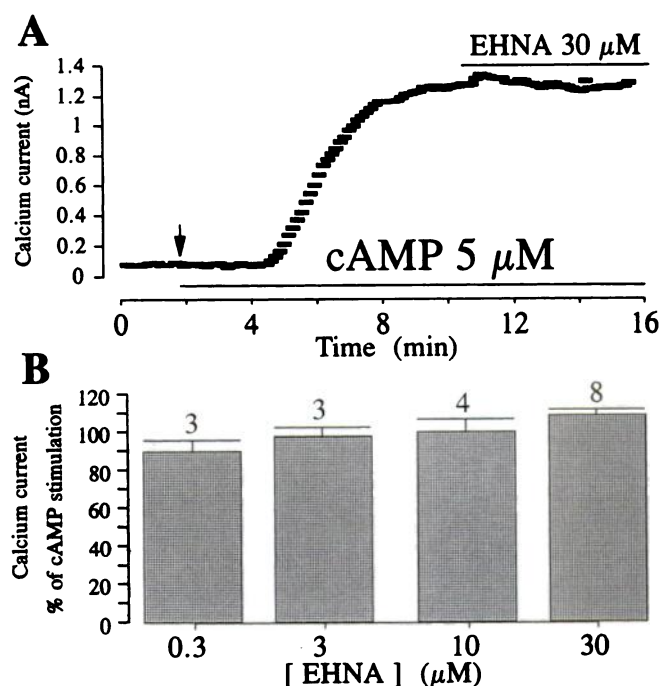


Fig. 2. Effects of EHNA on cAMP-stimulated I_{Ca} . A, A frog ventricular cell was initially superfused with control cesium-containing Ringer solution and internally dialyzed with control intracellular cesium-containing solution. Arrow, 5 μM cAMP was added to the intracellular solution, which then perfused the cell throughout the rest of the experiment. During the period indicated, the cell was exposed to 30 μM EHNA. B, A summary of the effects of EHNA on cAMP (10 μM)-stimulated I_{Ca} is shown. Bars, means and standard errors of the number of experiments indicated (numbers above the bars).

On average, 30 μM EHNA induced a nonsignificant inhibition of cAMP (5 μM)-stimulated I_{Ca} ($-7.4 \pm 10.5\%$, $n = 4$). A complete dose-response curve for the effects of EHNA on cAMP-elevated I_{Ca} is shown in Fig. 2B. The concentration of cAMP used was 10 μM , which induced, on average, a $695.1 \pm 43.7\%$ (mean $\pm$ standard error, $n = 34$) increase of I_{Ca} over its basal amplitude. As shown, EHNA, at concentrations ranging from 0.3 to 30 μM , exerted no significant effects on cAMP-elevated I_{Ca} . This suggests that EHNA does not act on I_{Ca} downstream from cAMP-dependent phosphorylation.

Next, we examined whether EHNA affects some step before the elevation of the cAMP concentration. To do this, the effects of EHNA were investigated on I_{Ca} that had been previously stimulated with isoprenaline, a β -adrenergic agonist. In the experiment shown in Fig. 3A, I_{Ca} was stimulated by 1 nM isoprenaline. This concentration of isoprenaline produced a submaximal stimulation of I_{Ca} ($187.2 \pm 32.0\%$, $n = 21$). As reported earlier (11), inhibitors of PDE3 and PDE4, i.e., milrinone (10 μM) and Ro 20-1724 (10 μM), respectively, or a large concentration of IBMX (200 μM) produced a substantial additional increase in I_{Ca} not maximally stimulated by cAMP phosphorylation. Under these conditions, however, EHNA (30 μM) was found to have no effect on I_{Ca} (Fig. 3A). The results of several similar experiments are summarized in Fig. 3B. Whereas milrinone (10 μM), Ro 20-1724 (10 μM), and IBMX (200 μM) produced substantial and significant increases of I_{Ca} on top of stimulation with 1 nM isoprenaline, the effect of EHNA (30 μM) was not significant (Fig. 3B). When the concentration of isoprenaline was increased to 1

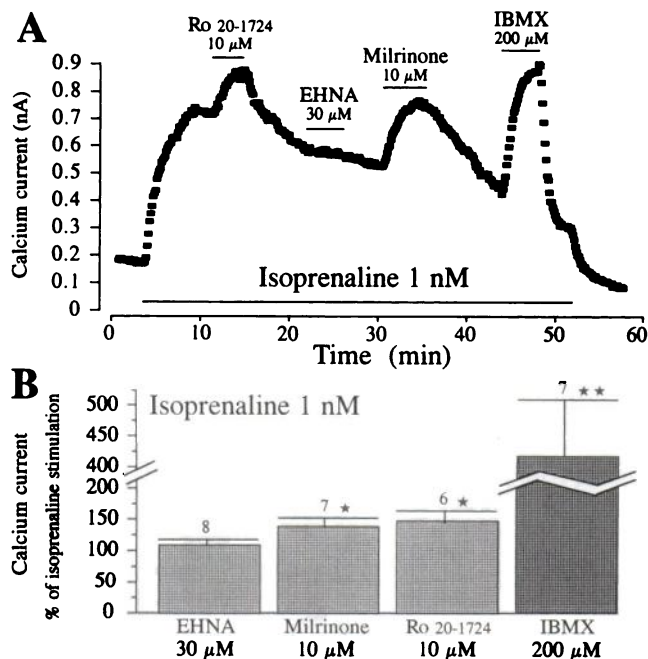


Fig. 3. Effects of EHNA and other PDE inhibitors on isoprenaline-stimulated I_{Ca} . **A**, A frog ventricular cell was initially superfused with control cesium-containing Ringer solution. During the periods indicated, the cell was successively exposed to isoprenaline (1 nM) alone and in the presence of either Ro 20-1724 (10 μM), EHNA (30 μM), milrinone (10 μM), or IBMX (200 μM). **B**, A 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} is shown. Bars, means and standard errors of the number of experiments indicated (*numbers above the bars*). Significant statistical differences from the isoprenaline-stimulated level (100%) are indicated; *, $p < 0.05$; **, $p < 0.005$.

