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EFFECT OF cAMP AND cGMP ON ENDOTHELIN-STIMULATED TYROSINE PHOSPHORYLATION IN RABBIT PLATELETS

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Summary: Activation of platelets by different agents results in the increased tyrosine phosphorylation of several substrate proteins. Thus, the effect of endothelin-1 on the stimulation of tyrosine phosphorylation in rabbit platelets can be inhibited by preincubation with forskolin, which increase the cAMP level. However, incubations of platelets with 8-Bromo-cGMP showed lower inhibitory effect. Forskolin produced a dose-dependent inhibition on three different protein substrates, with an IC_{50} of approximately 12.8, 4.0 and 8.0 μM in the three molecular mass ranges of 50, 60 and 100-200 kDa,respectively. These results show that the endothelin-stimulated tyrosine phosphorylation in rabbit platelets can be regulated by a novel pathway of platelet signal transduction in which the cAMP level could be more relevantly involved than cGMP in some molecular mass ranges of tyrosine phosphorylated proteins.

Endothelin-1, -2 and -3, which belong to a vasoactive peptide family [1], are produced by endotelial cells and present different structural and pharmacological properties. Endothelin has effects in several physiological systems by a feasible signal transduction pathway by activation of phospholipase C, enhancement of phosphatidylinositol turnover, elevation of cytosolic free Ca^{2+} levels and protein kinase C activation [2]. In rabbit platelets, endothelin inhibits "ex vivo" and "in vivo" aggregation [3-5], serotonin- and thrombin-induced aggregation [6], and calcium mobilization in human platelets [7]. Recently, we have demonstrated [8] that endothelin-1 has a direct effect on the stimulation of several tyrosine phosphorylated proteins in rabbit platelets through its receptor binding.

Although the role of tyrosine phosporylation in platelets signal transduction remains largely unknow, it has been correlated with aggregation, secretion and activation of protein kinase C [9,10]. Elevation of cyclic nucleotide levels has been proposed to inhibit phosphoinositide hydrolysis [11] and to activate protein kinase C in platelets [12]. On the other hand, sodium nitroprusside, a potent nitrovasodilator, inhibits platelet aggregation by stimulation of guanylate cyclase [13].

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We have previously proposed [8] that endothelin-1 affects the stimulation of tyrosine phosphorylation in rabbit platelets through specific receptor binding and, in consequence, plays a crucial role in platelet signal transduction. In regard to this case, and because cyclic nucleotide levels have been involved in several signal transduction pathways, our present study was undertaken to evaluate if agents which raise the intracellular level of cyclic nucleotides could affect endothelin-stimulation of tyrosine phosphorylation in rabbit platelets.

MATERIALS AND METHODS

Platelets isolation: Rabbit platelets were prepared as described earlier [14] from blood collected in 3.2% trisodium citrate. Platelets were suspended in HEPES-Tyrode's solution (10 mM N-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 5 mM glucose and 12 mM $NaHCO_3$, pH 7.4). The platelets number was estimated by the protein concentration determined by the Bradford method [15] using Bio-Rad protein assay reagent and bovine serum albumin as standard.

Platelets stimulation with endothelin-1: 140 μg of platelet protein ($\approx 7.5 \times 10^7$ platelets) resuspended in Tyrode's buffer, pH 7.4, were stimulated by incubation with 1 μM endothelin-1 for 1 min in the absence or presence of the appropriate concentrations of forskolin and 8-Bromo-cGMP (8-Br-cGMP), which are agents that raise cyclic nucleotide levels, cAMP (adenosine 3'5'-cyclic monophosphate) and cGMP (guanosine 3'5'-cyclic monophosphate) respectively. The platelets were preincubated with forskolin or 8-Br-cGMP for 3 and 15 min respectively. At the end of each stimulation period, activation was quenched by adding an equal volume of stopping buffer consisting of 3% SDS, 5% ß–mercaptoethanol, 10% glycerol, 60 mM Tris-HCl, pH 6.8.

