Calcium-activated potassium channels in human platelets

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1. The effect of intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) on human platelet ion channels was studied using the nystatin whole-cell patch clamp recording technique.

2. Ionomycin-induced increases in $[Ca^{2+}]_i$ rapidly activated a voltage-independent K^+-selective channel with a slope conductance of 30 pS in 154 mM K^+ saline. The single-channel conductance decreased in proportion to the square root of the external K^+ concentration such that the estimated conductance in 5 mM K^+ was approximately 5 pS.

3. The peak current under conditions expected to increase $[Ca^{2+}]_i$ to micromolar levels indicated that each platelet possesses a small number (5–7) of 30 pS Ca^{2+}-dependent K^+ channels (K_{Ca} channels).

4. Spontaneous $[Ca^{2+}]_i$ spiking was observed in many patch-clamped platelets using fura-2 fluorescence measurements. Each Ca^{2+} spike triggered up to five K_{Ca} channels at any one time. K_{Ca} channels were not active at resting levels of $[Ca^{2+}]_i$.

5. The results suggest that platelet K_{Ca} channels are not active under resting conditions but may have an important role in determining the membrane potential during Ca^{2+} signalling.

It is now well established that changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) play an important role during platelet activation (Hallam & Rink, 1985; Davies, Drotts, Weil & Simons, 1989; for reviews see Siess, 1989; Rink & Sage, 1990). The exact molecular mechanism by which Ca^{2+} acts is complex and involves interaction with other second messengers (Siess, 1991). Several proteins are known to be regulated by $[Ca^{2+}]_i$ directly or via binding to Ca^{2+}-binding proteins (e.g. calmodulin), including myosin light chain kinase, phospholipase A_2, ATPase, Ca^{2+}-dependent proteases and tyrosine kinases (Siess, 1989, 1991; Vostal, Jackson & Shulman, 1991). Little is known however, about the effects of $[Ca^{2+}]_i$ on ion channel proteins in the plasma membrane, partly because of the technical difficulties of electrophysiological recordings from these minute blood cells.

Indirect studies of platelet membrane conductances using potential-sensitive dyes have provided some evidence for Ca^{2+}-dependent K^+ channels (K_{Ca} channels). The Ca^{2+} ionophore A23187 evokes a hyperpolarization which is inhibited by quinine and charybdotoxin, blockers of K_{Ca} channels in other cells (Fine, Hansen, Salcedo & Aviv, 1989). In response to Ca^{2+}-elevating agonists such as ADP, thrombin and platelet-activating factor, the membrane potential recorded by potentiometric dyes shows a small Na^+-dependent depolarization (Horne & Simons, 1978; Pipili, 1985). Following removal of external Na^+, an underlying agonist-evoked hyperpolarization is observed which may be due to a Ca^{2+}-dependent K^+ conductance (Pipili, 1985). However, the mechanism of the agonist-evoked depolarization and its dependence on $[Ca^{2+}]_i$ remain unclear. In addition, results obtained with potential-sensitive dyes must be viewed with some caution considering that factors other than membrane potential can influence the dye signal (Rink, 1982).

Two laboratories have now obtained direct electrophysiological recordings from mammalian platelets (Maruyama, 1987; Mahaut-Smith, 1990; Mahaut-Smith, Rink, Collins & Sage, 1990) using the gigaseal patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Human platelets have a high density of voltage-gated K^+ channels (K_V channels) (Maruyama, 1987; Mahaut-Smith *et al.* 1990), which probably account for the resting potential of about −60 mV (MacIntyre & Rink, 1982; Maruyama, 1987; Mahaut-Smith *et al.* 1990). The effect of Ca^{2+} on K_V channels is unknown, since $[Ca^{2+}]_i$ elevation by Maruyama (1987) led to loss of recordings. Mahaut-Smith (1990) observed Cl^- channels in whole-cell recordings, with some evidence that they were stimulated by an increase in $[Ca^{2+}]_i$. However, it was not shown whether physiological levels of internal Ca^{2+} could induce Cl^- channel activity nor whether the Cl^- channels were directly dependent upon $[Ca^{2+}]_i$. In order to look more directly at the effect of raising $[Ca^{2+}]_i$ on platelet conductances, the present study used the nystatin perforated patch technique (Horn & Marty, 1988) to record whole-cell membrane currents during ionophore-induced increases in cytosolic $[Ca^{2+}]$.

A preliminary report of this work has been presented (Mahaut-Smith, 1994).

METHODS

Solutions and reagents

Nominally Ca^{2+}-free platelet saline contained (mM): 145 NaCl, 5 KCl, 10 Hepes, 2 $MgCl_2$, 10 D-glucose, titrated to pH 7·35 with NaOH (final Na^+ concentration approximately 149 mM). For K^+-free Na^+ saline, 5 mM NaCl replaced the KCl. For high-K^+ saline the 145 mM NaCl was replaced by 145 mM KCl and titrated to pH 7·35 with KOH (final K^+ concentration approximately 154 mM). Other levels of external K^+ were obtained by mixing K^+-free and high-K^+ saline solutions. Saline solutions containing 1 mM $CaCl_2$ were obtained by equimolar substitution of $MgCl_2$ for $CaCl_2$. Where indicated, 1 mM EGTA was added to the external saline to lower $[Ca^{2+}]$ further and the solution retitrated to the required pH. Pipette saline contained (mM): 50 KCl, 70 K_2SO_4, 1 $MgCl_2$, 0·1 EGTA, 10 Hepes, titrated to pH 7·2 with KOH and 100 μg ml^{-1} nystatin added, from a 500× stock, immediately prior to an experiment. Ionomycin was obtained from Calbiochem Novabiochem (UK) Ltd and the acetoxymethylester form of fura-2 (fura-2 AM) was from Molecular Probes (Eugene, OR, USA). Aspirin, apyrase (type V), bovin serum albumin (BSA), EGTA and nystatin were obtained from Sigma Chemical Co. (UK). Stock solutions of ionomycin, fura-2 AM and nystatin were all prepared using Me_2SO_4 (Aldrich, UK); stocks of aspirin and apyrase used absolute alcohol and water respectively.

