Extracellular Na⁺, but not Na⁺/H⁺ exchange, is necessary for receptor-mediated arachidonate release in platelets

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The effect of extracellular Na⁺ removal and replacement with other cations on receptor-mediated arachidonate release in platelets was studied to investigate the role of Na⁺/H⁺ exchange in this process. Replacement with choline⁺, K⁺, *N*-methylglucamine⁺ (which abolished the thrombin-induced pH_i rise) or Li⁺ (which allowed a normal thrombin-induced pH_i rise) significantly decreased arachidonate release in response to all concentrations (threshold to supra-maximal) of thrombin and collagen. This inhibition was not reversed by NH₄Cl (10 mM) addition, which raised the pH_i in the absence of Na⁺, but, on the contrary, NH₄Cl addition further decreased the extent of thrombin- and collagen-induced arachidonate release, as well as decreasing 'weak'-agonist (ADP, adrenaline)-induced release and granule secretion in platelet-rich plasma. No detectable pH_i rises were seen with collagen (1–20 µg/ml) and ADP (10 µM) in bis-(carboxyethyl)carboxyfluorescein-loaded platelets. Inhibition of thrombin-induced pH_i rises was seen with 0.5–5 µM-5-*NN*-ethylisopropylamiloride (EIPA), but at these concentrations EIPA had little effect on thrombin-induced arachidonate release. At higher concentrations such as those used in previous studies (20–50 µM), EIPA inhibited aggregation/release induced by collagen and ADP in Na⁺ buffer as well as in choline⁺ buffer (where there was no detectable exchanger activity), suggesting that these concentrations of EIPA exert 'non-specific' effects at the membrane level. The results suggest that (i) Na⁺/H⁺ exchange and pH_i elevations are not only not necessary, but are probably inhibitory, to receptor-mediated arachidonate release in platelets, (ii) inhibition of receptor-mediated release in the absence of Na⁺ is most likely due to the absent Na⁺ ion itself, and (iii) caution should be exercised in the use of compounds such as EIPA, which, apart from inhibiting the Na⁺/H⁺ exchanger, have other undesirable and misleading effects in platelets.

INTRODUCTION

Receptor stimulation in platelets leads to the activation of two phospholipid-hydrolysing enzymes, whose actions result in the generation of three second-messenger molecules with varying and important functions in platelet signal transduction. One of these enzymes, a phospholipase C, acts primarily on phosphatidylinositol 4,5-bisphosphate (PtdInsP₂), and its cleavage products, the second-messenger molecules 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (InsP₃) [1,2] participate in signal transduction via activation of the Ca²⁺- and phospholipid-dependent enzyme, protein kinase C [3,4] and via Ca²⁺ release from intracellular stores [5,6] respectively. The second enzyme, a phospholipase A₂, acts primarily on phosphatidylcholine and to a smaller extent on other phospholipids, including PtdIns and phosphatidylethanolamine [7–9], releasing free arachidonic acid, which is converted into the third second-messenger molecule, thromboxane A₂. The platelet membrane has been shown to possess specific receptors [10,11] through which thromboxane A₂ can activate phospholipase C, elevate intracellular Ca²⁺ ([Ca²⁺]_i) levels [12,13] and generally reinforce the actions of other 'initiating' re-

ceptor-operating agonists such as thrombin and collagen [14,15].

Phospholipase C activation can occur at resting [Ca²⁺]_i levels [16–18], whereas phospholipase A₂ activation, in general, is believed to be a step requiring raised [Ca²⁺]_i levels [7,17,19]. The exception to the latter in platelets is the release of arachidonate induced by collagen in the absence of raised [Ca²⁺]_i levels [20,21]. Recent studies have also suggested that Na⁺/H⁺ exchange across the plasma membrane and the resulting increase in intracellular pH (pH_i) may be a controlling factor in receptor-stimulated arachidonate release. Thus Limbird and co-workers [22,23] have suggested, using the Na⁺ substitute *N*-methylglucamine (NMG⁺) and an amiloride analogue (ethylisopropylamiloride; EIPA) inhibiting the exchanger, that this ion-exchange mechanism is obligatory in arachidonate release induced by 'weak' agonists such as ADP and adrenaline, although not essential for release induced by a 'strong' agonist such as thrombin.

