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Chloride Channels in Human Platelets: Evidence for Activation by Internal Calcium

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Summary. Whole-cell patch-clamp recordings were made from freshly isolated human platelets. The pipette contained a high concentration of divalent cations, which permitted easy disruption of cell-attached membrane patches by suction. Single-channel currents were measured when the pipette contained isotonic $BaCl_2$ or $MgCl_2$ saline; over 30 sec – 5 min an increasing number of channels appeared until conductance steps through individual channels could no longer be distinguished. The current-voltage relationship was curvilinear; chord conductance at −35 mV was 25 pS increasing to 45 to 52 pS at +45 mV. Ion substitution experiments showed the current to be primarily carried by Cl^-. E_{rev} was shifted 30 mV/10-fold change in external Cl^- (replaced by gluconate), was similar with $BaCl_2$ or $MgCl_2$ in the pipette and was not significantly shifted by replacing external Na^+ with K^+. Addition of 1 mM BAPTA to the $MgCl_2$ pipette saline prevented activation of Cl^- currents; with isotonic $CaCl_2$ internal saline, current appeared immediately upon patch rupture, suggesting that the Cl^- channels are dependent on internal Ca^{2+}. 5-nitro-2-(3-phenylpropylamino)-benzoate, reported to block a Cl^- conductance in studies of rat epithelial cells, caused a potent flickery block and may be a useful tool with which to investigate the physiological role of Cl^- currents in human platelets.

Key Words Cl^- channels · platelet · calcium · patch clamp

Introduction

The importance of ion channels in platelet responses has only recently been addressed due to the technical difficulties of direct electrophysiological recordings from these minute cells, only 1 by 3 μm in diameter. Membrane potential measurements using the potential-sensitive dye diS-C_3-(5), show a predominantly K-selective plasma membrane and a smaller Cl^- conductance (MacIntyre & Rink, 1982) but this technique cannot conclusively distinguish between a rheogenic carrier and a conducting pore. Electrical recordings from platelet membranes reconstituted into lipid bilayers have also suggested the presence of divalent cation-selective channels in thrombin-stimulated membranes (Zschauer et al., 1988) and anion-selective channels in unstimulated membranes (Manning & Williams, 1989).

Recently, the patch-clamp technique (Hamill et al., 1981) has been successfully applied to freshly isolated mammalian platelets. Maruyama (1987) showed that rabbit, rat and human platelets have several hundred voltage-gated potassium channels with a single-channel conductance of approximately 9 pS. It is likely that this conductance establishes the resting membrane potential of −60 to −70 mV (MacIntrye & Rink, 1982; Pipili, 1985). Channels activated by the agonist ADP have also been recorded in cell-attached patches from human platelets (Mahaut-Smith, Rink & Sage, 1989). These channels are permeable to monovalent and divalent cations and likely represent the pathway for Ca influx reported in quin 2 and fura-2 loaded platelets in response to ADP (Hallam & Rink, 1985; Sage & Rink, 1986).

In the present study the first direct measurements of chloride channels in human platelets were made using the whole-cell mode of patch-clamp recording. Channel activity appeared to require internal Ca^{2+}; therefore, this conductance may have a role during platelet activation. A brief report of this work has been presented to the Physiological Society (Mahaut-Smith, 1990).

Materials and Methods

Platelet Preparation

Blood was drawn from healthy subjects and 8.5-ml aliquots were mixed with 1.5 ml acid citrate dextrose anticoagulant. The anticoagulant contained 2.5 g/100 ml trisodium citrate, 1.5 g/100 ml citric acid and 2 g/100 ml D-glucose. The mixture was immediately centrifuged at 700 × g for 5 min and platelet-rich plasma (PRP) pipetted off. 100 μM aspirin and 20 μg/ml apyrase were added to prevent activation by spontaneously released thromboxane and ADP, respectively. 1 ml of PRP was then spun at "low-speed" in a MSE microcentaur and resuspended in nominally Ca-free platelet saline with 20 μg/ml apyrase.

