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THE INFLUENCE OF ACTIVATING HORMONES ON HUMAN PLATELET MEMBRANE Ca^{2+}-ATPase ACTIVITY

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SUMMARY: Intact platelets were pretreated with hormones and thereafter membranes were prepared and Ca^{2+}-ATPase activity determined. Thrombin decreased the V_{max} of Ca^{2+}-ATPase after pretreatment of intact platelets. Platelet activating factor, vasopressin and ADP also decreased Ca^{2+}-ATPase activity. 12-0-tetradecanoylphorbol-13-acetate (TPA) or A23187 or ionomycin alone had no effect, whilst the simultaneous pretreatment with TPA and Ca^{2+}-ionophore decreased Ca^{2+}-ATPase activity. cAMP elevating agents prostaglandin E_1 (PGE_1) and forskolin had no influence per se on Ca^{2+}-ATPase, but antagonized the inhibitory effect of thrombin. The data suggest a close connection between phosphoinositide metabolism and the Ca^{2+}-ATPase system.

Transcompartmental movements of calcium ions play a decisive role in regulating physiological and biochemical processes in platelets. There is a good correlation between the ability of agonists to elicit elevation of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and to induce phosphoinositide hydrolysis (1) suggesting that agonist induced hydrolysis of inositol lipids may be the molecular basis underlying Ca^{2+}-influx and Ca^{2+}-mobilization. For example, one product of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, namely myo-inositol 1,4,5-trisphosphate (IP_3), induces release of Ca^{2+} from intracellular pools (2).

In addition to Ca^{2+}-influx and mobilization counter-regulatory systems of Ca^{2+}-efflux/sequestration also operate in platelets (3,4). Evidence suggests that polyphosphoinositides themselves may modulate cellular Ca^{2+}-efflux, and it has been shown that PIP_2 is a powerful stimulator of the Ca^{2+}-ATPase in erythrocyte membranes, platelet microsomes and brain synaptic plasmalemma (5-7). It has further been suggested that PIP_2 breakdown leads to inactivation of Ca^{2+}-ATPase (8). Such inactivation of Ca^{2+}-transport in liver plasma membranes after agonist stimulation has been reported (9) and was associated with breakdown of PIP_2 and phosphatidylinositol 4-phosphate (PIP) (10).

We have studied whether platelet membrane Ca^{2+}-ATPase is influenced following stimulation of intact platelets with agonists known to effect activation via receptor-coupled phosphoinositide turnover.

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METHODS: Blood was drawn from healthy volunteers and washed platelets prepared as described previously (3). Platelets were finally resuspended (2x 10^8 cells/ml) in 145 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM Hepes-NaOH (pH 7.4) and incubated at 37°C for at least 1 hr prior to experimentation. From platelet isolates following prelabelling with ^{32}P-orthophosphate the preparative and equilibration procedures were judged to yield fully "rested" platelets on the basis of negligible (3%) ^{32}P-phosphatidic acid (PA) formation and insignificant ^{32}P incorporation into M_r 47000 and M_r 20000 proteins. Prior to experimental procedures $CaCl_2$ (0.5 mM) was added to platelet suspensions and the cells incubated for a further 5 min at 37°C. 4 ml aliquots of platelets were treated (with stirring) for various times at 37°C with compounds as indicated in legends. Reactions were terminated by freezing in liquid N_2. Native membranes were prepared after thawing of platelet lysates as previously described (3) and were assayed colorimetrically for Mg^{2+}-ATPase and Ca^{2+}-ATPase activities as described in detail previously (3). Briefly, incubations were performed at 37°C under the following conditions: 120 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM ATP, 20 mM TES-NaOH (pH 7.5), membrane protein (15-30 µg/ml) and in the absence and presence of 0.8 µM free Ca^{2+} (unless otherwise stated). Mg^{2+}-ATPase activity was determined from assays performed in the absence of $CaCl_2$. Ca^{2+}-ATPase activity was determined by subtracting activity measured in the absence of $CaCl_2$ from that in the presence of $CaCl_2$ (3). Activities are expressed as U/mg membrane protein where 1 U is defined as 1 nmol P_i released from ATP per min at 37°C. Protein concentrations were estimated colorimetrically (11). Statistical analysis was performed using Student's t-test for unpaired data.

