Vol. 141, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 26, 1986 Pages 13-19

A PHORBOL ESTER AND 1-OLEOYL-2-ACETYLGLYCEROL INDUCE Na^+/H^+ EXCHANGE IN HUMAN PLATELETS

Winfried Siffert and Peter Scheid

Institut für Physiologie, Ruhr-Universität, 4630 Bochum, F.R.G.

Received September 25, 1986

SUMMARY: This study aimed at investigating the mechanisms by which stimulation of human platelets results in activation of Na^+/H^+ exchange. Platelets were suspended in a slightly buffered medium and the stimulus-induced, amiloride-sensitive H^+ release, reflecting Na^+/H^+ exchange, was estimated from changes in the medium pH. H^+ release could be evoked by thrombin and by activators of protein kinase C such as 1-oleoyl-2-acetylglycerol (OAG) or 12-0-tetradecanoylphorbol-13-acetate (TPA). Both the thrombin- and the OAG-induced Na^+/H^+ exchange could be blocked by trifluoperazine, a protein kinase C inhibitor. The thrombin-induced H^+ release was also sensitive to increased intracellular cAMP levels, probably due to inhibition of phospholipase C activation, whereas the OAG-induced activation of Na^+/H^+ exchange was unaffected. Our data suggest that activation of Na^+/H^+ exchange is mediated by protein kinase C.

The stimulation of human platelets by thrombin leads to a rapid breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) forming 1,2- diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3; 1,2). DG activates protein kinase C (Pk-C; 3), whereas IP_3 has been demonstrated to induce Ca^{2+} release from internal membranes (4,5). Another early event following stimulation of platelets consists of activation of a Na^+/H^+ antiport (6) with subsequent cytoplasmic alkalinization (7). A similar Na^+/H^+ exchange has been

Abbreviations:
Bt_2-cAMP, dibutyrylic cAMP; PGE_1, prostaglandin E_1; PGI_2, prostaglandin I_2; TFP, trifluoperazine; Pk-C, protein kinase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; OAG, 1-oleoyl-2acetylglycerol; TPA, 12-0-tetra-decanoylphorbol-13-acetate; DMSO, dimethylsulfoxide

0006-291X/86 $1.50

shown to be induced in a variety of cells (for review see 8) and it was suggested that Pk-C might be involved in the activation mechanism (9-12).

To investigate the involvement of Pk-C in platelet Na^+/H^+ exchange we have measured thrombin-induced Na^+/H^+ exchange in the presence of compounds known to block Pk-C (trifluoperazine, 3, 12) or substances which elevate intracellular cAMP levels and thereby inhibit the stimulus-induced breakdown of PIP_2 (dibutyrylic cAMP, prostaglandins E_1 and I_2; 3, 13). In a more direct approach we investigated the ability of the Pk-C activators 12-O-tetradecanoylphorbol-13-acetate (TPA) and 1-oleoyl-2-acetylglycerol (OAG) to induce Na^+/H^+ exchange.

MATERIALS AND METHODS

Experimental. Blood was drawn by venipuncture from human volunteers and anticoagulated with ACD (1:7 by vol.). Platelet-rich plasma was obtained by centrifugation at 150 xg for 12 min. and platelets were then pelleted by centrifugation at 2000 xg for 20 min. The supernatant was discarded and the platelets were washed twice in a medium containing 136 mM NaCl, 2.7 mM KCl, 0.18 mM KH_2PO_4, 5 mM glucose, 0.01 % (w/v) albumine, pH 6.5. Finally, the cell suspension was adjusted to pH 7.4 with 0.02 M NaOH. Final platelet concentration ranged from 1 to 2 · 10^{11} cells/L. Platelet Na^+/H^+ exchange was quantitated as described earlier by measuring the Na^+-dependent, amiloride-sensitive H^+ efflux in response to stimulating agents (6). The H^+ release measured under these conditions was clearly not related to the extrusion of acidic granular contents since it also occured in the presence of substances known to inhibit the release reaction, e.g. aspirin and indomethacin (data not shown).

