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Endothelin-3, Ca^{2+} mobilization and cyclic GMP content in human platelets

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Abstract

As previously described for endothelin-3, platelet exposure to cyclic GMP-elevating agents such as sodium nitroprusside and M&B-22948 (2-*o*-propoxyphenyl-8-azapurin-6-one), a cGMP phosphodiesterase inhibitor, lowered Ca^{2+} mobilization in response to thrombin. Interestingly, when cGMP phosphodiesterases were blocked, endothelin-3 produced a dose-dependent cGMP accumulation ($P < 0.001$). Since endothelin-3 has been proposed to decrease the activity of Ca^{2+} accumulating pumps, we examined whether this latter effect could be mediated by a rise in cGMP content. Cyclic GMP decreased in a dose-dependent manner the initial rate and plateau value of the ATP-dependent $^{45}Ca^{2+}$ uptake in platelet membrane vesicles ($P = 0.006$ for each). Furthermore, combined treatment with endothelin-3 and M&B-22948 or a moderate concentration of Na^{+}-nitroprusside further reduced the thrombin-evoked Ca^{2+} discharge ($P = 0.004$ and 0.01, respectively), suggesting that endothelin-3 pre-exposure had reduced the amount of mobilizable Ca^{2+}. We propose that the depletion of platelet Ca^{2+} stores and the reduction of Ca^{2+} release evoked by endothelin-3 could be due, at least in part, to the elevation of cGMP content and to a decrease in Ca^{2+} accumulating pump activity.

Keywords: Platelet; Endothelin-3; Sodium nitroprusside; cGMP; Phosphodiesterase inhibitor; Ca^{2+}, cytosolic; Ca^{2+} pump

1. Introduction

In blood flow, platelets are mostly marginalized close to the vessel wall. They are thereby preferentially located near the vascular endothelium that has potent antithrombogenic effects, mediated in part by the release of prostacyclin, endothelium-derived relaxing factor and endothelins. Although the potent antiaggregating role of prostacyclin and nitric oxide have been demonstrated several years ago, direct inhibitory effects of endothelins on the platelet responses to thrombin, ADP, adrenaline or serotonin has been recognized only recently (Astarie-Dequeker et al., 1992, 1995a; Dockrell et al., 1992; Pietrazek et al., 1992; Touyz and Schiffrin, 1995). It is now widely accepted that prostacyclin and endothelium-derived relaxing factor essentially act through a rise in cyclic AMP and cGMP, respectively. In contrast, the mechanisms involved in platelet response to endothelins have not been clearly identified (Astarie-Dequeker et al., 1995a,b; Touyz and Schiffrin, 1995; Halim et al., 1995).

We have recently reported that endothelin-3 depletes resting Ca^{2+} store content (Astarie-Dequeker et al., 1995b), in agreement with the reduced thrombin-evoked Ca^{2+} release (Astarie-Dequeker et al., 1992, 1995a). Interestingly, platelets with elevated cGMP but not cAMP have also been described to have less stored Ca^{2+} (Johansson and Haynes, 1992). This prompted us to compare the direct effects of endothelin-3 and those of cGMP increasing agents on cytosolic Ca^{2+} movements and to examine endothelin-3 effect on cyclic GMP content. Two conditions under which cGMP content increases, treatments by sodium nitroprusside, a nitric oxide donor, and by M&B-22948 (2-*o*-propoxyphenyl-8-azapurin-6-one), a cGMP phosphodiesterase inhibitor, were selected.

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2. Materials and methods

2.1. Materials

Fura-2 acetoxymethylester (Fura-2AM) was obtained from Molecular Probes (Eugene, OR, USA). M&B-22948, purchased from Rhone-Poulenc Rorer (England), was dissolved in ethanol at a 10 mM concentration and the effects of the vehicle were checked in each experiment. Cyclic [^{125}I-cGMP] radioimmunoassay and $^{45}CaCl_2$ were obtained from Amersham (Les Ulis, France). All other chemicals were from Sigma Chemical Company.