RESULTS: The Ca^{2+}-ATPase but not Mg^{2+}-ATPase activity, of membranes isolated from platelets pretreated with thrombin was significantly decreased (Fig. 1). This rapid effect reached maximum levels of inhibition within 10-20 sec and was maintained after prolonged treatment periods. Thrombin pretreatment

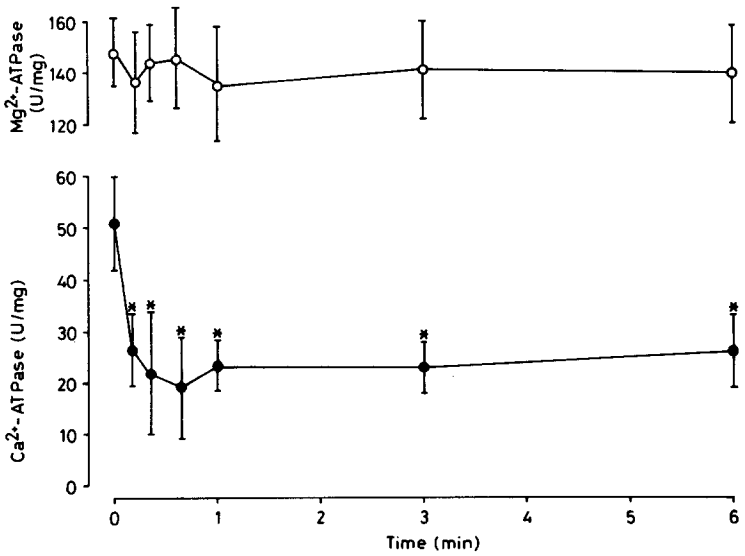

Figure 1. Thrombin pretreatment of human platelets inhibits membrane Ca^{2+}-ATPase; time dependency: Intact platelets were treated with thrombin (0.06 U/ml) for the indicated times and subsequently isolated membranes assayed for Mg^{2+}-(o) and Ca^{2+}-(●) ATPase activities as described in Materials and Methods. Data are given as mean ± SD from at least 10 separate experiments. * $p < 0.001$ and represents significance of difference from the zero time control.

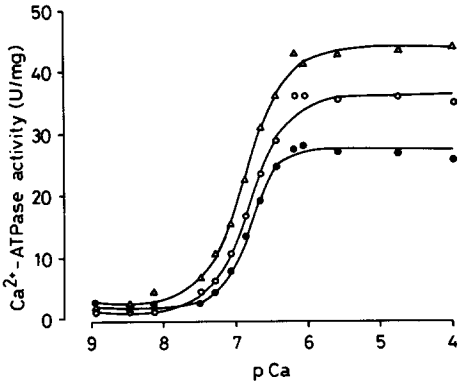

Figure 2. Influence of thrombin on Ca^{2+}-sensitivity of Ca^{2+}-ATPase: Intact platelets were treated for 2 min with solvent (saline) (△) or with thrombin (o, 0.03 U/ml; ●, 0.06 U/ml) and subsequently isolated membranes assayed for Ca^{2+}-ATPase activity as described in Methods in the absence or presence of varying free calcium concentrations (3.3×10^{-9} M to 1.01×10^{-4} M). pCa represents the negative logarithm of the molar free calcium concentration (3). Data represent mean values from 4 separate experiments. Half-maximal stimulatory calcium concentrations (K_a) are given in the text and were obtained by individual analysis of data from each separate experiment using the weighted non-linear regression method of De Lean et al (41).

