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AGONIST-EVOKED CHANGES IN CYTOSOLIC pH AND CALCIUM CONCENTRATION IN HUMAN PLATELETS: STUDIES IN PHYSIOLOGICAL BICARBONATE

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SUMMARY

1. Cytosolic pH (pH_i) and calcium concentration ($[Ca^{2+}]_i$) have been investigated in the presence and absence of physiological HCO_3^- in human platelets co-loaded with the fluorescent indicators BCECF and Fura-2. Basal pH_i and changes evoked by butyrate, thrombin, platelet activating factor (PAF), ADP and phorbol ester were investigated, as were the effects of removing external Na^+.

2. In the presence of physiological HCO_3^- and CO_2, basal pH_i was $7{\cdot}02 \pm 0{\cdot}04$ compared with $7{\cdot}15 \pm 0{\cdot}05$ in the absence of HCO_3^-. Estimated cytosolic buffering power was reduced from $35{\cdot}6 \pm 3{\cdot}0$ to $14{\cdot}5 \pm 0{\cdot}4$ mM/pH unit by the omission of HCO_3^-.

3. Thrombin evoked an immediate acidification of $0{\cdot}03 \pm 0{\cdot}01$ pH units in the presence of HCO_3^- and $0{\cdot}07 \pm 0{\cdot}01$ pH units in its absence. The acidifications were followed by a slow alkalinization. The final pH_i was $0{\cdot}10 \pm 0{\cdot}01$ units above basal in the presence of HCO_3^- and $0{\cdot}08 \pm 0{\cdot}02$ units above basal in the absence of HCO_3^-. The initial acidification was significantly greater in the absence of HCO_3^-. The subsequent increase in pH_i was similar in the presence and absence of this ion, but the calculated loss of proton equivalents was greater in the presence of HCO_3^-.

4. Replacement of extracellular Na^+ with *N*-methyl-D-glucamine resulted in a fall in basal pH_i and abolished recovery from thrombin-evoked acidification in both the presence and absence of HCO_3^-.

5. In the presence of HCO_3^-, PAF and ADP evoked an intracellular acidification similar to that caused by thrombin. However, with PAF and ADP, the subsequent recovery in pH_i was slow and did not rise above basal levels. Phorbol dibutyrate, an activator of protein kinase C, evoked a similar elevation in pH_i of $0{\cdot}04 \pm 0{\cdot}01$ units over 3 min in the presence and absence of HCO_3^-.

6. Stopped-flow fluorimetric measurements were made of both BCECF and Fura-2 fluorescence in the presence of HCO_3^-. In the presence and absence of external Ca^{2+}, thrombin-evoked rises in $[Ca^{2+}]_i$ peaked before any cytoplasmic alkalinization occurred. ADP evoked rapid elevations in $[Ca^{2+}]_i$, but caused no alkalinization.

7. These results (i) indicate that the absence of physiological HCO_3^- does not

qualitatively alter agonist-evoked changes in pH_i, although the presence of this ion affects basal pH_i and increases cytosolic buffering power, (ii) show that, even in the presence of HCO_3^-, agonist-evoked alkalinization is dependent on external Na^+, and (iii) provide further evidence that activation of Na^+–H^+ exchange and an elevation in pH_i are not a prerequisite for elevation of $[Ca^{2+}]_i$ in platelets.

INTRODUCTION

Stimulus-evoked increases in cytosolic pH (pH_i) or H^+ efflux have been widely reported in platelets (Horne, Norman, Schwartz & Simons, 1981; Zavoico, Cragoe & Feinstein, 1986; Siffert & Akkerman, 1987*a*; Simpson & Rink, 1987; Siffert, Siffert & Scheid, 1987; Zavoico & Cragoe, 1988). Similarly, thrombin or activators of protein kinase C have been demonstrated to promote Na^+–H^+ exchange across the platelet plasma membrane (Siffert *et al.* 1987). Yet, although stimulus–response coupling in platelets is comparatively well understood, with Ca^{2+}, inositol phosphates, diacylglycerol and cyclic nucleotides recognized as having important signalling roles (e.g. Rink & Hallam, 1984), there is disagreement as to the part stimulus-evoked changes in pH_i may play in activation. Siffert & Akkerman (1987*a*) have proposed that cytosolic alkalinization is a prerequisite of Ca^{2+} mobilization in platelets, whilst others (e.g. Simpson & Rink, 1987; Zavoico & Cragoe, 1988; Alonso, Collazos & Sanchez, 1988) have concluded that changes in pH_i do not form a necessary component of the pathways leading to Ca^{2+} mobilization or the stimulation of shape change or aggregation. Moreover, these earlier reports were of experiments conducted in HEPES-buffered salines which lacked HCO_3^-. In many cell types the absence of HCO_3^- profoundly affects pH_i regulation (Thomas, 1984; Ganz, Boyarsky, Boron & Sterzel, 1987; Gillespie & Greenwell, 1988; Kettermann & Schlue, 1988; Ganz, Boyarsky, Sterzel & Boron, 1989; Thomas, 1989; Grinstein, Rotin & Mason, 1989), and at least three types of HCO_3^- transport across cell membranes have been identified (e.g. Boron, 1986). A very recent preliminary report shows data suggesting that HCO_3^- potentiates thrombin-evoked elevation of pH_i in human platelets (Ozaki, Yatomi, Kariya & Kume, 1989). We have therefore studied basal pH_i and the changes following addition of butyrate (to acidify the cytosol) and various agonists in platelets in the presence and absence of a physiological concentration of HCO_3^-. To assess the possible role of changes in pH_i in generating changes in cytosolic calcium concentration ($[Ca^{2+}]_i$) we have investigated both variables by stopped-flow fluorimetry (Rink & Sage, 1985; Sage & Rink, 1987) using cells co-loaded with the fluorescent indicators BCECF and Fura-2. In addition, we have examined changes in pH_i evoked by the Ca^{2+} mobilizing agonists ADP and platelet activating factor (PAF), and compared them with changes evoked by the more powerful activator, thrombin, on which earlier studies have concentrated.

METHODS

Preparation of cells

Blood was drawn from healthy volunteers and 8·5 ml aliquots were added to 1·5 ml acid citrate dextrose anticoagulant in plastic test tubes. The anticoagulant contained: trisodium citrate (2·5 g/

100 ml), citric acid (1·5 g/100 ml) and D-glucose (2 g/100 ml). This procedure gave a final whole anticoagulated blood citrate concentration of 22 mM and a pH of 6·5. Platelet-rich plasma was prepared by centrifugation for 5 min at 700 *g* at room temperature and 100 μM-aspirin added. Platelets were then co-loaded with Fura-2 and BCECF by incubation for 45 min at 37 °C with 3 μM each of the respective acetoxymethylesters added from 5 mM stocks in dimethylsulphoxide (DMSO).The cells were then pelleted by centrifugation at 350 *g* for 20 min and as much of the supernatant as possible removed prior to resuspension.

In the present work, as in previous studies with platelets co-loaded with Fura-2 and BCECF, there was no evidence of significant cross-talk between the signals from the two dyes. The excitation wavelength for BCECF gives no Fura-2 emission (see e.g. Zavoico & Cragoe, 1988).The Fura-2 signals seen in the present work were closely similar to those we have previously seen in studies of singly loaded cells, as also found by Simpson & Rink (1987).