Platelet preparation

Blood was drawn from nineteen healthy unpaid volunteers, with their informed consent. Aliquots of 8·5 ml blood were mixed with 1·5 ml acid citrate dextrose anticoagulant. The anticoagulant contained 2·5 g $(100 ml)^{-1}$ trisodium citrate, 1·5 g $(100 ml)^{-1}$ citric acid and 2 g $(100 ml)^{-1}$ D-glucose. Platelet-rich plasma (PRP) was prepared by centrifugation at 700 *g* for 5 min. Aspirin (100 μM) and apyrase (20 μg ml^{-1}) were added to prevent activation by spontaneously released thromboxane and ADP respectively. Aliquots of 0·5–1 ml PRP were centrifuged at 'low speed' in a 'Microcentaur' microcentrifuge (MSE Scientific Instruments, UK) for 20–30 s and resuspended in nominally Ca^{2+}-free saline (see above) with 20 μg ml^{-1} apyrase and 0·1% BSA. The albumin further reduced spontaneous platelet activation. Platelets were used for up to 3 h after resuspension in Ca^{2+}-free saline. Aliquots of PRP were stored for up to 8 h at room temperature on a vertical wheel rotating at approximately 0·3 Hz. Platelets were loaded with fura-2 by incubating PRP with 5 μM fura-2 AM for 45–60 min at 37 °C.

Electrophysiology

The electrophysiological recording chamber (volume ~1 ml) was filled with Ca^{2+}-free normal saline containing 0·1% BSA. Ten to fifty microlitres of platelet suspension was then added, allowed to settle and perfused with serum-free normal saline. Solution flow was slower at the base of the chamber so that by adjusting the platelet density and the solution flow rate, a layer of platelets remained floating just above the coverslip forming the bottom of the chamber. Patch pipettes with a filled resistance of 5–10 MΩ were pulled from borosilicate glass (Clark Electromedical Instruments, UK). Under ×1000 magnification, a pipette was lowered to within 5 μm of a floating platelet which was then drawn to the pipette by suction. Transition to the whole-cell mode was indicated by the appearance of K_V channels following depolarization to −67 or −57 mV from −97 mV. Current was measured under voltage clamp with an Axopatch 200A patch clamp amplifier (Axon Instruments, CA, USA) at 2 kHz bandwidth and stored on videotape after digitization at 11 kHz by a Medical Systems PCM-4 (Greenvale, NY, USA) A/D VCR recorder adapter. Data were low-pass filtered at 0·9–2 kHz and stored on a Viglen PC (Viglen, UK) equipped with a Digidata interface (Axon Instruments) for analysis by pCLAMP software (Axon Instruments). Potentials have been corrected for the liquid:junction offset between pipette and normal saline solutions (−7 mV, measured with respect to a 3 M KCl reference electrode). Any Donnan potential that developed between pipette saline and cytoplasm was assumed to be negligible since the pipette $[Cl^-]$ was close to cytoplasmic $[Cl^-]$ and nystatin pores are impermeant to SO_4^{2-} (Horn & Marty, 1988). Single-channel current amplitudes were measured either from Gaussian fits to 'all-points' histograms or directly from the current records in which the baseline and open channel levels were set by eye. Experiments were conducted at the ambient temperature (22–26 °C). Statistical values are expressed as means ± standard deviation. Ionomycin was injected over a patch-clamped platelet from a nearby glass pipette using a pressure-controlled injection system (model PLI-100, Medical Systems Incorp., Greenvale, NY, USA).

Fluorescence experiments

Fura-2 fluorescence was measured by single-cell photometry using a Cairn spectrophotometer system (Cairn Research Ltd, Kent, UK) coupled to a Nikon Diaphot inverted microscope (Nikon, UK). Excitation light passed through a spinning filter wheel assembly containing four 340 nm and two 380 nm bandpass excitation filters. The 340/380 nm excitation light was focused onto the cells via a Nikon CF Fluor ×100 1·30 NA objective lens. Emitted light longer than 400 nm and shorter than 600 nm was selected by two dichroic mirrors and further filtered by a 485 nm long-pass filter before collection by the photomultiplier tube (Thorn EMI, UK). Cells were also illuminated by far-red light using a 785 nm long-pass filter (Comar Instruments, Cambridge, UK) which was processed by a JVC CCD camera and provided an image of the field of view during fluorescence recording. A variable iris placed in the emission path allowed light to be collected from an area slightly larger than the platelet held at the end of the patch pipette. The Cairn spectrophotometer subtracted background light from the photomultiplier output and summed the signal from filters at the same wavelength to provide a combined output at 340 and 380 nm excitation for each revolution of the filter wheel. Cell autofluorescence was negligible, thus background fluorescence could be measured by removing the cell from the selected area during an experiment and was subtracted before calculation of the 340/380 nm ratio, used as an indication of cytosolic free $[Ca^{2+}]$ (Grynkiewicz, Poenie & Tsien, 1985). The filter wheel rotated at 35–60 Hz and the signal from four or eight revolutions was averaged to provide fluorescence and ratio values every 67–229 ms.

RESULTS

Ionomycin-induced membrane currents

The effect of an increase in $[Ca^{2+}]_i$ on membrane currents in human platelets was tested in the nystatin whole-cell recording mode by application of the Ca^{2+} ionophore, ionomycin, from a nearby puffer pipette. Platelets have been previously shown to possess a high density of K_V channels (Maruyama, 1987; Mahaut-Smith *et al.* 1990) so to test for activation of other channel types, the potential

was held at −97 mV, well below the threshold for activation of K_V channels. To enable K^+ currents to be studied at this potential, the bath contained a high-K^+ saline (see Methods), which shifted the K^+ equilibrium potential to about −6 mV. Under these conditions, application of 1 μM ionomycin rapidly activated an inward current which was sustained throughout the ionomycin injection in saline containing 1 mM Ca^{2+} (Fig. 1*A*). During the initial few seconds of the response, the current could be seen to be carried by ion channels

