Journal of Physiology (1991), **441**, *pp.* 559–573
With 6 figures
Printed in Great Britain

RESTING AND ADP-EVOKED CHANGES IN CYTOSOLIC FREE SODIUM CONCENTRATION IN HUMAN PLATELETS LOADED WITH THE INDICATOR SBFI

BY STEWART O. SAGE, TIMOTHY J. RINK* AND MARTYN P. MAHAUT-SMITH†

From the Physiological Laboratory, University of Cambridge, Downing Street, Cambridge, CB2 3EG

(*Received 28 August 1990*)

SUMMARY

1. Cytosolic free Na^+ concentration, $[Na^+]_i$, was investigated in human platelets loaded with the fluorescent indicator SBFI (sodium-binding benzofuran isophthalate).

2. SBFI fluorescence from platelet suspensions was measured at excitation wavelengths of 340 and 385 nm and the 340/385 nm fluorescence ratio was calibrated in terms of $[Na^+]_i$ *in situ*. $[Na^+]_i$ was set to known values by resuspending cells in media with various $[Na^+]$, in the presence of the Na^+–K^+ ionophore, gramicidin.

3. Basal free $[Na^+]_i$ was $5{\cdot}5 \pm 0{\cdot}3$ mM ($n = 50$). This is considerably lower than estimates of total platelet Na^+, suggesting that much intracellular Na^+ is sequestered or bound.

4. ADP (40 μM) evoked a rise in $[Na^+]_i$ from $6{\cdot}4 \pm 0{\cdot}7$ to $18{\cdot}3 \pm 1{\cdot}1$ mM ($n = 8$). The ADP-evoked rise in $[Na^+]_i$ was abolished when external Na^+ was replaced with *N*-methyl-D-glucamine. This indicates that the rise in $[Na^+]_i$ was due to Na^+ entry.

5. In platelets loaded with the fluorescent pH indicator, BCECF, 40 μM-ADP was shown to evoke a fall in cytosolic pH (pH_i) from $7{\cdot}21 \pm 0{\cdot}03$ to $7{\cdot}12 \pm 0{\cdot}03$ ($n = 10$). Three minutes after ADP addition pH_i had only recovered to $7{\cdot}15 \pm 0{\cdot}03$. The recovery was dependent on external Na^+, suggesting it was mediated by Na^+–H^+ exchange. However, this would only account for an increase in $[Na^+]_i$ of approximately 0·5 mM, indicating most of the ADP-evoked Na^+ entry occurred by other mechanisms.

6. Stopped-flow fluorimetry showed that the ADP-evoked rise in $[Na^+]_i$ commenced without measurable delay and peaked within 1 s. The initial kinetics were thus similar to those reported for ADP-evoked rises in $[Ca^{2+}]_i$.

7. Cell-attached patch-clamp recordings showed that ADP evoked single-channel inward currents when included in the pipette-filling solution. The currents were similar whether Ca^{2+} was present or absent from the pipette. The slope conductance

* Present address: Amylin Corporation, 9373 Towne Center Drive, Suite 250, San Diego, CA 92121, USA.

† Present address: Howard Hughes Medical Institute, Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093, USA.

MS 8756

was 11 pS in the presence of external Ca^{2+} and 10 pS in its absence. Current–voltage relationships were similar and the reversal potentials were close to 0 mV under both conditions.

8. SK & F 96365 (20 μM), a blocker of receptor-mediated Ca^{2+} entry in several non-excitable cells, blocked the ADP-evoked rise in $[Na^+]_i$. This compound has been shown to only partly block the biphasic ADP-evoked rise in $[Ca^{2+}]_i$, being selective for the fast, receptor-operated phase of entry.

9. These data suggest that ADP rapidly activates a channel in that platelet plasma membrane which is permeable to Na^+ and divalent cations.

INTRODUCTION

In recent years, the availability of fluorescent indicators for Ca^{2+} has led to intensive study of the role of this ion in the regulation and activation of many cell types, including platelets (Rink & Sage, 1990). However, one of the events first reported to be associated with platelet activation was an influx of Na^+, demonstrated isotopically (Feinberg, Sandler, Scorer, Le Breton, Grossman & Born, 1977) in cells stimulated by ADP, an important early activator during haemostatic plug formation (e.g. Born, Cusack, Darwin & McClure, 1986). This Na^+ influx has, however, received relatively little subsequent attention.

Agonists evoke a small depolarization of the platelet membrane, which appears to be Na^+-dependent since it is reversed in the absence of extracellular Na^+ (Pipili, 1985). This depolarization does not appear to be sufficient for activation since depolarization with high K^+ or the Na^+–K^+ ionophore gramicidin, are without effect (MacIntyre & Rink, 1982). Moreover, agonist-evoked responses are little affected by the replacement of external Na^+ by large non-permeant cations (Pipili, 1985; Sage & Rink, 1986). Na^+ influx appeared not to be essential for activation in several reports (Feinberg *et al.* 1977; Sandler, Le Breton & Feinberg, 1980; Pipili, 1985; Sage & Rink, 1986). However, an elevation in cytosolic $[Na^+]$ ($[Na^+]_i$) might still influence Na^+-dependent processes such as Na^+–H^+ exchange, which is involved in the regulation of platelet cytosolic pH (e.g. Zavoico, Cragoe & Feinstein, 1986; Siffert & Akkerman, 1988); and a recent report has raised again the possibility that Na^+ conductance may be important in ADP- (and thrombin-) induced responses in human platelets (Pales, Palacious-Araus, Lopez & Gual, 1989).

The mechanism by which ADP evokes Na^+ entry is unknown. Voltage-operated and epithelial-type Na^+ channels do not appear to be present in platelets, since tetrodotoxin and amiloride are without effect on membrane-potential or the small ADP-evoked depolarization (Pipili, 1985; Pales *et al.* 1989). The activation of Na^+–H^+ exchange following an initial ADP-evoked cytosolic acidification seems unlikely to make more than a small contribution to Na^+ entry, since there is only very slow pH recovery, at least in the presence of bicarbonate (Sage, Jobson & Rink, 1990*a*).

It is also unlikely that there is a significant contribution from Na^+–Ca^{2+} exchange. Removal of external Na^+ is without effect on basal or agonist-evoked changes in $[Ca^{2+}]_i$, or on resting or stimulated $^{45}Ca^{2+}$ efflux (Rink & Sage, 1987). Similarly, total platelet Ca^{2+} is unchanged when external Na^+ is removed (Brass, 1984). However,

the ADP-evoked rise in $[Na^+]_i$ could be associated with the rise in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) evoked by this agonist (Hallam & Rink, 1985).

Using stopped-flow fluorimetry, we have shown that ADP evokes a biphasic Ca^{2+} influx in human platelets (Sage, Reast & Rink, 1990*c*). The first component of the ADP response, which is activated without measurable delay, appears to be mediated by receptor-operated channels in the plasma membrane, which we have demonstrated using the patch-clamp technique (Mahaut-Smith, Sage & Rink, 1990*b*). These channels carry an inward cationic current which could be responsible for conducting an appreciable Na^+ entry. The delayed component of the ADP-evoked Ca^{2+} influx appears to be associated with the discharge of an intracellular Ca^{2+} store (Sage *et al.* 1990*c*). The nature of this second pathway, and its ionic selectivity, are unknown.