Chemicals. Trifluoperazine (TFP), dibutyrylic cAMP (Bt_2-cAMP), prostaglandins E_1 and I_2 (PGE_1, PGI_2), 1-oleoyl-2-acetylglycerol (OAG), 12-O-tetradecanoylphorbol-13-acetate (TPA), thrombin (bovine), and the Ca^{2+} ionophore A 23187 were all obtained from Sigma (Munich, F.R.G.). Amiloride was obtained as a gift from MSD SHARP AND DOHME (Munich, F.R.G.)

RESULTS AND DISCUSSION

Trifluoperazine, which has been shown to block Pk-C in human platelets (14, 15), inhibited thrombin-induced H^+ release in a concentration-dependent manner as shown in Fig. 1. Half-maximal inhibition (IC_{50}) was obtained at about 20 µM TFP which agrees

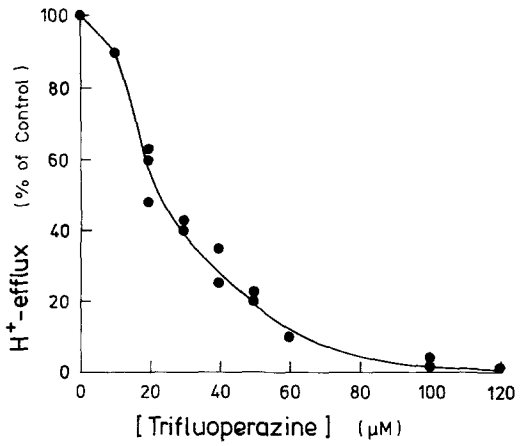

Fig. 1. Effect of trifluoperazine on H^+ release from activated platelets.
Platelets were pretreated with the inhibitor for 1 min. before addition of thrombin (0.1 U/ml). In the absence of TFP H^+ release amounted to 4.7 ± 0.3 μmol $H^+/10^{11}$ cells (± S.D., n = 6). Each point represents one single measurement.

well with values obtained by Owen and Villereal for serum stimulation of Na^+ influx in fibroblasts (16) and the inhibition of phorbol ester-induced activation of Na^+/H^+ exchange in lymphocytes (12).

The effects of intracellular cAMP on thrombin-induced Na^+/H^+ exchange are compiled in Fig. 2. Preincubation of platelets with

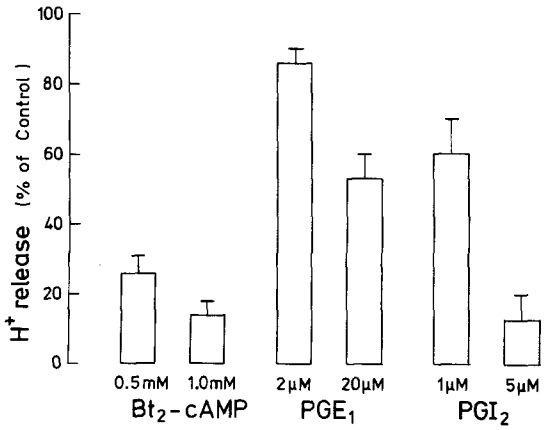

Fig. 2. Inhibition of thrombin-induced H^+ release by Bt_2-cAMP and prostaglandins E_1 and I_2.
Displayed is the amount of amiloride-sensitive H^+ release upon activation by thrombin (0.2 U/ml) as % of control. Each column represents the mean of five determinations (± S.D.) from at least two preparations.

0.5 and 1.0 mM Bt_2-cAMP led to a reduction of H^+ release by 75% and 86% of the control, respectively. Similarly, elevation of intracellular cAMP by PGI_2 or PGE_1 significantly reduced H^+ release in response to thrombin, the extent of which depended on the amount of prostaglandins added. The most likely explanation for these findings is that cAMP prevents the receptor-mediated breakdown of PIP_2 (3, 13) thereby reducing the formation of DG and the resultant activation of Na^+/H^+ exchange by Pk-C.