Solutions and Reagents

Platelet saline was nominally Ca free (to slow spontaneous activation of platelets that occurs in vitro) and contained (in mM) 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, titrated to pH 7.4 with NaOH. In substitution experiments, NaCl was replaced by KCl or by Na-gluconate; 150 mM Na-gluconate saline also contained no KCl. Stock Na-gluconate was made with NaOH and D-gluconic acid lactone. The high divalent cation pipette saline contained (in mM): 110 $BaCl_2$ or 110 $MgCl_2$ or 110 $CaCl_2$ and 10 HEPES buffered to pH 7.4 with *n*-methyl D-glucamine. Solution osmolarities were measured with a vapor pressure osmometer (Chemlab. Scientific, UK). There was no significant difference in osmotic strength between internal salines and normal external (Ca-free platelet) saline: (in mmol/kg, SD, $n = >5$) 110 mM $BaCl_2$ saline, 293 ± 1; 110 mM $CaCl_2$ saline, 298 ± 4; 110 $MgCl_2$ saline, 299 ± 3; Ca-free platelet saline, 295 ± 3. The tetrapotassium salt of 1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (K_4BAPTA) was obtained from Sigma Chemical, Poole, Dorset, and 5-nitro-2-(3-phenylpropylamine)-benzoic acid (NPPB) from Dr. D.J. Keeling at Smith Kline & French Research, Welwyn, UK.

Patch-Clamp Methodology

The glass coverslip forming the base of the electrophysiological recording chamber was siliconized before each experiment to prevent platelets adhering to the glass. "Sigmacote" (Sigma Chemical) was spread over the coverslip, allowed to dry, then washed with alcohol and saline. 20–50 μl of platelet suspension were pipetted into the chamber filled with Ca-free saline, allowed to settle and then perfused with more saline. Patch pipettes with a filled resistance of 5–10 Ω were pulled from VWR micropipettes (Drummond Scientific, USA). A pipette was lowered into the chamber, manipulated to within 5 μm of a floating platelet and the platelet drawn to the pipette using suction. Recordings were made from experiments in which the glass : membrane seal developed to at least 20 GΩ. Current was measured under voltage clamp with an EPC-7 patch-clamp amplifier (List Electronic, FDR) and stored on video tape after digitization by a PCM adaptor (SONY). Data were low-pass filtered at 1 kHz or 400 Hz (−3dB) and analyzed on a Tandon computer using Satori software (Intracel, UK).

The chamber was grounded through an agar bridge made with 1–2% agar and $BaCl_2$ pipette saline. Offset potentials were dialed out at the start of the experiment; therefore, upon formation of a giga-seal the potential in the pipette is shifted by the value of the liquid : liquid (pipette saline : bath saline) potential. Potential offsets will also occur upon changing the bath saline. Offsets were measured by reference to a 3 M KCl pipette or agar bridge. $BaCl_2$, $MgCl_2$ and $CaCl_2$ salines were all 5 mV with respect to platelet saline and 7 mV with respect to KCl saline. The bath potential was shifted −5, −9 and −12 mV when the normal platelet saline in the bath was changed to 77 mM Cl^- (70 gluconate) saline, 37 mM Cl^- (110 mM gluconate) saline and 150 mM Na-gluconate (2 mM Cl^-), respectively. All potentials have been corrected for these offsets.

Results

Conditions Required for Whole-Cell Recordings

Cell-attached platelet patches proved difficult to disrupt by suction when the pipette contained monovalent cation salines. Maruyama (1987) has also reported this problem and facilitated formation of whole-platelet recordings by combining ATP with a low divalent cation concentration in the pipette saline. In the present study, whole-cell recordings were easily obtained by suction when the pipette contained a high divalent cation saline (110 mM $MgCl_2$, $BaCl_2$ or $CaCl_2$; *see* Materials and Methods).