In an attempt to elucidate the mechanism of ADP-evoked Na^+ entry and to understand better agonist-evoked Ca^{2+} entry mechanisms in these cells, we have used the newly developed fluorescent Na^+ indicator, SBFI (sodium-binding benzofuran isophthalate) (Minta & Tsien, 1989), to make the first study of cytosolic free $[Na^+]$ in human platelets. SBFI has been shown to report $[Na^+]_i$ essentially independently of changes in cytosolic pH and $[K^+]_i$ in fibroblasts and lymphocytes (Harootunian, Kao, Eckert & Tsien, 1989), smooth muscle (Moore, Tsien, Minta & Fay, 1988) and gastric glands (Negulescu, Harootunian, Tsien & Machen, 1990). A preliminary account of some of these results has been presented (Sage, Mahaut-Smith & Rink, 1990*b*).

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 (25 mg ml^{-1}), citric acid (15 mg ml^{-1}) and D-glucose (20 mg ml^{-1}). This procedure gave a final whole blood citrate concentration of 22 mM and a pH of 6·5. Platelet-rich plasma (PRP) was prepared by centrifugation for 5 min at 700 *g* at room temperature and 100 μM-aspirin and 20 μg ml^{-1} apyrase were added.

Platelets were loaded with SBFI by incubation with 10 μM-SBFI AM (acetoxymethyl ester) for 1 h at 37 °C. The ester was dissolved in dimethylsulphoxide (DMSO) at a concentration of 5 mM and was mixed with an equal volume of 25% (w/v) Pluronic F-127 in DMSO before addition to PRP. Pluronic F-127 is a non-ionic surfactant which has been used to improve delivery of hydrophobic dyes including Fura-2 AM (Poenie, Alderton, Steinhardt & Tsien, 1986) and SFBI AM (Harootunian *et al.* 1989). Premixing with Pluronic greatly improved platelet loading with SBFI from the ester. The intracellular concentration of SBFI, estimated by comparing the fluorescence of a lysate of loaded cells with that of SBFI free acid added to a similar digitonin lysate of unloaded cells, was approximately 2 mM. After incubation, 0·6–0·8 ml aliquots of PRP were placed in 1·5 ml polypropylene tubes and centrifuged at 2900 *g* for 45 s in an MSE Micro Centaur centrifuge. As much of the supernatant as possible was removed and then the cells were resuspended in 1 ml of buffer of composition as required.

For determination of cytosolic pH (pH_i), cells were loaded with the fluorescent indicator BCECF by incubation of PRP for 45 min at 37 °C after the addition of 3 μM-BCECF AM. 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.

Measurement of $[Na^+]_i$

The cell suspension was placed in a cylindrical cuvette in a specially constructed thermostatted holder in a Perkin-Elmer MPF-44A spectrophotometer. The suspension could be stirred by means

of a magnetic stir bar. SBFI fluorescence, measured alternately at excitation wavelengths of 340 nm and 385 nm with emission at 500 nm, was recorded on a chart-recorder. The 340/385 nm excitation ratio was calculated after subtraction of the signal from external dye (determined by centrifugation to remove cells) and the autofluorescence (determined from unloaded cells) at each wavelength. Experimental aliquots of cells were resuspended in a HEPES-buffered saline of composition (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, 10 D-glucose, 1 $CaCl_2$. The HEPES was half neutralized by addition of 5 mM-NaOH, giving a pH of 7·4 at 37 °C. In some experiments, *N*-methyl-D-glucamine (NMDG) completely replaced Na^+; 150 mM-NMDG was neutralized by the addition of 10 mM-HEPES and sufficient HCl to give a pH of 7·4 at 37 °C. Apyrase (20 μg ml^{-1}) was added to all suspensions.

In platelets, as in other cells (Harootunian *et al.* 1989), the fluorescence of SBFI in the intracellular environment was markedly enhanced compared with that in cell lysate, making it necessary to calibrate the dye intracellularly by changing the external $[Na^+]$ in the presence of Na^+ ionophores to set $[Na^+]_i$ to known values. This procedure is comparatively straightforward with single adherent cells but problematic with cell suspensions. Platelets pose a particular difficulty since the cells are easily activated by repetitive handling or washing. We have therefore relied on calibration signals from aliquots of each preparation of SBFI-loaded platelets resuspended in media of different $[Na^+]$.

Aliquots of cells were suspended in media containing 1, 5, 10, 20, 50 and 150 mM-Na^+, prepared by appropriate mixing of a solution of composition (in mM): 30 NaCl, 120 sodium gluconate, 1 $MgCl_2$, 1 $CaCl_2$, 10 Na-HEPES, pH 7·4 at 37 °C and a similar solution in which K^+ completely replaced Na^+. Gramicidin (5 μM), a Na^+–K^+ ionophore, was added to set $[Na^+]_i = [Na^+]_o$. The low $[Cl^-]$ was chosen to be approximately equal to the intracellular $[Cl^-]$ (Feinberg *et al.* 1977), to minimize Cl^- flux and consequent changes in cell volume when the transmembrane monovalent cation gradients are collapsed in the presence of gramicidin. Also, if the concentration of semipermeable anions (Cl^-) and impermeant anions (proteins *vs.* gluconate) are balanced across the plasma membrane, any Donnan effect should be minimized so that gramicidin should equalize intracellular and extracellular $[Na^+]$ (Harootunian *et al.* 1989).

$[Na^+]_i$ was calculated as described by Harootunian *et al.* (1989). Briefly, fluorescence ratios R_1, R_2, R_3 at three known $[Na^+]_i$, C_1, C_2, C_3 were used to solve the three simultaneous equations:

$$R_o = [R_1R_2C_3(C_1-C_2)+R_2R_3C_1(C_2-C_3)+R_3R_1C_2(C_3-C_1)]/$$
$$[R_1C_1(C_3-C_2)+R_2C_2(C_1-C_3)+R_3C_3(C_2-C_1)],$$

$$R_\infty = [R_1R_2(C_2-C_1)+R_2R_3(C_3-C_2)+R_3R_1(C_1-C_3)]/[R_1(C_2-C_3)+R_2(C_3-C_1)+R_3(C_1-C_2)],$$

$$(K_dS_{f2}/S_{b2}) = C_i(R_\infty-R_i)/(R_i-R_o) \text{ for } i = 1, 2, \text{ or } 3.$$

In these equations, R_o is the 340/385 nm fluorescence ratio at 0 Na^+, R is the ratio at saturating Na^+, K_d is the Na^+ dissociation constant of the dye and S_{f2}/S_{b2} is the ratio of excitation efficiencies of free indicator to Na^+-bound indicator at 385 nm. The unknown $[Na^+]_i$ given by 340/385 nm fluorescence ratio, R, was then calculated using the equation:

$$[Na^+]_i = (K_dS_{f2}/S_{b2})(R-R_o)/(R_\infty-R).$$

Figure 1 shows the effects on SBFI fluorescence excited at 340 nm and 385 nm of adding 5 μM-gramicidin to platelets suspended in calibration media containing 1, 5, 10, 20, 50 and 150 mM-Na^+. The resulting 340/385 nm ratios, corrected for autofluorescence and external dye fluorescence, are shown against each pair of traces.