2.2. Platelet isolation

Platelet suspension was prepared as previously described (Astarie et al., 1989). Briefly, platelets were isolated from freshly obtained human blood, washed and resuspended in a buffer containing 145 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 5 mM glucose and 25 mM Hepes, pH 7.4 at 37°C (buffer A), at a final concentration of 10^8 cells/ml. Where stated, platelets were pretreated at 37°C with 0.1 mM M&B-22948 and/or various endothelin-3 and Na^+-nitroprusside concentrations for 5, 10 and 1 min, respectively.

2.3. Cytosolic $[Ca^{2+}]_i$ measurements

Cytosolic Ca^{2+} concentration was determined with fura-2 (Molecular Probes, Eugene, OR, USA) as previously described (Astarie et al., 1989; Astarie-Dequeker et al., 1995a). Fura-2-loaded platelets were washed by centrifugation and resuspended in buffer A containing either 1 mM or 30 nM free Ca^{2+} (adjusted with a Ca-EGTA buffer). Fluorescence intensities were measured on a Spex Fluorolog CM111 equipped with a dual excitation wavelength system.

2.4. Platelet cyclic GMP content

Platelets were pretreated or not with M&B-22948, endothelin-3 and Na^+-nitroprusside as described above. Their cGMP content was determined by a ^{125}I-cGMP radioimmunoassay. Its sensitivity was increased by an acetylation step. A 50% inhibition of ^{125}I-cGMP binding was obtained with 176 ± 6 fmol cGMP per ml.

2.5. $^{45}Ca^{2+}$ uptake into platelet membrane vesicles

Ca^{2+}-uptake experiments were performed on membrane vesicles prepared as described by Käzer-Glanzmann et al. (1977), with some modifications (Astarie-Dequeker et al., 1995b). Briefly, platelets were washed three times with a buffer containing 100 mM KCl, 15 mM NaCl, 2 mM $MgCl_2$, 12 mM sodium citrate, 10 mM glucose and 25 mM K-Hepes, pH 7.5 at 37°C, and resuspended in the same buffer. Cells were then disrupted by controlled ultrasonication and centrifuged at 19000 × g for 20 min at 4°C. The supernatant was sedimented at 100000 × g for 1 h. The pellet was resuspended in 100 mM KCl, 20 mM Hepes and 7.5 mM $MgCl_2$, pH 7.2 at 37°C (buffer B), at a final concentration of 0.3–0.4 mg protein per ml. Platelet membrane vesicles were stored at −80°C until use. The time course of $^{45}Ca^{2+}$ uptake into membrane vesicles was studied in buffer B containing 200 nM Ca^{2+} (10 μM EGTA, 4.72 μM $CaCl_2$ and 5 μCi $^{45}CaCl_2$). Membrane vesicles were preincubated with endothelin-3 or cGMP for 6 and 3 min, respectively. $^{45}Ca^{2+}$ uptake was initiated by the addition of 2.5 mM Mg-ATP. At different time intervals, aliquots were withdrawn and filtered through Millipore GSWP filters (0.22 μm pore size), washed twice with 5 ml of 0.1 M ice-cold $CaCl_2$ and counted.

2.6. Statistical analysis

Results are expressed as means ± S.E.M. Experiments involving multiple groups were subjected to analysis of variance (ANOVA) and differences between groups assessed by paired or unpaired Student's t-test. Ca^{2+}-uptake data were analyzed by computed second order polynomial regressions.

3. Results

3.1. Effects of Na^+-nitroprusside, M&B-22948 and endothelin-3 on the cytosolic Ca^{2+} transients induced by thrombin

In the first set of experiments, we confirmed that pretreatment with Na^+-nitroprusside did not affect resting $[Ca^{2+}]_i$ values but reduced thrombin-induced Ca^{2+} mobilization, independently of Ca^{2+} influx (Fig. 1). In the absence of Ca^{2+} influx, the Na^+-nitroprusside-induced inhibition of Ca^{2+} mobilization was dose-dependent ($F(5,49) = 19.14$, $P < 0.001$) with a maximal inhibition of 70% reached at 1 μM Na^+-nitroprusside. When tested at a low concentration (10 nM), the effects of Na^+-nitroprusside were similar to those of 100 μM M&B-22948 or 0.5 μM endothelin-3 (Fig. 1). These observations are consistent with the ability of the 3 compounds to reduce thrombin-evoked Ca^{2+} release from internal stores.

