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Inhibition of delayed rectifier K^+-current by levcromakalim in single intestinal smooth muscle cells: effects of cations and dependence on K^+-flux

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1 Whole-cell voltage-clamp recordings were made from single smooth muscle cells isolated from the longitudinal layer of the guinea-pig small intestine.

2 Levcromakalim ((-)Ckm) inhibited delayed rectifier K-current ($I_{K(DR)}$) and induced a voltage-independent K-current ($I_{K(-Ckm)}$). Both effects were inhibited similarly by glibenclamide. In some cells, however, $I_{K(-Ckm)}$ could be induced without any effect on $I_{K(DR)}$.

3 Ba^{2+} caused a voltage-dependent block of $I_{K(-Ckm)}$. The IC_{50} was 0.2 mM at -40 mV (6 cells), but at 0 mV 2 mM Ba^{2+} caused only a $26 \pm 7\%$ inhibition ($n = 5$). Ba^{2+} had much less effect on $I_{K(DR)}$, 2 mM Ba^{2+} having no inhibitory effect on current elicited by depolarization to -30 mV ($n = 6$) or 0 mV ($n = 5$).

4 Low concentrations of Zn^{2+} blocked $I_{K(-Ckm)}$ while having little effect on $I_{K(DR)}$. Zn^{2+} (40 μM) caused a $77 \pm 1\%$ reduction of $I_{K(-Ckm)}$ at -30 mV ($n = 4$) but $I_{K(DR)}$ was inhibited by only $10 \pm 3\%$ at the same voltage ($n = 4$).

5 Inward current amplitudes were compared in 135 mM Rb^+ and 135 mM K^+ bath solutions. (-)Ckm-activated Rb^+-current was only 4% of the K^+-current, whereas delayed rectifier Rb^+-current was larger than K^+-current.

6 (-)Ckm did not inhibit $I_{K(DR)}$ if $I_{K(-Ckm)}$ was blocked. In the presence of 2 mM Ba^{2+} or 135 mM Rb^+, (-)Ckm did not induce current nor did it inhibit the delayed rectifier. When $[Rb^+]_o$ was 25 mM and $[K^+]_i$ was 130 mM, (-)Ckm elicited outward current and inhibited outward delayed rectifier current (at voltages positive of the reversal potential) but it did not elicit inward current or inhibit inward delayed rectifier current (at voltages negative of the reversal potential).

7 These experiments indicate that (-)Ckm-activated K channels are more sensitive to inhibition by Ba^{2+} and Zn^{2+} and pass inward Rb^+ current less well than delayed rectifier K channels. They also suggest that (-)Ckm does not modulate delayed rectifier K channels directly or via an intermediate protein but that the inhibitory effect of (-)Ckm on $I_{K(DR)}$ arises as a consequence of K^+-flux through (-)Ckm-activated K channels.

Keywords: Levcromakalim; smooth muscle; K channel

Introduction

Levcromakalim and other K channel opener drugs are potent activators of K channels in smooth muscle cells (Hamilton *et al.*, 1986; Beech & Bolton, 1989a; reviewed by Edwards & Weston, 1993a). The target channel has a small conductance and is related to the ATP-sensitive K channels of other cell types (Kajioka *et al.*, 1991). It is notable for its apparent requirement for intracellular nucleoside diphosphates in order to open and for it sensitivity to inhibition by nanomolar concentrations of glibenclamide (Kajioka *et al.*, 1991; Beech *et al.*, 1993a,b). An additional effect of levcromakalim, acting in the same concentration range, is *inhibition* of delayed rectifier K-current (Beech & Bolton, 1989a; Edwards *et al.*, 1993). The effect also occurs with other K channel opener drugs (Ibbotson *et al.*, 1993a) and has been observed in ventricular myocytes (Heath & Terrar, 1994) and in an insulinoma cell line (Edwards & Weston, 1993b). It is of importance for our understanding of the mechanism of action of levcromakalim because it is the foundation of the 'conversion hypothesis' which proposes that levcromakalim- and ATP-sensitive K channels are modulated states of a delayed rectifier K channel (Edwards *et al.*, 1993; Edwards & Weston, 1993b; Ibbotson *et al.*, 1993b); inhibition of delayed rectifier K-current occurring as delayed rectifier K channels are shifted into a voltage-independent gating mode. If conversion does not occur the implication would appear to be that K channel opener drugs activate a regulatory protein which activates ATP-sensitive K channels and inhibits delayed rectifier K channels.