Immunoblotting for tyrosine-phosphorylated proteins: Stimulation reaction mixtures were subjected to standard SDS-PAGE on 12% polyacrylamide gels. Proteins (60-100 μg/track) were separated and then transferred to nitrocellulose paper by a wet blotting method (overnight at 200 mA). The non-specific binding was blocked with 5% low fat powder in 10 mM Tris-HCl, 100 mM NaCl and 0.1% Tween 20, pH 7.5 buffer. Platelet phosphotyrosine proteins were analyzed by Western blot utilizing a monoclonal anti-phosphotyrosine antibody [16]. The nitrocellulose blots were washed in the blocking buffer and reacted for two hours with goat anti-mouse peroxidase conjugated IgG and developed with an enhanced chemiluminescence (ECL) detection system.

Materials: Endothelin-1, forskolin and 8-Br-cGMP were purchased from Sigma. Nitrocellulose paper (0.2 mm) was obtained from Renner GmbH. Monoclonal anti-phosphotyrosine antibody (PY20) was from Affinity Research Products Ltd. Goat anti-(mouse IgG)-horseradish-peroxidase conjugate was purchased from Nordic Immunology. ECL system was acquired from Amersham International.

RESULTS

Endothelin-1 produces a rise in the phosphotyrosine proteins mainly affecting those in the molecular mass ranges of 50-60 kDa, 70-100 kDa and 100-200 kDa. These bands which rise in the presence of endothelin-1 and which are inhibited in the presence of genistein, a typical protein tyrosine kinase inhibitor, closely correspond to those previously reported in our laboratory [8].

Figures 1 and 2 show the effect of endothelin-1 on tyrosine phosphorylation of proteins by Western blotting in the presence or absence of forskolin and 8-Br-cGMP respectively. 100 μM forskolin produces an important inhibition of tyrosine phosphorylation: 64%, 75% and 80% in the three molecular mass ranges of proteins, pp50, pp60 and pp(100-200) kDa respectively compared to the levels obtained after endothelin-1 stimulation. A slight effect is observed for

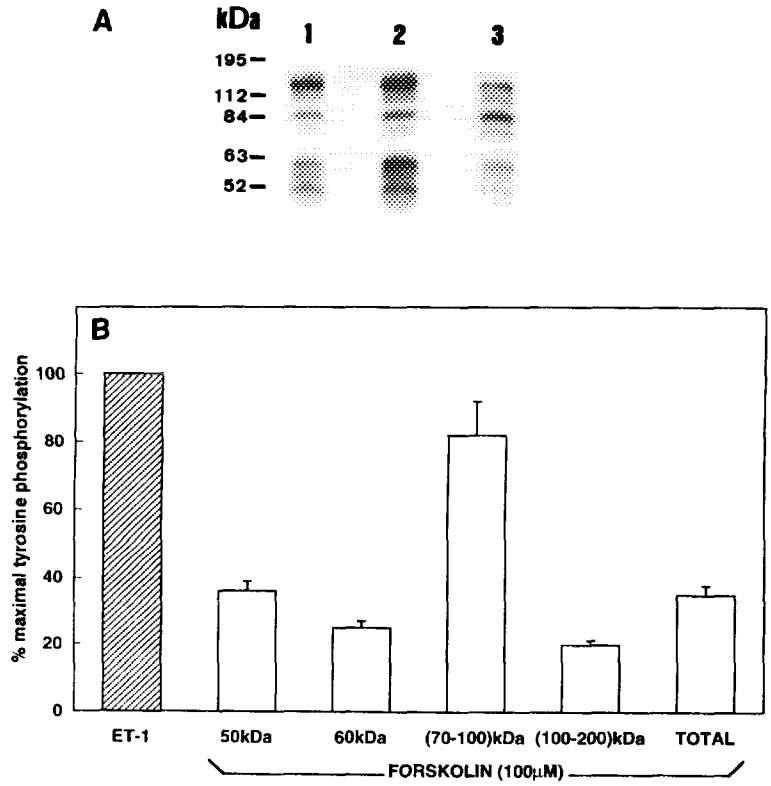

FIGURE 1. Endothelin-stimulated tyrosine phosphorylation and its inhibition by forskolin.

