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Characterization of Ca^{2+}-activated $^{86}Rb^{+}$ fluxes in rat C6 glioma cells: a system for identifying novel IK_{Ca}-channel toxins

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1 The pharmacological characteristics of a putative Ca^{2+} activated K^+ channel (IK_{Ca} channel) in rat glioma C6 cells were studied in the presence of the Ca^{2+} ionophore, ionomycin and various K^+ channel blockers, $^{86}Rb^+$ being used as a radioisotopic tracer for K^+.

2 The resting $^{86}Rb^+$ influx into C6 cells was 318 ± 20 pmol s^{-1}. The threshold for ionomycin activation of $^{86}Rb^+$ influx was approx. 100 nM. At ionomycin concentrations above the activation threshold, the initial rate of $^{86}Rb^+$ influx was proportional to ionophore concentration. Ionomycin-activated $^{86}Rb^+$ flux was saturable ($EC_{50} = 0.62 \pm 0.03$ μM) and was not inhibited by ouabain.

3 Intracellular Ca^{2+} increased within 30 s from a basal level of 42 ± 2 nM to 233 ± 17 nM, after addition of 2 μM ionomycin. During this period, intracellular pH fell from 7.03 ± 0.04 to 6.87 ± 0.03 and the cell hyperpolarized from -34 ± 10 mV to -76 ± 2 mV.

4 Single channel conductance measurements on inside-out patches in physiological K^+ solutions identified a 14 ± 3 pS Ca^{2+}-activated K^+ current between -25 mV and $+50$ mV. In symmetrical (100 mM) K^+, the single channel conductance was 26 pS.

5 Externally applied quinine ($IC_{50} = 0.12 \pm 0.34$ mM) and tetraethylammonium chloride ($IC_{50} = 10 \pm 1.9$ mM) inhibited $^{86}Rb^+$ influx into C6 cells in a concentration-dependent manner. Charybdotoxin ($IC_{50} = 0.5 \pm 0.02$ nM) and iberiotoxin ($IC_{50} = 800 \pm 150$ nM), as well as the crude venoms from the scorpions *Leiurus quinquestriatus* and *Mesobuthus tamulus*, also inhibited $^{86}Rb^+$ influx. In contrast, apamin and toxin I had no inhibitory effects on $^{86}Rb^+$ flux. A screen of fractions from cation exchange h.p.l.c. of *Mesob. tamulus* venom revealed the presence of at least four charybdotoxin-like peptides. One of these was iberiotoxin; the other three are novel toxins.

6 The ionomycin-activated $^{86}Rb^+$ influx into rat C6 glioma cells has proved to be a valuable pharmacological assay for the screening of toxins and crude venoms which modify intermediate conductance, Ca^{2+} activated K^+ channel activity.

Keywords: Ca^{2+}-activated K^+ channel; $^{86}Rb^+$; C6 glioma cells; ionomycin; charybdotoxin; iberiotoxin; *Mesobuthus tamulus* scorpion venom

Introduction

Potassium channels which are activated by increases in the level of intracellular Ca^{2+} (K_{Ca} channels) are a heterogeneous group, found in a wide variety of both electrically excitable and non-excitable cells (Latorre *et al.*, 1989). Three subtypes of K_{Ca} channels can be distinguished on the basis of their unitary conductance. The best characterized is the high conductance, or maxi-channel (BK_{Ca} channel, conductance typically 100–200 pS); this channel is involved in muscle contractility, action potential repolarization, the regulation of secretion in a variety of endocrine and exocrine cells, together with the control of intracellular osmolarity and cellular electrolyte concentration (Petersen & Maruyama, 1984). A second class of K_{Ca} channel has a small single channel conductance (5–18 pS, SK_{Ca} channel); in nerve and muscle, SK_{Ca} channels are responsible for the after-hyperpolarization which follows an action potential (Hugues *et al.*, 1982), while in a variety of non-excitable cells, these channels provide a link between intracellular messengers and membrane potential (Capiod *et al.*, 1986). A third class of channel, the intermediate conductance IK_{Ca} channel, has single channel conductances typically between 20 and 80 pS. It has a more restricted distribution than the former two subtypes and was originally identified in molluscan neurones (where it is probably responsible for the termination of action potential bursts in pacemaker cells – see Hermann & Erxleben 1987) and mammalian erythrocytes (Hamill, 1981) where it is activated upon metabolic exhaustion, the 'Gárdos effect'.

Apart from a biophysical classification, K_{Ca} channels can be characterized on the basis of their selectivity to different naturally-occurring peptide toxins, isolated from the venoms of a variety of scorpions and insects (Strong, 1990; Brewster & Strong, 1992). These toxins also provide invaluable tools for investigating structural differences between different channel subtypes and for evaluating the physiological contribution of an individual channel subtype to the behaviour of a cell. BK_{Ca} channels are typically blocked by charybdotoxin, a peptide toxin (M_r 4300 Da) isolated from the venom of the Israeli scorpion *Leiurus quinquestriatus hebraeus* (Miller *et al.*, 1985; Gimenez-Gallego *et al.*, 1988; Strong *et al.*, 1989) and iberiotoxin (M_r 4300 Da) isolated from the venom of the Indian scorpion, *Mesobuthus tamulus* (Galvez *et al.*, 1990). SK_{Ca} channels on the other hand, are specifically blocked by apamin (Blatz & Magleby, 1986; Capiod & Ogden, 1989; Park, 1994), an octadecapeptide (M_r 2000 Da) isolated from the venom of the European honey bee, *Apis mellifera* (Gauldie *et al.*, 1976; Strong & Brewster, 1992). IK_{Ca} channels have a pharmacology that is closer in characteristics to that of BK_{Ca} channels, than SK_{Ca} channels. IK_{Ca} channels are blocked by charybdotoxin (Hermann & Erxleben, 1987, Wolff *et al.*, 1988) but show a much reduced affinity for tetraethylammonium ion (TEA). IK_{Ca} channels are not blocked by apamin (Burgess *et al.*, 1981; Hermann & Hartung, 1983). More recently, other K_{Ca} channel toxins have been isolated with properties that are similar to, or complement, those of apamin and charybdotoxin (see, *inter*

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alia, Castle & Strong, 1986; Chicchi *et al.*, 1988; Auguste *et al.*, 1990; Crest *et al.*, 1992; Garcia-Calvo *et al.*, 1993; Sabatier *et al.*, 1993; Garcia *et al.*, 1994).

