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Direct block of native and cloned (Kir2.1) inward rectifier K^+ channels by chloroethylclonidine

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1 We have investigated the inhibition of inwardly rectifying potassium channels by the α-adrenergic agonist/antagonist chloroethylclonidine (CEC). We used two preparations; two-electrode voltage-clamp of rat isolated flexor digitorum brevis muscle and whole-cell patch-clamp of cell lines transfected with Kir2.1 (IRK1).

2 In skeletal muscle and at a membrane potential of −50 mV, chloroethylclonidine (CEC), an agonist at α_2-adrenergic receptors and an antagonist at α_{1x}-receptors, was found to inhibit the inward rectifier current with a K_i of 30 μM.

3 The inhibition of skeletal muscle inward rectifier current by CEC was not mimicked by clonidine, adrenaline or noradrenaline and was not sensitive to high concentrations of α_1-(prazosin) or α_2-(rauwolscine) antagonists.

4 The degree of current inhibition by CEC was found to vary with the membrane potential (approximately 70% block at −50 mV *c.f.* ~10% block at −190 mV). The kinetics of this voltage dependence were further investigated using recombinant inward rectifier K^+ channels (Kir2.1) expressed in the MEL cell line. Using a two pulse protocol, we calculated the time constant for block to be ~8 s at 0 mV, and the rate of unblock was described by the relationship $\tau = \exp((Vm + 149)/22)$ s.

5 This block was effective when CEC was applied to either the inside or the outside of patch clamped cells, but ineffective when a polyamine binding site (aspartate 172) was mutated to asparagine.

6 The data suggest that the clonidine-like imidazoline compound, CEC, inhibits inward rectifier K^+ channels independently of α-receptors by directly blocking the channel pore, possibly at an intracellular polyamine binding site.

Keywords: Potassium channel; inward rectifier; Kir; IRK1; skeletal muscle; CEC; chloroethylclonidine; imidazoline; imidazolidine; polyamine

Abbreviations: CEC, chloroethylclonidine; CHO, Chinese Hamster Ovary; D172N, aspartate$_{172}$ mutated to asparagine; EGTA, ethylene glycol-bis[beta-aminoethyl ether]-N,N,N′,N′-tetraacetic acid; E_k, equilibrium potential for potassium; FDB, flexor digitorum brevis; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; IRK1, synonym for Kir2.1, used in older papers; $Kd_{(v)}$, apparent dissociation constant at v mV; K_i, inhibition constant; Kir, inwardly rectifying potassium channel; MEL, murine erythroleukaemia cell line; *pKa*, apparent dissociation constant; TEVC, two-electrode-voltage clamp; *Vc*, command voltage; *Vm*, membrane potential

Introduction

Chloroethylclonidine (CEC) has been reported to have actions both as an agonist (Bültmann & Starke, 1993) and antagonist (Ford *et al*., 1994) at α-adrenergic receptors. The α-receptor mediated effects of CEC are largely irreversible and a recent study by Marjamäki *et al*. (1998) has located a site on the α_2-receptor which can be alkylated by CEC. In the heart, CEC interacts with α-receptors to increase the incidence of fatal cardiac arrhythmia during coronary occlusion (Geller *et al*., 1995). A recent brief report by Stadnicka *et al*. (1997) suggests that at least part of this arrhythmogenic action of CEC may involve an α-receptor mediated inhibition of cardiac inward rectifier K^+ channels and a consequent increase in cellular excitability.

Inward rectifier K^+ channels represent a significant conductance in skeletal muscle, where they may facilitate the re-uptake of K^+ ions from K^+ loaded transverse tubules after each action potential (Hille, 1992). Mammalian skeletal muscle is also known to express α-adrenergic receptors (Martin *et al*., 1990; Eason & Liggett, 1993) and we were interested in investigating the effect of CEC on this preparation, possibly establishing a link between α-receptors and inward rectifier channels. However, in voltage-clamp experiments, we find that CEC inhibits skeletal muscle inward rectifier currents in a reversible, dose-dependent manner which appears to be independent of α-adrenergic receptors. This effect is evident over a similar range of CEC concentrations (1–100 μM) to that used elsewhere in the study of α-adrenoceptors (Wang *et al*., 1991; Low *et al*., 1994; Tran & Forster, 1997). Inward rectifier channels of skeletal muscle are thought to be formed from subunits expressed from the Kir2.1 gene (Doupnik *et al*., 1995). We therefore further investigated this channel inhibition by recording whole-cell currents from a cell line expressing recombinant Kir2.1 inward rectifier channels, but lacking α-receptors. Our results are best explained by supposing that in addition to its actions on α-adrenoceptors, CEC may also directly inhibit inward rectifier channels by blocking the channel pore.

