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Extracellular Na^+ removal enhances granule secretion in platelets – evidence that Na^+/H^+ exchange is inhibitory to secretion induced by some agonists

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The effect of extracellular Na^+ ($[Na^+]_e$) removal on agonist-induced granule secretion in platelets in relation to $[pH]_i$ and $[Ca^{2+}]_i$ changes was investigated. Substitution of $[Na^+]_e$ with choline$^+$ of K^+ resulted in a significant enhancement of 5HT secretion induced by thrombin, collagen, U46619 and the protein kinase C activators, PMA and diC_8. Increases in $[Ca^{2+}]_i$ induced by thrombin and U46619 were slightly inhibited or unaffected in these buffers, but $[pH]_i$ increases induced by thrombin, U46619, PMA and diC_8 were abolished and a drop in $[pH]_i$ (0.05–0.1 units below resting) was observed. Although preincubation with potassium acetate produced a big drop in $[pH]_i$ and greatly increased secretion with all the agonists, particularly in the absence of $[Na^+]_e$, clear evidence that $[pH]_i$ rises due to Na^+/H^+ exchange are inhibitory to secretion was obtained only with thrombin. Thus, (i) NH_4Cl, which restored the increase in $[pH]_i$ in the absence of $[Na^+]_e$ reduced the potentiated secretory response to thrombin, (ii) no increase in thrombin-induced secretion was observed when Na^+ was replaced with Li^+, which allowed a normal increase in $[pH]_i$ and (iii) ethyl isopropyl amiloride (EIPA) abolished the $[pH]_i$ rise and potentiated thrombin-induced secretion. With collagen and U46619, the results suggest that removal of $[Na^+]_e$ per se rather than inhibition of Na^+/H^+ exchange results in enhanced secretion. It is concluded that $[Na^+]_e$ per se and $[pH]_i$ elevations via Na^+/H^+ exchange both have important inhibitory roles in the control of platelet granule secretion.

Na^+/H^+ exchange; Platelet; Secretion

1. INTRODUCTION

Many cells respond to stimulation with activation of an Na^+/H^+ exchanger located in the plasma membrane [1–4]. This results in an increase in the cytosolic pH $[pH]_i$, which can affect many intracellular processes and control cellular function. Recent work in platelets has suggested an obligatory role for Na^+/H^+ exchange in arachidonate release induced by 'weak' agonists such as ADP and adrenaline [5–8], as well as a potentiatory, if not obligatory, role for the ion-exchange mechanism in agonist-induced calcium mobilisation [9–11].

As the role of Na^+/H^+ exchange in the induction of platelet granule secretion is not known, we have used different approaches, namely, Na^+ substitutes and $[pH]_i$-altering agents such as ammonium chloride (NH_4Cl) and potassium acetate, to examine this problem. By correlating the extent of secretion with changes in $[pH]_i$ and $[Ca^{2+}]_i$, we have observed that an elevation in $[pH]_i$ via Na^+/H^+ exchange is inhibitory to platelet granule secretion under some conditions.

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Abbreviations: $[pH]_i$, intracellular pH; $[Ca^{2+}]_i$, intracellular Ca^{2+}; PMA, phorbol 12-myristate 13-acetate; diC_8, 1,2-dioctanoylglycerol; $[Na^+]_e$, extracellular Na^+

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2. METHODS

2.1. *Preparation of washed platelet suspensions*

For all the experiments described, the following standard procedure for preparing washed platelet suspensions was used, except that labelling of platelets with the various markers was carried out at different stages of the washing procedure. Citrated blood drawn from apparently healthy volunteers was centrifuged at 600 × *g* for 15 min to obtain platelet-rich plasma (PRP).