Glial cells are known to play an important role in the regulation of K^+ homeostasis in the brain (Pollen & Trachenberg, 1970). One regulatory mechanism, known as spatial buffering (Gardner-Medwin, 1983), may involve the Ca^{2+}-activated shunting of K^+ fluxes from a region of high $[K^+]$ to a region of low $[K^+]$. Evidence suggests that outward K^+ fluxes through K_{Ca} channels are triggered by glial cell depolarization and opening of voltage-activated Ca^{2+} channels, which leads to an increase in intracellular Ca^{2+} (MacVicar, 1984; Quant & MacVicar, 1986). TEA-sensitive K_{Ca} channels in inside-out patches of glial cell membranes grown in primary culture have a single channel conductance of 25 pS in physiological K^+ gradients (Quant & MacVicar, 1986). This suggests that these channels are of the IK_{Ca} subtype.

The discovery and characterization of novel toxins which selectively target different K_{Ca} channels is expected to further our understanding of the structural and functional relationships of this class of ion channel proteins. With this objective in mind, we have sought to devise a sensitive, specific and rapid *functional* assay to screen for new toxins which modulate IK_{Ca} channel activity. One problem in devising such an assay (as distinct from binding assays) is that the functional consequences of blockade of one channel can often be confused with the activation of another channel. Similar problems can arise if the functional effects of a minor component are masked by the opposite effects of a predominant component in a crude venom mixture. Here we characterize K_{Ca} fluxes in a rat glioma cell line (Benda *et al.*, 1968) using $^{86}Rb^+$ as a radioactive tracer and demonstrate that this system provides an extremely useful new method to screen crude venoms for IK_{Ca} channel activity and to identify IK_{Ca} channel toxins.

Methods

Cell culture

C6 rat glioma cells were seeded at a density of approx. 400 cells cm^{-2} in untreated six-well (35 mm diam.) plastic tissue culture dishes (Nunc) containing Eagle's-modified minimum essential medium and supplemented with 10% (v/v) foetal calf serum, 1% (w/v) non-essential amino acids, glutamine (2.92 mg ml^{-1}), gentamycin (50 μg ml^{-1}), penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and neomycin (50 μg ml^{-1}). Cells were grown at 37°C in a 95% humidified atmosphere with 5% CO_2. Cells were fed every other day and used 4–5 days after seeding, at which point the monolayers were confluent.

$^{86}Rb^+$ influx assays

Cell monolayers were pre-incubated (5 min, ambient temperature) in buffer A (mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgSO_4$ 0.4, glucose 25, Tris 25, pH 7.4 (Tas *et al.*, 1989). After aspiration, the cells were incubated at 37°C in buffer A containing $^{86}Rb^+$ (0.4 μCi ml^{-1}), ionomycin and potassium channel blockers, as indicated in the figure legends. After specified times, the incubation solution was removed by aspiration and the cells rapidly washed (< 10 s) with ice cold buffer A (3 × 3 ml). These washing conditions have been typically shown to remove 99% of extracellular space markers (see, *inter alia* Brown & Simmons, 1982). Cells were then dissolved in 0.1 M NaOH for the determination of total cell protein (Lowry *et al.*, 1951) and the cell $^{86}Rb^+$ content measured by determining Cerenkov radiation in a scintillation counter.

Determination of intracellular pH and Ca^{2+}

Cell monolayers were loaded with the pH indicator BCECF by incubation in buffer A containing 8 μM of the acetoxymethyl ester of the dye together with 100 mg l^{-1} of Pluronic F-127 for 30 min at 34°C, then placed on the stage of an inverted microscope at room temperature. Fields of cells were illuminated in turn with 430 nm and 500 nm light and the light emitted by intracellular BCECF collected by an intensified CCD camera. The ratio of emitted light intensities (I_{430}/I_{500}), a measure of intracellular pH, was calculated for each of ten cells in a field of view and converted to pH by comparison with *in vitro* solutions of known pH using a pK_a of 7.0. Results from three replicate experiments on a total of 30 cells were combined.

Intracellular free Ca^{2+} was measured similarly except that Fura-2 replaced BCECF and excitation wavelengths were 350 nm and 380 nm. However, we found that the ratio (I_{350}/I_{380}) measured from cells was often lower than that measured from a Ca^{2+}-free buffer solution, suggesting interference from intracellular viscosity (Poenie, 1990). Ion-sensitive electrode measurements indicate that the free Ca^{2+} concentration in resting C6 glioma cells is 40 nM (Erecinska *et al.*, 1991). Applying a viscosity correction factor of 0.52 to the measurements from resting cells gave a mean Ca^{2+} concentration of 40 nM, and we therefore applied this factor throughout, using a K_d of 135 nM.

Measurement of resting membrane voltage

Membrane voltage of glioma cells was measured by the amphotericin-B perforated patch technique (Rae *et al.*, 1991), a variant of the whole cell patch clamp in which solutes of mol. wt. > 180 cannot exchange between pipette and cytosol. A high resistance seal was formed between a cell bathed in buffer A and a micropipette containing (mM): KCl 55, K_2SO_4 70, $MgCl_2$ 7, HEPES 10, pH 7.3 and 240 μg ml^{-1} amphotericin-B. When sufficient amphotericin-B had partitioned into the patch of membrane at the pipette tip to reduce the access resistance below 40 MΩ, the recording amplifier was switched to current clamp to record membrane voltage.

Single channel measurements

Cells were bathed in calcium buffer containing KCl 100 mM, MOPS 10 mM (pH 7.2), EGTA 10 mM, plus calcium to a free concentration of 1.35 μM and magnesium to a free concentration of 1 mM (Molecular Probes, Eugene, Oregon, U.S.A.). Inside-out patches in symmetrical solution were created by forming a high-resistance seal between the cell and a micropipette containing the same buffer solution. An Axopatch 200 (Axon Instruments, Burlingame, CA, U.S.A.) in capacitance mode was used both to set the membrane voltage and to record current. In order to measure single-channel currents with a more physiological potassium gradient, pipettes were filled with Na_2SO_4 79 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM and HEPES 5 mM, pH 7.2 (low chloride medium). Under these ionic conditions any channels selective for sodium or chloride will generate a current out of the pipette at zero voltage.

