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Nitrendipine is a potent inhibitor of the Ca^{2+}-activated K^+ channel of human erythrocytes

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Nitrendipine, a classical blocker of L-type Ca^{2+} channels, is shown to be a potent inhibitor of the Ca^{2+}-activated K^+ channel of human erythrocytes. In erythrocytes suspended in a solution with physiological Na^+ and K^+ concentrations and in which the channel was activated using the Ca^{2+} ionophore ionomycin, nitrendipene inhibited K^+($^{86}Rb^+$) influx with an I_{50} of around 130 nM. Similar results were obtained for K^+($^{86}Rb^+$) efflux, and for K^+($^{86}Rb^+$) influx into cells suspended in a high-K^+ medium.

Nitrendipine; Ca^{2+}-activated K^+ channel; Erythrocyte; Sickle cell

1. INTRODUCTION

Ca^{2+}-activated K^+ ($K^+(Ca^{2+})$) channels were first identified in human erythrocytes [1] and are now known to be present in most cell types [2–4]. They show a wide range of single-channel conductances and differ in their dependence on Ca^{2+} and voltage. The most potent and selective known inhibitors of these channels are invertebrate toxins. Apamin, from bee venom, blocks low conductance $K^+(Ca^{2+})$ channels at nanomolar concentrations; it has no effect on high or intermediate conductance $K^+(Ca^{2+})$ channels, nor on other types of K^+ channels [4]. Charybdotoxin, from the scorpion *Leiurus quinquestriatus* is also highly potent but has a wider spectrum of action; it blocks high and intermediate conductance $K^+(Ca^{2+})$ channels (such as the 'Gardos channel' of human erythrocytes) at nanomolar concentrations [5], but other types of K^+ channels are also affected. These toxins serve as useful experimental tools but their use is restricted by their expense and limited availability. Consequently, much of the work on $K^+(Ca^{2+})$ channels has involved the use of low-molecular-weight organic inhibitors, such as quinine, quinidine [6] and tetra-alkylammonium derivatives [7]. These are generally inexpensive and are widely available; however, when used at the relatively high concentrations that are necessary for full inhibition of $K^+(Ca^{2+})$ channels (usually > 0.1 mM), such compounds are notoriously non-specific and are known to influence a wide range of membrane processes.

Nitrendipine is well established as a blocker of L-type Ca channels [8]. We report here that it inhibits the $K^+(Ca^{2+})$ channel of human erythrocytes at sub-micromolar concentrations and is therefore the most potent low-molecular-weight inhibitor of this pathway reported to date.

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Abbreviations: BAPTA-AM, bis-(*o*-aminophenoxyl-ethane-*N,N,N',N'*-tetraacetic acid, tetra(acetoxymethyl)-ester; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TCA, trichloroacetic acid.

2. MATERIALS AND METHODS

2.1. *Chemicals*

$^{86}RbCl$ was from NEN (UK). Ouabain and nifedipine were from Sigma Chemical Co. and bumetanide was a gift from Leo Laboratories (Aylesbury, Bucks.). Nitrendipine was a gift from M.W. Wolowyk. BAPTA-AM and ionomycin were from Calbiochem Corporation (CA, USA). All buffer reagents were of analytical grade.

2.2. *Erythrocytes*

Freshly drawn heparinised blood from three healthy donors was washed three times (by centrifugation for 4 min at 800 × *g*) in MOPS-buffered saline (134.4 mM NaCl, 5 mM KCl, 0.24 mM $MgCl_2$, 15 mM MOPS, 10 mM glucose, pH 7.4) and the buffy coat was removed by aspiration.

2.3. *K^+($^{86}Rb^+$) flux measurements*

Aliquots of the washed erythrocyte suspension were dispensed into microcentrifuge tubes containing either the low-K^+ washing solution (as above) of a high-K^+ saline (140 mM KCl, 0.24 mM $MgCl_2$, 15 mM MOPS, 10 mM glucose, pH 7.4) so as to give final extracellular K^+ concentrations of 5 or 80 mM. The final samples had a volume of 1.0 ml and a haematocrit of around 3%. All samples contained ouabain (0.1 mM), bumetanide (0.1 mM) and $CaCl_2$ (0.4 mM). The $K^+(Ca^{2+})$ channel was activated by the addition of the Ca ionophore ionomycin (1 mM in DMSO) to a final concentration of 1 μM. For cells in a low-K medium, activation of the channel with a Ca^{2+} ionophore leads to an immediate membrane hyperpolarisation and a subsequent cell shrinkage and intracellular acidification [9]; in the experiments in which cells were suspended at an extracellular K^+ concentration of 5

