

# Na<sup>+</sup>/H<sup>+</sup> exchange is not necessary for protein kinase C-mediated effects in platelets

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The role of Na<sup>+</sup>/H<sup>+</sup> exchange in protein kinase C-mediated effects in platelets was investigated by studying the effect of removal of extracellular Na<sup>+</sup> ([Na<sup>+</sup>]<sub>o</sub>) on the different responses induced by phorbol 12-myristate 13-acetate (PMA) and 1,2-dioctanoylglycerol (diC<sub>8</sub>). None of the responses studied, namely, protein phosphorylation, translocation of enzyme activity to the membrane fraction, potentiatory and inhibitory effects on platelet activation ([Ca<sup>2+</sup>]<sub>i</sub>), arachidonate and granule release) showed an absolute dependence on [Na<sup>+</sup>]<sub>o</sub>. With the exception of dense-granule release, which was clearly potentiated by the removal of [Na<sup>+</sup>]<sub>o</sub> and showed a negative correlation with exchanger activity, the other effects of PMA and diC<sub>8</sub> were not affected by [Na<sup>+</sup>]<sub>o</sub> removal. It is concluded that Na<sup>+</sup>/H<sup>+</sup> exchange is not essential for protein kinase C activation in platelets.

Na<sup>+</sup>/H<sup>+</sup> exchange; Platelet; Protein kinase C

## 1. INTRODUCTION

Recent studies in different cell systems have underlined the central role of the C<sup>2+</sup> and phospholipid-dependent enzyme, protein kinase C (PKC) in the control of cellular function [1]. These reports have described stimulatory as well as inhibitory effects of PKC activators on a variety of cellular processes. Cell functions stimulated by PKC activation include secretory events [2–5], 1,2-diacylglycerol (DAG) formation [6], Na<sup>+</sup>/H<sup>+</sup> exchange [7–10], [Ca<sup>2+</sup>]<sub>i</sub> mobilisation [11], as well as cell differentiation and growth [12,13]. Some of

these processes such as secretion, DAG formation and [Ca<sup>2+</sup>]<sub>i</sub> mobilisation can also be inhibited by PKC activation [4,5,14–17], and it is believed that activation of the enzyme can, depending on the cell system and response involved, act as a dual regulator of cellular functions. There is some evidence to suggest that the effects of PKC may in part, if not wholly, be mediated via Na<sup>+</sup>/H<sup>+</sup> exchange and the resulting elevation in intracellular pH, [pH]<sub>i</sub>. Thus, cell division and growth induced by mitogens and PKC activators is believed to be at least partially dependent on exchanger-mediated elevations in [pH]<sub>i</sub> [9,10,18,19]. Additionally, in platelets, Na<sup>+</sup>/H<sup>+</sup> exchange has been reported to be responsible for the effects of PKC on [Ca<sup>2+</sup>]<sub>i</sub> mobilisation [20]. Our continuing interest in the elucidation of the role of PKC as a stimulatory vs inhibitory signal in platelet activation led us to examine in detail the role of the Na<sup>+</sup>/H<sup>+</sup> mechanism in PKC mediated effects in platelets. Using the PKC activators, phorbol 12-myristate 13-acetate (PMA) and 1,2-dioctanoylglycerol (diC<sub>8</sub>), we report here that Na<sup>+</sup>/H<sup>+</sup> exchange is not necessary

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*Abbreviations:* [Na<sup>+</sup>]<sub>o</sub>, extracellular Na<sup>+</sup>; [pH]<sub>i</sub>, intracellular pH; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup>; PMA, phorbol 12-myristate 13-acetate; diC<sub>8</sub>, 1,2-dioctanoylglycerol; 5HT, 5-hydroxytryptamine; PKC, protein kinase C

for any of the effects mediated by PKC activation in platelets.

