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Discrimination between subtypes of apamin-sensitive Ca^{2+}-activated K^+ channels by gallamine and a novel *bis*-quaternary quinolinium cyclophane, UCL 1530

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1 Gallamine, dequalinium and a novel *bis*-quaternary cyclophane, UCL 1530 (8,19-diaza-3(1,4),5(1,4)-dibenzena-1(1,4),7(1,4)-diquinolina-cyclononadecanephanedium) were tested for their ability to block actions mediated by the small conductance, apamin-sensitive Ca^{2+}-activated K^+ (SK_{Ca}) channels in rat cultured sympathetic neurones and guinea-pig isolated hepatocytes.

2 SK_{Ca} channel block was assessed in sympathetic neurones by the reduction in the slow afterhyperpolarization (AHP) that follows an action potential, and in hepatocytes by the inhibition of the SK_{Ca} mediated net loss of K^+ that results from the application of angiotensin II.

3 The order of potency for inhibition of the AHP in sympathetic neurones was UCL 1530 > dequalinium > gallamine, with IC_{50} values of 0.08 ± 0.02, 0.60 ± 0.05 and 68.0 ± 8.4 μM respectively, giving an equi-effective molar ratio between gallamine and UCL 1530 of 850.

4 The same three compounds inhibited angiotensin II-evoked K^+ loss from guinea-pig hepatocytes in the order dequalinium > UCL 1530 > gallamine, with an equi-effective molar ratio for gallamine to UCL 1530 of 5.8, 150 fold less than in sympathetic neurones.

5 Dequalinium and UCL 1530 were as effective on guinea-pig as on rat sympathetic neurones.

6 UCL 1530 at 1 μM had no effect on the voltage-activated Ca^{2+} current in rat sympathetic neurones, but inhibited the hyperpolarization produced by direct elevation of cytosolic Ca^{2+}.

7 Direct activation of SK_{Ca} channels by raising cytosolic Ca^{2+} in hepatocytes evoked an outward current which was reduced by the three blockers, with dequalinium being the most potent.

8 These results provide evidence that the SK_{Ca} channels present in guinea-pig hepatocytes and rat cultured sympathetic neurones are different, and that this is not attributable to species variation. UCL 1530 and gallamine should be useful tools for the investigation of subtypes of apamin-sensitive K^+ channels.

Keywords: Calcium -activated potassium channels; hepatocytes; sympathetic neurones; gallamine; dequalinium

Introduction

Ca^{2+} -activated K^+ channels are found in a wide variety of cell types and can be divided into three main classes on the basis of single channel conductance and sensitivity to blocking agents (for review see Haylett & Jenkinson, 1990). Of these, the SK_{Ca} channel is characterized by its small single channel conductance (6-20 pS) and high sensitivity to the bee venom toxin, apamin. Though this channel has yet to be cloned and expresssed, work with apamin has already provided some indirect evidence to suggest the existence of subtypes. Thus, estimates of the apamin concentration required to cause 50% block of SK_{Ca} channels vary considerably between cells, ranging from 0.2 to 20 mM (Maas *et al.*, 1980; Grissmer *et al.*, 1992). Also, the action of apamin may reverse rapidly (Hall & Morton, 1991) or very slowly (Bourque & Brown, 1987; Grissmer *et al.*, 1992; Park, 1994), depending on the cell type. Ligand binding studies with labelled apamin also suggest differences in both affinity (see Schmid-Antomarchi *et al.*, 1986) and kinetics (Hughes *et al.*, 1982; Cook & Haylett, 1985), though these findings are difficult to interpret because the conditions used in the experiments varied greatly.

Though single channel recordings of SK_{Ca} activity have now been obtained from a variety of cells (Blatz & Magleby, 1986; Capiod & Ogden, 1989; Lang & Ritchie, 1990; Leinders & Vijverberg, 1992; Park, 1994), these studies have not been detailed enough to reveal differences. In another approach, some progress has been made towards the biochemical characterization of apamin binding proteins-presumed to form part of the SK_{Ca} channel (Schmid-Antomarchi *et al.*, 1984; Marqueze *et al.*, 1987; Auguste *et al.*, 1989). An important recent study by Wadsworth *et al.* (1994) has provided evidence that these proteins vary between species and in the same work it was shown that the neuromuscular blocking agent, gallamine, is less effective in displacing labelled apamin from its binding site in rat brain than in rabbit and guinea-pig liver. However, it is as yet unclear how these differences will be reflected in the functional properties of the channel.

In the present study, we have extended this approach by examining the ability of a novel compound, 8,19-diaza-3(1,4),5(1,4)-dibenzena-1(1,4),7(1,4)-diquinolina-cyclononadecanephanedium (UCL 1530), to block the SK_{Ca} channel in rat sympathetic neurones and guinea-pig hepatocytes and we have also compared it with gallamine and another well characterized blocker of this channel, dequalinium (Castle *et al.*, 1993; Dunn, 1994). We have found a large difference in the relative potency of these compounds between the two tissues, as well as a change in their rank order. This provides strong functional evidence for SK_{Ca} channel heterogeneity.

A preliminary account of some of this work has been published (Dunn *et al.*, 1994).

