Volume 222, number 1, 144–148 FEB 05144 September 1987

Elevation of pH_i is not an essential step in calcium mobilisation in fura-2-loaded human platelets

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Received 29 July 1987

Human platelets were co-loaded with the fluorescent indicators BCECF and fura-2 to mesure pH_i and $[Ca^{2+}]_i$ and incubated with aspirin to block cyclooxygenase. Either pH_i and shape change and aggregation or pH_i and $[Ca^{2+}]_i$ were measured simultaneously in the same stirred cuvette, at 37°C. In Hepes-buffered saline containing 1 mM Ca^{2+}, mean resting pH_i was 6.98 ± 0.01 (SE, $n = 59$). Changes of pH_i up to ± 0.35 units, imposed by additions of NH_4Cl, CO_2 or nigericin, produced no shape change or aggregation and only insignificant changes in $[Ca^{2+}]_i$. Sufficient thrombin to raise $[Ca^{2+}]_i$ over 1 μM and cause rapid shape change and aggregation increased pH_i by no more than 0.05 units, and the increase in pH_i lagged behind the elevation of $[Ca^{2+}]_i$. We conclude that changes in pH_i do not form a necessary or sufficient component of the pathways leading to receptor-mediated Ca^{2+} mobilisation or the stimulation of shape change or aggregation.

Platelet; pH_i; Ca^{2+}; Fura-2; BCECF

1. INTRODUCTION

There is general agreement that Ca^{2+}, inositol phosphates, diacylglycerol and cAMP have important second messenger functions in platelets (e.g. [1]) as they do in many cell types. Stimulus-evoked elevations of pH_i have also been seen in many cell types and a number of reports have described small changes in pH_i or H^+ extrusion in activated platelets and have demonstrated that thrombin or activators of C-kinase can promote Na^+-H^+ exchange (e.g. [2–4]). However, there is disagreement as to whether the evoked changes in pH_i play a causal role in activation. For instance, Zavoico et al. [4] concluded that the changes in pH_i could be dissociated from the mobilisation of Ca^{2+} in platelets, while Siffert and Ackkerman [5] have recently proposed that Na^+-H^+ exchange and elevation of pH_i "is a prerequisite of Ca mobilisation".

This paper reports experiments which examined the relationship between pH_i and three easily monitored early events in platelet activation, i.e. elevated $[Ca^{2+}]_i$, shape change and aggregation. We used human platelets co-loaded with fura-2 [6] and BCECF [7] so that $[Ca^{2+}]_i$ and pH_i could be monitored simultaneously by using dual-wavelength excitation. Combining fluorescence measurement with absorbance [8] provided a simultaneous measurement of pH_i with shape change and aggregation. Since the presence of Na^+-H^+ exchange in platelets is well established, as is the ability of thrombin and phorbol ester to activate this exchange [3,4], we did not make analysis of this mechanism a major focus of the work. The main objectives were: to impose changes of pH_i to see if this evoked Ca^{2+} mobilisation or functional responses; to impose changes in $[Ca^{2+}]_i$to determine whether this changed pH_i; and to observe pH_i, $[Ca^{2+}]_i$ and function in response to agonists

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Published by Elsevier Science Publishers B.V. (Biomedical Division)

acting at surface receptors. In view of the previously reported influence of external Na^+ and of inhibitors of Na^+-H^+ exchange on arachidonate release and TxA_2 formation [9], the cells were pretreated with aspirin to prevent formation of prostaglandins and thromboxane.

2. MATERIALS AND METHODS

2.1. *Preparation of platelets*

Washed human platelets were prepared from blood freshly drawn from volunteers who gave informed consent, as described [8]. The washed platelet suspension (1×10^9 cells/ml) was incubated with 0.4 μM fura-2 acetoxy methyl ester, and 2.0 μM BCECF acetoxy methyl ester and 100 μM aspirin for 45 min at 37°C. After incubation, 15 μl/ml acid-citrate dextrose stock [8] was added to the suspension to prevent activation of the platelets during the subsequent centrifugation at $350 \times g$ for 15 min. The pellets were resuspended in a solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM dextrose, 20 μg/ml apyrase, 0.05 U/ml hirudin and 10 mM Hepes (pH 7.40) at 37°C. The final cell count was approx. 1.5×10^8 cells/ml. The platelet suspensions were kept at room temperature until required. In the isotonic K^+ solution, 140 mM KCl replaced NaCl and the Hepes was neutralised with KOH instead of NaOH.