Platelets were stimulated with endothelin-1 (ET-1) in the absence or presence of 100 μM forskolin (see Materials and Methods). **A.** Western blot analysis of rabbit platelet proteins on 12% polyacrylamide gel, transferred and immunoblotted with anti-phosphotyrosine antibody. Lane 1, control assay in the absence of agonists; lane 2, endothelin-stimulated assay and lane 3 platelet preincubated with forskolin for 3 min before incubation with endothelin-1. **B.** Percentage of maximal tyrosine phosphorylation in the different molecular mass ranges of proteins: pp50, pp60, pp(70-100) and pp(100-200) kDa compared to endothelin-stimulation assay (lane 2). The results shown are mean ± standard error of three separate experiments.

pp(70-100) kDa (18%). In contrast (Fig. 2), in the presence of 100 μM 8-Br-cGMP the inhibitory effect was lower: 52%, 24%, 14% and 28% for pp50, pp60, pp(70-100) and pp(100-200) kDa respectively. The total decrease of tyrosine phosphorylation level was of 65% and 25% in the presence of forskolin and 8-Br-cGMP respectively.

The dose-response for inhibition of endothelin-1-induced tyrosine phosphorylation by forskolin and 8-Br-cGMP was studied. Figure 3 shows that forskolin inhibited tyrosine phosphorylation produced by 1 μM endothelin-1 for 1 min, with an IC_{50} of approximately 12.8, 4.0 and 8.0 μM for the proteins of three molecular mass ranges, 50, 60 and 100-200 kDa respectively. Forskolin produced no significant inhibition of tyrosine phosphorylation for the pp(70-100) kDa. 8-Br-cGMP produces, on rabbit platelets, a slight inhibition on tyrosine phosphorylation stimulated by endothelin-1 in the concentration range studied (Fig. 4). Only a

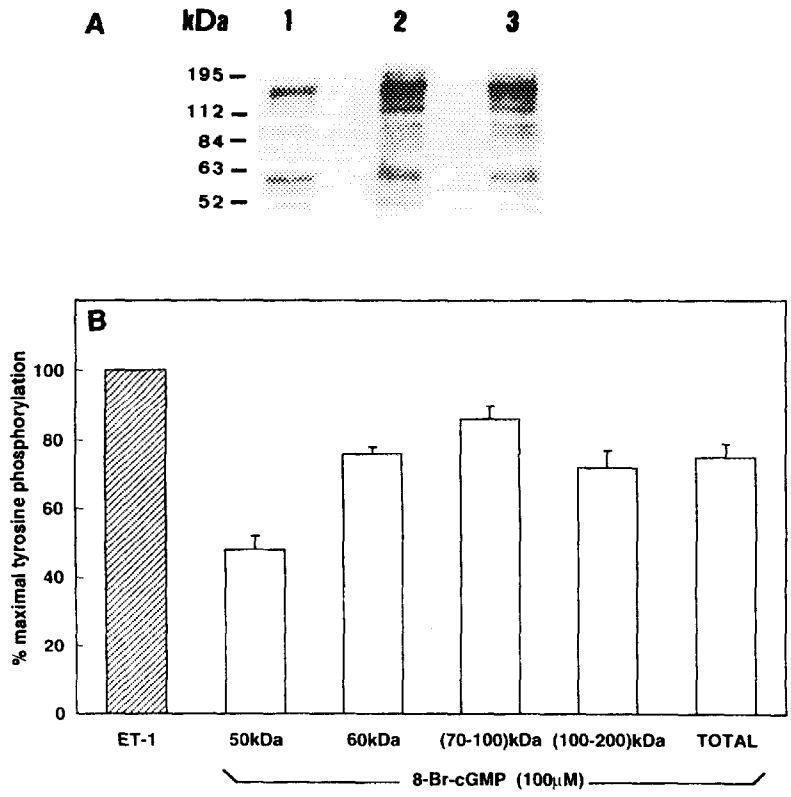

FIGURE 2. Endothelin-stimulated tyrosine phosphorylation and its inhibition by 8-Br-cGMP.