Methods

Preparation of guinea-pig hepatocytes

Hepatocytes were prepared by a modification of the collagenase disaggregation procedure described by Seglen (Seglen, 1973; Burgess *et al.*, 1981). Briefly, Hartley guinea-pigs (either sex) were anaesthetized with i.p fentanyl (0.1 mg kg^{-1}), droperidol (10 mg kg^{-1}) and pentobarbitone (30 mg kg^{-1}) and the

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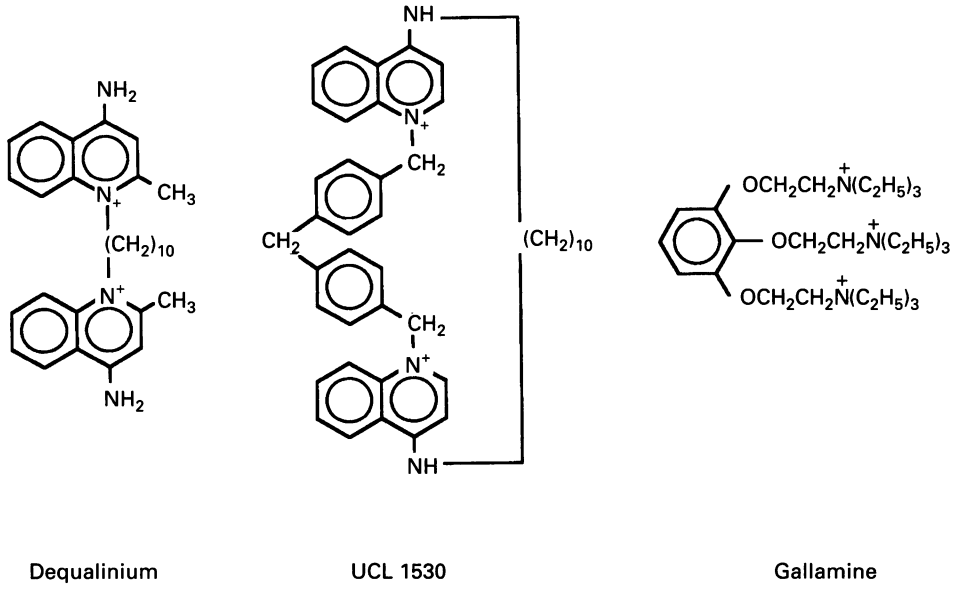

Dequalinium UCL 1530 Gallamine

Figure 1 Structures of the three blockers used in this study.

hepatic vein was cannulated. The liver was then excised and placed in a warmed chamber in which it was perfused at a rate of 20 ml min^{-1} for 10 min with Ca^{2+} free 'minimum essential medium' (MEM) containing (mM): NaCl 116, KCl 5.4, $MgSO_4$ 0.81, NaH_2PO_4 0.96, $NaHCO_3$ 25, EGTA 0.52 and glucose 5, equilibrated with 95% O_2 and 5% CO_2 at a temperature of 37°C. Perfusion was then continued for a further 6–10 min with MEM containing 5 mM Ca^{2+} and 50 mg collagenase in 120 ml. Next the liver was minced with a razor blade, and shaken in a conical flask with the collagenase solution for a further 4–7 min to improve the disaggregation. Enzyme activity was stopped by the addition of new-born calf serum (15% by volume), and single cells were separated from debris and undissociated tissue by filtration and centrifugation. The cells were finally suspended at a density of approximately 10^7 ml^{-1} in Eagle's medium supplemented with 2% bovine serum albumin, 10% new-born calf serum, penicillin (200 units ml^{-1}) and streptomycin (100 μg ml^{-1}). The cells were shaken gently in a conical flask at 37°C and were used within 2 h for measurement of K^+ loss via SK_{Ca} channels (see below); 80–90% of cells excluded Trypan blue. For electrophysiological experiments 20 μl aliquots of the cell suspension were added to 2 ml of supplemented Eagle's medium in 35 mm diameter plastic Petri dishes. The dishes were incubated at 37°C in an atmosphere of 95% O_2: 5% CO_2 for at least 90 min before the experiments were commenced and were then used in the following 6 h.

Cultured sympathetic neurones

Cultures of sympathetic neurones were prepared essentially as described by Smart (1987). Seventeen-day old Sprague Dawley rats were killed by inhalation of a rising concentration of CO_2, and the superior cervical ganglia were removed. Guinea-pig ganglia were obtained from adult animals killed for the preparation of hepatocytes (see above). The ganglia were desheathed, cut into small pieces and incubated in Ca^{2+} and Mg^{2+} -free Hanks's balanced salt solution (Gibco) containing 370 u ml^{-1} collagenase and 6 mg ml^{-1} bovine serum albumin at 37°C for 15 min. This was followed by incubation in Hanks's balanced salt solution containing 1 mg ml^{-1} trypsin and 6 mg ml^{-1} bovine serum albumin for 30 min. Ganglia were then dissociated with a fire-polished Pasteur pipette, and the resultant cell suspension plated onto laminin coated plastic culture dishes. Cells were grown in L-15 medium supplemented with 10% foetal calf serum, 0.2 mM glutamine, 0.6%(w/v) D-glucose, 0.19%(w/v) $NaHCO_3$, penicillin (100 u ml^{-1}), streptomycin (100 μg ml^{-1}) and nerve growth factor (mouse submaxillary gland 50 ng ml^{-1}). Cells were maintained at 37°C in an atmosphere of 95% O_2: 5% CO_2 and used between 6 h and 10 days in culture.

Measurement of K^+ loss from hepatocytes

K^+ loss from hepatocytes treated with angiotensin II (100 nM) was measured with an extracellular K^+ sensitive electrode made and used as described by Cook and Haylett (1985). The application of angiotensin to 2 ml aliquots of the cell suspension (containing $\sim 10^7$ cells ml^{-1}) caused an increase in cytosolic Ca^{2+} and thus activated SK_{Ca} channels, so that the cells lost K^+. The amount lost after 30 s was expressed as a percentage of the total K^+ of the cells, as estimated by the subsequent release in response to 200 μM digitonin. Blockers were added 2 min before the application of angiotensin II.

