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HIGHLY CO-OPERATIVE Ca^{2+} ACTIVATION OF INTERMEDIATE-CONDUCTANCE K^+ CHANNELS IN GRANULOCYTES FROM A HUMAN CELL LINE

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SUMMARY

1. To study Ca^{2+}-activated K^+ currents in dimethyl sulphoxide (DMSO)-differentiated HL-60 cells (HL-60 granulocytes), we have combined the patch clamp technique with microfluorimetric measurements of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$).

2. Elevations of $[Ca^{2+}]_i$ induced by the receptor agonist *N*-formyl-L-methionyl-L-phenylalanine (f-MLP), by cellular spreading or by the Ca^{2+} ionophore ionomycin, activated whole-cell currents. The kinetics of the current elevations closely paralleled the kinetics of the elevations in $[Ca^{2+}]_i$. Cellular spreading induced oscillations in $[Ca^{2+}]_i$ and parallel oscillatory changes in the amplitude of the recorded currents.

3. The reversal potential of the Ca^{2+}-activated current was a function of the extracellular K^+ concentration (56·1 mV per log $[K^+]$), demonstrating that the underlying conductance was selective for K^+.

4. The current was blocked by charybdotoxin, but insensitive to apamin.

5. The whole-cell current was inwardly rectifying. No time-dependent activation or inactivation of the current could be observed within the range of voltages tested (-100 to $+100$ mV).

6. The dependence of the current amplitude on the measured $[Ca^{2+}]_i$ revealed a half-maximal activation at approximately 350 nM $[Ca^{2+}]_i$, and a highly co-operative activation by $[Ca^{2+}]_i$ with an apparent Hill coefficient of approximately 8. Neither the half-maximal activation by $[Ca^{2+}]_i$ nor the apparent Hill coefficient depended on the voltage, and they were identical for Ca^{2+} elevations caused by the ionophore and the receptor agonist.

7. Analysis of Ca^{2+}-activated single-channel events in cell-attached recordings revealed an inwardly rectifying K^+ channel with a slope conductance of 35 pS. Fluctuation analysis of the Ca^{2+}-activated whole-cell current suggested an underlying single-channel conductance of a similar size (28 pS).

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8. In summary, we describe a charybdotoxin-sensitive, intermediate-conductance Ca^{2+}-activated K^+ channel in HL-60 granulocytes. The characteristics of the Ca^{2+} activation of this current (i.e. sensitivity to submicromolar $[Ca^{2+}]_i$, high co-operativity and voltage independence) are similar to the Ca^{2+} activation of the apamin-sensitive small-conductance K^+ channel. Our results also suggest that $[Ca^{2+}]_i$ elevations are the predominant, if not the only, activators of this channel during physiological stimulation of HL-60 granulocytes.

INTRODUCTION

Neutrophil granulocytes are mobile phagocytes that play a central role in the host defence against various micro-organisms. Despite being non-excitable cells, neutrophils undergo a large plasma membrane depolarization in response to agonists (for review see Nanda & Grinstein, 1991*a*). The ionic basis of this depolarization is thought to be electron transport through NADPH oxidase (Henderson, Chappell & Jones, 1987), an enzyme crucial for the generation of microbicidal oxygen derivatives. After stimulation with physiological agonists, neutrophil plasma membrane depolarization is followed by a repolarization. Three types of ion channel have been suggested to play a role in this repolarization: (i) voltage-dependent K^+ channels (Krause & Welsh, 1989); (ii) voltage-dependent H^+ channels (Henderson *et al.* 1987; Nanda & Grinstein, 1991*b*; Demaurex, Grinstein, Jaconi, Schlegel, Lew & Krause, 1993); and (iii) Ca^{2+}-activated K^+ channels (Krause & Welsh, 1989).

Ca^{2+}-activated K^+ channels can be divided into three classes on the basis of their single-channel conductance: BK, SK and IK channels (i.e. big, small and intermediate conductance K^+ channels; Cook, 1988; Castle, Haylett & Jenkinson, 1989). BK channels show a large single-channel conductance (> 100 pS), are in general sensitive to charybdotoxin and may be activated by voltage and by Ca^{2+}. The Ca^{2+} activation of BK channels is only moderately co-operative (McManus, 1991). SK channels show a small single-channel conductance (< 20 pS) and are generally sensitive to apamin. SK channels are activated by submicromolar Ca^{2+} concentrations in a highly co-operative manner (see for example Grissmer, Lewis & Cahalan, 1992). The Ca^{2+} activation of SK channels is voltage independent. IK channels form a very heterogeneous group and may be voltage dependent or independent and show single-channel conductances between 20 and 100 pS. The parameters of Ca^{2+} activation of the K^+ channels classified as IK are poorly defined. IK channels are in general insensitive to apamin, but were found in some cases to be charybdotoxin sensitive (Cook, 1988; Castle *et al.* 1989; McManus, 1991; Moczydlowski, Lucchesi & Ravindran, 1993).

In this study we investigated Ca^{2+}-activated K^+ channels in HL-60 cells that were induced to differentiate into neutrophil-like cells (HL-60 granulocytes). We demonstrated intermediate-conductance Ca^{2+}-activated K^+ channels and corresponding whole-cell currents. The currents are activated with high co-operativity by submicromolar $[Ca^{2+}]_i$, characteristics that have not been previously observed for IK channels.

METHODS

Culture of HL-60 cells

The human promyelocytic cell line HL-60 was cultured in the tissue culture medium RPMI 1640 supplemented with 10% fetal calf serum, penicillin (5 units ml^{-1}) and streptomycin (50 μg ml^{-1}). The cells were passaged two times per week and differentiated by adding dimethylsulphoxide (DMSO; final concentration 1·3% v/v) to the cell suspension 7 days before the experiments. The DMSO-differentiated cells (Collins, Ruscetti, Gallagher & Grallo, 1978) displayed a neutrophil phenotype, as assessed by the following criteria: (i) stimulation by the chemoattractant *N*-formyl-L-methionyl-L-phenylalanine (f-MLP) activated phosphoinositide turnover and induced $[Ca^{2+}]_i$ elevations (Krause, Schlegel, Wollheim, Anderson, Waldvogel & Lew, 1985; Lew, Monod, Krause, Waldvogel, Biden & Schlegel, 1986); (ii) stimulation by the chemoattractant f-MLP, Ca^{2+} ionophores and phorbol esters activated microbicidal mechanisms typical of neutrophils, such as superoxide generation and release of granular enzymes (Krause *et al.* 1985); and (iii) DMSO-differentiated, but not the undifferentiated, cells demonstrated the properties of adherence, chemotaxis and phagocytosis (microscopic observations, not shown; see also Fig. 1).