μM, which induced a strong stimulation of I_{Ca} of $661 \pm 80\%$ ($n = 6$), EHNA still had no effect on I_{Ca} ($1.3 \pm 6.5\%$ increase at 30 μM, $n = 6$).

Effects of EHNA on I_{Ca} in the presence of cGMP. If, as suggested (19, 20), EHNA acts as a cGS-PDE inhibitor, one would expect this compound to increase I_{Ca} only after cGMP levels are elevated. We, therefore, examined the effects of EHNA on I_{Ca} after intracellular perfusion with cGMP. As shown earlier, intracellular perfusion of a frog ventricular cell with cGMP strongly antagonizes the stimulatory action of cAMP (3, 4). Fig. 4A shows such an experiment, in which I_{Ca} was first stimulated by 10 μM cAMP and then 20 μM cGMP was added to the intracellular perfusion solution containing cAMP. Intracellular perfusion with cGMP antagonized by ~80% the stimulatory effect of cAMP. EHNA (30 μM) was then applied extracellularly to that cell and, as shown, the inhibitory effect of cGMP was fully antagonized by this compound (Fig. 4A). This effect was totally reversible upon washout of the drug. 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. 4B shows the concentration-response curve for the stimulatory effect of EHNA on I_{Ca} . The data were obtained in the presence of 10 μM cAMP and 20 μM cGMP and represent the percentage of cAMP-stimulated I_{Ca} in the absence of cGMP. In the presence of 20 μM cGMP, the stimulation of I_{Ca} was on average only $30.9 \pm 12.9\%$ ($n = 27$) (Fig. 4B) of its value with cAMP alone (100%). At concentrations above 0.3

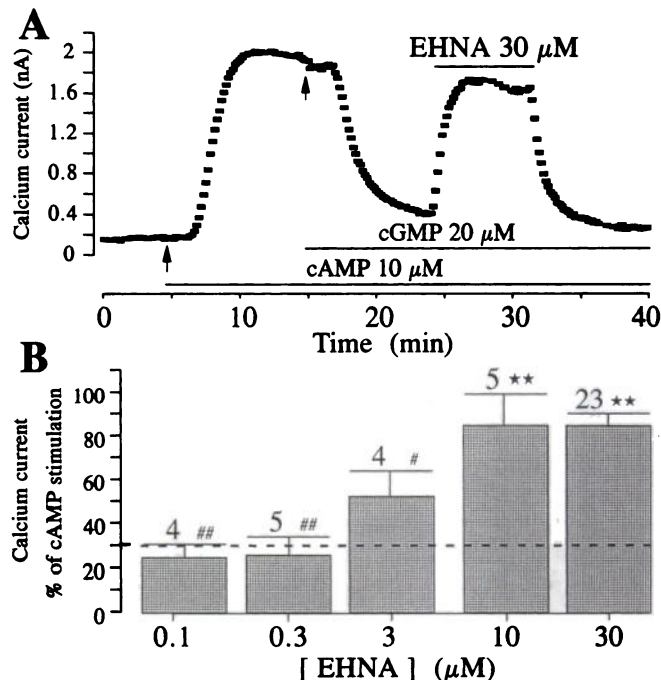


Fig. 4. Effects of EHNA on cGMP-inhibited I_{Ca} . **A**, A frog ventricular cell was initially superfused with control cesium-containing Ringer solution and internally dialyzed with control intracellular cesium-containing solution. First arrow, 10 μM cAMP was added to the intracellular solution, which then perfused the cell throughout the rest of the experiment. 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**, A summary of the effects of EHNA on I_{Ca} in the presence of cAMP (10 μM) and cGMP (20 μM) in the intracellular solution is shown. Bars, means and standard errors of the number of experiments indicated (*numbers above the bars*). Dashed line, addition of cGMP reduced the cAMP-induced stimulation (100%) to the percentage level indicated. Significant statistical differences from the cAMP-stimulated level (# and ##) or the cAMP plus cGMP level (**) are indicated; #, $p < 0.05$; ** or ##, $p < 0.005$.

μM, EHNA significantly increased I_{Ca} . At 3 μM, 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 EHNA. Increasing the concentration to 100 μM produced no additional effect on I_{Ca} (data not shown). These data demonstrate that EHNA is a total antagonist of the inhibitory effect of cGMP on I_{Ca} in frog ventricular cells.