Solutions

The HEPES-buffered saline (HEPES) had the composition (mM): NaCl, 145; KCl, 5; $MgSO_4$, 1; HEPES, 10; D-glucose, 10. The HEPES was half-neutralized by the addition of 5 mM-NaOH, giving a final pH of 7·4 at 37 °C. The HCO_3^--containing buffer (HCO_3^-) had the composition (mM): NaCl, 122; $NaHCO_3$, 23; KCl, 5; $MgSO_4$, 1; HEPES, 10; D-glucose, 10. The HEPES was half-neutralized by the addition of 5 mM-NaOH prior to the addition of the $NaHCO_3$ and the solution was gassed with 5% CO_2–95% O_2 to give a final pH of 7·4 at 37 °C. Nominally Na^+-free HCO_3^--containing buffer (0 Na^+–HCO_3^-) had the following composition (mM): *N*-methyl-D-glucamine (NMDG), 127, HCl, 122; $MgSO_4$, 1; HEPES, 10; $KHCO_3$, 23. The HEPES was thus half-neutralized by NMDG and the final pH was 7·4 at 37 °C when the solution was gassed with 5% CO_2. Nominally Na^+-free HEPES-buffered saline (0 Na–HEPES) had the composition (mM): NMDG, 127; HCl, 122; $MgSO_4$, 1; HEPES, 10; KCl, 23; pH 7·4 at 37 °C. Potassium was elevated in this buffer so as to be comparable with the concentration necessitated in 0 Na–HCO_3^- by the use of $KHCO_3$. To control for effects of elevated K^+, modified Na^+-containing media were prepared in which $NaHCO_3$ was replaced by $KHCO_3$ (23 mM-K^+–HCO_3^-) or NaCl was partially replaced by 23 mM-KCl (23 mM-K^+–HEPES). The additional 5 mM-KCl present in control buffers was omitted from all four buffers used in the low-Na^+ experiments (i.e. [K^+] was 23 mM in each). Apyrase (20 μg/ml) was added to all suspensions to prevent activation by residual traces of ADP. Solutions containing HCO_3^- were bubbled with 5% CO_2–95% O_2 for at least 30 min prior to use. Platelet suspensions containing HCO_3^- were kept in equilibrium with a 5% CO_2–95% CO_2 mixture at all times, the gas being delivered by polythene tubing to the plastic vessels containing stock suspensions, the reaction cuvettes and the holding reservoirs of the stopped-flow apparatus. Suspensions were maintained at 37 °C at all times. In some experiments, 1 mM-$CaCl_2$ was added to the cell suspension prior to use.

Measurement of pH_i

The cell suspension was placed in cylindrical glass cuvettes in a specially constructed thermostatted holder in a Perkin-Elmer MPF 44A spectrophotometer. The suspension could be stirred with a magnetic stir-bar. If agonists were added, stirring was usually stopped 5 s after the addition to prevent aggregation. The BCECF fluorescence, with excitation at 490 nm, emission at 530 nm and a slit width of 5 nm, was recorded on a chart recorder. Dye leakage was assessed by measuring at intervals the supernatant fluorescence after centifugation to remove the cells. The leak signal, which was found to increase linearly at $0{\cdot}44 \pm 0{\cdot}02$%/min (S.E.M., $n = 50$), was corrected for pH if necessary and subtracted from all experimental values. The remaining signal was then scaled to that which would have been obtained if all the dye had been intracellular. The corrected signals were then calibrated by comparison with a calibration curve obtained from an aliquot of the same cell suspension which had been lysed to release trapped dye by the addition of 50 μM-digitonin. The lysate pH was stepped from pH 6·0 to pH 8·0 by the addition of 1 M-HCl or 1 M-NaOH as required. The pH was monitored in the cuvette by a WPA EL26 pH electrode (WPA Ltd, Linton, Cambridgeshire) connected to a Universal PTI-6 digital pH meter. The pH value obtained was then corrected for the red shift which occurs in the intracellular environment by the method of Rink, Tsien & Pozzan (1982). Briefly, dye-loaded cells were suspended in a modified HEPES-buffered saline containing 135 mM-KCl and 15 mM-NaCl. The pH of the medium was then stepped from 8·0 to 6·0 by the addition of 1 M-NaOH or 1 M-HCl as required, after the addition of

nigericin (0·2 μg/ml; from 0·2 mg/ml stock in DMSO). Nigericin is a H^+–K^+ antiporter which sets $[H^+]_i/[H^+]_o = [K^+]_i/[K^+]_o$ (Thomas, Buchsbaum, Zimniak & Racker, 1979). The cells were then lysed by the addition of 50 μM-digitonin and the pH returned stepwise to 8·0 by the addition of 1 M-NaOH, and correction curves comparing lysate and intracellular fluorescence plotted after adjustment for dye leakage as detailed above.

The results are expressed as mean ± S.E. of mean (S.E.M.).

Measurement of $[Ca^{2+}]_i$, shape change and aggregation

Fura-2 fluorescence, with excitation at 339 nm and emission at 500 nm, was monitored as for BCECF above. The signals were corrected for dye leakage according to the method of Rink & Pozzan (1985) and calibrated in terms of $[Ca^{2+}]_i$ as previously described (Pollock, Rink & Irvine, 1986).

The optical density of the stirred platelet suspension could be measured simultaneously with fluorescence. Shape change and aggregation could thus be monitored by the turbidimetric method of Born (1970). Stirring was maintained in experiments assessing these parameters.

The results are expressed as mean ± S.E.M..

Stopped-flow fluorimetry

Stopped-flow kinetic measurements of Fura-2 fluorescence were made as previously described (Sage & Rink, 1987). For BCECF fluorescence, the procedure was modified since an artificial fluorescence increase occurred on mixing cell and agonist solutions. The artifact was presumably due to slight pH imbalance between the cell suspension and agonist solution. Traces were corrected for this artifact by subtracting the fluorescence change determined on mixing cell suspension with agonist-free buffer.

The results are expressed as mean ± S.E.M.

Materials

The acetoxymethyl esters of Fura-2 and BCECF were from Molecular Probes, Eugene, OR, USA. The Na_2-ADP, apyrase, aspirin, butyric acid, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), *N*-methyl-D-glucamine, nigericin, phorbol dibutyrate, sodium butyrate and bovine thrombin were from Sigma, Poole, Dorset. Platelet activating factor and HEPES were from Calbiochem, San Diego, CA, USA. Digitonin was from BDH Ltd, Poole, Dorset.

RESULTS

Basal pH_i and butyrate-induced acidification in the presence and absence of bicarbonate

In HCO_3^--containing medium, the basal pH_i determined from BCECF fluorescence was 7·02 ± 0·04 (S.E.M., $n = 10$), compared with 7·15 ± 0·05 ($n = 10$) in the absence of HCO_3^-. The difference was significant (Student t test, difference means, $0·05 > P > 0·02$). Basal pH_i was similarly elevated in the absence of HCO_3^- in Na^+-containing and Na^+-free media containing 23 mM-K^+ (Table 1).