Electrophysiological recordings

We used the patch clamp technique either in the cell-attached or in the whole-cell configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch clamp electrodes were pulled from borosilicate glass (external diameter 1·2 mm, Mecanex, Nyon, Switzerland) using a BB-CH-PC puller (Mecanex, Nyon, Switzerland). Pipette resistance varied between 5 and 15 MΩ and seal resistance between 5 and 50 GΩ. Patch clamp recordings were made using a List EPC-7 amplifier (List Medical, Darmstadt, Germany) in the voltage clamp mode. Junction potentials were less than 5 mV in all experiments. The cell input resistance varied between 0·3 and 5 GΩ. Series resistance was between 10 and 20 MΩ and was not compensated. The cell capacitance of the differentiated HL-60 cells ranged between 3 and 6 pF. The whole-cell currents were filtered at 3 kHz and recorded either at 40 Hz (low resolution) or at 10 kHz (high resolution) using two 12-bit A–D boards (Acqui and Daqsys, Informatic Division, Centre Medical Universitaire, Geneva) interfaced to a PC/AT computer. For cell-attached recordings the currents were filtered at 10 kHz on the List EPC-7 amplifier, digitized at 50 kHz on a 12-bit A–D interface (Daqsys, Informatic Division, Centre Medical Universitaire, Geneva), and subsequently filtered at 2·5 kHz using a moving average procedure. Agonists and blockers were applied either through a perfusion pipette (10 μm inner diameter) located 30 μm from the cell, or through a continuous solution exchange system. All experiments were performed at room temperature.

Measurement of cytosolic free Ca^{2+} in patched cells

Intracellular calcium concentration in patched cells was measured as previously described (Demaurex, Schlegel, Varnai, Mayr, Lew & Krause, 1992). Cells (2×10^7 ml^{-1}) were incubated for 30 min at room temperature with 5 μM fluorescent calcium indicator indo-1 AM, centrifuged, resuspended at 2×10^7 cells ml^{-1} in bath solution 1 (the solutions used in this study are shown in Table 1) and kept at room temperature. Just before use, an aliquot of 10^6 cells was placed on a glass coverslip and allowed to adhere. Experiments were performed within 1 h. On the stage of a Nikon Diaphot inverted microscope (Nikon Corp., Tokyo, Japan), cells were illuminated at 360 nm using a mercury lamp. The emitted fluorescence was split at 455 nm using a dichroic mirror, and light intensity measured at 405 and 480 nm on two P1 photometers (Hamamatsu, Japan). Recording of the data was performed with the same A–D interface used for electrophysiological measurements. R, the ratio of the emitted fluorescence at 405 and 480 nm, was calculated, and $[Ca^{2+}]_i$ was derived using the equation:

$$[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R),$$

where K_d is the dissociation constant and β is the fluorescence dynamic range of the dye. R_{min}, R_{max}, and $K_d\beta$ were determined in dye-loaded cells using the patch-pipette method (Almers & Neher, 1985). $R_{min}(= 0{\cdot}025 \pm 0{\cdot}005; n = 6)$ was measured as the R value following break-in with a pipette solution containing 10 mM EGTA. $R_{max}(= 0{\cdot}35 \pm 0{\cdot}01; n = 8)$ was determined as the R value of intact cells incubated with 2 μM ionomycin in the presence of 10 mM $CaCl_2$. $K_d\beta(= 1280 \pm 40;$

$n = 6$) was determined after break-in with a pipette solution heavily buffered at 300 nM Ca^{2+} (9·2 mM EGTA and 5·4 mM $CaCl_2$).

Reagents

Apamin, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine and DMSO were purchased from Sigma (St Louis, MO, USA), fetal calf serum from Gibco (Paisley, Scotland, UK) and indo-1 AM from Molecular Probes (Eugene, OR, USA). Charybdotoxin (synthetic peptide) was from Peninsula Laboratories (Belmont, CA, USA). All other chemicals were of analytical grade and were obtained from Sigma, Merck and Fluka. Apamin (stock concentration of 10 μM) was dissolved in 0·05 M acetic acid (prepared with deoxygenated water) and stored tightly sealed at −20 °C. Charybdotoxin (stock concentration of 10 μM) was dissolved in 150 mM NaCl and stored in glass containers at −20 °C.

TABLE 1. Solutions used in this study

	Concentration (mM)					
Solution	NaCl	KCl	KAsp	$MgCl_2$	$CaCl_2$	Hepes/pH
Ba 1	140	5	0	2	2	20/7·2
Ba 2	115	30	0	2	2	20/7·2
Ba 3	5	140	0	2	2	20/7·2
Ba 4	0	40	100	2	2	20/7·2
Pi 1	2	140	0	2	0	20/7·2
Pi 2	0	40	100	2	0	20/7·2

Ba, bath solutions; Pi, pipette solutions. In addition to the ions shown in the table, the pipette solutions contained (mM): 1 ATP, 0·02 GPT, 0·2 EGTA, and 25 μM indo-1 free acid. The free Ca^{2+} concentration in the pipette solution was approximately 200 nM, as measured with a Ca^{2+}-sensitive electrode. Osmolarity was controlled at 280 ± 5 mosmol l^{-1} for all solutions. KAsp, potassium aspartate.

RESULTS

Whole-cell currents in HL-60 granulocytes activated by an increase in $[Ca^{2+}]_i$

To study whether $[Ca^{2+}]_i$ elevations activate membrane conductances in HL-60 granulocytes, we simultaneously measured $[Ca^{2+}]_i$ and whole-cell currents. Experiments were initially performed using intra- and extracellular solutions mimicking physiological ionic conditions (Ba 1, Pi 1; see Table 1 for definition). HL-60 cells were voltage clamped at −60 mV in the whole-cell configuration and brief voltage pulses to two target voltages were repeatedly applied to allow the measurement of the whole-cell conductance throughout the experiment (Fig. 1*Ac* and *Bc*).

A brief application of the chemotactic peptide f-MLP through a superfusion pipette (Fig. 1*Aa*) led to a transient $[Ca^{2+}]_i$ increase. The f-MLP-induced increase in $[Ca^{2+}]_i$ was paralleled by the activation of a whole-cell current (Fig. 1*Ab*). The currents were outward at all the three voltages applied in this experiment (−60 mV, −30 mV and 0 mV), suggesting that the current reverses at a potential more negative than −60 mV.

In many cells, 'spontaneous' $[Ca^{2+}]_i$ oscillations were observed during the time course of an experiment (Fig. 1*Ba*). As shown previously in human neutrophils, these oscillations are due to activation of adherence receptors during cellular spreading (Jaconi, Theler, Schlegel, Appel, Wright & Lew, 1991). The $[Ca^{2+}]_i$ oscillations were accompanied by oscillatory increases in the whole-cell current (Fig. 1*Bb*). The currents were outward at 0 mV and −60 mV, but inward at −90 mV, suggesting

that the current was carried by K^+ ions (see below). At all voltages, the kinetic features of the currents closely paralleled those of the $[Ca^{2+}]_i$ signal, displaying the

