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Chloride channels in excised membrane patches from human platelets: effect of intracellular calcium

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Abstract

Human platelets were studied by patch clamp recordings from inside-out membranes; these were formed by briefly dipping the platelet, in cell-attached mode, into silicone grease. At 20°C, in symmetrical 150 mM NaCl, spontaneous channel openings were rarely observed at negative potentials, whereas depolarised potentials (+60 to +100 mV) elicited sustained channel activity in 38% of patches. The single channel conductance was 53 ± 1 pS at +80 mV (outward current), decreasing to 20 ± 2 pS at −80 mV (inward current). Ion substitution experiments indicated that this channel conducts Cl^- and not Na^+. Furthermore, 5-nitro-2-(3-phenylpropylamino)benzoate (100 μM), a recognized inhibitor of anion channels, induced a reversible 'flickery' channel block. We estimate that each platelet possesses ≥ 30 such channels. Kinetic analysis suggested at least two open channel states ($\tau = 0.8 \pm 0.2$ ms, $\tau = 22 \pm 14$ ms, $n = 4$) and two closed states ($\tau = 0.8 \pm 0.2$ ms, $\tau = 12 \pm 0.6$ ms, $n = 4$). Increasing $[Ca^{2+}]_i$ to 10 μM, following channel activation by depolarisation, had no significant effect on channel kinetics or open probability, however, elevated $[Ca^{2+}]_i$ (300 nM–10 μM) increased the number of anion channels activated by subsequent depolarisation. This study represents the first recordings of ionic currents in excised, inside-out membrane patches from human platelets, and provides further evidence for the existence of chloride channels in these cells.

Keywords: Patch clamp; Platelet; Chloride channel; Calcium ion, intracellular

1. Introduction

The regulation of ion channels in human platelets has proved difficult to study, largely because of the technical problem of obtaining direct electrophysiological recordings from these cell fragments, which average only 1 by 3 μm. Potentiometric dyes have provided an indirect method of assessing membrane conductances in suspensions of platelets [1,2], but this technique cannot distinguish between rheogenic carriers and ion channels, and the dye signal is often influenced by factors other than membrane potential. Platelet ion channels have also been studied following insertion of membrane vesicles into phospholipid bilayers; these studies give evidence for a thrombin-evoked cation channel [3] and an anion-selective channel [4]. However, there is always the possibility that this technique severely alters channel properties; furthermore the channel orientation in the bilayer and the original membrane compartment from which the channels are derived are often uncertain.

The giga-ohm patch clamp recording technique [5] has now been used by two groups to obtain direct recordings of ion channel activity from mammalian platelets [6,7]. These recordings have allowed characterisation of voltage-gated K^+ channels, ADP-gated nonselective cation channels, chloride channels and calcium-activated K^+ channels [8–10,6,7]. The effect on these channels of intracellular calcium (Ca_i^{2+}), established as a pivotal second messenger during platelet activation [11], has been only briefly addressed. Mahaut-Smith [8] showed ionomycin and spontaneously-induced increases in $[Ca^{2+}]_i$ reversibly activated a small number of potassium channels (up to 7 per platelet). The appearance of chloride channels in whole-cell recordings was also shown to be dependent on the presence of intracellular calcium [10], although it was unclear whether calcium acts directly on the Cl^- channel or via one or more calcium-dependent pathways. The cell-attached and whole-cell recording configurations, the only patch clamp modes so far used to study platelet ion

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channels, are restricted by their lack of access to the cytosolic face of the channel, which limits studies on the actions of second messengers. In this work we have established a method that has enabled the first recordings of single channel events in excised, inside-out membrane patches from human platelets, thereby allowing assessment of the effect of calcium on ion channel activity. Preliminary reports of this work have been presented in abstract form [12,13].