## 2. EXPERIMENTAL

PMA and quin-2 ester were purchased from Sigma (England); DiC<sub>8</sub> and bis(carboxyethyl)carboxyfluorescein (BCECF) from Novabiochem (UK) and Calbiochem (UK), respectively; and all radiochemicals and  $\beta$ -thromboglobulin assay kits from Amersham (England).

### 2.1. Preparation of washed platelet suspensions

Washed platelet suspensions resuspended in a pH 7.4 buffer (buffer B) composed of 10 mM Hepes, 5 mM KCl, 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.35% BSA, 0.09% glucose, 0.05 U/ml hirudin and 145 mM Na<sup>+</sup> or choline<sup>+</sup> as the chloride salt were prepared from citrated platelet-rich plasma following a centrifugation-wash step in a pH 6.5 buffer (buffer A), comprising 35 mM citric acid, 5 mM KCl, 0.5 mM CaCl<sub>2</sub>, 0.35% bovine serum albumin (BSA), 0.09% glucose, 0.05 U/ml hirudin and 103 mM Na<sup>+</sup> or choline as described [17]. Loading of platelets with quin-2 and [<sup>14</sup>C]5HT was carried out by incubating 20  $\mu$ M quin-2 ester or 1  $\mu$ Ci/ml [<sup>14</sup>C]5HT with PRP for 30 min at 37°C. For experiments on [pH]<sub>i</sub> determinations, protein phosphorylation and arachidonate release, a concentrated platelet suspension (10<sup>9</sup> platelets/ml) in buffer A (minus Ca<sup>2+</sup> and BSA for the phosphorylation experiments) containing Na<sup>+</sup> was incubated with 5  $\mu$ M BCECF ester for 45 min, 0.25 mCi/ml carrier-free [<sup>32</sup>P]orthophosphate for 90 min, or 0.75  $\mu$ Ci/ml [<sup>3</sup>H]arachidonic acid for 90 min at 37°C following which platelets were washed by centrifugation and resuspended in buffer B of the appropriate ionic composition. For all experiments, washed platelets were pre-treated with indomethacin (10  $\mu$ M) to avoid effects of endogenously formed thromboxane A<sub>2</sub>.

### 2.2. Measurement of [<sup>14</sup>C]5HT, [<sup>3</sup>H]arachidonate and $\beta$ -thromboglobulin release and <sup>32</sup>P-protein phosphorylation

[<sup>14</sup>C]5HT and [<sup>3</sup>H]arachidonate release were evaluated 3 min after addition of the appropriate agonist by counting platelet supernatants for released radioactivity [17].  $\beta$ -Thromboglobulin ( $\beta$ -TG) levels in platelet supernatants were measured by radioimmunoassay using commercially available kits. Protein phosphorylation following addition of PMA or diC<sub>8</sub> was monitored in <sup>32</sup>P-labelled platelets by SDS-polyacrylamide gel electrophoresis and autoradiography [17].

### 2.3. Measurement of [Ca<sup>2+</sup>]<sub>i</sub> and [pH]<sub>i</sub>

Fluorescence measurements on quin-2 and BCECF-loaded platelets (unstirred) and calibration of the quin-2 and BCECF signals to calculate [Ca<sup>2+</sup>]<sub>i</sub> and [pH]<sub>i</sub> were carried out essentially as in [21]. Absolute [pH]<sub>i</sub> values were determined by using the nigericin/KCl method to correct the values obtained from lysing platelet suspensions (at external pH 6.0–8.0) with digitonin.