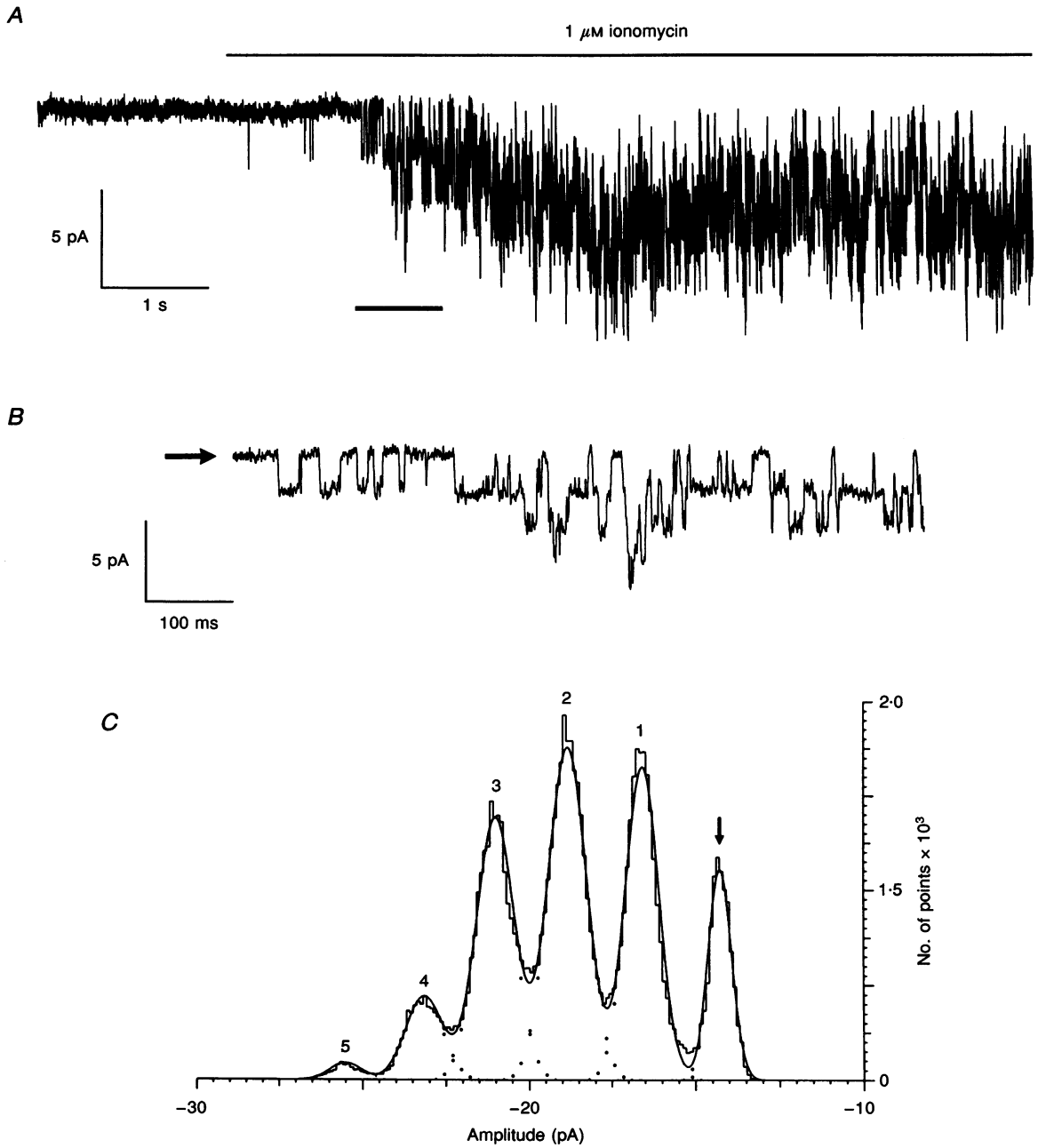

Figure 1. Ionomycin-induced whole-cell membrane currents

Holding potential, −97 mV. Bath saline: 154 mM K^+, 1 mM Ca^{2+} (see Methods for details). *A*, membrane currents activated by 1 μM ionomycin applied from a puffer pipette. *B*, expanded section of current record indicated in *A* by bar. Baseline current is indicated by the arrow. Downward current deflections are inward currents. *C*, all-points amplitude histogram of the first 10 s of the ionomycin response with a 6th-order Gaussian (least squares) fit superimposed. Arrow indicates the peak representing baseline (all channels closed); the difference between baseline and one channel open was 2·41 ± 0·41. The difference between successive peaks was not significantly different: 1 − 2, 2·22 ± 0·53 pA; 2 − 3, 2·14 ± 0·59 pA; 3 − 4, 2·13 ± 0·64 pA; and 4 − 5, 2·39 ± 0·64 pA. Bandwidth, 1·5 kHz in *A* and *B* and 2 kHz for *C*.

with a single-channel current amplitude of about 2·4 pA in this cell (Fig. 1*B*). Rapidly, multiple channels were activated and individual channel events could not be distinguished in the record. However, it was possible to fit a 6th-order Gaussian distribution to an all-points amplitude histogram of the first 10 s of the ionomycin response (Fig. 1*C*). The amplitudes between successive peaks were not significantly different suggesting the existence of five channels of the same type. At −97 mV the peak current in response to 1 μM ionomycin, in the presence of 1 mM external Ca^{2+}, indicated that a maximum of five to seven channels were activated at any one time (data from 5 cells).

Prolonged exposure to ionomycin in either the presence or absence of external Ca^{2+} invariably resulted in the delayed appearance of membrane currents other than those described by Fig. 1, or loss of the recording. In order to restrict this study to the Ca^{2+}-dependent channels observed in the initial phase of the response, cells were bathed in Ca^{2+}-free medium and ionomycin applied briefly (1–2 s) from the puffer pipette. To maintain a Ca^{2+} gradient for ionomycin and permit repetitive increases in $[Ca^{2+}]_i$, the injection pipette saline also contained 1 mM Ca^{2+}. In this way, the Ca^{2+}-dependent channel activity could be compared at different membrane potentials in the same cell (Fig. 2). A 2 s application of ionomycin and