2. Materials and methods

2.1. Platelet isolation

Venous blood was obtained from healthy volunteers, with their informed consent, 8.5 ml aliquots being mixed with 1.5 ml anticoagulant (2.5 g/100 ml trisodium citrate, 1.5 g/100 ml citric acid and 2 g/100 ml D-glucose). Platelet-rich plasma (PRP) was prepared by centrifugation at $700 \times g$ for 5 min. Spontaneous activation by thrombin and ADP was prevented by addition of aspirin (100 μM) and 20 μg/ml apyrase, respectively. Aliquots (0.5–1 ml) of PRP were centrifuged for 25 s at 'low speed' in a Microcentaur microcentrifuge (MSE Scientific Instruments, UK) and the platelets resuspended in nominally Ca^{2+}-free standard platelet saline (see below) containing 20 μg/ml apyrase and 0.1% bovine serum albumin (BSA). The addition of BSA further reduced spontaneous platelet activation. PRP was stored at room temperature, for up to 8 h, on a vertical wheel rotating at approx. 0.3 Hz, but after resuspension, the platelets were used within 3 h.

2.2. Solutions and reagents

The standard platelet saline, unless otherwise stated, was Ca^{2+}-free and contained (mM) 150 NaCl, 1 $MgCl_2$, 10 Hepes, 1 EGTA, titrated to pH 7.3 with 1 M NaOH. Low-Na^+ platelet saline contained (mM) 137 *N*-methyl-D-glucamine Cl, 17 NaCl, 1 $MgCl_2$, 10 Hepes, 1 EGTA, titrated with 1 M *N*-methyl-D-glucamine to pH 7.35. A stock 150 mM sodium gluconate solution was made with 150 mM sodium gluconate lactone and 150 mM NaOH, stirred for 48 h until the pH reached 7.35. Low-Cl^- saline contained (mM) 133.05 sodium gluconate, 17 NaCl, 1 $MgCl_2$, 10 Hepes, 1 EGTA titrated with NaOH to pH 7.35. A Ca^{2+}:EGTA buffer system was used to set the free [Ca^{2+}] in the range 300 nM to 10 μM and was calculated by a Fortran IV program based on the method of Robertson and Potter [14]. Aspirin, apyrase, BSA, EGTA and nystatin were obtained from Sigma (UK) and 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB) was obtained from Calbiochem Novabiochem (UK). Stock solutions of nystatin and NPPB were both prepared using dimethylsulfoxide (Aldrich, UK). Aspirin and apyrase were made up in absolute alcohol and water, respectively.

