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Activation of cGMP-stimulated phosphodiesterase by nitroprusside limits cAMP accumulation in human platelets: effects on platelet aggregation

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cGMP enhances cAMP accumulation in platelets via cGMP-inhibited phosphodiesterase (PDE3) [Maurice and Haslam (1990) Mol. Pharmacol. **37**, 671–681]. However, cGMP might also limit cAMP accumulation by activating cGMP-stimulated phosphodiesterase (PDE2). We therefore evaluated the role of PDE2 in human platelets by using *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) to inhibit this enzyme selectively. IC_{50} values for the inhibition of platelet PDE2 by EHNA, with 10 μM cAMP as substrate in the absence and in the presence of 1 μM cGMP, were 15 and 3 μM respectively. Changes in platelet cyclic [^{3}H]nucleotides were measured after prelabelling with [^{3}H]adenine and [^{3}H]guanine. Nitroprusside (NP) caused concentration-dependent increases in [^{3}H]cGMP and a biphasic increase in [^{3}H]cAMP, which was maximal at 10 μM (49 ± 6 %) and smaller at 100 μM (32 ± 6 %) (means ± S.E.). In the presence of EHNA (20 μM), which had no effects alone, NP caused much larger increases in platelet [^{3}H]cAMP (125 ± 14 % at 100 μM). EHNA also enhanced [^{3}H]cGMP accumulation at high NP concentrations. In accord with these results, EHNA markedly potentiated the inhibition of thrombin-induced platelet aggregation by NP. The roles of cAMP and cGMP in this effect were investigated by using 2′,5′-dideoxyadenosine to inhibit adenylate cyclase. This compound decreased the accumulation of [^{3}H]cAMP but not that of [^{3}H]cGMP, and diminished the inhibition of platelet aggregation by NP with EHNA. We conclude that much of the effect of NP with EHNA is mediated by cAMP. Lixazinone (1 μM), a selective inhibitor of PDE3, increased platelet [^{3}H]cAMP by 177 ± 15 %. This increase in [^{3}H]cAMP was markedly inhibited by NP; EHNA blocked this effect of NP. Parallel studies showed that NP suppressed the inhibition of platelet aggregation by lixazinone. EHNA enhanced the large increases in [^{3}H]cAMP seen with 20 nM prostacyclin (PGI_2), but had no effect with 1 nM PGI_2. NP and 1 nM PGI_2 acted synergistically to increase [^{3}H]cAMP, an effect attributable to the inhibition of PDE3 by cGMP; EHNA greatly potentiated this synergism. In contrast, NP decreased the [^{3}H]cAMP accumulation seen with 20 nM PGI_2, an effect that was blocked by EHNA. The results show that, provided that cGMP is present, PDE2 plays a major role in the hydrolysis of low cAMP concentrations and restricts any increases in cAMP concentration and decreases in platelet aggregation caused by the inhibition of PDE3. At high cAMP, PDE2 plays the major role in cAMP breakdown, whether cGMP is present or not.

INTRODUCTION

Activators of adenylate cyclase, such as prostacyclin (PGI_2) or prostaglandin E_1, or of soluble guanylate cyclase, such as nitroprusside (NP) and other NO donors, have long been known to inhibit blood platelet aggregation [1–3]. Although it was initially assumed that the cAMP and cGMP formed by these enzymes acted independently by stimulating cAMP-dependent and cGMP-dependent protein kinases respectively, it is now clear that there are several potential sites of interaction between these cyclic nucleotides. These include effects of cGMP on cAMP hydrolysis by cyclic nucleotide phosphodiesterases [4,5], cross-talk between the effects of cAMP and cGMP on the cyclic nucleotide-dependent protein kinases [6,7] and phosphorylation of the same substrate in response to increases in the concentration of either cAMP or cGMP [8].

The cGMP-inhibited phosphodiesterase (PDE3) seems to be particularly important in platelets. Three cyclic nucleotide phosphodiesterases, PDE2, PDE3 and PDE5, have been clearly identified in these cells [9,10] but PDE3 is thought to be responsible for at least 80 % of the hydrolysis of cAMP at the relatively low cAMP concentrations required to inhibit platelet function [10]. Several workers have noted that low concentrations of PGI_2 (or prostaglandin E_1) and NP (or other nitrovasodilator drugs) act synergistically to inhibit platelet aggregation (see, for example, [11,12]). In 1990, Maurice and Haslam [5] showed that in rabbit platelets this synergism can be attributed to increases in the accumulation of cAMP as a result of the inhibition of PDE3 by cGMP. Subsequent studies with human platelets have confirmed this finding [13,14], although at high platelet cAMP concentrations the addition of an NO donor (SIN-1) decreased cAMP [13].

Experiments in this laboratory have shown that NO donors not only enhance the increases in platelet cAMP caused by activators of adenylate cyclase, but also increase platelet cAMP by themselves [2,5]. This observation has been confirmed by some (see, for example, [14,15]), but not others (see, for example, [16,17]). In either case, it is clear that these increases in cAMP are small, perhaps smaller than would be expected given that cGMP is a potent inhibitor of PDE3, and may be close to the limit of detection by most radioimmunoassays [13]. In some of these experiments, we found that 100 μM NP seemed to be less effective than 1–10 μM NP in increasing cAMP in human platelets (M. M. L. Davidson and R. J. Haslam, unpublished work). This observation, together with the inhibitory effect of SIN-1 at high platelet cAMP levels [13], suggested a possible involvement of the cGMP-stimulated phosphodiesterase (PDE2) in the regulation of platelet cAMP concentrations. This enzyme has been purified

Abbreviations used: DDA, 2′,5′-dideoxyadenosine; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; NP, nitroprusside; PDE, phosphodiesterase; PGI_2, prostacyclin.