Cl^- Channels in Whole-Cell Recordings

Figure 1 illustrates the main type of channel activity that appeared in the whole-cell mode with $BaCl_2$ pipette saline. Current traces are shown at different pipette potentials (V_p). In the cell-attached condition V_p must be subtracted from the membrane potential (approximately −60 to −70 mV; MacIntyre & Rink, 1982; Pipili, 1985) to give the patch potential; in the whole-cell mode, the potential across the platelet membrane is set directly by V_p. The cell-attached patch was usually devoid of channel activity within the applied potential (V_p) range +45 to −75 mV. After application of light suction to break through into whole-cell mode, current steps due to individual channel openings developed in 14 out of 14 experiments (the appearance of these channels with time is described in detail later). Current was outward at positive potentials, that is anions being conducted into the cell or cations flowing out, and reversed direction close to 0 mV. Similar currents developed in whole-cell recordings with isotonic $MgCl_2$ pipette saline (11 out of 11 experiments). Single-channel current-voltage relationships for the predominant conductance level are plotted in Fig. 1*B* (*i,ii*). E_{rev} was similar for both divalent cations (average values, ± SD: $BaCl_2$: 2 ± 2 mV, $n = 6$; $MgCl_2$: 3 ± 1 mV; $n = 5$) and both relationships rectified such that more current was carried through the channel at positive membrane potentials. The chord conductances at +45 mV were 45 ± 5 pS (SD $n = 5$) for $BaCl_2$ and 52 ± 3 pS ($n = 4$) for $MgCl_2$; at −35 mV the conductance was 25 ± 2 pS for both $BaCl_2$ ($n = 6$) and $MgCl_2$ ($n = 3$).

Replacing all NaCl in the bath with KCl did not significantly affect the reversal potential of the single-channel current (Fig. 1*B* (*iii*); E_{rev}, 5 ± 1 mV, $n = 5$), whereas replacing all NaCl and KCl with Na-gluconate shifted E_{rev} to more positive potentials (Fig. 1*B* (*iv*)). Clear single-channel outward currents were not observed in 150 mM Na-gluconate saline but reversed current was recorded in salines containing 37 mM Cl^- (110 mM gluconate) and 77 mM Cl^- (75 mM gluconate). E_{rev} shifted by 30 mV/10-fold change ($n = 3$) in external [Cl^-]. The results suggest that the current activated in these whole-cell conditions is primarily carried by Cl^-. Cl^- channels in