The addition of 50 mM-NMDG butyrate to platelets suspended in Na^+-free medium induced an initial fall in pH_i of 0·48 ± 0·04 units ($n = 6$) in the presence of HCO_3^- and 0·92 ± 0·06 units ($n = 6$) in the absence of HCO_3^- (not shown). Cytosolic buffering power, calculated as described by Szatkowski & Thomas (1986), was 35·6 ± 3·0 and 14·5 ± 0·04 mM/pH unit in the presence and absence of HCO_3^- respectively. The difference was significant (paired Student t test, $P < 0·001$).

Thrombin-evoked changes in pH_i in the presence and absence of bicarbonate

Figure 1 shows responses evoked by thrombin (0·5 unit/ml) in platelets co-loaded with BCECF and Fura-2 and suspended in HEPES or HCO_3^- media. Figure 1*A*

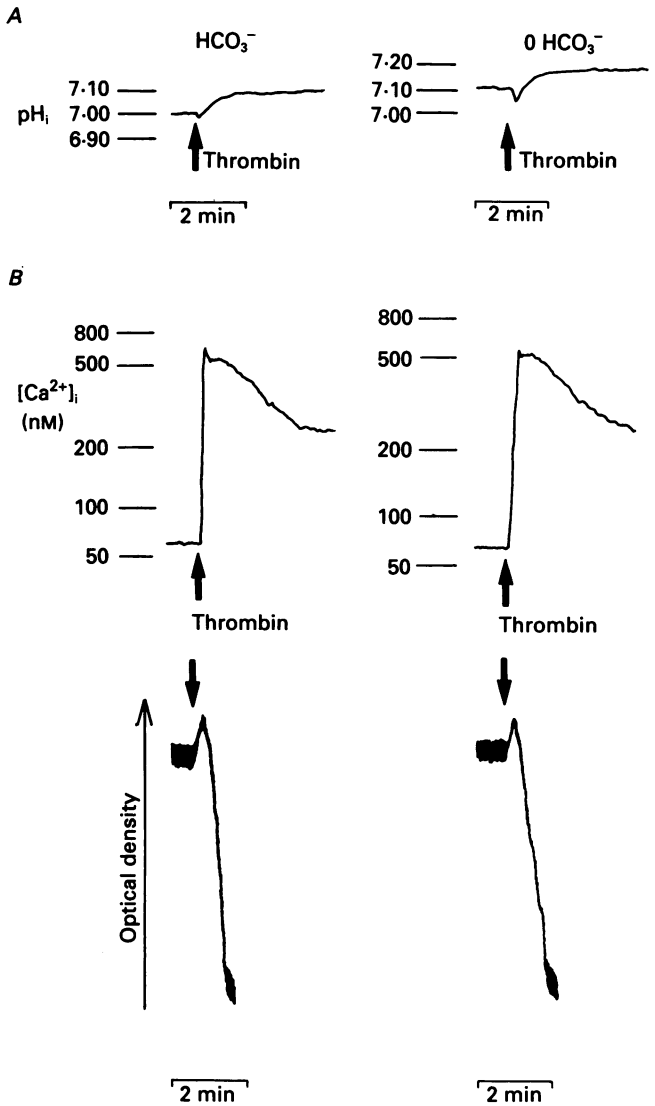

Fig. 1. Responses evoked by thrombin (0·5 units/ml) in the presence and absence of HCO_3^-. *A*, BCECF fluorescence calibrated in terms of pH_i. *B*, Fura-2 fluorescence calibrated in terms of $[Ca^{2+}]_i$ (upper traces) and optical density (lower traces). Traces on the left are from experiments conducted in 23 mM-HCO_3^- and those on the right in the absence of HCO_3^-.

shows BCECF fluorescence. Stimulation evoked an initial acidification followed by a later alkalinization. The fall below basal pH_i was $0{\cdot}03 \pm 0{\cdot}01$ units (S.E.M., $n = 10$) in cells in the presence of HCO_3^- and $0{\cdot}07 \pm 0{\cdot}01$ units ($n = 10$) in the absence of HCO_3^-. The difference was significant (Student t test, difference means, $0{\cdot}01 > P > 0{\cdot}001$). Three minutes after addition of thrombin, the pH_i had risen $0{\cdot}10 \pm 0{\cdot}01$ units ($n = 10$) above the basal level in HCO_3^- and $0{\cdot}08 \pm 0{\cdot}02$ units ($n = 10$) above basal in HEPES. The difference in the increment in pH_i was not significant ($P > 0{\cdot}05$). The

initial rate of loss of proton equivalents, calculated using the cytosolic buffering powers determined above were $11{\cdot}1 \pm 1{\cdot}1$ and $5{\cdot}8 \pm 0{\cdot}7$ mM/min (S.E.M., $n = 10$ for each) in the presence and absence of HCO_3^- respectively.

Thrombin-evoked elevation in $[Ca^{2+}]_i$

The upper traces of Fig. 1*B* show Fura-2 fluorescence. Thrombin evoked similar rises in $[Ca^{2+}]_i$ in the presence and absence of HCO_3^-. In the presence of external

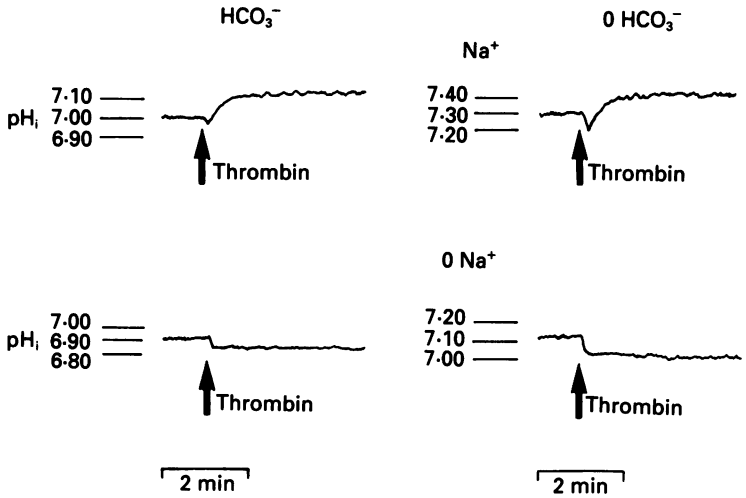

Fig. 2. Effect of Na^+ removal on pH_i responses evoked by thrombin (0·5 units/ml) in the presence and absence of HCO_3^-. The upper traces show responses in control medium and lower traces responses in medium in which Na^+ was completely replaced with NMDG. Traces on the left are from experiments conducted in 23 mM-HCO_3^-, those on the right in the absence of HCO_3^-. $[K^+]$ was 23 mM in all media (see Methods).

Ca^{2+}, $[Ca^{2+}]_i$ was elevated by 628 ± 30 nM (S.E.M., $n = 5$) in HCO_3^- and 611 ± 28 nM ($n = 5$) in HEPES. The lower traces of Fig. 1*B* show the optical density of the platelet suspension. There was no discernable difference in the shape change or aggregation evoked by thrombin in the presence or absence of HCO_3^-.