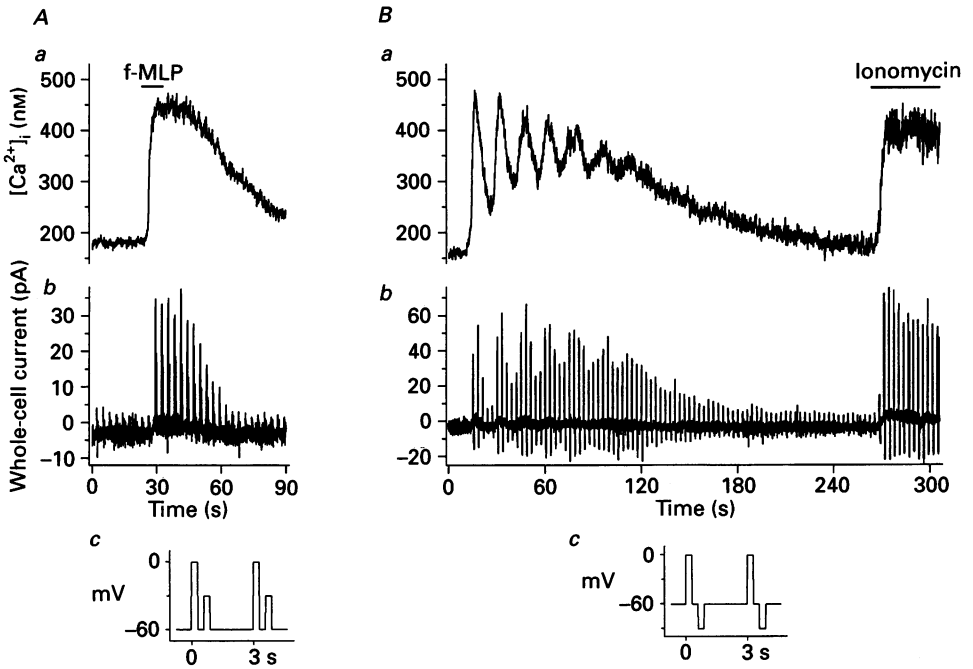

Fig. 1. Effect of $[Ca^{2+}]_i$ elevations on whole-cell currents in HL-60 cells. Indo-1-loaded cells were voltage clamped at −60 mV in the whole-cell configuration, and brief pulses to target voltages were repeatedly applied. Bath solution was Ba 1 and pipette solution was Pi 1, as defined in Table 1. *Aa* and *Ba*, $[Ca^{2+}]_i$; *Ab* and *Bb*, whole-cell currents; *Ac* and *Bc*, the continuously applied voltage protocol (note the different time scale). *A*, $[Ca^{2+}]_i$ was increased by f-MLP (1 μM) applied through a second micropipette in the vicinity of the cell. *B*, spontaneous $[Ca^{2+}]_i$ oscillations due to cellular spreading; after termination of the spontaneous $[Ca^{2+}]_i$ oscillations, ionomycin (1 μM) was added to the bath solution. Note that the changes in current closely parallel the $[Ca^{2+}]_i$ signal. Traces are representative of six similar experiments.

same oscillating pattern and decreasing to near basal levels upon the return of $[Ca^{2+}]_i$ to baseline. A subsequent $[Ca^{2+}]_i$ increase produced by application of the Ca^{2+} ionophore ionomycin (1 μM) to the bath solution caused a subsequent activation of the whole-cell currents. As in the case of the spontaneous $[Ca^{2+}]_i$ oscillations, the currents associated with the ionomycin-induced $[Ca^{2+}]_i$ increase were outward at 0 mV and −60 mV, but inward at −90 mV.

Thus, $[Ca^{2+}]_i$ elevations induced by application of a chemotactic peptide, by cellular spreading or by application of a Ca^{2+} ionophore were associated with the activation of whole-cell currents that reversed sign close to the equilibrium potential for K^+, suggesting that the current was carried by K^+ ions.

Ionic selectivity of the currents

To study the selectivity of the Ca^{2+}-activated conductance, we varied the extracellular K^+ concentration and measured the reversal potential of the whole-cell

currents (E_{rev}). The cells were voltage clamped at −60 mV and voltage pulses to +30 mV, 0 mV and −90 mV were applied every 5 s. The conductance was activated by application of ionomycin (1 μM) to bath solutions with a $[K^+]$ of 5 mM (Ba 1, Pi 1), 30 mM (Ba 2, Pi 1) and 140 mM (Ba 3, Pi 1). Varying extracellular K^+ concentration had no effect on basal $[Ca^{2+}]_i$ levels, and did not significantly alter the $[Ca^{2+}]_i$ levels

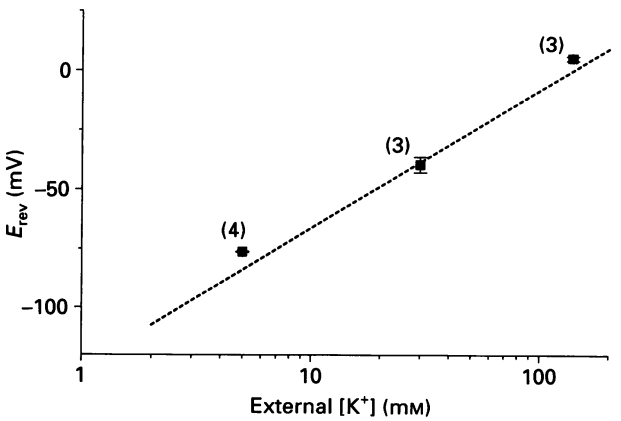

Fig. 2. Reversal potentials of the Ca^{2+}-activated currents, measured at different external K^+ concentrations. The current was induced by application of ionomycin (1 μM) to bath solutions with a $[K^+]$ of 5 mM (Ba 1, Pi 1), 30 mM (Ba 2, Pi 1) and 140 mM (Ba 3, Pi 1). Currents at different voltages (−90, −60, 0 and +30 mV) were measured at basal and high $[Ca^{2+}]_i$. Currents at basal $[Ca^{2+}]_i$ were subtracted from currents at high $[Ca^{2+}]_i$ and the differences, representing the Ca^{2+}-activated currents, were plotted as a function of membrane voltage. The reversal potential (E_{rev}) was taken as the intersection of the voltage axis with a line connecting the experimental points, and is plotted as a function of bath $[K^+]$. Symbols are means±S.E.M. with the number of determinations in parentheses. The dashed line is the predicted Nernst potential for K^+ ions at 20 °C.

reached after ionomycin addition (not shown). To determine E_{rev}, the currents recorded before ionomycin addition were subtracted from the currents measured at maximally elevated $[Ca^{2+}]_i$ and the differences, representing the Ca^{2+}-activated currents, were plotted against the membrane voltage. The experimental points were connected by lines, and E_{rev} was taken as the intersection of these lines and the voltage axis.

As shown in Fig. 2, changing external K^+ concentration from 5 mM to 140 mM caused E_{rev} to shift from −78 to 0 mV. The plot of E_{rev} as a function of bath $[K^+]$ agreed well with the predicted Nernstian relation for a K^+-selective conductance (Fig. 2, dotted line); a fit to the experimental points yielded a slope of 56·1 mV per log $[K^+]$, whereas the slope of the Nernst equation for a perfectly K^+-selective conductance at 20 °C is 58·17 mV per log $[K^+]$. These results show that the Ca^{2+}-activated conductance is K^+ selective and we therefore refer to it as the $G_{K(Ca)}$ conductance, and to the corresponding currents as $I_{K(Ca)}$.