Fractionation of venoms

All chromatographic procedures were carried out with a Gilson 715 Semi-Autoprep h.p.l.c. system. Crude scorpion venoms (either *Leiurus quinquestriatus hebraeus* or *Mesobuthus tamulus*) were extracted in water acidified with acetic acid (pH 3.0, 20 min, 4°C) and insoluble material was removed by centrifugation (30,000 *g*, 4°C, 20 min). Any mucoid pellets remaining were re-extracted and the combined extracts diluted to 500 ml with 20 mM NH_4OAc (pH 7.0) (buffer A). Extracts were filtered (0.2 μm nylon filter) and loaded at 10 ml min^{-1} on to a 4 ml cation exchange h.p.l.c. column (Neobar CS) equilibrated with buffer A. After elution of unbound material, retained *L. quinquestriatus* peptides were eluted with a 0–40% linear gradient of buffer B (1 M ammonium acetate pH 7.0) at 4.75 mM min^{-1} and a flow rate of 4 ml min^{-1}. Column fractions (4 ml) were monitored at 280 nm. Retained *Mesob. ta-*

mulus peptides were eluted with a 0–40% linear gradient of buffer B at 5 mM min^{-1} and a flow rate of 8 ml min^{-1}; 8 ml fractions were collected. Crude *Oxyuranus scutellatus* snake venom (100 mg) was suspended in 1 ml ammonium acetate buffer (150 mM, pH 9.0) and allowed to stand at ambient temperature (1 h). The extract was filtered and then loaded on to a Superdex 75 gel exclusion column (60 cm × 2.6 cm diam.; Pharmacia Hi-load). The column was eluted at 1 ml min^{-1} with the same ammonium acetate buffer. Fractions were monitored at 280 nm and peak fractions were pooled.

Materials

The C6 glioma cell line was obtained from the PHLS, Porton Down, U.K. (ECACC No: 85040101). BCECF and Fura-2 were obtained from Calbiochem. *Oxyuranus scutellatus* and *Leiurus quinquestriatus hebraeus* venoms, as well as charybdotoxin, were purchased from Latoxan (Rosans, France). Amphotericin-B, apamin and *Mesob. tamulus* venom were purchased from Sigma (Poole, Dorset). A sample of *Mesob. tamulus* venom was also kindly provided by Dr P. Gopalakrishnakone (Singapore). Toxin I was a gift from Professor A. Harvey (Strathclyde University, Glasgow) and iberiotoxin was a gift from Dr G. Kaczorowski (Rahway, New Jersey, U.S.A.). $^{86}Rb^+$ was purchased from Amersham International (Amersham, Bucks). All other chemicals were of Analar quality.

Statistics

Unless otherwise indicated, all data in the text and figures are expressed as mean ± s.e.mean of n observations.

Results

The Ca^{2+} ionophore, ionomycin, was used to activate Ca^{2+}-dependent $^{86}Rb^+$ fluxes (via a putative K_{Ca} channel) in confluent monolayer cultures of rat glioma C6 cells. In the absence of ionomycin, the steady-state basal influx of $^{86}Rb^+$ into the cells was estimated to be 318 ± 20 pmol s^{-1} (n = 5). Activation of $^{86}Rb^+$ fluxes was studied as a function of both ionophore concentration and incubation period in the presence of ionophore (Figure 1a). The effect of increasing ionomycin concentration on the total $^{86}Rb^+$ accumulated within the cell was saturable (EC_{50} = 0.62 ± 0.03 μM ionomycin, n = 5). Uptake (in the presence or absence of ionomycin) of $^{86}Rb^+$ was linear for the first 60 s and under these initial rate conditions, efflux of radioisotope was negligible and the accumulation of isotope within the cells could be considered to be entirely due to an influx component. An ionomycin concentration of approx 0.1 μM was necessary to activate $^{86}Rb^+$ flux over basal levels and during the initial 30 s of $^{86}Rb^+$ flux, the influx rate was proportional to log ionomycin concentration (Figure 1b). Similar data were obtained with another Ca^{2+} ionophore, A23187 (results not shown).

These results suggested that $^{86}Rb^+$ flux was activated by elevated levels of intracellular Ca^{2+} and in order to confirm this hypothesis, intracellular Ca^{2+} levels were measured directly with Fura-2. When ionomycin was applied to C6 cells, intracellular Ca^{2+} rose within 30 s to 233 ± 17 nM (30 cells) and then fell back to a maintained plateau (Figure 2). Intracellular pH fell in the 30 s after ionophore application, from 7.03 ± 0.04 to 6.87 ± 0.03 (30 cells), with no significant recovery. A pH fall subsequent to a Ca^{2+} rise is seen in a wide variety of cells and probably reflects proton release from Ca^{2+} buffers (Bolsover *et al.*, 1986).

Membrane voltage was measured during ionophore application, using the perforated patch technique. In all cells studied, inomycin caused a hyperpolarization from an average resting membrane potential of −34 ± 10 mV to a maximum of −76 ± 2 mV (n = 4), that was accompanied by a fall in membrane resistance (Figure 3). This suggests that the rise of intracellular Ca^{2+} observed in Figure 2 activated a K_{Ca} channel in the C6 cells, which in turn, resulted in the increased permeability of the cell to $^{86}Rb^+$ that was seen in Figure 1.