During a series of studies involving various Na⁺ substitutes, pH_i-elevating agents, EIPA and different platelet agonists, we discovered that (1) arachidonate release induced by all thrombin concentrations (threshold

Abbreviations used: EIPA, 5-*NN*-ethylisopropylamiloride; NMG, *N*-methylglucamine; [Ca²⁺]_i, intracellular Ca²⁺ concn.; pH_i, intracellular pH; BCECF, 2',7'-bis-carboxyethyl-5(6)-carboxyfluorescein.

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to supramaximal) was inhibited by removal of Na^+ and not reversed by pH_i -elevating agents, and (2) the Na^+ substitute, NMG^+ , and EIPA are inhibitors of aggregation at concentrations used in earlier platelet studies. We describe, in this report, our results from such studies, and present a new hypothesis on the role of extracellular Na^+ , and not of Na^+/H^+ exchange, in receptor-mediated arachidonate release in platelets.

MATERIALS AND METHODS

EIPA was kindly given by Dr. W. Siffert, Max-Planck-Institut für Biophysik, Frankfurt, West Germany. Quin 2 ester and bis(carboxyethyl)carboxyfluorescein (BCECF) ester were obtained from Sigma, Poole, Dorset, U.K., and Calbiochem, Cambridge, U.K. respectively. [^3H]Arachidonic acid (200 mCi/mmol) was obtained from Amersham International, Amersham, Bucks., U.K.

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 different markers, namely [^3H]arachidonic acid, BCECF and quin 2, was carried out during different stages of the washing procedure.

Citrated blood drawn from apparently healthy human volunteers was centrifuged at 600 *g* for 15 min to obtain platelet-rich plasma (PRP). After acidification of the platelet-rich plasma with 0.1 M-citric acid and addition of 20 nM-prostacyclin, the platelet-rich plasma was centrifuged at 1500 *g* for 10 min (room temperature) 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, 0.09% glucose, 0.05 unit of hirudin (Biopharm, Swansea, Wales, U.K.)/ml and 103 mM- Na^+ , -choline $^+$, - K^+ , - NMG^+ or - Li^+ as the chloride salt (buffer A). After addition of 20 nM-prostacyclin, these platelet suspensions were centrifuged at 1500 *g* for 10 min, and the platelet pellets 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% albumin (except where otherwise mentioned), 0.09% glucose, 0.05 unit of hirudin/ml and 145 mM of any one of the cations mentioned above (buffer B) at a count of $(2-4) \times 10^8$ platelets/ml.

Platelets were loaded with quin 2 by incubating 20 μM -quin 2 ester with platelet-rich plasma for 30 min at 37 °C. For the experiments on arachidonate release and pH_i determinations, a concentrated platelet suspension (10^9 platelets/ml) in buffer A containing Na^+ was incubated with 5 μM -BCECF ester for 45 min or with 0.75 μCi of [^3H]arachidonic acid for 90 min 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 any one of the other cations, centrifuged at 1500 *g* for 10 min, and the pellets were resuspended in buffer B of the desired ionic composition. For all the experiments, the washed platelets were pre-treated with indomethacin (10 μM) (unless otherwise mentioned) to avoid effects of endogenously formed thromboxane A_2 . Additionally, the platelets were pre-treated with phenidone (250 μM) to inhibit lipoxygenase activity for experiments on arachidonate release.

Measurement of [^3H]arachidonate release

Platelet supernatants were counted for released radioactivity 3 min after addition of the inducing agonist as described previously [21].

Measurement of $[\text{Ca}^{2+}]_i$ and pH_i

Fluorescence measurements on quin 2- and BCECF-loaded platelets (unstirred) and calibration of the signals were carried out essentially as described previously [24]. Excitation wavelengths for the quin 2 and BCECF experiments were respectively 339 and 490 nm, and emission wavelengths respectively 500 and 520 nm. Absolute pH_i values were calculated by using the nigericin/KCl method to correct the values obtained by lysing the platelets with digitonin at different external pH values.

