Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 178-186

THE Na^+/H^+ ANTIPORTER IS NOT INVOLVED IN POTENTIATION OF THROMBIN-INDUCED RESPONSES BY EPINEPHRINE

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Received January 20, 1988

SUMMARY: Stimulation of platelets with thrombin, ADP and epinephrine has recently been shown to activate a Na^+/H^+ antiporter, with a resulting alkalinization of the cytoplasm. Unlike thrombin, however, epinephrine is incapable of directly activating phospholipase C, but is well known to potentiate the effects of thrombin on this enzyme and other subsequent steps of platelet activation. Therefore, we have studied the involvement of the Na^+/H^+ antiporter in this aspect of epinephrine action to see whether alkalinization of platelet cytosol could be a requirement for agonists to stimulate inositol phospholipid hydrolysis and mobilize cellular Ca^{2+} stores. Alpha-thrombin induced the rapid formation of inositol trisphosphate with a parallel mobilization of intracellular Ca^{2+} stores. Epinephrine alone had no effect on either of these parameters. The response to thrombin desensitized over a period of minutes, and after this had occurred, epinephrine was able to activate phospholipase C and induce the release of intracellular Ca^{2+}. This showed that epinephrine was able to recouple thrombin receptors to phospholipase C, and this appeared to be mediated by the same mechanism which is involved in potentiation by epinephrine of thrombin-stimulation of phospholipase C. These effects of epinephrine were not altered by inhibition of the Na^+/H^+ antiporter with ethylisopropylamiloride or by use of the Na^+/H^+ ionophore, monensin. We conclude that epinephrine potentiates thrombin-induced responses by a mechanism which is unrelated to its effects on the Na^+/H^+ antiporter, and this is not a requirement for thrombin-induced phospholipase C activation.

Activation of human platelets with either thrombin or phorbol ester has been shown to induce an increase in the pH of the cytosol (1). These effects appeared to be mediated by stimulation of a Na^+/H^+ antiporter, and the results of this study implied that agonist-induced protein kinase C stimulation was responsible (1). In contrast, Zavoico et al. (2) reported that thrombin induced an initial acidification of platelet cytosol with a subsequent alkalinization, and that phorbol ester had little effect on this response. The acidification was also produced using Ca^{2+} ionophore (2).

0006-291X/88 $1.50

Although somewhat contradictory, these results suggested that thrombin-stimulated inositol phospholipid hydrolysis preceded and caused the activation of the antiporter. Moreover, Siffert and Akkerman (3) have further suggested that cytoplasmic alkalinization of platelet cytosol induced by thrombin is a prerequisite for thrombin-induced increases in the cytosolic Ca^{2+} concentration.

In contrast to this apparently inositide hydrolysis-dependent stimulation of the antiporter, Sweatt et al. (4,5) have suggested that epinephrine activates Na^+/H^+ exchange independently of inositol phospholipid hydrolysis, and the alkalinization of the cytoplasm activates a phospholipase A_2. The resulting cyclooxygenase products are then proposed to activate phospholipase C. On the other hand, recent evidence indicates that stimulation of the Na^+/H^+ antiporter was a consequence of fibrinogen receptor expression in epinephrine-activated platelets (6).

Epinephrine has also been shown by several groups (7-10) to potentiate stimulation of platelets by other agonists, such as thrombin, but alone epinephrine is ineffective in the absence of cyclo-oxygenase metabolites. We have examined in this report the involvement of the Na^+/H^+ antiporter in epinephrine-induced potentiation of thrombin-stimulated inositol phospholipid hydrolysis and Ca^{2+}-mobilization. Our results show that there is no involvement of cytosolic alkalinization in either the initial action of thrombin, or potentiation by epinephrine.

MATERIALS AND METHODS

Materials. Alpha-thrombin was a generous gift of Dr. John Fenton II, Division of Laboratories and Research, New York State Department of Health, Albany, NY. Drs. Gerhard Burckhardt and Thomas Friedrich (Max-Planck-Institut fur Biophysik, Frankfurt, West Germany) kindly supplied us with ethylisopropylamiloride. Epinephrine and monensin were from Sigma Chemical Co., myo-[2-^{3}H]inositol (10-20 Ci/mmol) was from Amersham, and Indo-1 AM from Behring Diagnostics.

