Activation of Na^+/H^+ exchange in human platelets stimulated by thrombin and a phorbol ester

Winfried SIFFERT, Gerlinde SIFFERT and Peter SCHEID

Institut für Physiologie, Ruhr-Universität, 4630 Bochum, Federal Republic of Germany

We have investigated changes in cytoplasmic pH (pH_i) in activated human platelets, using the fluorescent probe 2,7-biscarboxyethyl-5(6)-carboxyfluorescein. Stimulation of platelets by thrombin or 12-*O*-tetradecanoylphorbol 13-acetate increased pH_i by about 0.11 pH unit above the resting value. This increase in pH_i depended on the presence of external Na^+ and was inhibited by ethylisopropylamiloride. The data suggest that protein kinase C mediates Na^+/H^+ exchange in human platelets.

INTRODUCTION

Stimulation of human platelets results in an uptake of Na^+ ions (Feinberg *et al.*, 1977; Sandler *et al.*, 1980) and an increase in cytoplasmic pH (pH_i) (Horne *et al.*, 1981; Simons *et al.*, 1982). We have previously demonstrated a Na^+ -dependent amiloride-sensitive H^+ efflux from activated platelets, suggesting that stimulation of human platelets induces Na^+/H^+ exchange (Siffert *et al.*, 1984). Since in that study only extracellular pH was measured, the corresponding changes in pH_i , which are expected to be of major significance for concomitant changes in cell function (e.g. Busa & Nuccitelli, 1984), could only be roughly estimated. We have therefore used a fluorescent-dye technique in the present study to measure stimulus-evoked changes in pH_i of human platelets, and have used their Na^+ -dependence and amiloride-sensitivity to characterize them as being due to a Na^+/H^+ antiport in the plasma membrane.

METHODS

Preparation of cells

Platelets were prepared from freshly drawn human blood anti-coagulated with $\frac{1}{6}$ vol. of ACD (2.5 g of sodium citrate, 1.5 g of citric acid and 2.0 g of glucose in 100 ml of water). The blood was then centrifuged at 200 g for 15 min to obtain platelet-rich plasma, from which platelets were then pelleted by centrifugation at 700 g for 20 min at room temperature and resuspended at 37 °C in 2.5 ml of buffer [145 mM-NaCl, 5 mM-KCl, 1 mM- MgSO_4 , 10 mM-Hepes (free acid), 5 mM-glucose, 0.01% bovine serum albumin, pH 6.5]. Apyrase (10 $\mu\text{g}/\text{ml}$; Sigma, grade I) was added to remove extracellular ADP. The cell density of this cell suspension was approx. $5 \times 10^9/\text{ml}$.

Determination of cytoplasmic pH

The pH_i was determined with the fluorescent 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF; Rink *et al.*, 1982) as its tetra-acetoxymethyl ester (BCECF-am), which is a membrane-permeant intracellularly trappable pH indicator.

The above cell suspension was incubated with 10 μM -BCECF-am (added from a 5 mM stock solution in dimethyl sulphoxide) at 37 °C for 30 min. The final concentration of the solvent never exceeded 0.2% (v/v). Thereafter, the loaded cells were washed twice by centrifugation (600 g, 20 min) and resuspension in Hepes buffer in order to remove non-internalized or leaking dye. The final pellet was suspended in 1 ml of Hepes buffer (pH 7.4, 37 °C; cell density about 1.5×10^{10} cells/ml). The concentrated suspension was stored at 37 °C until use.

Fluorescence measurements were performed in a Jobin-Yvon 3D fluorescence spectrophotometer. The wavelengths for excitation and emission were 495 ± 10 and 530 ± 5 nm respectively. Samples (25 μl) of labelled platelets were added to pre-warmed buffer (2.5 ml) and gently mixed with a silicone-treated pipette. After establishment of a stable baseline, the platelet-stimulating agent was added and the suspension was again gently agitated. This procedure required about 10–20 s and accounts for the gaps in the original recordings. Leakage of dye during the time of measurement (about 1 h) was less than 3% and could be neglected. It was estimated by comparing the fluorescence of digitonin-lysed cells with that of the supernatant after removal of the cells by centrifugation.

pH_i determinations were calibrated as described by Horne *et al.* (1981). Briefly, samples of platelets were suspended in Hepes buffer containing 120 mM-KCl, 25 mM-NaCl and 2 μM -nigericin. The ionophore sets $[\text{K}^+]_i/[\text{K}^+]_o = [\text{H}^+]_i/[\text{H}^+]_o$, so that if cells are suspended in media where $[\text{K}^+]_o \approx [\text{K}^+]_i$, $[\text{H}^+]_i$ will equal $[\text{H}^+]_o$. In this way, pH_i can be manipulated by adjusting the external pH (pH_o) to certain values. The fluorescence of platelets recorded at various known pH_i ($= \text{pH}_o$) values yielded a calibration curve, which was then used to estimate pH_i in resting and stimulated cells.