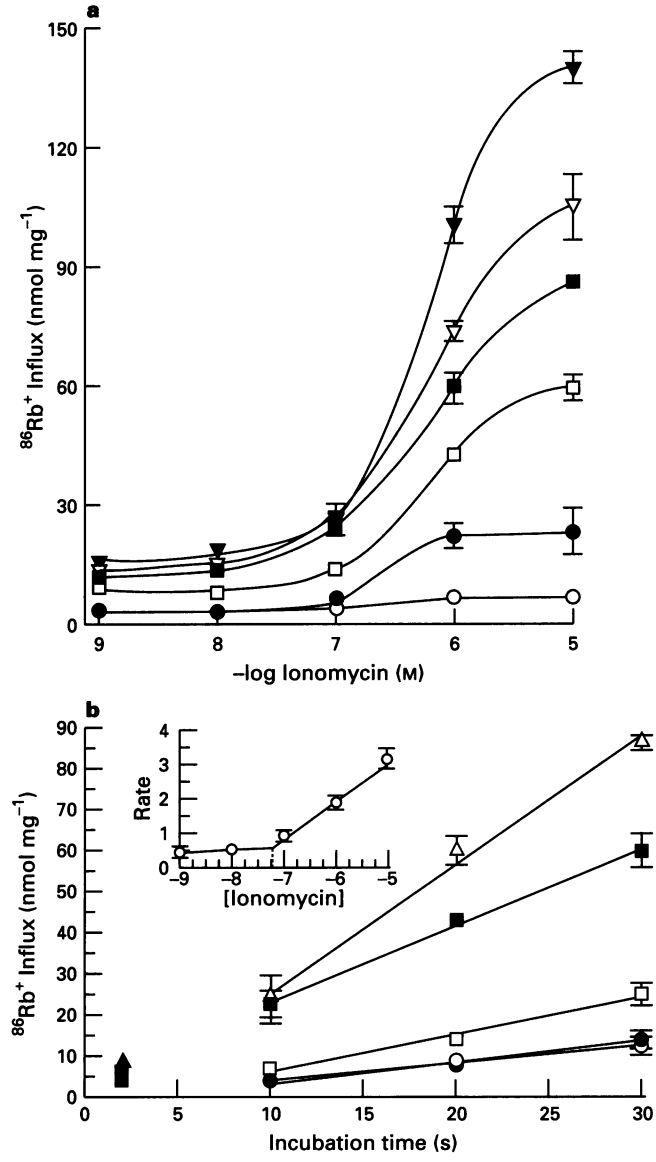

Figure 1 Kinetics of $^{86}Rb^+$ influx into C6 glioma cells and dependence on ionomycin concentration. (a) Uptake of $^{86}Rb^+$ as function of ionomycin concentration at different time periods. Monolayers of C6 glioma cells in 6-well tissue culture plates were pre-incubated for 5 min in 3 ml buffer A (see Methods). After pre-incubation, the solution was removed by aspiration, and the cells were incubated in 3 ml buffer A containing $^{86}Rb^+$ at 0.4 μCi ml^{-1} and different concentrations of ionomycin (0.001–10 μM). The uptake of $^{86}Rb^+$ into the C6 cells was measured after 2 s (○); 10 s (●); 20 s (□); 30 s (■); 60 s (▽) and 90 s (▼). Data points represent the mean ± s.d. (n = 4). All other procedures are as described in Methods. (b) Kinetics of $^{86}Rb^+$ influx. The same $^{86}Rb^+$ influx data from (a) during the initial 30 s, replotted as a function of time. Concentrations of ionomycin are: 0.001 μM (○); 0.01 μM (●); 0.1 μM (□); 1 μM (■); 10 μM (△). Inset: Influx rate (nmol $^{86}Rb^+$ mg^{-1} cell protein s^{-1}) as a function of ionomycin concentration. The intersection of the two lines indicates the approx. concentration of ionomycin required to activate $^{86}Rb^+$ influx.

It should be pointed out that there is no contradiction in our observation of a cell hyperpolarization and $^{86}Rb^+$ *influx*, as a consequence of channel activation by ionomycin. Although the *net* current flow is outward (hence cell hyperpolarization), $^{86}Rb^+$ initially flows *into* the cell when the channel is opened, down its ionic concentration gradient. Since $^{86}Rb^+$ is only used as an isotopic tracer, the extracellular con-

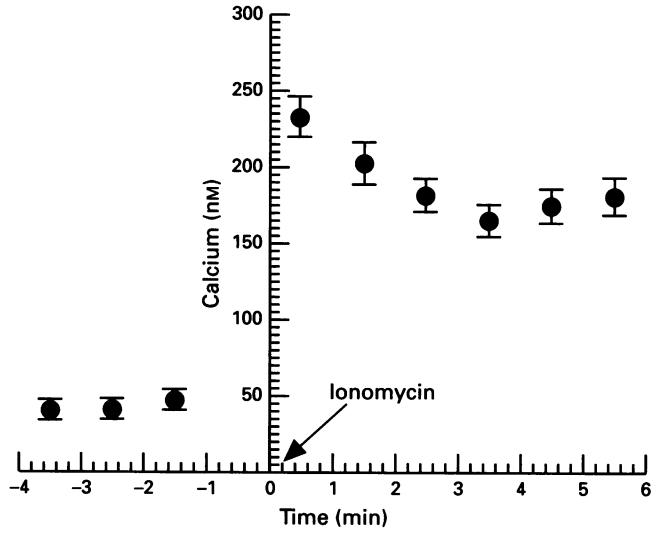

Figure 2 Determination of intracellular free Ca^{2+} in C6 glioma cells before and after treatment with ionomycin. Intracellular free Ca^{2+} was determined by the Fura-2 technique. Cell monolayers were grown as described in the Methods and loaded with dye. Emitted light intensities at 350 nm and 380 nm were collected by an intensified CCD camera. Levels of free intracellular Ca^{2+} (nM) were determined as described in the Methods. Ionomycin (2 μM) was added at time zero (indicated by arrow). Data points are the mean ± s.e. mean from three experiments, each experiment averaging the results from 10 cells in a field of view.

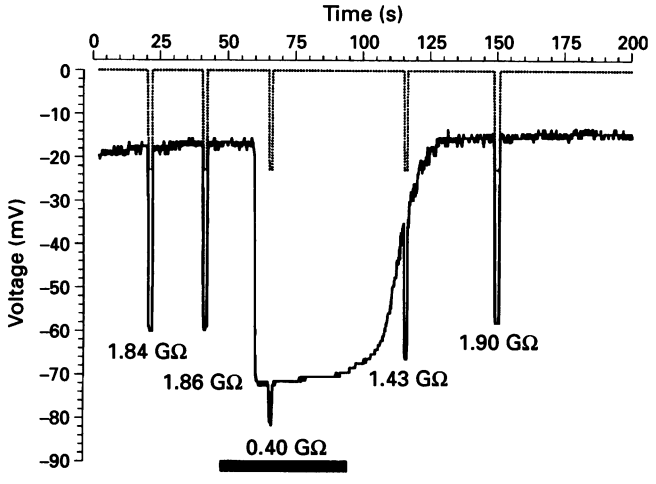

Figure 3 Membrane potential in C6 glioma cells before and after treatment with ionomycin. Cell monolayers were grown as described in the Methods. Membrane potentials were measured by the amphotericin-B perforated patch technique. A high resistance seal was formed between the C6 cell and micropipette, the latter containing 240 $\mu g\,ml^{-1}$ amphotericin-B. For further details see Methods. The continuous trace shows the membrane voltage. The dotted trace indicates when hyperpolarizing current pulses were applied to measure cell input resistance. Calculated values of input resistance are shown below each voltage excursion. The solid bar indicates application of ionomycin (2 μM). Input resistance was determined by injecting current pulses.

centration of $^{86}Rb^+$ at the beginning of the influx period (maximum 0.5 μM) will have a negligible influence on membrane potential.