### 2.4. Measurements of protein kinase C activity in platelet cytosol and membrane fractions

Platelets (0.8–1  $\times$  10<sup>9</sup> in 320  $\mu$ l buffer B, containing Na<sup>+</sup> or

choline), incubated with PMA (160 nM) without stirring for 1–15 min at 37°C, were collected by centrifugation (1500  $\times$  g, 5 min, 4°C) by discarding the supernatant, resuspension of the pellet in 100  $\mu$ l of 20 mM Tris-HCl (pH 7.4) containing 2 mM EDTA, 0.5 mM EGTA, 50  $\mu$ g/ml leupeptin, 1 mM phenylsulphonyl fluoride and 0.1%  $\beta$ -mercaptoethanol (buffer C) and subsequent sonication (4  $\times$  10 s bursts). The suspension was centrifuged at 35000  $\times$  g for 60 min at 2°C. The pellet, following brief sonication (4  $\times$  5 s bursts) in buffer C and the cytosolic supernatant, were subjected to chromatography on DEAE-cellulose (DE52, Whatman) equilibrated with buffer C and the enzyme eluted with 300  $\mu$ l buffer C containing 0.12 M NaCl. PKC activity in the eluates from the membrane and cytosol fractions was assayed immediately by measuring <sup>32</sup>P incorporation into histone substrate using [ $\gamma$ -<sup>32</sup>P]ATP as the phosphate donor [22].

## 3. RESULTS

### 3.1. Effect of [Na<sup>+</sup>]<sub>e</sub> removal on diC<sub>8</sub>/PMA-induced aggregation, 5HT and $\beta$ -TG secretion

Platelet aggregation and 5HT secretion induced by diC<sub>8</sub> and PMA were significantly enhanced upon substitution of [Na<sup>+</sup>]<sub>e</sub> with choline<sup>+</sup> (fig.1,

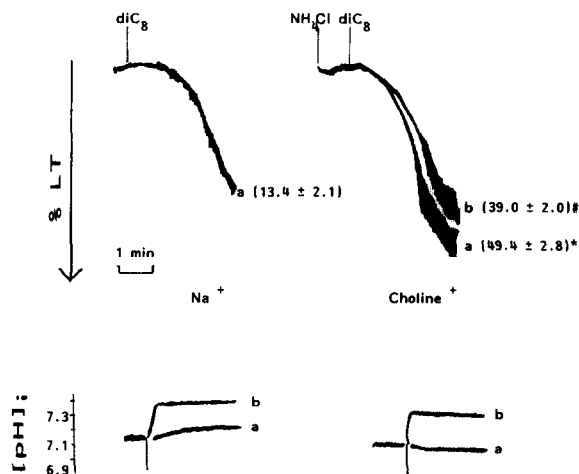


Fig.1. DiC<sub>8</sub> (60  $\mu$ M)-induced platelet aggregation, [<sup>14</sup>C]5HT secretion and [pH]<sub>i</sub> changes in Na<sup>+</sup> and choline<sup>+</sup> buffer. (Upper) Aggregation traces showing the increase in light transmission after addition of diC<sub>8</sub>; values in parentheses beside each trace represent % [<sup>14</sup>C]5HT released 3 min after diC<sub>8</sub> addition (means  $\pm$  SE of 12 determinations from 4 experiments). (Lower) Fluorescence changes representing the [pH]<sub>i</sub> induced by diC<sub>8</sub> in BCECF-loaded platelets. In both panels, traces a and b represent the respective effects of diC<sub>8</sub> alone in Na<sup>+</sup> or choline<sup>+</sup> buffer and of pre-incubating platelets with NH<sub>4</sub>Cl (10 mM) for 1 min before diC<sub>8</sub> addition. \*  $p$  < 0.01 vs diC<sub>8</sub> control in Na<sup>+</sup> buffer (Student's  $t$ -test for unpaired data). #  $p$  < 0.01 vs diC<sub>8</sub> control in choline<sup>+</sup> buffer.