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Methods

Preparation of rat flexor digitorum brevis (FDB) muscle followed the methods of McKillen (1993). Briefly, Wistar rats were killed by stunning followed by cervical dislocation. The FDB muscle was removed to cold 1.2 mM Ca^{2+} bathing solution (see Solutions), then incubated in a 3 mg ml^{-1} collagenase buffer solution (1.2 mM Ca^{2+}). Individual fibres were then isolated by gentle trituration in a nominally Ca^{2+}-free bathing solution. Skeletal muscle membrane currents were recorded with two-electrode-voltage clamp (TEVC) of these fibres using an NPI Turbo-TEC 10C as described previously (Barrett-Jolley & McPherson, 1998). The stable expression of wildtype Kir2.1 channels and the recording of currents from murine erythroleukaemia (MEL) cells using whole cell patch clamp has also been described previously (Stanfield *et al*., 1994), transient expression of wildtype and D172N mutant Kir2.1 in Chinese Hamster Ovary K1 (CHO) cells followed the methods of Dart *et al*. (1998). Whole-cell currents were recorded using an Axopatch 200B amplifier and a DigiData 1200 interface, and series resistance compensation was used at approximately 90% (always $>85\%$).

TEVC experiments on skeletal muscle fibres were performed at 26–28°C and patch-clamp experiments at 22–24°C. The voltage protocols are shown in schematic diagrams above the current records in each of the relevant figures, and capacity transients have been removed digitally. Recordings were either filtered at 5 kHz (8-pole Bessel) and digitized at 10 kHz (patch clamp), or filtered 1 kHz, digitized at 3 kHz (two-electrode voltage-clamp), except where stated.

Solutions and materials

Skeletal muscle experiments During dissection and collagenase treatment, muscles were bathed in a solution containing (in mM): NaCl 154, KCl 5, Na_2HPO_4 1, HEPES 10, $MgCl_2$ 1.2 and $CaCl_2$ 1.2; pH 7.4 with NaOH. After collagenase treatment, the muscle was triturated and then stored for up to 6 h in (mM): NaCl 154, KCl 5, Na_2HPO_4 1, HEPES 10 and $MgCl_2$ 1.2; pH 7.4 with NaOH. The bathing solution used during the electrophysiological experiments contained (mM): Na_2SO_4 77, Na_2HPO_4 1, K-gluconate 40, $MgSO_4$ 1.2, $CaSO_4$ 8, HEPES 10 and glucose 10. pH was raised to 7.4 with NaOH (1 M stock – approximately 2.5 ml l^{-1}). All electrode glass was from Clarke Electromedical, Pangbourne, U.K. Voltage electrodes were pulled from thick walled borosilicate glass (o.d. 1.2 mm), and had resistances of approximately 20 MΩ when filled with 1 M KCl. Current electrodes were pulled from thin walled borosilicate glass (o.d. 1.2 mm), and had resistances of 3–8 MΩ when filled with 1 M K_3-citrate, 0.2 M KCl, and 2 mM EGTA.

Patch clamp experiments Electrodes were pulled from borosilicate glass (o.d. 1.5 mm), and fire polished to give a final resistance of 5 MΩ when filled. The pipette-filling solution contained (mM): KCl 140, $MgCl_2$ 1, EGTA 10, HEPES 10, pH 7.2. The external solution used for recording contained (mM): KCl 70, NaCl 70, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10; pH 7.3.

Drugs

All drugs were purchased from Sigma, (St. Louis, U.S.A.). Chloroethylclonidine (CEC, chemical name: 2-[2,6-dichloron-beta-chloroethyl-N-methyl-4-aminomethyl]phenylimino-2-imidazolidine) is a member of the 'clonidine-like imidazolidine' family and these compounds are largely positively charged under physiological conditions, with *pKa* in the region of 7.4 to 10.5 (Rouot *et al*. 1976, Timmermans & Van Zwieten 1978, Kim & Martin 1991). CEC is, however, *di*-basic and although the ionization constants themselves have not been published *pKa* prediction with pKalc gives values of 4.2 and 8.3 (PALLAS 2.1, CompuDrug). In fact, it is likely that the compound is significantly more basic than this (personal communication from Y. Martin, Abbot Labs, Illinois) and there could be up to about 40 or 50% *di*-protonated CEC in solution at physiological pH.

Both in patch-clamp and TEVC experiments, drug application was by rapid bath perfusion. Solution exchange was complete within 2–4 or 10–20 s for patch-clamp and TEVC experiments respectively (from control measurement of junction potentials at the recording site).