Effects of Na^+ *substitution on thrombin-evoked changes in* pH_i

Figure 2 shows the effects of Na^+ substitution in the presence and absence of HCO_3^-. On complete replacement of Na^+ with NMDG, basal pH_i fell significantly in both the presence and absence of HCO_3^- (Table 1). Thrombin-evoked acidification was slightly, but not significantly, increased in the absence of external Na^+, whilst the subsequent alkalinization was completely abolished in both the presence and absence of HCO_3^- (Table 1). To allow replacement of $NaHCO_3$ with $KHCO_3$, $[K^+]$ was elevated to 23 mM in control and Na^+-free media (see Methods). This manoeuvre did not significantly alter basal or thrombin-evoked changes in pH_i in the presence or absence of HCO_3^- (Table 1).

The effects on thrombin-evoked changes in pH_i of DIDS, which blocks the three known HCO_3^- transport systems in other cell types, were also tested. In HCO_3^--containing medium, 0·5 mM-DIDS slowed the rate of alkalinization following initial acidification evoked by thrombin (0·5 units/ml) from $0{\cdot}22 \pm 0{\cdot}03$ to $0{\cdot}12 \pm 0{\cdot}03$ units/min (S.E.M., both $n = 9$) (data not shown). The difference was

significant (paired Student t test, $0{\cdot}05 > P > 0{\cdot}02$). However, thrombin-evoked alkalinization was also significantly ($0{\cdot}01 > P > 0{\cdot}001$) slowed by 0·5 mM-DIDS in HEPES-buffered, HCO_3^--free medium from $0{\cdot}46 \pm 0{\cdot}09$ to $0{\cdot}16 \pm 0{\cdot}03$ pH units/min. Since similar effects of DIDS were seen in the presence and absence of HCO_3^-, these results suggest that the DIDS-induced slowing of

TABLE 1. Basal and thrombin-evoked changes in pH_i in the presence and absence of HCO_3^-

Medium	Basal pH_i	Initial fall in pH_i	Final change in pH_i	n
HCO_3^-	$7{\cdot}02 \pm 0{\cdot}04$	$-0{\cdot}03 \pm 0{\cdot}01$	$+0{\cdot}10 \pm 0{\cdot}01$	10
	*	***	n.s.	
HEPES	$7{\cdot}15 \pm 0{\cdot}05$	$-0{\cdot}07 \pm 0{\cdot}01$	$+0{\cdot}08 \pm 0{\cdot}01$	10
23 mM-K^+/HCO_3^-	$7{\cdot}07 \pm 0{\cdot}03$	$-0{\cdot}02 \pm 0{\cdot}00$	$+0{\cdot}10 \pm 0{\cdot}01$	14
	**	n.s.	****	
0 Na^+/HCO_3^-	$6{\cdot}92 \pm 0{\cdot}05$	$-0{\cdot}04 \pm 0{\cdot}01$	$-0{\cdot}03 \pm 0{\cdot}01$	14
23 mM-K^+/HEPES	$7{\cdot}28 \pm 0{\cdot}05$	$-0{\cdot}10 \pm 0{\cdot}01$	$+0{\cdot}08 \pm 0{\cdot}02$	14
	*	n.s.	****	
0 Na^+/HEPES	$7{\cdot}13 \pm 0{\cdot}05$	$-0{\cdot}12 \pm 0{\cdot}01$	$-0{\cdot}14 \pm 0{\cdot}02$	14

n.s. = not significantly different ($P > 0{\cdot}05$); * = $0{\cdot}05 > P > 0{\cdot}02$; ** = $0{\cdot}02 > P > 0{\cdot}01$; *** = $0{\cdot}01 > P > 0{\cdot}001$; **** = $P < 0{\cdot}001$.

alkalinization is due to an action or actions other than on HCO_3^- transport. Somewhat surprisingly, 0·1 mM-DIDS increased the rate of alkalinization evoked by thrombin (0·5 units/ml) from $0{\cdot}20 \pm 0{\cdot}03$ to $0{\cdot}34 \pm 0{\cdot}03$ (both $n = 6$) pH units/min in the presence of HCO_3^- and from $0{\cdot}29 \pm 0{\cdot}05$ to $0{\cdot}44 \pm 0{\cdot}07$ (both $n = 6$) pH units/min in the absence of HCO_3^-. The rate of recovery from an intracellular acidification evoked by the addition of 50 mM-sodium butyrate in the presence of HCO_3^- was also increased by the addition of DIDS (0·5 mM) from $0{\cdot}02 \pm 0{\cdot}00$ ($n = 9$) to $0{\cdot}03 \pm 0{\cdot}00$ ($n = 8$) pH units/min. At present we can offer no explanation for the acceleration of alkalinization by the lower concentration of DIDS.

Changes in pH_i evoked by platelet activating factor, ADP and phorbol ester

Figure 3 shows changes in pH_i evoked by ADP, platelet activating factor (PAF) and phorbol dibutyrate in HCO_3^--containing medium in the presence or absence of external Ca^{2+}. Figure 3*A* shows, in this batch of cells, the usual biphasic pH_i response to thrombin. Figure 3*B* and 3*C* show that in response to PAF (20 ng/ml), an initial acidification was followed by a slow recovery in pH_i to around the basal level; following ADP (20 μM), pH_i was still below basal levels 3 min after stimulation. Figure 3*D* shows that phorbol dibutyrate evoked a slow rise in pH_i which was not preceded by a measurable acidification. Changes in pH_i evoked by all four agonists were not significantly different in the presence or absence of external Ca^{2+} (Table 2).

Stopped-flow kinetic measurements in the presence of bicarbonate

Figure 4 shows changes in BCECF and Fura-2 fluorescence evoked by thrombin (0·5 units/ml) or ADP (40 μM) in the presence of HCO_3^- and recorded by stopped-flow fluorimetry. Cells were stimulated in both the presence and absence of external Ca^{2+} and responses recorded with a sampling interval of 100 or 10 ms. The agonist-evoked falls in pH_i were variable in size. Acidification, when detectable, proceeded on a similar time course to agonist-evoked rises in $[Ca^{2+}]_i$. Thrombin-evoked alkalinization was markedly slower in onset.

Figure 4*A* shows that in the presence of external Ca^{2+}, the thrombin-evoked rise in $[Ca^{2+}]_i$, indicated by increased Fura-2 fluorescence, was delayed in onset by