Toxin sensitivity of $I_{K(Ca)}$

Two toxins have been found useful for the characterization of Ca^{2+}-activated K^+ channels. Charybdotoxin, derived from a scorpion venom, blocks most BK channels at concentrations between 1 and 10 nM, and some IK channels at concentrations

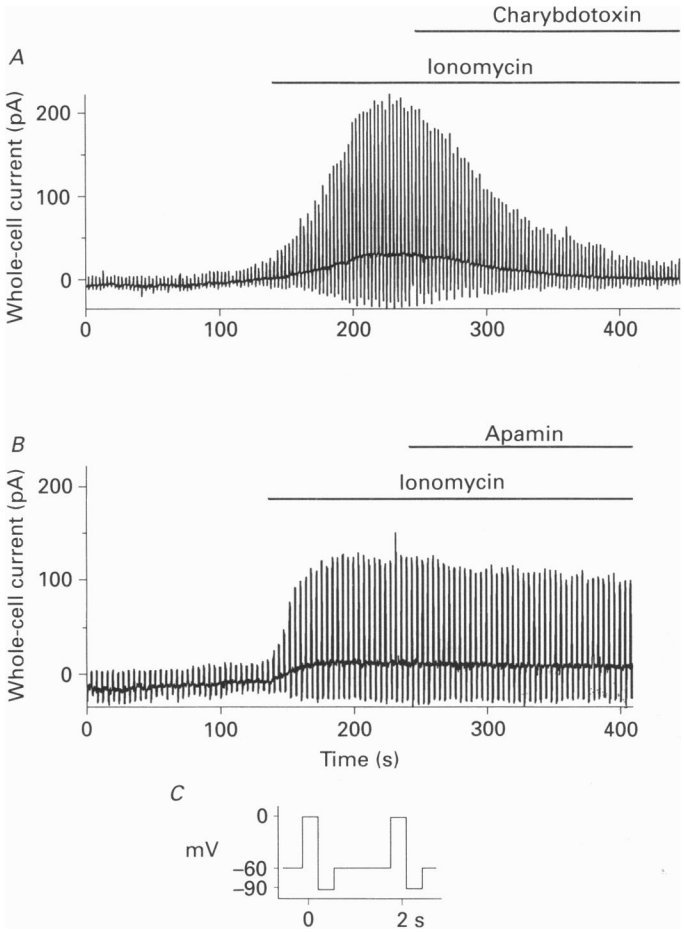

Fig. 3. Toxin sensitivity of $I_{K(Ca)}$. HL-60 cells were voltage clamped at −60 mV, and brief pulses to target voltages (*C*) were repeatedly applied to monitor the plasma membrane conductance (solutions Ba 1, Pi 1). When indicated, $[Ca^{2+}]_i$ was increased by application of ionomycin (1 μM) to the bath solution. *A*, whole-cell currents in a cell that was exposed first to ionomycin (1 μM), then to charybdotoxin (100 nM). Charybdotoxin reduced the ionomycin-induced current by $\simeq 80\%$. The residual current was not blocked by subsequent addition of apamin (not shown). *B*, whole-cell currents in a cell that was exposed first to ionomycin (1 μM), then to apamin (100 nM). Apamin did not significantly reduce the current. (A small, time-dependent decrease in current amplitude was also observed in the absence of the toxin: see Fig. 1*B*.) Similar results were obtained in five (charybdotoxin) or four (apamin) different experiments.

between 10 and 100 nM, while apamin, derived from bee venom, blocks SK channels at concentrations between 1 and 10 nM (Castle *et al.* 1989). We therefore tested the effect of the two toxins on the Ca^{2+}-activated K^+ currents in HL-60 granulocytes.

Charybdotoxin (100 nM) blocked the current by more than 80% (Figs 3*A* and 4*B*; $n = 5$). In contrast, no significant effect of apamin could be observed (Fig. 3*B*); $n = 4$), even when very high concentrations were tested (200 nM, not shown). Also, no additional effect of apamin after block by charybdotoxin was observed (not shown).

Whole-cell current–voltage relationship of $I_{K(Ca)}$

To obtain a detailed current–voltage relationship, we stepped from a holding voltage of 0 mV to target voltages between −100 and +100 mV (Fig. 4*A*), and recorded the currents with a high sampling rate (10 kHz). To detect a possible rectifying behaviour of the whole-cell conductance, the recordings were performed in symmetrical potassium aspartate (Ba 4, Pi 2).

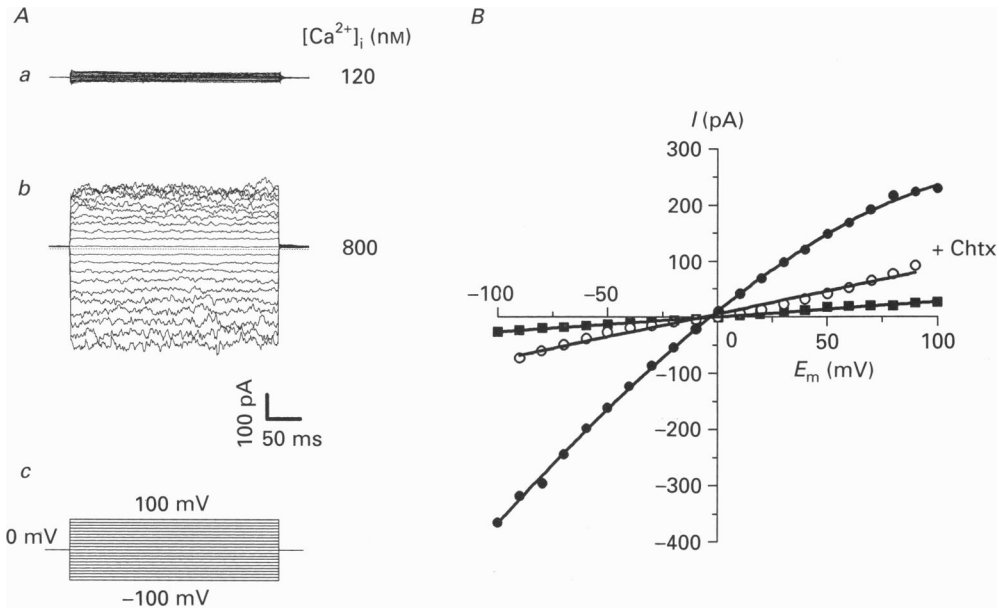

Fig. 4. Whole-cell current–voltage relationship of $I_{K(Ca)}$. An indo-1-loaded HL-60 cell was voltage-clamped at 0 mV in the whole-cell configuration, and 300 ms steps to voltages ranging from −100 mV to +100 mV were applied every 2 s (*Ac*). Bath and pipette contained 140 mM K^+ (solutions Ba 4, Pi 2). *A*, high-resolution recording of currents at basal $[Ca^{2+}]_i$ (*Aa*, $[Ca^{2+}]_i$ = 120 nM) and after application of 1 μM ionomycin to the bath solution (*Ab*, $[Ca^{2+}]_i$ = 800 nM). The dotted line indicates the zero-current level. *B*, current–voltage relationship of the Ca^{2+}-activated K^+ currents. ■, currents at basal $[Ca^{2+}]_i$ (120 nM). ●, currents at high $[Ca^{2+}]_i$ (800 nM). ○, currents at high $[Ca^{2+}]_i$ (800 nM) in the presence of 100 nM charybdotoxin (Chtx). Traces and plots are shown without any correction for a potential leak current; lines were fitted to the data by eye. Similar results were obtained in seven (ionomycin) and three (charybdotoxin) different experiments.