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from platelets [18] and, like the bovine heart PDE2 [19], shows homotropic co-operativity with high half-maximal velocities at approx. 40 μM cAMP or cGMP and the capacity to respond to low cGMP concentrations with a strong stimulation of cAMP hydrolysis. Recently, *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) has been shown to inhibit heart PDE2 in a potent and highly selective manner [20]. We have now used this compound to investigate the role of PDE2 in human platelets and have found that the activation of PDE2 by cGMP on the addition of NP to platelets has marked and functionally important effects on platelet cAMP. Some of these results have been published in a preliminary form [21].

EXPERIMENTAL

Materials

[2,8-^{3}H]Adenine (26.9 Ci/mmol), [2,8-^{3}H]cAMP (27.0 Ci/mmol), [8-^{3}H]cGMP (9.3 Ci/mmol) and [U-^{14}C]AMP (590.4 mCi/mmol) were obtained from NEN Products (Mississauga, ON, Canada). [8-^{3}H]Guanine (11.9 Ci/mmol) and [8-^{14}C]GMP (50 mCi/mmol) were from Moravek Biochemicals (Brea, CA, U.S.A.) and [8-^{14}C]ATP (50 mCi/mmol) and [8-^{14}C]cAMP (54 mCi/mmol) from ICN (Costa Mesa, CA, U.S.A.). [8-^{14}C]GTP (52 mCi/mmol), [8-^{14}C]cGMP (52 mCi/mmol) and ACS (aqueous counting scintillant) were obtained from Amersham (Oakville, ON, Canada). Zaprinast, pepstatin and PMSF were purchased from Sigma (St. Louis, MO, U.S.A.), and leupeptin from Boehringer–Mannheim (Laval, PQ, Canada). DEAE-Sephacel was supplied by Pharmacia Biotech (Montreal, Canada) and Affi-Gel 601 boronate affinity gel by Bio-Rad Laboratories (Mississauga, ON, Canada). 2′,5′-Dideoxyadenosine (DDA) was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.). All nucleotides, NP, BSA, heparin, neutral alumina (WN-3) and Dowex-50 resin were supplied by sources listed previously [5]. Lixazinone (Syntex Corporation, Palo Alto, CA, U.S.A) and PGI_2 (Upjohn, Kalamazoo, MI, U.S.A.) were gifts. EHNA was generously supplied by Dr. T. Podzuweit (Max Planck Institute for Physiological and Clinical Research, Bad Nauheim, Germany), and thrombin by Dr. J. W. Fenton II (New York State Department of Health, Albany, NY, U.S.A.).

Partial purification of human platelet phosphodiesterases

Platelets were isolated from 200 ml of fresh human blood and washed by the method of Mustard et al. [22], with some modifications. After centrifugation of platelet-rich plasma, the platelets were first washed in Ca^{2+}-free Tyrode's solution (10 ml) containing 5 mM Pipes buffer, pH 6.5, apyrase (90 μg/ml), BSA (3.5 mg/ml) and heparin (50 i.u./ml) and were then washed in the same medium without heparin and BSA. The platelets (approx. 2×10^{10}) were then resuspended in 8 ml of ice-cold buffer containing 50 mM Tris (pH adjusted to 6.0 with acetic acid), 1 mM EDTA, 4 μM pepstatin, 80 μM chymostatin, 400 μM leupeptin, 1 mM PMSF and 5 mM 2-mercaptoethanol and sonicated six times (15 s each) at 0 °C. Any intact platelets and the platelet granules were quickly removed by centrifugation at 19000 ***g*** for 20 min and soluble protein was then isolated by ultracentrifugation at 100000 ***g*** for 1 h. Supernatant (approx. 8 mg of protein) was applied to a DEAE-Sephacel column (2.0 ml) that had been washed with buffer containing 50 mM Tris/acetate (pH 6.0) and 1 mM EDTA. The column was then washed with 10 ml of the same buffer containing 2 μM pepstatin, 40 μM chymostatin, 50 μM leupeptin, 0.5 mM PMSF and 5 mM 2-mercaptoethanol; platelet proteins were eluted with a linear sodium acetate gradient (0–0.8 M) dissolved in 40 ml of this solution. Fractions (0.5 ml) were collected and assayed for phosphodiesterase activities, with 10 μM [^{3}H]cGMP, 10 μM [^{3}H]cAMP and 0.5 μM [^{3}H]cAMP as substrates. Three peaks of enzyme activity were observed and identified as PDE5 (eluted by 0.01–0.07 M sodium acetate), PDE2 (eluted by 0.25–0.40 M sodium acetate) and PDE3 (eluted by 0.44–0.61 M sodium acetate) from their relative activities with [^{3}H]cAMP and [^{3}H]cGMP as substrates and from the selective inhibitory effects of 10 μM zaprinast, 50 μM EHNA and 0.5 μM lixazinone.