Platelets were stimulated with endothelin-1 (ET-1) in the absence or presence of 100 μM 8-Br-cGMP (see Materials and Methods). **A.** Western blot analysis of rabbit platelet proteins on 12% polyacrylamide gel, transferred and immunoblotted with anti-phosphotyrosine antibody. Lane 1, control assay in the absence of agonists; lane 2, endothelin-stimulated assay and lane 3 platelet preincubated with 8-Br-cGMP for 15 min before incubation with endothelin-1. **B.** Percentage of maximal tyrosine phosphorylation in the different molecular mass ranges of proteins: pp50, pp60, pp(70-100) and pp(100-200) kDa compared to endothelin-stimulation assay (lane 2). The results shown are mean ± standard error of three separate experiments.

significant reduction of endothelin-1 effect was observed in the presence of this agent for pp50 with an IC_{50} of 80 μM.

DISCUSSION

The role of tyrosine phosphorylation in platelets signal transduction cascade is not totally understood. Since cyclic nucleotides have been involved in several steps of this cascade. Here, we have studied in platelets, the possible effect of these cyclic nucleotides in the stimulation of tyrosine kinase by endothelin.

Our findings show that forskolin, which increase the cAMP level, produces in a dose-dependent way, a marked inhibition of the tyrosine phosphorylation stimulated by endothelin mainly in three molecular mass ranges of proteins pp50, pp60 and pp(100-200) kDa, and a slight

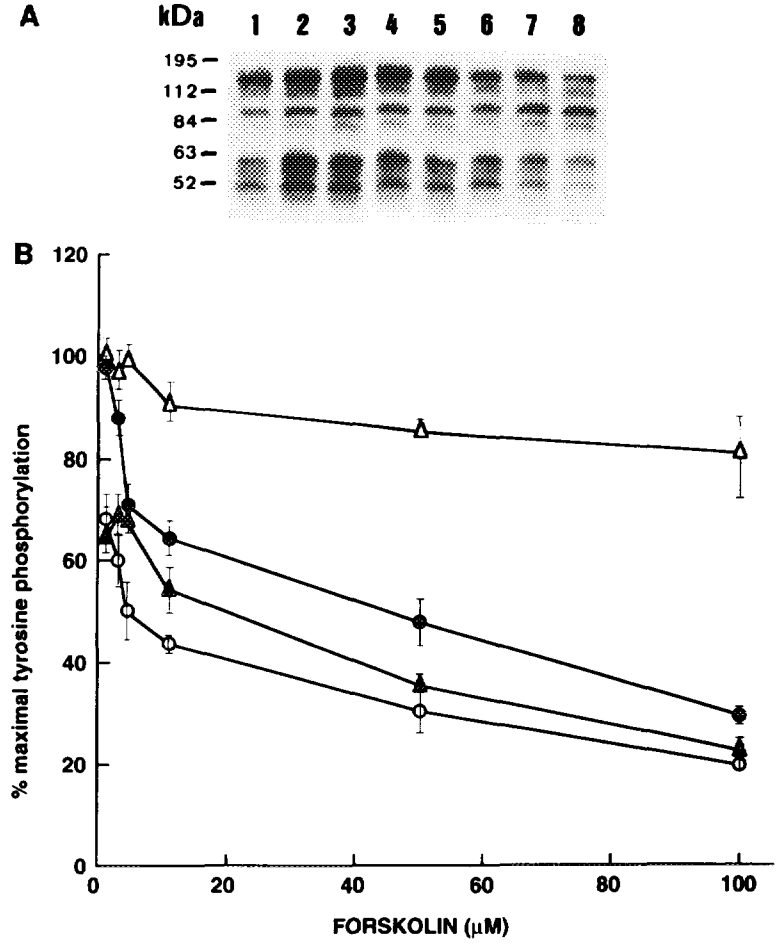

FIGURE 3. Dose-response for forskolin inhibition of endothelin-stimulated tyrosine phosphorylation of rabbit platelet proteins.