If EHNA acts as a cGS-PDE inhibitor, its effect should be qualitatively similar to that of IBMX, a nonselective PDE inhibitor that blocks cGS-PDE in the high micromolar range of concentrations (29). We showed previously that IBMX could antagonize the inhibitory effect of cGMP on I_{Ca} (3, 4). Here we compared the effects of EHNA and IBMX in the same cell. In the experiment shown in Fig. 5A, intracellular perfusion with 20 μM cGMP began after 10 μM cAMP had stimulated I_{Ca} by ~8-fold. cGMP induced a strong inhibition of I_{Ca} , which could be blocked by about 60% in this cell with 30 μM EHNA. After washout of EHNA, the current returned to its level with cAMP plus cGMP, and the cell was exposed to a massive (500 μM) concentration of IBMX. This high concentration of IBMX produced a similar effect on I_{Ca} as did a ~17 times lower concentration of EHNA (Fig. 5C). As shown in Fig. 5, B and C, the effects of EHNA and IBMX were

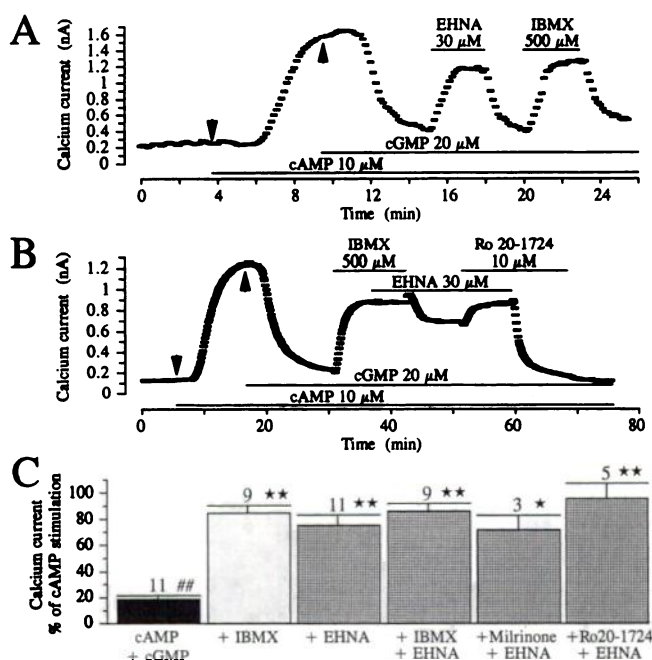


Fig. 5. Comparison of the effects of EHNA and IBMX on I_{Ca} . A and B, A frog ventricular cell was initially superfused with control cesium-containing Ringer solution and internally dialyzed with control intracellular cesium-containing solution. First arrowhead, 10 μ M cAMP was added to the intracellular solution, which then perfused the cell throughout the rest of the experiment. Second arrowhead, 20 μ M cGMP was added to the cAMP-containing intracellular solution. A, During the periods indicated, the cell was exposed to 30 μ M EHNA and, after washout of EHNA, to 500 μ M IBMX. B, During the periods indicated, the cell was exposed to 500 μ M IBMX, 30 μ M EHNA, 10 μ M Ro 20-1724, or a combination of IBMX plus EHNA or EHNA plus Ro 20-1724. C, A summary of the effects of EHNA (30 μ M), IBMX (500 μ M), EHNA plus IBMX, EHNA plus milrinone (10 μ M), and EHNA plus Ro 20-1724 (10 μ M) on I_{Ca} in the presence of cAMP (10 μ M) and cGMP (20 μ M) in the intracellular solution is shown. Bars, means and standard errors of the number of experiments indicated (numbers above the bars). Addition of cGMP reduced the cAMP-induced stimulation (100%) to the percentage level indicated (cAMP + cGMP). Significant statistical differences from the cAMP-stimulated level (##) or the cAMP plus cGMP level (* and **) are indicated; *, $p = 0.01$; ** or ##, $p < 0.001$.

not additive. Although not statistically significant, the effect of IBMX often appeared somewhat larger than that of EHNA (Fig. 5B). This was likely due to the fact that IBMX not only antagonized the effect of cGMP on cGS-PDE but also blocked other PDE isoforms, particularly PDE4, which was shown earlier to regulate I_{Ca} in frog heart cells (11). We tested this hypothesis by investigating the effects of Ro 20-1724, an inhibitor of PDE4 (11), in the presence of EHNA. As shown in Fig. 5, B and C, Ro 20-1724 (10 μ M) induced a small additional increase in I_{Ca} . However, under similar conditions milrinone, an inhibitor of PDE3 (11), had no effect on I_{Ca} (Fig. 5C). This suggests that PDE3 was already fully blocked by the concentration of cGMP (20 μ M) present in the pipette. Neither milrinone ($n = 3$) nor Ro 20-1724 ($n = 5$) had any effect on I_{Ca} in the presence of cAMP plus cGMP alone, i.e., in the absence of EHNA (11).

Effects of EHNA on I_{Ca} in the presence of NO donors. We next examined the effects of EHNA on SIN-1- and SNP-induced inhibitions of I_{Ca} in frog ventricular cells. In the experiment shown in Fig. 6A, the cell was first exposed to 1 μ M isoprenaline, which produced a large increase in I_{Ca} . The cell was then challenged with 30 μ M SIN-1, which reduced

the isoprenaline stimulation by about 50%. In the continuing presence of SIN-1, the cell was exposed to 30 μ M EHNA, which completely reversed the effect of SIN-1. Fig. 6B shows a similar type of experiment using another NO donor, SNP. In this experiment, exposure of the cell to 1 mM SNP also produced a ~50% reduction of the stimulatory effect of 0.1 μ M isoprenaline. The effects of two different concentrations of EHNA (10 and 30 μ M), in the presence of isoprenaline and SNP, are shown; 30 μ M EHNA fully reversed the inhibitory effect of SNP on I_{Ca} . Fig. 6C summarizes the results obtained in four different experiments using SIN-1 and five different experiments using SNP. These data demonstrate that, at a 30 μ M concentration, EHNA fully antagonizes the inhibitory effect of SIN-1 or SNP on I_{Ca} .