3.2. Comparison of the effects of Na^+-nitroprusside, M&B-22948 and endothelin-3 on cGMP content

Under our experimental conditions, in contrast to Na^+-nitroprusside and M&B-22948 which increased basal cGMP levels, endothelin-3 alone up to 5×10^{-7} M had only weak effects. However, as illustrated in Fig. 2, when cGMP breakdown was minimized by pretreatment with 100 μM M&B-22948, endothelin-3 dose dependently

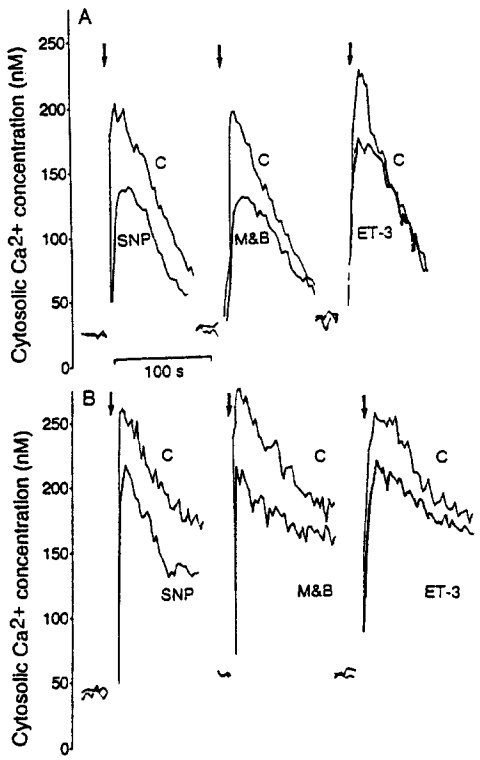

Fig. 1. Effects of Na^+-nitroprusside, M&B-22948, a cGMP phosphodiesterase inhibitor, and endothelin-3 on the $[Ca^{2+}]_i$ transients evoked by 0.05 U/ml thrombin in the presence of 30 nM (A) and 1 mM (B) extracellular free Ca^{2+}.

caused cGMP accumulation ($F(4,49) = 9.7$, $P = 0.001$). A maximal increase of $30 \pm 6\%$ above the effect of M&B-22948 itself was reached at 10 nM endothelin-3 (from 2.11 ± 0.45 to 2.79 ± 0.60 pmol cGMP/10^9 platelets, $n = 10$, $P = 0.004$ by paired Student's t-test).

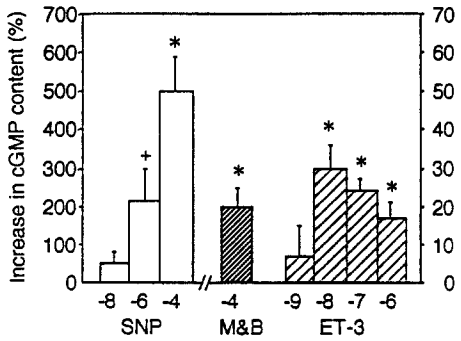

Fig. 2. Increase in platelet cyclic GMP content induced by Na^+-nitroprusside, M&B-22948 and endothelin-3. Platelets from 4–17 donors were preincubated with these compounds for 1, 3 and 10 min, respectively. The concentrations studied are indicated by their logarithms on the abscissa. The stimulations are expressed as percentage of the basal values. The endothelin-3-induced increase in platelet cyclic GMP content is expressed as percentage of the basal value obtained in the presence of 100 μM M&B-22948. Note the different scales used for the stimulation of cGMP by Na^+-nitroprusside (left ordinate) and by M&B-22948 or endothelin-3 (right ordinate). $^+$ $P < 0.05$, * $P < 0.001$ when compared to cGMP levels in untreated platelets.