Experimental approaches

These included the use of (1) Na^+ substitutes such as K^+ , choline $^+$ and NMG^+ , which are not transported by the Na^+/H^+ exchanger, as well as Li^+ , which is transported by the exchanger [25], (2) pH_i -elevating agents such as NH_4Cl , methylamine, which can diffuse inside the cell and directly elevate the pH_i even in the absence of extracellular Na^+ , and monensin, which can transport Na^+ from the outside to the inside of the cell, and (3) EIPA, which inhibits the exchanger [26].

Statistical analysis was performed by Student's *t* test for unpaired data.

RESULTS

Fig. 1 shows some of the effects seen in Na^+ , choline $^+$ and Li^+ buffers upon addition of thrombin, ionomycin, ADP, EIPA plus thrombin, or NH_4Cl . Thrombin (2 units/ml) and ionomycin (6 μM) induced a large increase in $[\text{Ca}^{2+}]_i$ (> 800 nM) and a relatively small increase in pH_i (0.1–0.15 unit) over resting levels in Na^+ as well as in Li^+ buffer (Fig. 1). Rises in pH_i but not $[\text{Ca}^{2+}]_i$ induced by thrombin (2 units/ml) and ionomycin (6 μM) were abolished in choline $^+$ buffer. Addition of NH_4Cl alone caused no change in the resting $[\text{Ca}^{2+}]_i$ level, but caused a small increase in threshold thrombin-induced $[\text{Ca}^{2+}]_i$ rises in Na^+ buffer (Table 1). This contrasted with the much larger and statistically significant increases in threshold ionomycin-induced $[\text{Ca}^{2+}]_i$ rises caused by all the pH_i -elevating agents in Na^+ buffer (Table 1). Large increases in pH_i in all buffers were seen with NH_4Cl on its own, and were slightly accentuated by addition of thrombin or ionomycin in Na^+ buffer (Fig. 1). EIPA (0.5–5 μM) abolished the thrombin (2 units/ml)- and ionomycin (6 μM)-induced pH_i rises without affecting the resting pH_i or the $[\text{Ca}^{2+}]_i$ elevations induced by these concentrations of thrombin and ionomycin in Na^+ buffer (effect of NH_4Cl and EIPA on thrombin-induced pH_i rise in Na^+ buffer shown in Fig. 1). Collagen (1–20 $\mu\text{g}/\text{ml}$) (results not shown) and ADP (10 μM) (Fig. 1) induced no significant change in the resting pH_i in Na^+ buffer in five experiments, even though clear activation (shape change, aggregation) by both agonists of BCECF-loaded washed platelets was observed.

Agonist-induced arachidonate release in Na^+ -containing and Na^+ -free buffer and the effect of NH_4Cl

(i) Na^+ versus choline $^+$ buffer. Table 2 summarizes the

Fig. 1. (a) $[Ca^{2+}]_i$ elevations induced by thrombin and ionomycin in quin 2-loaded platelets resuspended in Na⁺, choline⁺ and Li⁺ buffers; (b) pH_i changes induced by thrombin, ionomycin, ADP, NH₄Cl and EIPA plus thrombin in BCECF-loaded platelets resuspended in Na⁺, choline⁺ and Li⁺ buffers

Traces a, b, c, d and e in each of the buffer systems in both panels represent the effect of 2 units of thrombin/ml, 6 μ M-ionomycin, 10 mM-NH₄Cl (the effect of thrombin added 1 min after NH₄Cl is shown by the broken line), 5 μ M-EIPA plus 2 units of thrombin/ml and 10 μ M-ADP respectively.

effects of thrombin, collagen and ionomycin (at maximal concentrations) on arachidonate release in Na⁺ and choline⁺ buffer. Essentially, the results show that receptor (thrombin, collagen)-mediated arachidonate release was significantly less, but ionomycin-induced release was significantly greater, in choline⁺ buffer than that in Na⁺ buffer. Similarly, NH₄Cl or methylamine addition inhibited thrombin- and collagen-induced release in Na⁺

and choline⁺ buffer, but greatly enhanced that induced by ionomycin. NH₄Cl also significantly inhibited ADP- and adrenaline-induced granule secretion and adrenaline-induced thromboxane B₂ formation in platelet-rich plasma (Table 2). Decreased arachidonate release in choline⁺ buffer was seen at all concentrations of thrombin and collagen from threshold to supra-maximal (thrombin dose-response curves in Na⁺ and choline⁺ buffers and the effect of NH₄Cl are shown in Fig. 2).