Phosphodiesterase assays

A modification of published methods [23,24] was used. Assays (100 μl) contained 5 mM $MgCl_2$, 50 mM Hepes (adjusted to pH 7.5 with KOH), 1 mM EGTA, 0.1 mM leupeptin and 0.1 μCi of [^{3}H]cAMP (0.5 or 10 μM) or [^{3}H]cGMP (10 μM). Reactions were initiated by the addition of an appropriate volume of enzyme (6–20 μl), to give approx. 10% substrate hydrolysis. Incubations were for 5 min at 30 °C and were terminated by addition of 0.1 ml of 10 mM EDTA (pH 7.6) followed by heating at 100 °C for 2 min. [^{14}C]AMP (2000 d.p.m.) or [^{14}C]GMP (4000 d.p.m.) was added to appropriate samples as a recovery marker. Labelled AMP or GMP was isolated by chromatography on Affi-Gel 601 boronate affinity columns (0.8 ml of gel). Non-hydrolysed cyclic nucleotides were removed with 16 ml of solution containing 50 mM Hepes, pH 8.5, and 100 mM $MgCl_2$; 5′-nucleotides were then eluted with 2 ml of 250 mM acetic acid, which was counted for ^{3}H and ^{14}C in 10 ml of ACS. Assays were performed in triplicate and any [^{3}H]AMP or [^{3}H]GMP isolated from samples without enzyme was subtracted. IC_{50} values for the inhibition of phosphodiesterase activity were calculated with GraFit version 3.0 (Erithacus Software Ltd., Staines, U.K.).

Prelabelling assays for changes in platelet cyclic nucleotides

Washed platelets were isolated as described above, except that 10 ml of Tyrode's solution containing 5 mM Hepes, pH 7.4, apyrase (30 μg/ml) and BSA (3.5 mg/ml) was used for the second resuspension [at (2.0–2.5)$\times 10^9$ platelets/ml]. Platelet metabolic nucleotide pools were then labelled by a modification of the method of Maurice and Haslam [5]. [^{3}H]Adenine (1 μM, 20 Ci/mmol) was added and the suspension was incubated at 37 °C for 30 min. The platelets were then resuspended in 20 ml of the same medium and incubated with 1.5 μM [^{3}H]guanine (9.3 Ci/mmol) for 1 h at 37 °C. The labelled platelets were finally resuspended at 4.6×10^8/ml in Tyrode's solution containing 5 mM Hepes, pH 7.4, apyrase (6 μg/ml) and BSA (3.5 mg/ml).

Incubations of prelabelled platelets were started by the mixing of 0.445 ml of labelled platelet suspension with 0.055 ml of additions. NP and DDA were dissolved in 0.154 M NaCl, and EHNA, lixazinone or zaprinast in DMSO (final concentration 0.2% v/v). PGI_2 was dissolved in 0.139 M NaCl containing 9.4 mM Na_2CO_3 and was added to samples 12 s before the platelets. Incubations were for 1 min at 37 °C and were terminated by the addition of 0.5 ml of 20% (w/v) trichloroacetic acid. [^{14}C]cAMP and [^{14}C]cGMP (1000 d.p.m. of each) were added to all samples as recovery markers. Platelet cAMP and cGMP were isolated by chromatography on neutral alumina and separated on Dowex 50 anion-exchange resin as described previously [5]. Platelet [^{3}H]ATP and [^{3}H]GTP were isolated from samples of the prelabelled platelet suspension (0.445 ml), which were mixed with 0.5 ml of 20% trichloroacetic acid and 30000 d.p.m. of [^{14}C]ATP and [^{14}C]GTP [25]; the amounts of [^{3}H]ATP and [^{3}H]GTP obtained were corrected for the recovery of

[14C]ATP and [14C]GTP. Platelet [3H]cAMP and [3H]cGMP were then expressed as percentages of the total platelet [3H]ATP and [3H]GTP respectively [5]. All incubations for measurement of changes in [3H]cAMP and [3H]cGMP were performed in triplicate. Individual experiments were repeated at least three times, with minor variations.

Platelet aggregation studies

Platelets were isolated as described for the prelabelling assays of cyclic nucleotides, except that after the second wash the platelets were resuspended at $(4\text{–}5)\times10^8$/ml in Tyrode's solution containing 5 mM Hepes, pH 7.4, apyrase (3 μg/ml) and BSA (3.5 mg/ml). Samples of this suspension were stirred with additions in an aggregometer (Payton Associates, Scarborough, ON, Canada) before the addition of human thrombin to give a final volume of 1.0 ml. Aggregation was recorded as the decrease in attenuance of the platelet suspension and was followed for 2 min after the addition of thrombin.

RESULTS

Effects of EHNA on partly purified platelet cyclic nucleotide phosphodiesterases

EHNA has been reported to be a selective inhibitor of PDE2 isolated from human and pig myocardium [20]. We studied the effects of this compound on platelet cyclic nucleotide phosphodiesterases to evaluate the suitability of EHNA for the investigation of the role of PDE2 in platelet function. The three platelet phosphodiesterases PDE5, PDE2 and PDE3, were separated by a modification of an earlier method [9]. The activity of PDE2 (substrate 10 μM [3H]cGMP) was potently inhibited by EHNA (IC_{50} 1.4 μM), whereas the activities of PDE5 (substrate 10 μM [3H]cGMP) and PDE3 (substrate 0.5 μM [3H]cAMP) were not significantly affected by EHNA concentrations up to 100 μM (Figure 1). The same result was obtained when PDE5 was assayed with 0.5 μM [3H]cGMP. The cAMP phosphodiesterase activities of bovine heart and platelet PDE2 are strongly stimulated by low concentrations of cGMP [10,11]. The effects of EHNA on basal and cGMP-stimulated platelet PDE2 activities, using 10 μM [3H]cAMP as substrate without or with 1 μM cGMP, were therefore investigated (Figure 2). The results show that EHNA was more effective as an inhibitor of enzyme activity in the presence than in the absence of cGMP (IC_{50} values 3.3 and 14.6 μM respectively). These findings indicated that EHNA is a selective inhibitor of platelet PDE2 that is particularly effective in the presence of cGMP. In contrast, lixazinone, which at 0.5 μM inhibited platelet PDE3 by 97 ± 1% (three experiments), had very little effect on PDE2 activity with 10 μM [3H]cAMP (9 ± 3% inhibition with 1 μM lixazinone).