Stimulation conditions and general procedures were as described in Materials and Methods. **A.** Immunoblotting for tyrosine phosphorylated proteins after stimulation with endothelin-1 in the absence (lane 2) or presence of forskolin at the different concentrations: 0.1 (lane 3), 1.0 (lane 4), 2.0 (lane 5), 10.0 (lane 6), 50.0 (lane 7) and 100.0 μM (lane 8). Lane 1, control assay. **B.** Percentage of maximal tyrosine phosphorylation in the different molecular mass ranges of proteins: pp50 (●), pp60 (○), pp(70-100) (△) and pp(100-200) kDa (▲). Results are expressed as percentage of the endothelin-stimulated rise in phosphotyrosine and given as mean ± standard error of three separate experiments.

effect was seen for pp(70-100) kDa. In contrast, with 8-Br-cGMP, a stable cGMP analog, the inhibitory effect was lower in all molecular mass ranges of proteins. These results are in agreement with those earlier described in which the elevation of cAMP level [17,18] but not cGMP [18] was found to inhibit thrombin-stimulated tyrosine phosphorylation in human platelets. Elevation of cAMP levels may inhibit tyrosine phosphorylation by its well know ability to block the activation of phospholipase C [11].

Synthetic inhibitors of certain tyrosine kinases [19] completely inhibit thrombin-stimulated tyrosine phosphorylation but have no effect on phosphatidylinositol 4,5,-bisphosphate