Following acidification of the PRP with 0.1 N citric acid and addition of 20 nM PGI_2, the PRP was centrifuged at 1500 × *g* for 10 min to obtain a pellet which was resuspended in a pH 6.5 buffer composed of 36 mM citric acid, 5 mM KCl, 0.5 mM $CaCl_2$, 0.35% bovine serum albumin (BSA), 0.09% glucose, 0.05 U/ml hirudin (Biopharm, England) and 103 mM NaCl/choline chloride/KCl/LiCl (buffer A). Following addition of 20 nM PGI_2, these suspensions were centrifuged at 1500 × *g* for 10 min and the platelet pellets were resuspended in a pH 7.4 buffer composed of 10 mM Hepes, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.35% BSA, 0.09% glucose, 0.05 U/ml hirudin and 145 mM NaCl/choline chloride/KCl/LiCl (buffer B) at a count of 2–4 × 10^8 platelets/ml.

Loading of platelets with quin 2 and [^{14}C]5HT was carried out by incubating 20 μM quin 2 ester (Sigma, England) or 1 μCi/ml [^{14}C]5HT (50 Ci/mmol, Amersham, England) with PRP for 30 min at 37°C. For the experiments on $[pH]_i$ determinations and protein phosphorylation, a concentrated platelet suspension (10^9 platelets/ml) in buffer A (minus Ca^{2+} and BSA for the phosphorylation experiments) containing Na^+, was incubated with 5 μM bis-(carboxyethyl) carboxy fluorescein (BCECF, Calbiochem, England) ester or 0.25 mCi/ml carrier-free [^{32}P]orthophosphate for 45 min or 90 min respectively, at 37°C. At the end of this incubation period, the platelet suspension was diluted 2-fold with the same buffer or with buffer A containing choline$^+$/K^+/Li^+, centrifuged at 1500 × *g* for 10 min and the pellets were resuspended in buffer B of the appropriate ionic composition. For all the experiments, the washed platelets were pre-treated with indomethacin (10 μM) to avoid effects of endogenously formed thromboxane A_2.

2.2. *Measurement of [^{14}C]5HT secretion*

[^{14}C]5HT release was measured 3 min after addition of the appropriate agonist by counting the platelet supernatants for released radioactivity [12].

2.3. *Measurement of $[Ca^{2+}]_i$ and $[pH]_i$*

Fluorescence measurements on quin 2 and BCECF-loaded platelets (unstirred) and calibration of the quin 2 and BCECF signals to calculate $[Ca^{2+}]_i$ and $[pH]_i$ were carried out essentially as previously described [9]. Excitation wavelengths for the quin 2 and BCECF experiments were respectively 339 and 490 nM, emission wavelengths respectively 500 and 520 nM. Absolute $[pH]_i$ values were calculated by using the nigericin/KCl method to correct the values obtained from lysing the platelet suspensions (at pH 6.0–8.0) with digitonin.

2.4. *Measurement of protein phosphorylation*

Incubations of ^{32}P-labelled platelets containing the various additions were terminated with 0.5 vol. of an SDS stopping solution (9% SDS, 2-mercaptocthanol, 15% glycerol, 0.186 M Tris pH 6.8 and 0.03% bromophenol blue) and subjected to SDS polyacrylamide gel electrophoresis (4% stacking and 7–20% separating gels) [12]. Following electrophoresis, dried gels were subjected to autoradiography for assessment of ^{32}P-labelling in the different protein bands.

3. RESULTS

3.1. *Effect of $[Na^+]_e$ removal on 5HT secretion induced by various platelet agonists*

Earlier workers [2,9,13] have shown that thrombin as well as protein kinase C activators can stimulate the plasma membrane Na^+/H^+ exchanger and thereby cause an increase in $[pH]_i$. To evaluate the role of this exchange mechanism in