2.2. *Experimental procedures*

Fluorescence was monitored simultaneously with absorbance in an Aminco Bowman spectrofluorimeter specially modified as in [8]. The simultaneous monitoring of BCECF and fura-2 signals was carried out in a Spex Flurolog dual-excitation fluorimeter; the excitation wavelength used for BCECF was 490 nm and for fura-2 340 nm, the emission being monitored at 520 nm. The fura-2 fluorescence signal was calibrated as described by Pollock et al. [10]. The BCECF signals were calibrated essentially according to Rink et al. [7]. The amount of dye that had leaked from the cells was calculated from the immediate shift in signals seen on rapidly changing the pH of the suspension by approx. 0.5 pH units. At the end of the experiment the platelets were lysed with 50 μM digitonin and the signal recorded while the pH was varied stepwise between pH 6 and 8. We also corrected the signals for the spectral shift which BCECF undergoes on loading into an intracellular environment [7]. To do this platelets were resuspended in isotonic K^+ buffer and treated with 2 μg/ml nigericin thus allowing the external pH to set pH_i. The external pH was then decreased stepwise from pH 8 to pH 6 and the signal recorded. The cells were then lysed and the pH increased stepwise back to pH 8. The two calibrations, intracellular and post-lysis, were then plotted and the difference between the two calibration curves showed that the signal in the digitonin lysate underestimated pH_i by approx. 0.15 units. The pH_i values presented in this paper have all been appropriately corrected from this analysis. In each experiment 0.8-ml aliquots of the suspension were allowed to equilibrate in the fluorimeter for a few minutes at 37°C; then 1 mM $CaCl_2$ and 1 mg/ml fibrinogen were added and the suspension was continuously stirred unless otherwise indicated. Thrombin and ADP were added from saline. Nigericin, TPA and ionomycin were added from DMSO; DMSO never exceeded 0.2% (v/v). NH_4Cl was added from 1 M stock. pCO_2 was increased by addition of 8 μl of 1 M $NaHCO_3$ to which excess dry ice had been added, to give a final concentration of 10 mM $NaHCO_3$ and an external pH of 7.50. The pCO_2 in the cuvette could therefore be calculated from the Henderson-Hassellbach equation to be 12 mmHg.

The results shown in the figures or quoted in the text are representative of those of at least three similar trials with different batches of cells.

2.3. *Materials*

Fura-2-AM was obtained from Molecular Probes (Eugene, OR); BCECF-AM from the Research Development Corp., Hospital for Sick Children; ionomycin from Calbiochem; and nigericin, TPA, thrombin, hirudin, apyrase and fibrinogen from Sigma.

3. RESULTS AND DISCUSSION

3.1. *Resting pH_i and $[Ca^{2+}]_i$*

Resting pH_i measured under the conditions of these experiments was 6.98 ± 0.01 (SE) based on 59 determinations in 11 different experiments. This value is typical for mammalian cells at 37°C (see e.g. [11]) and is close to that previously reported in

BCECF-loaded human platelets [4,5]. The mean measured resting $[Ca^{2+}]_i$ in these experiments was 101 ± 6 nM (n = 30). This is in the range previously reported for quin-2- or fura-2-loaded platelets [1,10].

