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Activation of sodium-proton exchange is not a prerequisite for Ca^{2+} mobilization and aggregation in human platelets

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Recently it has been suggested [(1987) Nature 325, 456–458; (1987) FEBS Lett. 212, 123–126] that the activation of Na^+/H^+ exchange is a prerequisite for platelet aggregation and the development of the Ca^{2+} signal. As direct evidence for the role of the Na^+/H^+-exchange pathway the inhibition of the Ca^{2+} signal by EIPA, a specific inhibitor of Na^+/H^+ exchange, was offered. Here we demonstrate that low concentrations of EIPA (below 1 μM) completely block Na^+/H^+ exchange while EIPA inhibits aggregation or Ca^{2+} mobilization only in concentrations 100-times greater than 1 μM. Moreover, another amiloride analogue, CBDMB, developed to act predominantly on Na^+/Ca^{2+} exchange, does not affect Na^+/H^+ exchange in platelets but blocks aggregation and Ca^{2+} mobilization. We conclude that while Na^+/H^+ exchange has a fundamental role in platelet functions it is not prerequisite for the development of Ca^{2+} signal and aggregation.

Ca^{2+} signal; Na^+/H^+ exchange; Thrombin; Amiloride analogue; (Human platelet)

1. INTRODUCTION

By now it is generally accepted that cell activation by a variety of stimulating agents is mediated by a rapid rise in $[Ca^{2+}]_i$ and involves the specific hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield $InsP_3$ and DAG. $InsP_3$ liberates Ca^{2+} from a non-mitochondrial intracellular store while DAG may act through the activation of protein kinase C which, in turn, can activate Na^+/H^+ exchange in the plasma membrane [3–7]. The actual timing of all these intracellular events is still poorly understood and platelets provide a relatively well manageable model system for studying these phenomena. Several studies in platelets have indicated that a thrombin-induced Ca^{2+} signal precedes the activation of Na^+/H^+ exchange [8–10]. However, in their recent communications Siffert et al. [1,2] concluded that the activation of sodium-proton exchange is a prerequisite for Ca^{2+} mobilization and aggregation in human platelets. They showed that removal of external Na^+ strongly reduced the thrombin-induced increase in $[Ca^{2+}]_i$ as measured in quin2-loaded platelets, both in the presence or absence of external Ca^{2+}. An amiloride analogue, EIPA, inhibitor of the Na^+/H^+-exchange transport, also inhibited this Ca^{2+} signal, while the resting $[Ca^{2+}]_i$ was unchanged. The authors further showed that removal of Na^+ inhibited H^+ extrusion, while intracellular

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Abbreviations: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; CBDMB, 5-(*N*-4-chlorobenzyl)-*N*-(2′,4′-dimethyl)benzamil; DMB, *N*-(2′,4′-dimethyl)benzamil; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; $InsP_3$, inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol

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alkalinization potentiated the thrombin-induced Ca^{2+} signal and platelet aggregation. Moreover, thrombin induced an intracellular alkaline shift and this was prevented by the removal of external Na^+ or by the addition of EIPA.

The only direct evidence for the role of the Na^+/H^+-exchange pathway in Ca^{2+} mobilization is the inhibition of the Ca^{2+} signal by EIPA in quin2-loaded platelets. However, in these experiments [1,2] the authors used 40–100 μM of this inhibitor, while the half-maximal inhibitory concentration of EIPA on Na^+/H^+ exchange, as measured in a variety of tissues at physiological Na^+ concentrations, is about 0.1 μM [11–14]. Thus a non-specific effect of this amiloride analogue in these high concentrations may occur.

In order to resolve these questions we have measured the concentration-dependent effects of EIPA and another amiloride analogue, CBDMB, on Ca^{2+} mobilization, aggregation and on Na^+/H^+ exchange as reflected by a Na-propionate-induced swelling in human platelets. The amiloride analogue CBDMB was developed to act predominantly on Na^+/Ca^{2+} exchange, because as a consequence of substitution of the guanidino group of amiloride it has a negligible effect on Na^+/H^+ exchange (K_i above 500 μM) [13–15], while its K_i for Na^+/Ca^{2+} exchange is 7.3 μM as demonstrated on pituitary plasma membrane vesicles using the method described in [16] (Kaczorowski, G.J., personal communication).

2. MATERIALS AND METHODS

Platelets were isolated from freshly drawn citrated blood by centrifugation at 400 × *g* for 10 min. Platelet-rich plasma was centrifuged for 15 min at 1200 × *g* and the platelets were resuspended in a nominally Ca^{2+}-free Tyrode solution (pH 7.25). Platelets were loaded with quin2 by incubating them in the same solution containing 15 μM quin2-acetoxymethyl ester (Calbiochem) for at least 30 min at room temperature. Then the cells were centrifuged for 10 s at 10000 × *g*, rinsed twice and resuspended (10^8 cells/ml) in the same solution. Fluorescence was measured by a Hitachi F-4000 fluorescent spectrophotometer at 37°C with continuous stirring. Fluorescence was recorded at an excitation wavelength of 337 nm (5 nm slit) and an emission wavelength of 490 nm (5 nm slit). Calibration of $[Ca^{2+}]_i$ was carried out as described in [17,18] using an apparent K_d of 115 nM for Ca^{2+}-quin2 (cf. [17]). CBDMB gave a concentration-dependent background fluorescence independent of divalent cations or thrombin and this fluorescence has been subtracted. Aggregation was measured in the same quin2-loaded cells in the presence of 1% plasma in a Lumi-aggregometer (Chrono-log, model 460, PICA), at 37°C. EIPA, DMB and CBDMB were synthesised by the methods described [19,20].