Table 1

DiC<sub>8</sub> and PMA-induced granule secretion in platelets resuspended in Na<sup>+</sup> and choline<sup>+</sup> buffer

		Release (%) (means $\pm$ SE)	
		[ <sup>14</sup> C]5HT	$\beta$ TG ( $\mu$ g/ml)
DiC <sub>8</sub> (60 $\mu$ M)	Na <sup>+</sup>	13.4 $\pm$ 2.1	0.35 $\pm$ 0.10
	choline <sup>+</sup>	49.4 $\pm$ 2.8 <sup>a</sup>	0.63 $\pm$ 0.21
PMA (16 nM)	Na <sup>+</sup>	2.8 $\pm$ 0.7	1.2 $\pm$ 0.24
	choline <sup>+</sup>	12.7 $\pm$ 1.8 <sup>a</sup>	1.4 $\pm$ 0.29

<sup>a</sup>  $p < 0.01$  vs release in Na<sup>+</sup> buffer

[<sup>14</sup>C]5HT and  $\beta$ TG secretion was measured in platelet supernatants 3 min after addition of diC<sub>8</sub> (60  $\mu$ M) or PMA (16 nM). The release in resting platelets was subtracted from the values shown

table 1). This was accompanied by the absence of a [pH]<sub>i</sub> rise and cytoplasmic acidification (0.05 pH units below resting) in choline<sup>+</sup> buffer. Pre-

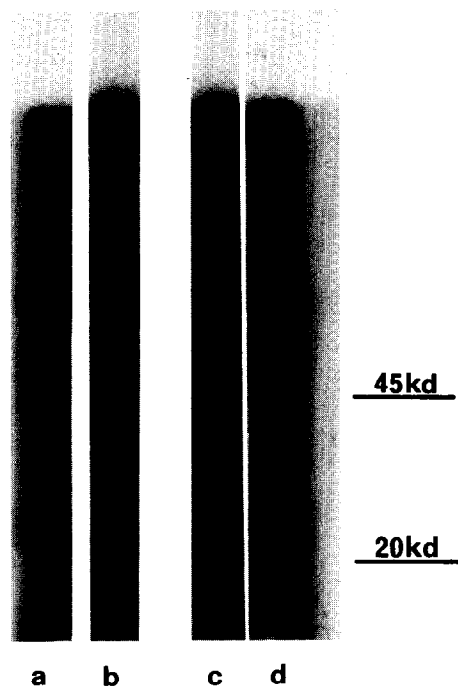


Fig.2. DiC<sub>8</sub>-induced protein phosphorylation in Na<sup>+</sup> and choline<sup>+</sup> buffer. Autoradiograph of a dried gel (from an experiment in which 20 kDa protein labelling was enhanced in choline<sup>+</sup> buffer) showing changes in <sup>32</sup>P labelling of the 45 and 20 kDa substrates. Lanes: (a,b) resting and diC<sub>8</sub> (60  $\mu$ M)-treated platelets (3 min) respectively, in Na<sup>+</sup> buffer; (c,d) as for a,b in choline<sup>+</sup> buffer.

treatment of platelets with NH<sub>4</sub>Cl (10 mM) elevated the [pH]<sub>i</sub> (0.01–0.2 pH units above resting) in Na<sup>+</sup> and choline<sup>+</sup> buffer and partially reversed the potentiated secretory response to diC<sub>8</sub> in choline<sup>+</sup> buffer (fig.1). In contrast to 5HT secretion,  $\beta$ -TG secretion induced by diC<sub>8</sub> and PMA was unaffected by removal of [Na<sup>+</sup>]<sub>e</sub> (table 1).

### 3.2. Effect of [Na<sup>+</sup>]<sub>e</sub> removal on protein phosphorylation and translocation of PKC activity induced by diC<sub>8</sub>/PMA

Phosphorylation states of the various proteins in resting platelets were not significantly different in Na<sup>+</sup> vs choline<sup>+</sup> buffer. In three out of five experiments, diC<sub>8</sub> and PMA-induced phosphorylation of the major 45 and 20 kDa substrates was not