Table 1

Effect of Na^+ removal on agonist-induced [^{14}C]5HT secretion

	[^{14}C]5HT secretion (%) (means ± SE)			
	Na^+	Choline$^+$	K^+	Li^+
Thrombin (0.05 U/ml)	20.5 ± 2.7	34.7 ± 4.1*	45.8 ± 3.4**	11.5 ± 4.5
diC_8 (60 μM)	13.4 ± 2.1	49.4 ± 2.8**	32.4 ± 2.8**	13.7 ± 3.3
PMA (16 nM)	2.8 ± 0.7	12.7 ± 1.8*	8.3 ± 0.2*	–
Collagen (20 μg/ml)	22.2 ± 1.4	37.1 ± 1.9**	52.3 ± 2.8**	34.0 ± 2.5**
U46619 (1 μM)	7.8 ± 1.8	38.4 ± 4.4**	51.4 ± 2.1**	42.2 ± 1.8**

Washed platelets resuspended in Hepes (pH 7.4) buffer containing Na^+, choline$^+$, K^+ or Li^+, and pre-loaded with [^{14}C]5HT were used in these experiments. The extent of [^{14}C]5HT released was measured 3 min after addition of the various agents (number of determinations, *n*, in four separate experiments was 12–16). * $P < 0.01$, ** $P < 0.001$ compared with the response in Na^+ buffer (Student's *t*-test for unpaired data)

platelet granule secretion, we examined the effect of Na^+ removal and replacement with two types of Na^+ substitutes: (i) choline$^+$ and K^+, which are not transported by the exchanger and cause cytoplasmic acidification [2,14]; (ii) Li^+, which is known to be transported by the exchanger and causes cytoplasmic alkalinisation [14]. Table 1 summarises the effects of 1,2-dioctanoylglycerol and phorbol 12-myristate 13-acetate (PMA), two agents that directly activate protein kinase C in intact platelets [12,15], and agonists acting through distinct receptors, namely, thrombin, collagen and the thromboxane mimetic, U46619. Essentially, replacement of Na^+ with choline$^+$ or K^+ resulted in a significant enhancement of 5HT secretion with all agents tested. Additionally, increased secretion with collagen and U46619 but not thrombin and diC_8 was observed in Li^+ buffer. To test whether prolonged exposure to ions other than Na^+ was responsible for the enhanced secretion, a concentrated (30-fold) platelet suspension resuspended in Na^+ buffer was prepared. This was then diluted in Na^+ or choline$^+$ buffer, and used at various times after dilution to monitor agonist-induced secretion. The final concentration of $[Na^+]_e$ following dilution in the choline$^+$ buffer was estimated to be approx. 5 mM. It was found that time of exposure to the choline$^+$ ion did not influence the secretory response to any of the agonists tested.

3.2. *Effect of $[Na^+]_e$ removal on agonist-induced $[pH]_i$ and $[Ca^{2+}]_i$ changes*

Apart from thrombin, diC_8 and PMA, which induced a detectable rise in $[pH]_i$ in Na^+ buffer