Electrophysiology

Culture dishes were placed on the stage of an inverted microscope (Diaphot, Nikon), and perfused with normal physiological salt solution (see below). When drugs were applied by bath perfusion, the flow rate was 5–7 ml min^{-1}, but when micro-perfusion was used, the rate was reduced to 0.5–1 ml min^{-1}. Experiments on the afterhyperpolarization (AHP) were carried out at 30°C while other electrophysiological experiments were conducted at room temperature.

The micro-perfusion system was based on that described by Konnerth *et al.* (1987). It consisted of seven reservoirs feeding under gravity to a glass manifold formed from seven pieces of 1.5 mm thin wall electrode glass. This assembly had been 'pulled' under heat and then broken back to give a tip of 400 μm diameter which was in turn cemented into another glass or plastic tube drawn out to give a tip 200 μm diameter, with a dead space of less then 5μl. Flow through each tube was controlled manually with 3-way Luer fitting taps. Control solution was allowed to flow through one channel and drug applications were effected by simultaneously turning the control off, and the drug solution on.

Inhibition of the AHP of cultured sympathetic neurones The culture dish was perfused with a solution (hereafter refered to as normal physiological solution) of the following composition (mM): NaCl 154, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 5.6, HEPES 10, adjusted to pH 7.4 with NaOH. Intracellular recordings were made with conventional 'sharp' microelectrodes filled with 1 M KCl (resistance 80–120 MΩ) connected to the

headstage of a Neurolog NL102 amplifier. Action potentials were evoked by injection of a 30 ms depolarizing current pulse every 5 s. The test compounds were applied by bath perfusion for long enough (1–3 min) to cause a steady reduction in the AHP. The extent of the inhibition was expressed as a percentage (for further details see Dunn, 1994).

Ca^{2+} currents: The extracellular solution contained (mM): tetraethylammonium chloride (TEA) 141, CsCl 3, $MgCl_2$ 1.2, glucose 5.6, HEPES 10, adjusted to pH 7.4 with CsOH. Whole cell voltage clamp recording was carried out with a List EPC 7 amplifier. Electrodes were fabricated from glass capillaries (Clark Electromedical GC15OTF) and after fire polishing had a resistance of 2–5 MΩ when filled with the intracellular solution containing (mM): CsCl 110, $MgCl_2$ 2, HEPES 40, EGTA 3, ATP 2, adjusted to pH 7.4 with CsOH. Voltage clamp commands were generated, and data capture and analysis carried out using the pClamp suite of programmes (Axon Instruments). On-line leak subtraction was carried out using a P/8 protocol, in which the currents evoked by eight small hyperpolarizing voltage commands (an eighth the size of the depolarizing jump) were digitally subtracted from the current produced by the depolarizing jump.

Direct activation of SK_{Ca} channels by raising cytosolic Ca^{2+}: Experiments on direct activation of SK_{Ca} channels in sympathetic neurones were carried out using a two electrode protocol as follows. Cells were bathed in normal physiological solution (see above), impaled with a 'sharp' microelectrode, and a few action potentials recorded to confirm the presence of a stable AHP. Next a patch electrode was applied to the cell, and a gigaseal formed. This electrode was filled with solution containing: (mM) K_3 citrate 76, HEPES 30, EGTA 1, $CaCl_2$ 5, adjusted pH 7.4 with KOH. The free Ca^{2+} concentration of this solution (20 μM) was calculated by use of an iterative computer programme ('REACT', G.L. Smith, Dept of Chemistry, University College London), with binding constants given in Sillen & Martell (1964) . The bath solution was then changed to Ca^{2+} -free solution, with or without blocking drug, so that the only available source of Ca^{2+} was the patch electrode. The membrane under the patch electrode was then ruptured, allowing Ca^{2+} to diffuse into the cell. The resulting changes in the intracellularly recorded membrane potential and conductance could then be observed.

Electrophysiological experiments on hepatocytes were carried out by the whole cell patch clamp technique, with electrodes (resistance 1–3 MΩ) filled with the same high Ca^{2+} solution used for the experiments on sympathetic neurones (see above). Cells were bathed in a chloride-free extracellular solution of the following composition (mM) Na isethionate 137, K gluconate 4.7, Ca $(gluconate)_2$ 2.5, $MgSO_4$ 0.8, HEPES 10, glucose 11 adjusted to pH 7.4 with NaOH. The use of chloride-free solutions prevented complications due to the activation of Ca^{2+}-dependent chloride channels (see Field & Jenkinson, 1987; Capiod *et al.*, 1987; Koumi *et al.*, 1994). After formation of a gigaseal, the pipette potential was set to −20 mV. On attaining the whole cell configuration, there was intially little holding current, but over the next 20–180 s, an outward current developed as Ca^{2+} diffused into the cell and activated the Ca^{2+} sensitive K^+ channels (see Figure 6a). Drugs were then applied by micro-perfusion to determine their ability to block this outward current. Repeated attempts to conduct identical experiments on sympathetic neurones were unsuccessful as the outward currents were very transient, declining to zero in less then 30 s.

Data analysis

Values are given as the mean ±s.e. mean except where otherwise stated. It was generally not possible to obtain a full dose-response curve and determine an IC_{50} for each cell. Instead, the procedure we adopted was to pool the data from a number of cells, and then fit the Hill equation to the combined data using an iterative least squares fitting routine (CVFIT, written by Professor D. Colquhoun, Department of Pharamcology, University College London) which gives a fitted parameter ± an approximate standard deviation (see Colquhoun *et al.*, 1974 for details).