When micropipettes containing a low chloride medium (Na^+ 158 mM, K^+ 5 mM, Cl^- 11 mM, see Methods) were held

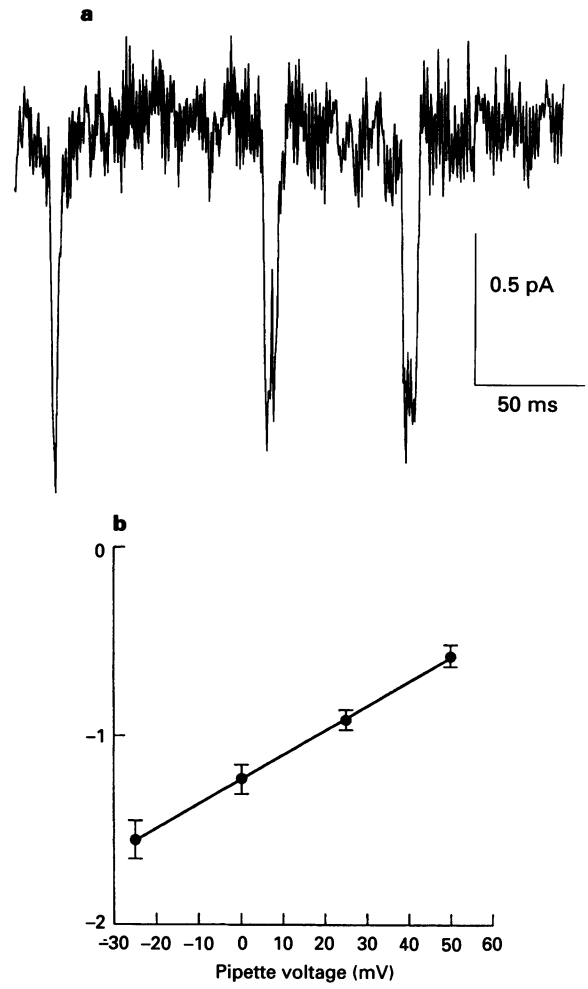

Figure 4 Single channel analysis: (a) Records from an inside-out patch. The cytosolic face of the membrane was exposed to a calcium buffer containing KCl 100 mM, MOPS 10 mM (pH 7.2), EGTA 10 mM plus calcium to a free concentration of 1.35 μM and magnesium to a free concentration of 1 mM. The extracellular face was exposed to low chloride medium comprising Na_2SO_4 79 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM and HEPES 5 mM, pH 7.2. There was no transmembrane voltage difference. The current, digitally filtered at 1 kHz, shows three channel openings producing current into the pipette, indicating activity of a potassium-selective channel. Although no channel activity was recorded before pulling the patch off the cell, openings were seen immediately upon exposing the cytosolic face of the membrane to Ca^{2+} 1.35 μM, consistent with the channels being calcium-activated. (b) *I-V* relationship for channels from one patch, conditions as (a). Each point is the mean of 10 single channel events, ± s.e. mean, recorded with the pipette set to the indicated voltages. In this patch the best-fit line indicated a conductance of 13 pS. In the six patches studied, the mean conductance was 14 ± 3 pS.

at bath voltage and sealed onto cells bathed in KCl 100 mM, free Ca^{2+} 1.35 μM, no single channel events were recorded. However, when the pipettes were pulled away from the cell to give inside-out patches in which the cytosolic face of the membrane was exposed to 1.35 μM free Ca^{2+}, single channel currents of amplitude 0.98 ± 0.09 pA (6 patches) appeared immediately (Figure 4a). Currents were into the pipette, ruling out sodium or chloride channels as the source of the current. Single channel current showed an ohmic dependence on pipette voltage (Figure 4b) and indicated a conductance of 14 ± 3 pS (6 patches). Three additional experiments were performed under symmetrical conditions with KCl 100 mM, free Ca^{2+} 1.35 μM on each side of the membrane. In these experiments, the single channel conductance was measured as 25, 25 and 29 pS (mean 26 pS).

In order to confirm that the ionomycin-stimulated $^{86}Rb^+$

flux measured in Figure 1 was not an active transport process, the effect of ouabain (a blocker of Na/K ATPase) on $^{86}Rb^+$ flux was studied. There was a negligible difference in the time course of $^{86}Rb^+$ flux over a 4 min period in either the presence or absence of 10 mM ouabain (Figure 5). Only 5% of the total measured flux after 4 min was ouabain-sensitive and therefore likely to be energy-dependent. However, since rat tissues are known to be peculiarly insensitive to the effects of ouabain (Detweiler, 1967; Cooke, 1981), cells were also pre-incubated with the drug for different time periods prior to a flux experiment. Figure 5 (inset) shows that pre-incubation with 10 mM ouabain for up to 30 min has absolutely no effect on the resultant ionomycin-stimulated $^{86}Rb^+$ flux over a subsequent 30 s assay period, confirming the passive nature of the flux process. Figure 5 (inset) also shows that approx 90% of the measured flux during this assay period was ionomycin-dependent.

Having characterized the basic properties of the flux process, the effects of K^+ channel blockers on ionomycin-activated $^{86}Rb^+$ fluxes were studied with 2 μM ionophore during a 30 s incubation period. TEA and quinine, agents which block a variety of K_{Ca} channels in many tissues and cells (Rudy, 1988), completely blocked ionomycin-activated $^{86}Rb^+$ influx in C6 cells (Table 1). Although both charybdotoxin and iberiotoxin, well characterized high affinity blockers of BK_{Ca} channels, inhibited ionomycin-activated $^{86}Rb^+$ influx in a dose-dependent manner (Table 1, Figure 6a), charybdotoxin was three orders of magnitude more potent. However apamin, a highly specific blocker of SK_{Ca} channels was completely ineffective at concentrations up to 100 μM. Toxin I (a close homologue of