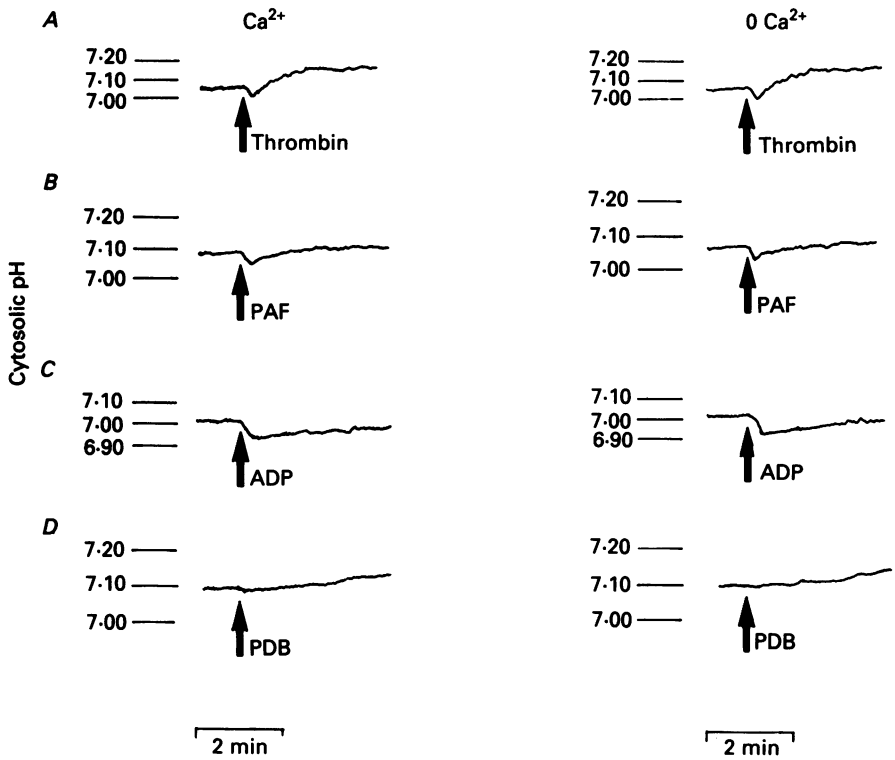

Fig. 3. Cytosolic pH responses evoked in the presence of HCO_3^- by: *A*, thrombin (0·5 units/ml), *B*, PAF, 20 μl/ml) *C*, ADP (20 μM) and *D*, phorbol dibutyrate (PDB; 200 nM). Traces on the left are from experiments conducted in the presence of 1 mM-external Ca^{2+} and those on the left in the absence of added Ca^{2+}.

TABLE 2. Changes in pH_i evoked by thrombin, PAF, ADP and phorbol dibutyrate (PDB) in the presence of HCO_3^-

Agonist	Ca_o^{2+}	Basal pH_i	Initial fall in pH_i	Final change in pH_i	n
Thrombin	+	7·04±0·03	−0·06±0·01	+0·14±0·01	16
(0·5 units/ml)	−	7·04±0·03	−0·05±0·01	+0·11±0·01	16
PAF	+	7·07±0·02	−0·04±0·01	+0·01±0·01	17
(20 ng/ml)	−	7·08±0·02	−0·05±0·01	0·00±0·01	17
ADP	+	7·03±0·03	−0·07±0·01	−0·04±0·01	16
(20 μM)	−	7·03±0·02	−0·07±0·01	−0·05±0·01	16
PDB	+	7·12±0·04	—	+0·04±0·01	13
(200 nM)	−	7·11±0·05	—	+0·04±0·01	14

0·57±0·3 s (S.E.M., $n = 8$) and peaked at 4·4±0·4 s ($n = 8$). The pH_i, indicated by BCECF fluorescence, did not rise back to the basal level until 6·0±0·3 s ($n = 25$) after stimulation, by which time $[Ca^{2+}]_i$ had peaked. In the absence of external Ca^{2+} (Fig. 4*B*), the onset of rise in $[Ca^{2+}]_i$ was delayed by 0·67±0·3 s ($n = 8$) and peaked at 3·9±0·5 s ($n = 8$), whilst pH_i did not rise back to the basal level until 5·7±0·6 s ($n = 8$) after stimulation.

Figure 4*C* shows that in the presence of external Ca^{2+}, ADP evoked a rise in $[Ca^{2+}]_i$ without measurable delay ($n = 5$). In the absence of external Ca^{2+} (Fig. 4*D*), the delay in onset of ADP-evoked rise in $[Ca^{2+}]_i$ was 210 ± 20 ms ($n = 5$). These Fura-2 signals are closely similar to those seen with these concentrations of thrombin and ADP in singly loaded cells in the absence of HCO_3^- (Sage & Rink, 1987). The small ADP-evoked acidifications, which were not followed by alkalinization, were not resolvable by the stopped-flow system and are not shown.

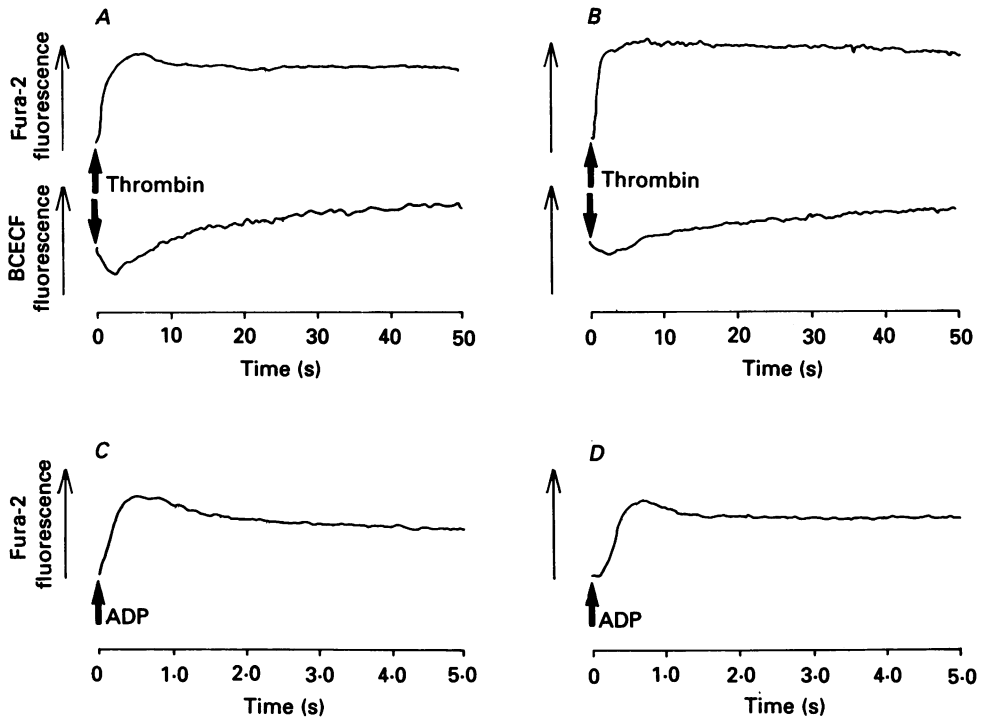

Fig. 4. Stopped-flow recordings. *A* and *B* show Fura-2 and BCECF fluorescence responses evoked by thrombin (0·5 units/ml) in the presence of 1 mM-external Ca^{2+} and HCO_3^- (*A*) or the absence of added Ca^{2+} (*B*). *C* and *D* show Fura-2 fluorescence responses evoked by 40 μM-ADP in the presence and absence of external Ca^{2+}. Note different time bases of thrombin and ADP traces.

DISCUSSION

These results provide the first reported measurements of $[Ca^{2+}]_i$ and pH_i in human platelets resuspended in media containing physiological concentrations of CO_2 and HCO_3^-. Fortunately, for us and the many other groups who have studied these variables in resting and stimulated platelets, we found no significant differences in the extent or kinetics of $[Ca^{2+}]_i$ rises evoked by thrombin, PAF or ADP in the presence and absence of HCO_3^-. Nor were the changes in pH_i caused by these agonists markedly affected, being modified mainly in the way expected by the additional cytosolic buffering capacity in HCO_3^--containing medium. We find no

evidence for an important role for HCO_3^--dependent H^+ transport in human platelets under the conditions and within the time frame of our experiments.