Drugs and reagents

Standard salts were of 'AnalaR' grade and obtained from BDH, except Na isethionate (Fluka Chemie AG); HEPES, EGTA, K gluconate and Ca $(gluconate)_2$ (Sigma). UCL 1530 was synthesized in our laboratories by reacting 4-chloroquinoline with 1,10-diaminodecane, followed by cyclization of the product with di(p-bromomethylphenyl) methane. It was purified by preparative high performance liquid chromatography and obtained as a hydrated trifluoroacetate salt. Full details of the synthesis will be published subsequently. Dequalinium was a kind gift from the Laboratories for Applied Biology Ltd (London). Tissue culture media were obtained from Gibco. With the exception of gallamine, pentobarbitone (May & Baker), fentanyl, droperidol (Janssen Pharamceuticals) and collegenase (Worthington, CLS 2) all other drugs were purchased from Sigma.

Results

Inhibition of the AHP in rat sympathetic neurones

Recording the AHP which follows the action potential in sympathetic neurones provides a relatively simple and convenient, albeit somewhat indirect, means of testing compounds

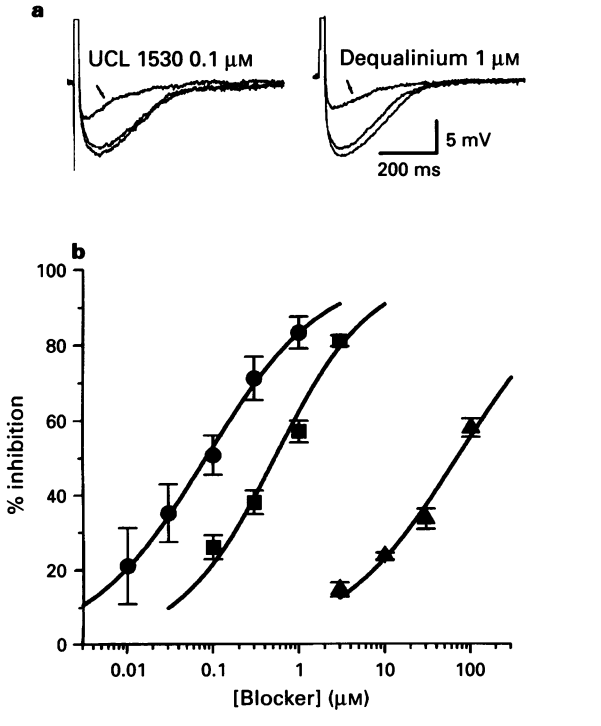

Figure 2 Inhibition of the slow afterhyperpolarization (AHP) in rat sympathetic ganglion neurones. (a) Recordings from the same neurone showing inhibition of the AHP by 0.1 μM UCL 1530 (left) and 1μM dequalinium (right). Each panel shows three superimposed traces recorded before, after 1.5 min in the presence of the drug (arrow), and after 5 min wash out. At the gain used, the peaks of the action potentials are off scale. (b) Concentration-response curves for inhibition of the AHP by UCL 1530 (●), dequalinium (■) and gallamine (▲). Points represent the mean ± s.e. mean from 4 to 20 cells. The curves are least squares fits of the data to the Hill equation with unconstrained slope.

for their ability to block neuronal SK_{Ca} channels. Dequalinium has previously been shown to be a potent and selective inhibitor of the AHP in sympathetic neurones, with an IC_{50} of 1 μM (Dunn, 1994). In the present study it was slightly more effective with an IC_{50} of 0.60 ± 0.05 μM (fitted value ± s.d. data from 17 cells). As Figure 2 illustrates, the novel compound UCL 1530 was found to be considerably more active than dequalinium with an IC_{50} of 0.08 ± 0.02 μM (fitted value ± s.d. data from 12 cells). The tris-quaternary neuromuscular blocker gallamine has previously been shown to inhibit the SK_{Ca} channel in guinea-pig hepatocytes with an IC_{50} of 12 μM (Cook & Haylett, 1985). However, when this compound was tested for its ability to inhibit the AHP, it was over 100 times less potent than dequalinium, with an IC_{50} of 68.0 ± 8.4 μM (fitted value ± s.d. data from 5 cells) (Figure 2).

Inhibition of K^+ efflux from guinea-pig hepatocytes

Inhibition of K^+ efflux from guinea-pig hepatocytes has often been used for the pharmacological characterization of the SK_{Ca} channel (Burgess *et al.*, 1981; Cook & Haylett, 1985; Dunn *et al.*, 1991; Castle *et al.*, 1993), and for several compounds, there is good agreement in their ability to block the channel in hepatocytes and sympathetic neurones (Dunn *et al.*, 1991). However, when UCL 1530 was tested as a blocker of the SK_{Ca} channel in hepatocytes it was found to be less potent than dequalinium, with IC_{50} of 2.6 ± 1.0 μM (Figure 3a), compared with 1.7 ± 0.7 μM (fitted values ± s.d., $n = 3$) for dequalinium. As gallamine had been found to be less potent than expected when tested as a blocker of the AHP in rat sympathetic neurones, it was again tested for its ability to inhibit K^+ loss from guinea-pig hepatocytes. In this series of experiments, dequalinium was slightly less potent (IC_{50} 3.7 ± 0.1 μM) while the IC_{50} for gallamine was 15.1 ± 0.6 μM (fitted values ± s.d., $n = 4$) (Figure 3b), which is close to the value reported by Cook & Haylett (1985).