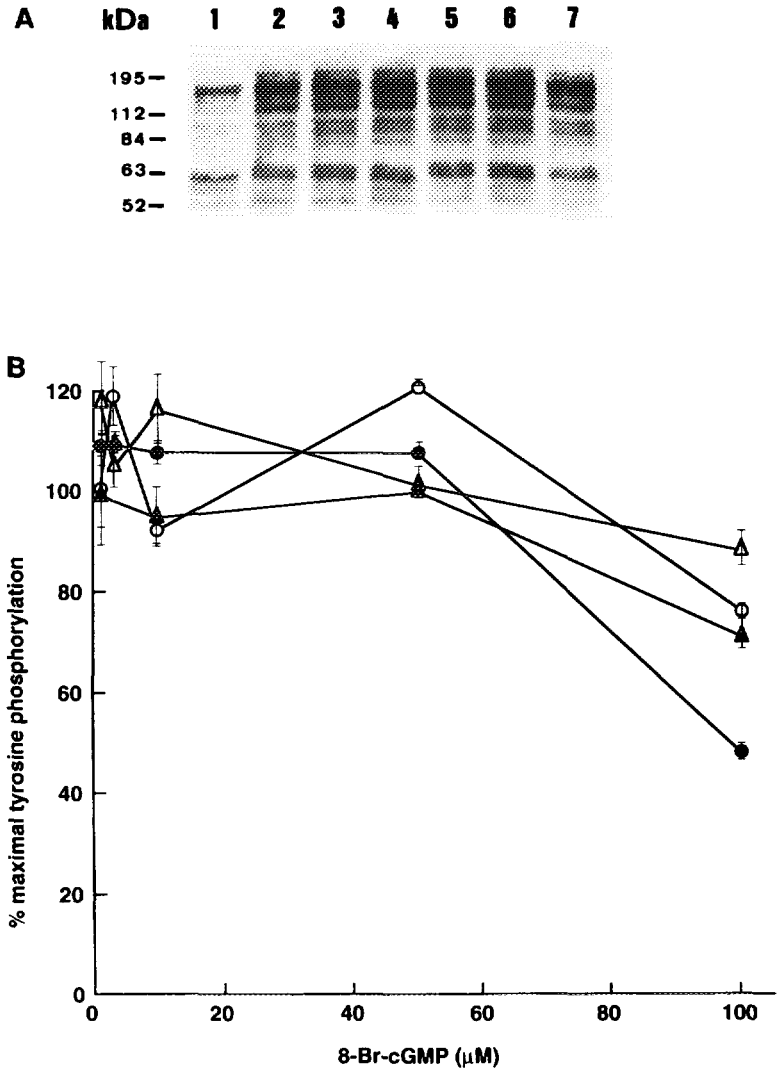

FIGURE 4. Dose-response for 8-Br-cGMP inhibition of endothelin-stimulated tyrosine phosphorylation of rabbit platelet proteins.

Stimulation conditions and general procedures were as described in Materials and Methods. **A.** Immunoblotting for tyrosine phosphorylated proteins after stimulation with endothelin-1 in the absence (lane 2) or presence of 8-Br-cGMP at the different concentrations: 0.5 (lane 3), 1.0 (lane 4), 10.0 (lane 5), 50.0 (lane 6) and 100.0 μM (lane 7). Lane 1, control assay. **B.** Percentage of maximal tyrosine phosphorylation in the different molecular mass ranges of proteins: pp50 (●), pp60 (○), pp(70-100) (△) and pp(100-200) kDa (▲). Results are expressed as percentage of the endothelin-stimulated rise in phosphotyrosine and given as mean ± standard error of three separate experiments.

hydrolysis. In consequence, we could postulate that the enhanced tyrosine phosphorylation due to endothelin-1 may be a secondary response to the activation of phospholipase C and can be modulated by cAMP levels. It has been reported that a cGMP analog have a preferential inhibitory effect on the release and subsequent metabolism of arachidonic acid [20]. Therefore,

phospholipase A_2 pathway appears to be an important target for the physiological action of cGMP in platelets. Our results, which demonstrate that endothelin-stimulated tyrosine phosphorylation is slightly affected by changes in cGMP levels, could involve the phospholipase C pathway and not the phospholipase A_2 as an early event prior to stimulation of tyrosine phosphorylation.

Another possible explanation of these results is the involvement of cyclic nucleotides on platelet tyrosine phosphatase activities. The different endothelin-stimulation effects and the IC_{50} values found on the substrate proteins could be related to this fact. In this regard, changes in cyclic nucleotide levels, could affect the coordinated action or the spatiotemporal distribution of protein kinase and phosphatase activities in the signal transduction cascade [21,22]. Recently Cirri et al. [23] have described the activation mechanism of the rat liver isoenzyme phosphotyrosine protein phosphatase II (AcP_2) by cGMP enhancing the rate of the enzyme-phosphate hydrolysis by directly acting on the limiting step [complex thiol-phosphate covalent intermediate] of the catalytic process. Thus, the elevation of intracellular concentration of cyclic nucleotides could stimulate some tyrosine phosphatase isoenzyme activities *in vivo*. On the other hand, it has been recently described that modulation of cGMP metabolism is a mechanism through which tyrosine kinase pathway can interact with other signal transduction pathways and indirectly influence cellular processes normally regulated by cGMP [24].

In conclusion, this paper shows that endothelin-1-stimulated tyrosine phosphorylation in rabbit platelets can be significantly inhibited by high cAMP levels and, sensibly less by high cGMP levels. Further investigation must be performed to understand the endothelin effect on the mechanism of tyrosine phosphorylation in platelets and its role on platelet signal transduction cascades. In this regard, the evaluation of the effect of endothelin-1 on the tyrosine phosphatase activities and the possible influence of cyclic nucleotide levels, are in progress in our laboratory.

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