3.2. *Imposed changes of pH_i*

Fig.1 shows typical responses to addition of 10 mM NH_4Cl, 0.2 μg/ml nigericin and 12 mmHg pCO_2. NH_4Cl produced an abrupt increase of pH_i which ranged from 0.17 to 0.3 units in different preparations and was maintained for several minutes. Nigericin gave the acid shift expected from the induced exchange of intracellular K^+ for external H^+. Under the conditions of these experiments pH_i returned only slowly, part way to the basal value. As shown by previous investigators (e.g. [4]), subsequent addition of thrombin or TPA could promote a more rapid restoration of pH_i which was dependent on external Na^+ and blocked by 100 μM amiloride (not shown). Fig.1 also shows that increasing pCO_2 gave the expected fall in pH_i, despite a small increase of 0.1 unit in external pH (see section 2). These results show that BCECF was giving a satisfactory indication of cytosolic pH. In fig.1A, absorbance was monitored simultaneously as an index of shape change and aggregation, and clearly neither imposed acidification nor imposed alkalinization gave any measurable response. Similarly, fig.1B shows that the changes in pH_i produce no significant changes in $[Ca^{2+}]_i$ simultaneously monitored from the fura-2 signals. The tiny change in signal seen with NH_4Cl and nigericin indicates alterations in $[Ca^{2+}]_i$ of no more than 15–20 nM. The result obtained with nigericin further suggests that there is very little dischargeable free Ca^{2+} in the mitochondria since this ionophore is a highly effective uncoupling agent.

3.3. *Effects of thrombin, ionomycin and ADP*

Fig.2 shows pH_i responses to three agents known to activate platelets in different ways. Thrombin acts at specific surface receptors and stimulates hydrolysis of phosphatidylinositol bisphosphate, formation of diacylglycerol and the rapid entry and internal release of Ca^{2+} [1]. Fig.2A confirms that thrombin promotes rapid shape change and aggregation; however, the associated

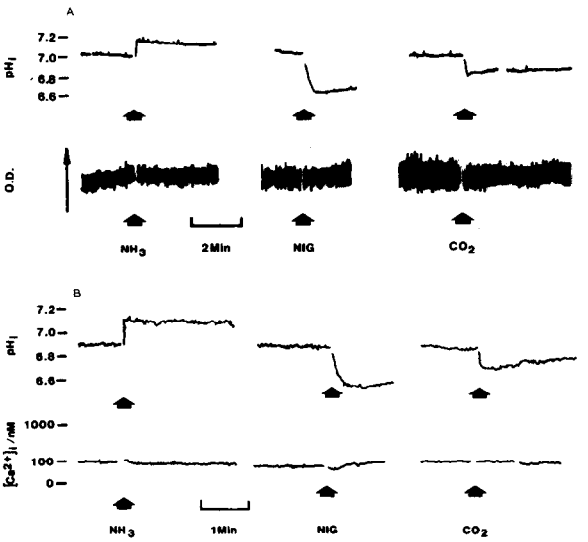

Fig.1. Representative traces showing the effects of changing pH_i on either (A) absorbance or (B) $[Ca^{2+}]_i$, measured simultaneously with pH_i in the same stirred cuvette. 10 mM NH_4Cl (NH_3), 0.2 μg/ml nigericin (NIG) and 10 mM HCO_3^- with 12 mmHg pCO_2 (CO_2) were added to perturb pH_i. (NH_4Cl addition had no measurable effect on pH_o; addition of the HCO_3^-/CO_2 buffer caused a rise in pH_o of ~0.1 unit.)

pH_i changes were extremely small. There was a barely measurable, but consistently seen, initial fall of pH_i of ~0.02 units. This was followed by a slower increase of pH_i to between 0.025 and 0.05 units above the initial level. This pattern of response is similar to, but a little less extensive than, that reported by Zavoico et al. [4] for BCECF-loaded platelets stimulated by thrombin at room temperature. We suppose that the initial fall reflects an increased metabolic production of acid, and that the rise is due to some stimulation of Na^+-H^+ exchange. The main point is that the net result is a remarkably stable pH_i following thrombin stimulation. The first pair of traces in fig.2B confirms that under our experimental conditions thrombin evoked the well-known rapid increase in $[Ca^{2+}]_i$ to over 1 μM which peaked before pH_i had risen above the basal level. A similar rapid increase in $[Ca^{2+}]_i$ with either no measurable change in pH_i or a very small decrease was also seen with ADP (see fig.2) and the thromboxane mimetic U46619 (not shown). Fig.2B also shows that the Ca^{2+} ionophore ionomycin rapidly increased $[Ca^{2+}]_i$ and