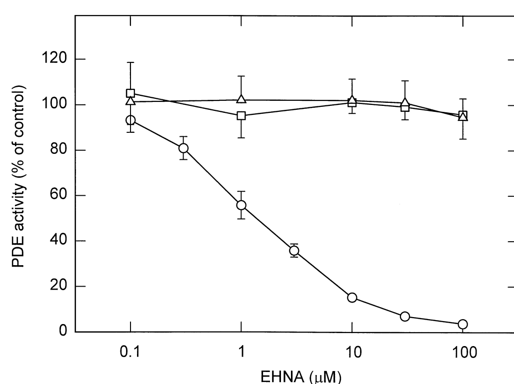

Figure 1 Effects of EHNA on the three soluble cyclic nucleotide phosphodiesterases isolated from human platelets

Three PDE activities were separated from the platelet supernatant fraction as described in the Experimental section, and identified as PDE2, PDE3 and PDE5. Samples of each enzyme were then incubated for 5 min at 30 °C with the following substrates: PDE2, 10 μM [3H]cGMP (○); PDE3, 0.5 μM [3H]cAMP (□); PDE5, 10 μM [3H]cGMP (△), in each case in the absence or presence of the indicated concentrations of EHNA. Values for cyclic nucleotide hydrolysis in the presence of EHNA are expressed as percentages of the values obtained without EHNA and are the means ± S.E. of three determinations in a single experiment.

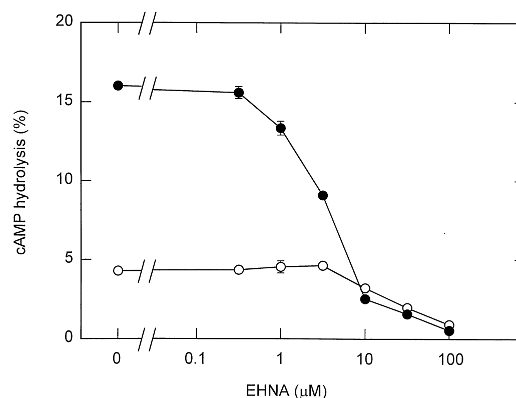

Figure 2 Effects of EHNA on the activity of human platelet PDE2 in the absence or presence of cGMP

PDE2 was isolated from platelets as described in the Experimental section. Samples were incubated for 5 min at 30 °C with 10 μM [3H]cAMP (○) or 10 μM [3H]cAMP and 1 μM cGMP (●), in each case in the presence of the indicated concentration of EHNA. Values for the percentage of cAMP hydrolysed are shown and are the means ± S.E. three determinations in a single experiment.

Effects of NP, EHNA and lixazinone on platelet cyclic nucleotide concentrations; relationship to the inhibition of platelet aggregation

Experiments in this laboratory have suggested that NP may have a biphasic effect on platelet cAMP content, causing optimal increases at 1–10 μM and smaller increases at higher concentrations of NP (M. M. L. Davidson and R. J. Haslam, unpublished work). We have examined this phenomenon in detail, using EHNA to investigate the possibility that the inhibitory component reflects activation of PDE2.

Platelet metabolic nucleotide pools were prelabelled with [3H]adenine and [3H]guanine, as described in the Experimental section, and incubated for 1 min with 0.1–100 μM NP. Platelet [3H]cAMP accumulation was maximal with 10 μM NP (Figure 3a). In the total number of experiments on which the present study is based, the increases in [3H]cAMP were as follows (means ± S.E.): 29 ± 5% with 0.1 μM NP ($n=3$), 47 ± 5% with 1 μM NP ($n=8$), 49 ± 6% with 10 μM NP ($n=9$) and 32 ± 6% with 100 μM NP ($n=13$). The decrease in [3H]cAMP accumulation on increasing NP from 10 μM to 100 μM was significant ($P<0.05$, two-sided paired t test, $n=8$). Platelet [3H]cGMP increased rapidly as the concentration of NP was increased (Figure 3b), reaching 1.3 ± 0.2% of platelet [3H]GTP with 100 μM NP ($n=15$). The increases in [3H]cAMP caused by NP are consistent with an inhibition of platelet PDE3 by cGMP.

The possible role of PDE2 in the inhibitory component of the biphasic effect of NP was investigated with EHNA (Figures 3

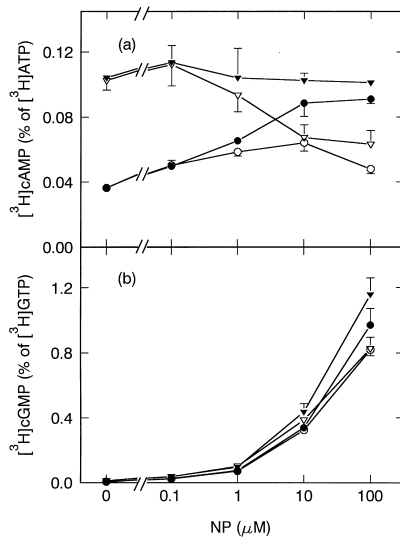

Figure 3 Effects of different NP concentrations on the accumulation of cyclic nucleotides in human platelets in the presence or absence of lixazinone and EHNA

Metabolic nucleotide pools were prelabelled by incubation of platelets with [^{3}H]adenine and [^{3}H]guanine as described in the Experimental section. Samples of platelet suspension were incubated for 1 min at 37 °C with the indicated concentrations of NP and either no further additions (○), 20 μM EHNA (●), 1 μM lixazinone (▽) or 20 μM EHNA + 1 μM lixazinone (▼). Platelet [^{3}H]cAMP (**a**) and [^{3}H]cGMP (**b**) were isolated and expressed as percentages of the total platelet [^{3}H]ATP and [^{3}H]GTP respectively, as described in the Experimental section. Results are the means ± S.E. of triplicate measurements in a single experiment.