Voltage dependence of the effects of EHNA. The voltage dependence of the effects of EHNA was investigated by comparing individual current recordings and current-voltage relationships in the presence and absence of EHNA. Fig. 7, A and C, shows three sets of current traces each, which were obtained at three different membrane potentials (-25, 0, and +30 mV). The traces in Fig. 7A were obtained in a cell intracellularly perfused with either 5 μ M cAMP alone or cAMP plus 10 μ M cGMP, with or without 30 μ M EHNA added to the extracellular solution. The traces in Fig. 7C were obtained in a cell exposed to 0.1 μ M isoprenaline alone, isoprenaline plus 1 mM SNP, and isoprenaline plus SNP plus 30 μ M EHNA. It appears that the addition of EHNA in the presence of cGMP (Fig. 7A) or SNP (Fig. 7C) mainly restores the fast kinetics of I_{Ca} inactivation, which are characteristic of an I_{Ca} that is stimulated by cAMP-dependent phosphorylation. The complete current-voltage relationships for I_{Ca} and I_{200} corresponding to the three different conditions of Fig. 7, A and C, are shown in Fig. 7, B and D, respectively. It appears that the effect of EHNA occurs in an essentially voltage-independent manner, which makes it unlikely that EHNA binds directly to or interacts with the calcium channel protein. In addition, EHNA does not modify the integrity of the cell membrane, because I_{200} , a representation of the leak current, is not affected by the drug over the whole range of potentials explored (Fig. 7, B and D). The results shown in Fig. 7 are typical of three to five different experiments. These data favor the hypothesis that EHNA affects I_{Ca} exclusively via an increase in cAMP-dependent phosphorylation.

Mechanism of action of EHNA. So far, our results demonstrate that EHNA antagonizes in a similar manner the inhibitory effects of cGMP, SIN-1, and SNP on stimulated I_{Ca} in frog ventricular cells. These findings favor the hypothesis that EHNA does so by inhibiting cGS-PDE. To test this hypothesis, we examined the effects of EHNA on PDE activity in frog ventricle particulate fraction and on the purified soluble cGS-PDE. As shown in Fig. 8A, EHNA did not modify basal PDE activity in the particulate fraction. In contrast, it reduced cGMP-stimulated PDE activity in a dose-dependent manner via a decrease in the maximal velocity (V_{max}). A maximal 75% reduction in V_{max} was observed with 30 μ M EHNA in the presence of 5 μ M cGMP, with half-maximal inhibition occurring at 4 μ M EHNA. EHNA also induced a dose-dependent inhibition of the purified soluble cGS-PDE (Fig. 8B), whereas, at up to 30 μ M, the drug had little effect on the activity of soluble PDE1, -3, and -4 isoforms (Fig. 8C). As for the particulate PDE, the effect of EHNA on the soluble cGS-PDE resulted from a decrease in V_{max} , with a compara-

