

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 $\text{Na}^+/\text{Ca}^{2+}$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ was unchanged. The authors further showed that removal of Na^+ inhibited H^+ extrusion, while intracellular

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Abbreviations: $[\text{Ca}^{2+}]_i$, 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

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 $\text{Na}^+/\text{Ca}^{2+}$ 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 $\text{Na}^+/\text{Ca}^{2+}$ 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 $400 \times g$ for 10 min. Platelet-rich plasma was centrifuged for 15 min at $1200 \times g$ 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 $10000 \times g$, 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ (fig.1). This effect was independent of the addition of thrombin (not shown) and caused a delayed, non-specific, slow aggregation.

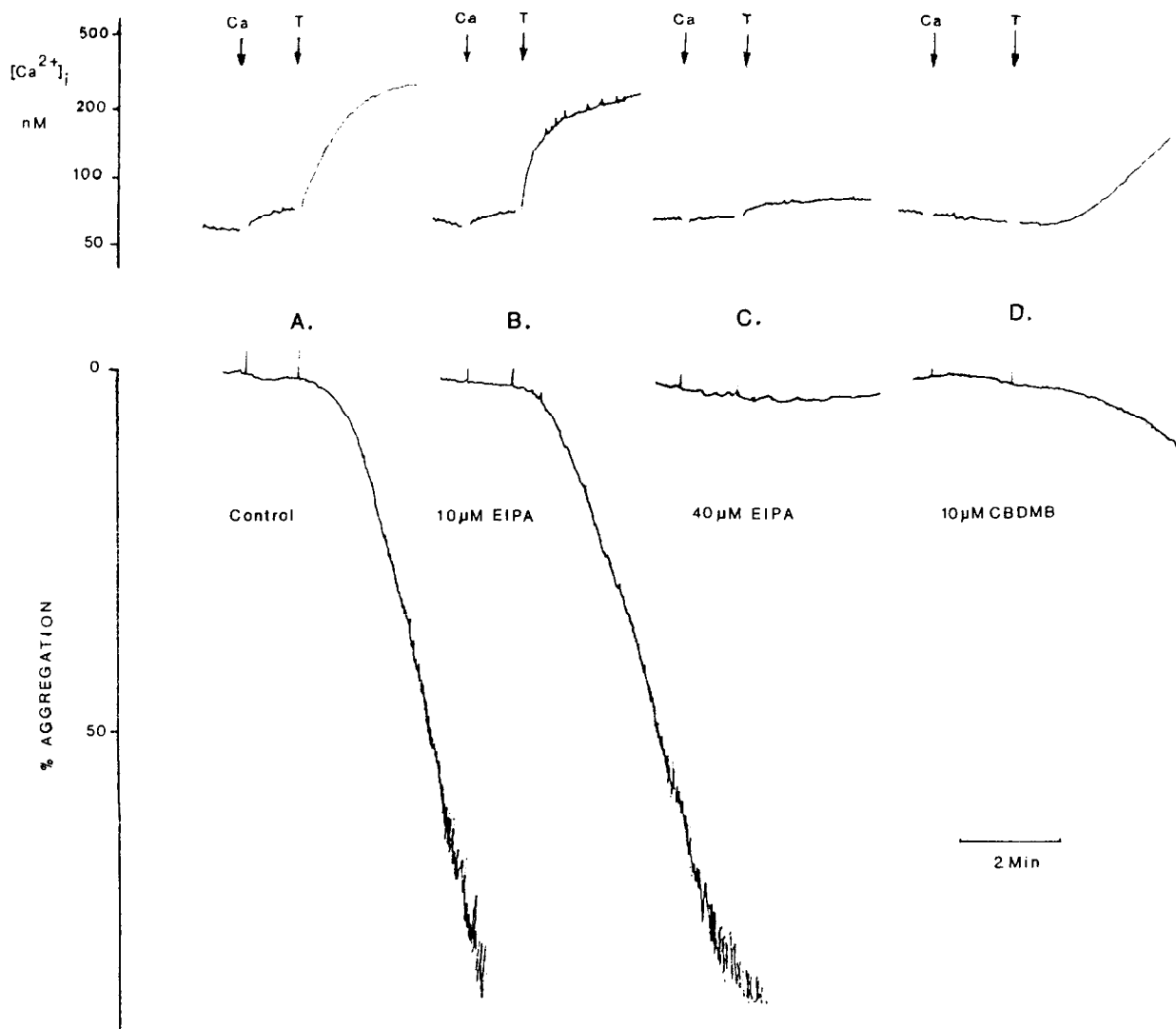


Fig.1. Effects of EIPA and CBDMB on the thrombin-induced increase in $[Ca^{2+}]_i$ (upper panel) and aggregation (lower panel) in human platelets loaded with the fluorescent Ca^{2+} indicator quin2. (A) Control; (B) 10 μ M EIPA; (C) 40 μ M EIPA; (D) 10 μ M CBDMB. The arrows indicate the addition of $CaCl_2$ (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

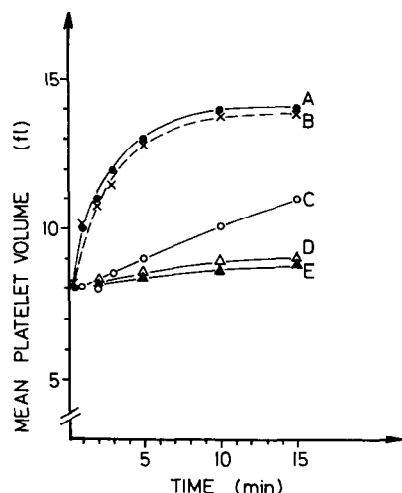


Fig.2. Effects of EIPA and CBDMB on Na^+/H^+ exchange as measured by platelet swelling in a Na-propionate medium. Results are representative of four independent experiments. (A) Control; (B) $10 \mu\text{M}$ CBDMB; (C) $0.1 \mu\text{M}$ EIPA; (D) $2 \mu\text{M}$ EIPA; (E) $10 \mu\text{M}$ EIPA.

four different experiments the half-maximal inhibition was obtained between 0.1 and $0.2 \mu\text{M}$ 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 $10 \mu\text{M}$ (higher concentrations produced platelet degradation).

Another amiloride analogue, DMB, specific for inhibition of $\text{Na}^+/\text{Ca}^{2+}$ 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 $\text{Na}^+/\text{Ca}^{2+}$ 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 ag-

gregation 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 $\text{Na}^+/\text{Ca}^{2+}$ exchange or other, as yet unidentified Na^+ -dependent processes.

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