Table 1. Effect of pH_i -elevating agents on thrombin- and ionomycin-induced $[Ca^{2+}]_i$ elevations

Quin 2-loaded platelets (resuspended in the absence of albumin for experiments with ionomycin) and threshold concentrations of thrombin (0.05 unit/ml) and ionomycin (10 nM) were used for these experiments. pH_i -elevating agents were added 1 min before the agonist. Values represent means $\pm$ S.E.M. of 9–12 determinations from 3–4 separate experiments; * P < 0.01 compared with the agonist control.

Agonist	pH_i -elevating agent	$[Ca^{2+}]_i$ (nM)
None (resting)	—	90 $\pm$ 10
	Monensin (10 μ M)	85 $\pm$ 15
Thrombin	—	364 $\pm$ 67
	Monensin (10 μ M)	344 $\pm$ 41
	NH ₄ Cl (10 mM)	673 $\pm$ 240
	Methylamine (20 mM)	522 $\pm$ 155
Ionomycin	—	237 $\pm$ 30
	Monensin (10 μ M)	1792 $\pm$ 560*
	NH ₄ Cl (10 mM)	419 $\pm$ 22*
	Methylamine (20 mM)	562 $\pm$ 171*

(ii) Na⁺ versus Li⁺ buffer. Table 2 also shows that, despite normal pH_i elevations in Li⁺ buffer (Fig. 1), thrombin-induced arachidonate release was significantly decreased in Li⁺ compared with that in Na⁺ buffer. Collagen-induced release was also inhibited, but ionomycin-induced release was significantly greater in Li⁺ than in Na⁺ buffer.

(iii) K⁺ versus NMG⁺ buffer. Arachidonate release induced by thrombin, collagen and ionomycin followed the same pattern in K⁺ and NMG⁺ buffer as in choline⁺ buffer. However, one notable and important difference between the results obtained in these different buffer systems was the decreased aggregation seen with thrombin, collagen and ionomycin only in NMG⁺ buffer. In K⁺ buffer (Fig. 3), as well as in choline⁺ buffer (results not shown), the extent of platelet aggregation induced by these agonists was indistinguishable from that in Na⁺ buffer.

Effect of EIPA on thrombin-induced arachidonate release

At concentrations that abolished the thrombin-induced pH_i rise (0.5–5 μ M), EIPA had very little effect on thrombin-induced arachidonate release (Table 3).

Table 2. Agonist-induced arachidonate release in Na⁺-containing and Na⁺-free buffer

NH₄Cl (10 mM) was preincubated for 1 min before addition of the agonist. The release in resting platelets was subtracted from the release in agonist-stimulated platelets shown. The values represent means $\pm$ S.E.M. of 12–16 determinations from 3–4 separate experiments; **P* < 0.01–0.001 compared with the response in Na⁺ buffer; ***P* < 0.01 compared with the agonist control.

(a) Washed platelets			
	³ H]Arachidonate release (%)		
	Na ⁺	Choline ⁺	Li ⁺
Thrombin (2 units/ml)	21.2 $\pm$ 0.1	14.1 $\pm$ 0.3*	10.1 $\pm$ 0.1*
NH ₄ Cl + thrombin	18.1 $\pm$ 0.4**	12.5 $\pm$ 0.2**	—
Collagen (20 μ g/ml)	7.0 $\pm$ 0.2	5.1 $\pm$ 0.5*	2.1 $\pm$ 0.2*
NH ₄ Cl + collagen	5.2 $\pm$ 0.3**	4.1 $\pm$ 0.2**	—
Ionomycin (6 μ M)	3.3 $\pm$ 0.1	10.1 $\pm$ 0.6*	13.1 $\pm$ 0.4*
NH ₄ Cl + ionomycin	9.1 $\pm$ 0.6**	18.1 $\pm$ 0.6**	—