The reliability of this technique was assessed by the use of ratios obtained at 1, 10 and 150 mM-Na^+ to calibrate the signals obtained at 5, 20 and 50 mM-Na^+. In a small number of preparations, the deviation of the interpolated from the true value was greater than 15% and these were rejected. With practice in the rapid resuspension of many individual aliquots of cells, very reliable calibration was possible. In nine experiments, the values of known $[Na^+]_i$ set at 5, 20 and 50 mM were estimated by interpolation of ratios at 1, 10 and 150 mM-Na^+ as $5·6 \pm 0·3$, $19·9 \pm 1·2$ and $49·1 \pm 3·3$ mM. The results indicate that good estimates of $[Na^+]_i$ can be obtained from SBFI fluorescence in cell suspensions. Nevertheless, it must be borne in mind that all values given for $[Na^+]_i$ in this work are estimates which are subject to an error of around 10% (as assessed from the deviation of estimated values of calibration standards from the true values as described above). Possible sources of error include failure of gramicidin to fully equilibrate internal and external $[Na^+]$ (although as discussed above, this should be minimized by equalizing the internal and

external $[Cl^-]$) and the effect of K^+ on the indicator. The selectivity of SBFI for Na^+ over K^+ is only about 20:1 in calibration media (Minta & Tsien, 1989), however, this may not reflect the selectivity in the intracellular environment. In rat embryo fibroblasts, in the presence of gramicidin, nigericin and monensin, changes in $[K^+]$ of 40 mM had no measurable effect on the fluorescence ratio, whilst changes in $[Na^+]$ of 4 mM evoked a substantial rise in the 340/385 nm ratio (Harootunian *et al.* 1989).

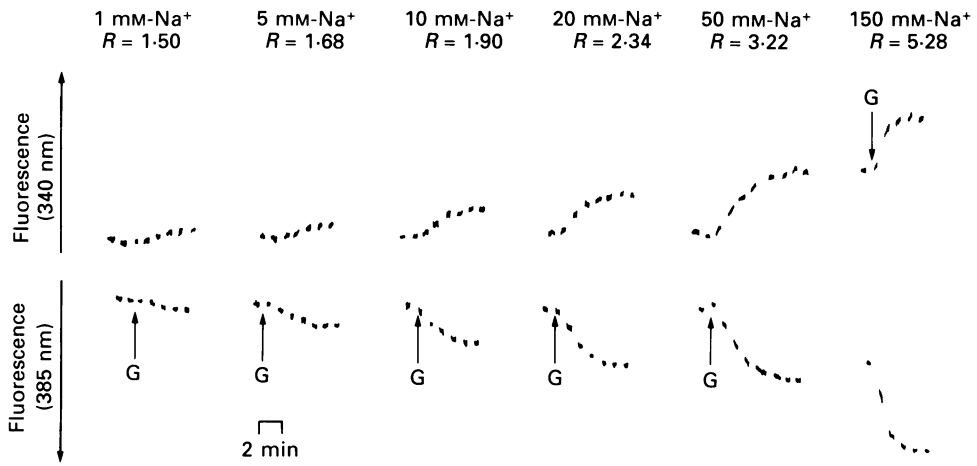

Fig. 1. Calibration of SBFI fluorescence. SBFI fluorescence from dye-loaded platelets was recorded alternately with excitation wavelengths of 340 and 385 nm with emission at 500 nm. Aliquots of cells were resuspended in calibration media containing the $[Na^+]$ indicated. Gramicidin (G; 5 μM) was added as indicated. The resulting 340/385 nm fluorescence ratios (R), after equilibration of $[Na^+]_i$ with $[Na^+]_o$, are shown. Ratios have been corrected for external dye and autofluorescence as described under Methods.

Measurement of pH_i

BCECF fluorescence, with excitation wavelength 490 nm and emission at 530 nm, was measured and calibrated in terms of pH_i as previously described (Sage *et al.* 1990*a*).

Stopped-flow fluorimetry

Stopped-flow kinetic measurements of SBFI fluorescence, with excitation at 340 nm and emission 500 nm, were made as previously described for platelets loaded with Fura-2 and BCECF (Sage & Rink, 1987; Sage *et al.* 1990*a*). A small increase in SBFI fluorescence was seen in agonist-free control experiments. Such an artifactual rise in fluorescence is not seen in Fura-2-loaded cells (Sage & Rink, 1990). The fluorescence rise may reflect an increase in $[Na^+]_i$ during the rapid injection of cells into the recording chamber or the lysis of a fraction of the cells. The presence of such artifacts in SBFI-loaded but not Fura-2-loaded cells might be due to the use of pluronic in the case of the former. Agonist-stimulated fluorescence changes were corrected for this artifact by subtraction of agonist-free controls obtained using the same cell preparation.

Electrophysiology

Cell-attached patch-clamp recordings were made as previously described (Mahaut-Smith, Rink & Sage, 1989; Mahaut-Smith *et al.* 1990*b*). Briefly, cells were collected from PRP by microcentrifugation and resuspended in Ca^{2+}-free, Na-HEPES-buffered saline (145 mM-Cl^-) of composition as above; 20–50 μl of the platelet suspension was added to Na-HEPES-buffered saline in the siliconized electrophysiological recording chamber. After the cells had settled, the chamber was perfused with more saline. Patch pipettes with a filled resistance of 5–10MΩ were pulled from VWR micropipettes (Drummond Scientific Co., USA) on a Narishige PP-93 patch pipette puller. Pipettes were filled with Na-HEPES-buffered saline after the addition of 1 mM-$CaCl_2$ or 1 mM-EGTA without added Ca^{2+} and back-filled with ADP (40 μM–1 mM in Na-HEPES saline) to reduce

desensitization to the agonist during seal formation (Mahaut-Smith *et al.* 1990*b*). The concentration of ADP at the patch was therefore unknown. A pipette was lowered into the chamber, manipulated to within 5 μm of a floating platelet and the cell drawn to the pipette using suction. Recordings were made from experiments in which the glass–membrane seal developed to a resistance of at least 10 GΩ. Current was measured under voltage clamp with an EPC-7 patch-clamp amplifier (List Electronic, FRG) and stored on videotape after digitization by a PCM adaptor (SONY). Data were low-pass filtered at 400 Hz to 1 kHz (−3 dB) and analysed on a Tandon computer using Sartori software (Intracel Ltd, Cambridge, UK). Patch-clamp experiments were carried out at room temperature (20–24 °C). The recording chamber was grounded through an agar bridge made with 1–2% agar and Na-HEPES-buffered saline.

Materials

The acetoxymethyl esters of SBFI and BCECF, and Pluronic F-127 were from Molecular Probes, Eugene, OR, USA. Na_2-ADP, apyrase, aspirin, EGTA and *N*-methyl-D-glucamine were from Sigma, Poole, Dorset. HEPES was from Calbiochem, San Diego, CA, USA. SK & F 96365 (1-(*β*-(3-(*p*-methoxyphenyl)-propyloxyl]-*p*-methoxyphenethyl)-1H-imidazole hydrochloride) was synthesized by Dr W. P. Armstrong, Department of Medicinal Chemistry, Smith Kline and French Research Ltd, Welwyn, Hertfordshire.

RESULTS

Basal $[Na^+]_i$

Resting platelet $[Na^+]_i$ in control medium containing 145 mM-Na^+ and 5 mM-K^+ was $5{\cdot}5 \pm 0{\cdot}3$ mM ($n = 50$). The results shown in Fig. 1 indicate that resting $[Na^+]_i$ is little affected by variation in external $[Na^+]$ or membrane potential (due to changes in $[K^+]_i$), except at the highest $[Na^+]$. The elevation in resting $[Na^+]_i$ in the 150 mM-Na^+ calibration medium reflects the effect of the prolonged (1–2 h) absence of external K^+, which would be expected to inhibit the Na^+–K^+-ATPase leading to a rise in $[Na^+]_i$.