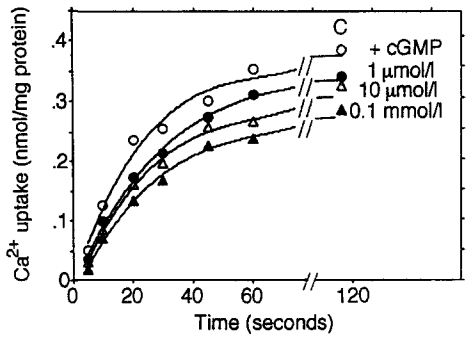

Fig. 3. Time course of ATP-dependent Ca^{2+} uptake in the absence and presence of cGMP. Platelet membrane vesicles were preincubated for 3 min at 37°C with various concentrations of cGMP, as indicated, before the addition of 2.5 mM ATP. The inhibitory effect of these compounds on the initial rate of Ca^{2+} uptake was determined on samples from 6 separate blood donors.

3.3. Influence of cGMP on ATP-dependent $^{45}Ca^{2+}$ uptake into platelet membrane vesicles

Even if an increase in cGMP could be a reason for the antiaggregating properties of endothelin-3, it remained to be determined whether cGMP accumulation could participate in the endothelin-3 inhibitory effects on Ca^{2+} store release (Astarie-Dequeker et al., 1992, 1995a,b) through the activity of Ca^{2+}-ATPases of the dense tubular system (Pernollet et al., 1995). We therefore investigated the influence of cGMP on ATP-dependent $^{45}Ca^{2+}$ uptake into platelet membrane vesicles. As shown in Fig. 3, cGMP concentrations, ranging from 1 μM to 0.1 mM, decreased in a dose-dependent manner the initial rate and plateau value of the ATP-dependent $^{45}Ca^{2+}$ uptake ($F(12,3) = 6.95$ and 6.98, respectively, $P = 0.006$ for each). In comparison, the active $^{45}Ca^{2+}$ uptake was almost totally abolished by 1 μM thapsigargin, a specific inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase (Thastrup et al., 1990), indicating that the major part of $^{45}Ca^{2+}$ was

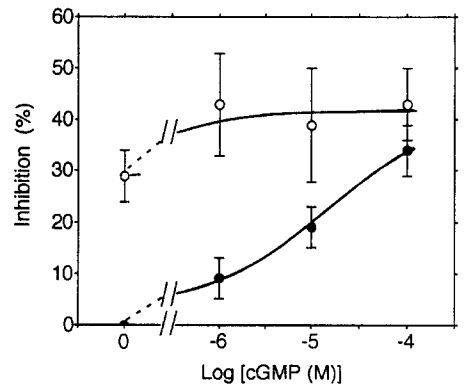

Fig. 4. Inhibitory effect of cGMP on the initial rate of ATP-dependent Ca^{2+} uptake into platelet membrane vesicles, in the presence (○) and absence (●) of 5 nM thapsigargin. Cyclic GMP and/or thapsigargin was added 3 min before ATP addition. ANOVA analysis of cGMP effects indicated a significant dose dependence in the absence of thapsigargin ($F(3,18) = 13.1$, $P < 0.001$) but not in its presence.

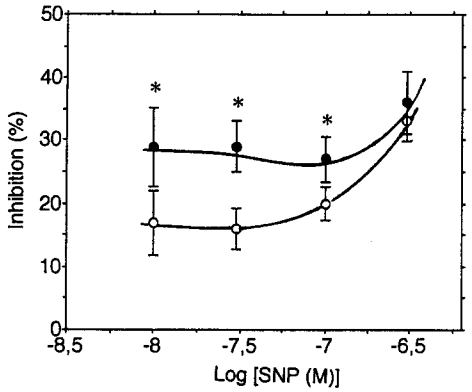

Fig. 5. Effect of combined endothelin-3 and Na^+-nitroprusside treatment on thrombin-induced Ca^{2+} mobilization. The inhibitory effects of Na^+-nitroprusside, alone (○) or combined with 5×10^{-7} M endothelin-3 (●), on Ca^{2+} mobilization induced by 0.05 U/ml thrombin were studied in the absence of Ca^{2+} influx. Percentages ± S.E.M. of the thrombin-evoked Ca^{2+} peak in control platelets, determined in 5–7 independent experiments. * $P < 0.03$ by paired t-test. The response to Na^+-nitroprusside ($F(3,20) = 3.99$) was no longer dose-dependent in the presence of endothelin-3 ($F(3,20) = 1.07$).

driven by Serca pumps (Astarie-Dequeker et al., 1995b). Combined treatments with a moderate thapsigargin concentration (5 nM) and cGMP concentrations ranging from 1 to 100 μM did not increase further the inhibitory effect of thapsigargin on the initial rate of active $^{45}Ca^{2+}$ uptake, which reached the same equilibrium value as with thapsigargin alone (Fig. 4). The observation that the effects of thapsigargin and cGMP were not additive suggests that cGMP affects essentially the Ca^{2+} transport driven by Serca pumps, at a site interacting with the thapsigargin inhibitory effect.