Analysis

It was not possible to complete full dose-response curve experiments on each fibre, and so sigmoidal curves were fit to the mean data points using the following relationship:

$$f = 1 - \frac{[c]^h}{[c]^h + 10^{h.K_1}}$$

where f is the fractional current, i.e., the fraction of current remaining, $[c]$ is the concentration of drug, K_i is the concentration for half block, and h is the Hill coefficient. This equation assumes geometric, rather than arithmetic distribution of errors (see Black & Shankley 1985). The values for K_i and h (and the confidence intervals) quoted in the text are the best fit parameters calculated by the curve fitting protocol (Microcal Origin) after the appropriate logarithmic transformation. All other averaged data are expressed as the mean ± s.e.mean (n = number of measurements).

Results

Inward rectifier K^+ currents were present in all skeletal muscle fibres studied. Muscle fibres were voltage-clamped at the estimated equilibrium potential for K^+ (E_K, −32 mV) then stepped to different test potentials (Figure 1). The membrane potential was then returned to E_K in three steps to reduce muscle twitching and the associated dislodging of the microelectrodes. There was little outward current in response to small depolarizing pulses, but hyperpolarization resulted in large inward currents. Steps to voltages more positive than about 0 mV resulted in the additional activation of delayed rectifier currents. Using the estimations of fibre surface area made previously (2×10^{-3} cm^2, Barrett-Jolley & McPherson, 1998), chord conductance density at a command potential of −150 mV, is equivalent to approximately 1 mS cm^{-2}.

Inward rectifier K^+ channels from mammalian skeletal muscle are inhibited by CEC

To analyse the effect of CEC, we adopted a protocol similar to that used previously by Barrett-Jolley & McPherson (1998) to investigate K_{ATP} currents in these fibres. Figure 2a shows one such experiment. Application of CEC led to a dose-related inhibition of the inward current at −60 mV, and this inhibition was largely reversed within 10 min of drug removal. In fact, whole-cell inward rectifier currents

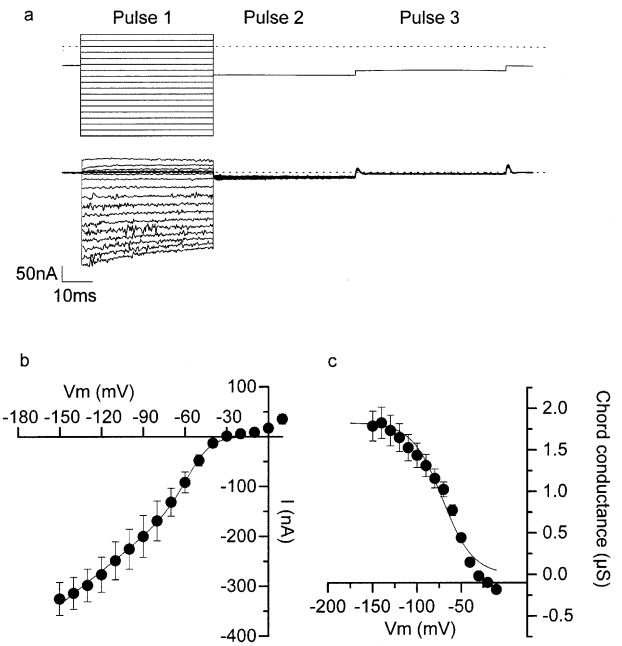

Figure 1 Inward rectifier potassium currents in mammalian skeletal muscle. (a) Muscle fibres were held at E_k (−32 mV) and stepped first to a test potential (Pulse 1: between −150 mV and +20 mV), and then, returned progressively to E_k (Pulse 2: −48 mV, Pulse 3: −40 mV). The upper panel shows the voltage protocol, the lower panel shows the resultant membrane currents, sampled at 10 kHz, filtered at 5 kHz. Dotted lines represent the zero current and voltage levels. (b) The current-voltage curve constructed from 15 experiments similar to that shown in part (a). (c) The Boltzmann transformation of the current-voltage data presented in part (b). Data has been fitted with a single component Boltzmann curve; maximum conductance is 1.98 μS, midpoint −71 mV, slope 17 mV and χ^2 0.03.