decreased only the V_{max} of membrane Ca^{2+}-ATPase without altering Ca^{2+}-sensitivity (Fig. 2). Calculated K_a (Ca^{2+}) values for platelets treated in the absence of hormone or in the presence of 0.03 and 0.06 U/ml thrombin were 6.86 ± 0.09, 6.82 ± 0.11 and 6.76 ± 0.06 respectively (pCa, mean ± SD, n=4). The inhibitory effect of thrombin on the V_{max} of Ca^{2+}-ATPase was dose-dependent with half-maximal and maximal inhibitory thrombin concentrations of 0.04 U/ml and 0.15 U/ml respectively (complete dose profile not shown). When calmodulin-depleted membranes were prepared (3) from thrombin pretreated platelets and then assayed for Ca^{2+}-ATPase activity in the presence of exogenous calmodulin (10^{-9} to 10^{-6} M; data not shown) it was also evident that thrombin did not influence the affinity (K_m) of the enzyme for calmodulin (control vs thrombin: 3.5 ± 1.0 nM vs 4.0 ± 1.2 nM (mean ± SD, n=6)). Thrombin treatment of isolated plasma membranes (in presence or absence of $CaCl_2$ and/or GTP did not result in the changes described above (data not shown).

In order to determine whether inhibition of Ca^{2+}-ATPase might be a characteristic activation response, the effect of platelet pretreatment with several other stimulatory agonists on membrane Mg^{2+} and Ca^{2+}-ATPase activities was studied. Platelet activating factor, vasopressin and ADP significantly decreased Ca^{2+}-ATPase activity, although the inhibitory effects of these agonists, particularly in the case of ADP were less potent that that of thrombin (Table 1). Mg^{2+}-ATPase activities were unaltered.

Since the activation of platelets by most agonists is biochemically characterized by an increase in $[Ca^{2+}]_i$ and the production of diacylglycerol

TABLE 1

Effect of platelet pretreatment with various platelet activators on membrane ATPase activities

Stimulant	Mg^{2+}-ATPase (U/mg)	Ca^{2+}-ATPase (U/mg)
None	105.6 ± 16	71.8 ± 17
Platelet activating factor (50 nM)	100.8 ± 22	42.7 ± 18 ($p<0.001$)
Vasopressin (100 nM)	93.7 ± 18	50.3 ± 22 ($p<0.005$)
ADP (50 μM)	98.9 ± 15	58.5 ± 14 ($p<0.05$)
Thrombin (0.11 U/ml)	93.8 ± 13	26.3 ± 11 ($p<0.001$)

Intact platelets were treated for 5 min with the indicated platelet activators and subsequently isolated membranes assayed for Mg^{2+} and Ca^{2+}-ATPase activities as described in Methods. Data are given as mean ± SD from at least 8 separate experiments. p values represent significance of difference from control unstimulated platelets.

via phosphoinositide breakdown we investigated the influence of platelet pretreatment with the Ca^{2+}-ionophores, A23187 and ionomycin, and TPA. The concentrations of ionophores and TPA were selected as being subthreshold in terms of eliciting phosphoinositide turnover, arachidonic acid metabolism or dense granule release and aggregation. Neither Mg^{2+} nor Ca^{2+}-ATPase activities were altered following platelet pretreatment with either TPA, or A23187 or ionomycin alone (Table 2), while Ca^{2+}-ATPase activity was significantly decreased in response to the simultaneous addition of TPA plus A23187 or TPA plus ionomycin (Table 2).

Because platelets are bidirectionally regulated by Ca^{2+} (activation stimulated) and cAMP (activation inhibited) we also studied Ca^{2+}-ATPase activity following platelet pretreatments with cAMP-elevating agents. Neither PGE_1 nor forskolin *per se* had any effect on Ca^{2+}-ATPase (Fig. 3). However, the inhibitory effect of thrombin was antagonized, albeit incompletely, by both PGE_1 and forskolin (Fig. 3).

DISCUSSION: The study demonstrates for the first time an inhibition of platelet membrane Ca^{2+}-ATPase activity following pretreatment of intact platelets with stimulatory hormones. The changes in Ca^{2+}-ATPase may involve the inositolphospholipids either via a direct influence on the enzyme (5-7) or via modulation of polyphosphoinositide Ca^{2+}-binding sites (12-15). Furthermore, since decreased membrane Ca^{2+}-ATPase activity was not observed following

TABLE 2

Effect of platelet pretreatment with Ca^{2+}-ionophores and/or TPA on membrane ATPase activities