Basal pH_i and cytosolic buffering power in the presence and absence of bicarbonate

Basal pH_i was significantly higher in the absence than in the presence of HCO_3^-. A similar elevation in basal pH_i in the absence of HCO_3^- has recently been reported in embryonic chick somitic cells (Gillespie & Greenwell, 1988). This difference may partly be due to a rise in pH_i caused by the loss of CO_2 from cells when resuspended in HCO_3^--free, HEPES-buffered medium from platelet-rich plasma. In addition, the platelet plasma membrane may have an appreciable HCO_3^- conductance which would result in the expulsion of this ion from the cytosol under the influence of a negative membrane potential, equivalent to an entry of H^+. A small, progressive decline in basal pH_i was observed in all media, which was more pronounced in HCO_3^--free solutions. This may reflect a recovery following an elevation in pH_i, presumably a result of preparation of cells in the absence of CO_2. These results indicate that caution is required to allow recovery from disturbances in pH_i when changing solutions and that basal pH_i may be elevated under the unphysiological conditions of absence of HCO_3^-. Our results suggest that HCO_3^--dependent transport processes are not required for maintaining basal pH_i at levels far higher than electrochemical equilibrium in platelets; this contrasts with some other cells, in which pH_i is higher in the presence of HCO_3^- than in its absence (Ganz *et al.* 1987; Gillespie & Greenwell, 1988).

The absence of HCO_3^- significantly reduced cytosolic buffering power. Hence many earlier reports may have overestimated changes in pH_i resulting from the activation of cells under unphysiological conditions.

Thrombin-evoked changes in pH_i in the presence and absence of bicarbonate

Thrombin evoked a small initial fall in pH_i followed by a sustained rise above basal levels in the presence and absence of HCO_3^-. Similar responses, in the absence of HCO_3^-, have been reported previously (e.g. Zavoico *et al.* 1986; Simpson & Rink, 1987; Siffert & Akkerman, 1987*a*; Zavoico & Cragoe, 1988). The thrombin-evoked acidification was twice as large in the absence of HCO_3^- as in its presence, reflecting the greater cytosolic buffering power in cells suspended in HCO_3^--containing medium. Siffert, Siffert, Scheid & Akkerman (1989) have recently claimed that agonist-evoked intracellular acidifications are an artifact of platelet shape change. The present data do not support this hypothesis, since the absence of HCO_3^- increased the thrombin-evoked intracellular acidification but had no measurable effect on shape change. The reported absence of thrombin-induced acidification in platelets which had already changed shape (Siffert *et al.* 1989) suggests that the cytoskeletal changes or an associated increased metabolism are the main cause of the acidification. The alternative idea that the elevation in $[Ca^{2+}]_i$ causes the fall in pH_i seems unlikely in view of the observation that ionophore-induced elevation of $[Ca^{2+}]_i$ in platelets had no detectable effect on pH_i (Simpson & Rink, 1987).

Thrombin-evoked alkalinization is believed to be mediated by the activation of Na^+–H^+ exchange via the production of diacylglycerol and the activation of protein kinase C (e.g. Siffert *et al.* 1987; Siffert, Fox, Muckenoff & Scheid, 1984; Zavoico

et al. 1986). In the present experiments, similar thrombin-evoked increases in pH_i were observed in the presence and absence of HCO_3^-. However, the initial rate of loss of proton equivalents was greater in HCO_3^--containing medium. This might partly reflect the lower pH_i in the presence of HCO_3^-, since the Na^+–H^+ exchanger is enhanced by lowered pH_i; however, another role for HCO_3^- in speeding proton efflux cannot be ruled out.

Replacement of external Na^+ with NMDG resulted in a fall in basal pH_i in both the presence and absence of HCO_3^- (Table 1). Replacement of Na^+ slightly, but not significantly, increased the thrombin-evoked acidification in both the presence and absence of HCO_3^-. There was no recovery following acidification in Na^+-free medium with or without HCO_3^-, confirming the Na^+ dependence of the alkalinization. Our results contrast with those of Ozaki *et al.* (1989), who reported (i) that the presence of 10 mM-HCO_3^- enhanced thrombin-induced alkalinization and more strikingly, (ii) that the addition of 10 mM-HCO_3^- to Na^+-free medium could restore a thrombin-evoked elevation of pH_i. We do not understand the basis of the apparent contradiction between those results and those of the present study. However, we note that Ozaki *et al.* (1989) used Tris rather than HEPES buffer and that they give no details of their control of P_{CO_2} and external pH in HCO_3^--containing media. These authors could not explain their data in terms of Na^+-coupled HCO_3^- transport since the effects occurred in Na^+-free media; thus they appear to favour a role for 'anion channels'.

Thrombin-evoked rises in $[Ca^{2+}]_i$ were the same in the presence and absence of HCO_3^-, indicating that the observed differences in basal pH_i were without effect on the mechanisms gating Ca^{2+} fluxes.

Comparison of changes in pH_i evoked by thrombin, platelet activating factor, ADP and phorbol ester

Thrombin, PAF and ADP all evoked an early fall in pH_i in HCO_3^--containing medium, in the presence or absence of external Ca^{2+}. Subsequent recovery of pH_i varied between agonists. Only thrombin evoked a significant elevation in pH_i above basal levels. After PAF-evoked acidification, pH_i returned slowly to the basal level whilst after stimulation by ADP, pH_i was still below basal levels 3 min after stimulation. Recovery was not dependent on external Ca^{2+}.

Phorbol dibutyrate evoked a slow alkalinization in the presence and absence of external Ca^{2+}, confirming previous reports that Na^+–H^+ exchange is activated by protein kinase C (Zavoico *et al.* 1986; Siffert *et al.* 1987). The lack of acidification following stimulation by phorbol esters may be correlated with the lack of shape change evoked by these agents (e.g. Rink, Sanchez & Hallam, 1983). Physiologically, protein kinase C is activated by diacylglycerol, a product of the agonist-evoked hydrolysis of phosphatidylinositol bisphosphate. Thrombin is a more powerful stimulus for phosphatidylinositol bisphosphate hydrolysis than is PAF, whilst ADP is reported to evoke much less, or no, hydrolysis of it (Fisher, Bakshian & Baldassare, 1985; Daniel, Dangelmaier, Selak & Smith, 1986). The ability of thrombin alone to stimulate a sustained cytoplasmic alkalinization in platelets most probably reflects the greater effectiveness of this agonist in promoting a sustained production of diacylglycerol. The weak effect of PAF in promoting Na^+–H^+ exchange mirrors the

weak effect of this agonist in promoting Ca^{2+} efflux under conditions where thrombin or phorbol ester markedly speed Ca^{2+} extrusion (Rink & Sage, 1987). The much slower rises in pH_i evoked by PAF and ADP, which do not exceed basal levels, may reflect activation of H^+ extrusion by the fall in pH.