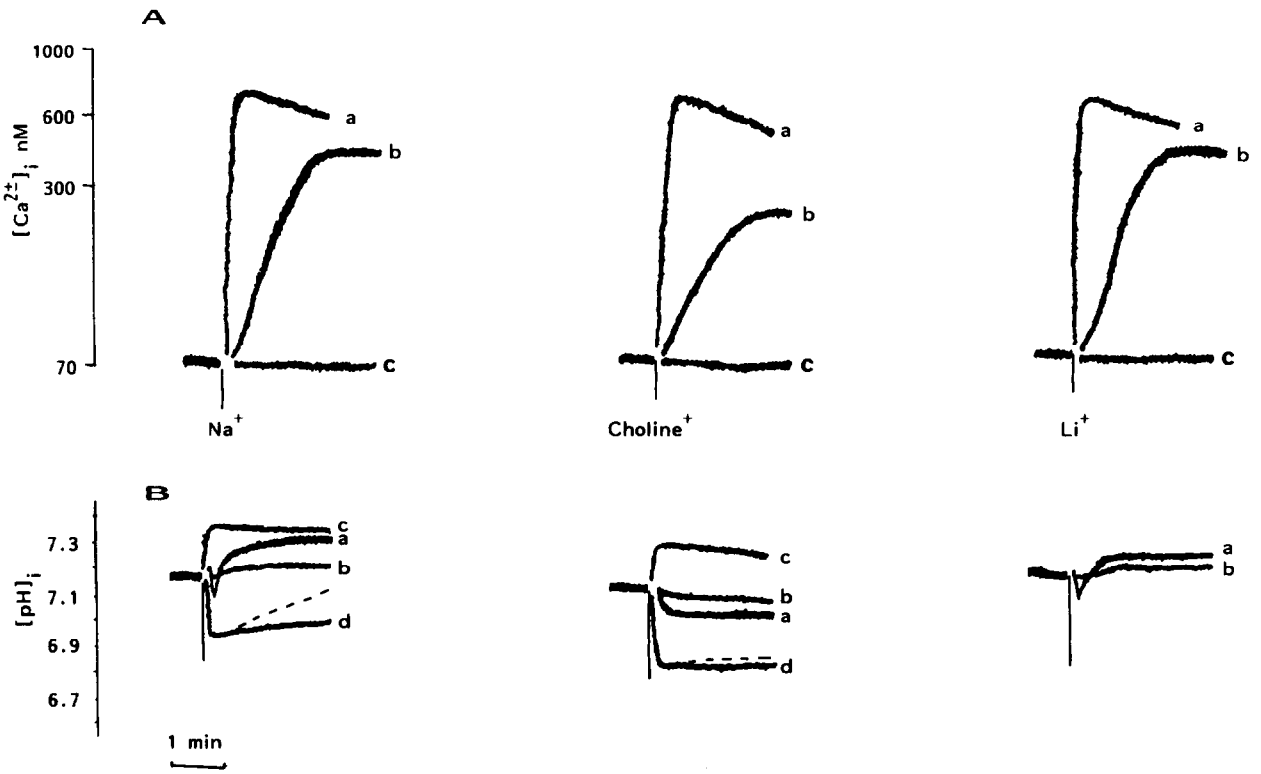

Fig.1. (A) Thrombin and diC_8-induced $[Ca^{2+}]_i$ changes in platelets resuspended in Na^+, choline$^+$ and Li^+ buffers. (B) Effect of thrombin, diC_8, NH_4Cl and potassium acetate on $[pH]_i$ in platelets resuspended in Na^+, choline$^+$ and Li^+ buffers. Fluorescence recordings representing the $[Ca^{2+}]_i$ and $[pH]_i$ levels are shown. Quin-2-loaded platelets resuspended in the different buffers were used for the experiments in panel A. Traces a, b and c in each of the buffer systems represent the effect of 0.05 and 0.2 U/ml thrombin and 60 μM diC_8, respectively. BCECF-loaded platelets resuspended in the different buffers were used for the experiments in panel B. Traces a, b, c, d in each of the buffer systems represent the effect of thrombin (2 U/ml), diC_8 (60 μM), NH_4Cl (10 mM) and potassium acetate (20 mM). The dashed line above trace d represents the effect of thrombin (0.05 U/ml) addition to platelets treated with potassium acetate for 1 min. Addition of thrombin (0.05–2 U/ml) 10 s–1 min after NH_4Cl did not affect the NH_4Cl-induced $[pH]_i$ rise in Na^+ or choline$^+$ buffers.