Responses to imposed changes in $[Na^+]_i$

The effects of agents known to elevate $[Na^+]_i$ were tested on SBFI-loaded cells. Gramicidin (5 μM), which allows transmembrane Na^+ and K^+ flux, caused a rise in the 340/385 nm ratio, which peaked in about 2 min (Fig. 2*A*). Monensin (5 μM), which mediates Na^+–H^+ exchange, evoked a similar, though more rapid elevation in the 340/385 nm fluorescence ratio (Fig. 2*B*). Ouabain (20 μM), which inhibits the Na^+–K^+-ATPase, evoked a slow rise in the 340/385 nm ratio, indicating a rise in $[Na^+]_i$ from $3{\cdot}9 \pm 0{\cdot}3$ mM to $9{\cdot}8 \pm 1{\cdot}0$ mM ($n = 6$) over 10 min (Fig. 2*C*). If the Na^+–K^+-ATPase was completely blocked, this result indicates a basal Na^+ leak giving an increase in $[Na^+]_i$ of 0·6 mM min^{-1} and a plasma membrane flux of 50 pM cm^{-2} s^{-1} assuming no buffering of cytosolic Na^+, a platelet surface membrane area of 20 μm^2 and volume of 10^{-14} l.

ADP-evoked changes in $[Na^+]_i$

Figure 3 shows the effects of ADP on SBFI fluorescence. In control medium (145 mM-Na^+), 40 μM-ADP evoked a rapid increase in the 340/385 nm ratio (Fig. 3*A*), indicating an elevation in $[Na^+]_i$ from $6{\cdot}4 \pm 0{\cdot}7$ mM to $18{\cdot}3 \pm 1{\cdot}1$ mM ($n = 8$). The $[Na^+]_i$ then declined slowly back towards basal levels. In nominally Na^+-free medium, in which Na^+ had been completely replaced with NMDG for 20 min, 40 μM ADP was without effect on the 340/385 nm ratio (Fig. 3*B*). The estimated $[Na^+]_i$ was

essentially unchanged, being $0{\cdot}3 \pm 0{\cdot}1$ mM before and $0{\cdot}2 \pm 0{\cdot}0$ mM ($n = 8$) after the addition of 40 μM-ADP.

We have reported before that ADP evokes biphasic Ca^{2+} influx in platelets (Sage *et al.* 1990*c*). We have also shown that the compound SK & F 96365 (20 μM), an

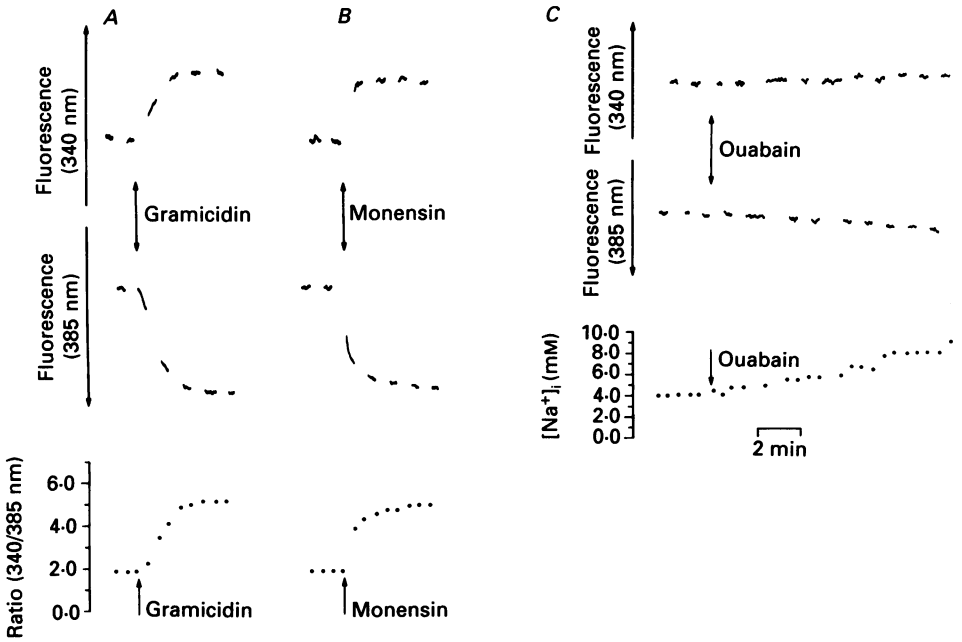

Fig. 2. Effects of gramicidin, monensin and ouabain on SBFI fluorescence. The upper pair of traces in each section show SBFI fluorescence recorded as for Fig. 1. The lower traces in each section show the calculated 340/385 nm fluorescence ratio (*A*, *B*) or the estimated $[Na^+]_i$ (*C*). Gramicidin (5 μM), monensin (5 μM) or ouabain (20 μM) were added as indicated by the arrows.

inhibitor of receptor-mediated Ca^{2+} entry in a number of non-excitable cells (Merritt, Benham, Armstrong, Jaxa-Chamiec, Hallam, Jacob, Leigh, McCarthy, Moores & Rink, 1990), blocks the fast, receptor-operated phase of ADP-evoked Ca^{2+} entry, but not the delayed phase, which appears dependent on the discharge of the intracellular Ca^{2+} stores (Sage *et al.* 1990*c*). Interestingly, the ADP-evoked elevation in 340/385 nm ratio in control medium was completely abolished by 20 μM-SK & F 96365 (Fig. 3*C*). The $[Na^+]_i$ in the presence of 20 μM-SK & F 96365 was $6{\cdot}1 \pm 0{\cdot}5$ mM before and $5{\cdot}7 \pm 0{\cdot}3$ mM ($n = 8$) after the addition of ADP. The difference was not significant (Student's *t* test, $0{\cdot}5 > P > 0{\cdot}1$).

ADP-evoked changes in cytosolic pH: minimal contribution of sodium–proton exchange to ADP-evoked rises in $[Na^+]_i$

Figure 4 shows fluorescent signals from BCECF-loaded platelets calibrated in terms of pH_i. In control medium (145 mM-Na^+), ADP (40 μM) evoked a rapid cytosolic acidification, with pH_i falling from the basal level of $7{\cdot}21 \pm 0{\cdot}03$ to a minimum of $7{\cdot}12 \pm 0{\cdot}03$ ($n = 10$). The pH_i then rose slowly back towards the basal