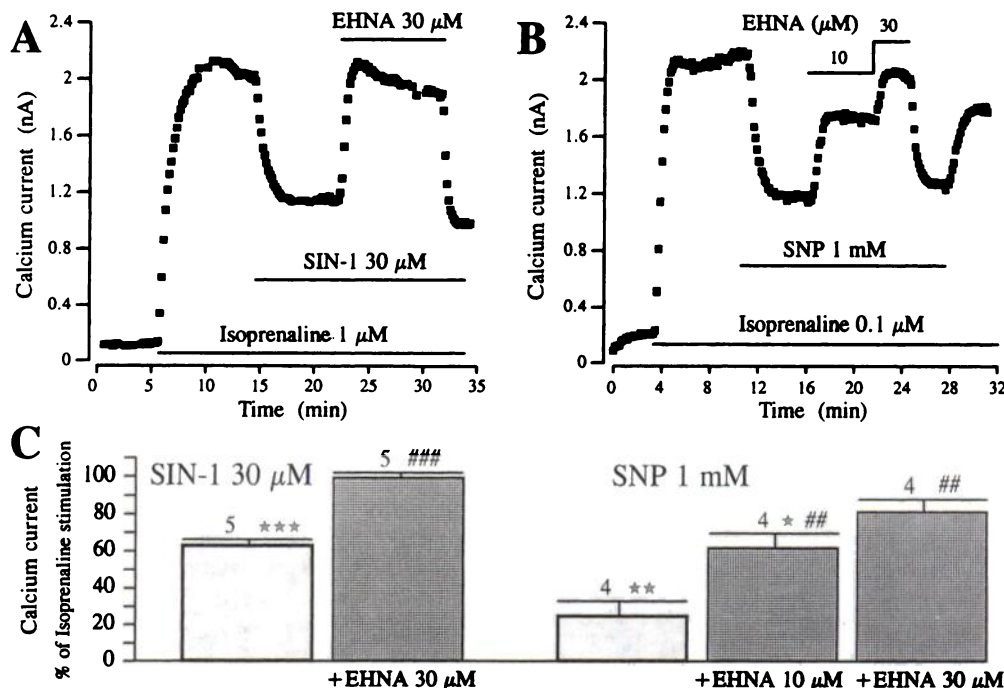


Fig. 6. Effects of EHNA in the presence of NO donors. **A**, Effect of EHNA on SIN-1-induced inhibition of I_{Ca} . A frog ventricular cell was initially superfused with control cesium-containing Ringer solution. During the periods indicated, the cell was superfused with isoprenaline (1 μ M), isoprenaline plus SIN-1 (30 μ M), and isoprenaline plus SIN-1 plus EHNA (30 μ M). **B**, Effect of EHNA on SNP-induced inhibition of I_{Ca} . A frog ventricular cell was initially superfused with control cesium-containing Ringer solution. During the periods indicated, the cell was superfused with isoprenaline (0.1 μ M), isoprenaline plus SNP (1 mM), and two concentrations of EHNA (10 and 30 μ M), in the presence of isoprenaline and SNP. **C**, Summary of the effects of EHNA on I_{Ca} in the presence of isoprenaline and the NO donors SIN-1 or SNP. Isoprenaline was used at 1 μ M and 0.1 μ M for the experiments with SIN-1 and SNP, respectively. On average, 0.1 μ M and 1 μ M isoprenaline induced $721.1 \pm 55.6\%$ ($n = 5$) and $1326.6 \pm 209.1\%$ ($n = 5$) increases of I_{Ca} , respectively. Bars, means and standard errors of the number of experiments indicated (numbers above the bars). Addition of SIN-1 or SNP reduced the isoprenaline-induced stimulation (100%) to the percentage level indicated (white bars). Significant statistical differences from the isoprenaline-stimulated level (*, **, and ***) or the isoprenaline plus SIN-1 or SNP level (## and ###) are indicated; *, $p < 0.05$; **, $p < 0.005$; *** or ###, $p < 0.001$.

ble potency, with half-maximal inhibition occurring at 5 μ M EHNA. Interestingly, inhibition by EHNA of soluble cGS-PDE occurred in a noncompetitive 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 (Fig. 8B).

We sought to confirm this finding in intact cells, and we examined the potency of EHNA to reverse the inhibitory effect of two different concentrations of cGMP on I_{Ca} . In the experiment shown in Fig. 9A, the cell was intracellularly perfused with 5 μ M cAMP, which induced a ~ 7 -fold increase in I_{Ca} . cGMP at 10 μ M was then added to the intracellular perfusion solution so that the cAMP stimulation was strongly reduced. As shown in Fig. 9, exposing the cell to 30 μ M EHNA fully reversed the inhibitory effect of cGMP. Then, in the continuing presence of EHNA, the intracellular cGMP concentration was increased to 100 μ M, which did not induce any modification of I_{Ca} . This suggests that a 30 μ M concentration of EHNA was equipotent in antagonizing the inhibitory effects of 10 and 100 μ M cGMP on cAMP-elevated I_{Ca} . This is further demonstrated in the summary data shown in Fig. 9B. Indeed, 30 μ M EHNA reversed the inhibitory effect of cGMP on I_{Ca} to the same extent whether cGMP was used at a 5, 10, or 100 μ M concentration. Together with the apparent non-competitiveness with cGMP of EHNA effects on purified soluble cGS-PDE activity, these findings support the hypothesis that cGMP and EHNA bind to different sites on the cGS-PDE.

Participation of adenosine deaminase in the effects of EHNA? The results obtained from the experiments described above led us to hypothesize that EHNA selectively inhibits cGS-PDE activity. However, because EHNA is an inhibitor of adenosine deaminase, it seemed essential to rule out a possible indirect effect of EHNA due to adenosine accumulation. Adenosine (10 μ M) had no effect on PDE activity in frog ventricle particulate fraction, measured under basal conditions or in the presence of 5 μ M cGMP to stimulate the cGS-PDE activity (data not shown). At the same concentration, adenosine induced an inhibitory effect on isoprenaline-stimulated I_{Ca} in two of four cells (data not shown; see also Ref. 30). 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} .

Discussion

In the present study, we examined the effects of EHNA on I_{Ca} and PDE activity in frog ventricular cells. Several main conclusions can be drawn from our experiments. (i) EHNA has no effect on basal I_{Ca} or on basal PDE activity. (ii) EHNA has no effect on I_{Ca} elevated by either isoprenaline or intracellular cAMP, whereas inhibitors of PDE3 and PDE4 induce a further stimulation of I_{Ca} . (iii) EHNA reverses the inhibitory effect of cGMP and NO donors on cAMP- or isoprenaline-