Treatment	Mg^{2+}-ATPase	Ca^{2+}-ATPase
Control	118.1 ± 31	83.6 ± 31
TPA (5 nM)	98.8 ± 16	87.9 ± 38
A23187 (10 nM)	141.1 ± 44	69.4 ± 19
Ionomycin (5 nM)	101.5 ± 22	67.4 ± 10
A23187 (10 nM) plus TPA (5 nM)	129.9 ± 35	43.7 ± 7*
Ionomycin (5 nM) plus TPA (5 nM)	116.7 ± 29	56.3 ± 13*

Platelets were treated for 5 min with the indicated compounds and membrane preparations subsequently assayed for Mg^{2+}- and Ca^{2+}-ATPase activities as described in Methods. Values are given as mean ± SD from at least 8 separate experiments. * $p < 0.01$ and represents significance of difference from control untreated platelets.

hormone treatment of isolated membranes the involvement of a soluble cytosolic component(s) (e.g. phospholipase C, phospholipase A_2 or protein kinase C) was implicated.

Kinetically the inhibition we describe is unlikely to involve the rapid elevation in $[Ca^{2+}]_i$ associated with hormone-receptor binding (1-2 sec)

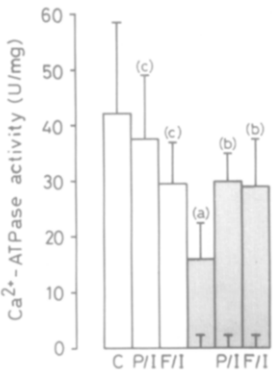

Figure 3. PGE_1 and forskolin antagonize inhibitory effects of thrombin on Ca^{2+}-ATPase: Intact platelets were preincubated at 37°C for 1 min with solvent (0.001% ethanol) [C] or PGE_1 (10 μM) plus isobutylmethylxanthine (100 μM) [P/I] or forskolin (5 μM) plus isobutylmethylxanthine (100 μM) [F/I] (all open bars) and then treated with thrombin (0.11 U/ml) [T] (hatched bars) for 2 min. Ca^{2+}-ATPase activity measurements were performed on subsequently isolated membrane preparations as described in Methods. Values are given as mean ± SD from 8 separate experiments. (a), $p < 0.001$ and represents significance of difference from control; (b), $p < 0.01$ and represents significance of difference from platelets treated with thrombin alone; (c) no significant difference from either control untreated platelets or from platelets treated with thrombin plus cAMP elevating agents.

(13-18) but rather the metabolism of phosphatidylinositol (PIP_2, 10-20 sec; 19,20). The inhibitory effects of vasopressin and platelet activating factor were much less potent than thrombin. While receptor coupled breakdown of PIP_2 occurs in response to all of these hormones, the measured extent of PIP_2 hydrolysis in platelets stimulated by platelet activating factor ($\sim$ 30% (21)) and vasopressin is less ($\sim$ 15% (22,23)) than that in response to thrombin ($\sim$ 40-50% (19)). ADP elicited the smallest decrease in Ca^{2+}-ATPase activity. In the presence of aspirin ADP does not induce PIP_2 hydrolysis (19) while in the absence of cycloxygenase inhibitors a limited decrease (7%) in PIP_2 occurs (24). Therefore, the inhibitory effect of ADP on Ca^{2+}-ATPase observed in this study (performed in the absence of cycloxygenase inhibitors) may be due to a secondary thromboxane-induced PIP_2 hydrolytic effect (25,26). Thromboxane is also produced in response to vasopressin (27), platelet activating factor and thrombin (25). However, the inhibition of Ca^{2+}-ATPase is unlikely to be a function of stimulated arachodonic acid metabolism since a comparable inhibition of Ca^{2+}-ATPase in response to thrombin occurred even in the presence of acetylsalicylate or indomethacin (data not shown) which completely abolish thrombin-induced thromboxane generation (25). A further explanation for the inhibitory effects of ADP may be that under the given experimental procedures ADP also induces release of serotonin (25) which itself provokes a minor decrease ($\sim$ 8%) in PIP_2 (28) All these findings together with the observation that thrombin-induced inhibition of Ca^{2+}-ATPase was dose-dependent suggest a possible cause-effect relationship between extent of PIP_2 hydrolysis and the degree of inhibition of Ca^{2+}-ATPase activity. Such an association has already been made with respect to the regulation of rat liver Ca^{2+}-transport by vasopressin, angiotensin II and epinephrine (9,10).