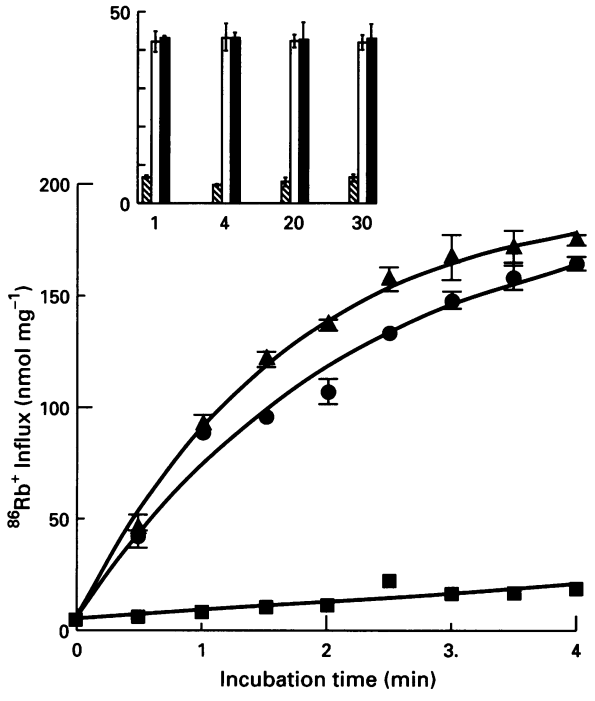

Figure 5 Effects of ouabain on the time course of ionomycin-induced $^{86}Rb^+$ influx. Monolayers of C6 glioma cells in 6-well tissue culture plates were pre-incubated for 5 min in 3 ml buffer A (see Methods). After pre-incubation, the solution was removed by aspiration and the cells were incubated in 3 ml buffer A containing $^{86}Rb^+$ at 0.4 μCi ml^{-1} with or without ouabain. $^{86}Rb^+$ influx into the cells was initiated by the addition of ionomycin. $^{86}Rb^+$ uptake was followed for incubation periods up to 4 min. Data represent means ± s.d. (n=4) for $^{86}Rb^+$ influx measured in the absence of ionomycin (■); the presence of 2 μM ionomycin (▲); the presence of 2 μM ionomycin and 10 mM ouabain (●). Inset represents $^{86}Rb^+$ influx after varying pre-incubation periods (1–30 min) in the presence of 10 mM ouabain. The pre-incubation medium was removed by aspiration before the incubation media was added. Basal influx (hatched columns); influx in the presence of 2 μM ionomycin (open columns); influx in the presence of 2 μM ionomycin and 10 mM ouabain (solid columns). Data points represent the mean ± s.d. (n=6).

Table 1 Inhibition of ionomycin-induced $^{86}Rb^+$ fluxes in C6 glioma cells

Inhibitor	IC_{50} (M)	(n)
TEA	1.0 ± 0.19 × 10–2	(4)
Quinine	1.2 ± 0.34 × 10–4	(4)
Iberiotoxin	8.0 ± 1.5 × 10–7	(4)
Charybdotoxin	5.0 ± 0.20 × 10–10	(4)

Cells were incubated in influx buffer containing 0.4 mCi ml^{-1} $^{86}Rb^+$ and inhibitors. At time zero, ionomycin (final concentration 2 μM) was added and $^{86}Rb^+$ accumulated in the cells was measured after 30 s. For further details, see Methods.

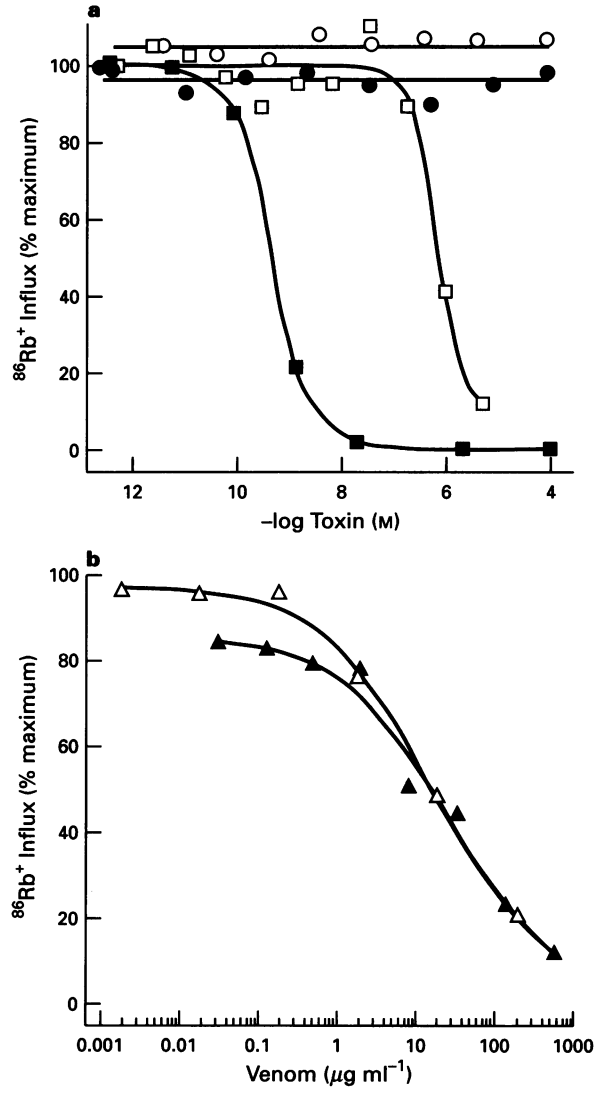

Figure 6 Effects of potassium channel toxins and crude venoms on ionomycin-stimulated $^{86}Rb^+$ influx into C6 glioma cells. C6 cells were grown as described in the Methods and the $^{86}Rb^+$ flux assay was carried out as previously described, using 2 μM ionomycin and a 30 s uptake period. Each point represents data of the means (n=4). (a) Inhibition of ionomycin-induced $^{86}Rb^+$ influx by increasing concentrations of charybdotoxin (■), iberiotoxin (□), toxin I (○) and apamin (●). (b) Inhibition of ionomycin-induced $^{86}Rb^+$ influx by increasing concentrations of the crude venoms from the scorpions *L. quinquestriatus* (△) and *Mesob. tamulus* (▲). For preparation of the crude venom samples, see Methods.

dendrotoxin and a blocker of A-type, voltage-activated K^+ channels) was also unable to block iomomycin-activated $^{86}Rb^+$ influx at concentrations up to 100 μM (Figure 6a).