Relationship between changes in pH_i and $[Ca^{2+}]_i$

Siffert & Akkerman (1987*a*) have suggested that a rise in pH_i is a prerequisite for Ca^{2+} mobilization in platelets. Their argument is based on their finding that replacement of external Na^+ can suppress thrombin-evoked discharge of internal Ca^{2+} stores and slows and reduces Ca^{2+} influx. We have reported previously, however, that Na^+ replacement with choline has no significant effect on Ca^{2+} influx or release from internal stores evoked by thrombin or PAF (Sage & Rink, 1986). These conflicting results are not easily explained, although there were some differences in the methods of platelet preparation (Rink, 1987). Siffert & Akkerman (1987*b*) have also stated that thrombin-evoked Ca^{2+} mobilization is pH sensitive only at low doses of thrombin. A similar sensitivity of thrombin-evoked responses to Na^+ removal has been reported by Connolly & Limbird (1983), who attributed the effect to a requirement for Na^+ in the generation of thromboxane A_2, itself a stimulus to hydrolysis of phosphatidylinositol bisphosphate and the elevation of $[Ca^{2+}]_i$ in platelets (Hallam, Rink & Sanchez, 1983; Seiss, Siegel & Lapetina, 1983; Watson, Reep, McConnell & Lapetina, 1985). It has since been suggested that low doses of thrombin, as well as the weaker platelet agonists ADP and adrenaline, stimulate arachidonic acid release and so thromboxane A_2 production by evoking Na^+–H^+ exchange which in turn activates a phospholipase A_2 (Sweatt, Blair, Cragoe & Limbird, 1986; Sweatt, Connolly, Cragoe & Limbird, 1986). Our results do not indicate any essential role for such a mechanism, since all the data were obtained using platelets in which cyclooxygenase, and so production of thromboxane A_2, was blocked by prior treatment with aspirin.

Strong evidence against an essential role for an elevation in pH_i in platelet activation also comes from the present results. Only thrombin was found to evoke an eventual rise in pH_i, whilst after stimulation with PAF and ADP, pH_i remained below basal levels for several minutes. All three agonists are known to elevate $[Ca^{2+}]_i$ by stimulating both Ca^{2+} influx and the release of Ca^{2+} from intracellular stores (Rink, Smith & Tsien, 1982; Hallam, Sanchez & Rink, 1984; Hallam & Rink, 1985). Further, we believe compelling, evidence against a causal role for elevation of pH_i in agonist-evoked rises in $[Ca^{2+}]_i$ comes from the stopped-flow studies. In both the presence and absence of external Ca^{2+}, the thrombin-evoked rise in $[Ca^{2+}]_i$, as indicated by Fura-2 fluorescence, was under way at least 5 s before pH_i, as indicated by BCECF fluorescence, exceeded basal values. In fact, in both the presence and absence of external Ca^{2+}, thrombin-evoked rises in $[Ca^{2+}]_i$ had peaked before pH_i was above basal levels. We have reported previously that ADP, in the presence of external Ca^{2+}, evokes rises in $[Ca^{2+}]_i$ without measurable delay, whilst in the absence of external Ca^{2+} ADP-evoked rises in $[Ca^{2+}]_i$ are delayed by only 200 ms (Sage & Rink, 1987). Here we have demonstrated similar ADP-evoked rises in $[Ca^{2+}]_i$ in the presence of HCO_3^-. These rapid Ca^{2+} signals are generated during the period of agonist-evoked cytoplasmic acidification. It has previously been reported that

changes in pH_i imposed by addition of NH_4Cl, CO_2 or nigericin do not significantly alter $[Ca^{2+}]_i$ (Simpson & Rink, 1987).

We therefore conclude that an elevation in cytoplasmic pH_i is not a *requirement* for the mobilization of Ca^{2+} in platelets. Agonist-evoked alkalinization, when present, may, however, serve to protect the cell from severe cytosolic acidification following a rapid increase in metabolic activity upon activation, as previously suggested (Simpson & Rink, 1987). In our hands, thrombin, but not PAF or ADP, evokes significant elevation in pH_i by activating Na^+–H^+ exchange. All three agonists, however, mobilize internal Ca^{2+} (e.g. Hallam *et al.* 1984; Hallam & Rink, 1985; Rink & Sage, 1987). The role of agonist-evoked alkalinization, if any, does not thus appear to be *regulation* of $[Ca^{2+}]_i$. Even a role for changes in pH_i in *modulating* Ca^{2+} fluxes, for example by enhancing inositol trisphosphate-evoked discharge of the internal Ca^{2+} stores (Brass & Joseph, 1985), seems unlikely in view of the relative time courses revealed by stopped-flow fluorimetry.

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REFERENCES

ALONSO, M. T., COLLAZOS, J. M. & SANCHEZ, A. (1988). Thrombin-induced cytoplasmic alkalinization in human platelets is not a prerequisite for Ca mobilization. *Journal of Physiology* **396**, 45*P*.

BORN, G. V. R. (1970). Observations on the change in shape in blood platelets brought about by adenosine diphosphate. *Journal of Physiology* **209**, 487–511.

BORON, W. F. (1986). Intracellular pH regulation in epithelial cells. *Annual Review of Physiology* **48**, 377–388.

BRASS, L. F. & JOSEPH, S. K. (1985). A role for inositol trisphosphate in intracellular Ca^{2+} mobilisation and granule secretion in platelets. *Journal of Biological Chemistry* **260**, 15172–15179.

CONNOLLY, T. M. & LIMBIRD, L. E. (1983). Removal of extraplatelet Na^+ eliminates indomethacin-sensitive secretion from human platelets stimulated by epinephrine, ADP and thrombin. *Proceedings of the National Academy of Sciences of the USA* **80**, 5320–5324.

DANIEL, J. L., DANGELMAIER, C. A., SELAK, M. & SMITH, J. B. (1986). ADP stimulates IP_3 formation in human platelets. *FEBS Letters* **206**, 299–303.

FISHER, G. J., BAKSHIAN, S. & BALDASSARE, J. J. (1985). Activation of human platelets by ADP causes a rapid rise in cytosolic free Ca^{2+} without hydrolysis of phosphatidylinositol-4,5-bisphosphate. *Biochemical and Biophysical Research Communications* **129**, 958–964.

GANZ, M. B., BOYARSKY, G., BORON, W. F. & STERZEL, R. B. (1987). pH regulation in cultured glomerular mesangial cells. *Federation Proceedings* **46**, 1285.

GANZ, M. B., BOYARSKY, G., STERZEL, R. B. & BORON, W. F. (1989). Arginine vasopressin enhances pH_i regulation in the presence of HCO_3^- by stimulating three acid–base transport systems. *Nature* **337**, 648–651.

GILLESPIE, J. I. & GREENWELL, J. R. (1988). Changes in intracellular pH and pH regulating mechanisms in somitic cells of the early chick embryo: a study using fluorescent pH-sensitive dye. *Journal of Physiology* **405**, 385–395.

GRINSTEIN, S., ROTIN, D. & MASON, M. J. (1989). Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochimica et biophysica acta* **988**, 73–97.

HALLAM, T. J. & RINK, T. J. (1985). Responses to adenosine diphosphate in human platelets loaded with the fluorescent calcium indicator quin2. *Journal of Physiology* **368**, 131–146.