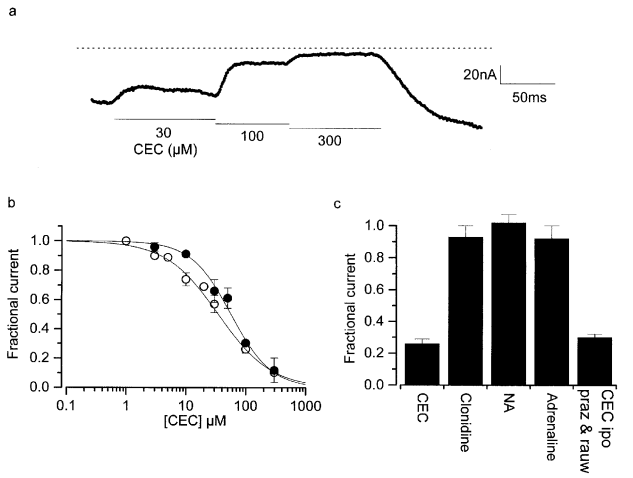

Figure 2 The inhibition of inward rectifier currents by CEC in skeletal muscle. (a) Inward rectifier K^+ currents were measured by holding the membrane potential at E_k (−32 mV), and stepping (at 5 s intervals) to −60 mV (100 ms), −48 mV (100 ms) and −40 mV (100 ms) before returning to E_k. The record shows that the currents measured at the end of the 50 ms pulses to −60 mV were rapidly inhibited by the application of increasing concentrations of CEC. Dotted lines represent the zero current level. (b) Mean dose-response data recorded in a number of experiments such as that shown in (a) (hollow circles are control data, filled circles are data recorded in the presence of rauwolscine and prazosin). The smooth lines are from fits to the mean data: Control, K_i 37 μM (95% CI: 24–55 μM) and h=1.1 (95% CI: 0.75–1.4, χ^2 0.75, n=8), IPO prazosin and rauwolscine K_i 55 μM (95% CI: 46–66 μM) and h=1.3 (95% CI: 1.1–1.7, χ^2 0.57, n=8). (c) Mean fractional current remaining (three or more experiments each) after the addition of 100 μM of the given agonist. 'CEC ipo praz & rauw' represents CEC added in the presence of 3 μM prazosin and rauwolscine.

were often observed to run up somewhat during the course of longer experiments; this phenomenon was not related to the application of CEC. Application of CEC, in a number of experiments similar to that shown in Figure 2a, allowed construction of a full concentration-effect curve (Figure 2b). The block of inward rectifier current occurred with a K_i of approximately 37 μM and Hill coefficient of unity (see Figure 2 legend).

Channel inhibition by CEC is independent of α-adrenergic receptors

If the CEC block was dependent on an action at α-adrenoceptors it should be abolished or reduced by α-adrenoceptor antagonists. However the addition of high concentrations of α_1- and α_2-antagonists (3 μM prazosin and rauwolscine) had little effect on either the inward rectifier current itself or the inhibition of this current by CEC. For example, 100 μM CEC inhibited inward currents by 74±3% (n=6), in the absence of antagonists, and by 70±3% (n=5) in the presence of 3 μM prazosin and rauwolscine (P>0.05, unpaired t-test). Figure 2b shows that the concentration-effect curve for CEC addition was not significantly affected by the presence of both 3 μM prazosin and 3 μM rauwolscine (see Figure 2 legend). If CEC did act through α-adrenoceptors, its blocking action should be mimicked by other α-adrenoceptor agonists. We, however, saw no inhibition of the inward current on addition of either 100 μM clonidine, adrenaline or noradrenaline, as shown in Figure 2c.

The CEC inhibition of inward rectifier current is voltage dependent

The results described above suggest that CEC does not act *via* interaction with α-receptors. An alternative possibility is that CEC blocks by a direct interaction with the channel. Such a mechanism often results in a voltage dependent block if the blocking substance is charged or the block is state dependent. We therefore compared current-voltage relations in the presence and absence of 100 μM CEC. Figure 3 shows that the blockade of the inward rectifier current is indeed voltage dependent, the degree of block reducing as the membrane potential becomes more negative. Qualitatively, control inward rectifier currents tended to saturate negative to about −150 mV, whereas currents in the presence of CEC continued to increase until approximately −190 mV, at which point they are about the same size as the control currents. From the relationship between membrane potential and the dissociation constant for a charged, pore-blocking ion at that potential (Woodhull, 1973), we estimate δz ('effective valency') to be 0.56 (Figure 2d). This implies that CEC blocks from the inside (assuming the active species to be positively charged, see Methods), and thus block is relieved by hyperpolarization.