Venoms from the Israeli scorpion *Leiurus quinquestriatus hebraeus* and the Indian red scorpion, *Mesobuthus tamulus* are known to contain toxins which block K_{Ca} channels (e.g. charybdotoxin, leiurotoxin, iberiotoxin). Both of these venoms inhibited ionomycin-stimulated $^{86}Rb^+$ influx into C6 cells (Figure 6b). Separation of the crude venoms by cation exchange h.p.l.c. revealed that both venoms contained several fractions that inhibited $^{86}Rb^+$ fluxes. For example, there appeared to be at least four distinct fractions in *Mesob. tamulus* venom that blocked $^{86}Rb^+$ fluxes in a charybdotoxin-like manner (Figure 7). The second of these four fractions (Fraction 37) represents iberiotoxin (Galvez *et al.*, 1990) and the remainder (fractions 11, 55 and 63) are novel toxins, yet to be fully characterized. A similar screening of *L. quinquestriatus hebraeus* venom revealed the presence of at least two different toxins, closely related to charybdotoxin (data not shown); it is most likely that the latter probably correspond to charybdotoxin-2 (Lucchesi *et al.*, 1989) and other similar toxins recently characterized in the same venom (Marshall *et al.*, 1994).

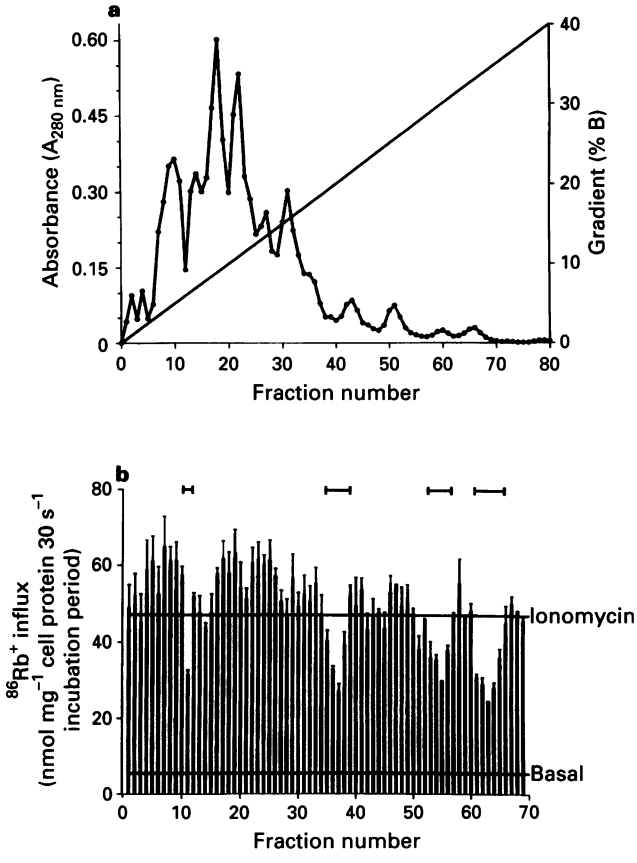

Figure 7 Cation exchange chromatography of *Mesobuthus tamulus* venom and $^{86}Rb^+$ influx assays on individual column fractions. (a) Elution profile of retained soluble components of the crude venom from *Mesob. tamulus*, separated on a Neobar CS h.p.l.c. cation exchange column (see Methods). Column fractions were monitored at 280 nm. (b) The effects of individual fractions obtained from (a) on ionomycin-induced $^{86}Rb^+$ influx into C6 glioma cells. Fractions were lyophilised and resuspended in 200 μl water: 10 μg protein (assuming 1 mg = 1 $AU_{280\,nm}$) from each fraction was tested in the influx assay (total volume 2 ml) described earlier, using 2 μM ionomycin and a 30 s incubation period. Horizontal lines represent control influx measurements (no venom fractions) in either the presence or absence of ionomycin (Ionomycin/Basal, respectively). All other procedures were as described in Methods. Data represent the mean ± s.d. ($n = 4$).

Venom from the Australian taipan snake *Oxyuranus scutellatus* has been shown to block Ca^{2+}-activated K^+ fluxes in human erythrocytes in a rather non-specific, optical dispersion assay (Alvarez & Sancho, 1989). However, blocking activity was atypical in that it was irreversible and with an extremely slow onset; additionally, the putative blocker was remarkably heat stable. These observations led us to question whether this snake venom would in fact possess the highly basic, rapidly acting polypeptides which are typical of IK_{Ca} and BK_{Ca} channel blockers; neither crude venom, nor venom fractions separated on a Superdex-75 gel exclusion column, were able to inhibit ionomycin-stimulated $^{86}Rb^+$ influx into C6 cells (data not shown).

Discussion

Application of ionomycin to C6 glioma cells caused a rise of cytosolic Ca^{2+} and a membrane hyperpolarization accompanied by an increase in the permeability of the membrane to $^{86}Rb^+$ ions, consistent with the opening of K_{Ca} channels. The determination of a single channel K_{Ca} conductance of 14 pS (26 pS in symmetrical 100 mM K^+ solutions), together with the pharmacological demonstration of inhibition of $^{86}Rb^+$ flux by both charybdotoxin and TEA but not by either apamin or toxin I (a close structural and functional homologue of dendrotoxin), suggests that this channel is most closely related to the IK_{Ca} subtype. The value of the single channel conductance in the rat C6 glioma cell line is very similar to the 25 pS K_{Ca} channel already identified in primary cultures of rat glial cells. Although K_{Ca} currents in C6 cells have not been identified previously, several groups have provided indirect evidence for their existence (Tas *et al.*, 1988; Reiser *et al.*, 1989).

The saturable nature of the flux process (with respect to ionomycin concentration), suggested the presence of a Ca^{2+} binding site associated with the intracellular face of the channel. The Ca^{2+} sensitivity of the K_{Ca} channel (our data indicate the channels spend a significant fraction of time open at a membrane voltage of -76 mV when cytosolic Ca^{2+} is about 250 nM) is typical of that seen with other IK_{Ca} channels, such as in erythrocytes, lymphocytes and granulocytes, which are all activated by sub-micromolar concentrations of Ca^{2+} (Wolff *et al.*, 1988; Partiseti *et al.*, 1992; Varnai *et al.*, 1993). The cytosolic free Ca^{2+} concentration inducing 50% of the maximal current response is typically 350 nM in granulocytes (Varnai *et al.*, 1993). This is in distinction to the Ca^{2+}-sensitivity of BK_{Ca} channels, which characteristically open either when the cytosolic Ca^{2+} concentration is several micromolar or at strongly depolarized voltages (Barrett *et al.*, 1982; Hille, 1992).