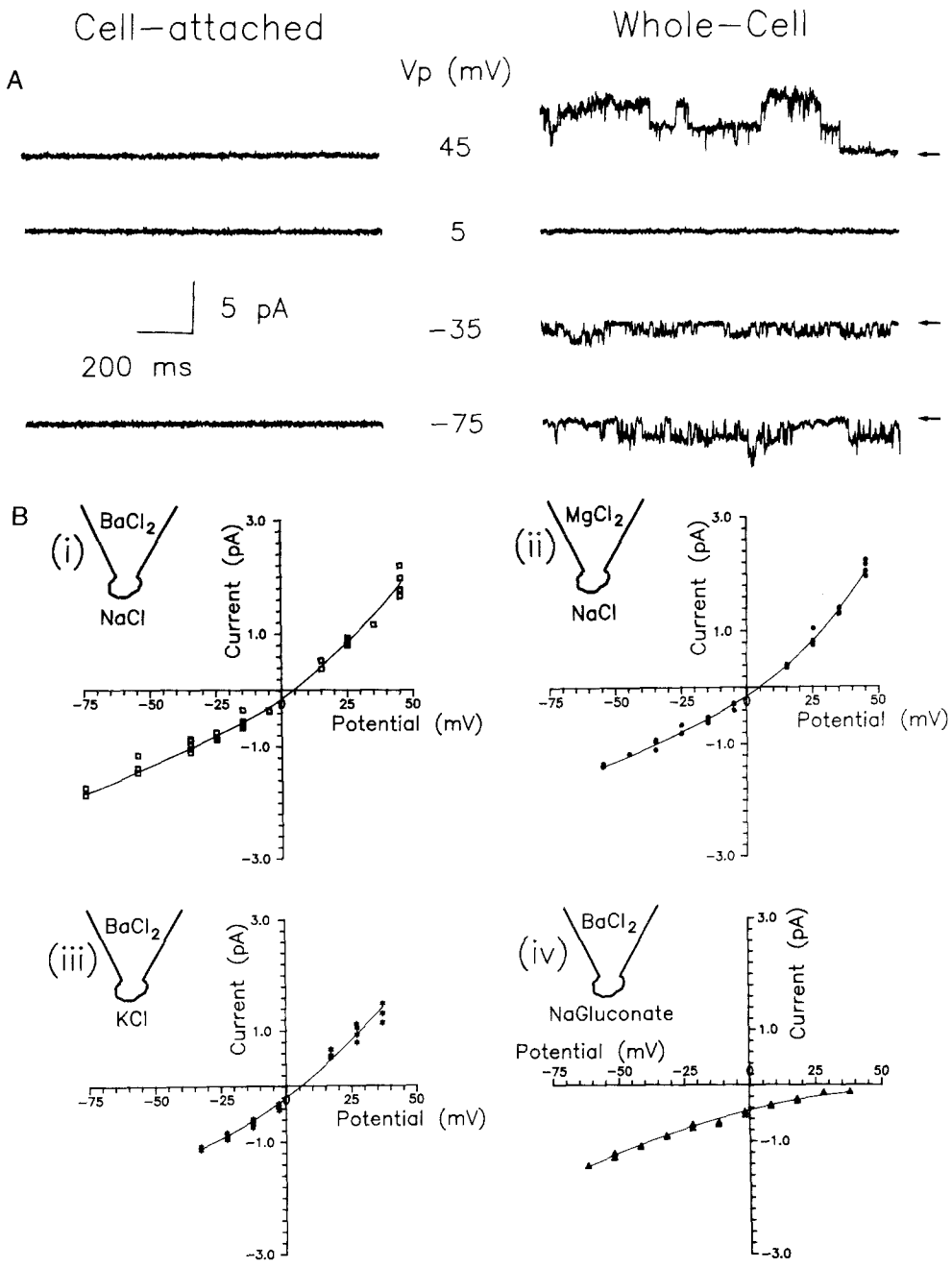

Fig. 1. Single-channel currents activated during whole-cell recordings. (*A*) Examples of current activity at different applied potentials (V_p) from a cell-attached patch (left panel) and between 1 min 20 sec and 2 min 20 sec after patch rupture (right panel). Inward current is shown by a deflection below the baseline which is indicated by the arrow. Filtered at 400 Hz. Pipette filled with 110 mM $BaCl_2$ saline. (*B*) Single-channel current : voltage relationships for the currents shown in *A*. Pipette: 110 mM $BaCl_2$ saline (*i*), (*iii*) and (*iv*); 110 $MgCl_2$ saline (*ii*). Bath: nominally Ca-free saline (*i*), (*ii*); high K saline (*iii*) and 150 mM Nagluconate saline (*iv*). Each symbol is an averaged current value from a single cell

other preparations have smaller, yet significant permeabilities to anions with larger molecular weights (for example, *see* Bosma, 1989; Manning & Williams, 1989), and permeability to gluconate is the most likely explanation for why E_{rev} shifted by less than the Nernstian ideal. No activity value was available for gluconate; therefore, the permeability of gluconate relative to Cl^- cannot be calculated.

The Cl^- equilibrium potential, calculated from the equation

$$E_{Cl} = RT/F \cdot \ln[a_{Cl_i}[Cl^-]_i/a_{Cl_o}[Cl^-]_o]$$

(where a is the activity coefficient for Cl^- in internal or external salines obtained from Robinson and Stokes (1955)) was -4 mV for $BaCl_2$ internal saline and normal external saline. The measured reversal potential (3 mV, *see above*) was more positive than E_{Cl}, which can be explained by a significant permeability to Na^+ or K^+. In fact, Manning and Williams (1989) have measured P_{Cl}/P_K for reconstituted platelet Cl^- channels to be 4:1. The absolute permeability to cations cannot be derived from the present experiments since different cation species were present at the two membrane surfaces. The lack of shift on replacing NaCl with KCl suggests that the channel is equally permeable to these two monovalent cations.