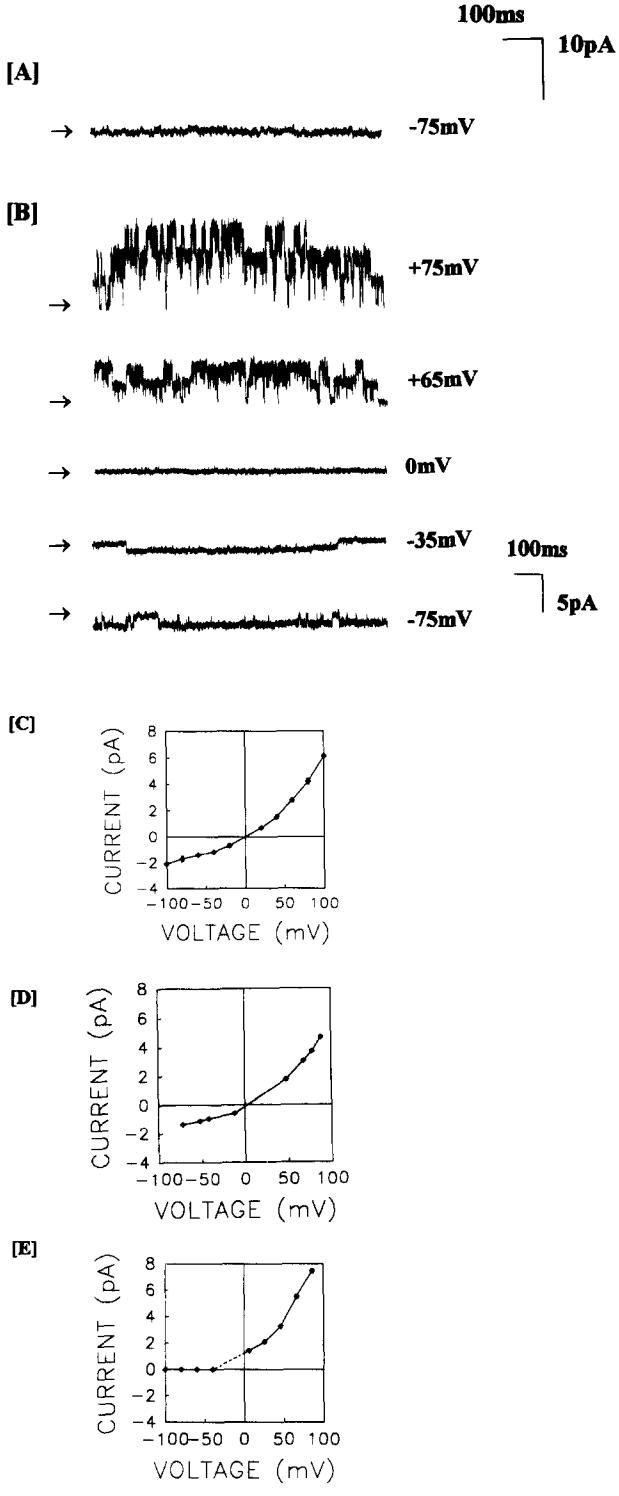
2.3. Electrophysiology

A 250 μl recording chamber, constructed from perspex with a glass coverslip forming its base, was mounted on an inverted microscope and initially filled with Ca^{2+}-free standard saline containing 0.1% BSA. 50–100 μl of platelet suspension were added and the platelets allowed to settle; the chamber was then perfused with standard saline. The perfusion removed platelets from the upper part of the chamber more rapidly than from the bottom, so by adjusting the length and rate of perfusion, a layer of unattached platelets could be left just above the coverslip. Patch electrodes (filled resistance, 5–10 MΩ) were pulled from borosilicate filamented glass capillaries (Clark Electromedical Instruments, UK). A filled pipette was lowered into the chamber, slight positive pressure being applied. It was moved, under $1000 \times$ magnification, to within ≈ 5 μm of a floating platelet, the positive pressure was released and suction used to draw the platelet onto the pipette tip. This frequently resulting in the formation of a multigigaohm resistance seal between the pipette glass and the platelet membrane. The chamber was grounded via a 2% agar bridge made up in 150 mM NaCl platelet saline. All potentials were corrected for liquid junction potentials, measured by reference to a 3 M KCl bridge. Data were filtered by a 3-pole Bessel filter at 3 kHz within the L/M EPC-7 patch amplifier (List, Germany) and stored on videotape following digitization at 18 kHz by a Digital data recorder (Instrutech, USA). Data were replayed and low-pass filtered at 1–3 kHz (Bessel) before acquisition to computer at 10 kHz through a Digidata interface for analysis by pClamp6 software (Axon Instruments, CA, USA). A digital Gaussian filter (1 kHz) within pClamp6 was also used occasionally. Channel amplitudes were determined either using Gaussian fits to all point histograms or directly, by eye, from the current records. To determine the distribution of open and closed time durations of the channel, a threshold was set halfway between the open and closed state and each time a current crossed this threshold, a transition was assumed. Lifetime histograms were plotted as a function of the number of events per bin against time and fitted with a simplex least squares method; data less than twice the rise time of the filter were disregarded [15]. All experiments were performed at the ambient temperature (18 to 20°C). Data were expressed as means $\pm$ S.D. with the number of observations (n) in parenthesis. Statistical significance was evaluated by the Student's unpaired t-test, $P < 0.05$ being considered significant.

2.4. Formation of inside-out configuration

The conventional method of obtaining the inside-out patch clamp configuration, when cells are not adherent to the coverslip, is to briefly pass a cell sealed in cell-attached mode across the air/water interface. However, in human platelets, this approach invariably resulted in rapid

deterioration of the giga-ohm seal. We therefore developed the following technique which appeared less traumatic to the membrane:glass seal. Before addition of platelets to the chamber, a drop of silicone grease (Type M494, Imperial Chemical Industries, Ambersil Ltd, UK) was placed on the base of the bath. Following formation of a cell-attached seal, the platelet was rapidly dipped into and withdrawn from the drop of silicone grease. Upon removal, the 'outer vesicle' of the platelet remained in the grease, resulting in formation of an excised inside-out membrane patch.