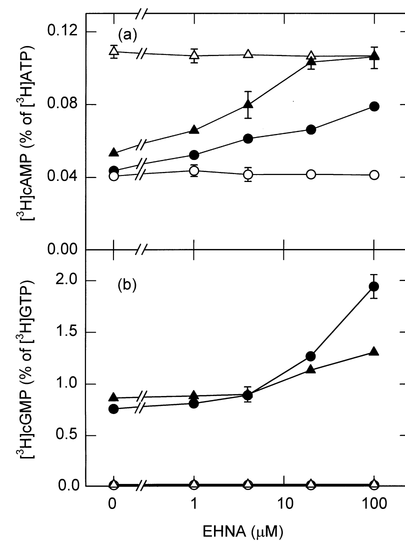

Figure 4 Effects of EHNA on the accumulation of cyclic nucleotides in human platelets caused by NP and lixazinone

Metabolic nucleotide pools were prelabelled by incubation of platelets with [^{3}H]adenine and [^{3}H]guanine as described in the Experimental section. Samples of platelet suspension were incubated for 1 min at 37 °C with the indicated concentrations of EHNA and either no further additions (○), 100 μM NP (●), 1 μM lixazinone (△) or 100 μM NP + 1 μM lixazinone (▲). Platelet [^{3}H]cAMP (**a**) and [^{3}H]cGMP (**b**) were isolated and expressed as percentages of the total platelet [^{3}H]ATP and [^{3}H]GTP respectively, as described in the Experimental section. Results are the means ± S.E. of triplicate measurements in a single experiment.

and 4). When NP was absent, or present at 0.1 μM, the addition of 20 μM EHNA had no significant effect on platelet [^{3}H]cAMP accumulation. However, with higher NP concentrations, EHNA increased the accumulation of [^{3}H]cAMP, which reached a plateau above 10 μM NP (Figure 3a). Relative to the increases in [^{3}H]cAMP seen with NP alone, the effect of EHNA was most marked with 100 μM NP. Under these conditions, NP plus EHNA increased [^{3}H]cAMP by $125 \pm 14\%$ ($n = 11$), much more than seen with NP alone ($P < 0.001$, two-sided paired t test, $n = 11$). The actions of increasing concentrations of EHNA on [^{3}H]cAMP accumulation in the presence of 100 μM NP are shown in Figure 4(a). An effect of EHNA was detectable with 1 μM compound; progressively larger increases in [^{3}H]cAMP were seen at higher concentrations. These results indicate that high concentrations of NP (cGMP) activate PDE2 and greatly diminish the increases in [^{3}H]cAMP caused by the inhibition of PDE3. When NP was present at 100 μM, 20 μM EHNA caused rather variable increases in [^{3}H]cGMP accumulation (Figures 3b and 4b), amounting to $113 \pm 28\%$ of the value seen with NP alone (mean ± S.E.M., $n = 12$). However, EHNA had much less effect on the [^{3}H]cGMP accumulation caused by lower NP concentrations.

To distinguish the effects of NP on PDE2 activity from its effects on PDE3, prelabelled platelets were studied in which the latter enzyme was inhibited by lixazinone (Figures 3 and 4). In the absence of NP, 1 μM lixazinone increased [^{3}H]cAMP by $177 \pm 15\%$ (mean ± S.E., $n = 4$), considerably more than seen with the most effective concentration of NP. At concentrations greater than 1 μM, NP inhibited this effect of lixazinone (Figures 3a and 4a). This action of NP was greatest at 100 μM, when platelet [^{3}H]cAMP decreased to $55 \pm 5\%$ of the value observed with lixazinone alone (mean ± S.E., $n = 4$). To determine whether this effect of NP was caused by activation of PDE2, prelabelled platelets were incubated with EHNA in addition to lixazinone. In the presence of 20 μM EHNA, NP had no significant inhibitory effects on the [^{3}H]cAMP accumulation caused by lixazinone (Figures 3 and 4). These results confirm that, at high concentrations (10–100 μM), NP stimulates cAMP hydrolysis by PDE2, and that this markedly attenuates the increase in [^{3}H]cAMP attributable to the inhibition of PDE3. With the highest concentration of EHNA tested (100 μM), the accumulation of [^{3}H]cGMP induced by 100 μM NP was increased by $128 \pm 23\%$ (mean ± S.E., $n = 4$). This suggests that PDE2 also plays a major role in the breakdown of cGMP in the platelet.

The functional significance of the activation of PDE2 by NP was examined in platelet aggregation studies. In each experiment, the concentration of thrombin used to induce aggregation was adjusted to permit detection of a full range of inhibitory effects. Under conditions in which 10 μM NP alone caused a slight inhibition of thrombin-induced aggregation (compare Figure 5, a and b), the further addition of 20 μM EHNA abolished platelet aggregation, leaving only a thrombin-induced change in platelet shape (increase in attenuance) (Figure 5, f). EHNA alone had no effect on platelet aggregation (Figure 5, e). These results show that when the activity of PDE2 was blocked, the inhibitory action of NP on aggregation was greatly enhanced. At 1 μM, lixazinone alone completely prevented platelet aggregation but, in the simultaneous presence of 10 μM NP, this inhibitory effect of lixazinone was greatly diminished (Figure 5, c and 5, d). On the addition of 20 μM EHNA, this apparent stimulation of aggregation by NP was abolished (Figure 5h) and an inhibition of aggregation similar to that seen with lixazinone alone was observed. These results are consistent with stimulation of PDE2 by NP. Each of the effects described above was seen in at least