Time-Dependent Channel Activation

After establishing a whole-cell recording, the Cl^- currents developed in a complex manner. This is illustrated in Fig. 2 for a $MgCl_2$ saline-filled pipette; a similar pattern was observed for $BaCl_2$ except that currents generally appeared more rapidly. The current started as irregular fluctuations on top of the baseline, as illustrated in Fig. 2*A* by the trace at 45 mV taken approximately 12 sec into whole-cell recording. These initial events were due to partial opening of the "square" 52-pS Cl^- channel described above. They reversed close to 0 mV and developed into square channel events as shown in the trace at 15 sec. With time, the number of active channels increased as shown in the recording at 1 min 45 sec. Figure 2*B* is an amplitude histogram, constructed from a 9.2-sec recording with multiple current levels. Five peaks can be distinguished which coincide with multiples of the single-channel conductance (48 pS in this cell) and confirm the presence of four individual Cl^- channels. Eventually, as more current developed, steps due to individual channel events could not be distinguished. Presumably, this is due to activation of more Cl^- channels as the reversal potential of the whole-cell current did not change. The peak-to-peak current was used to estimate the total number of Cl^- channels activated in an individual platelet. The value depended on how long the recording was maintained; at least five channels were activated in all recordings with $MgCl_2$ or $BaCl_2$ pipette salines. The maximum number (before loss of stable recording) ranged from 6 to 30 channels per platelet (average 14). The peak-to-peak current is most likely an underestimate of channel density because not all channels will be either closed or open simultaneously.

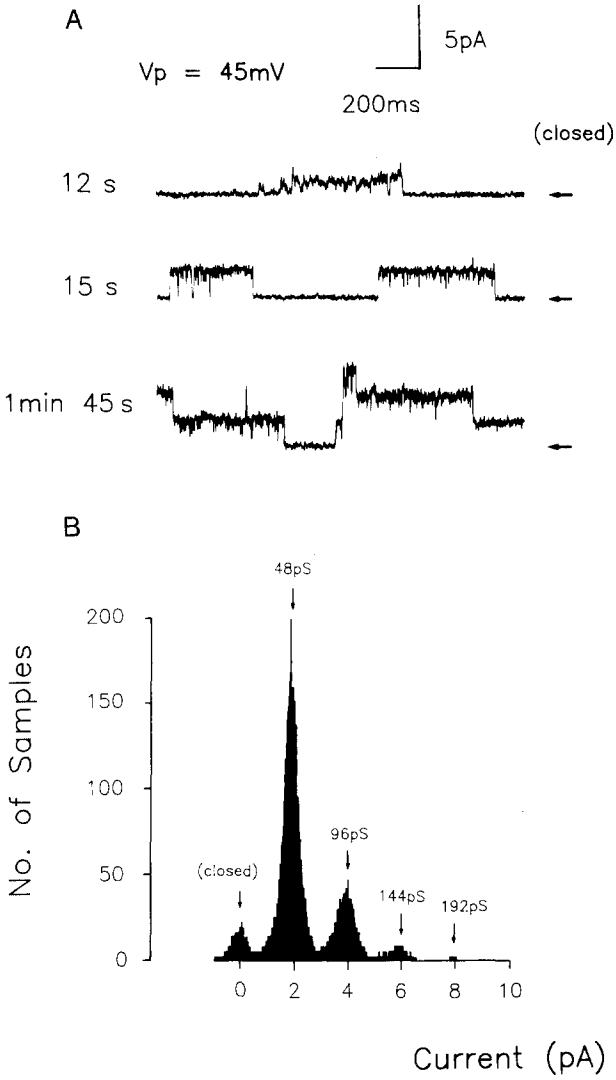

Fig. 2. Time-dependent increase in whole-cell currents. Pipette filled with 110 mM $MgCl_2$ saline. (*A*) Records of current activity at 12 sec, 15 sec and 1 min 45 sec after rupture of the membrane patch. Pipette potential: 45 mV. Filtered at 400 Hz. (*B*) Amplitude histogram of a 9.2-sec current record displaying multiple current levels (same cell as *A*). Pipette potential: 45 mV. Ordinate: number of data points (record digitized at 5 kHz). Abscissa: current (pA) from the baseline (closed state). Arrows indicate the conductance levels expected for summation of individual channels measured at approximately 15 sec (48 pS)