The action of CEC on recombinant Kir2.1 channels

A more detailed analysis of the voltage-dependence of CEC block of inward rectifier channels was possible using a MEL cell line expressing the strong inward rectifier Kir2.1 (IRK1,

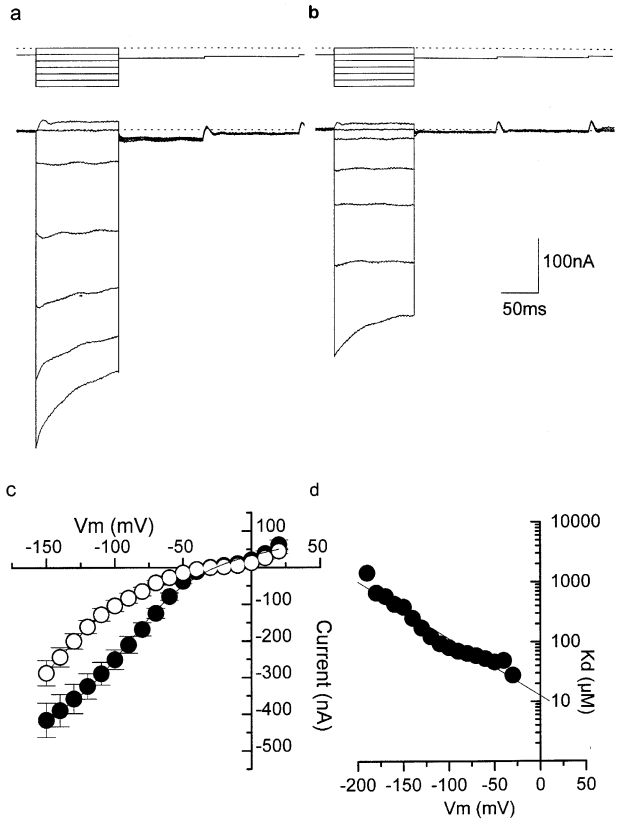

Figure 3 CEC block of inward rectifier currents is voltage-dependent. (a) Control currents and (b) current recorded in the presence of 100 μM CEC, the upper panel of each represents the voltage protocol and the lower panel shows the recorded membrane currents. (c) Current-voltage curves recorded in control (solid circles) and 100 μM CEC solutions (hollow circles), from five fibres similar to that shown in (a) and (b). Currents were measured at the end of each test pulse. (d) Estimation of the distance of the CEC blocking site through the channel pore, *delta* (see Woodhull 1973; Aidley & Stanfield, 1996). $Kd_{(Vm)}$ is calculated from part (c) by the equation; $Kd_{(Vm)} = [CEC] \times F_{(Vm)}/B_{(Vm)}$, where $F_{(Vm)}$ is the fractional activity of the current in [CEC] at membrane potential Vm (calculated from (c)) and $B_{(Vm)}$ is $1 - F_{(Vm)}$. The data has been fitted with the line; $\ln Kd_{(Vm)} = -(\delta\, z\, F\, Vm/RT) + \ln Kd_{(0)}$, where z, F, R and T have their usual meanings. $\delta\, z$ (also known as the 'effective valency') was 0.56, $Kd_{(0)} = 12.3$.

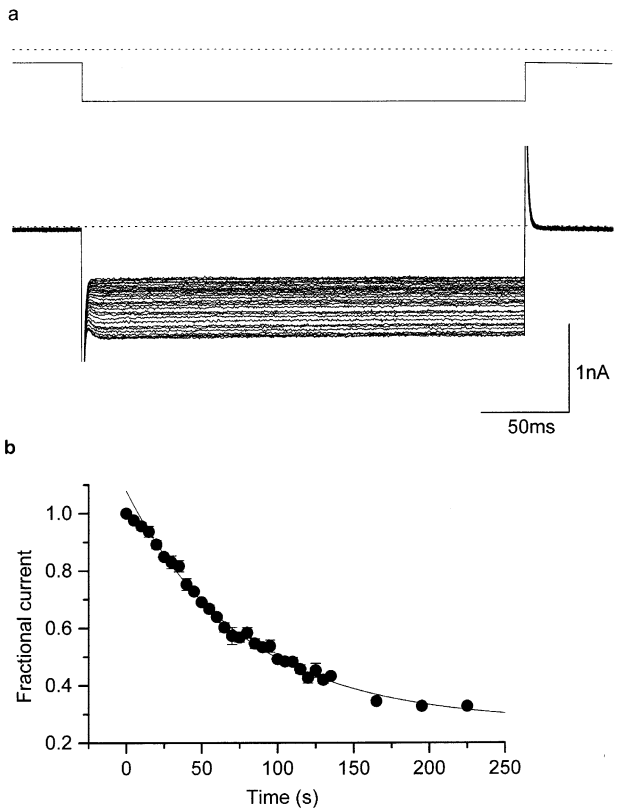

Figure 4 CEC inhibition of whole cell Kir2.1 currents recorded in the MEL cell line. (a) Shows a cell held at E_k (-17 mV under these recording conditions) and stepped to -67 mV at 5 s intervals whilst CEC (100 μM) is added. (b) Time course of the onset of inhibition from four experiments similar to that shown in (a). The smooth line is an exponential, fitted to data points from t = 15 s onward; $\tau = 78$ s.