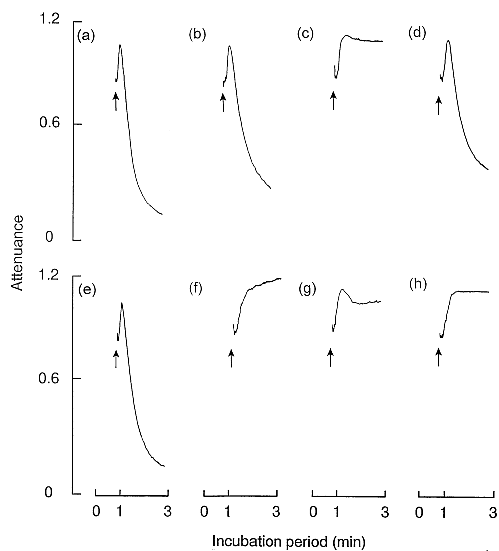

Figure 5 Interactions between NP, lixazinone and EHNA in their effects on thrombin-induced platelet aggregation

Human platelets were isolated and washed as described in the Experimental section. Samples of platelet suspension were incubated for 1 min at 37 °C in the absence (**a**–**d**) or presence (**e**–**h**) of 20 μM EHNA and the following other additions: (**a**, **e**) none; (**b**, **f**) 10 μM NP; (**c**, **g**) 1 μM lixazinone; (**d**, **h**) 10 μM NP + 1 μM lixazinone. Platelet aggregation was then initiated by the addition of human thrombin (final concentration 0.3 i.u./ml) (arrow) and recorded with an aggregometer. Tracings of the recordings are shown.

Table 1 Effects of DDA on the accumulation of cyclic nucleotides in human platelets caused by NP and EHNA

Metabolic nucleotide pools were prelabelled by the incubation of platelets with [^{3}H]adenine and [^{3}H]guanine as described in the Experimental section. Samples of platelet suspension were incubated for 1 min at 37 °C in the absence or presence of 100 μM DDA and either no further additions, 10 μM NP, 20 μM EHNA or 10 μM NP + 20 μM EHNA, as indicated. Platelet [^{3}H]cAMP and [^{3}H]cGMP were isolated and expressed as percentages of the total platelet [^{3}H]ATP and [^{3}H]GTP respectively; values are the means ± S.E. of triplicate measurements in a single experiment.

Additions	[^{3}H]cAMP (% of [^{3}H]ATP)	[^{3}H]cGMP (% of [^{3}H]GTP)
None	0.025 ± 0.001	0.009 ± 0.002
DDA	0.023 ± 0.001	0.012 ± 0.003
NP	0.041 ± 0.001	0.389 ± 0.006
NP + DDA	0.034 ± 0.001	0.427 ± 0.032
EHNA	0.025 ± 0.001	0.008 ± 0.001
EHNA + DDA	0.026 ± 0.001	0.008 ± 0.001
NP + EHNA	0.055 ± 0.002	0.397 ± 0.012
NP + EHNA + DDA	0.041 ± 0.001	0.457 ± 0.018

two additional experiments, differing only in the concentrations of thrombin used.

Because EHNA could cause an increase in both cyclic nucleotides in the presence of NP, we sought to determine whether the potentiation of the inhibitory effect of NP by EHNA was mediated by cAMP or cGMP, by using the adenylate cyclase inhibitor DDA [26] to inhibit [^{3}H]cAMP formation (Table 1). An NP concentration of 10 μM was used in these experiments (as in Figure 5) to minimize the effect of EHNA on platelet [^{3}H]cGMP. DDA (100 μM) diminished the accumulation of [^{3}H]cAMP by

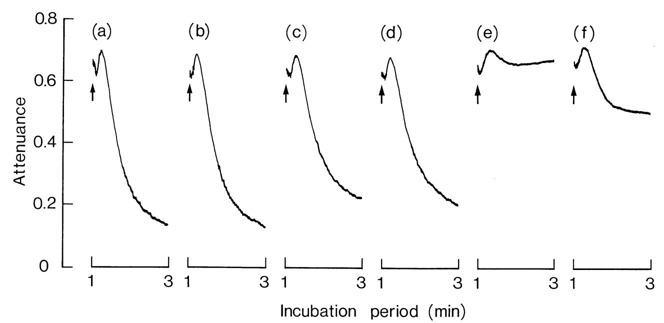

Figure 6 Effect of DDA on the inhibition of thrombin-induced platelet aggregation by NP and EHNA

Human platelets were isolated and washed as described in the Experimental section. Samples of platelet suspension were incubated for 1 min at 37 °C in the absence (**a**, **c**, **e**) or presence (**b**, **d**, **f**) of 100 μM DDA and the following other additions: (**a**, **b**) none; (**c**, **d**) 10 μM NP; (**e**, **f**) 10 μM NP + 20 μM EHNA. Platelet aggregation was then initiated by the addition of human thrombin (final concentration 0.125 i.u./ml) (arrow) and recorded with an aggregometer. Tracings of the recordings are presented. The addition of 20 μM EHNA or 20 μM EHNA + 100 μM DDA had no effect on platelet aggregation (results not shown).

Table 2 Effects of a low PGI_2 concentration and NP on platelet cyclic nucleotides in the absence or presence of EHNA

Metabolic nucleotide pools were prelabelled by the incubation of platelets with [^{3}H]adenine and [^{3}H]guanine as described in the Experimental section. Samples of platelet suspension were incubated for 1 min at 37 °C with 20 μM EHNA, 1 nM PGI_2 and 10 μM NP in the combinations indicated. Platelet [^{3}H]cAMP and [^{3}H]cGMP were isolated and expressed as percentages of total platelet [^{3}H]ATP and [^{3}H]GTP respectively. Results are the means ± S.E. of triplicate measurements in a single experiment.