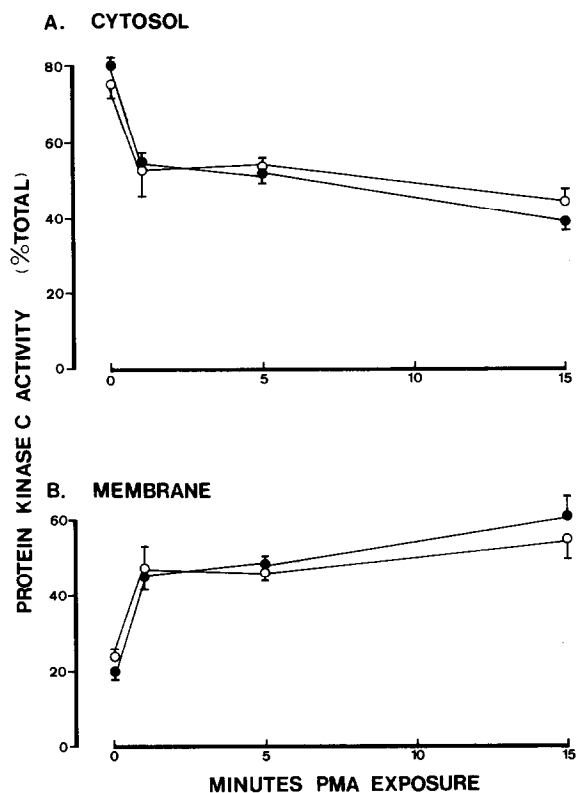


Fig.3. PMA-induced translocation of protein kinase C activity in platelets resuspended in Na<sup>+</sup> and choline<sup>+</sup> buffer. Unstirred platelets resuspended in Na<sup>+</sup> (●) or choline<sup>+</sup> (○) buffer were treated with PMA (160 nM) for 1, 5 or 15 min following which PKC activity in the membrane and cytosol fractions was assayed. Enzyme activity in resting platelets is shown as zero time.

significantly different in  $\text{Na}^+$  vs choline $^+$  buffer (fig.2), although in two other experiments, the 20 kDa substrate showed a greater increase in  $^{32}\text{P}$  labelling in choline $^+$  buffer upon  $\text{diC}_8$  addition. Fig.3 shows that the extent and time course of PMA-induced translocation of PKC activity from cytosol to membrane were virtually identical in  $\text{Na}^+$  and choline $^+$  buffer. In resting platelets, the distribution of enzyme activity in cytosol vs membrane was approx. 80 vs 20%, respectively, in both buffers, and this changed to an approximately equal distribution of PKC activity in cytosol and membrane 1 min after addition of PMA. This was maintained for up to 5 min after PMA addition; thereafter, a small increase (10%) in membrane-bound enzyme activity was observed in both buffers.

### 3.3. Effect of $[\text{Na}^+]_e$ removal on potentiatory/-inhibitory effects of PMA/ $\text{diC}_8$ on receptor-mediated responses

Our previous studies [17] have shown that PMA

and  $\text{diC}_8$  have potentiatory effects on collagen-induced arachidonate release with incubation times 1–5 min. Substitution of  $[\text{Na}^+]_e$  with choline $^+$  reduced the extent of collagen (20  $\mu\text{g}/\text{ml}$ )-induced arachidonate release but did not affect its enhancement by PMA (increase from 6.11 to 8.7% in  $\text{Na}^+$  buffer; increase from 4.04 to 7.04% in choline $^+$  buffer, with a 5 min pre-incubation of 16 nM PMA before addition of collagen;  $p < 0.01$  in both cases). Fig.4 demonstrates that although the extent of thrombin (0.08 U/ml)-induced  $[\text{Ca}^{2+}]_i$  elevations was slightly different in  $\text{Na}^+$  vs choline $^+$  buffer, the inhibition of this response by  $\text{diC}_8$  was similar in both buffer systems (75.5 vs 80% inhibition in  $\text{Na}^+$  vs choline $^+$  buffer).  $\text{DiC}_8$  alone did not change resting  $[\text{Ca}^{2+}]_i$  in  $\text{Na}^+$  or choline $^+$  buffer.