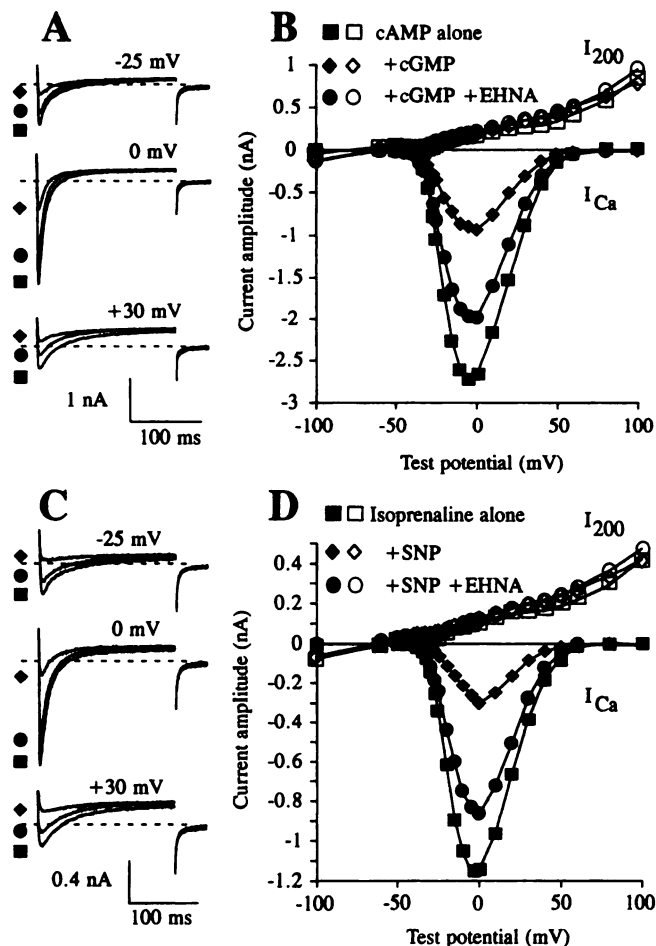


Fig. 7. Voltage dependence of the effects of EHNA on I_{Ca} . **A** and **B**, The effect of EHNA ($30 \mu\text{M}$) was examined in the presence of cAMP ($5 \mu\text{M}$) and cGMP ($5 \mu\text{M}$). **C** and **D**, The effect of the same concentration of EHNA was examined in the presence of isoprenaline ($0.1 \mu\text{M}$) and SNP (1 mM). **A** and **C**, Individual current traces obtained by depolarization of the cell for 200 msec at three different test potentials (-25 , 0 , and $+30 \text{ mV}$) from a -80-mV holding potential (see Materials and Methods) are superimposed for the three experimental conditions tested in each experiment, i.e., cAMP (squares), cAMP plus cGMP (diamonds), and cAMP plus cGMP plus EHNA (circles) (**A**) and isoprenaline (squares), isoprenaline plus SNP (diamonds), and isoprenaline plus SNP plus EHNA (circles) (**B**). Dotted lines in **A** and **C**, the zero-current level for each series of current traces. **B** and **D**, Current-voltage relationships for I_{Ca} (filled symbols) measured during 200-msec depolarizations to various potentials and for I_{200} (open symbols), the current measured at the end of the 200-msec depolarizations (see Materials and Methods), in the same experiments as in **A** and **C**, respectively, are shown. Symbols in **B** and **D**, same conditions as in **A** and **C**, respectively.

elevated I_{Ca} . (iv) EHNA specifically inhibits the cGS-PDE isoform. (v) The effects of EHNA on I_{Ca} and cGS-PDE activity occur at the same concentrations ($IC_{50} = 3\text{--}5 \mu\text{M}$), appear noncompetitive with cGMP, and cannot be mimicked by adenosine. We conclude that EHNA acts primarily to inhibit cGS-PDE in intact frog ventricular myocytes.

Since the discovery by Beavo *et al.* (31) of a stimulatory effect of cGMP on cAMP hydrolysis in various tissues, including heart, much information has accumulated on the properties (32), structure (32, 33), and amino acid sequence (33, 34) of the cGS-PDE isoforms (for review, see Refs. 17, 29, and 35–37). Although cGS-PDE is expressed in a large number of tissues, relatively little is known regarding its function. One reason for this is that at the present time no agent has been

described as a truly selective inhibitor of cGS-PDE (17, 35). Various compounds, such as dipyridamole or the isoquinoline derivatives HL-725 (trequinsin) and papaverine (29, 38), have been shown to produce a somewhat greater inhibition of cardiac cGS-PDE than of PDE1 and PDE3 (29, 35). However, in platelets dipyridamole inhibits PDE1 and cGS-PDE activities to comparable degrees (35), and in frog heart dipyridamole and papaverine were shown to inhibit PDE4 with K_i values lower than those for cGS-PDE (39). For these reasons, the evidence demonstrating a physiological function for cGS-PDE in various tissues, such as human fibroblasts (40), bovine adrenal glomerulosa cells (41), rat pheochromocytoma PC-12 cells (38), and frog cardiac myocytes (3, 4, 12), has been obtained by means other than direct and selective inhibition of cGS-PDE. These means included a demonstration by immunological methods of a high level of expression of cGS-PDE (41), a comparison of the effects of various cyclic nucleotide analogs and of their relative rates of *in vitro* hydrolysis by purified cGS-PDE (3, 4, 41), and the use of nonselective PDE inhibitors such as IBMX (3, 4, 12). In the frog ventricle, this type of approach has led to an accumulation of data supporting a predominant role for cGS-PDE in the regulation of I_{Ca} by cGMP and NO donors (3, 4, 10–12, 16). For this reason, the frog ventricle is an ideal preparation with which to investigate the effects of a potential cGS-PDE inhibitor.