The ability of thrombin to induce Na^+/H^+ exchange could be mimicked by stimulation of platelets with the phorbol ester TPA (Fig. 3). Thrombin induced, after a short lag time, a rapid extrusion of H^+. TPA and the Ca^{2+} ionophore A 23187 also provoked H^+ liberation although the total amount of H^+ released was only 50% of that released by thrombin. If a combination of TPA and

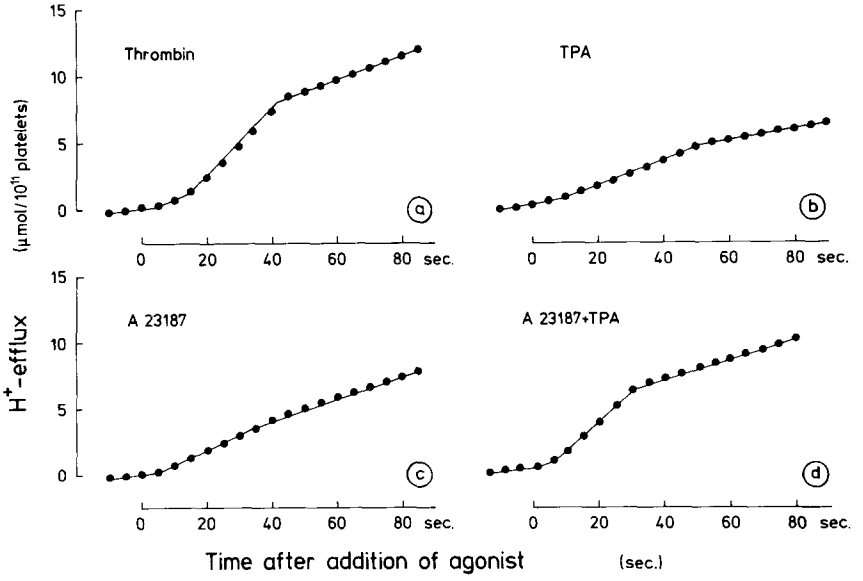

Fig. 3. Effect of thrombin, TPA, and Ca^{2+}-ionophore A 23187 on H^+ release.
Platelets were stimulated by a) 0.1 U/ml thrombin, b) 100 nM TPA c) 100 nM A 23187, and d) 100 nM TPA plus 100 nM A 23187. Graphs were drawn after calculating the total amount of H^+ released from stimulated platelets using the original pH registrations and the buffer capacity of the suspending medium as described earlier (6). Each trace is representative for at least seven determinations yielding almost identical results.

TABLE 1

Stimulation by thrombin or OAG of Na^+/H^+ exchange in human platelets

Assay conditions	H^+ release
Thrombin (0.1 U/ml)	4.8 ± 0.5 (7)
OAG (10 µg/ml)	2.2 ± 0.3 (6)
OAG (20 µg/ml)	3.1 ± 0.2 (5)
OAG (60 µg/ml)	4.9 ± 0.4 (8)
OAG (60 µg/ml), Na^+-free medium	0.2 ± 0.1 (7)
OAG (60 µg/ml) + TFP (25 µM)	1.8 ± 0.2 (7)
OAG (60 µg/ml) + PGI_2 (5 µM)	4.7 ± 0.5 (8)

Values represent means (± S.D.) of amiloride-sensitive (1 mM amiloride) H^+ release (µmol $H^+/10^{11}$ cells). The number of experiments, performed in at least two different preparations, is given in parantheses. OAG was added from a stock solution (50 mg/ml) in 100% DMSO. The final concentration of the solvent did not exceed 0.015 vol.%. DMSO alone did not induce H^+ release.

A 23187 was used, proton liberation equalled that observed after treatment of platelets with thrombin. A similar synergism between phorbol ester and Ca^{2+} has also been described for the activation of Na^+/H^+ exchange in fibroblasts (17) and obviously reflects the requirement of Pk-C for Ca^{2+} and phospholipid for full activation (3).