Blockade of Na^+/H^+ exchange

In some experiments, Na^+/H^+ exchange was blocked by removal of extracellular Na^+ (Na_o^+). Various concentrations of Na_o^+ were established by mixing Hepes buffer containing NaCl with buffer in which NaCl was iso-osmotically replaced by choline chloride or by

Abbreviations used: BCECF, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein; EIPA, ethylisopropylamiloride; Na_i^+ , Na_o^+ , intra- and extra-cellular Na^+ (similarly for other ions); pH_i , cytoplasmic pH; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Fig. 1. Effect of thrombin on cytoplasmic pH in human platelets

Representative fluorescence traces are superimposed, and one trace shows the control pH_i before stimulation of BCECF-loaded platelets by thrombin (Thr; arrow). The thrombin concentrations used are (a) 0.5 unit/ml, (b) 0.1 unit/ml, (c) 0.05 unit/ml and (d) 0.02 unit/ml. Platelet concentration was 10^8 /ml, and temperature, 37°C .

Table 1. Effect of thrombin on cytoplasmic pH in human platelets

Platelets, loaded with BCECF, were stimulated by various thrombin concentrations. Total changes in pH_i (ΔpH_i) were obtained as described in the Methods section. Maximal rates of changes in pH_i , $(dpH_i/dt)_{\max}$, were derived from the steepest slope of original recordings. Values represent means $\pm$ S.D. (n = number of experiments).

Thrombin (unit/ml)	$(dpH_i/dt)_{\max}$ (min^{-1})	ΔpH_i	n
0.5	0.78 ± 0.2	0.12 ± 0.03	15
0.1	0.12 ± 0.2	0.11 ± 0.02	12
0.05	0.05 ± 0.01	0.10 ± 0.03	14
0.02	0.02 ± 0.005	0.06 ± 0.02	8

N-methyl-D-glucamine. Since the platelets were always stored in Na^+ -containing medium to prevent cytoplasmic acidification, the lowest Na_o^+ concentration that could be established was approx. 2 mM. In other experiments Na^+/H^+ exchange was inhibited by ethylisopropyl-amiloride (EIPA), a potent amiloride analogue (Vigne *et al.*, 1983). A 20 mM stock solution of EIPA was prepared in dimethyl sulphoxide.

Materials

Thrombin (bovine), TPA, nigericin, apyrase and bovine albumin were obtained from Sigma (Munich, Germany). BCECF-am was purchased from HSC Research Development Co. (Toronto, Canada). EIPA was a gift from Dr. Th. Friedric and Dr. G. Burckhardt (Frankfurt, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

RESULTS AND DISCUSSION

Cytoplasmic pH of unactivated platelets averaged 7.15 ± 0.08 (mean $\pm$ S.D.; $n = 64$), which is in agreement with the values reported by others (Wencel *et al.*, 1980; Horne *et al.*, 1981; Simons *et al.*, 1982; Zavoico *et al.*, 1986). Stimulation of platelets by thrombin led to an

Fig. 2. Effect of Na_o^+ on thrombin-induced cytoplasmic alkalization

The maximal rate of change of pH_i , $(dpH_i/dt)_{\max}$, in response to stimulation with 0.25 unit of thrombin/ml is plotted as a function of Na_o^+ . $(dpH_i/dt)_{\max}$ was obtained from the steepest slopes of original fluorescence tracings. Na_o^+ was lowered by iso-osmotic replacement with choline $^+$ or *N*-methyl-D-glucamine. Cell concentration was 0.9×10^8 /ml. Each point represents the mean ($\pm$ S.D.) of at least four determinations on three different preparations.

Fig. 3. Effect of EIPA on thrombin-induced cytoplasmic alkalization

The maximal rate of pH change, $(dpH_i/dt)_{\max}$, is plotted as a function of the inhibitor concentration. Stimulus: 0.1 unit of thrombin/ml; cell concentration 1.1×10^8 /ml. Results are means of duplicate determinations.

increase in the fluorescence of BCECF, indicating cytoplasmic alkalization (Fig. 1). The final increase in pH_i did not significantly depend on agonist concentration in the range of 0.05–0.5 unit of thrombin/ml, whereas the maximum rate of alkalization, $(dpH_i/dt)_{\max}$, increased with increasing thrombin concentration (Fig. 1 and Table 1). Cytoplasmic pH was raised on the average by 0.11 pH unit (range 0.05–0.15) above the resting value, confirming previous reports in the literature (Horne *et al.*, 1981; Simons *et al.*, 1982; Zavoico *et al.*, 1986).