3.4. Effect of combined treatment by endothelin-3 with M&B-22948 or Na^+-nitroprusside on thrombin-induced Ca^{2+} release from internal stores

The inhibitory effects of 0.1 mM M&B-22948 and 0.5 μM endothelin-3 on thrombin-induced Ca^{2+} discharge were additive (from 36 ± 11 in the presence of M&B-22948 alone, to 45 ± 9% inhibition with the two compounds combined, $P = 0.04$ by paired t-test, $n = 6$). As shown on Fig. 5, pretreatment with endothelin-3 also further increased the inhibitory effect of Na^+-nitroprusside up to 0.1 μM (from 16 ± 3 to 26 ± 4%, $P < 0.02$ by paired t-test, $n = 6$ for 0.03 μM Na^+-nitroprusside). However, endothelin-3 failed to potentiate the effects of Na^+-nitroprusside at concentrations higher than 0.1 μM (Fig. 5), which induced a dramatic rise in cGMP content (see Fig. 2).

4. Discussion

This study of the in vitro effects of endothelin-3 on human platelets demonstrates that endothelin-3 increases platelet cGMP content and reduces thrombin-induced cytosolic Ca^{2+} transients, similarly to sodium nitroprusside and M&B-22948. In addition, the observations that cGMP reduces the ATP-dependent Ca^{2+} uptake into platelet membrane vesicles and that this inhibition was not additive to that of thapsigargin, indicate that cGMP decreases Serca-pump activity at the level of the dense tubular system. Taken together, these results strongly suggest that the reduction of platelet internal Ca^{2+} stores by endothelin-3 (Astarie-Dequeker et al., 1995b) could be mediated, at least partially, through an increased cGMP content.

Interestingly, the maximally effective endothelin-3 concentration was 10 nM for both its anti-aggregatory effect (Astarie-Dequeker et al., 1992) and the rise in cGMP. The proposal that endothelin-3 effects in platelets are partially mediated by a rise in cGMP is compatible with the previously reported effects of membrane-permeable analogs of cGMP and cGMP-elevating substances other than those used in the present study. As was observed for endothelin-3, these compounds behave as anti-aggregants and reduce the cytosolic Ca^{2+} discharge from internal stores caused by various agonists (Nguyen et al., 1991; Brüne and Ullrich, 1992). The stimulation of endothelin receptors has been reported to increase cGMP levels in some tissues, such as rat aorta or mesentery (Fujitani et al., 1993; Warner et al., 1989), glomeruli (Edwards et al., 1992) and porcine kidney epithelial (Ishii et al., 1991) or neuronal cells (Reiser, 1990). In most cases, this effect was mediated through an increased production of nitric oxide, which in turn activates guanylate cyclase. NO synthases are present in platelets (Metha et al., 1995; Muruganandam and Mutus, 1994) and could be involved in the cGMP accumulation evoked by endothelin-3. However, neither the nature of endothelin receptors, nor their relation to NO synthases has been characterized in platelets. The maximum rise in cGMP levels reached after endothelin-3 pretreatment was of the same order of magnitude as that observed in M&B-22948-treated platelets. It was far below that observed in the presence of high Na^+-nitroprusside concentrations. This could suggest an influence on cGMP degradation rather than on its synthesis. However, the endothelin-3-induced increase in cGMP concentration was observed only when phosphodiesterase activity was inhibited. Furthermore, the observation that endothelin and Na^+-nitroprusside inhibitory effects on thrombin-evoked Ca^{2+} mobilization were additive only for the low Na^+-nitroprusside concentrations, favors the involvement of endothelin-3 in cGMP synthesis rather than its breakdown. The observation that N^{ω}-monomethyl-L-arginine reduces the endothelin-3-induced cGMP accumulation (Gagnet et al., unpublished data) strongly supports this last proposal.