Only very small leak currents were observed prior to application of ionomycin (Fig. 4*Aa*). $I_{K(Ca)}$ currents were induced by application of ionomycin to the bath solution (Fig. 4*Ab* and *c*). Under these conditions, the currents appeared instantaneously after the voltage pulse, within the time resolution of the voltage clamp, and did not activate during the 500 ms depolarizing pulse. The whole-cell conductance was asymmetrical, with larger currents at negative than at corresponding positive voltages (Fig. 4*B*, ●). When charybdotoxin (100 nM) was added to the bath solution, the Ca^{2+}-activated current was blocked by approximately 80% (Fig. 4*B*, ○). Thus, the Ca^{2+}-activated whole-cell conductance showed an inward rectification with no detectable voltage-dependent activation or inactivation. This

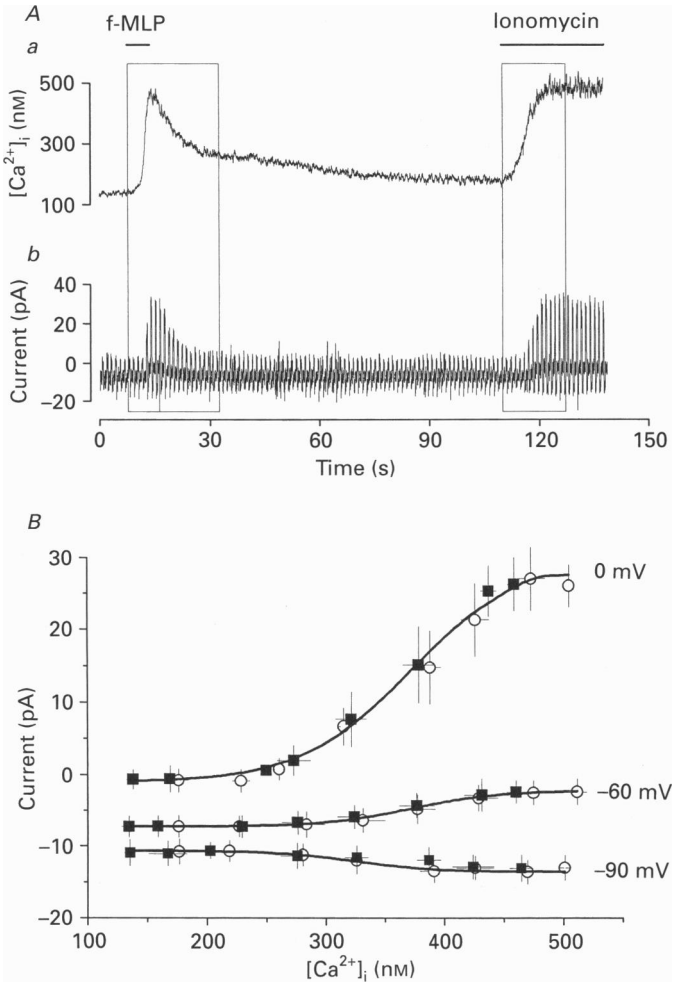

Fig. 5. $[Ca^{2+}]_i$ dependence of $I_{K(Ca)}$. HL-60 cells loaded with indo-1 were voltage clamped at −60 mV in the whole-cell configuration, and 300 ms pulses to two target voltages (−90 and 0 mV) were applied every 3 s. *A*, original traces (*a*, $[Ca^{2+}]_i$; *b*, whole-cell current) of a cell exposed first to f-MLP (1 μM) and then to ionomycin (1 μM). The portions indicated by the lines were selected to calculate the Ca^{2+} dependence of current activation. *B*, the $[Ca^{2+}]_i$ values measured in the selected portions were averaged at 50 nM intervals (from 100 to 550 nM), and the corresponding currents averaged separately for each voltage values (−60, 0 and −90 mV). The averaged current values were then plotted against the averaged $[Ca^{2+}]_i$ values for both the f-MLP-induced (■) and the ionomycin-induced (○) $[Ca^{2+}]_i$ increase. Note that for f-MLP, the analysed segment includes both the rising and the falling phase of the $[Ca^{2+}]_i$ transient The bars indicate means ± S.E.M.

instantaneous inward rectification is consistent with an inward rectification of the underlying unitary conductance (see below).

Calcium activation of $I_{K(Ca)}$

To characterize the Ca^{2+} activation of the currents, we applied successively f-MLP (1 μM) and ionomycin (1 μM) to the bath solution, and measured simultaneously

$[Ca^{2+}]_i$ and the whole-cell currents (Fig. 5*A*). Steps to depolarizing and hyperpolarizing voltages were continuously applied from a holding voltage of −60 mV, and the currents recorded at these three voltages were plotted against the measured $[Ca^{2+}]_i$ (Fig. 5*B*).

The threshold $[Ca^{2+}]_i$ for activation of the current was around 250 nM. Between 250 and 450 nM the current amplitude then increased as a function of $[Ca^{2+}]_i$. Intracellular calcium concentrations higher than 450 nM did not lead to any further current increases. In some recordings, $[Ca^{2+}]_i$ values of up to 800 nM were observed, and did not increase the whole-cell currents above levels observed at 450 nM (not shown). Thus, the conductance was activated within a relatively narrow range of physiological $[Ca^{2+}]_i$ values. In the case of f-MLP, no difference in the Ca^{2+} sensitivity

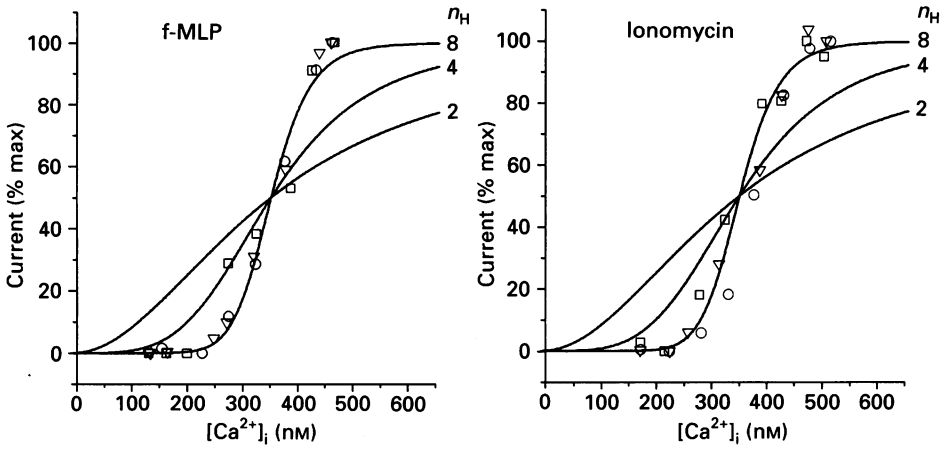

Fig. 6. Normalized current *versus* $[Ca^{2+}]_i$ relationships at different voltages. Data from Fig. 5*B*. For the three voltages tested, the measured current was normalized to its maximal value and plotted against the corresponding $[Ca^{2+}]_i$. ○, −60 mV, ▽, 0 mV, □, −90 mV. The modified Hill equation was fitted to the data, using different values of n_H:

$$y = y_{min} + y_{max}/[1 + (K_D/[Ca^{2+}]_i)^{n_H}],$$

where n_H is the Hill coefficient and K_D is the dissociation constant. The best fits were obtained with n_H values of 8.