Additions	[^{3}H]cAMP (% of [^{3}H]ATP)	[^{3}H]cGMP (% of [^{3}H]GTP)
None	0.023 ± 0.004	0.004 ± 0.001
NP	0.036 ± 0.005	0.582 ± 0.021
PGI_2	0.045 ± 0.003	0.002 ± 0.002
PGI_2 + NP	0.096 ± 0.008	0.559 ± 0.023
EHNA	0.028 ± 0.000	0.003 ± 0.001
EHNA + NP	0.065 ± 0.008	0.779 ± 0.042
EHNA + PGI_2	0.039 ± 0.001	0.005 ± 0.002
EHNA + PGI_2 + NP	0.184 ± 0.014	0.834 ± 0.041

40–50 % whenever NP was present, but did not affect the [^{3}H]cGMP content of the platelets (Table 1). As shown in Figure 6, the inhibition of aggregation observed on the addition of both 10 μM NP and 20 μM EHNA was decreased by the addition of DDA. This result suggests that the inhibition of platelet aggregation by NP plus EHNA is mediated more by cAMP than by cGMP.

Roles of cyclic nucleotide phosphodiesterases in the interactions between PGI_2 and NP in platelets

In the present study, the addition of NP (10 μM) and PGI_2 (1 nM) had super-additive effects on the accumulation of [^{3}H]cAMP, consistent with the inhibition of PDE3 by cGMP. In three identical experiments, the increases in [^{3}H]cAMP (means ± S.E.) were; 46 ± 12 % with NP, 181 ± 47 % with PGI_2 and 456 ± 34 % with NP plus PGI_2. We also studied the effects of EHNA on platelet cyclic nucleotide concentrations

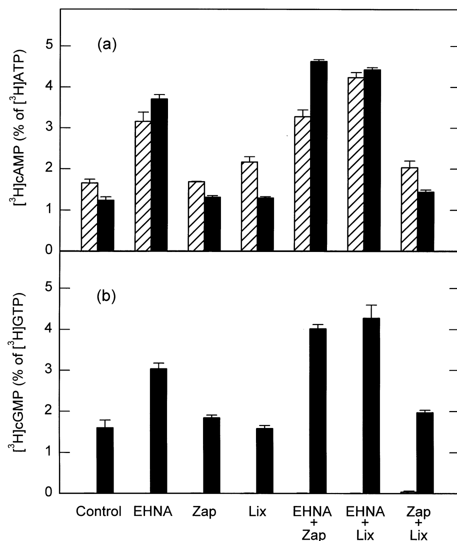

Figure 7 Inhibition by NP of the increase in platelet [^{3}H]cAMP caused by 20 nM PGI_2; suppression of this effect by EHNA

Metabolic nucleotide pools were prelabelled by incubation of platelets with [^{3}H]adenine and [^{3}H]guanine as described in the Experimental section. Samples were incubated for 1 min at 37 °C with 20 nM PGI_2 in the absence (hatched columns) or presence (solid columns) of 100 μM NP and other additions as follows: none (control); 20 μM EHNA; 10 μM zaprinast (Zap); 1 μM lixazinone (Lix); 20 μM EHNA + 10 μM zaprinast; 20 μM EHNA + 1 μM lixazinone; 10 μM zaprinast + 1 μM lixazinone. Platelet [^{3}H]cAMP (**a**) and [^{3}H]cGMP (**b**) were isolated and expressed as percentages of the total platelet [^{3}H]ATP and [^{3}H]GTP respectively; values are the means ± S.E. of triplicate measurements in a single experiment.

under these conditions to ascertain whether PDE2 affects the result (Table 2). EHNA (20 μM) had no effect on the [^{3}H]cAMP accumulation when PGI_2 alone was present but, as before, enhanced that seen in the presence of NP. When both PGI_2 and NP were present, EHNA had an even greater effect on [^{3}H]cAMP accumulation, with the result that the synergism between these compounds was enhanced. These results show that the synergistic interaction between NP and PGI_2 does not require PDE2 and is in fact diminished by the activation of PDE2. This phosphodiesterase seems to have no role in the breakdown of low cAMP concentrations in the absence of an increase in cGMP concentration.

We then investigated whether the same conclusions applied in the presence of a higher PGI_2 concentration (20 nM). Under these conditions, EHNA (20 μM) increased [^{3}H]cAMP accumulation, indicating that at high PGI_2 (cAMP) concentrations, PDE2 is active in the absence of cGMP (Figure 7). Lixazinone caused a smaller increase in [^{3}H]cAMP, indicating a lesser role for PDE3. In the presence of 20 nM PGI_2 and 100 μM NP, the synergistic effects of these compounds on [^{3}H]cAMP accumulation were no longer detectable. Instead, NP inhibited the accumulation of [^{3}H]cAMP caused by PGI_2, suggesting that the activation of PDE2 had a larger effect than any inhibition of PDE3. This decrease in [^{3}H]cAMP accumulation was not seen in the presence of EHNA (Figure 7). In contrast, neither zaprinast (10 μM) nor lixazinone (1 μM) prevented the inhibition of [^{3}H]cAMP accumulation by NP, indicating that this was mediated exclusively by the activation of PDE2. When both EHNA and zaprinast were present with PGI_2, NP tended to increase platelet [^{3}H]cAMP (29 ± 12 % in three experiments), probably as a result of the inhibition of PDE3 by cGMP. However, NP had no effect with PGI_2 in the presence of EHNA and lixazinone, probably because both PDE2 and PDE3 were fully inhibited (Figure 7). We conclude that with 20 nM PGI_2, when platelet [^{3}H]cAMP concentrations are high, PDE2 and to a lesser extent PDE3 participate in the hydrolysis of cAMP, whereas in the presence of NP only PDE2 is involved.