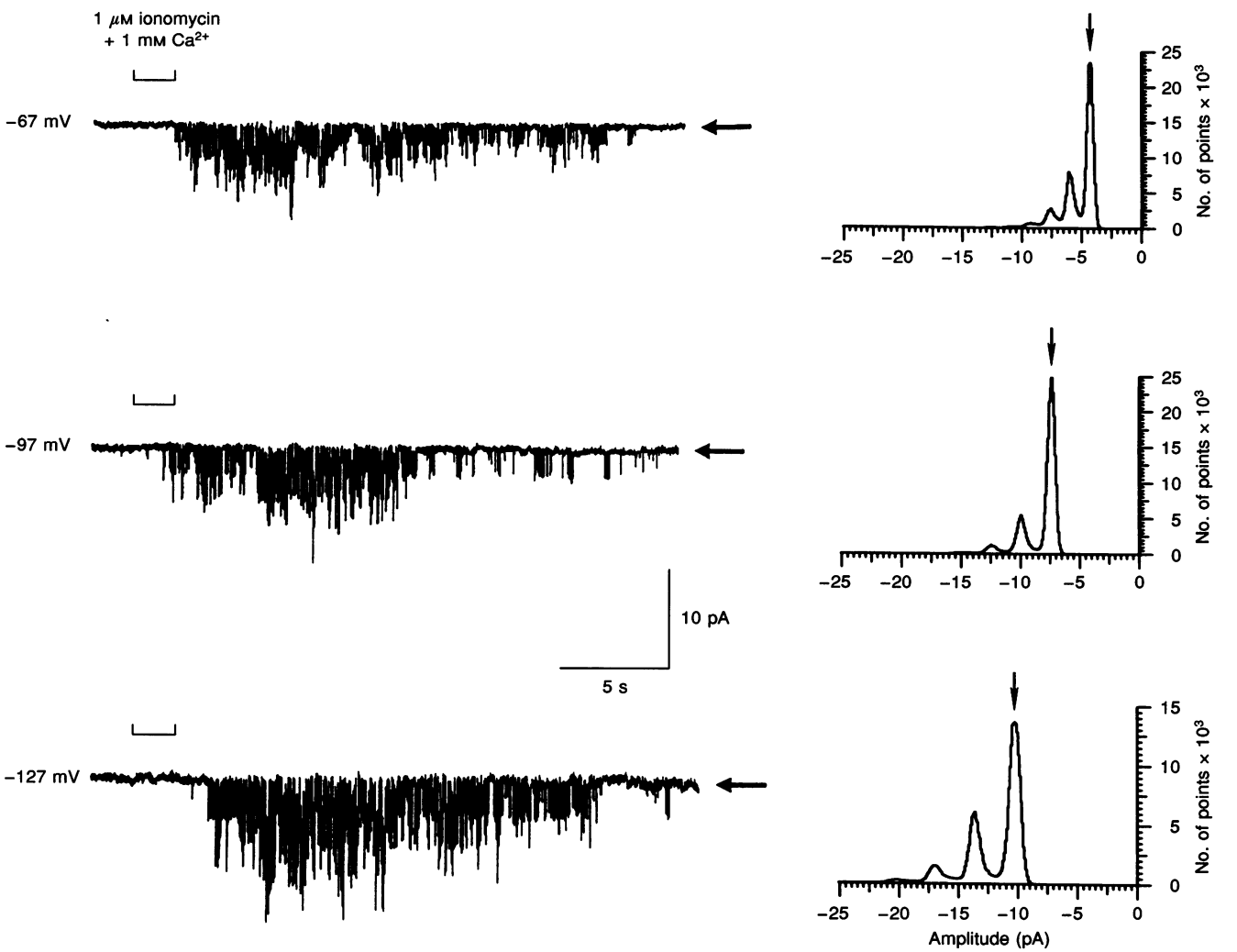

Figure 2. Effect of membrane potential on the Ca^{2+}-dependent channel

One micromolar ionomycin and 1 mM Ca^{2+} were applied together for 2 s while holding at −67, −97 and −127 mV in the same cell. Injections were approximately 1 min apart. Bath saline: nominally Ca^{2+}-free, 154 mM K^+. Membrane current records at each potential are shown to the left. Amplitude histograms of the 27 s following addition of the ionomycin and Ca^{2+} are shown to the right. The arrows indicate the baseline (all channels closed) in both current and amplitude histograms. Bandwidth, 0·9 kHz.

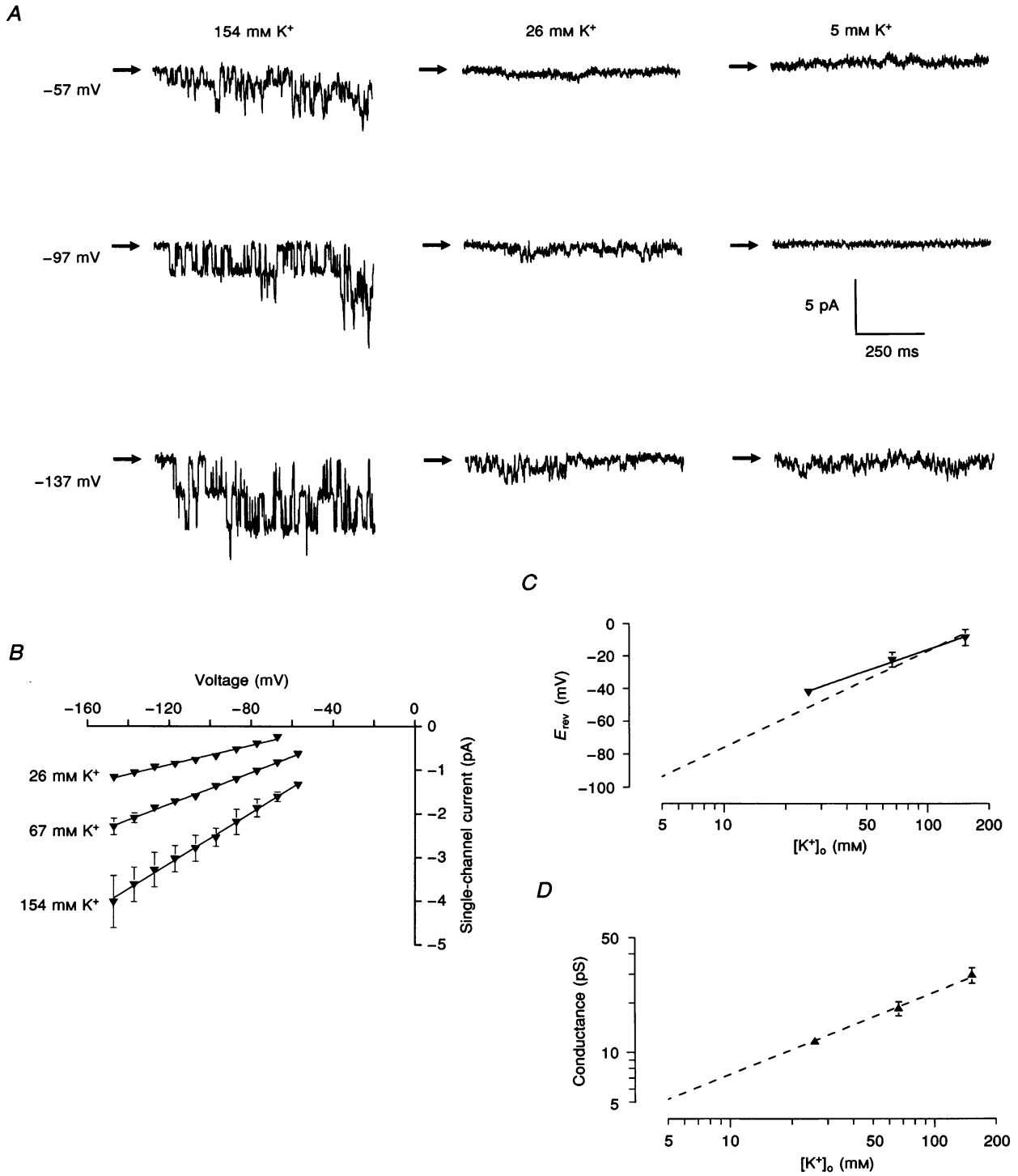

Figure 3. Effect of external [K^+] on the ionomycin-induced single-channel currents