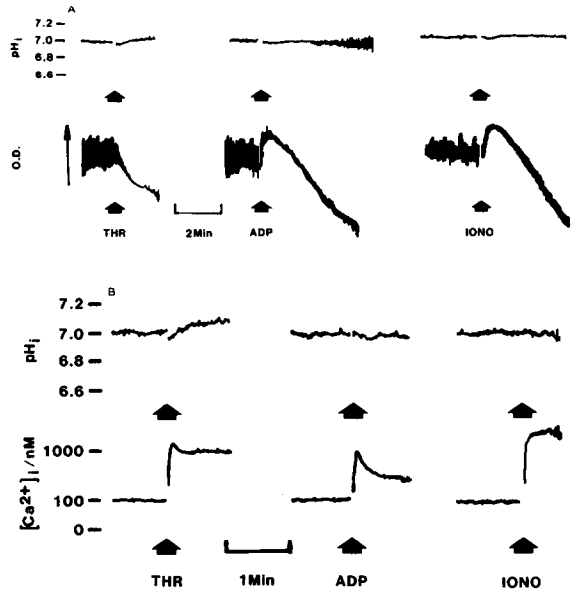

Fig.2. Representative traces showing the effects of the agonists thrombin (0.5 U/ml), ADP (20 μM) and ionomycin (100 nM) on (A) pH_i and absorbance and (B) pH_i and $[Ca^{2+}]_i$.

evoked shape change and aggregation, but caused no shift in pH_i. TPA caused the expected slow aggregation in the absence of any rise in $[Ca^{2+}]_i$ [13]; there was no measurable increase in pH_i under these conditions. In our experiments thrombin and TPA evoked distinct elevations in pH_i only when the cells had first been acidified by nigericin or increased pCO_2.

3.4. *Conclusions*

One of the main conclusions from our observations of platelet pH_i, and indeed of previous workers, is the remarkable stability of this physiological variable during the massive cellular upheaval of shape change and aggregation, which goes along with a 10–20-fold increase in $[Ca^{2+}]_i$. It is also evident from our data, and from previous work, that an increase in pH_i is neither necessary nor sufficient to evoke an increase in $[Ca^{2+}]_i$. An imposed increase of pH_i larger than that evoked by natural agonists evoked no increase in $[Ca^{2+}]_i$. Agonists such as thrombin and ADP which can elevate $[Ca^{2+}]_i$ within a fraction of a second gave only small rises in pH_i which occurred after $[Ca^{2+}]_i$ was declining towards resting levels. We have previously shown that complete replacement of external Na^+ by choline has no effect on the extent of the $[Ca^{2+}]_i$ rise produced by thrombin or by platelet-activating factor [12] and only a small effect on the time course of the increase. This result in itself refutes the hypothesis that Na^+-H^+ exchange is a prerequisite for Ca^{2+} mobilisation.

Notwithstanding this conclusion, there is evidently an effective Na^+-H^+ exchange system in human platelets which is activated by thrombin and TPA, presumably by a protein kinase C, but its relevance to platelet activation has yet to be elucidated. One possibility is that it serves to prevent a possibly damaging cytosolic acidification that may follow the metabolic burst associated with activation. One way to examine this idea would be to analyse the responsiveness of platelets when the cytosol has been acidified with pCO_2 or nigericin; possibly pCO_2 would be the better approach since nigericin has other effects including uncoupling mitochondria.

It is important to point out that our experiments were performed in cells pretreated with aspirin to obviate effects on thromboxane formation. The results obtained by Limbird and co-workers [9] clearly indicate that the removal of external Na^+ or treatment with amiloride can inhibit arachidonate release and thromboxane formation and thereby have effects on platelet responsiveness similar to those produced by cyclooxygenase inhibitors. Our reading of the results of Siffert and Ackkerman [5,13] suggests to us that they were misled by such effects into thinking that Na^+-H^+ exchange played a fundamental part in the mobilisation of Ca^{2+}. In fact their figures [5] show that higher concentrations of thrombin can give full responses in an Na^+-free medium, and they also show that the thrombin-induced elevation of pH_i is somewhat slower than the increase in $[Ca^{2+}]_i$.

ACKNOWLEDGEMENTS

We thank Mrs M. Bowden for help in preparing this manuscript and Dr J.E. Merritt for helpful comments.

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