## 4. DISCUSSION

A number of recent studies [7–10] have described stimulatory effects of PKC activators on the  $\text{Na}^+/\text{H}^+$  exchange mechanism, and it has been

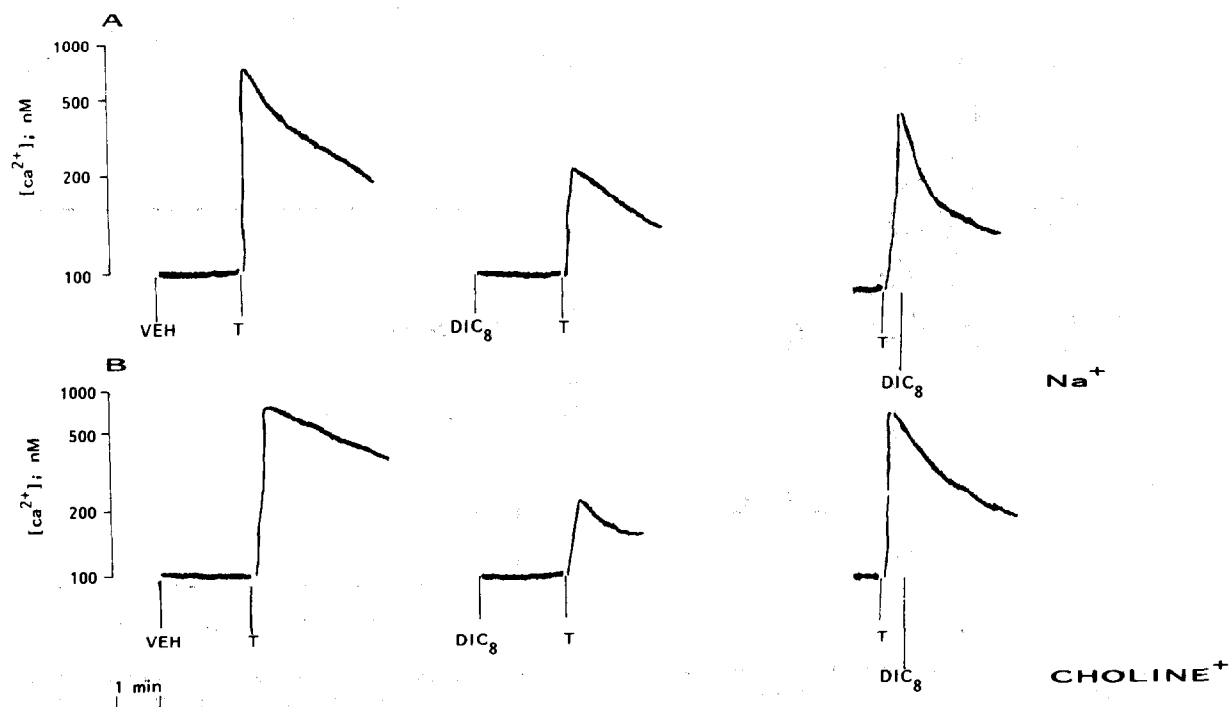


Fig.4. Effect of  $\text{diC}_8$  (60  $\mu\text{M}$ ) on thrombin (0.08 U/ml)-induced  $[\text{Ca}^{2+}]_i$  mobilisation in quin-2-loaded washed platelets resuspended in  $\text{Na}^+$  or choline $^+$  buffer.  $\text{DiC}_8$  was added 2 min before thrombin or at the peak of the thrombin-induced  $[\text{Ca}^{2+}]_i$  rise. Experiments were carried out with platelets resuspended in  $\text{Na}^+$  and choline $^+$  buffer (as these were different batches of platelets, the scales for the  $[\text{Ca}^{2+}]_i$  concentrations are different).

suggested that receptor-mediated exchanger activity is due partially or totally to PKC activation by endogenously formed diacylglycerol. The increase in  $[pH]_i$  caused by  $Na^+$  entering the cell has been described as an important mediator of the effects caused by PKC and/or receptor activation, namely, cell differentiation and proliferation [9,10,18,19] and  $[Ca^{2+}]_i$  mobilisation [20]. As PKC is an important signalling system in platelets, exerting positive and negative control over different platelet responses, and since activation of the enzyme leads to  $Na^+/H^+$  exchange in platelets, we considered it vital to examine the role of  $Na^+/H^+$  exchange in PKC activation, as well as responses mediated by activation of this enzyme in platelets.