(0.05–0.1 units above resting levels) (fig.1b), U46619 (1 μM) was also found to consistently elicit a small rise in $[pH]_i$ (~0.05 units) in Na^+ buffer (data not shown). Collagen (Horm, 10–20 μg/ml), however, consistently produced a big drop in BCECF fluorescence without a subsequent increase (data not shown). This was attributed to quenching of the BCECF signal by the buffer solution in which the collagen was made up. Rises in $[pH]_i$ with thrombin, diC_8, PMA and U46619 were also observed in Li^+ buffer (data with thrombin and diC_8 in fig.1b). These were indistinguishable from the $[pH]_i$ rises seen in Na^+ buffer. Resting $[pH]_i$ levels in choline$^+$ and K^+ buffer were not significantly different to that in Na^+ buffer, but all agents that caused an increase in $[pH]_i$ over resting in Na^+ buffer, caused a significant drop in $[pH]_i$ in choline$^+$ or K^+ buffer (0.05–0.15 units below resting levels, fig.1b).

Resting $[Ca^{2+}]_i$ levels were not significantly different in Na^+, choline$^+$, K^+ or Li^+ buffer. $[Ca^{2+}]_i$ increases induced by thrombin at the concentration used for the secretion studies (0.05 U/ml) were greatly reduced in choline$^+$ and K^+ buffers but unaffected in Li^+ buffer (fig.1a). DiC_8, PMA and collagen induced no increase over the resting $[Ca^{2+}]_i$ level in Na^+, choline$^+$, K^+ or Li^+ buffer, while U46619-induced $[Ca^{2+}]_i$ rise was unaffected or significantly inhibited in choline$^+$ and K^+ buffer respectively (225 ± 15, 216 ± 21, and 91 ± 8 nM over resting in Na^+, choline$^+$ and K^+ buffer respectively).

3.3. *Role of $[pH]_i$ in 5HT secretion*

To assess whether changes in $[pH]_i$ were responsible for the potentiated secretory responses seen in the absence of $[Na^+]_e$, the effects of NH_4Cl (10 mM) and potassium acetate (20 mM), which respectively raise and lower $[pH]_i$ on their own (fig.1b), were tested. NH_4Cl was found to partially or totally reverse the potentiated responses to thrombin and diC_8 while not affecting the responses to collagen or U46619 in choline$^+$ buffer (table 2). Potassium acetate was found to significantly enhance 5HT secretion in response to all agents, particularly in choline$^+$ buffer, while not inducing any secretion on its own.

3.4. *Effect of EIPA on agonist-induced secretion*

At concentrations that abolished thrombin and diC_8-induced $[pH]_i$ rises (20–50 μM), EIPA significantly potentiated thrombin but not diC_8 or collagen-induced secretion (table 3). The potentiated response to thrombin in the presence of EIPA was partially reversed by NH_4Cl (10 mM) addition. EIPA alone caused no change in resting $[pH]_i$ in Na^+ buffer, but in combination with thrombin caused pronounced acidification over a 4 min incubation (0.1–0.2 units below resting levels). No such acidification was observed when

Table 2

Effect of NH_4Cl and potassium acetate on agonist-induced [^{14}C]5HT secretion in Na^+ and choline$^+$ buffer

		[^{14}C]5HT secretion (%) (means ± SE)		
		Control	+ NH_4Cl	+ pot. acetate
Thrombin (0.05 U/ml)	Na^+	20.5 ± 2.7	19.8 ± 2.5	29.7 ± 1.7
	Choline$^+$	34.7 ± 4.1	19.1 ± 2.8*	46.6 ± 3.9*
DiC_8 (60 μM)	Na^+	13.4 ± 2.1	14.3 ± 1.9	21.4 ± 3.3
	Choline$^+$	49.4 ± 2.8	39.0 ± 2.0*	69.3 ± 2.0**
Collagen (20 μg/ml)	Na^+	22.2 ± 1.4	19.9 ± 1.1	–
	Choline$^+$	37.1 ± 1.9	33.5 ± 1.8	–
U46619 (1 μM)	Na^+	7.8 ± 1.8	9.1 ± 1.8	47.5 ± 3.4**
	Choline$^+$	38.4 ± 4.4	38.9 ± 4.3	55.5 ± 2.4*