Our data indicate that after the addition of ionomycin, there was a 10 s latency period before the C6 cell hyperpolarized, presumably reflecting diffusion of the ionophore through the cell membrane. (Intracellular Ca^{2+} had attained a maximum value by the time the first set of measurements were made, 30 s after addition of the ionophore). This may represent a limiting factor for the activation of $^{86}Rb^+$ uptake and probably accounts for the low level of $^{86}Rb^+$ accumulated after 2 s, independent of ionophore concentration.

Although the single channel conductance K_{Ca} conductance that we observe in C6 cells is on the borderline between accepted values for IK_{Ca} and SK_{Ca} channels, the pharmacological profile of the C6 channel is clearly of the IK_{Ca} subtype (high affinity for charybdotoxin ($IC_{50} = 0.5$ nM), low affinity for TEA ($IC_{50} = 10$ mM) and complete insensitivity to 100 μM apamin). Although the C6 channel is blocked by charybdotoxin, it does not have a pharmacological profile typical of BK_{Ca} channels, which are normally much more sensitive to TEA (affinities typically less than 1 mM). On the other hand, SK_{Ca} channels are typically not blocked by TEA, even at high concentrations. Iberiotoxin (Galvez *et al.*, 1990) has been shown to be much more selective for BK_{Ca} channels than charybdotoxin (which also blocks several voltage-activated K channels). Our data suggest that iberiotoxin has a relatively low affinity for IK_{Ca}

channels (to the best of our knowledge, this is the first time the affinity of an IK_{Ca} channel for iberiotoxin has been determined); the low affinity of iberiotoxin for the C6 channel (IC_{50} = 800 nM), three orders of magnitude less potent than charybdotoxin, attests to the high selectivity of iberiotoxin for BK_{Ca} channels.

Charybdotoxin is an extremely basic polypeptide and studies by Miller, MacKinnon and colleagues (Anderson *et al.*, 1988; MacKinnon & Miller, 1989) have convincingly demonstrated that there is a strong electrostatic interaction between the cationic toxin and anionic amino acid residues at the mouth of the K_{Ca} channel. It is not surprising therefore that there is a large difference in the affinity of charybdotoxin for its binding site on isolated plasma membranes in low ionic strength medium (e.g. K_i = 0.02 nM for charybdotoxin displacement of labelled toxin from binding sites on aortic plasma membranes in 20 mM Tris/ 20 mM NaCl, Vazquez *et al.*, 1989), compared to its ability to block K_{Ca} currents in physiological saline (e.g. K_d = 50 nM for charybdotoxin inhibiting K_{Ca} currents in *Helix* neurones, Crest *et al.*, 1992). Our present studies (IC_{50} = 0.5 nM for charybdotoxin block), would imply that even under conditions of physiological ionic strength, charybdotoxin has a very high affinity for the IK_{Ca} channel in C6 cells and therefore the nature of the anionic toxin binding sites at the mouth of the pores of the IK_{Ca} and BK_{Ca} channels are most probably different. A similar conclusion has been reached by Wolff *et al.* (1988) in their study of IK_{Ca} channels in erythrocytes; these authors also demonstrated that charybdotoxin was unusually potent in blocking K_{Ca} efflux (IC_{50} = 0.8 nM) in physiological saline.

Dendrotoxin-sensitive, voltage-activated potassium currents have recently been identified in C6 cells (Wang *et al.*, 1992). However, it is extremely unlikely that this channel is activated under our particular experimental conditions because the $^{86}Rb^+$ fluxes measured here are specifically activated by Ca^{2+} ionophores and not inhibited by toxin I. Under our normal assay conditions, the basal flux in the absence of ionomycin is typically less than 10% of the flux in the presence of the ionophore. A K^+ channel assay procedure that can distinguish between charybdotoxin-like activity and dendrotoxin-like activity is very useful because charybdotoxin has been shown to block certain dendrotoxin-sensitive, voltage-dependent K^+ channels (e.g. dorsal root ganglion cells, Schweitz *et al.*, 1989b). In addition, charybdotoxin and dendrotoxin binding sites in certain tissues (e.g. brain) have been shown to be mutually competitive (Harvey *et al.*, 1989; Schweitz *et al.*, 1989a). The TEA-sensitivity of the dendrotoxin-sensitive currents in C6 cells is considerably greater (IC_{50} = 0.5 mM, Wang *et al.*, 1992) than of the ionomycin-sensitive $^{86}Rb^+$ fluxes that we observe (IC_{50} = 10 mM).

In a previous study, A_{23187}-induced $^{86}Rb^+$ uptake in C6 cells (over a 4 min incubation period) was activated by 200 nM intracellular Ca^{2+} with full activation at 400 nM Ca^{2+} (Tas *et al.*, 1988). Although these results are not directly comparable with those presented here (our techniques enable a much finer time resolution), intracellular Ca^{2+} levels required for channel activation are very similar. Although these workers also showed that $^{86}Rb^+$ fluxes were sensitive to charybdotoxin (albeit with a reduced affinity), their fluxes were not blocked by TEA. The reasons for these discrepancies are not clear, although it is possible that in the previous study, either initial rate conditions did not apply and/or active transport processes became significant and complicated quantitative measurements. It can be seen from our results that by 4 min (the standard incubation period used by Tas and colleagues), the flux rate had levelled off and a significant ouabain-sensitive component to the $^{86}Rb^+$ flux process had crept in.

Toxins play an important role in the molecular characterization of potassium channels (Rehm & Lazdunski, 1988; Scott *et al.*, 1990; Garcia-Calvo *et al.*, 1991), which is complementary to cDNA cloning approaches. The discovery and characterization of novel toxins which selectively modulate different channel subtypes will no doubt continue to contribute to our understanding of structural and functional relationships between different members of this large family of ion channel proteins. In our attempts to characterize novel potassium channel toxins which block Ca^{2+}-activated K^+ channels (and more recently, to identify monoclonal antibodies that selectively modulate K^+ channel function), we have sought to establish a sensitive, rapid and specific functional screening assay. Here we have shown that the ionomycin-stimulated $^{86}Rb^+$ influx measured in C6 cells provides an excellent model system for our purpose. The validity and use of this assay has been proven by identifying charybdotoxin and possible homologues in *L. quinquestriatus* venom. At least three new toxins have been targeted in fractions from *Mesob. tamulus* venom and purification is currently in progress. Equally, this assay has been shown to be capable of distinguishing between crude venoms which have been proven to have K_{Ca} channel blocking activity (e.g. *L. quinquestriatus* and *Mesob. tamulus*) and other venoms (e.g. *Oxyuranus scutellatus*), the ability of which to block K_{Ca} channels has proved more controversial.

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