While these differences in the relative potencies of dequalinium, UCL 1530 and gallamine in the two cell types could indicate SK_{Ca} channel heterogeneity, the experiments were carried out under different conditions and the SK_{Ca} channels were activated indirectly. We therefore thought it necessary to eliminate certain other possible explanations.

UCL 1530 has no effect on Ca^{2+} currents in sympathetic neurones

The AHP in rat sympathetic neurones results from the elevation of cytosolic Ca^{2+} levels by the influx of Ca^{2+} through voltage-gated Ca^{2+} channels which open during the action potential (McAfee & Yarowsky, 1979; Freschi, 1983). If UCL 1530 interfered with this process then its potency as a blocker of the neuronal SK_{Ca} channel would have been overestimated. This compound was therefore tested for its ability to inhibit the voltage-activated Ca^{2+} current which was recorded in Na^+-free, TEA-containing bathing solution, using patch pipettes filled with CsCl (see Methods). As Figure 4 shows, 1 μM UCL 1530 (a concentration producing near maximal inhibition of the AHP) produced no detectable inhibition of the Ca^{2+} current in sympathetic neurones, while the subsequent application of 5 μM Cd^{2+} to the same cells reduced the current by over 50%.

Direct activation of neuronal SK_{Ca} channels.

To confirm that inhibition of the AHP in sympathetic neurones by UCL 1530 was due to an interaction with the SK_{Ca} channel, a series of experiments was carried out in which the cytosolic Ca^{2+} concentration was elevated directly. In these experiments, the membrane potential was monitored with a microelectrode, and Ca^{2+} was allowed to diffuse into the cell from a patch electrode (see Methods). There was usually a small almost instantaneous change in the membrane potential when the membrane under the patch pipette was finally ruptured to establish the 'whole cell' recording configuration. This was presumably due to slight mechanical disturbance of the microelectrode impalement, and it was followed by a rapid hyperpolarization of about 10 mV associated with an increase in membrane conductance.

This response reached a peak in approximately 2 s and then declined (Figure 5a). When the same protocol was carried out with a patch electrode containing Ca^{2+}-free solution, no hy-

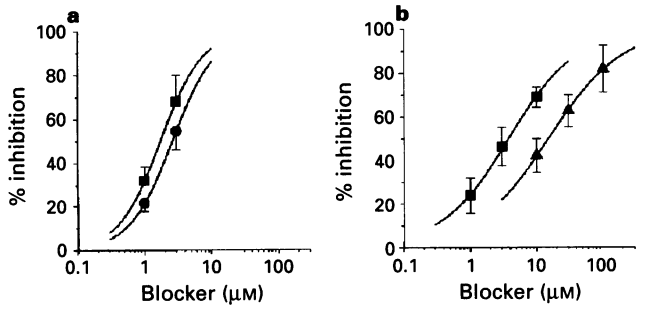

Figure 3 Inhibition of SK_{Ca} channels in hepatocytes. The graphs show concentration-response curves for inhibition of angiotensin II-evoked K^+ release from a suspension of guinea-pig hepatocytes in two series of experiments. The curves are least squares fits of the data to the Hill equation. Because of the difference in experimental design and in the potency of dequalinium, the two sets of data have not been combined. (a) Comparison of the effects of dequalinium (■) and UCL 1530 (●). The points represent the mean ± s.e. mean of observations from 3 experiments. (b) Comparison of the effects of dequalinium (■) and gallamine (▲). Data from 4 experiments.

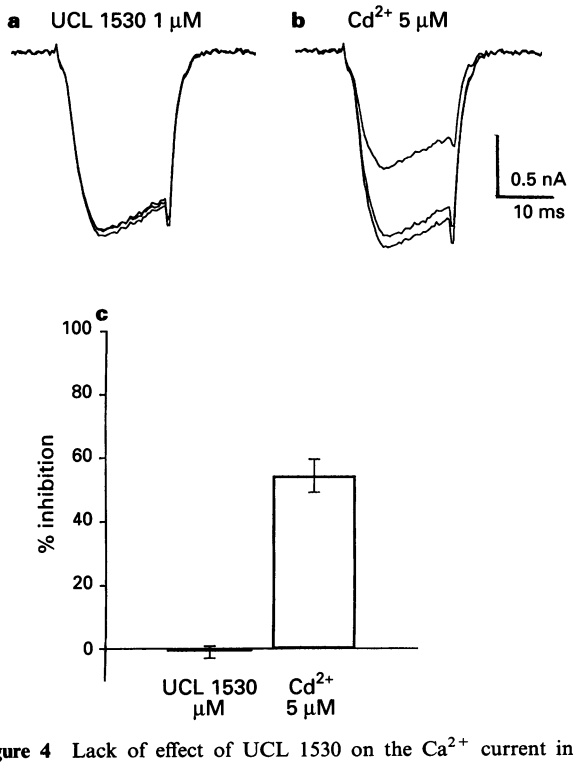

Figure 4 Lack of effect of UCL 1530 on the Ca^{2+} current in rat sympathetic neurones. (a) Three superimposed calcium currents evoked by depolarization from −60 to +10 mV first in control solution, then after 1.5 min in the presence of 1 μM UCL 1530, and finally after washing out UCL 1530. (b) Three superimposed currents recorded from the same cell as in (a), again, first in control solution, then after 0.5 min in the presence of 5 μM Cd^{2+} (arrow), and lastly after washing out the Cd^{2+}. (c) Histogram showing the effect of UCL 1530 and Cd^{2+} on the Ca^{2+} current recorded from the same cells. Columns represent the mean ± s.e. mean inhibition of the current in 4 cells.

perpolarization was observed (Figure 5b), confirming the role of Ca^{2+} in the response. When however the bathing solution contained 1 μM dequalinium or 0.2 μM UCL 1530 the hyperpolarization and conductance increase were greatly attenuated (Figure 5c). After correcting for the small depolarization observed in the 0 Ca^{2+} controls, the inhibition of the AHP amounted to 80% and 78% respectively.