Evidence for Activation by Internal Ca^{2+}

The strong time-dependent activation in whole-cell mode suggested that chloride channels were somehow induced by the high divalent cation concentration as it dialyzed the platelet cytosol. Chloride currents in certain cell types are activated by internal Ca^{2+} (for review, *see* Marty, 1989, although the relevant potency of other divalent cations has not been

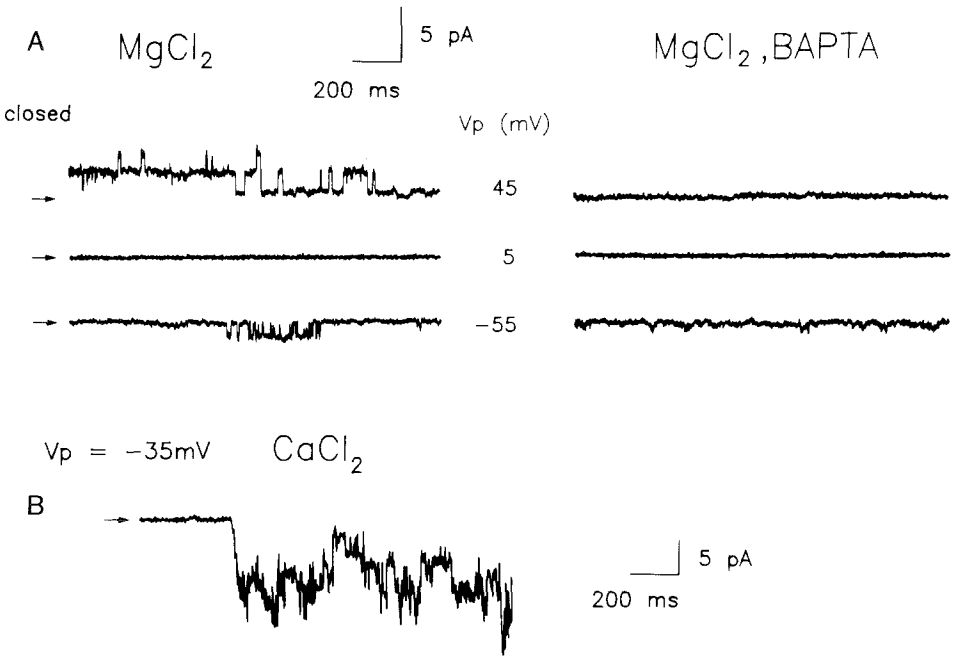

Fig. 3. Evidence for activation by internal Ca^{2+}. (*A*) Current traces at different pipette potentials (V_p). Left panel: pipette filled with 110 mM $MgCl_2$ saline; records taken approximately 1 min after patch rupture. Right panel: pipette filled with 110 mM $MgCl_2$ saline and 1 mM K_4BAPTA; records taken at least 4 min after patch rupture. Arrow indicates level where all channels are in the closed state. (*B*) Current associated with patch rupture at −35 mV with 110 mM $CaCl_2$ pipette saline. The arrow indicates the baseline current in the cell-attached mode. Current polarity is inverted upon patch rupture; in whole-cell mode downward deflection is an inward current. All currents filtered at 400 Hz