For measuring Na^+/H^+ exchange quin2-loaded or control platelets were suspended at 0 min in a Na-propionate medium (140 mM Na-propionate, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 5.5 mM glucose, 20 mM Tris-HCl, pH 6.8). Cell volume was measured by an electronic sizing (Coulter-type) instrument (Laborscale, Medicor). Cell volume was calibrated by latex beads. There was no difference observed in this experiment between control or quin2-loaded platelets.

3. RESULTS AND DISCUSSION

3.1. *Effects of EIPA and CBDMB on Ca^{2+} signal and aggregation*

Fig.1 demonstrates the effects of EIPA and CBDMB on the thrombin-induced increase in $[Ca^{2+}]_i$ in quin2-loaded human platelets. Up to 10 μM concentration EIPA has no effect on aggregation or on the Ca^{2+} signal. It was previously reported by Siffert et al. [21] and others [10] that EIPA at this concentration (10 μM) almost completely abolishes thrombin-induced alkalinization. As shown in fig.1, 10 μM CBDMB or 40 μM EIPA inhibit the Ca^{2+} signal and aggregation. By a detailed analysis of these effects we found that half-maximum inhibition was obtained by about 30 μM EIPA and 3–5 μM CBDMB. Both EIPA and CBDMB had the above concentration-dependent effects on the thrombin-induced rise of $[Ca^{2+}]_i$ in quin2-loaded platelets in the absence of external Ca^{2+} as well (not shown). It has to be mentioned that 10 μM CBDMB, in the presence of 1 mM external Ca^{2+}, produced a slow rise in platelet $[Ca^{2+}]_i$ (fig.1). This effect was independent of the addition of thrombin (not shown) and caused a delayed, non-specific, slow aggregation.