Direct activation of hepatocyte SK_{Ca} channels

The determination of the potency of compounds as SK_{Ca} channel blockers in hepatocytes had been carried out with a suspension of cells at a relatively high density ($\sim 10^7$ ml^{-1}), and at a temperature of 37°C, while experiments on sympathetic neurones were done at 30°C with isolated cells adhering to a culture dish. To rule out the possibility that the different results observed were a consequence of the dissimilar experimental conditions, electrophysiological experiments were carried out with guinea-pig hepatocytes. For these experiments, whole cell patch clamp recordings were made, using electrodes filled with a solution containing 20 μM free Ca^{2+}. A Cl^--free bathing fluid was employed, to avoid the complication of Ca^{2+}-activated Cl^- currents. On achieving the whole cell configuration, an outward current associated with an increase in membrane conductance developed, due to the opening of SK_{Ca} channels (Figure 6a). This reached a peak of 0.47 ±0.03 nA ($n=8$) in 2 to 5 s and was maintained for between 2 and 10 min. Application of SK_{Ca} channel blockers produced a rapid and reversible reduction in this current (Figure 6a). While the action of the non-peptide blockers reversed rapidly, the action of apamin was considerably slower in both onset and recovery.

Dequalinium (0.1 to 1 μM) produced a concentration-dependent reduction in the outward current in Ca^{2+} loaded hepatocytes. Fitting the Hill equation to the data, and assuming a maximal response equal to that produced by 25 nM apamin, a concentration which should have blocked almost all of the SK_{Ca} channels (Burgess *et al.*, 1981), yielded an IC_{50} of 0.28 ± 0.05 μM (fitted value ± s.d. data from 8 cells) (Figure 6b), which is lower than that observed both in our K^+ loss experiments, and in previous work with intact hepatocytes (Castle *et al.*, 1993). UCL 1530 at a concentration of 0.3 μM produced only about 20% reduction in the outward current, while 3 μM gallamine caused near maximal inhibition (Figure 6b). From these results, both UCL 1530 and gallamine were clearly less potent that dequalinium. Although a range of concentrations of gallamine and UCL 1530 would have to be tested to allow a precise comparison, these results are sufficient to show that, regardless of the increased absolute potency of dequalinium in the single-hepatocyte experiments, the potency order of dequalinium > UCL 1530 ⩾ gallamine is similar to that found in the K^+ loss experiments and very different from that observed with the sympathetic neurones.

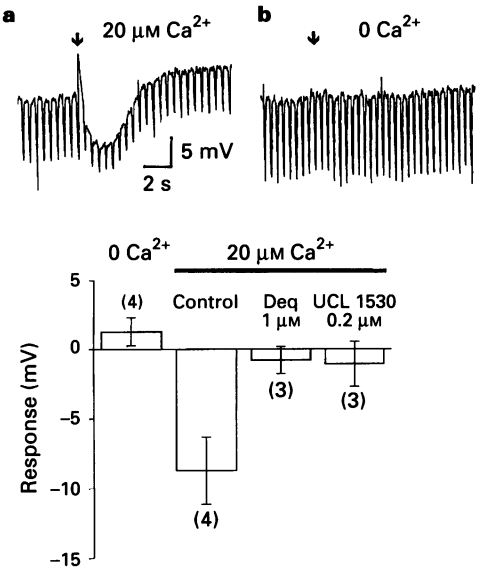

Figure 5 Direct activation of SK_{Ca} channels in rat sympathetic neurones. (a) Intracellular recording of membrane potential from a rat sympathetic neurone, in Ca^{2+}-free bathing solution, and with a patch electrode containing 20 μM free Ca^{2+} sealed onto the cell throughout. The initial resting potential was −52 mV. Downward deflections are electronic potentials produced by injection of 0.2 nA current pulses through the recording electrode. On establishing the whole cell configuration with the patch electrode (at the arrow), there was a rapid hyperpolarization associated with a decrease in resistance. (b) When the same procedure was carried out with other cells, but using a patch electrode containing Ca^{2+}-free solution no hyperpolarization was observed. (c) Histogram showing the combined results from 14 neurones which were patched either with Ca^{2+}-free or Ca^{2+}-containing electrodes in the absence of blockers, or while bathed in a Ca^{2+}-free solution containing either 1 μM dequalinium or 0.2 μM UCL 1530. These concentrations of the blockers produced approximately equal reductions in the response to Ca^{2+}. The values plotted are the means ± s.e.mean of the observed changes in membrane potential and the numbers of cells tested are indicated in parentheses.