A, representative current traces at three potentials in different levels of external K^+ (Na^+ substitution). The arrow indicates the baseline current at which no channels were open, assessed prior to ionomycin application. *B*, current–voltage relationships in 26, 67 and 154 mM external K^+ (indicated against each curve); data from 5, 6 and 11 cells respectively. *C*, semilogarithmic plot of external [K^+] against extrapolated reversal potential. The dashed line is the theoretical reversal potential predicted from the Nernst equation for a K^+-selective channel at 24 °C. *D*, double logarithmic plot of external [K^+] against single-channel slope conductance. The dashed line was drawn from eqn (1). Data in *C* and *D* from 5, 6 and 8 cells in 26, 67 and 154 mM K^+, respectively. Error bars are not shown if smaller than the height of the symbol.

Ca^{2+} evoked channel activity lasting about 25–30 s, presumably the time taken for the Ca^{2+} regulatory mechanisms to clear the Ca^{2+} load from the cytoplasm. Figure 2 shows three consecutive responses from the same cell, approximately 1 min apart, at −67, −97 and −127 mV. In the amplitude histograms, peaks due to the baseline (indicated by the arrow) and opening of one, two or three channels could be discerned at each potential. (A small additional peak due to the opening of a fourth channel was also present in the response at −67 mV.) A similar result was obtained in three other cells and suggests that the Ca^{2+}-dependent channel has little or no requirement upon voltage for its activation. More quantitative measurements of voltage dependency were not possible in these nystatin whole-cell experiments because of the difficulty of maintaining a constant $[Ca^{2+}]_i$.

Channel conductance and selectivity

Figure 3*A* illustrates the effect of varying external $[K^+]$ on the ionomycin-evoked single-channel currents

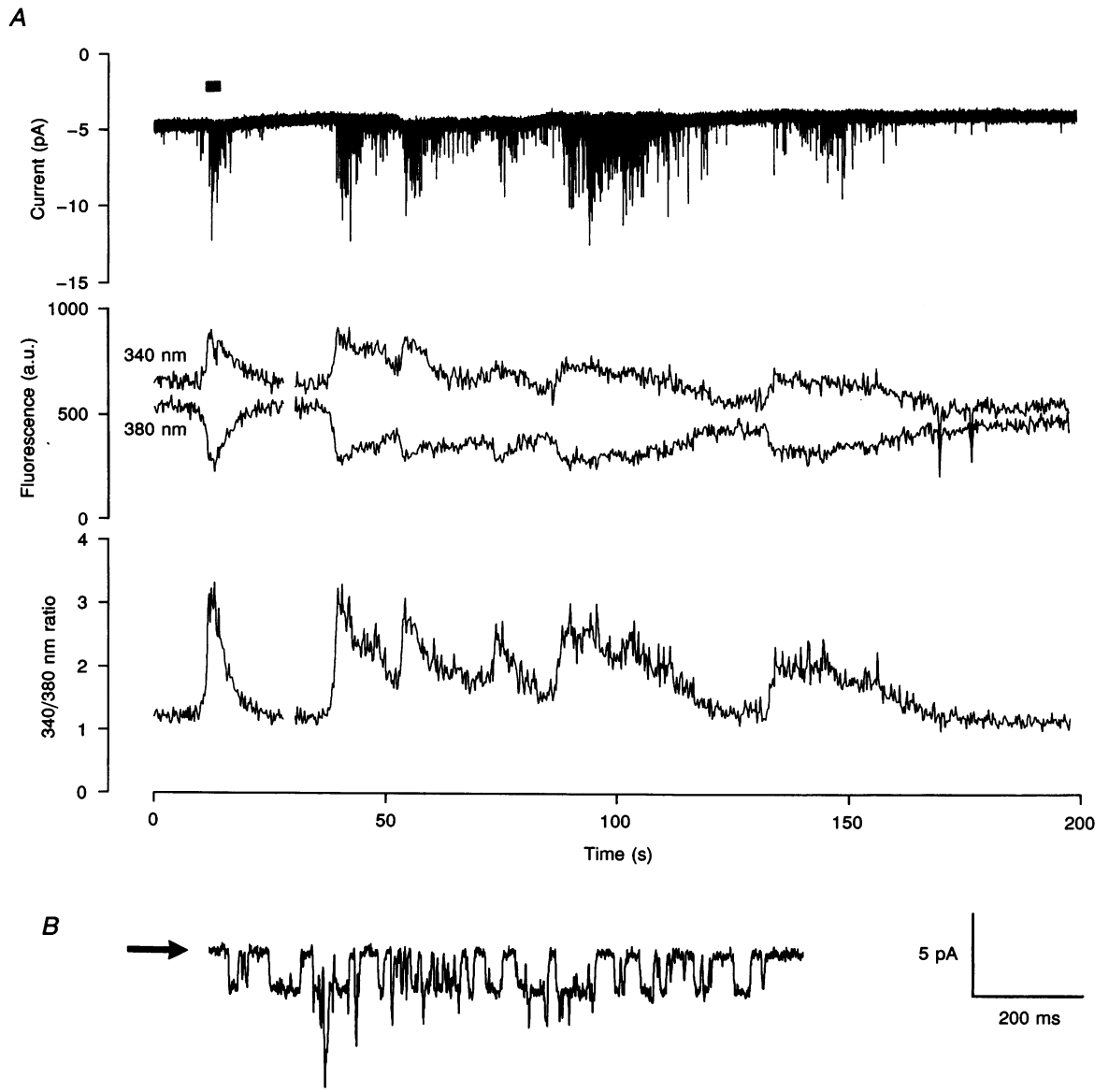

Figure 4. Spontaneous oscillations of $[Ca^{2+}]_i$ and membrane current

A fura-2-loaded platelet was clamped at −97 mV, and the emission from 340 and 380 nm excitation alternately monitored by photometry. Background fluorescence was measured by moving the platelet out of the collection window, at about 30 s into the recording (gap in fluorescence traces). The background-corrected single-wavelength fluorescence signals are shown in the centre graph (a.u., arbitrary units) together with the corrected 340/380 nm ratio (lower graph) and the whole-cell membrane currents (upper graph). *B*, expanded section of current record, indicated by the bar in *A*. Bandwidth 1 kHz. External saline: 154 mM K^+, Ca^{2+} free (1 mM EGTA saline).