HALLAM, T. J., RINK, T. J. & SANCHEZ, A. (1983). Effects of arachidonate and thromboxane A_2 analogue on human platelets studied with a fluorescent indicator for cytoplasmic free calcium. *Journal of Physiology* **343**, 96*P*.

HALLAM, T. J., SANCHEZ, A. & RINK, T. J. (1984). Stimulus–response coupling in human platelets.

Changes evoked by platelet activating factor in cytoplasmic free Ca^{2+} monitored with the fluorescent calcium indicator quin2. *Biochemical Journal* **218**, 819–827.

HORNE, W. C., NORMAN, N. E., SCHWARTZ, D. B. & SIMONS, E. R. (1981). Changes in cytoplasmic pH and in membrane potential in thrombin-stimulated human platelets. *European Journal of Biochemistry* **120**, 295–302.

KETTERMANN, H. & SCHLUE, W-R. (1988). Intracellular pH regulation in cultured mouse oligodendrocytes. *Journal of Physiology* **406**, 147–162.

OZAKI, Y., YATOMI, Y., KARIYA, T. & KUME, S. (1989). Anion channels contribute to the regulation of intracellular pH in human platelets. *Thrombosin Research* **53**, 221–230.

POLLOCK, W. K., RINK, T. J. & IRVINE, R. F. (1986). Liberation of [^{3}H]arachidonic acid and changes in cytoplasmic free calcium in fura-2-loaded human platelets stimulated by ionomycin and collagen. *Biochemical Journal* **235**, 869–877.

RINK, T. J. (1987). Intracellular pH and cytoplasmic free Ca^{2+}. *Nature* **327**, 375–376.

RINK, T. J. & HALLAM, T. J. (1984). What turns platelets on? *Trends in Biochemical Sciences* **9**, 215–219.

RINK, T. J. & POZZAN, T. (1985). Using quin2 in cell suspensions. *Cell Calcium* **6**, 133–144.

RINK, T. J. & SAGE, S. O. (1985). Stopped-flow fluorescence measurements of fura-2-loaded human platelets. *Journal of Physiology* **369**, 115*P*.

RINK, T. J. & SAGE, S. O. (1987). Stimulated calcium efflux from fura-2-loaded human platelets. *Journal of Physiology* **393**, 513–524.

RINK, T. J., SANCHEZ, A. & HALLAM, T. J. (1983). Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature* **305**, 317–319.

RINK, T. J., SMITH, S. W. & TSIEN, R. Y. (1982). Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca^{2+}-independent activation for shape change and secretion. *FEBS Letters* **148**, 21–26.

RINK, T. J., TSIEN, R. Y. & POZZAN, T. (1982). Cytoplasmic pH and free Mg^{2+} in lymphocytes. *Journal of Cell Biology* **95**, 189–196.

SAGE, S. O. & RINK, T. J. (1986). Effects of ionic substitution on $[Ca^{2+}]_i$ rises evoked by thrombin and PAF in human platelets. *European Journal of Pharmacology* **128**, 99–107.

SAGE, S. O. & RINK, T. J. (1987). The kinetics of changes in intracellular calcium concentration in fura-2-loaded human platelets. *Journal of Biological Chemistry* **262**, 16364–16369.

SIESS, W., SIEGEL, F. L. & LAPETINA, E. G. (1983). Arachidonic acid stimulates the formation of 1,2-diacylglycerol and phosphatidic acid in human platelets. *Journal of Biological Chemistry* **258**, 11236–11242.

SIFFERT, W. & AKKERMAN, J. W. N. (1987*a*). Activation of sodium–proton exchange is a prerequisite for Ca^{2+} mobilisation in human platelets. *Nature* **325**, 456–458.

SIFFERT, W. & AKKERMAN, J. W. N. (1987*b*). Intracellular pH and cytoplasmic free Ca^{2+}. *Nature* **327**, 376.

SIFFERT, W., FOX, G., MUCKENHOFF, K. & SCHEID, P. (1984). Thrombin stimulates Na^+–H^+ exchange across the human platelet plasma membrane. *FEBS Letters* **172**, 272–274.

SIFFERT, W., SIFFERT, G. & SCHEID, P. (1987). Activation of Na^+/H^+ exchange in human platelets stimulated by thrombin and phorbol ester. *Biochemical Journal* **241**, 301–303.

SIFFERT, W., SIFFERT, G., SHEID, P. & AKKERMAN, J. W. N. (1989). Activation of Na^+/H^+ exchange and Ca^{2+} mobilisation start simultaneously in thrombin-stimulated platelets. Evidence that platelet shape change disturbs early rises of BCECF fluorescence which causes an underestimation of actual cytosolic alkalinisation. *Biochemical Journal* **258**, 521–527.

SIMPSON, A. W. M. & RINK, T. J. (1987). Elevation of pH_i is not an essential step in calcium mobilisation in fura-2-loaded human platelets. *FEBS Letters* **222**, 144–148.

SWEATT, J. D., BLAIR, I. A., CRAGOE, E. J. & LIMBIRD, L. E. (1986). Inhibitors of Na^+/H^+ exchange block epinephrine- and ADP-induced stimulation of human platelet phospholipase C by blockade of arachidonic acid release at a prior step. *Journal of Biological Chemistry* **261**, 8660–8666.

SWEATT, J. D., CONNOLLY, T. M., CRAGOE, E. J. & LIMBIRD, L. E. (1986). Evidence that Na^+/H^+ exchange regulates receptor-mediated phospholipase A_2 activation in human platelets. *Journal of Biological Chemistry* **261**, 8667–8673.

SZATKOWSKI, M. S. & THOMAS, R. C. (1986). New method for calculating pH_i from accurately measured changes in pH_i induced by a weak acid and base. *Pflügers Archiv* **407**, 59–63.

THOMAS, J. A., BUCHSBAUM, R. N., ZIMNIAK, A. & RACKER, E. (1979). Intracellular pH measurements in Ehrlich ascites tumour cells utilising spectroscopic probes generated in situ. *Biochemistry* **18**, 2210–2218.

THOMAS, R. C. (1984). Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. *Journal of Physiology* **354**, 3–22*P*.

THOMAS, R. C. (1989). Bicarbonate and pH_i response. *Nature* **337**, 601.

WATSON, S. P., REEP, B., MCCONNELL, R. T. & LAPETINA, E. G. (1985). Collagen stimulates [^{3}H]inositol trisphosphate formation in indomethacin-treated human platelets. *Biochemical Journal* **226**, 831–837.

ZAVOICO, G. B. & CRAGOE, E. J. (1988). Ca^{2+} mobilisation can occur independent of acceleration of Na^+/H^+ exchange in thrombin-stimulated human platelets. *Journal of Biological Chemistry* **263**, 9635–9639.

ZAVOICO, G. B., CRAGOE, E. J. & FEINSTEIN, M. B. (1986). Regulation of intracellular pH in human platelets. Effects of thrombin, A23187 and ionomycin and evidence for activation of Na^+/H^+ exchange and its inhibition by amiloride analogues. *Journal of Biological Chemistry* **261**, 13160–13167.