[^{14}C]5HT-labelled platelets were pre-incubated with NH_4Cl (10 mM) or potassium acetate (20 mM) for 1 min before addition of the various agents and reactions were terminated 3 min later (n = 12–16 in four separate experiments). * $P < 0.01$, ** $P < 0.001$ compared with the agonist control

Table 3

Effect of EIPA on agonist-induced [^{14}C]5HT secretion

	[^{14}C]5HT secretion (%) (means ± SE)
Thrombin	31.1 ± 5.4
EIPA + thrombin	64.1 ± 2.9*
NH_4Cl + EIPA + thrombin	42.7 ± 4.36**
diC_8	12.3 ± 1.5
EIPA + diC_8	10.0 ± 1.3
Collagen	23.7 ± 2.9
EIPA + collagen	33.3 ± 4.7

EIPA (20 μM) was added 1 min before thrombin (0.05 U/ml), collagen (20 μg/ml) or diC_8 (60 μM) and reactions were terminated 3 min after addition of the agonist. NH_4Cl (10 mM) was added 30 s before EIPA (n = 9–12 from four separate experiments). * $P < 0.001$ compared with thrombin control, ** $P < 0.001$ compared with EIPA + thrombin

EIPA was added along with diC_8 (results not shown). Long incubations with EIPA alone (6–8 min) resulted in up to 30% [^{14}C]5HT release; hence the pre-incubation time with EIPA was limited to 1 min.

3.5. *Effect of $[Na^+]_e$ removal on diC_8 and thrombin-induced protein phosphorylation*

Contrasting results on diC_8 and thrombin-induced protein phosphorylation were obtained upon removal of $[Na^+]_e$. While diC_8 (18–60 μM)-induced 45 kDa protein phosphorylation was unchanged by removal of $[Na^+]_e$ i.e. in choline$^+$ buffer, thrombin (0.05 U/ml)-induced protein phosphorylation was significantly reduced (fig.2). Preincubation of the platelets with potassium acetate (20 mM) for 1 min before addition of diC_8 was found to significantly enhance the extent of phosphorylation of the 45 kDa protein in the choline$^+$ buffer, and pre-incubation with NH_4Cl (10 mM) had no effect.

Fig.2. Effect of Na^+ removal and $[pH]_i$ manipulations on thrombin and diC_8-induced protein phosphorylation in ^{32}P-labelled platelets. An autoradiograph of a dried gel following electrophoresis showing changes in ^{32}P-labelling of different proteins is shown. Lanes a and e represent resting platelets in choline$^+$ and Na^+ buffer, respectively. Lanes b, c, d and f, g, h represent in choline$^+$ and Na^+ buffer, respectively, the effect of diC_8 (18 μM), NH_4Cl (10 mM) + diC_8 and potassium acetate (20 mM) + diC_8. Lanes i, j, k and l, m, n represent in choline$^+$ and Na^+ buffer, respectively, the effect of thrombin (0.05 U/ml), NH_4Cl + thrombin and potassium acetate + thrombin. NH_4Cl and potassium acetate were added 1 min before diC_8 and thrombin and reactions were terminated 3 min later (similar results were obtained when reactions were terminated at 30, 60 and 90 s after addition of thrombin or diC_8). These agents on their own induced no significant changes in ^{32}P-labelling of the different proteins over a 4 min period.