(b) Platelet-rich plasma		
	5-Hydroxy[¹⁴ C]tryptamine secretion (%)	Thromboxane B ₂ (pmol/10 ⁸ platelets)
ADP (10 μ M)	40.8 $\pm$ 1.6	—
NH ₄ Cl + ADP	9.0 $\pm$ 1.4**	—
Adrenaline (10 μ M)	33.9 $\pm$ 3.9	15.3 $\pm$ 1.6
NH ₄ Cl + adrenaline	5.4 $\pm$ 0.8**	1.6 $\pm$ 0.4**

Approx. 10-fold higher concentrations (20–40 μ M) caused a 20–25% decrease in the thrombin-induced release; however, these high concentrations were found to inhibit ‘non-specifically’ collagen-induced aggregation/arachi-

donate release (Fig. 4) and ADP-induced aggregation in Na⁺ as well as in choline⁺ buffer. As the platelets were not treated with indomethacin in this set of experiments, larger responses and no apparent differences in Na⁺ and choline⁺ buffer were seen.

Fig. 2. Dose-response curves of thrombin-induced [³H]-arachidonate release in Na⁺ (●) and choline⁺ (▲) buffer

The effect of NH₄Cl (10 mM) preincubation for 1 min before thrombin addition is shown by (○) (Na⁺) and (△) choline⁺. The results represent means $\pm$ S.E.M. of 12–18 determinations in 3–4 different experiments; **P* < 0.001 compared with the thrombin response in Na⁺ buffer; ***P* < 0.01 compared with the respective thrombin control in Na⁺ or choline⁺ buffer.

DISCUSSION

Many recent studies have focussed on the Na⁺/H⁺ exchange mechanism and its role in cellular processes. In platelets it has been suggested that the ion-exchange

Fig. 3. Thrombin (2 units/ml)- and ionomycin (6 μ M)-induced aggregation and [³H]arachidonate release in platelets resuspended in K⁺ and NMG⁺ buffers

The aggregation traces are representative of 10–12 determinations in 4 different experiments, and the values in parentheses beside each trace represent the percentage of [³H]arachidonate released (means $\pm$ S.E.M. of 12 determinations).

Table 3. Effect of EIPA on thrombin-induced pH_i-elevation and [³H]arachidonate release

EIPA at different concentrations was incubated with BCECF- or [³H]arachidonic acid-labelled platelets for 1 min before addition of thrombin (2 units/ml), and reactions were allowed to run for 3 min. Addition of thrombin to resting platelets in the absence of EIPA resulted in a pH_i rise of 0.18 units above resting (pH 7.14), and 16.3 ± 0.8 % release of [³H]arachidonate over that in resting platelets; **P* < 0.001–0.01 compared with the thrombin control.

[EIPA] (μM)	Inhibition (%)	
	pH _i rise	[³ H]Arachidonate release
0.25	30 ± 10*	0
0.5	62 ± 15*	0
1.0	91 ± 8*	16 ± 2*
5.0	100*	15 ± 3*
10.0	100*	11 ± 4*
20.0	100*	25 ± 5*
40.0	100*	21 ± 3*

mechanism and the resulting rise in pH_i is obligatory in arachidonate release induced by 'weak' agonists such as ADP and adrenaline [22,23]. These studies have utilized NMG⁺ as the Na⁺ substitute and EIPA as an inhibitor of exchanger activity. Our studies using NMG⁺ and EIPA have enabled us to conclude that results obtained using these two tools can be misleading, as both are inhibitors of platelet aggregation independently of any effect on Na⁺/H⁺ exchange. Such a conclusion was made from two important findings: (i) aggregation induced by all the agonists tested was inhibited only in NMG⁺-containing buffer, of all the cations tested, and (ii) EIPA, at concentrations used in previous studies [22,23], inhibited platelet aggregation and arachidonate release induced by collagen, as well as ADP-induced aggregation, not only