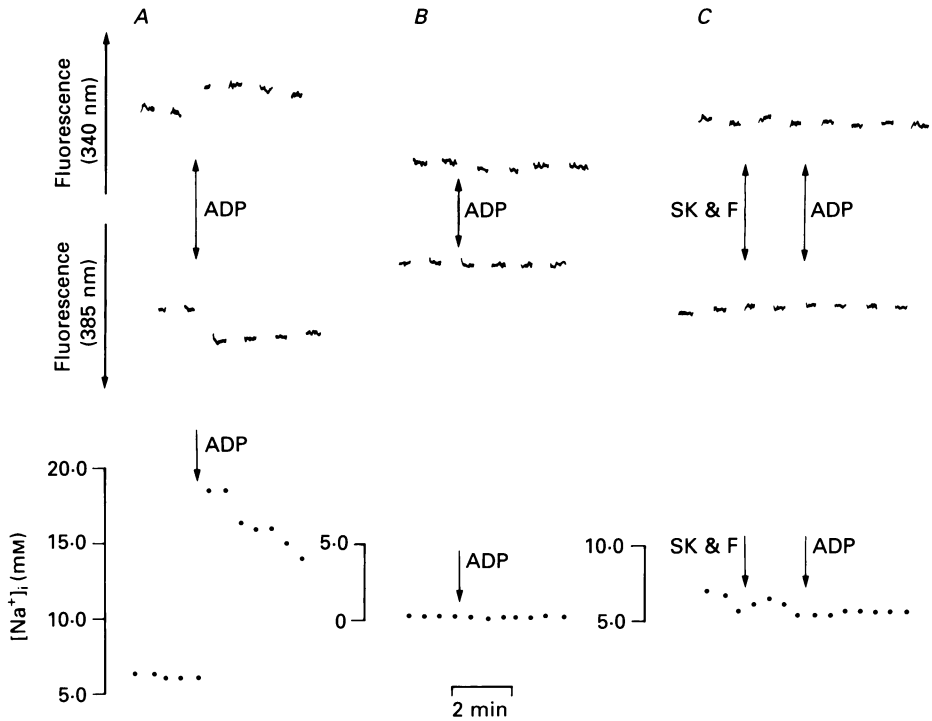

Fig. 3. Effects of ADP on SBFI fluorescence. The upper pair of traces in each section show SBFI fluorescence recorded as for Fig. 1. The lower traces show the calculated $[Na^+]_i$. Dye-loaded cells were resuspended in control, Na^+-containing medium (*A*, *C*) or Na^+-free medium in which NMDG completely replaced Na^+ (*B*). ADP (40 μM) of SK & F 96365 (SK & F; 20 μM) were added as indicated by the arrows.

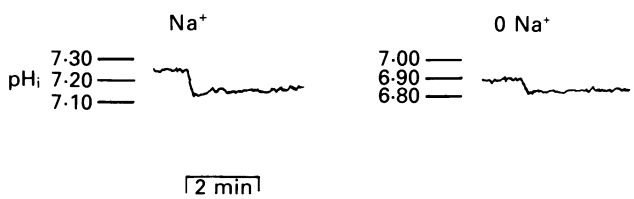

Fig. 4. Effects of ADP (40 μM) on cytosolic pH. Traces show fluorescence records from BCECF-loaded cells with excitation at 490 nm and emission at 530 nm, calibrated in terms of pH_i. The trace on the left was obtained from cells resuspended in control, Na^+ medium and that on the right in Na^+-free medium.

level, reaching $7{\cdot}15 \pm 0{\cdot}03$ ($n = 10$) 3 min after the addition of ADP. The recovery was dependent on external Na^+. In nominally Na^+-free medium, in which NMDG completely replaced Na^+, ADP (40 μM) evoked a fall in pH_i from a basal level of $6{\cdot}90 \pm 0{\cdot}04$ to $6{\cdot}83 \pm 0{\cdot}05$ ($n = 10$). Three minutes after agonist addition, no recovery had occurred. The ADP-evoked changes in pH_i and the effects of Na^+ removal in HEPES-buffered, HCO_3^--free media are similar to those we have previously reported in HCO_3^--containing media (Sage *et al.* 1990*a*).

These results indicate that there is slow Na^+-dependent alkalization following the

initial cytosolic acidification evoked by ADP. The rise in pH_i of only $0{\cdot}02 \pm 0{\cdot}01$ units ($n = 10$) means that the contribution of Na^+–H^+ exchange to the ADP-evoked rise in $[Na^+]_i$, assuming a cytosolic buffering power of 14·5 mM (pH unit)$^{-1}$ (Sage *et al.* 1990*a*), would be no more than 0·5 mM. This is not likely to be an under estimate

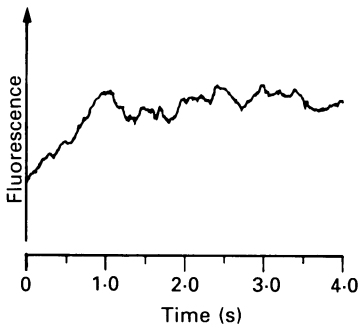

Fig. 5. Stopped-flow recording of ADP-evoked changes in SBFI fluorescence. The early events of the ADP-evoked change in SBFI fluorescence, with excitation at 340 nm and emission at 500 nm, were recorded by stopped-flow fluorimetry (see Methods). ADP (40 μM) was added at time zero. The trace is typical of five experiments.

because of an early activation of Na^+–H^+ exchange at the time of cytosolic acidification, since the ADP-evoked fall in pH_i was small and of similar magnitude to that seen in the absence of external Na^+.

Stopped-flow fluorimetry

The early kinetics of the ADP-evoked rise in $[Na^+]_i$ were investigated by stopped-flow fluorimetry. Figure 5 shows that ADP evoked a rise in SBFI-fluorescence which began without measurable delay and peaked within 1 s.

Patch-clamp studies

We have previously reported that ADP activates cation channels in cell-attached patches of intact platelets (Mahaut-Smith *et al.* 1990*b*). When the pipette filling solution lacked ADP, the records showed only a few or no spontaneous inward single-channel currents (Mahaut-Smith *et al.* 1990*b*). Back-filling the pipettes with an ADP-containing saline resulted in the transient appearance of single-channel inward currents whilst addition of ADP to the bath was without effect. The single-channel conductance was 11 pS at the resting potential and the estimated reversal potential of the ADP-evoked current was near 0 mV. The replacement of external chloride by gluconate had little effect on the slope conductance or reversal potential and ADP evoked single-channel events when the pipette contained isotonic $BaCl_2$. These earlier results indicate that ADP evokes the opening of a cation-selective receptor-operated channel. We have also demonstrated, using stopped-flow fluorimetry, that ADP evokes the entry of Ba^{2+} in platelets without measurable delay (< 30 ms), and have proposed that the ADP-evoked channel accounts for this. We have further proposed that, under physiological conditions, Ca^{2+} entry occurs by this pathway (Mahaut-Smith *et al.* 1990*b*) and that this may mediate the rapid phase of Ca^{2+} entry evoked by ADP (Sage *et al.* 1990*c*).