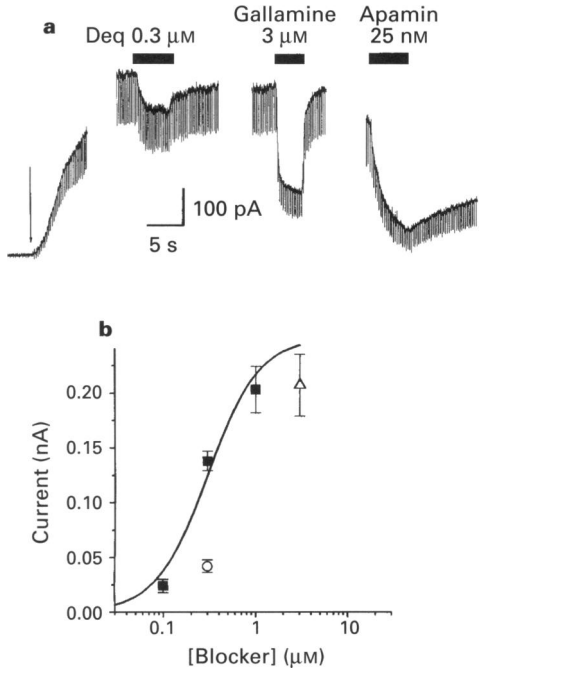

Figure 6 Direct activation of SK_{Ca} channels in hepatocytes. (a) Sections of continuous whole cell voltage clamp recording from a single guinea-pig hepatocyte, made with a recording pipette containing 20 μM free Ca^{2+}. The holding potential was −20 mV. On attaining the whole cell configuration (arrow), there was no instantaneous current, but an outward current developed with time, associated with an increase in membrane conductance. Downward deflections are responses to 10 mV hyperpolarizing voltage commands 200 ms in duration. Application of dequalinium (Deq, 0.3 μM), gallamine (3 μM) and apamin (25 nM), for the times indicated by the horizontal bars, reversibly reduced the outward current and membrane conductance. (b) Concentration-response curve for the effect of dequalinium (■) on the Ca^{2+}-activated current. The ordinate scale plots the reduction in current caused by increasing concentrations of dequalinium (abscissa scale). For comparison, single concentrations of gallamine (△) and UCL 1530 (○) were also tested on the same cells. Points represent the mean ± s.e. mean from 3 cells. The curve is a least squares of the dequalinium data to the Hill equation, assuming a maximum effect equal to that produced by a near maximal (25 nM) concentration of apamin.

One of the major differences between these experiments and those on K^+ loss was the use of Cl^--free solutions. To see whether this might account for the increased potency of dequalinium, its action was compared in normal and Cl^--free solutions. Because of the variability of the Ca^{2+} -activated K^+ current, and the presence of a large Ca^{2+}-activated Cl^- current, it was necessary to normalize the response with respect to that produced by 25 nM apamin. Using this approach, dequalinium was found to be approximately twice as potent in reducing the outward current when applied in the absence of external Cl^- (Figure 7).

Effect of UCL 1530 on guinea-pig sympathetic neurones

The difference in the pharmacology of the SK_{Ca} channels in rat sympathetic neurones and guinea-pig hepatocytes described above could conceivably have been due to inter-tissue or inter-species variability. Rat hepatocytes do not possess Ca^{2+} -activated K^+ channels (Burgess *et al.*, 1981; Takanashi *et al.*, 1992), so it was not possible to look for inter-species differences with this tissue. Dequalinium and UCL 1530 were however compared for their ability to inhibit the AHP in guinea-pig sympathetic neurones. When tested on three neurones at a single concentration of 0.3 μM, dequalinium produced 41 $\pm$ 2% inhibition, close to that observed in rat cells (38 $\pm$ 3%). The same concentration of UCL 1530 produced 77 $\pm$ 13% inhibition in the guinea-pig neurones as compared with 71 $\pm$ 6% in the rat. It is clear that the SK_{Ca} channel in guinea-pig sympathetic neurones resembles that in the corresponding rat cells more closely than the type present in guinea-pig hepatocytes.

Discussion

Our initial observation that the relative potencies of UCL 1530 and dequalinium as blockers of SK_{Ca} channel-mediated responses differed between hepatocytes and sympathetic neurones came as a surprise, since previous work with simpler analogues of dequalinium had shown them to be equally effective in the two tissues (Dunn *et al.*, 1991). A possible explanation, and one that we favour, is that the SK_{Ca} channels in the two tissues are distinct, and that the difference is such that some feature of the structure of UCL 1530 confers the ability to discriminate between them. Before this conclusion can be accepted, it is necessary to consider other ways of accounting for the findings. This is particularly important because of the very different experimental conditions used in the measurements with hepatocytes and sympathetic neurones. One possibility relates to the indirectness of the tests with neurones in which the SK_{Ca} channels were activated by evoking an action potential that allowed Ca^{2+} to enter the cells. Our observations could be explained if UCL 1530 were to have interfered with the influx of Ca^{2+} during the action potential. We think this unlikely for several reasons. In the first place, the structurally related compound, dequalinium, has previously been shown not to block voltage-gated Ca^{2+} channels in sympathetic neurones at concentrations up to 10 μM (Dunn, 1994). In addition, we have shown in the present study that UCL 1530 at concentrations up to 1 μM (sufficient to greatly reduce the AHP) also fails to affect the Ca^{2+} current initiated by a voltage step. Finally, in experiments where the SK_{Ca} channel was activated directly by loading the neurones with Ca^{2+}, UCL 1530 appeared to be approximately 5 times more potent as a blocker than dequalinium. However, because of the technical difficulty of these experiments, each blocker was tested at only a single concentration, which produced approximately 80% inhibition. With such a high level of block, caution is needed in interpretation of the data. Nevertheless they are consistent with a potency ratio similar to that observed for inhibition of the AHP.