A major drawback in the use of EHNA to inhibit cGS-PDE is that EHNA is a potent inhibitor of adenosine deaminase. This raises the possibility that inhibition of adenosine deaminase, possibly via an accumulation of adenosine, may participate in the effects of EHNA described here. Although we could not totally eliminate this possibility, a series of arguments makes this hypothesis most unlikely. First, adenosine does not mimic the effects of EHNA on either PDE or I_{Ca} . When applied extracellularly to isolated frog ventricular cells, adenosine either has no effect on or induces an inhibition of isoprenaline-stimulated I_{Ca} , likely mediated by the activation of A_1 adenosine receptors, which are negatively coupled to adenylyl cyclase (30). The intracellular accumulation of adenosine upon exposure to EHNA might also affect the regulation of I_{Ca} . In particular, adenosine could inhibit adenylyl cyclase by binding to the intracellular P-site of the enzyme. However, such an effect would result in an inhibition of I_{Ca} , which has not been observed with EHNA. Second, it is most unlikely that adenosine deaminase is functioning under our experimental conditions, even in the absence of EHNA. Indeed, adenosine deaminase is hardly detectable in cardiac myocytes, whereas it is present at high levels in other cardiac cell types (42). In addition, the K_m of adenosine deaminase for adenosine is in the range of $20\text{--}50 \mu\text{M}$ (42). Thus, unless adenosine deaminase activity is present in a compartment not readily available for intracellular perfusion, e.g., in the close vicinity of the membrane, the continuous dialysis of the cell in our whole-cell patch-clamp experiments would prevent such a large accumulation of adenosine within the cell. Finally, the cell is also continuously superfused with fresh extracellular solution, which does not contain adenosine. This would tend to rapidly dilute from the extracellular surface of the membrane any adenosine released by the cell, hence eliminating possible activation of membrane adenosine receptors. Therefore, for all of these reasons, we conclude that inhibition of adenosine deaminase

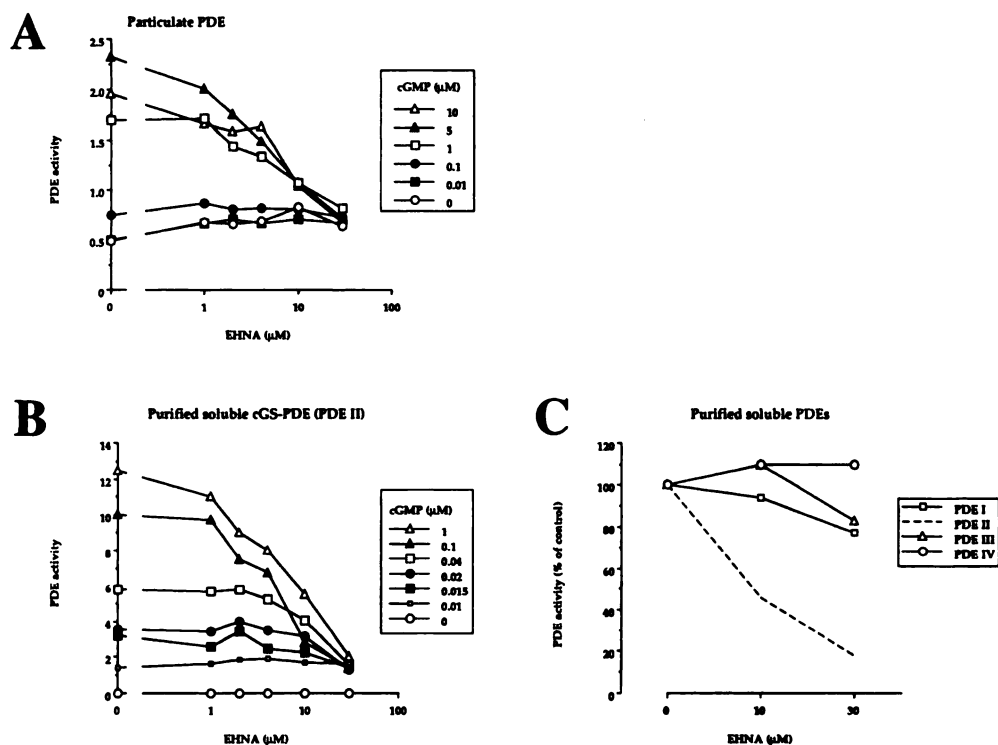


Fig. 8. A, Dose-dependent inhibition by EHNA of cGMP-stimulated PDE activity in frog ventricle particulate fraction. PDE activity was measured in the presence of 2μ M cAMP, with different cGMP concentrations and varying EHNA concentrations, as described in Materials and Methods. B, Examination of dose-dependent inhibition by EHNA of purified soluble cGS-PDE activity under the same conditions as used for the particulate fraction. C, Specificity of EHNA effect. The effects of 10 and 30 μ M EHNA on the purified soluble PDE1–4 isoforms were examined under the same conditions as for A and B. The data are normalized with respect to the PDE activities in the absence of EHNA. These were as follows: PDE1, 14.2 ± 2.5 nmol of cAMP/mg/10 min; PDE3, 7.0 ± 0.6 nmol of cAMP/mg/10 min; PDE4, 1.0 ± 0.1 nmol of cAMP/mg/10 min. Dotted line, effect of EHNA on cGS-PDE activity in the presence of 0.1 μ M cGMP (taken from B).

does not participate to any significant extent in the effects of EHNA described in the present study.

We also examined the possibility that EHNA might have other effects in our preparation, in addition to the inhibitory effects on cGS-PDE and adenosine deaminase, that could account for some of the observed effects of EHNA on I_{Ca} . However, we found that, when applied in the absence of cGMP or NO donors, EHNA did not modify the amplitude, kinetics, or voltage dependence of I_{Ca} . This was found when I_{Ca} was at its basal level or when I_{Ca} was stimulated by isoprenaline or intracellular cAMP, suggesting that EHNA does not directly interact with the Ca^{2+} channels in their phosphorylated or nonphosphorylated states. This also suggests that EHNA does not interact with any intermediate step between β -adrenergic receptor activation and phosphorylation of the Ca^{2+} channels. This latter hypothesis is further supported by the finding that EHNA did not modify basal or isoprenaline-stimulated adenylyl cyclase activity in frog ventricle particulate fraction (data not shown). In addition to inhibition of adenosine deaminase and cGS-PDE, EHNA has been found to be an inhibitor of dynein ATPase activity, actin assembly, and cell motility (43, 44). Although we did not address these questions specifically, it is hard to anticipate how these alternative mechanisms could account for the observed effects of EHNA on I_{Ca} and PDE activity.