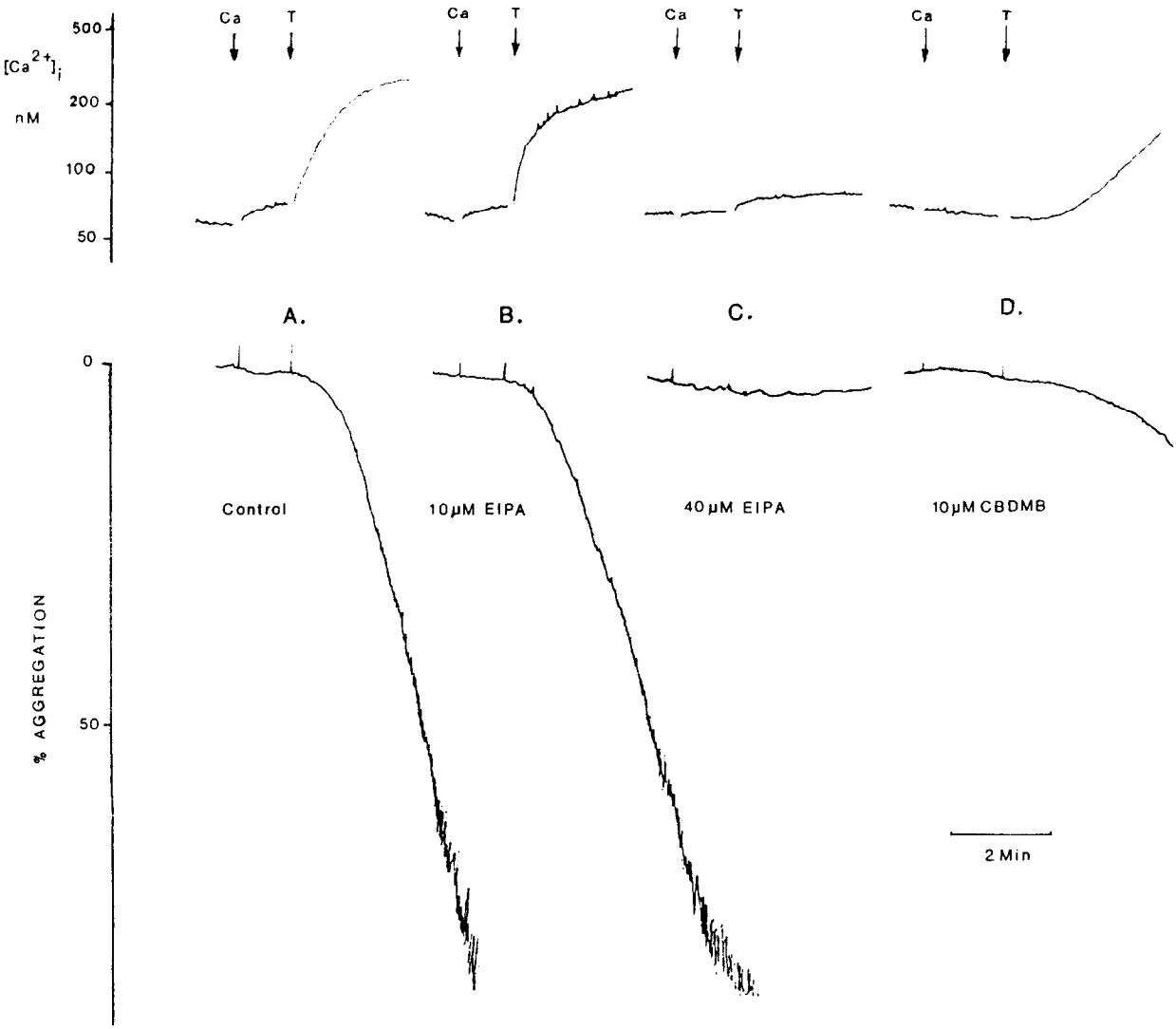

Fig.1. Effects of EIPA and CBDMB on the thrombin-induced increase in $[Ca^{2+}]_i$ (upper panel) and aggregation (lower panel) in human platelets loaded with the fluorescent Ca^{2+} indicator quin2. (A) Control; (B) 10 μM EIPA; (C) 40 μM EIPA; (D) 10 μM CBDMB. The arrows indicate the addition of $CaCl_2$ (final conc. 1 mM) and thrombin (0.5 U/ml). Each trace represents experiments repeated with three or four different preparations.

3.2. *Effects of EIPA and CBDMB on Na^+/H^+ exchange*

Fig.2 shows the effects of EIPA and CBDMB on Na^+/H^+ exchange in human platelets. It was studied by using a simple test-system: swelling in a Na-propionate medium. This method has been used in various cell types to follow the functioning of the Na^+/H^+-exchange system [22–25] as in a Na-propionate medium undissociated propionic acid enters the cells and cytoplasmic acidification initiates Na^+/H^+ exchange. These processes result in net Na^+ and propionate entry and cell swelling. The increase in cell volume can be followed by electronic sizing and any inhibitor of Na^+/H^+ exchange stops this swelling [23–25]. This swelling can only occur if a coupled Na^+/H^+ exchange is in operation and, as shown in fig.2, is strongly inhibited by low concentrations of EIPA. Based on

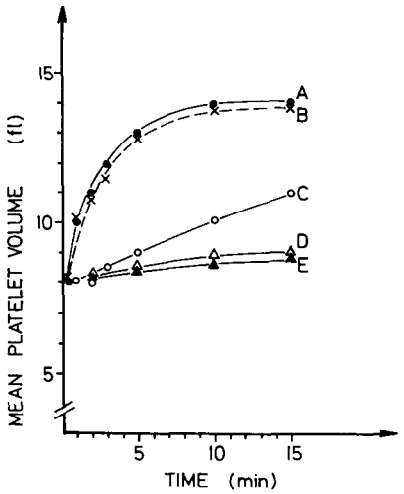

Fig.2. Effects of EIPA and CBDMB on Na^+/H^+ exchange as measured by platelet swelling in a Na-propionate medium. Results are representative of four independent experiments. (A) Control; (B) 10 μM CBDMB; (C) 0.1 μM EIPA; (D) 2 μM EIPA; (E) 10 μM EIPA.

four different experiments the half-maximal inhibition was obtained between 0.1 and 0.2 μM EIPA, in good agreement with the data for the inhibition of Na^+/H^+ exchange by EIPA in other tissues [11–14]. CBDMB was found to be ineffective on Na^+/H^+ exchange-dependent platelet swelling up to the concentration of 10 μM (higher concentrations produced platelet degradation).

Another amiloride analogue, DMB, specific for inhibition of Na^+/Ca^{2+} exchange in different tissues [16] acted similarly to CBDMB (not shown).

4. CONCLUSIONS

From the above experimental findings we conclude that EIPA inhibits Ca^{2+} mobilization and aggregation of human platelets only in concentrations more than 100-times greater than that required to block Na^+/H^+ exchange. In contrast the amiloride analogue CBDMB, presumed to act dominantly on Na^+/Ca^{2+} exchange, inhibits the signal and aggregation without any effect on the Na^+/H^+-exchange system. Certainly, the inhibition of Na^+/H^+ exchange does not prevent aggregation and Ca^{2+} mobilization, thus the activation of Na^+/H^+ exchange is not a prerequisite for these processes. The modulating effects of external Na^+ and the inhibition of platelet activation by amiloride derivatives may indicate a certain role for Na^+/Ca^{2+} exchange or other, as yet unidentified Na^+-dependent processes.

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