The present results obtained with the Na^+ indicator suggest that the rapidly activated, ADP-evoked pathway is also permeable to Na^+. To test this hypothesis, ADP-evoked channel activity was compared with and without Ca^{2+} in the pipette-filling saline, when Na^+ was the major external cation. ADP transiently evoked

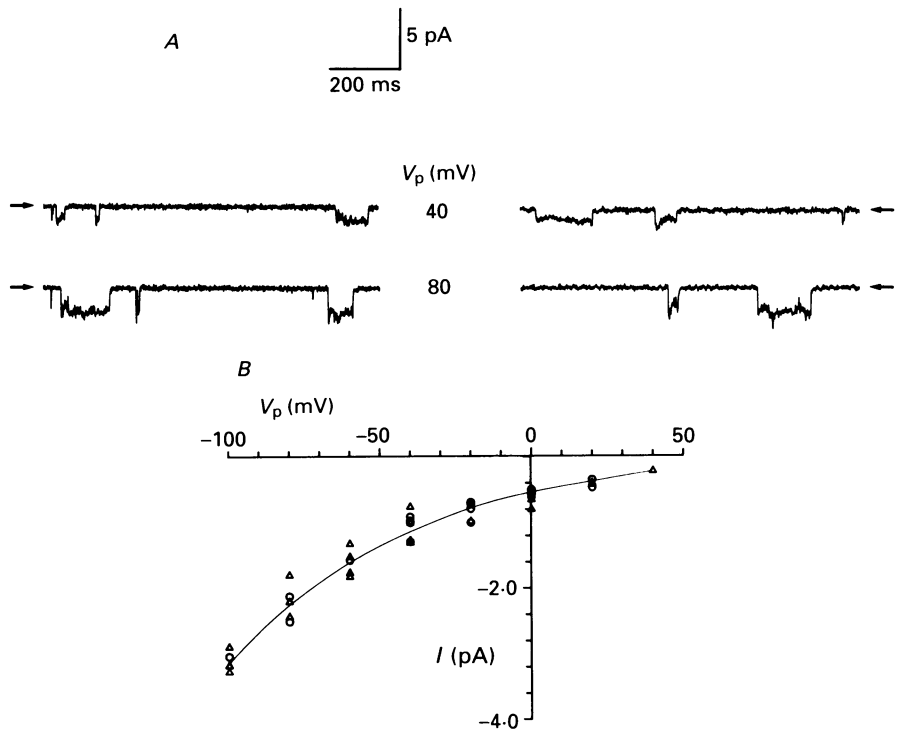

Fig. 6. Effect of removing Ca^{2+} on ADP-evoked single channel currents. Currents were recorded from cell-attached patches in the presence of 40 μM–1 mM-ADP, back-filled into the pipette. *A*, current records of ADP-evoked activity at pipette potentials (V_p) of 40 and 80 mV when the pipette was filled with 1 mM-Ca^{2+}-containing saline (left panel) or Ca^{2+}-free saline with 1 mM-EGTA (right panel). The arrow beside each trace indicates the level at which all channels appeared to be closed and a deflection below the baseline represents inward current. *B*, single-channel current plotted as a function of $-V_p$ (total potential across the patch is the sum of the platelet membrane potential and $-V_p$) for 1 mM-Ca^{2+}-containing saline (△; data from 8 cells) and Ca^{2+}-free, EGTA-containing saline (○; data from 7 cells). Each symbol is the average from an individual patch. The line was drawn by hand through the data points for Ca^{2+}-containing experiments.

single-channel inward currents of comparable amplitude and duration when the pipette saline contained 1 mM-$CaCl_2$ (Fig. 6*A*, left panel) or 1 mM-EGTA without added Ca^{2+} (Fig. 6*A*, right panel). As previously demonstrated (Mahaut-Smith *et al.* 1990*b*), only occasional or no channel openings were detectable under control conditions when the pipette-filling solution lacked ADP (data not shown).

Figure 6*B* shows a plot of single-channel current amplitude as a function of pipette potential, taken from a number of experiments in Ca^{2+}-free saline (○) and 1 mM-Ca^{2+}-containing saline (△). The slope conductance at 0 mV applied potential was

similar for the two conditions: Ca^{2+}-free, 10 pS; 1 mM-Ca^{2+}, 11 pS, and displayed a similar degree of curvilinearity. The extrapolated reversal potentials were also similar: −65 mV (V_p) in the presence and −60 mV in the absence of external Ca^{2+}. Since the platelet resting potential is around −60 to −70 mV (MacIntyre & Rink, 1982; Pipili, 1985; Mahaut-Smith, Rink, Collins & Sage, 1990*a*), the reversal potentials for these currents (resting potential: V_p) lie close to 0 mV. We have previously shown the reversal potential to be similar when the pipette contains 110 mM-$BaCl_2$ (Mahaut-Smith *et al.* 1990*b*). These results suggest that the ADP-gated channel shows little selectivity between monovalent and divalent cations and, with normal physiological gradients, these channels would be expected to carry a significant amount of Na^+.

DISCUSSION

These results provide the first measurements of free $[Na^+]_i$ in human platelets. We have demonstrated that the fluorescent indicator SBFI can be loaded into platelets and report $[Na^+]_i$ when calibrated by resuspension in media containing different $[Na^+]$ and treated with the Na^+–K^+ ionophore, gramicidin.

Resting $[Na^+]_i$ was estimated to be 5·5 ± 0·3 mM ($n = 50$). This value is similar to the estimates of resting $[Na^+]_i$ obtained using SBFI of 4·2 mM in fibroblasts and 9·4 mM in lymphocytes (Harootunian *et al.* 1989), and 8–10 mM in gastric gland cells (Negulescu *et al.* 1990). Our determination of free $[Na^+]_i$ is, however, considerably lower than the estimate for total platelet Na^+ of 42 mM obtained using $^{22}Na^+$ (Feinberg *et al.* 1977). Such isotopic determinations may overestimate total intracellular Na^+ due to difficulties in compensating for contaminating extracellular Na^+, although the study of Feinberg and co-workers takes rigorous precautions to minimize this problem. Even allowing for an overestimate of total Na^+, our results indicate that free $[Na^+]_i$ is substantially lower, suggesting that much of the intracellular Na^+ must be bound or sequestered. Hinke (1959, 1960) suggested that substantial amounts of intracellular Na^+ must be bound following the first determinations of intracellular Na^+ activity using microelectrodes in squid axons and crab and lobster muscle cells. Estimates for free $[Na^+]_i$ well below total Na^+ have also been reported for other cells including lymphocytes (Smith, Morris, Hesketh and Metcalfe, 1986; Harootunian *et al.* 1989), salamander oocytes (Horowitz & Paine, 1979) and *Xenopus* embryos (Slack, Warner & Warren, 1973).

ADP evoked a peak increase in $[Na^+]_i$ of 12 mM above basal levels. This is in good agreement with earlier estimates made from $^{22}Na^+$ entry (see Fig. 2 of Feinberg *et al.* 1977). The ADP-evoked increase in $[Na^+]_i$ was completely abolished by removal of external Na^+. This indicates that under these conditions ADP evokes no detectible mobilization of intracellular bound Na^+ and that the rise in $[Na^+]_i$ is attributable to Na^+ entry from the external medium. The failure of ADP to evoke a change in $[Na^+]_i$ in Na^+-free medium is not due to an inability of the cells to respond. ADP-evoked Ca^{2+} influx and release of Ca^{2+} from intracellular stores are unaffected under these conditions (Sage, 1988).

Possible entry mechanisms for Na^+ include permeation of the pathways for agonist-evoked Ca^{2+} entry, Na^+–H^+ and Na^+–Ca^{2+} exchange. Conflicting evidence for

Na^+–Ca^{2+} exchange in platelets has been reported. However, direct assessments, rather than those relying on effects on functional responses to agonists, suggest against a significant role of Na^+–Ca^{2+} in these cells. Brass (1984) reported that the removal of extracellular Na^+ was without effect on basal $[Ca^{2+}]_i$ or total Ca^{2+} content. Similarly, we have reported that Na^+ substitution with NMDG is without effect on basal $[Ca^{2+}]_i$, the decline in $[Ca^{2+}]_i$ following stimulation with thrombin or on basal and agonist-evoked $^{45}Ca^{2+}$ effluxes (Rink & Sage, 1987). Even if a small Na^+–Ca^{2+} exchange has gone undetected in these earlier studies, it is unlikely to make a significant contribution to the ADP-evoked rise in $[Na^+]_i$ reported here. The stopped-flow studies indicate that the ADP-evoked rise in $[Na^+]_i$ commences without measurable delay and peaks within 1 s, whereas the decline in $[Ca^{2+}]_i$ occurs over many minutes (e.g. Hallam & Rink, 1985).