Preparation of human platelets. Platelets were isolated from blood of healthy human donors who had not taken medication for at least the previous two weeks. The blood was anticoagulated with 3.8% trisodium citrate (6 ml in 60 ml blood) and centrifuged at 200 g for 20 min. The platelet-rich plasma was then centrifuged for 20 min at 800 g and the platelets resuspended in the appropriate volume of buffer. The buffers contained (mM): NaCl, 138;

KCl, 2.9; Hepes, 20; NaH_2PO_4, 3.3; $MgCl_2$, 1.0. When platelets were loading with labelled inositol, and in all cases when platelets were washed, the buffer also contained 1 mM EGTA. In all experiments, platelets were treated with aspirin (1 mM) for 20 min and resuspended in the presence of an ADP scavenger (apyrase, 0.6 ADP'ase units/ml). During all experiments, just before centrifugation of platelet samples and during periods of radioactive labelling or loading with Indo-1, prostacyclin (100 ng/ml) was added to inhibit platelet activation.

Measurement of inositol phospholipid hydrolysis. Platelets from 200 ml of blood were resuspended in 1 ml of EGTA-containing buffer and 1 ml of [^{3}H]inositol (0.5 mCi) was added. After incubation for 3 hours to label inositol phospholipids, platelets were diluted to 30 ml with buffer, centrifuged for 10 min and resuspended in final buffer without EGTA. Aliquots were incubated with or without agonist and, after the times indicated in the figures, samples (0.5 ml) were taken and the reactions were stopped by addition of 1.8 ml $CHCl_3/CH_3OH/HCl$ (100:200:2) and the phases separated by addition of 0.6 ml each of $CHCl_3$ and H_2O and centrifugation. The water-soluble inositol phosphates produced were separated by anion exchange chromatography, as described previously (11).

Measurement of Intracellular Ca^{2+} concentration. Platelets from 100 ml blood were incubated in plasma containing 5 μM Indo-1 AM for 45 min. The cells were then centrifuged and resuspended in buffer containing EGTA and 1 mM aspirin. After 20 min, the cells were again centrifuged and suspended in buffer without EGTA. Changes in the intracellular cellular Ca^{2+} concentration of aliquots (2 ml) were monitored continuously using a fluorescence spectrophotometer (Perkin Elmer) with excitation and emission wavelengths of 340 and 390 nm, respectively. Changes in the fluorescence were calibrated to changes in cellular Ca^{2+} levels as previously described (12).

RESULTS

Thrombin-induced inositol trisphosphate production. Alpha-thrombin stimulated a rapid formation of inositol trisphosphate in [^{3}H]inositol-labelled human platelets (fig 1). This response desensitized over the next few minutes, such that a second application of alpha-thrombin (10 nM) was ineffective (not shown). Epinephrine (100 μM), which alone did not stimulate any inositol phosphate formation (not shown), produced a marked restoration of the formation of inositol trisphosphate in response to alpha-thrombin following its desensitization (fig 1).

Addition of ethylisopropylamiloride, 40 μM, a concentration which abolishes Na^+/H^+ antiporter activity (4,5), had no effect on the ability of epinephrine to elicit the formation of inositol trisphosphate when added after thrombin desensitization (fig 1).

Thrombin-induced mobilization of Ca^{2+} from intracellular stores. Alpha-thrombin (10 nM) caused a rapid increase in platelet intracellular Ca^{2+}

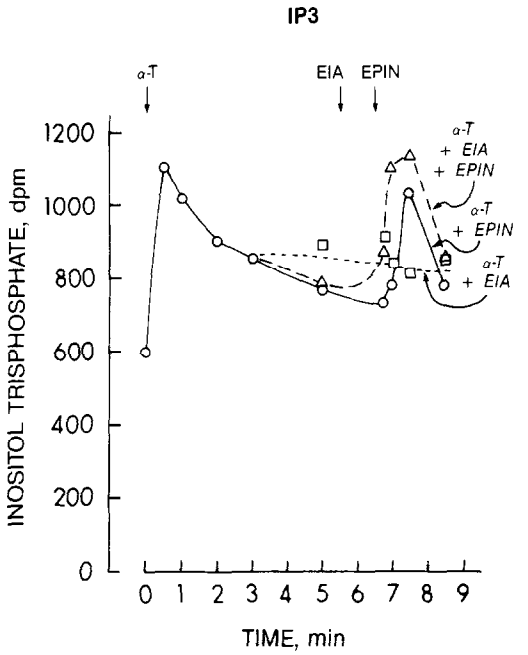

Fig. 1. Alpha-thrombin stimulation of inositol trisphosphate formation-desensitization and resensitization by epinephrine. Inositol phospholipids of aspirin-treated platelets were labelled with myo-[2-^{3}H]inositol and then stimulated with alpha-thrombin (α-T,t=0, 10 nM). Either buffer, ethylisopropylamiloride (EIA, 40 μM) or epinephrine (EPIN,100 μM) were added at the indicated times. Aliquots were taken at each time point to assess the formation of inositol phosphates. Inositol monophosphate, bisphosphate and trisphosphate were all measured, but only the levels of inositol trisphosphate (IP3) are presented. The other species showed similar changes, but with longer lag time consistent with precursor-product relationship. Results are from one experiment, but quantitatively identical results were observed on two separate platelet preparations.