3. Results

3.1. Single channel recordings from excised, inside-out membrane patches

When the pipette and bath contained 150 mM NaCl, spontaneous channel events were rarely observed at negative membrane potentials in cell-attached recordings or following patch excision into the inside-out configuration (Fig. 1A). In 15 out of 39 patches (38%), depolarisation to potentials of +60 mV or greater resulted in activation of channels carrying outward current, after a delay of between 30 s and 5 min. Once channels had appeared at these positive potentials, they remained active throughout a wide voltage range (−75 to +75 mV); Fig. 1B shows typical current traces at different membrane potentials. Up to three channels of similar amplitude could be distinguished in the current traces from each patch and an average density of 0.48 channels was calculated from all excised patches. Assuming a random distribution of channels in the membrane, and taking the area under the pipette to be 1/67th of the total platelet surface (12.5 μm^2), we estimate that each platelet has a total of at least thirty of these channels.

In symmetrical 150 mM NaCl, the single channel current–voltage relationship was outwardly rectifying (Fig. 1C) with chord conductances of 53.5 ± 1.5 pS ($n = 5$) at +80 mV decreasing to 20.9 ± 2.7 pS ($n = 5$) at −80 mV. The ratio of single channel conductances, $g_{Cl^-,+80}/g_{Cl^-,-80} = 2.56$, gives a relative measure of the degree of rectification. Replacement of Na^+ in the bath with *N*-methyl-D-glucamine did not significantly affect the current–voltage relationship (Fig. 1D), while replacement of chloride with gluconate, shifted E_{rev} −40 mV per 10-fold change in $[Cl]_i$ (Fig. 1E). This indicates that the channel is selective for anions over monovalent cations and has a relatively high selectivity for Cl^- over gluconate. At the ambient temperature (average 19°C) one

Fig. 1. Current–voltage relationship and ionic selectivity of channels induced by depolarisation in inside-out patches. (A,B) Current traces at −75 mV immediately after excision (A), and at various potentials in the range −75 to +75 mV after depolarisation to +60 mV for 5 min (B). Data recorded in symmetrical NaCl with outward current shown as upward deflections from the baseline current level where all channels are closed (arrow). Bandwidth 1 kHz. (C–E) Single channel current–voltage relationships with 150 mM NaCl pipette saline and bath saline containing 150 mM NaCl (C), 136 mM *N*-methyl-D-glucamine Cl (D), 17 mM NaCl and 133 mM sodium gluconate, 17 mM NaCl (E). Each value (■) is the mean of five experiments. Error bars are not shown if smaller than the size of the symbol.

would predict a shift of −57 mV per 10-fold change in [Cl^-] if the channel were completely selective to chloride. Chloride channels in other cell types have been found to possess significant permeabilities to larger molecular weight anions [16]. Therefore significant permeability to gluconate is the most likely explanation for E_{rev} shifting less than expected for the Nernstian ideal. No activity coefficient is available for gluconate, thus permeability of gluconate relative to chloride could not be determined.

During development of the 21–52 pS channel described above, current activity consisting of irregular current fluctuations from the baseline (closed) level was also observed, as illustrated in Fig. 2A. These currents appeared to be due to the partial opening of the 21–52 pS channel conductance since they reversed at the same potential and were observed alongside openings of the latter. Amplitude histograms of these partial events (Fig. 2B) and the full conductance (Fig. 2C) revealed one predominant open level with no distinct sub-conductance levels.

3.2. Analysis of single channel kinetics

For analysis of the chloride channel kinetics, we used excised patches with only one active channel. The current trace in Fig. 3 illustrates typical activity from such a patch at +65 mV. The open time histogram, constructed from longer current recordings (Fig. 3B), could be fitted by a double exponential ($\tau = 0.8 \pm 0.2$ ms, $n = 4$; $\tau = 22 \pm 14$ ms, $n = 4$). The closed time histogram (Fig. 3C) was also best fit by a double exponential ($\tau = 0.8 \pm 0.2$ ms, $n = 4$; $\tau = 12 \pm 6$ ms, $n = 4$). These data therefore suggest the presence of two open and two closed channel states.