measured at three potentials. The voltage range over which the ionomycin-induced response could be studied was restricted to approximately −57 mV and more hyperpolarized potentials. At more depolarized potentials, sustained activation of K_V channels (Maruyama, 1987; Mahaut-Smith *et al.* 1990) obscured the ionomycin-induced currents. Current–voltage relationships for three different external K^+ concentrations are shown in Fig. 3*B*. The relationships were reasonably linear over the potential range −147 to −57 mV, with slopes of 29·7 ± 3·2 pS ($n = 11$); 18·6 ± 1·8 pS ($n = 6$); 11·7 ± 0·7 pS ($n = 5$) in 154, 67 and 26 mM external K^+, respectively. At lower $[K^+]_o$, the channel conductance was reduced further such that single-channel current amplitudes could not be measured accurately over the range of potentials used here.

Extrapolation of the current–voltage relationships in Fig. 3*B* gave an estimate of the reversal potential (E_{rev}) which is plotted against $\log_{10}[K^+]$ in Fig. 3*C*. The gradient was less than that predicted by the Nernst equation for a perfectly K^+-selective ion channel (shown by the dashed line in Fig. 3*C*), thus one would predict some permeability to Na^+ or Cl^-. However in 145 mM NaCl, 5 mM KCl external saline, the channels appeared to reverse at approximately −90 to −100 mV (Fig. 3*A*), close to the K^+ equilibrium potential. The most likely explanation for this apparent discrepancy is that E_{rev} is not accurately estimated by extrapolation of the *I–V* curves. This would be the case if the channel displays inward rectification, because the curves would become more curvilinear at higher $[K^+]$. Overall, the data suggest that this channel displays a high selectivity for K^+, which is a property shared by K_{Ca} channels of similar conductance in other blood cells (Grygorczyk, Schwarz & Passow, 1984; Gallin, 1989; Mahaut-Smith & Mason, 1991).

In Fig. 3*D*, the single-channel conductance (γ) is plotted against $[K^+]_o$ on a double logarithmic plot. The values were well described by the dashed line drawn to eqn (1):

$$\gamma = c[K^+]^{0.5}, \quad (1)$$

where $c = 2{\cdot}33$ pS. A similar square root function has been reported for K^+ conductances in other cells (Hagiwara & Takahashi, 1974; Fukushima, 1982; Sakmann & Trube, 1984; Sauve, Simoneau, Monette & Roy, 1986; Shibasaki, 1987). The single-channel conductance in 5 mM external K^+ calculated using eqn (1) is 5·2 pS.

Ca^{2+} oscillations and K_{Ca} channels

Spontaneous bursts of K_{Ca} channel activity were observed during many whole-platelet recordings. In the experiment of Fig. 4, whole-cell membrane currents and $[Ca^{2+}]_i$ were simultaneously recorded from a fura-2-loaded platelet (see Methods). The cell was held at −97 mV and bathed in 154 mM KCl saline. As in previous studies of spontaneous and agonist-evoked Ca^{2+} oscillations in single platelets (Nishio, Ikegami & Segawa, 1991; Heemskerk, Hoyland, Mason & Sage, 1992), $[Ca^{2+}]_i$ increased in the form of single spikes or multiple spikes on top of a plateau of elevated $[Ca^{2+}]_i$. Between the spikes or plateaux, $[Ca^{2+}]_i$ returned to what appeared to be a set level (ratio 1·27 ± 0·24, $n = 18$), similar to that observed in non-spiking cells (ratio 1·28 ± 0·15, $n = 9$). As shown by the whole-cell current recording, ion channels carrying inward current were activated during each Ca^{2+} spike, but not when $[Ca^{2+}]_i$ was at its resting level. These spontaneous currents were not observed in normal (145 mM NaCl, 5 mM KCl) saline solutions at −97 mV, confirming that they are K^+ currents. The single-channel current (Fig. 4*B*) measured 2·55 ± 0·09 pA at −97 mV (data from 4 cells) which was not significantly different from the single-channel current amplitude induced by ionomycin at this potential (2·56 ± 0·21; data from 9 cells). The maximum current activated by spontaneous Ca^{2+} spikes corresponded to between three and five K_{Ca} channels being simultaneously open (data from 5 cells).

DISCUSSION

This study provides the first direct evidence that human platelets possess K^+ channels stimulated by an increase in $[Ca^{2+}]_i$. Applications of ionomycin at a dose previously reported to rapidly elevate $[Ca^{2+}]_i$ to micromolar levels in the presence of external Ca^{2+} (Hallam & Rink, 1985; Davies *et al.* 1989; Rink & Sage, 1990), activated a current whose reversal potential shifted with alterations in external $[K^+]$. The fact that ionomycin could evoke channel activity within 1 s of application in many cells (see Fig. 1), in a repetitive manner (Fig. 2), suggested that channel activation occurred as a direct result of an increase in cytosolic $[Ca^{2+}]$ or via a Ca^{2+}-binding protein, rather than as a consequence of a Ca^{2+}-dependent platelet response such as exocytosis. This conclusion is supported by measurements from cells displaying repetitive Ca^{2+} spikes, where K_{Ca} channels were activated during each brief Ca^{2+} spike. Platelets also possess K_V channels which are activated by depolarization with a threshold of about −60 to −70 mV (Maruyama, 1987; Mahaut-Smith *et al.* 1990). In contrast to the K^+ channels activated by $[Ca^{2+}]_i$ in this study, where only one channel conductance of 30 pS in high-K^+ saline was observed, platelet K_V channels have at least five different conductance levels in the range 5–34 pS in high-K^+ saline. The effect of $[Ca^{2+}]_i$ was not due to activation of K_V channels at one conductance level since the Ca^{2+}-dependent K^+ channel lacked voltage sensitivity and platelet K_V channels are inhibited by ionomycin-induced increases in $[Ca^{2+}]_i$ (M. P. Mahaut-Smith, unpublished observations), as previously shown for K_V channels in lymphocytes (Bregestovski, Redkozubov & Alexeev, 1986). This study therefore confirms the earlier conclusions of Fine *et al.* (1989) and Pipili (1985), using potential-sensitive dyes, that platelets possess K_{Ca} channels.