Activation of platelets by the exogenous diacylglycerol OAG also induced amiloride-sensitive, Na^+ dependent H^+ release as summarized in Table 1. The amount of H^+ liberated depended on the final concentration of OAG applied and, at 60 µg/ml, was identical to that released by 0.1 U/ml thrombin. Blocking Pk-C by TFP led to a distinct inhibition of OAG-induced H^+ release, whereas elevation of intracellular cAMP by PGI_2 was without any effect. Taken together, our findings support the hypothesis that activation of

Na^+/H^+ exchange in stimulated platelets is mediated by activation of Pk-C: 1) The thrombin-induced H^+ release could be inhibited by TFP (Fig. 1) and this compound was also effective when OAG was used as a stimulus (Table 1) which might rule out effects of TFP on calmodulin (15) or phospholipases (18). 2) The thrombin-induced H^+ release was sensitive to increases in intracellular cAMP levels (Fig. 2) which probably prevents the stimulus-induced formation of DG (3, 13). This effect was bypassed if Pk-C was directly activated by OAG (Table 1). 3) The thrombin-induced activation of Na^+/H^+ exchange could be mimicked by a combination of TPA and a Ca^{2+} ionophore (Fig. 3) suggesting a role of Ca^{2+} and DG in the activation process under physiological conditions.

Activation of Na^+/H^+ exchange in stimulated human platelets appears to be of physiological significance for platelet function since inhibition of this ion transport system also inhibits the formation of arachidonic acid, the release reaction and aggregation (19-21). We suggest that the antiport is activated by Pk-C which has also been observed for other cells and tissues (8-12).

ACKNOWLEDGEMENT

This study was supported by the Deutsche Forschungsgemeinschaft, grant No. Sche 46/1-5.

REFERENCES

1. Agranoff, B.W., Murthy, P., and Seguin, P. (1983) J. Biol. Chem. 259, 2076-2078
2. Kawahara, Y., Takai, Y., Minakuchi, R., Sana, K., and Nishizuka, Y. (1980) Biochem. Biophys. Res. Commun. 97, 309-317
3. Nishizuka, Y. (1984) Nature 308, 693-698
4. Authi, K.S. and Crawford, N. (1985) Biochem. J. 230, 247-253
5. O'Rourke, F.A., Halenda, S.P., Zavoico, G.B., and M.B. J. Biol. Chem. 260, 956-962
6. Siffert, W., Fox, G., Mückenhoff, K., and Scheid, P. FEBS Lett. 172, 272-274
7. Horne, W.C., Norman, N.E., Schwartz, D.B., and Simons, E.R. (1981) Eur. J. Biochem. 120, 295-302
8. Grinstein, S. and Rothstein, A. (1986) J. Membrane Biol. 90, 1-12
9. Rosoff, P.M., Stein, L.F., and Cantley, L.C. (1984) J. Biol. Chem. 259, 7056-7060

10. Moolenaar, W.H., Tertoolen, L.G.J., and de Laat, S.W. (1984) Nature 312, 371-374
11. Swann, K., and Whitaker, M. (1985) Nature 314, 274-277
12. Grinstein, S., Cohen, S., Goetz, J.D., and Rothstein, A. (1985) J. Cell Biol. 101, 269-276
13. Watson, S.P., McConnell, R.T., and Lapetina, E.G. (1984) J. Biol. Chem. 259, 13199-13203
14. Sanchez, A., Hallam, T.J., and Rink, T.J. (1983) FEBS Lett. 164, 43-46
15. Feinstein, M.B., and Hadijan, R.A. (1982) Molec. Pharmacol. 21, 422-431
16. Owen, N.E. and Villereal, M.L. (1982) Proc. Natl. Acad. Sci. USA 79, 3537-3541
17. Vicentini, L.M. and Villereal, M.L. (1985) Proc. Natl. Acad. Sci. USA 82, 8053-8056
18. Watanabe, T., Hashimoto, Y., Teramoto, T., Kume, S., Naito, C., and Oka, H. (1986) Arch. Biochem. Biophys. 246, 699-709
19. Sweatt, J.D., Johnson, S.L., Cragoe, E.J., and Limbird, L.E. (1985) J. Biol. Chem. 260, 12910-12919
20. Horne, W.C., and Simons, E.R. (1978) Thromb. Res. 13, 599-607
21. Connolly, T.M., and Limbird, L.E. (1983) Proc. Natl. Acad. Sci. USA 80, 5320-5324