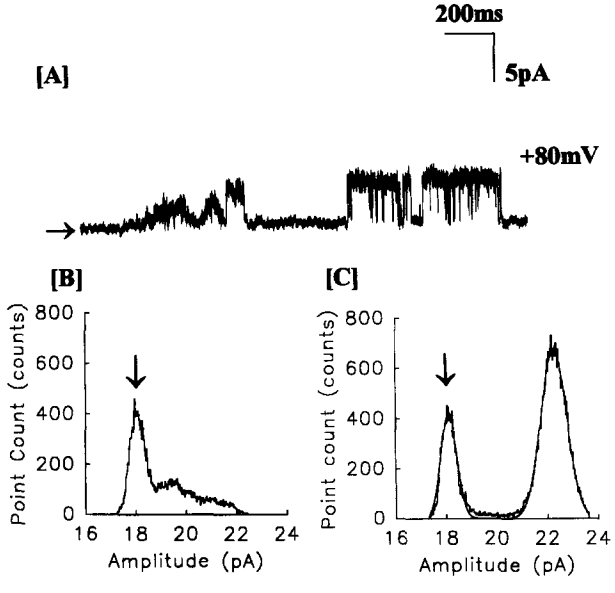


Fig. 2. Channel conductance during and after activation by positive potentials. (A) Current trace during the development of active channels at +80 mV in an excised, inside-out patch. (B) All points amplitude histogram of membrane currents during development of active channels. (C) All points amplitude histogram of membrane currents following full channel activation. The arrows indicate the level at which all channels are closed (baseline).

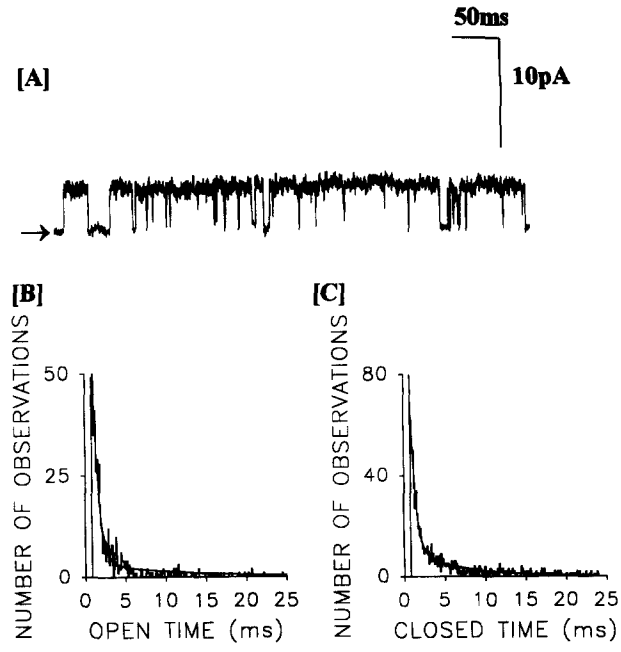


Fig. 3. Kinetic analysis of chloride channel. (A) Current trace showing typical channel events at +65 mV from an excised patch with a single active chloride channel. Bandwidth 3 kHz. Open time (B) and closed time (C) histograms have both been fitted by a double exponential: (B): $\tau = 0.6$ and 11 ms; (C): $\tau = 0.6$ and 19 ms.

3.3. The effect of intracellular calcium on chloride channel activity

In a previous patch clamp study of human platelets using whole-cell recordings [10], activation of an outwardly rectifying chloride channel was suggested to be dependent on the presence of intracellular calcium. However, it was unclear from these whole-cell recordings whether calcium was acting directly on the channel or indirectly, via a calcium-dependent process. To address this issue we applied increasing [Ca^{2+}] to the cytosolic face of excised membrane patches. The recordings described above were conducted in Ca^{2+}-free (1 mM EGTA) saline. Elevation of cytosolic calcium to 300 nM ($n = 10$), 1 μM ($n = 14$) or 10 μM ($n = 4$), by bath perfusion, failed to activate channel activity at negative potentials in symmetrical NaCl. Strong positive membrane potentials ($\geq +60$ mV) activated Cl^- channel currents confirming their presence in these excised membrane patches. The number activated was potentiated by increased [Ca^{2+}]$_i$ from an estimated 30 per platelet in 1 mM EGTA to 44, 54 and 61 channels in the presence of 300 nM, 1 μM and 10 μM Ca^{2+}, respectively. Addition of calcium (10 μM) to the cytosolic face of the channel, following channel activation by depolarisation in EGTA-containing bath saline, had no significant effect on the open probability, open or closed time. Mean open time, 4.9 ± 0.3 ms in 0 Ca^{2+} vs.