The currents induced by ionomycin were carried by a K^+ channel with a single-channel conductance of about 30 pS in 154 mM K^+ but with a much smaller conductance in normal external K^+ concentrations. During continuous application of a dose of ionomycin expected to maintain $[Ca^{2+}]_i$ well into the micromolar range (Hallam & Rink, 1985), the current was carried by a maximum of only five to seven channels. Despite its small conductance and low density when compared to the large numbers of K_V channels (>100 channels per cell) in these cells (Maruyama, 1987; Mahaut-Smith *et al.* 1990), activation of K_{Ca} channels can substantially hyperpolarize the membrane from its resting level (Fine *et al.* 1989). This does imply that very few channels are open in unstimulated platelets, which was a conclusion made previously by Maruyama (1987) following the observation that mammalian platelets have an extremely high input resistance (>50 GΩ). Under resting conditions, therefore, it may take only one or two open K^+ channels to set the potential at or near the potassium equilibrium potential.

Simultaneous measurement of $[Ca^{2+}]_i$ and membrane currents indicated that spontaneous Ca^{2+} spiking in patch-clamped platelets can also trigger the opening of K_{Ca} channels. The induction of Ca^{2+} spikes in platelets in the absence of an agonist is not well understood but here it was probably triggered by exposure to the glass surface of the patch pipette. Similar spontaneous and agonist-evoked oscillations have been observed by other groups using fluorescence imaging of platelets adhering to poly-ethyleneimine- or fibrinogen-coated glass coverslips (Nishio *et al.* 1991; Heemskerk *et al.* 1992). In the present photometric study, between or following these transient elevations, $[Ca^{2+}]_i$ returned to a set level, indistinguishable from the level in non-oscillating cells. While $[Ca^{2+}]_i$ was at this resting level, there was little or no activation of K_{Ca} channels (Fig. 4). This result would suggest that K_{Ca} channels will have little influence on the membrane potential in completely unstimulated platelets, but following activation will significantly influence the membrane potential. Unfortunately not enough is known at present about the relative pharmacology of K_{Ca} channels and K_V channels in human platelets for us to conclusively test their relative roles in platelet function before and after activation. A careful pharmacological study of each K^+ conductance is needed since preliminary results indicate they have overlapping sensitivities to certain blockers. For example, K_V channels are blocked by quinine (Maruyama, 1987) and charybdotoxin (Mahaut-Smith *et al.* 1990), and both are antagonists of the Ca^{2+}-dependent hyperpolarization measured in platelet populations (Fine *et al.* 1989). Mahaut-Smith *et al.* (1990) concluded that the negative resting membrane potential was set by K_V channels on the basis of charybdotoxin sensitivity, large numbers of K_V channels, the voltage threshold being close to the resting potential and the lack of appearance of other K^+ channels. Although platelets are now known to have both K_V and K_{Ca} conductances, K_V channels probably do set the membrane potential at rest since K_{Ca} channels are not activated until $[Ca^{2+}]_i$ is elevated above resting levels.

The present study provides only limited information on the properties of the platelet K_{Ca} channels because of the difficulty in controlling $[Ca^{2+}]_i$ in the nystatin whole-cell patch recordings and the small numbers of K_{Ca} channels in comparison to voltage-gated K^+ channels. However, in terms of its size and lack of voltage dependence, the platelet K_{Ca} channels resemble the Ca^{2+}-dependent K^+ channels in a number of blood cells, including human red blood cells (Grygorczyk *et al.* 1984), rat thymic and human B lymphocytes (Mahaut-Smith & Schlichter, 1989; Partiseti, Choquet, Diu & Korn, 1992), human T lymphocytes (Grissmer, Nguyen & Cahalan, 1993) and macrophages (Gallin, 1989). Smaller-conductance K_{Ca} channels, about 5–7 pS (in high-K^+ saline) were also observed in rat thymocytes and human leukaemic T cells (Mahaut-Smith & Schlichter, 1989; Grissmer, Lewis & Cahalan, 1992), but platelet K_{Ca} channels appeared to have only one main conductance level of about 30 pS in 154 mM KCl. Ca^{2+}-activated K^+ channels exist in a variety of tissues with single-channel conductances ranging from 4 pS up to 300 pS (for reviews see Blatz & Magleby, 1987; Latorre, Oberhauser, Labarca & Alvarez, 1989). This class of channel has been classified into either two or three categories based on channel conductance: large conductance (130–300 pS single-channel conductance in 100–140 mM KCl; abbreviated to BK or maxi K^+) and small conductance (SK; less than about 80 pS in symmetrical K^+), with the small-conductance channels being subdivided by Cook (1988) into small (10–14 pS) and intermediate (18–50 pS) channels. Platelet K_{Ca} channels should probably be grouped with the other blood cell K_{Ca} channels which are usually called intermediate (also referred to as 'other' K_{Ca} channels by Blatz & Magleby, 1987), despite the fact that as the external $[K^+]$ is reduced to more physiological levels, the conductance is decreased to that of small K_{Ca} channels.

In a previous patch clamp study of human platelets, a 45 pS outwardly rectifying Cl^- channel developed in the whole-cell configuration (Mahaut-Smith, 1990). The study used a high concentration of divalent cations in the pipette to permit stable whole-cell recordings, since resealing of the membrane under the pipette was a problem when using monovalent cation-based internal saline solutions (Mahaut-Smith *et al.* 1990). The appearance of these Cl^- channels was prevented by the Ca^{2+} chelator BAPTA being added to the internal saline solutions; thus it was concluded that the Cl^- channel was induced by the micromolar levels of contaminating Ca^{2+} present in the Ba^{2+} and Mg^{2+} salts. In the present experiments, where nystatin perforation of the cell-attached patch was used

to obtain whole-cell recordings and leave the cytoplasm relatively undisturbed, raising $[Ca^{2+}]_i$ initially evoked only a K^+-selective channel. In many experiments, prolonged exposure to ionomycin evoked other as yet undefined currents, which may include a Cl^- conductance. One possible explanation for these results is that platelet Cl^- channels are activated by higher levels of internal Ca^{2+} than K_{Ca} channels. Alternatively, they could be triggered indirectly as a result of a Ca^{2+}-dependent event such as secretion.