concentration to a maximum of about 1 μM (fig 2). Over the next 5 minutes the Ca^{2+} level returned to basal levels (fig 2). The same effects were seen in the presence of 1 mM Ca^{2+}, 1 mM EGTA or without either addition (as presented, fig 2), showing that the Ca^{2+} was predominantly coming from intracellular Ca^{2+} stores. After the decline to basal levels, a second challenge of platelets with alpha-thrombin (10 nM) was without effect on the cytosolic Ca^{2+} concentration, whereas the less effective agonist, platelet activating factor (0.1-2.5 μM) did induce some further release of Ca^{2+}. Addition of epinephrine (100 μM) at this time induced the release of a significant proportion of the remaining Ca^{2+} stores (fig 2). When added

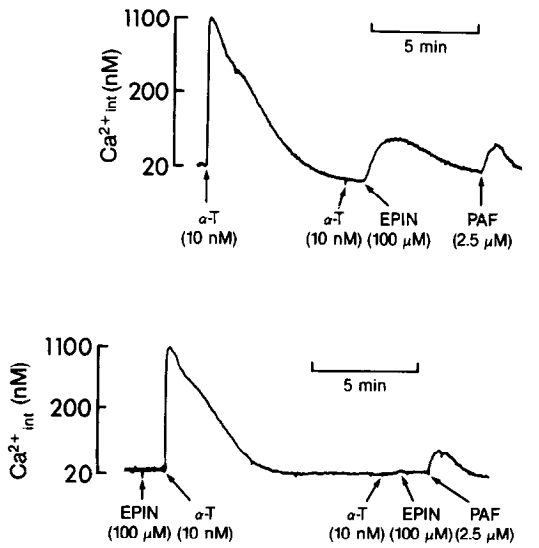

Fig. 2. Alpha-thrombin-stimulated release of intracellular Ca^{2+}stores-desensitization and resensitization by epinephrine. Indo-1-loaded platelets were stimulated with alpha-thrombin (α-T, 10 nM) and the fluorescence measured in a fluorimeter (excitation, 340 nm; emission, 390 nm). At the times indicated, epinephrine (EPIN, 100 μM) or platelet activating factor (PAF, 2.5 μM) were added. The tracings obtained were highly reproducible between different donors, and the same effects were seen on six other preparations.

alone, there was no effect of epinephrine on the cytosolic Ca^{2+} concentration (fig 2, lower panel).

The effects of epinephrine when added after the alpha-thrombin-induced Ca^{2+} release had desensitized were half maximal at 1 μM and maximal at 10 μM (fig 3), and were inhibited by the $alpha_2$-adrenergic receptor antagonist, yohimbine (not presented).

Monensin (10 μM) added 2 min prior to alpha-thrombin (10 nM) had no effect on the stimulated release of intracellular Ca^{2+} stores (fig 4). The subsequent effects of epinephrine (100 μM) were similarly unaffected by monensin when added either before or after the alpha-thrombin-induced Ca^{2+} release had desensitized (fig 4).

DISCUSSION

The role of the Na^+/H^+ antiporter in platelet responses to agonists is an unresolved issue at present. One complication of some of the previously

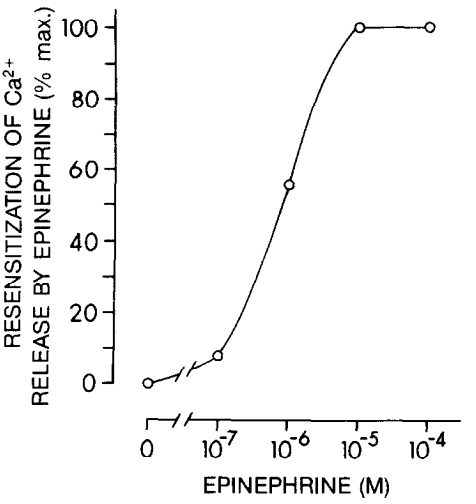

Fig. 3. Dose-response curve of epinephrine-induced resensitization of thrombin-stimulated Ca^{2+} release. Ca^{2+} release was measured as described in figure 2. After the alpha-thrombin (10 nM) response had desensitized, the degree of mobilization of Ca^{2+} by the subsequent addition of epinephrine (as shown in figure 2) was assessed using different epinephrine concentrations. The same dose-response relationship was seen using two different platelet preparations.