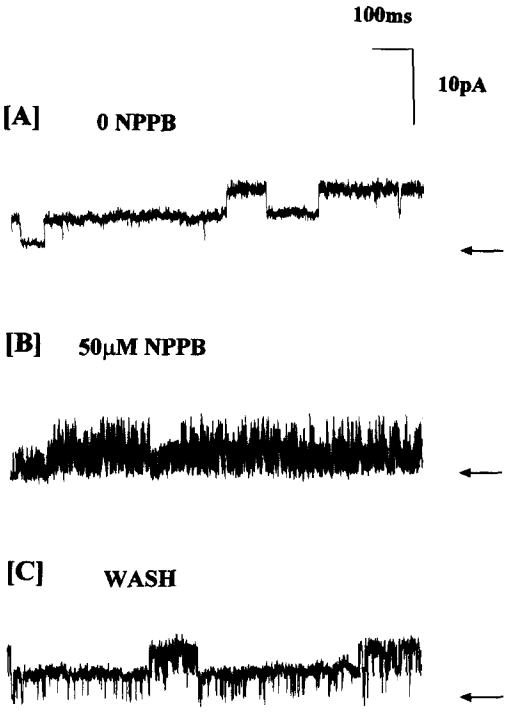


Fig. 4. Block of chloride channels by 5-nitro-2-(3-phenylpropylamino) benzoate (NPPB). Chloride channel activity in an inside-out patch at +70 mV (A) before addition of NPPB; (B) 1 min after addition of 50 μM NPPB to the bath and (C) 3 min after wash. Arrows indicate closed channel level. Bandwidth 1 kHz.

6.9 ± 2.2 ms in 10 μM Ca^{2+} ($n = 3$, $P > 0.1$); mean closed time, 3.6 ± 0.5 ms in 0 Ca^{2+} vs. 2.7 ± 0.5 ms in 10 μM Ca^{2+} ($n = 3$, $P > 0.1$); open probability 0.7 ± 0.21 in 0 Ca^{2+} vs. 0.7 ± 0.12 in 10 μM Ca^{2+} ($n = 3$, $P > 0.1$)

3.4. Inhibition of channel activity by NPPB

The carboxylate analogue, NPPB, is a potent blocker of chloride channels in epithelial cells [17]. Application of 50 μM NPPB (or 100 μM, not shown) to the cytosolic face of the patch, caused a 'flickery' block of the channel (Fig. 4B). Removal of NPPB resulted in the return of the longer duration channel openings (Fig. 4C) indicating that blockade by NPPB is reversible.

4. Discussion

This report describes the first recordings of single channel events in excised, inside-out membrane patches from human platelets. Using this configuration, we observed a channel with an outwardly rectifying *I–V* relationship that was activated by prolonged depolarisation (≥ +60 mV). Ion substitution experiments indicated that this channel is selective for anions over cations and has a higher permeability to Cl^- over larger anions such as gluconate. Cl^--selective channels in human platelets have previously been observed using whole-cell patch clamp recordings [10] and following insertion of membrane vesicles into lipid bilayers [4]. In whole-cell recordings, the chord conductance at +50 mV was 44 pS, decreasing to 28 pS at −50 mV, which is similar to the conductance in excised patches of 54 pS at +80 mV decreasing to 20.9 pS at −80 mV. Comparison with anion-selective channels reconstituted into planar lipid bilayers [4] is more difficult as orientation of the channel in the bilayer is uncertain. Assuming the 'trans' face of the channel is inwards then the *I–V* relationship of this channel is also outwardly rectifying and has a single channel conductance (estimated from Fig. 2 in [4] for 150 mM Cl^-) of 71 pS at +50 mV and 28 pS at −50 mV, which is close to that seen in patch clamp recordings. In our experiments, the channel density, estimated from the average number of channels per excised membrane patch, was shown to have a lower limit of thirty channels per platelet. This is also comparable to the number of Cl^- channels in whole cell recordings, where the peak to peak current indicated a total of between 6 and 30 per platelet. The lower estimate in whole-cell recordings probably results from the channels not all opening simultaneously.