Using two activators of the enzyme, PMA and  $diC_8$ , we have been able to demonstrate that neither the activation of PKC nor the effects due to enzyme activation in platelets have a dependence on  $Na^+/H^+$  exchange. In a previous study that addressed the role of the exchanger in PKC-mediated effects in platelets, namely,  $Ca^{2+}$  mobilisation [20], the authors concluded that PKC enhances  $Ca^{2+}$  mobilisation by activating  $Na^+/H^+$  exchange. Our view of this study is that the authors not only had insufficient evidence from which to draw their conclusion, but also failed to select the appropriate response to study. This is because their experiments involved the use of trifluoperazine and sphingosine as PKC inhibitors, which in a number of recent studies, including ours [23–25], have proved to be weak inhibitors of the enzyme with many undesirable 'side-effects' (on membrane stability/integrity, etc.), such that we regard them to be unsuitable reagents in this respect. Furthermore, there is no evidence implicating PKC as a positive modulator of  $Ca^{2+}$  mobilisation in platelets; rather, some studies [17,26,27] have convincingly shown PKC to be an inhibitor of receptor-mediated  $Ca^{2+}$  mobilisation in platelets. It is difficult therefore to see how PKC can enhance  $Ca^{2+}$  mobilisation via  $Na^+/H^+$  exchange, and it is our view, as judged from the present results, that none of the effects actually mediated by PKC activation in platelets are dependent on  $Na^+/H^+$  exchange. The only effect that was clearly altered by  $[Na^+]_e$  removal was 5HT secretion. This process, contrary to earlier views on the importance of exchanger activity for PKC activation, was enhanced by  $[Na^+]_e$  removal. As the poten-

tiated secretory response was at least partially reversed by addition of  $NH_4Cl$ , we conclude that  $[pH]_i$  elevation via  $Na^+/H^+$  exchange is inhibitory to platelet dense-granule secretion. Interestingly, our results suggest that  $\alpha$ -granule secretion may not be controlled in this way by  $Na^+/H^+$  exchange.

Regarding the mechanism underlying the increased secretion of 5HT in the absence of  $[Na^+]_e$ , it is unlikely that 45 kDa protein phosphorylation and  $[Ca^{2+}]_i$  are controlling factors, as these functions, unlike 5HT secretion, were unaffected by  $[Na^+]_e$  removal. However, the increased phosphorylation of the 20 kDa protein seen in some experiments in the absence of  $[Na^+]_e$  may be responsible for the increase in secretion and further studies assessing the contractile state of  $diC_8$ /PMA-treated platelets in the absence of  $[Na^+]_e$  may shed some light on this issue.

In conclusion, although  $Na^+/H^+$  exchange may be an important positive modulator of receptor-mediated  $Ca^{2+}$  mobilisation [28] as suggested earlier, the present study suggests that exchanger activation and elevations in  $[pH]_i$  are not necessary for PKC activation or the effects mediated by activation of this enzyme in platelets. Clearly the platelet must represent a cell system in which, far from  $Na^+/H^+$  exchange being the mediator of the effects of PKC, the two signalling systems affect cellular processes independently, and in some cases, such as  $Ca^{2+}$  mobilisation and 5HT secretion, have opposing effects on the same response.