mM, nitrendipine was added (at the appropriate concentration), the samples were transferred to a 37°C water bath for 10 min, then ionomycin was added immediately before commencing the flux. For cells suspended at an extracellular K^+ concentration of 80 mM the transmembrane K^+ distribution was close to electrochemical equilibrium and the cells therefore remained in an approximately steady state on activation of the channel [9]. In these experiments the ionophore was added, then the cells were incubated at 37°C for at least 10 min before adding nitrendipine, then commencing the flux by the addition of $^{86}Rb^+$.

In preliminary experiments it was found that the influx of $^{86}Rb^+$ into cells treated with ionomycin appeared approximately linear with time for up to ≤ 4 min (in both high-K and low-K media). Unidirectional K influx was therefore estimated from the $^{86}Rb^+$ accumulated within a fixed 1-min incubation period. $^{86}Rb^+$ was added at the final activity of approximately 5 $\mu Ci \cdot ml^{-1}$. The flux was terminated by the addition of quinine (0.87 mM in ice-cold saline, 300 μl) to the flux suspension, then four (300 μl) aliquots of the suspension were transferred immediately to microcentrifuge tubes containing 800 μl of ice-cold saline (with 0.1 mM quinine) layered over 250 μl of dibutylphthalate. The tubes were centrifuged (10 000 × g, 15 s) to sediment the cells below the oil, then the supernatant solution was aspirated. Each tube was rinsed three times with water, then the dibutylphthalate was aspirated. The cell pellet was lysed with 0.1% (v/v) Triton X-100 (0.5 ml), then deproteinised by the addition of 5% (w/v) TCA (0.5 ml) followed by centrifugation (10 000 × g, 10 min). The $^{86}Rb^+$ in the supernatant solutions was measured using Cerenkov counting. The extracellular radioactivity trapped in the cell pellets was estimated from aliquots taken from an ice-cold sample to which the quinine 'stopping solution' had been added prior to the $^{86}Rb^+$. $K(^{86}Rb^+)$ efflux measurements were carried out as has been described elsewhere [10].

2.4. *^{45}Ca influx timecourse measurements*

Washed erythrocytes were resuspended to a haematocrit of 10% in a solution containing 145 mM NaCl, 5 mM KCl, 10 mM MOPS, 5 mM glucose and 0.1 mM EGTA (pH 7.4). BAPTA-AM was added (final concentration 0.3 mM) and the cells incubated (37°C, 90 min) to allow for the uptake and hydrolysis intracellularly of the ester to the Ca^{2+}-chelating free acid (BAPTA; [11]). Following the incubation, the cells were washed twice in a solution containing 70 mM NaCl, 80 mM KCl, 0.15 mM $MgCl_2$ and 10 mM HEPES (pH 7.4), then dispensed into a microcentrifuge tube. Ionomycin was added to a final concentration of 1 μM. The final sample volume was 1.5 ml and the haematocrit was 5%. $CaCl_2$ (containing trace amounts of $^{45}Ca^{2+}$) was added to a concentration of 0.4 mM (and a final activity of 1 $\mu Ci \cdot ml^{-1}$), then the tube was placed at 37°C. Intracellular Ca^{2+} was chelated by BAPTA, preventing its extrusion by the Ca^{2+} pump [11]. At 20–30 s time intervals, aliquots (100 μl) of the suspension were taken from the flux tube and transferred to microcentrifuge tubes containing 800 μl of ice-cold saline layered over 250 μl of dibutylphthalate. The tubes were centrifuged immediately (10 000 × g, 15 s) then processed as described above for $^{86}Rb^+$ influx. The amount of $^{45}Ca^{2+}$ in the TCA supernatant was measured by β-scintillation spectroscopy.