The different findings with the two tissues could also be accounted for if the effectiveness of UCL 1530 was consistently underestimated in the net K^+ efflux experiments with hepatocytes. This could have occurred as a consequence of greater uptake or metabolism of the compound following its addition to the relatively concentrated cell suspensions used in these measurements. To rule this out, isolated hepatocytes were studied electrophysiologically using the same techniques as with sympathetic neurones. The SK_{Ca} channels were again directly activated by Ca^{2+} entry from a patch electrode. These experiments revealed a similar rank order of potency as that determined from measuring K^+ loss from suspensions of hepatocytes, namely, dequalinium > UCL 1530 $\geqslant$ gallamine, with gallamine only approximately 4 times less potent than dequalinium. However, the absolute potencies differed in the two sets of measurements, underlining the difficulty of comparing channel blocking data obtained under different experimental conditions, and from different cell types. For example, some of the measurements required use of Cl^--free solution, and our results showed that replacement of extracellular Cl^- by isethionate increases the potency of dequalinium as a blocker of the SK_{Ca} channel. The cationic composition of the extracellular solution is already known to influence the binding of apamin to the SK_{Ca} channel (Habermann & Fischer, 1979; Hugues *et al.*, 1982; Habermann, 1984) and more recent work has shown that intracellular anions also affect the activity of a number of K^+ channels, as well as their sensitivity to blocking agents (Zhang *et al.*, 1994; Heath & Terrar, 1994; McKillen *et al.*, 1994). In the light of these reports, the modest change in the potency of dequalinium observed on removing external Cl^- is perhaps not surprising. Its mechanism remains to be studied. It should be added here that membrane potential, channel open probability and the external K^+ concentration can all influence the potency of K^+ channel blockers. While this might account for some of the discrepancies observed in this study, it is unlikely that the differences between the tissues in the potency order of the three test compounds can be explained in such a way.

Our findings with gallamine agree with and complement those of Wadsworth *et al.* (1994) who found it to be less effective in displacing [^{125}I]-apamin from its binding sites on brain as compared with liver cell membranes. It might be ar-

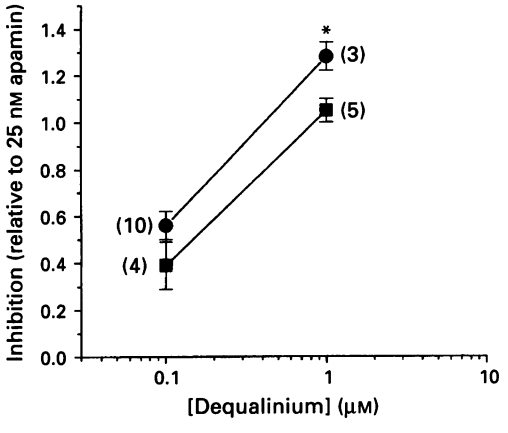

Figure 7 Comparison of the action of dequalinium in normal and chloride-free bathing solution. The graph shows the inhibition of the sustained outward current in Ca^{2+}-loaded guinea-pig hepatocytes by two concentrations of dequalinium, when the experiment was conducted either in normal Krebs solution (■), or in chloride free (equi-molar replacement with isethionate) solution (●). The mean values have been plotted $\pm$ s.e. mean, and the number of cells tested is indicated in parentheses. Because of the large variability in the size of the Ca^{2+}-activated current, and the presence of a large Ca^{2+}-activated Cl^- current in normal solution, the inhibitions have been normalized with respect to that produced by a standard application of 25 nM apamin during the same recording. Because of the slow onset of the action of apamin (see Figure 5a), these inhibitions may not have been maximal. *Statistically significant ($P<0.05$) by Student's t test.

gued that because both sets of evidence for the heterogeneity of the SK_{Ca} channels are based largely on comparisons between rat neurones and guinea-pig hepatocytes, the observed differences could have reflected inter-species variation. However, our finding that dequalinium and UCL 1530 are as active in guinea-pig sympathetic neurones as in those of the rat rules out such an explanation.

To summarise to this point, our work reveals differences in the relative potency of UCL 1530, dequalinium and gallamine, with the equi-effective molar ratio between gallamine and UCL 1530 differing by 150 fold between neurones and hepatocytes. This strongly suggests that the SK_{Ca} channels in these cells differ. Nevertheless, though we have been able to rule out certain other explanations, our evidence remains relatively indirect. Though either single channel or 'macro-patch' recordings from SK_{Ca} channels in outside-out patches isolated from sympathetic neurones could provide more decisive information, repeated attempts in our laboratory to obtain such records have been unsuccessful, and it is noteworthy that no recordings of this kind have yet been reported in the literature. Our tentative conclusions are however in keeping with other evidence that suggests that the SK_{Ca} channels are heterogeneous. Photoaffinity labelling and chemical crosslinking studies with [^{125}I]-apamin have already identified a number of apamin binding polypeptides (Schmid-Antomarchi *et al.*, 1984; Marqueze *et al.*, 1987; Auguste *et al.*, 1989), suggesting the SK_{Ca} channel is composed of several subunits. While all the cell types studied have yielded a protein of approximately 30 kDa, its size has been reported to range from 25 to 33 kDa, depending on the tissue. Furthermore, in rabbit hepatocytes and PC12 cells the electrophoretic mobility of this protein differs in reducing and non-reducing conditions, whereas the comparable proteins from rat brain and guinea-pig liver do not (Wadsworth *et al.*, 1994). This evidence is clearly consistent with the existence of SK_{Ca} channel subtypes.

In conclusion, we have compared the ability of gallamine, dequalinium and a novel cyclophane, UCL 1530, to block SK_{Ca} channels in rat sympathetic neurones and guinea-pig hepatocytes. Our finding that the rank order for the three blockers is different in these two kinds of cell adds to the evidence for the existence of subtypes of apamin-sensitive Ca^{2+}- activated K^+ channels. The work also shows that it should be possible to modify the activity of particular cells by the application of non-peptidic agents selective for SK_{Ca} channel subtypes.

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