We were unable to test directly the possible contribution of Na^+–H^+ exchange to the ADP-evoked rise in $[Na^+]_i$. Attempts to use the inhibitor ethylisopropylamiloride were unsuccessful because this compound interfered with SBFI fluorescence. However, we have shown that ADP evokes only a small fall in pH_i followed by a slow recovery towards the resting level. This is calculated to increase $[Na^+]_i$ by no more than 0·5 mM over the first three minutes after stimulation (see results). The contribution of Na^+–H^+ exchange to the ADP-evoked rise in $[Na^+]_i$ of about 12 mM, which peaks within 1 s, would therefore be negligible.

We have reported previously that, in the presence of bicarbonate, ADP evokes a small cytosolic acidification which recovers only slowly towards the basal pH_i (Sage *et al.* 1990*a*). Here we show a similar effect of ADP on pH_i in nominally bicarbonate-free, HEPES-buffered medium. The slow recovery of pH_i after the initial acidification evoked by ADP contrasts markedly with the response evoked by thrombin, where the acidification is followed by an alkalization in the presence and absence of bicarbonate (Sage *et al.* 1990*a*). This difference is probably due to thrombin being a much more potent stimulus to diacylglycerol (DAG) production, which activates Na^+–H^+ exchange via protein kinase C (Siffert, Siffert & Scheid, 1987). The failure of Na^+–H^+ exchange to evoke a rapid recovery in pH_i in the absence of a substantial production of DAG evoked by ADP, may be due to the substantial rise in $[Na^+]_i$ reducing the driving force for exchange.

The time course of the ADP-evoked rise in $[Na^+]_i$, resolved using stopped-flow fluorimetry, is very similar to that we have previously reported for ADP-evoked rises in $[Ca^{2+}]_i$ (Sage & Rink, 1987, 1990). We have suggested that the ADP-evoked rise in $[Ca^{2+}]_i$, which occurs without measurable delay, is conducted by non-selective, receptor-operated cation channels in the plasma membrane, which we have demonstrated in cell-attached patch-clamp recordings (Mahaut-Smith *et al.* 1990*b*). Here we have shown that removal of external Ca^{2+} has little effect on the conductance or reversal potential of current flow through ADP-evoked channels in cell-attached recordings, indicating that these channels mediate a substantial Na^+ influx. Hence the rapid ADP-evoked rise in $[Na^+]_i$, which commences without measurable delay, appears to be mediated by receptor-operated, non-selective cation channels in the plasma membrane.

Given that ADP evoked a rise in $[Na^+]_i$ of 12 mM in 1 s, and a single-channel conductance of 10 pS, one can estimate the number of ADP receptor-operated channels in the platelet membrane, assuming that this channel is solely or mainly responsible for conducting Na^+ influx. Assuming a membrane surface area of 20 μm^2 and a driving force for Na^+ entry of 100 mV, the data suggests that each platelet

would possess about ten channels if these were continuously open after activation, or fifty channels if, as observed (Mahaut-Smith *et al.* 1990*b*), these were open for about 20% of the time. This estimate is in general agreement with recent experiments we have conducted using the whole-cell patch-clamp technique (Mahaut-Smith, Rink & Sage, 1991*a*). At a holding potential of −70 mV, ADP evoked a transient whole-cell current which peaked at between 13 and 31 pA in different cells. Unitary ADP-evoked currents could be resolved during the decaying phase of the response, allowing the single channel current to be estimated as 0·9 pA. Thus these results indicate a channel density of 14–34 per cell (Mahaut-Smith, Rink & Sage, unpublished observations).

The ADP-evoked rise in $[Ca^{2+}]_i$ can be resolved into two phases using stopped-flow fluorimetry (Sage *et al.* 1990*c*). The use of Mn^{2+} as a tracer for Ca^{2+} (Sage, Merritt, Hallam & Rink, 1989; Merritt, Jacob & Hallam, 1989) indicates that both phases of the rise in $[Ca^{2+}]_i$ are associated with Ca^{2+} influx (Sage *et al.* 1990*c*). A fast phase, which commences without measurable delay and the timecourse of which is temperature insensitive, appears to be conducted by the receptor-operated channels described above. There is then a delayed phase which has a temperature sensitive timecourse and which occurs coincident with, and may be regulated by, the discharge of the intracellular Ca^{2+} store.

The compound SK & F 96365 has been shown to block receptor-mediated Ca^{2+} entry in a number in a number of cell types (Merritt *et al.* 1990). Interestingly, this compound blocks the fast, but not the delayed phase of ADP-evoked Ca^{2+} or Mn^{2+} entry in platelets, demonstrating the independence of the two pathways (Sage *et al.* 1990). Here we have shown that, at the same concentration that selectively blocks one of the two phases of Ca^{2+} entry, SK & F 96365 blocked completely the ADP-evoked rise in $[Na^+]_i$. This suggests that the delayed Ca^{2+} entry mechanism is considerably more Ca^{2+} selective than the fast, receptor-operated channel pathway, since it appears to admit Ca^{2+} and Mn^{2+} (Sage *et al.* 1990*c*), but not Na^+ (present results).

Although store-regulated pathways for Ca^{2+} entry have been proposed in a number of cell types (Rink & Hallam, 1989), the nature of such mechanisms is not understood. It may be that a physical structure, perhaps similar to a gap junction, links the plasma membrane to that of the intracellular Ca^{2+} store, and that this structure is regulated by the concentration of Ca^{2+} within the store (e.g. Merritt & Rink, 1987). Our results suggest that if such a mechanism operates in platelets, either the pathway into the store, or the release channel in the store membrane regulated by inositol 1,4,5-trisphosphate, must show considerable selectivity for Ca^{2+} over Na^+. In this respect, it will be interesting to learn of the ionic selectivity, under physiological conditions, of the inositol 1,4,5-trisphosphate receptor-regulated channel which mediates the release of Ca^{2+} from intracellular stores and which has recently been purified and reconstituted (Ferris, Huganir, Supattapone & Snyder, 1989).

In conclusion, we have demonstrated that usefulness of the new fluorescent indicator SBFI for the study of $[Na^+]_i$ in platelets, provided the first measurements of free $[Na^+]_i$ in these cells and further analysed the sub-second cation influxes evoked by ADP.

Note added in proof. Borin & Siffert have recently reported the use of SBFI in human platelets (*Journal of Biological Chemistry* **265**, 19543–19550). The higher resting value of $[Na^+]_i$ reported in their study may reflect the lower temperature (25 °C), a different platelet preparation protocol and the use of high-Cl^- calibration media.

S.O.S held a Royal Society 1983 University Research Fellowship. This work was funded in part by a research donation from Smith Kline & French Research Ltd.

REFERENCES

BORIN, M. & SIFFERT, W. (1990). Stimulation by thrombin increases the cytosolic free Na^+ concentration in human platelets. Studies with the novel fluorescent cytosolic Na^+ indicator sodium-binding benzofuran isophthalate. *Journal of Biological Chemistry* **265**, 19543–19550.