The results obtained from platelets treated with subthreshold concentrations of TPA or ionophores alone suggest that the effect of stimulatory hormones on platelet membrane Ca^{2+}-ATPase is not the consequence per se of either increased Ca^{2+}-mobilization or activation of protein kinase C. The moderate inhibition of Ca^{2+}-ATPase following simultaneous addition of TPA and ionophores may also reflect a secondary PIP_2 hydrolytic effect, which in this case arises from the synergistic role of protein kinase C and Ca^{2+}-mobilization in mediating serotonin release (29) and thromboxane synthesis (30).

TPA itself has been reported to promote polyphosphoinositide formation in platelets (PIP (31,32)) and in lymphocytes (PIP and PIP_2 (33)). This diacylglycerol mimetic was also shown to stimulate ATP-dependent Ca^{2+}-transport by neutrophil plasma membranes (34) and $^{45}Ca^{2+}$-efflux in macrophages (35). Nevertheless our inability to demonstrate a similar stimulatory effect of TPA on membrane Ca^{2+}-ATPase activity following treatment of control platelets is compatible with the observations that TPA neither alters resting platelet

$[Ca^{2+}]_i$ (36) nor increases PIP_2 (31,32). TPA does, however, suppress vasopressin, thrombin and platelet activating factor induced elevations in platelet (18,36) and this is associated with an inhibition of PIP_2 breakdown (18) and suppression of phosphatidate formation (36). We have not investigated the ability of TPA to counteract the inhibitory influence of stimulatory hormones on platelet Ca^{2+}-ATPase. However, inhibition of the same events occur when platelets are treated with agents known to elevate their cAMP content (16,17,38).

The inhibitory action of cAMP on platelet activation has been attributed to its effect on lowering $[Ca^{2+}]_i$ (17,37), although there is also evidence that cAMP inhibits platelet activation independently of a lowering effect on $[Ca^{2+}]_i$ (38). Our results demonstrating a lack of effect on Ca^{2+}-ATPase activity following PGE_1 or forskolin treatment of platelets support the latter viewpoint (38). While our results are discrepant from those of Kaser-Glanzmann et al (37) who reported a cAMP-dependent stimulation of Ca^{2+}-uptake in platelet membrane vesicles, they are nevertheless consistent with the hypothesis that PIP_2 is a biological modulator of Ca^{2+}-ATPase activity since the effects of elevated cAMP levels on phosphoinositide metabolism are to increase PIP (39) or to increase phosphatidylinositol (PI) and decrease PA (40) without influencing levels of PIP_2 (39,40). Our observation that both PGE_1 and forskolin could antagonize the inhibitory effect of thrombin on Ca^{2+}-ATPase may be explained by the fact that cAMP inhibits the stimulated breakdown of inositol lipids via inhibition of phospholipase C (18).

The findings reported herein, at least with respect to the maintenance of inhibition with prolonged periods of platelet treatment, are apparently contradictory to the reported observations that hormone-stimulated elevations in $[Ca^{2+}]_i$ are transient and reversible (16-18,36) and to the belief that activated platelets maintain Ca^{2+} homeostasis. In addition the membrane preparation used consists of both external and internal membranes making it impossible to draw unambiguous conclusions as to whether the Ca^{2+}-ATPase involved promotes Ca^{2+}-extrusion or Ca^{2+}-sequestration. Nevertheless, the data are strongly suggestive of the involvement of inositol lipids in modulating Ca^{2+}-ATPase activity. Since inhibition of Ca^{2+}-ATPase could be demonstrated under all conditions associated with PIP_2 breakdown without any significant alteration under other conditions associated with changes in PI or PIP, the most likely candidate mediating this regulation is PIP_2. Our observations support the previous suggestion (8) that breakdown of PIP_2 leads to inactivation of Ca^{2+}-pumping ATPase. This inhibition may act in concert with the IP_3 induced release of Ca^{2+} from intracellular stores to maintain elevated $[Ca^{2+}]_i$ and to promote or sustain a full platelet activation response.

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