In conclusion, this study demonstrates the existence, in human platelets, of a small number (5–7 per cell) of intermediate-conductance Ca^{2+}-activated K^+ channels which probably play an important role in maintaining the driving force for Ca^{2+} influx during activation.

Blatz, A. L. & Magleby, K. L. (1987). Calcium-activated potassium channels. *Trends in Neurosciences* **10**, 463–467.

Bregestovski, P., Redkozubov, A. & Alexeev, A. (1986). Elevation of intracellular calcium reduces voltage-dependent potassium conductance in human T cells. *Nature* **319**, 776–778.

Cook, N. S. (1988). The pharmacology of potassium channels and their therapeutic potential. *Trends in Biochemical Sciences* **9**, 21–28.

Davies, T. A., Drotts, D. L., Weil, G. J. & Simons, E. R. (1989). Cytoplasmic Ca^{2+} is necessary for thrombin-induced platelet activation. *Journal of Biological Chemistry* **264**, 19600–19606.

Fine, B. P., Hansen, K. A., Salcedo, J. R. & Aviv, A. (1989). Calcium-activated potassium channels in human platelets. *Proceedings of the Society for Experimental Biology and Medicine* **192**, 109–113.

Fukushima, Y. (1982). Blocking kinetics of the anomalous potassium rectifier of tunicate egg studied by single channel recording. *Journal of Physiology* **331**, 311–331.

Gallin, E. K. (1989). Evidence for a Ca-activated inward-rectifying K channel in human macrophages. *American Journal of Physiology* **257**, C77–85.

Grissmer, S., Lewis, R. S. & Cahalan, M. D. (1992). Ca^{2+}-activated K^+ channels in human leukemic T cells. *Journal of General Physiology* **99**, 63–84.

Grissmer, S., Nguyen, A. N. & Cahalan, M. D. (1993). Calcium-activated potassium channels in resting and activated human T lymphocytes. *Journal of General Physiology* **102**, 601–630.

Grygorczyk, R., Schwarz, W. & Passow, H. (1984). Ca^{2+}-activated K^+ channels in human red cells. *Biophysics Journal* **45**, 693–698.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

Hagiwara, S. & Takahashi, K. (1974). The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *Journal of Membrane Biology* **18**, 61–80.

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.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

Heemskerk, J. W. M., Hoyland, J., Mason, W. T. & Sage, S. O. (1992). Spiking in cytosolic calcium concentration in single fibrinogen-bound fura-2-loaded human platelets. *Biochemical Journal* **283**, 379–383.

Horn, R. & Marty, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *Journal of General Physiology* **92**, 145–159.

Horne, W. C. & Simons, E. R. (1978). Probes of transmembrane potentials in platelets: changes in cyanine dye fluorescence in response to aggregation stimuli. *Blood* **51**, 741–749.

Latorre, R., Oberhauser, A., Labarca, P. & Alvarez, O. (1989). Varieties of calcium-activated potassium channels. *Annual Review of Physiology* **51**, 385–399.

Livne, A., Grinstein, S. & Rothstein, A. (1987). Volume-regulating behavior of human platelets. *Journal of Cellular Physiology* **131**, 354–363.

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. (1990). Chloride channels in human platelets: Evidence for activation by internal calcium. *Journal of Membrane Biology* **118**, 69–75.

Mahaut-Smith, M. P. (1994). Ca^{2+}-activated K^+ channels in human platelets. *Journal of Physiology* **476.P**, 59*P*.

Mahaut-Smith, M. P. & Mason, M. J. (1991). Ca^{2+}-activated K^+ channels in rat thymic lymphocytes: activation by concanavalin A. *Journal of Physiology* **439**, 513–528.

Mahaut-Smith, M. P., Rink, T. J., Collins, S. C. & Sage, S. O. (1990). Voltage-gated potassium channels and the control of membrane potential in human platelets. *Journal of Physiology* **428**, 723–735.

Mahaut-Smith, M. P. & Schlichter, L. C. (1989). Ca^{2+}-activated K^+ channels in human B lymphocytes and rat thymocytes. *Journal of Physiology* **415**, 69–83.

Maruyama, Y. (1987). A patch-clamp study of mammalian platelets and their voltage-gated potassium current. *Journal of Physiology* **391**, 467–485.

Nishio, H., Ikegami, Y. & Segawi, T. (1991). Fluorescence digital image analysis of serotonin-induced calcium oscillations in single blood platelets. *Cell Calcium* **12**, 177–184.

Partiseti, M., Choquet, D., Diu, A. & Korn, H. (1992). Differential regulation of voltage and calcium-activated potassium channels in human B lymphocytes. *Journal of Immunology* **148**, 3361–3368.

Pipili, E. (1985). Platelet membrane potential: simultaneous measurement of $diSC_{3(5)}$ fluorescence and optical density. *Thrombosis and Haemostasis* **54**, 645–649.

Rink, T. J. (1982). Measurement of membrane potential with chemical probes. *Techniques in the Life Sciences. Lipid and Membrane Biochemistry* **B423**, 1–29.

Rink, T. J. & Sage, S. O. (1990). Calcium signaling in human platelets. *Annual Review of Physiology* **52**, 431–449.

Sakmann, B. & Trube, G. (1984). Conductance properties of single inwardly rectifying potassium channels in ventricular cells from guinea pig heart. *Journal of Physiology* **347**, 641–657.

SAUVE, R., SIMONEAU, C., MONETTE, R. & ROY, G. (1986). Single channel analysis of the potassium permeability in HeLa cancer cells: evidence for a calcium-activated potassium channel of small unitary conductance. *Journal of Membrane Biology* **92**, 269–282.

SHIBASAKI, T. (1987). Conductance and kinetics of delayed rectifier potassium channels in nodal cells of the rabbit heart. *Journal of Physiology* **387**, 227–250.

SIESS, W. (1989). Molecular mechanisms of platelet activation. *Physiological Reviews* **69**, 58–178.

SIESS, W. (1991). Multiple signal-transduction pathways synergize in platelet activation. *News in Physiological Sciences* **6**, 51–56.

VOSTAL, J. G., JACKSON, W. L. & SHULMAN, N. R. (1991). Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. *Journal of Biological Chemistry* **266**, 16911–16916.

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