BORN, G. V. R., CUSACK, N. J., DARWIN, C. & MCCLURE, M. (1986). Prolongation of bleeding time in rats by ADP receptor antagonists. *Journal of Physiology* **381**, 22*P*.

BRASS, L. F. (1984). The effect of Na^+ on Ca^{2+} homeostasis in unstimulated platelets. *Journal of Biological Chemistry* **259**, 12571–12575.

FEINBERG, H., SANDLER, W. C., SCORER, M., LE BRETON, G. C., GROSSMAN, B. & BORN, G. V. R. (1977). Movement of sodium into human platelets induced by ADP. *Biochimica et Biophysica Acta* **470**, 317–324.

FERRIS, C. D., HUGANIR, R. L., SUPATTAPONE, S. & SNYDER, S. H. (1989). Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. *Nature* **342**, 87–89.

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.

HAROOTUNIAN, A. T., KAO, J. P. Y., ECKERT, B. K. & TSIEN, R. Y. (1989). Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. *Journal of Biological Chemistry* **264**, 19458–19467.

HINKE, J. A. M. (1959). Glass microelectrodes for measuring intracellular activities of sodium and potassium. *Nature* **184**, 1257–1258.

HINKE, J. A. M. (1960). The measurement of sodium and potassium activities in the squid axon by means of cation-selective glass microelectrodes. *Journal of Physiology* **156**, 314–335.

HOROWITZ, S. B. & PAINE, P. L. (1979). Reference phase analysis of free and bound intracellular solutes. Isothermal and isotopic studies of cytoplasmic sodium, potassium and water. *Biophysical Journal* **25**, 45–62.

MACINTYRE, D. E. & RINK, T. J. (1982). The role of platelet membrane potential in the initiation of platelet aggregation. *Thrombosis and Haemostasis* **47**, 22–26.

MAHAUT-SMITH, M. P., RINK, T. J., COLLINS, S. C. & SAGE, S. O. (1990*a*). Voltage-gated potassium channels and the control of membrane potential in human platelets. *Journal of Physiology* **428**, 723–735.

MAHAUT-SMITH, M. P., RINK, T. J. & SAGE, S. O. (1989). Single channels in human platelets activated by ADP. *Journal of Physiology* **415**, 24*P*.

MAHAUT-SMITH, M. P., RINK, T. J. & SAGE, S. O. (1991*a*). 'Whole-cell' recordings of ADP-evoked currents and single-channel events in human platelets using nystatin-permeabilized patches. *Journal of Physiology* **434**, 38*P*.

MAHAUT-SMITH, M. P., SAGE, S. O. & RINK, T. J. (1990*b*). Receptor-operated single channels in intact human platelets. *Journal of Biological Chemistry* **265**, 10479–10483.

MERRITT, J. E., BENHAM, C. D., ARMSTRONG, W. P., JAXA-CHAMIEC, A., HALLAM, T. J., JACOB, R., LEIGH, B. K., MCCARTHY, S. A., MOORES, K. E. & RINK, T. J. (1990). SK & F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochemical Journal* **271**, 515–522.

MERRITT, J. E., JACOB, R. & HALLAM, T. J. (1989). Use of manganese to discriminate between calcium influx and mobilisation from internal stores in stimulated human neutrophils. *Journal of Biological Chemistry* **264**, 1522–1527.

MERRITT, J. E. & RINK, T. J. (1987). Regulation of cytosolic free calcium in fura-2-loaded rat parotid acinar cells. *Journal of Biological Chemistry* **262**, 17362–17369.

MINTA, A. & TSIEN, R. Y. (1989). Fluorescent indicators for cytosolic sodium. *Journal of Biological Chemistry* **264**, 19449–19457.

MOORE, E. D. W., TSIEN, R. Y., MINTA, A. & FAY, F. S. (1988). Measurement of intracellular sodium with SBFP, a newly developed sodium sensitive fluorescent dye. *FASEB Journal* **2**, A754.

NEGULESCU, P. A., HAROOTUNIAN, A., TSIEN, R. Y. & MACHEN, T. E. (1990). Fluorescence measurements of cytosolic free Na concentration, influx and efflux in gastric cells. *Cell Regulation* **1**, 259–268.

PALES, J., PALACIOS-ARAUS, L., LOPEZ, A. & GUAL, A. (1989). Influence of sodium conductances on platelet activation. *Biochimica et Biophysica Acta* **980**, 33–36.

PIPILI, E. (1985). Platelet membrane potential: simultaneous measurements of diS-C_3-(5) fluorescence and optical density. *Thrombosis and Haemostasis* **54**, 645–649.

POENIE, M., ALDERTON, J., STEINHARDT, R. & TSIEN, R. (1986). Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. *Science* **233**, 886–889.

RINK, T. J. & HALLAM, T. J. (1989). Calcium signalling in non-excitable cells: notes on oscillations and store refilling. *Cell Calcium* **10**, 385–395.

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. & SAGE, S. O. (1990). Calcium signalling in human platelets. *Annual Reviews of Physiology* **52**, 431–449.

SAGE, S. O. (1988). Stimulus–response coupling in human blood platelets: the generation and termination of calcium signals. Ph.D. Thesis, University of Cambridge.

SAGE, S. O., JOBSON, T. M. & RINK, T. J. (1990*a*). Agonist-evoked changes in cytosolic pH and calcium concentration in human platelets: studies in physiological bicarbonate. *Journal of Physiology* **420**, 31–45.

SAGE, S. O., MAHAUT-SMITH, M. P. & RINK, T. J. (1990*b*). ADP-evoked changes in cytosolic sodium concentration in human platelets loaded with the fluorescent indicator, SBFI. *Journal of Physiology* **429**, 85*P*.

SAGE, S. O., MERRITT, J. E., HALLAM, T. J. & RINK, T. J. (1989). Receptor-mediated calcium entry in fura-2-loaded human platelets stimulated with ADP and thrombin: dual wavelength studies with Mn^{2+}. *Biochemical Journal* **258**, 923–926.

SAGE, S. O., REAST, R. & RINK, T. J. (1990*c*). ADP evokes biphasic calcium influx in fura-2-loaded human platelets. Evidence for calcium entry regulated by the intracellular calcium store. *Biochemical Journal* **265**, 675–680.

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.

SAGE, S. O. & RINK, T. J. (1990). Reply to: 'Rapid blood platelet activation: continuous- and quenched-flow *versus* stopped-flow approaches'. *Biochemical Journal* **265**, 306–307.

SANDLER, W. C., LE BRETON, G. C. & FEINBERG, H. (1980). Movement of sodium into human platelets. *Biochimica et Biophysica Acta* **600**, 448–455.

SIFFERT, W. & AKKERMAN, J. W. N. (1988). Na^+/H^+ exchange as a modulator of platelet activation. *Trends in Biochemical Sciences* **13**, 148–151.

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.

SLACK, C., WARNER, A. E. & WARREN, R. L. (1973). The distribution of sodium and potassium in amphibian embryos during early development. *Journal of Physiology* **232**, 297–312.

SMITH, G. A., MORRIS, P. G., HESKETH, R. & METCALFE, J. C. (1986). Design of an indicator of intracellular free Na^+ concentration using ^{19}F-NMR. *Biochimica et Biophysica Acta* **889**, 72–83.

ZAVOICO, G. B., CRAGOE, E. J. & FEINSTEIN, M. B. (1986). Regulation of intracellular pH in human platelets. *Journal of Biological Chemistry* **261**, 13160–13167.