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## REFERENCES

- [1] Nishizuka, Y. (1986) *Science* 233, 305–312.
- [2] Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikara, T. and Nishizuka, Y. (1983) *J. Biol. Chem.* 258, 6701–6704.
- [3] Burgoyne, R.D., Morgan, A. and O'Sullivan, A.J. (1988) *FEBS Lett.* 238, 151–155.
- [4] Okano, Y., Yamada, K., Takagi, H. and Nozawa, Y. (1986) *Biochem. Biophys. Res. Commun.* 137, 1112–1118.
- [5] Barrowman, M.M., Cockcroft, S. and Gomperts, B.D. (1986) *FEBS Lett.* 201, 137–142.
- [6] Takuwa, N., Takuwa, Y. and Rasmussen, H. (1987) *Biochem. J.* 243, 647–653.
- [7] Besterman, J.M. and Cuatrecasas, P. (1984) *J. Cell Biol.* 99, 340–343.

- [8] Berk, B.C., Aronow, M.S., Brock, T.A., Cragoe, E., jr, Gimbrone, M.A., jr and Alexander, R.W. (1987) *J. Biol. Chem.* 262, 5057–5064.
- [9] Moolenaar, H. (1986) *Annu. Rev. Physiol.* 48, 363–376.
- [10] Rozengurt, E. (1986) *Science* 234, 161–66.
- [11] Albert, P.R., Wolfson, G. and Tashjian, A.H., jr (1987) *J. Biol. Chem.* 262, 6577–6581.
- [12] Morin, M.J., Kreutter, D., Rasmussen, H. and Sartorelli, A.C. (1987) *J. Biol. Chem.* 262, 11758–11763.
- [13] Rozengurt, E., Rodriguez-Pena, M., Coombs, M. and Sinnett-Smith, J. (1984) *Proc. Natl. Acad. Sci. USA* 81, 5748–5752.
- [14] Naccache, P.H., Molski, T.F.P., Borgeat, P., White, J.R. and Sha'afi, R.I. (1985) *J. Biol. Chem.* 260, 2125–2131.
- [15] Brock, T.A., Rittenhouse, S.E., Powers, C.W., Ekstein, L.S., Gimbrone, M.A., jr and Alexander, R.W. (1985) *J. Biol. Chem.* 260, 14158–14162.
- [16] McNiel, P.L., McKenna, M.P. and Taylor, D.L. (1985) *J. Cell Biol.* 101, 372–379.
- [17] Krishnamurthi, S., Joseph, S. and Kakkar, V.V. (1987) *Eur. J. Biochem.* 167, 585–593.
- [18] Soltoff, S.P. and Cantley, L.C. (1988) *Annu. Rev. Physiol.* 50, 207–223.
- [19] Whetton, A.D., Vallance, S.J., Mock, P.N., Cragoe, E.J., Dexter, T.M. and Heyworth, C.M. (1988) *Biochem. J.* 256, 585–592.
- [20] Siffert, W. and Akkerman, J.W.N. (1988) *J. Biol. Chem.* 263, 4223–4227.
- [21] Simpson, A.W.M. and Rink, T.J. (1987) *FEBS Lett.* 222, 144–148.
- [22] Uratsuji, Y., Nakanishi, H., Takeyama, Y., Kishimoto, A. and Nishizuka, Y. (1985) *Biochem. Biophys. Res. Commun.* 130, 654–661.
- [23] Pitet, D., Krause, K.H., Wollheim, C.B., Bruzzone, R. and Lew, D.P. (1987) *J. Biol. Chem.* 262, 10072–10076.
- [24] Krishnamurthi, S., Patel, Y. and Kakkar, V.V. (1989) *Biochim. Biophys. Acta* 1010, 258–264.
- [25] Hidaka, H. and Hagiwara, M. (1987) *Trends Pharmacol. Sci.* 8, 162–164.
- [26] Zavoico, G.B., Halenda, S.P., Sha'afi, R.I. and Feinstein, M.B. (1985) *Proc. Natl. Acad. Sci. USA* 82, 3859–3862.
- [27] MacIntyre, D.E., McNicol, A. and Drummond, A.H. (1985) *FEBS Lett.* 180, 160–164.
- [28] Siffert, W. and Akkerman, J.W.N. (1987) *Nature* 325, 456–458.