TABLE 2. Characteristics of K^+ currents activation by Ca^{2+}

	f-MLP			Ionomycin		
	−90 mV	−60 mV	0 mV	−90 mV	−60 mV	0 mV
EC_{50}	357±20	359±21	364±24	359±32	350±6	346±57
EC_{90}	466±37	475±43	490±41	496±51	470±34	506±112
Hill coefficient	8·9±2·4	8·2±1·5	7·7±1·6	7·1±1·3	7·8±1·4	6·2±1·2

Parameters of current activation for Ca^{2+} increases elicited by f-MLP (1 μM) or ionomycin (1 μM). The cytosolic free Ca^{2+} concentrations that induced 50% (EC_{50}), and 90% (EC_{90}) of the maximal current response, and the Hill coefficient of the $[Ca^{2+}]_i$ *versus* current plot were determined at three different voltages (−90, −60 and 0 mV). EC_{50} and EC_{90} were determined graphically. The Hill coefficient of the Ca^{2+} activation was determined using a Hill equation according to Grissmer *et al.* 1992. Data are shown as means±S.E.M. (n = 6 for ionomycin and 3 for f-MLP).

of the current was observed between the rise and decay of the Ca^{2+} response, even in experiments performed at higher time resolution (not shown).

When normalized to maximum current amplitude, the steep calcium dependency of the currents is striking, as is the absence of effect of the applied voltage (Fig. 6). From the normalized current–$[Ca^{2+}]_i$ relationship, we calculated the cytosolic free Ca^{2+} concentration that induced 50 % (EC_{50}), and 90 % (EC_{90}) of the maximal current response, and the Hill coefficient for different cells (Table 2). The EC_{50} was approximately 350 nM and the activation of the current by $[Ca^{2+}]_i$ was highly co-operative, with a calculated Hill coefficient ranging between 6 and 9. Comparison of the $[Ca^{2+}]_i$ increase caused by f-MLP and ionomycin (Fig. 6*A* and 6*B*, and Table 2) showed a comparable $[Ca^{2+}]_i$ dependency of current activation. Thus, for a given $[Ca^{2+}]_i$ the receptor agonist f-MLP did not have additional effects on $I_{K(Ca)}$ as compared with the Ca^{2+} ionophore. This suggests that the activation of the conductance by f-MLP is predominantly, if not exclusively, mediated by changes in $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$-*activated* K^+ *channels in cell-attached patches*

To identify the unitary conductance underlying the Ca^{2+}-activated whole-cell current, we performed cell-attached patch clamp experiments and measured in parallel $[Ca^{2+}]_i$.

No single-channel events were observed at resting $[Ca^{2+}]_i$ levels (Fig. 7*Aa*). After addition of ionomycin, $[Ca^{2+}]_i$ increased to values > 500 nM and single-channel events could be observed. (Fig. 7*Ab* shows a patch that contained 2 channels.) Similar to the whole-cell current, the unitary current displayed inward rectification (Fig. 7*C*), yielding values for the unitary conductance of 37 pS at −80 mV and of 20 pS at +80 mV. The slope conductance, derived from a linear fit ($R = 0{\cdot}99$) of the current–voltage relationship between −80 and 0 mV, was 35 ± 2 pS. Recordings from patches with only one channel and with more than 100 single-channel events at a constant $[Ca^{2+}]_i$ were used to calculate the opening probability (P_o) of the channel at $[Ca^{2+}]_i > 500$ nM. At −80 mV, the P_o values obtained were $0{\cdot}68 \pm 0{\cdot}02$ ($n = 6$; mean ± S.E.M.). In more than 100 single-channel recordings in HL-60 granulocytes, we have never observed large-conductance Ca^{2+}-activated K^+ channels.

Analysis of whole-cell current fluctuations

As an additional approach to obtaining an estimate of the unitary conductance of the Ca^{2+}-activated whole-cell currents, we have performed stationary fluctuation analysis of the Ca^{2+}-activated whole-cell currents. For a constant whole-cell current value, the relationship between the unitary current i and the fluctuation of the whole-cell current is defined by the equation:

$$i = \frac{\sigma_I^2}{I(1-P_o)},$$

where σ_I^2 is the variance of the whole-cell current, I the averaged whole-cell current, and P_o the opening probability of the channel (Hille, 1992).

Figure 8*A* shows current fluctuations at −80 mV in a whole-cell recording, at low $[Ca^{2+}]_i$ (*a*), high $[Ca^{2+}]_i$ (*b*) and high Ca^{2+} after addition of charybdotoxin (*c*). Figure

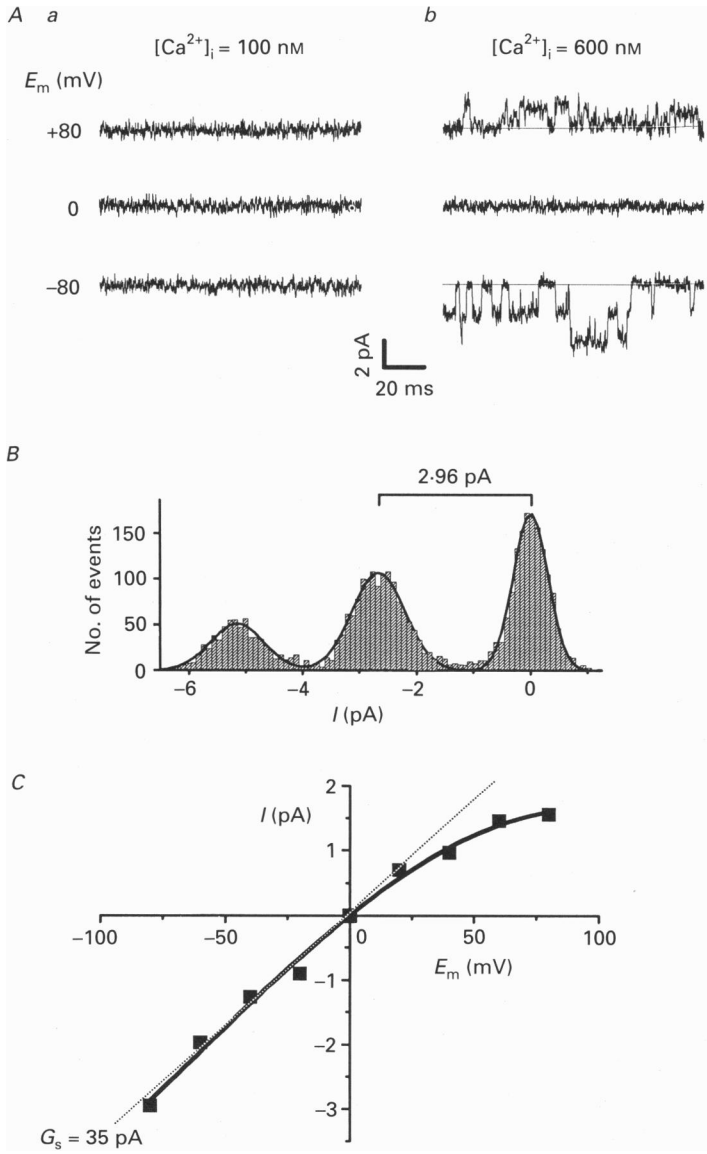

Fig. 7. Single calcium-activated K^+ channels recorded in the cell-attached configuration. Bath and pipette contained 140 mM K^+ (solutions Ba 4, Pi 2), and membrane potential was assumed to be 0 mV. *Aa*, currents at basal $[Ca^{2+}]_i$ (100 nM, measured with indo-1). *Ab*, currents in the same patch 2 min after addition of ionomycin (1 μM) to the bath solution (measured $[Ca^{2+}]_i$ = 600 nM). The patch contained two channels; the dotted line indicates the closed state. Data were collected at 50 kHz, and a running average procedure (continuous average of 4 neighbouring points) was used to smooth the current trace. *B*, amplitude histograms of the currents shown in *Ab*, bottom. Lines are Gaussian curves fitted to the data. The peak on the right represents the current when the channels are closed, the two peaks on the left the current when the channels are open. The difference between the peaks was used to calculate the unitary current amplitude. *C*, single-channel current–voltage relationship of the Ca^{2+}-activated currents shown in *A*. The unitary current amplitude was measured as shown in *B*. Inward rectification of the unitary