Chloride channels in epithelial cells can be blocked by a series of related compounds [17], the most potent of which was NPPB. In this study, 50–100 μM NPPB induced a reversible 'flickery' block of Cl^- channel openings, whereas the same concentration of NPPB gave rise to an almost complete channel block in whole-cell recordings [10]. It has been suggested that NPPB interacts with the extracellular face of the channel protein [18]. Thus in excised membrane patches, where NPPB is applied to the cytosolic surface, the drug may have to pass through the plasma membrane to be effective, hence the reduced sensitivity compared with whole-cell recordings, where NPPB was applied extracellularly.

Cl^- channels in excised membrane patches from lymphocytes and epithelial cells [19–21], have been shown to be activated by prolonged depolarising voltages in a similar manner to the platelet Cl^- channel. The mechanism of activation in all these studies is uncertain. It is possible that an inhibitory regulator is displaced from the channel, resulting in activation. Alternatively, the large electric field may cause the ion channel protein to cross an energy barrier and switch from an inactive to an active state. On the other hand, physiological agonists produce only relatively small depolarisations in membrane potential (E_m), for example thrombin, ADP and PAF transiently depolarise the membrane by 6–8 mV from the resting E_m of about −60 mV to −70 mV [2] a voltage change that would be insufficient to activate the channel. Consequently, imposing large electrical gradients is clearly a non-physiological means of activating the channel. Nevertheless, depolarisation is useful as a 'gauge' to verify and quantify the presence of chloride channels and was used in this study as an important control experiment when evalu-

ating the effect of second messengers on channel activation.

In whole-platelet recordings, activation of a Cl^- channel was shown to be dependent on the presence of internal calcium [10]; furthermore potentiometric studies provide evidence for the presence of a calcium-dependent chloride conductance [22]. In the present study, direct elevation of intracellular calcium concentration from sub-nanomolar (1 mM EGTA) up to 10 μM, at negative membrane potentials, failed to elicit channel activity, although increased $[Ca^{2+}]_i$ potentiated the number of channels activated by subsequent depolarisation. The finding that exposure to high $[Ca^{2+}]_i$, after activation of channels by depolarisation, had no effect on channel kinetics indicated that the chloride channel is not directly activated by intracellular calcium, but it can influence a stage during channel activation by strong positive membrane potentials. Lack of activation by internal Ca^{2+}, in contrast to previous findings [10,22], could be explained by loss of a calcium-dependent effector molecule, possibly a multifunctional protein kinase, during excised patch recordings.

The function of this chloride channel in the platelet remains unclear, although the reversible block by NPPB may prove a useful tool in future studies. A chloride conductance has been implicated in volume regulation, since exposure of platelets to a hypotonic medium increases chloride permeability from low resting levels [22,23]. Ca_i^{2+} is a pivotal second messenger in platelet activation [11], therefore previous studies indicating a dependence of Cl^- channels on Ca_i^{2+} [22,10] suggest that they may become more active after platelet stimulation. $[Cl^-]_i$ has not been directly measured in human platelets, however potentiometric dye studies suggest that the chloride equilibrium potential is about -35 mV [7]. Therefore, an increased Cl^- conductance would tend to depolarise the membrane from resting E_m of -60 to -70 mV and diminish calcium influx, thus these channels may have a negative feedback or modulatory role during platelet activation.

To our knowledge, this is the first reported use of silicone grease in the formation of stable excised inside-out patch recordings. In our hands this was the only method of ripping the patch from the outer platelet vesicle without destroying the giga-ohm glass:membrane seal. Brief exposure to the air/water interface led to a rapid reduction in seal resistance, perhaps as a result of the greater surface tension at an air:water versus a grease/water interface. This approach, which we term the 'silicone grease trap' technique may be useful in the formation of excised patches from other small cells in suspension or from organelles.

In conclusion, we have presented the first single channel recordings from inside-out patches of human platelets, excised by a new 'silicone grease trap' technique; these provide further evidence for the existence of an outwardly rectifying Cl^- channel of medium conductance in these cells.

Acknowledgements

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