in Na⁺ buffer but also in choline⁺ buffer in the absence of any detectable exchanger activity. Many compounds inhibiting platelet aggregation have also been shown to inhibit arachidonate release [27–29], and it is thought that a general feature of compounds acting at the level of the membrane is their ability to affect these two processes in platelets. It is not entirely surprising that NMG⁺ and EIPA are inhibitors of aggregation because of the free or substituted amino groups in both molecules, since several previous studies have shown the importance of these structural moieties in the inhibition of platelet aggregation [30–32]. It is our view therefore that the inhibition in NMG⁺ buffer and by EIPA of 'weak' agonist-induced arachidonate release, which is an aggregation-dependent event [15,33], cannot be interpreted as implying an important role for Na⁺/H⁺ exchange, as it is probably due to 'non-specific' inhibition of the aggregation process by NMG⁺ and EIPA. Moreover, the little or no pH_i rise seen with ADP and adrenaline in the absence of cyclooxygenase metabolites in two other studies [24,34] apart from ours suggests that activation of the exchanger by weak agonists may be secondary to arachidonate release and/or its conversion. In the context of NMG⁺ effects on aggregation, Agam *et al.* [35], during the preparation of this manuscript, have reported similar decreased aggregation responses in NMG⁺ buffer with thrombin.

Our studies with various substitutes have allowed us to confirm that receptor-mediated arachidonate release is inhibited by removal of extracellular Na⁺, but our interpretation is that this inhibition is due to removal of the Na⁺ ion itself. This is based on four main findings: (i) inhibition of thrombin-induced arachidonate release was seen not only in choline⁺ and K⁺ buffers in the absence of a pH_i rise but also in Li⁺ buffer, in which a normal pH_i rise in response to thrombin was observed; (ii) NH₄Cl addition, which raised the pH_i even in choline⁺ buffer, did not reverse the inhibited response in the absence of Na⁺, as might be expected if the inhibition was due to inhibition of Na⁺/H⁺ exchange, but caused further inhibition of thrombin- as well as collagen-induced arachidonate release in choline⁺ buffer; (iii) concentrations of EIPA that abolished the thrombin-induced pH_i rise had little or no effect on thrombin-induced arachidonate release; and (iv) collagen-induced arachidonate release was inhibited in the absence of Na⁺, despite there being no detectable pH_i rise in the presence of Na⁺. It seems that raising the pH_i to greater than resting levels under normal conditions in Na⁺ buffer is inhibitory to receptor-mediated arachidonate release, as NH₄Cl inhibited the release in response not only to thrombin and collagen but also to ADP and adrenaline.

The inhibition of receptor-mediated release by NH₄Cl contrasts with the significant enhancement of ionomycin-induced release reported in previous studies [23] and the present one, but, to our way of thinking, such a synergism between raised [Ca²⁺]_i and pH_i (implicating Na⁺/H⁺ exchange as a positive modulator of Ca²⁺-induced arachidonate release) is not relevant to receptor-mediated release. This is because both Na⁺ and raised pH_i probably affect steps in receptor-enzyme coupling that are not applicable to ionomycin-induced actions. It is interesting that ionomycin-induced [Ca²⁺]_i rises were potentiated to a significantly greater extent than thrombin-induced [Ca²⁺]_i rises by pH_i-elevating agents, which may explain the increased ionomycin-induced arachidonate release in the presence of these compounds. As the concentration

Fig. 4. Effect of EIPA (20 and 40 μM) on collagen (20 μg/ml)-induced aggregation and [³H]arachidonate release in platelets resuspended in Na⁺ and choline⁺ buffer

Platelets were not pre-treated with indomethacin or phenidone in these experiments, to allow a full aggregation response to collagen. All other details are as described for Fig. 3; **P* < 0.001 compared with the respective collagen control.

of ionomycin used for these experiments was sub-threshold for induction of any of the activation processes (aggregation, secretion or arachidonate release), elevation of pH_i may enhance $[\text{Ca}^{2+}]_i$ mobilization, not via potentiation of the actions of a specific second messenger such as InsP_3 , as suggested previously [36], but possibly via a general increase in Ca^{2+} flux across membranes etc.

In summary, our results have helped us make two important conclusions: firstly, that caution should be exercised in the use of compounds such as NMG^+ and EIPA in work relating to Na^+/H^+ exchange, as results with these compounds can be misleading, and secondly, that Na^+/H^+ exchange and rises in pH_i are not essential for receptor (weak or strong agonists)-mediated arachidonate release, although extracellular Na^+ probably is. Clearly, more work in this area will lead to a better understanding of the mechanisms controlling arachidonate release in platelets.

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