An important feature of the present study is the remarkable correspondence between our biochemical and electrophysiological data. For example, in frog myocytes EHNA had no effect on isoprenaline- or cAMP-elevated I_{Ca} , whereas other selective PDE inhibitors, such as milrinone or Ro 20–

1724, or the nonselective PDE inhibitor IBMX increased I_{Ca} . This suggests that, whereas different forms of PDE, more specifically the PDE3 and PDE4 isoforms (see also Refs. 11 and 16), were active under our experimental conditions, EHNA did not modify their activity. The data obtained with purified soluble PDE isoforms supported this observation, because EHNA (up to 30 μ M) was found to have no or little effect on PDE1, -3, or -4. Moreover, both in intact myocytes and in particulate fractions, an effect of EHNA was observed only after elevation of cGMP concentrations. 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 the particulate fraction or on purified soluble cGS-PDE. Finally, both biochemical and electrophysiological data support the hypothesis that EHNA inhibits cGS-PDE in a noncompetitive 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 cGS-PDE.

Our results also confirm the importance of cGS-PDE in mediating the response of frog ventricular cells to cGMP or NO donors. Indeed, EHNA fully antagonized the inhibitory effect of intracellular cGMP and NO donors, such as SIN-1 and SNP, on I_{Ca} . This supports our initial hypothesis that the inhibition of I_{Ca} by cGMP and NO is entirely due to a massive enhancement of cGS-PDE activity in this preparation (3, 4, 12). Interestingly, EHNA had no effect on I_{Ca} in the absence of cGMP or NO donors, whereas inhibitors of PDE3 (milrinone) and PDE4 (Ro 20–1724) stimulated the current elevated by nonmaximal concentrations of cAMP or isoprenaline

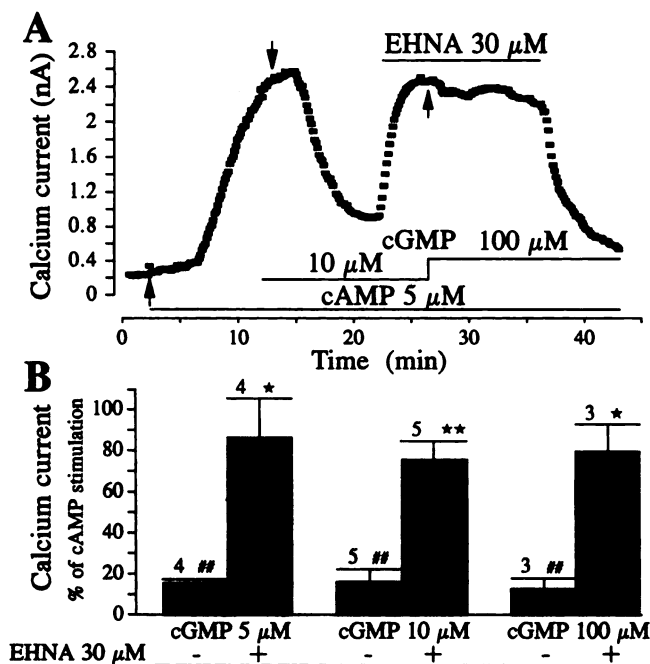


Fig. 9. Effects of EHNA on I_{Ca} in the presence of increasing concentrations of cGMP. **A**, A frog ventricular cell was initially superfused with control cesium-containing Ringer solution and internally dialyzed with control intracellular cesium-containing solution. First arrow, 5 μ M cAMP was added to the intracellular solution, which then perfused the cell throughout the rest of the experiment. Second arrow, 10 μ M cGMP was added to the cAMP-containing intracellular solution. During the period indicated, the cell was superfused with 30 μ M EHNA, which completely reversed the inhibitory effect of cGMP. Third arrow, cGMP concentration was increased to 100 μ M, which had no effect on I_{Ca} . **B**, A summary of the effects of EHNA (30 μ M) on I_{Ca} in the presence of cAMP (5 μ M) and three different concentrations of cGMP (5, 10, and 100 μ M) in the intracellular solution is shown. Bars, means and standard errors of the number of experiments indicated (numbers above the bars). Addition of cGMP reduced the cAMP-induced stimulation (100%) to the percentage level indicated ($-EHNA$). Significant statistical differences from the cAMP-stimulated level (##) or the cAMP plus cGMP level (* and **) are indicated; *, $p < 0.01$; ** or ##, $p < 0.005$.

(Ref. 11 and this study). It suggests that, in frogs, cGS-PDE does not participate in the basal PDE activity of cardiac myocytes. This aspect needs to be examined in other species, e.g., by investigating the effects of EHNA on cyclic nucleotide levels and electrical and mechanical activities of cardiac tissues or isolated cells and their modifications by cGMP activators, such as NO donors or natriuretic peptides. The use of EHNA should also lead to further understanding of the regulatory role of cGS-PDE in the function of other cell types and organs and in their responses to various physiological stimuli.

Acknowledgments

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