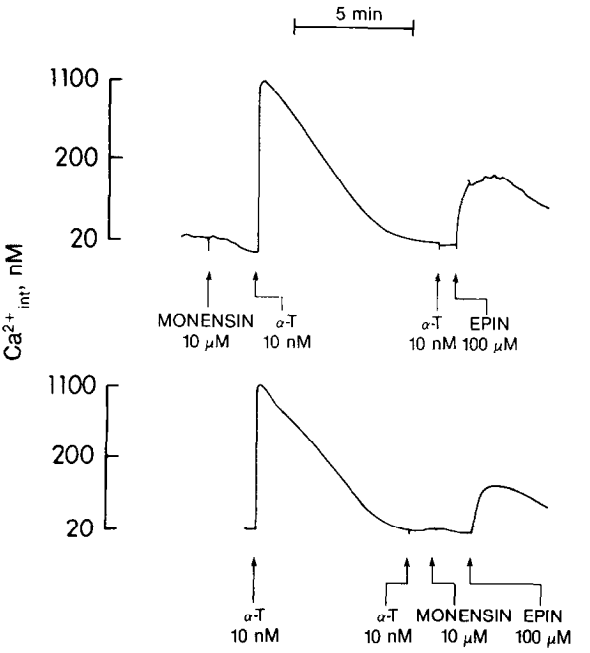

Fig. 4. The effect of monensin on alpha-thrombin-induced Ca^{2+} release and resensitization by epinephrine. Changes in platelet cytosolic Ca^{2+} levels were measured as described in figure 2.

published data is that platelets were used which had intact cyclooxygenase activity. Siffert and Akkerman (3) found that the Na^+/H^+ antiporter inhibitor, ethylisopropylamiloride, partially antagonized the thrombin stimulation of Ca^{2+} release, and concluded that the antiporter was essential for the Ca^{2+} release mechanism. As expressed by Rink (13), the study of Siffert and Akkerman (3) did not discount the possible involvement of arachidonic acid metabolites in activation of platelets stimulated by thrombin.

Sweatt et al. (4) suggest that epinephrine directly stimulates a Na^+/H^+ antiporter which is then responsible for activating a phospholipase A_2. This results in an arachidonic acid metabolite cascade which then activates phospholipase C. However, two other conditions could also explain these data (a) the basal level of arachidonate metabolites seen in their preparation was alone insufficient to activate platelets. However, addition of epinephrine could potentiate activation of phospholipase C, and result in an amiloride-sensitive feedback stimulation of phospholipase A_2, or (b) the activity of phospholipase A_2 in addition to the Na^+/H^+ antiporter may be directly inhibited by the conditions used, those of low extracellular Na or pH and the presence of ethylisopropylamiloride. This could explain both the inhibition of action of low concentrations of thrombin by ethylisopropylamiloride (known to be aspirin-sensitive) (14) and also that of epinephrine. The effect of conditions which inhibit the Na^+/H^+ antiporter on direct stimulation of phospholipase A_2 was not documented in these reports.

We attempted to resolve, using aspirin-treated platelets, whether the ability of epinephrine to potentiate thrombin stimulation of phospholipase C and Ca^{2+} mobilization was due to an effect on Na^+/H^+ exchange. Epinephrine was found to recouple the desensitized thrombin receptor to both phospholipase C and Ca^{2+} mobilization. This effect appeared to be mediated by the same mechanism involved in potentiation of agonist-induced responses since (a) potentiation by epinephrine of other agonists is due to stimulation of $alpha_2$ -adrenergic receptors (7-10), as found for its resensitizing

effects, and (b) the dose-response curve for potentiation by epinephrine is over the same concentration range (15) as that found for resensitization.

This manner of measuring the effects of epinephrine (i.e., added following thrombin desensitization) was used because it provided a more sensitive assay system than examining changes in the intensity of the thrombin response with or without preincubation with epinephrine.

Neither inhibition of the Na^+/H^+ antiporter with ethylisopropylamiloride nor dissipating the Na^+/H^+ gradient with the monovalent cation ionophore, monensin, had any effect on either the thrombin-induced hydrolysis of inositol phospholipids or the subsequent release of intracellular Ca^{2+} stores. The epinephrine-induced resensitization of these responses was similarly unaffected by either agent.

We can conclude from our results that (a) inositol trisphosphate production in activated platelets correlates extremely well with mobilization of intracellular Ca^{2+} stores, and so supports the contention (16) that inositol trisphosphate is the physiological mediator of this response (b) changes in the activity of the Na^+/H^+ antiporter or the cytoplasmic pH directly have no effect in intact platelets on the ability of inositol trisphosphate to mobilize intracellular Ca^{2+} stores (c) the $alpha_2$-adrenergic receptor and its coupling system are unaffected by changes in cellular pH (d) the potentiating and resensitizing effects of epinephrine do not require an intact cyclooxygenase activity and (e) epinephrine must be acting to potentiate and resensitize thrombin receptor activation of phospholipase C and Ca^{2+} mobilization by a mechanism which is unrelated to its ability to stimulate the Na^+/H^+ antiporter.

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