4. DISCUSSION

A great deal of recent interest in cellular signalling mechanisms has focussed on the Na^+/H^+ exchange mechanism and the role of elevated $[pH]_i$ in cellular responses. In platelets, it has been suggested that Na^+/H^+ exchange is obligatory in arachidonate release induced by 'weak' agonists [5–8], and potentiatory, if not obligatory, in receptor-mediated Ca^{2+} mobilisation [9–11]. In the present study, we have focussed on platelet granule secretion as a potential site for control by Na^+/H^+ exchange, and have found that contrary to earlier reports on $[pH]_i$ elevations being a positive signal in some platelet activation processes, the exchange mechanism is inhibitory to platelet granule secretion under some conditions. Evidence that this is the case in thrombin-induced secretion comes from three of our findings: (i) secretion is greatly enhanced in choline$^+$ and K^+ buffers in the absence of a $[pH]_i$ rise and this potentiated response is reversed by NH_4Cl, which raises $[pH]_i$; (ii) no increase in secretion is seen when $[Na^+]_e$ is replaced by Li^+, which allows a normal agonist-induced $[pH]_i$ rise and (iii) EIPA inhibits the $[pH]_i$ rise and potentiates secretion. In the case of collagen and U46619, the increased secretion even in Li^+ buffer, the lack of effect of NH_4Cl and EIPA suggest that, $[Na^+]_e$ per se, rather than Na^+/H^+ exchange may be the important factor inhibiting secretion. DiC_8-induced secretion may be modulated by both $[Na^+]_e$ as well as Na^+/H^+ exchange because NH_4Cl partially reverses the potentiated response in choline$^+$ buffer and no increase in secretion is seen in Li^+ buffer, even though EIPA has no potentiatory effect on secretion. It is interesting that pre-treatment with potassium acetate, which caused a big drop in

$[pH]_i$, increased secretion with all the agonists, particularly in the absence of $[Na^+]_e$, where the effect of this agent is less easily reversed by agonists able to activate the exchanger. This suggests that a large drop in $[pH]_i$ of several units favours dense-granule secretion via a general mechanism common to all agonists, although the absence of Na^+/H^+ exchange, which causes a smaller drop in $[pH]_i$ is probably potentiatory only to thrombin-induced secretion.

Whether altered receptor status in the absence of Na^+ is responsible for the enhanced secretory responses to collagen and U46619 (both receptor-operating stimuli) needs to be investigated, as increased receptor binding along with increased superoxide generation etc., in response to F-Met-Leu-Phe in the absence of $[Na^+]_e$ has been reported in neutrophils [16,17]. In the case of thrombin and diC_8-induced secretion, two possible mechanisms, which could be involved in the increased secretory responses in the absence of $[Na^+]_e$, namely, protein kinase C-mediated phosphorylations and $[Ca^{2+}]_i$, seem unlikely candidates. This is because (i) no significant difference in the extent of diC_8-induced protein phosphorylation was observed in Na^+ versus $choline^+$ buffer, and (ii) thrombin-induced protein phosphorylation and $[Ca^{2+}]_i$ elevations, in contrast to 5HT secretion, were significantly reduced in $choline^+$ compared with Na^+ buffer. These two findings suggest that although protein kinase C activation per se may be unaffected by the absence of $[Na^+]_e$ and an intact Na^+/H^+ exchanger, receptor-mediated protein kinase C activation (via diacylglycerol formation [18,19]), may be dependent on the presence of $[Na^+]_e$ and/or an intact Na^+/H^+ exchanger. Studies on thrombin-induced inositol phospholipid hydrolysis to test such a possibility are currently in progress. The enhanced protein phosphorylation in $choline^+$ buffer upon addition of potassium acetate may indicate that a large drop in resting $[pH]_i$ of several units may favour protein kinase C-mediated phosphorylation, even though a small drop in $[pH]_i$ caused by the lack of an intact exchanger may have no effect on it. It is also unlikely that changes in membrane depolarization could account for the increased secretion, as the responses in $choline^+$ and K^+ buffer were similar, despite different depolarisation states in the presence of these two ions [20].

In conclusion, the present study has revealed that $[Na^+]_e$ as well as the Na^+/H^+ exchange mechanism via increased $[pH]_i$ can negatively control platelet granule secretion; and although the site of action of both components is not known at present, the exchanger and $[Na^+]_e$ may play a similar inhibitory role in other cellular signal transduction systems leading to granule exocytosis.

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