studied. Neither Mg^{2+} nor Ba^{2+} can activate the large conductance $I_{K(Ca)}$ channel from skeletal muscle (Oberhauser, Alvarez & Latorre, 1988); therefore, it is possible that platelet Cl^- currents were activated by Ca^{2+} released from intracellular binding sites, since this effect has been shown for Ba^{2+} in molluscan neurones (Meech, 1980), or by Ca^{2+} contamination from the reagents and water. According to the manufacturer's specifications (BDH, AnalaR grade), 110 mM $MgCl_2$ salt is contaminated by up to 2.2 μM Ca^{2+}; the Ca impurity in $BaCl_2$ salt was much higher (Ca^{2+} and Sr^{2+} maximum impurities are listed combined as 0.1%). The water probably contained a few micromolar Ca^{2+}. BAPTA can be used to chelate free Ca^{2+} in $MgCl_2$ saline because it has a high selectivity (>10^5) for Ca^{2+} over Mg^{2+} (Tsien, 1980). This is not possible in the case of $BaCl_2$ saline; our own results indicate that the BAPTA family of buffers and fluorescent indicators bind Ba^{2+} and Ca^{2+} with a similar K_d (M.P. Mahaut-Smith, S.O. Sage & T.J. Rink, *unpublished observations*). Addition of 1 mM BAPTA to $MgCl_2$ pipette saline largely prevented the appearance of Cl^- channels in whole-cell recordings in four out of four cells (Fig. 3*A*). After 4 min whole cell, only small currents were observed on top of the baseline within the applied potential range tested (−55 to 45 mV). In experiments on cells from the same preparations with $MgCl_2$ pipette saline in the absence of BAPTA, Cl^- channels developed in the whole-cell mode as described earlier in seven out of seven cells. Further evidence for the Cl^- current being dependent on internal Ca was provided by recordings with 110 mM $CaCl_2$ saline in the pipette. The trace in Fig. 3*B* shows that current was instantly activated once the membrane patch had broken. Steps due to individual channel openings could not be distinguished but the current reversed close to 0 mV and displayed outward rectification similar to that observed with Ba^{2+} and Mg^{2+}-filled pipettes.

Block of the Cl^- Current

Wangemann et al. (1986) have examined the block of epithelial Cl^- channels by a series of related compounds; the most potent was (5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB). The effect of 10^{-4} M NPPB on whole-cell platelet Cl^- currents is illustrated in Fig. 4. In this experiment, recorded with a $MgCl_2$-filled pipette, three channels were active (control) at a potential of 45 mV before the blocker

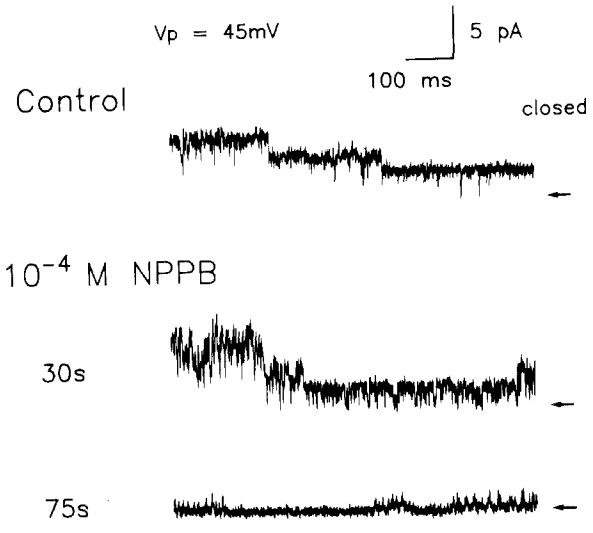

Fig. 4. Block of Cl^- currents by 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB). Whole-cell currents at an applied potential of 45 mV immediately before (control) and 30 and 75 sec after 10^{-4} M NPPB. NPPB was added to the bathing medium approximately 40 sec after attaining a whole-cell recording. Pipette filled with 110 mM $MgCl_2$ saline; bath contained Ca-free platelet saline. All records filtered at 1 kHz

was added to the bath. Mixing of the drug in the bath was rapid (within 1–2 sec), but the effect of NPPB took 30–60 sec to fully develop. NPPB produced a flickery channel block, as shown by the current record at 30 sec after addition. The full effect of NPPB is shown by the trace at 75 sec; for brief periods all channels were held in the closed state interspersed by periods of brief channel openings (a similar effect was obtained in three other cells). The exact time course of block is not apparent from these experiments since in control experiments in the absence of NPPB, the number of channels active increased over the first 1–5 min in all cells tested (*see above*). The effect of NPPB represents a substantial block considering the many channels that developed in control cells.