Stanfield *et al*., 1994), which is also known to be expressed in skeletal muscle (Doupnik *et al*., 1995). Figure 4a,b show the time course of block by CEC of the whole cell Kir2.1 current recorded at -60 mV. Application of 100 μM CEC to MEL cells by bath perfusion inhibited Kir2.1 currents by approximately 70%, a similar degree of inhibition to that described above for native skeletal muscle fibres. The development of the block occurred with a time constant (τ) of 78 ± 3 s ($n = 3$).

Current records and instantaneous current-voltage relations for Kir2.1 in the absence and presence of CEC are shown in Figure 5. Current-voltage curves constructed in the presence of 100 μM CEC (Figure 5c) show the same pronounced voltage dependence as that seen in skeletal muscle (see Figure 3). However with MEL cells it was possible to use a protocol with long (1 s) voltage steps and 30 s interpulse intervals. This allowed time-dependent current relaxations to be measured in the presence of 100 μM CEC. These relaxations result from the time and voltage-dependent unblock of the inward rectifier current. Each relaxation (negative to -60 mV) has been fit with a single exponential. Figure 5d shows τ plotted against voltage for three similar experiments. The rate of relaxation increased e-fold for a 22 mV hyperpolarization.

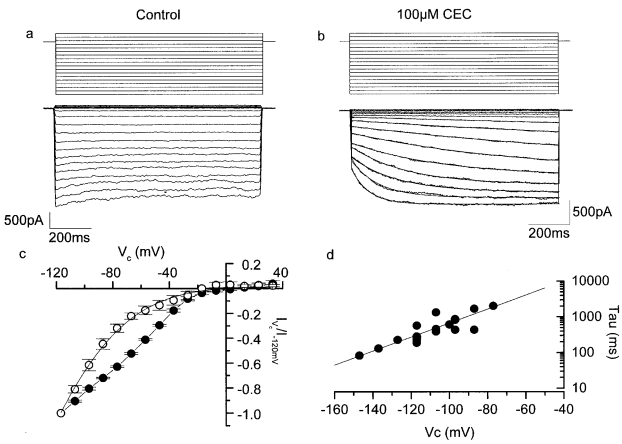

Figure 5 Kir2.1 inhibition by CEC: voltage- and time-dependent block. (a) Control whole cell currents recorded from MEL cells expressing Kir2.1 channels and (b) whole cell currents recorded in the presence of 100 μM CEC, both in response to long (1 s) voltage steps from a holding potential of E_k. The current data records in (b) are each fit with a single exponential, see (d). In both (a) and (b), the upper panel is a schematic representation of the voltage protocol, the command voltage is incremented by 10 mV for each step. (c) Mean instantaneous current-voltage data for four control and five test cells, normalized to the -120 mV current value of each cell (where conductance is maximum). The solid lines are smooth curves drawn through the points. (d) τ (from three experiments similar to (b)) plotted against membrane potential. The smooth line is a single exponential $\tau = \exp((V_c + 149)/22.14)$ s, where V_c is the command potential in mV.

The rate of channel block

Above (in Figure 4b), we have measured the time course of onset of CEC block. The time course of current block resulting from change of drug concentration may represent either the rate of binding to the block site (and associated channel occlusion), the rate of access to that site, limited by, for example, permeation of CEC across the cell membrane, or a combination of the two (solution exchange time is very small compared with on rate for block, see Methods). To measure the absolute rate of voltage-dependent block in the presence of 100 μM CEC, we used a two pulse protocol. MEL cells were hyperpolarized for sufficient time for the channels to unblock, then depolarized for a variable time to a voltage *Vc* mV, before finally returning to the hyperpolarized voltage again. The ratio of the instantaneous current of the second pulse to the steady-state current of the first therefore gives the fraction of blockage which has developed at *Vc* mV over the intervening period. Figure 6a shows an example of this protocol and Figure 6b shows a plot of the fractional current against time for several such experiments. The data (Figure 6b) are fit with a single exponential curve with $\tau = 7.8 \pm 1.8$ s. As described above, the rate at which CEC block of inward rectifier currents of both skeletal muscle and cloned Kir2.1 channels develops when CEC is applied to the outside of the cell is about an order of magnitude slower than this, consistent with CEC having to enter the cell to reach the site at which it blocks.

The site of CEC block

The nature of the voltage-dependence of block by CEC (see above), implies that the site of action is likely to be from the inside of the cell. Inward rectifier potassium channels are known to be blocked from the inside of the cell by a family of endogenous channel regulators, the polyamines (Ficker *et al*., 1994; see Discussion). The major site of action for these polyamines is believed to be the negatively charged amino acid residue D172 (aspartate), since mutation of this site to neutral asparagine markedly reduces the polyamine sensitivity of the channel (Yang *et al*., 1995). We therefore investigated whether, firstly, CEC could indeed act from the inside of the cell, and secondly whether the D172N mutation also prevented the action of CEC.