8*B* shows the variance of the whole-cell currents (σ_I^2, hatched bars) and the mean whole-cell current (I, filled bars) of seven different cells. The averaged σ_I^2/I of the charybdotoxin-sensitive, Ca^{2+}-activated currents was $0{\cdot}72 \pm 0{\cdot}02$ (mean ± S.E.M.). Assuming an opening probability of 0·68 (see previous section) and applying the above equation, the observed current fluctuations would predict a unitary current of 2·25 pA and a unitary conductance of 28 pS.

Thus, the values of the unitary conductance derived from fluctuation analysis are slightly smaller, but in the same order of magnitude, as the one derived from single-channel analysis. The results obtained with both methods clearly suggest that a channel of intermediate conductance (between 20 and 40 pS) is the basis of the observed Ca^{2+}-activated whole-cell currents. Concerning the relatively small but consistent discrepancy between the results of the single-channel and the fluctuation analysis, it has been previously noted that the unitary conductances measured at the single-channel level tended to be up to 40% larger than the conductance values determined by fluctuation methods in the same cell (Fenwick, Marty & Neher, 1982).

Based on the mean Ca^{2+}-activated whole-cell current of 262 pA at −80 mV (see Fig. 8), the mean unitary current of 2·25 pA at −80 mV (see above) and the opening probability of 0·68 (see above), we estimate an average number of 171 channels per cell. Taking an average capacitance of 5 pF for differentiated HL-60 cells (see Methods section), and a specific capacitance of 1 $\mu F\ cm^{-2}$ (Hille, 1992), the cell surface of an HL-60 cell is approximately 500 μm^2. The estimated channel density is therefore 0·34 channels μm^{-2} cell surface. This fits well with our observation in the single-channel recordings. We used patch pipettes with a resistance between 5 and 10 MΩ, which corresponds to a tip opening between 1 and 2 μm (Sakmann & Neher, 1983). Under these conditions, the probabilities of observing none, one or two Ca^{2+}-activated single channels in a cell attached patch were around 40, 40 and 20% respectively; no patches with three single channels were observed (not shown).

DISCUSSION

In this report we describe and characterize a Ca^{2+}-activated K^+ channel in HL-60 granulocytes. Analysis of single-channel events showed the presence of Ca^{2+}-activated intermediate-conductance K^+ (IK) channels. Whole-cell recordings revealed Ca^{2+}-activated K^+ currents. The whole-cell currents appeared to be due to opening of the IK channels because: (i) analysis of whole-cell current fluctuations revealed an underlying unitary conductance similar to that determined from single-channel recordings, (ii) both, single-channel and whole-cell currents, were inwardly rectifying, and (iii) both, single-channel and whole-cell currents, were activated at submicromolar $[Ca^{2+}]_i$. The properties of the Ca^{2+} activation of the whole-cell current, i.e. voltage-independence, high co-operativity, and sensitivity to submicromolar $[Ca^{2+}]_i$, had previously been described only for SK but not for IK channels.

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current occurs at voltages more positive than +30 mV. A linear fit of the data between −80 mV and 0 mV yielded a slope conductance of 35 pS. Similar results were observed in five different experiments.

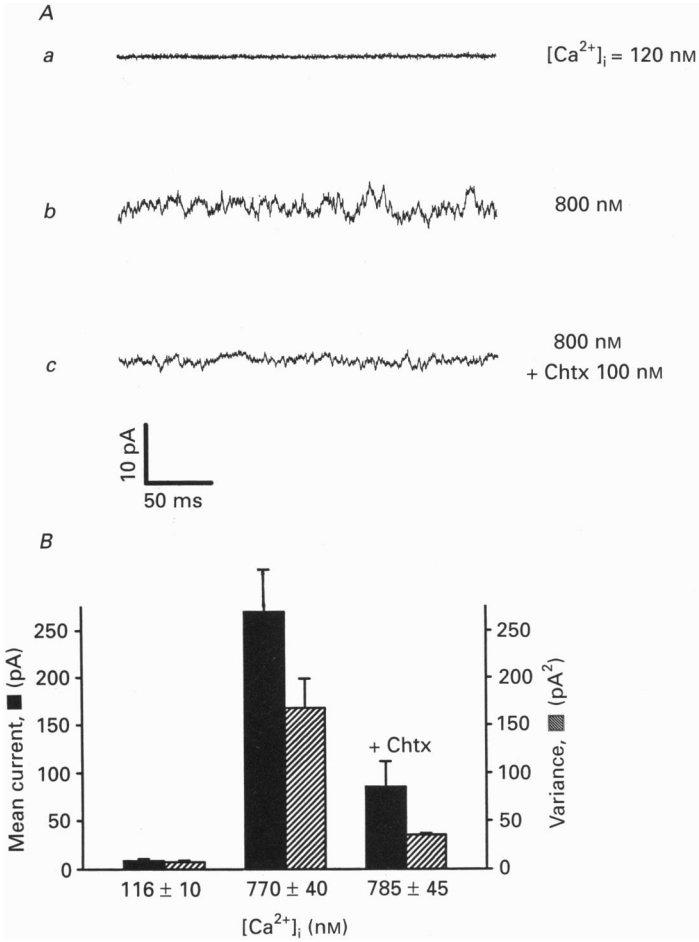

Fig. 8. Fluctuation analysis of the Ca^{2+}-activated K^+ current. *A*, examples of high-resolution recordings of whole-cell currents at −80 mV. The cell was voltage clamped at −60 mV and pulses to −80 mV of 500 ms duration were applied repeatedly (solutions Ba 4, Pi 2). *Aa*, currents at basal $[Ca^{2+}]_i$ (120 nM). *Ab*, currents after application of 1 μM ionomycin to the bath solution ($[Ca^{2+}]_i$ = 800 nM). *Ac*, current at high $[Ca^{2+}]_i$ after the application of charybdotoxin (Chtx 100 nM). The current fluctuations increase with the activation of $I_{K(Ca)}$, and are reduced by charybdotoxin. *B*, mean current (filled bars) and current variance (hatched bars) measured at −80 mV before and after activation of $I_{K(Ca)}$, and after block by charybdotoxin. For each cell, mean current and variance were determined on recordings of 500 ms duration sampled at 10 kHz. Data are means ± S.E.M. of seven (ionomycin) and three (charybdotoxin) different experiments.