We next determined the dependence on Na_o^+ of cytoplasmic alkalization. As shown in Fig. 2, lowering Na_o^+ by iso-osmotic replacement by choline distinctly

Fig. 4. Effect of phorbol ester on cytoplasmic pH in human platelets

Platelets were stimulated by TPA (arrow). A stock solution of TPA (20 mM) was prepared in dimethyl sulphoxide and further diluted to 20 μM in water. Traces: (a) 100 nM-TPA; (b) 50 nM-TPA; (c) 100 nM-TPA, Na^+ replaced by choline. The traces are representative of at least six independent determinations.

decreased the rate of the thrombin-induced increase in pH_i . Identical results were obtained when *N*-methyl-D-glucamine was used as a substitute for Na^+ (results not shown). Removal of Na_o^+ did not cause measurable cytoplasmic acidification in unstimulated platelets, because the cells were always stored in 145 mM- Na_o^+ medium and only briefly suspended in low- Na_o^+ media before addition of agonist.

The amiloride analogue EIPA, a potent inhibitor of Na^+/H^+ exchange, suppressed the thrombin-induced increase in pH_i (Fig. 3). Half-maximal inhibition was obtained at about 1 μM -EIPA, which is in satisfactory agreement with observations in other cell types (Vigne *et al.*, 1983, 1984).

Both findings, dependence on Na_o^+ and inhibition by EIPA of the stimulus-induced cytoplasmic alkalinization, reconfirm and extend previous reports (Horne *et al.*, 1981; Simons *et al.*, 1982; Siffert *et al.*, 1984; Zavoico *et al.*, 1986) that activation of human platelets results in activation of Na^+/H^+ exchange.

One of the earliest steps of activation of platelets consists of the phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate to 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (Agranoff *et al.*, 1983). Whereas the latter has been demonstrated to release Ca^{2+} from internal membranes (Authi & Crawford, 1985; Brass & Joseph, 1985), the former activates protein kinase C (Kaibuchi *et al.*, 1983). As this latter reaction has been suggested to be involved in the activation of Na^+/H^+ exchange (for reviews see Grinstein & Rothstein, 1986; Moolenaar, 1986), we were interested in its potential activation of Na^+/H^+ exchange in human platelets. We

have therefore stimulated human platelets with the phorbol ester TPA, a potent activator of protein kinase C (Nishizuka, 1984), and monitored changes in pH_i . Stimulation of platelets with TPA induced cytoplasmic alkalinization, which was sensitive to decreased external $[\text{Na}^+]$ (Fig. 4), indicating that it is due to Na^+/H^+ exchange.

In conclusion, our measurements confirm previous observations that the intracellular alkalinization induced in human platelets by thrombin stimulation is mediated by an amiloride-sensitive Na^+ -dependent Na^+/H^+ antiport. Protein kinase C may play a role in the link between receptor occupancy and activation of the antiport.

We are indebted to Dr. T. Friedrich and Dr. G. Burkhardt (Max-Planck-Institut für Biophysik, Frankfurt, Germany) for generously giving us EIPA. This study was supported by the Deutsche Forschungsgemeinschaft, grant no. Sche 46/1-5.

REFERENCES

- Agranoff, B. W., Murthy, P. & Seguin, E. B. (1983) *J. Biol. Chem.* **258**, 2076–2078
- Authi, K. S. & Crawford, N. (1985) *Biochem. J.* **230**, 247–253
- Brass, L. F. & Joseph, K. S. (1985) *J. Biol. Chem.* **260**, 15172–15179
- Busa, W. B. & Nuccitelli, R. (1984) *Am. J. Physiol.* **246**, R409–R438
- Feinberg, H., Sandler, W. C., Scorer, M., Le Breton, G. C., Grossmann, B. & Born, G. V. R. (1977) *Biochim. Biophys. Acta* **470**, 317–324
- Grinstein, S. & Rothstein, A. (1986) *J. Membr. Biol.* **90**, 1–12
- Horne, W. C., Norman, N. E., Schwartz, D. B. & Simons, E. R. (1981) *Eur. J. Biochem.* **120**, 295–302
- Kaibuchi, K., Takai, Y., Sawamura, M., Hashijima, M., Fujikura, T. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701–6704
- Moolenaar, W. H. (1986) *Annu. Rev. Physiol.* **48**, 363–376
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Rink, T. J., Tsien, R. Y. & Pozzan, T. (1982) *J. Cell Biol.* **95**, 189–196
- Sandler, W. C., Le Breton, G. C. & Feinberg, H. (1980) *Biochim. Biophys. Acta* **600**, 448–455
- Siffert, W., Fox, G., Mückenhoff, K. & Scheid, P. (1984) *FEBS Lett.* **172**, 272–274
- Simons, E. R., Schwartz, D. B. & Norman, N. E. (1982) in *Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Functions* (Nuccitelli, R. & Deamer, D. W., eds.), pp. 463–482, Alan R. Liss, New York
- Vigne, P., Frelin, C., Audinot, M., Borsotto, M., Cragoe, E. J. *Biochem. Biophys. Res. Commun.* **116**, 86–90
- Vigne, P., Frelin, C., Audinot, M., Borsotto, M., Cragoe, E. J. & Lazdunski, M. (1984) *EMBO J.* **3**, 2647–2651
- Wencel, J., Burt, T. & Feinberg, H. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 424
- Zavoico, G., Cragoe, E. J. & Feinstein, M. B. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 227