3. RESULTS AND DISCUSSION

Fig. 1 presents inhibition data for nitrendipine at concentrations from 10^{-8} to 10^{-5} M on ^{86}Rb influx through the erythrocyte $K^+(Ca^{2+})$ channel. Results are presented in both low-K (5 mM) and high-K (80 mM) media, and give similar inhibition, with an I_{50} value of around 130 nM. Confirmatory experiments measuring the Ca^{2+}-induced ^{86}Rb efflux from pre-loaded cells gave similar I_{50} values of 100–130 nM for nitrendipine inhibition. In a necessary control experiment, ^{45}Ca influx was measured

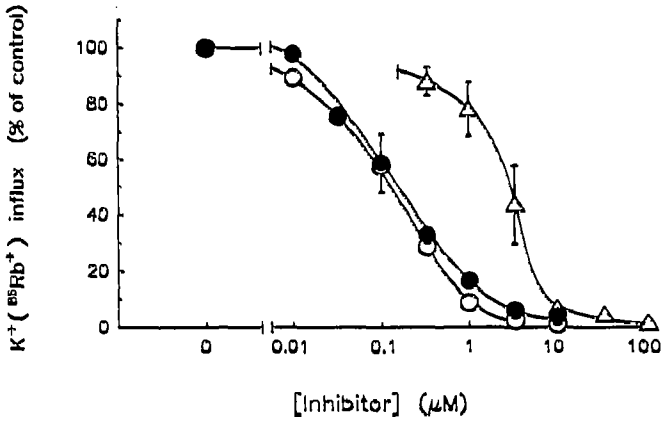

Fig. 1. The effect of nitrendipine (circles) and nifedipine (triangles) on $K^+(^{86}Rb^+)$ influx via the $K^+(Ca^{2+})$ channel of normal human erythrocytes suspended in solutions containing 5 mM (open symbols) or 80 mM (closed symbols) K^+. Influx rates are expressed as a percentage of those measured in the absence of inhibitor. The results are averaged from experiments on cells from at least three different donors. Error bars indicate ± SEM and, where not shown, fall within the symbols.

in ionomycin-treated erythrocytes, to establish that nitrendipine was not acting via an inhibition of ionomycin-mediated Ca entry. In experiments on cells from three different donors there was no inhibition of ^{45}Ca entry in cells treated with 1 μM nitrendipine.

Fig. 1 also shows data for the effect of nifedipine, another dihydropyridine, on $^{86}Rb^+$ influx through the $K^+(Ca^{2+})$ channel of erythrocytes suspended in a low-K^+ medium. The I_{50} was around 3 μM, somewhat less than the value of 18.7 μM reported by Kaji [12] for the inhibition by nifedipine of $K^+(^{86}Rb^+)$ efflux via the erythrocyte $K^+(Ca^{2+})$ channel, but still more than 20-fold higher than that observed for nitrendipine in the present study.

The present results identify nitrendipine as a convenient high-affinity blocker for studying Ca^{2+}-activated K^+ channels. Additionally, $K^+(Ca^{2+})$ channel blockade must be taken into account when considering the action of nitrendipine in other cell types. The I_{50} for the inhibition of the erythrocyte $K^+(Ca^{2+})$ channel is similar to the value of 154 nM determined for inhibiting L-type calcium channels in the heart [8], suggesting that nitrendipine may be as potent (or even more potent) an inhibitor of the K^+ channel as it is of Ca^{2+} channels.

Finally, nitrendipine may offer an effective therapy for maintaining sickle cells in their hydrated state. K^+ loss via the erythrocyte $K^+(Ca^{2+})$ channel is a potentially important cause of red cell dehydration in sickle-cell disease [13], the consequential increase in concentration of sickle heamoglobin causing an expotential increase in its rate of polymerisation and ioss of cell deformability [14]. Charybdotoxin, a highly potent inhibitor of the erythrocyte $K^+(Ca^{2+})$ channel [5], is known to protect against dehydration of sickle cells [15], as does

nitrendipine [16]. No trials of clinical efficacy of nitrendipine in sickle-cell disease have been reported, although a pilot study of 7 patients showed no improvement in blood parameters [17]. Nifedipine, in a study of 11 patients [18], improved retinal and conjunctival perfusion. The present results indicate the need for more substantive clinical evaluation of nitrendipine in sickle-cell disease.

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