As predicted, inclusion of 100 μM CEC in the patch pipette did block wildtype Kir2.1 channels (Figure 7). The rate of onset of this block was dependent on cell access resistance, but steady-state block at -100 mV (*Vc*) was to $10 \pm 3\%$ $n = 8$ of control. Interestingly, CEC block of inwardly rectifying potassium currents was significantly less in the D172N mutants (mean residual current $80 \pm 3\%$, $n = 6$, $P < 0.0005$, unpaired *t*-test, compared to wildtype, Figure 7b,c), suggesting that CEC block may involve an electrostatic interaction with D172.

Discussion

Inward rectification of skeletal muscle potassium currents was first observed by Katz (1949) and subsequently characterized in the frog by Leech & Stanfield (1981). These channels allow K^+ ions to move into the cell much more readily than they allow K^+ to efflux, due to the voltage-dependent block of the channel pore by intracellular Mg^{2+} and polyamines (Matsuda *et al*., 1987; Lopatin *et al*., 1994). They are involved in setting the resting membrane potential and help control cellular

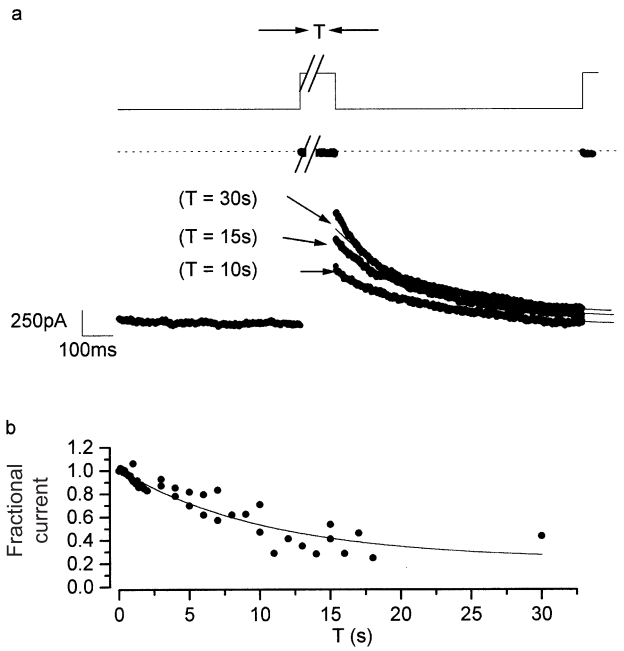

Figure 6 Two pulse protocol. (a) The upper panel is a schematic of the voltage protocol used. Cells held at -120 mV and stepped to 0 mV for a variable time *T* (to allow channels to block) and then stepped back to -120 mV to monitor how much block had developed in time *T*. (b) Data from five experiments similar to that shown in (a). The smooth line is an exponential with time constant 7.8 s.

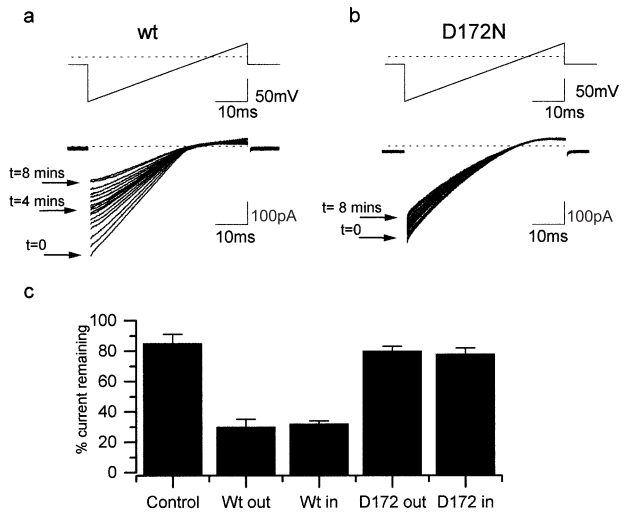

Figure 7 Site of CEC block. For these experiments, we transiently transfected CHO cells with wildtype or D172N-mutant Kir2.1 channels. We detected no differences between wildtype Kir expressed in MEL and CHO cells. (a) Current ramps recorded from CHO cells expressing wildtype Kir2.1 and with 100 μM CEC inside the patch pipette. The upper panel illustrates the voltage protocol and the lower panel shows the resulting currents recorded at 30 s intervals. t = 0 is the first ramp recorded after beginning whole-cell clamp of the cell, before there is time for significant CEC to have reached the interior of the cell. (b) Similar recording conditions to (a), however, using cells expressing the D172N Kir2.1 mutant. (c) Comparison of percentage steady-state current remaining (at -100 mV) during current ramps recorded from CHO cells expressing; wildtype Kir2.1, with no CEC present (control), wildtype Kir2.1 with 100 μM CEC in the patch pipette ('wt in'), wildtype Kir2.1 with 100 μM CEC in the bath ('wt out'), D172N mutants with 100 μM CEC in the bath ('D172N out') and D172N mutants with 100 μM CEC in the patch pipette ('D172N in').

excitability. In skeletal muscle these channels may serve the additional role of K^+ reabsorption from the T-tubules at times of high muscle activity.