Related IK channels in other cell types

In contrast to SK and BK channels, IK channels do not represent a homogeneous group of channels. IK channels of various cell types may differ markedly in terms of single-channel conductance, voltage activation and toxin sensitivity. However, the IK channels described in HL-60 granulocytes (this study), in macrophage-related cell

lines (McCann, Keller & Guyre, 1987; Kakuta *et al.* 1988; Gallin, 1989; Kanno & Takishima, 1990), in erythrocytes (the so-called Gardos-channel (Gardos, 1958; Hamill, 1981; Grygorczyk & Schwarz, 1983; Grygorczyk, Schwarz & Passow, 1984) and in lymphocytes (Mahaut-Smith & Schlichter, 1989; Mahaut-Smith & Mason, 1991; Grissmer *et al.* 1992) share several properties. These channels: (i) have a single-channel conductance between 20 and 40 pS, (ii) are inwardly rectifying, (iii) are voltage independent, (iv) are charybdotoxin sensitive and apamin insensitive (toxin sensitivity was previously studied only in lymphocytes and erythrocytes), and (v) are activated by Ca^{2+} concentrations in the submicromolar range in the intact cells and in whole-cell studies.

For the rest of the discussion, we will refer to this subgroup of IK channels as IK_b (the subgroup of IK channels that is commonly found in blood cells). IK_b channels are widely distributed among blood cells, but to our knowledge they have not been described in other cell types. This raises the interesting possibility that the expression of IK_b channels might be restricted to developmentally related blood cells; however, further studies will be necessary to clarify this point. It should be noted that the IK_b channel may co-exist in the same cell with other Ca^{2+}-activated K^+ channels. Lymphocytes express SK channels (Mahaut-Smith & Schlichter, 1989; Mahaut-Smith & Mason, 1991; Grissmer, Lewis & Cahalan, 1992), and mature macrophages express BK channels (Kakuta *et al.* 1988; Gallin, 1984, 1985; McCann *et al.* 1987) in addition to the IK_b channel. In contrast, in erythrocytes and granulocytes only IK_b channels have been described so far.

Parameters of Ca^{2+} activation

Our study demonstrates that the Ca^{2+} activation of HL-60 cell IK_b channel (i) is voltage independent, (ii) occurs at low (submicromolar) Ca^{2+} concentrations, and (iii) is highly co-operative. We determined these parameters of Ca^{2+} activation by measuring the averaged $[Ca^{2+}]_i$ over the entire cell. This value of $[Ca^{2+}]_i$ probably underestimates the submembraneous Ca^{2+} concentration. However, such a deviation could not account for the observed co-operativity and voltage independence. Thus, the observed parameters of Ca^{2+} activation most probably reflect the intrinsic properties of the channel. Comparable quantitative analysis has not been performed previously in cell-attached or whole-cell studies; however, some indirect evidence suggests that the Ca^{2+} activation of IK_b channels in other cell types also occurs with high co-operativity at submicromolar $[Ca^{2+}]_i$: (i) physiological agonists or lectins, which usually increase $[Ca^{2+}]_i$ in the submicromolar range, potently activate IK_b channels in cell-attached studies of macrophages (Gallin, 1989; Hara, Ichinose, Sawada, Imai & Maeno, 1990) and lymphocytes (Mahaut-Smith & Mason, 1991); (ii) Ca^{2+}-dependent K^+ efflux in erythrocytes (Wolff, Cecchi, Spalvins & Canessa, 1988) and Ca^{2+}-dependent K^+ currents in HL-60 promyelocytes (Wieland, Gong, Chou & Brent, 1992) are activated by submicromolar $[Ca^{2+}]_i$; and (iii) based on an observed 'all-or-none behaviour' of the Ca^{2+}-gating mechanism of erythrocyte K^+ efflux, a highly co-operative gating of the erythrocyte Ca^{2+} channel has been proposed (Lew, Muallem & Seymour, 1983). In excised-patch studies, generally higher Ca^{2+} concentrations were necessary to activate the channel and only a small amount of co-operativity was observed (Grygorczyk & Schwarz, 1983; Grygorczyk, Schwarz &

Passow, 1984; Kakuta *et al.* 1988), possibly because of a complex mechanism of co-operativity that requires intact cellular structures.

In addition to $[Ca^{2+}]_i$, G-proteins, second messengers or protein phosphorylation have been shown to regulate the activity of various Ca^{2+}-activated K^+ channels (Toro & Stefani, 1991). In our study, the $[Ca^{2+}]_i$–current relationship of the IK_b channel was similar for current activation by the receptor agonist f-MLP and by the Ca^{2+} ionophore ionomycin. This suggests that an increase in $[Ca^{2+}]_i$ is the predominant, if not the only, signal for IK_b channel activation by f-MLP.

The parameters of Ca^{2+} activation of the IK_b channel described in this study are very similar to results obtained with a lymphocyte SK channel (Grissmer *et al.* 1992). The Ca^{2+} activation of the lymphocyte SK channel was voltage independent, occurred at physiological $[Ca^{2+}]_i$ concentrations (EC_{50}, 400 nM), and was highly co-operative (Hill coefficient, 5). Thus, despite the clearly divergent single-channel conductance and toxin sensitivity of the SK and the IK_b channel, their characteristics of Ca^{2+} activation suggest that they are activated in the same biological context: (i) because of their threshold of activation, which is only slightly above the resting $[Ca^{2+}]_i$, virtually any biologically relevant $[Ca^{2+}]_i$ increase will activate these channels; (ii) because of the high co-operativity of the Ca^{2+} activation, $[Ca^{2+}]_i$ changes between 100 and 500 nM are exquisitely sensed by these channels; and (iii) because of their voltage independence, activation of these channels does not require depolarization of the plasma membrane.

Possible biological significance of IK_b channels

What might be the role of the very close coupling of the IK_b channels to the $[Ca^{2+}]_i$ signal? In granulocytes, the negative plasma membrane potential is required for a normal cellular response to agonists, and depolarization inhibits agonist-induced phospholipase C activation (Pittet, di Virgilio, Pozzan, Monod & Lew, 1990), Ca^{2+} influx (Mohr & Fewtrell, 1987; Di Virgilio, Lew, Andersson & Pozzan, 1987; Demaurex *et al.* 1992), and cellular functions (Mohr & Fewtrell, 1987; Martin, Nauseef & Clark, 1988). During neutrophil activation, the superoxide generation by the NADPH oxidase involves translocation of electrons from the cytosol to extracellular oxygen. The electron transport by the oxidase is electrogenic and has been shown to depolarize the plasma membrane (Henderson *et al.* 1987). The rate of superoxide generation during the neutrophil respiratory burst can reach 10 nmoles O_2^- min^{-1} $(10^6$ cells$)^{-1}$. Thus, one cell can transport up to 10^8 electrons per second through this oxidative system, which would generate a current of 16 pA. Human neutrophils and HL-60 cells are very small cells which at rest have a high input resistance (more than 10 GΩ, see Fig. 4*A*). The activation of an inward current of 16 pA would depolarize a cell with a 10 GΩ input resistance by 160 mV. Thus it is clear that there must be an activation of ion channels to support charge neutralization and repolarization. The presumed biological relevance of K^+ channel activation by Ca^{2+} is therefore to counteract the depolarization during neutrophil activation and to reconstitute the negative resting potential after the termination of the respiratory burst response.

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