As previously observed and confirmed herein for endothelin-3, both Na^+-nitroprusside and M&B-22948 decreased thrombin-evoked Ca^{2+} movements independently of an inward transmembrane Ca^{2+} gradient. Our results fit well with the proposal that, in addition to its inhibitory

effect on the opening of Ca^{2+} channels (Simon and Chap, 1989), an elevation of cyclic cGMP also diminishes the amount of Ca^{2+} mobilizable from internal stores (Johansson and Haynes, 1992). It has been suggested that cGMP may exert its antagonist effects through various mechanisms. They include the activation of cGMP-protein kinases and of cAMP-dependent pathways. Geiger et al. (1992) showed that nitric oxide generating drugs decrease calcium mobilization through an activation of cGMP-protein kinases, which lead to the inhibition of phospholipase C. Cyclic GMP could also inhibit cAMP breakdown by reducing the activity of the cGMP-inhibitable cAMP phosphodiesterase (Grant et al., 1992).

Although the above mechanisms could be involved in the reduction of thrombin-induced Ca^{2+} discharge by endothelin, they could not explain the depletion of internal Ca^{2+} stores (Astarie-Dequeker et al., 1995b). Our present findings demonstrate that cGMP reduced the initial rate of ATP-dependent $^{45}Ca^{2+}$ uptake, thereby suggesting an inhibitory effect on Ca^{2+}-ATPase activity. Since cGMP did not have additive effects with thapsigargin, a specific inhibitor of Serca-pumps, we propose that cGMP affects preferentially the Ca^{2+} pumps of the dense tubular system. The decrease in the steady-state value of Ca^{2+} uptake agrees with a reduced filling state of the internal stores and with a reduced capacity of further Ca^{2+} release. This agrees with the findings of Johansson and Haynes (1992) who observed that a rise in platelet cGMP content decreased the amount of stored Ca^{2+}. However, in contrast to our present findings, these authors proposed that this cGMP effect was mediated by an activation of Ca^{2+} extruding pump. Although we cannot rule out such possibility under stimulating conditions, our results strongly demonstrated that cGMP also inhibits Ca^{2+} reuptake in the dense tubular system in the absence of agonist stimulation.

However, the direct in vitro inhibitory effect of endothelin-3 on the activity of Ca^{2+} pumps of platelet membrane vesicles (Astarie-Dequeker et al., 1995b) can hardly be attributed to a raised cGMP level, as its synthesis by isolated membranes, in the absence of substrate and soluble guanylate cyclase, is unlikely. The proposal that endothelin-3 reduces the filling state of internal Ca^{2+} stores and Ca^{2+} mobilization in intact platelets only through a rise in cGMP constitutes therefore a too simplistic view. In particular, recent observations suggested that the protein kinase C could also play a role in endothelin-3 effects on Ca^{2+} mobilization from internal stores. Pietrazek et al. (1992) proposed that platelet pretreatment with endothelin induced protein kinase C activation through an unknown mechanism. This provokes a negative modulation of IP_3 production, as a consequence of a feedback inhibition of the coupling between the agonist subsequently added and receptor tightly coupled to phospholipase C. A similar conclusion was reached by Touyz and Schiffrin (1995), who demonstrated that staurosporine, a non-specific protein kinase C inhibitor and calphostin C, a highly selective protein kinase C inhibitor, reverses the inhibitory actions of endothelin-1 on thrombin-stimulated $[Ca^{2+}]_i$ rise. They thus proposed that endothelin-1 influences cytosolic calcium transients through protein kinase C inhibition of inositol triphosphate generation. This proposal and our observations can be in agreement if we distinguish the preconditioning and acute effects of endothelin. Under the first conditions, in the absence of stimulation, endothelin exerts a preconditioning effect including a reduction of the amount of Ca^{2+} stored and activation of protein kinase C. As a consequence, the decrease in IP_3-related signal, associated to a decreased content of Ca^{2+} stores, leads to a reduction of Ca^{2+} mobilization in response to an ulterior platelet stimulation.

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