Discussion

These experiments provide evidence for a chloride conductance in the plasma membrane of human platelets which is induced by internal Ca^{2+}. It remains to be determined from where these channels originate and whether Ca_i^{2+} activates directly or by initiating a series of biochemical events that leads to channel activity. If present in the plasma membrane of intact platelets and directly triggered by Ca_i^{2+} then these channels should be activated in excised, inside-out patches by Ca^{2+} at the cytosolic surface. Alternatively, it is possible that they originate from internal membranes inserted into the plasma membrane following a Ca-dependent fusion process. Cl^- currents were not detected in cell-attached patches from intact human platelets before or in response to ADP (Mahaut-Smith et al., 1989); however, vesicle fusion may not occur within such a patch. My attempts to seal onto intact platelets following stimulation by thrombin have not been successful and indeed this may not be possible after the shape change at activation.

Anion-selective channels have been reported in platelet membrane fragments inserted into lipid bilayers (Manning & Williams, 1989). The channel properties are difficult to compare with those observed in the present patch-clamp study because the polarity of reconstituted membranes is uncertain. Assuming that the "*trans*" surface faces inward, then the Cl^- channel in lipid bilayers displays a similarly rectifying current-voltage relationship and comparable conductance in symmetrical 150 mM Cl^- (60 pS at 40 mV from E_{rev}). Manning and Williams (1989) show that ATP blocks the chloride channel in the reconstituted membrane and propose that Maruyama (1987) failed to detect Cl^- currents in whole-cell recordings because ATP was present in the pipette. However, ATP blocked only from the "*cis*" surface and, as discussed above, the *cis* surface probably faces outward. The present results provide evidence that platelet Cl^- channels are dependent upon internal Ca^{2+}; thus, it is more likely that Maruyama (1987) did not observe Cl^- currents because EGTA was present in the pipette filling solution.

The physiological relevance of the Cl^- channel described here is not yet known. Under resting conditions, the membrane potential (−60 to −70 mV; MacIntyre & Rink, 1982; Pipili, 1985) is set by high K^+ selectivity. The normal Cl^- conductance is low but is greatly stimulated during regulatory volume responses to a hypotonic medium (Livne, Grinstein & Rothstein, 1987). Reducing the osmotic strength of the bathing medium initially causes platelets to swell which is followed by a volume decrease due to increased K^+ and Cl^- permeabilities. The internal and external salines used in the present patch-clamp study were of approximately equal osmolarities; therefore, it is not known whether the Cl^- channel can be stimulated by an osmotic gradient in addition to Ca_i^{2+}. A volume-sensitive Cl^- conductance in human colonic cells (Worrell et al., 1989) is stimulated during whole-cell recordings with isosmotic pipette filling salines due to the pipette saline behaving as if it is hypertonic. This effect, possibly due to

the presence of osmotically active cytosolic constituents which do not equilibrate with the pipette saline, was not tested in platelets. If the same mechanism was active in the present recordings from human platelets, it must have in addition been dependent upon intracellular Ca^{2+}.

Lymphocytes show a regulatory volume decrease similar to that observed in platelets (Grinstein et al., 1982) and interestingly, Cahalan and Lewis (1988) observed a time-dependent increase in Cl^- current during whole-cell patch recordings in response to dilution of the external medium. It is worth noting, however, that of the three types of Cl^- conductances so far detected in lymphocytes, the platelet channel most closely resembles the cAMP-regulated Cl^- channel (Chen, Schulman & Gardner, 1989), rather than the mini-condcutance (2.6 pS) Cl^- channel proposed as the anion permeability involved in regulatory volume decrease (Cahalan & Lewis, 1989) or the large conductance (400-pS) voltage-dependent anion channel with unknown function (Bosma, 1989; Cahalan & Lewis, 1988). The evidence that platelet Cl^- currents require Ca_i^{2+} does favor a role for this conductance during activation since Ca_i^{2+} is the primary second messenger triggering activation in platelets. However, it remains to be determined whether the $[Ca^{2+}]_i$ rises during platelet activation are sufficient to stimulate channel activity and whether these channels are vital to the ensuing hemostatic events. One approach would be to use a blocker such as NPPB to correlate block of the Cl^- channels with inhibition of platelet responses (for example, shape change or aggregation).

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