DISCUSSION

Increasing evidence indicates that the stimulation of PDE2 by cGMP is of physiological or pathophysiological importance in a variety of cell types in which the enzyme is expressed. Thus in adrenal glomerulosa cells, ANP decreases cAMP and steroidogenesis through the activation of PDE2 [27], whereas in frog ventricular cells, PDE2 seems to mediate the inhibitory effect of cGMP on the cAMP-stimulated Ca^{2+} current [28]. There is also evidence that in rat lung PDE2 plays a role in the hypoxic pressor response [29]. In addition, activation of PDE2 by atrial natriuretic factor or NP has been shown to decrease cAMP in PC12 cells [30]. In some of the more recent of these studies, EHNA was used as a tool to demonstrate the role of PDE2 [28,29]. This compound was originally designed as an inhibitor of adenosine deaminase [31] but was recently shown to be a selective inhibitor of myocardial PDE2 (as opposed to other phosphodiesterases) at concentrations in the micromolar range [20]. Although adenosine stimulates cAMP formation in platelets [32], it is very unlikely that adenosine mediates any of the effects we have described for several reasons. First, the concentrations of EHNA required for inhibition of adenosine deaminase are at least two orders of magnitude lower than those that inhibit PDE2. Secondly, incubations were brief (1–3 min) and were performed with freshly washed platelets.

In the present, as in previous studies (see, for example, [5]), we have used prelabelling assays for platelet cyclic nucleotides in which the metabolic ATP and GTP pools were first labelled by preincubation of the platelets with [^{3}H]adenine and [^{3}H]guanine. These methods have been fully validated by comparison with radioimmunoassays [5,25], and in the simple form used in the present study are easy to perform. Most importantly, the assays we have used permit the detection and accurate quantification of small increases in platelet cyclic nucleotides, in particular the approx. 50 % increases in platelet cAMP caused by NP. Thus, using radioimmunoassays, some workers have been able to detect increases in platelet cAMP with NP [5,14,15], whereas others have not [16,17] or have found increases that were not quite statistically significant [13]. The ability to measure small increases in platelet cAMP is of particular importance, because most of the basal cAMP in platelets is already bound to the tight-binding sites on cAMP-dependent protein kinases, with the result that a doubling of platelet cAMP can cause almost full activation of these enzymes [33].

Our measurement of the changes in cyclic nucleotide content of human platelets in response to increasing concentrations of NP show that activation of guanylate cyclase and the accumulation of [^{3}H]cGMP are associated with a biphasic increase in [^{3}H]cAMP concentration. Presumably this reflects the inhibition of PDE3 by cGMP, as demonstrated in rabbit platelets [5], but the effects of NP were substantially weaker than those of lixazinone, a selective inhibitor of PDE3. This discrepancy was resolved by our experiments with EHNA. Thus EHNA increased the accumulation of [^{3}H]cAMP caused by NP to a level close to that seen with lixazinone. Moreover, NP inhibited the increases in [^{3}H]cAMP caused by lixazinone, an effect that was blocked by EHNA. The results therefore show that in the presence of cGMP

there can be a simultaneous inhibition of PDE3 and activation of PDE2 in the platelet. With high NP concentrations (10–100 μM), the latter effect is of major significance, whereas with 0.1–1 μM NP little PDE2 activity was detected and the inhibition of PDE3 predominated. Our platelet aggregation studies showed that these changes in [^{3}H]cAMP concentration have major functional significance for the platelet and that, under the conditions of our experiments, the increases in platelet [^{3}H]cGMP caused by NP are alone insufficient to inhibit platelet aggregation. Thus we observed that NP suppressed the inhibition of platelet aggregation by lixazinone, a counter-intuitive effect given that both compounds are usually regarded as potent inhibitors of platelet function.

The experiments we have performed permit the identification of the phosphodiesterases responsible for the breakdown of cAMP under various conditions. Thus, in the absence of additions or in the presence of a low PGI_2 concentration, PDE3 alone seems to be responsible for cAMP hydrolysis because only PDE3 inhibitors increase [^{3}H]cAMP platelet under these conditions. When NP alone is present, PDE3 is inhibited and cAMP breakdown depends largely or entirely on the activation of PDE2 by cGMP. The same applies when NP is present with a low concentration of PGI_2 (1 nM). With a high concentration of PGI_2 alone (e.g. 20 nM), both PDE2 and PDE3 participate in cAMP hydrolysis because EHNA or, to a smaller extent, lixazinone could increase the cAMP concentration further. However, when NP is added with a high PGI_2 concentration, PDE3 is inhibited and PDE2 plays by far the more important role in cAMP breakdown.

Our observations have potentially interesting implications for platelet function and may be relevant to other cells and tissues that contain PDE2. Thus the effectiveness of nitric oxide, derived from endogenous sources or nitrovasodilator drugs, as an inhibitor of platelet function is partly compromised by the presence of PDE2 in the platelet and therefore could be enhanced by an inhibitor of PDE2. Moreover, the actions of PDE3 inhibitors on platelets and on other cells that contain both PDE3 and PDE2 may be suppressed if nitric oxide is also present. As a whole, our results suggest that the importance of PDE2 in the regulation of the cAMP concentration in the platelet has been underestimated and that drugs that inhibit PDE2, or combine this ability with other activities, could have useful effects.

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