This paper reports, for the first time, the properties of inward rectifier current measured from whole fibres of mammalian skeletal muscle. We find their properties to be similar to that of frog skeletal muscle (Leech & Stanfield, 1981), both in terms of maximum conductance density (approximately 1 mS cm^{-2}, in both cases) and in the dependence of conductance on membrane potential. Several subtypes of inward rectifier channel have been cloned (see Doupnik *et al*., 1995) and skeletal muscle is known to express the strong inward rectifier Kir2.1 (IRK1). As we anticipated, the properties of the inward rectifier currents from skeletal muscle were broadly similar to that of the recombinant Kir2.1 expressed in the MEL cell line. It is likely, however, that the skeletal muscle whole-fibre currents contained Kir2.1 along with other members of the inward rectifier family.

Block of inward rectifier currents by CEC, not mediated by α-receptors

Generally, the actions of CEC are mediated by α-adrenoceptors, which they either activate or inactivate irreversibly by alkylation of an extracellular site (see for example, Marjamaki *et al*., 1998). CEC is unusual in that it appears to act as an irreversible agonist at some receptors (α_2: Bültmann & Starke, 1993) and an irreversible antagonist at others (α_{1b}: Ford *et al*., 1994). However, we have several lines of evidence that in our experiments the inward rectifier blocking action of CEC was not modulated by α-receptors: (1) The effect of CEC was not mimicked by either the α_2 selective agonist clonidine, or by the non-selective α agonists adrenaline and noradrenaline; (2) In our experiments, the block of inward rectifier currents was fully reversible – this would not be expected for an α-receptor alkylation mechanism; (3) Any conventional α_1- or α_2-receptors should be blocked by a sufficient concentration of rauwolscine and prazosin (when applied together, these concentrations of rauwolscine and prazosin would shift an α_1- or α_2-receptor mediated dose-response curve by a factor of hundreds or thousands), but these two compounds were ineffective in preventing the CEC block of the skeletal muscle inward rectifier current; and (4) CEC was an effective blocker of recombinant inward rectifier channels expressed in the MEL cell line – a cell line not believed to express α-receptors.

Mechanism of block

The strongly voltage dependent nature of CEC inhibition of inward rectifier currents implies that CEC acts from within the electric field of the membrane, e.g., by blocking the channel pore. At physiological pH, clonidine-like imidazoline drugs exist largely in the positively charged state (Timmermans & Van Zweiten, 1978; Kim & Martin, 1991), and so membrane permeability will be low. It is possible however, that in the case of CEC, sufficient ligand enters the cell to block the pore from the inside, but is driven out of the pore at very negative potentials. If this were the case, one would expect that current relaxations resulting from change in CEC concentration would be slower than those resulting from change in membrane potential. This was indeed the case; τ for initial onset of CEC inhibition (Δ[CEC]) was approximately 80 s (Figure 4), whereas τ from the two pulse method (ΔVm) was $\sim$8 s (Figure 6). The idea that CEC block of inwardly rectifying potassium channels occurred from the inside of the cell, was further strengthened by our experiments showing that inclusion in the patch pipette resulted in similar voltage-dependent block.

Interestingly, the inward rectification properties of strong inwardly rectifying potassium channels are in part conferred by the intracellular actions of the family of positively charged (at physiological pH) polyamines (Ficker *et al*., 1994; Lopatin *et al*., 1994). We observed that the voltage-dependence of block of Kir2.1 by CEC was, in general, similar to that of the polyamines. For example, we calculated an 'effective valency' (Figure 3d) of 0.56, whereas Lopatin *et al*. (1994) found a *delta* value of 0.66 for spermine ($\delta z = 2.64$, $z = +4$). This would suggest that if the active species of CEC is the dominating CEC-H^+ form (see Methods), the blocking site for CEC is at a similar electrical distance to that for polyamines. Furthermore, our experiments showing CEC insensitivity on channels lacking a high affinity polyamine binding site (D172N mutants, Yang *et al*., 1995), suggest that the imidazoline compound CEC and polyamines may act through a common intracellular site.

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