If the 'conversion hypothesis' is true, it might be expected that levcromakalim-induced and delayed rectifier K-currents would be affected similarly by substances which block K channels and indeed this is true for 4-aminopyridine, quinidine and phencyclidine (Beech & Bolton, 1989a). In this study we have extended this approach, investigating the effects of Ba^{2+}, Zn^{2+} and Rb^+, cations which might be expected to interact in or near K channel pore regions (Armstrong *et al.*, 1982; Gilly & Armstrong, 1982; Kwok & Kass, 1993; see also Hille, 1992). As a result we have found marked differences in the properties of the levcromakalim-induced and delayed rectifier K-currents. We then used this information to investigate the mechanism underlying the effect of levcromakalim on the delayed rectifier and suggest an explanation where there is no direct action of levcromakalim (or a binding protein) on delayed rectifier K channels.

Methods

Male guinea-pigs (350-400 g) were killed by cervical dislocation followed by exsanguination. Longitudinal muscle was removed from the ileum and jejunum by peeling it off in 1 cm

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lengths and placing it immediately in Hanks solution containing (mM): NaCl 137, NaH_2PO_4 0.34, KCl 5.4, K_2HPO_4 0.44, glucose 8, HEPES 5, $CaCl_2$ 0.01 and titrated to pH 7.4 with NaOH. Four pieces of muscle (length = 2–4 mm) were then transferred into Hanks solution at 37°C for 10 min before being resuspended in a mixture of collagenase (0.4 mg ml^{-1}) and pronase (0.24–0.4 mg ml^{-1}) in Hanks solution for 20 min at 37°C. At the end of this period, the tissues were cleaned of the enzyme mixture by washing in Hanks solution and single cells were isolated by gentle trituration. Cells were stored at 2°C and used within 10 h.

Recordings were made using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) at room temperature (20–25°C). Patch pipettes were made from borosilicate glass (Clark Electromedical Instruments: o.d. 1.5 mm, i.d. 0.86 mm) and had resistances of 1–4 MΩ after fire-polishing. The patch-clamp amplifier was either an Axopatch-ID or an Axopatch-200A (Axon Instruments, Inc.) and data were captured to a 386 or 486 PC after digitization by a CED 1401 or a 1401 plus (Cambridge Electronic Design Ltd.). Currents were filtered at 0.5–1 kHz using a Bessel filter. Voltage-clamp command paradigms and data analysis were performed using CED software and Origin 2.8 (MicroCal Inc, Northampton, MA, U.S.A.). Results are expressed as mean ± s.e.mean. Statistical comparisons were made using the Students *t* test.

Solutions (Table 1) were titrated to pH 7.4 using NaOH (bath solutions) or KOH (pipette solution). Na_2ATP (1 mM) was added to the pipette solution and the pH re-titrated to pH 7.4. It was then filtered (pore size 0.2 μm; Gelman Sciences) and frozen in aliquots at − 18°C. Salts were purchased from either BDH, Sigma or Aldrich. EGTA (ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid), HEPES (N-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulphonic acid) and glibenclamide were purchased from Sigma. Glibenclamide and levcromakalim were prepared as 10 or 100 mM stocks in dimethylsulphoxide (DMSO). The final concentration of DMSO was ≤ 0.1% for all experiments. Levcromakalim ((-)Ckm) was a generous gift from Dr T.C. Hamilton (SKB). Junction potentials between bath and

Table 1 Composition of solutions (mM; A-E are bath solutions)

	NaCl	*KCl*	*RbCl*	*$CaCl_2$*	*$MgCl_2$*	*$CdCl_2$*	*Glucose*	*HEPES*	*EGTA*	*pH*
A	130	5	0	1.5	1.2	0.1	8	10	0	7.4
B	110	25	0	1.5	1.2	0.1	8	10	0	7.4
C	110	0	25	1.5	1.2	0.1	8	10	0	7.4
D	0	135	0	1.5	1.2	0.1	8	10	0	7.4
E	0	0	135	1.5	1.2	0.1	8	10	0	7.4
Pipette	0	130	0	0	2	0	0	10	10	7.4

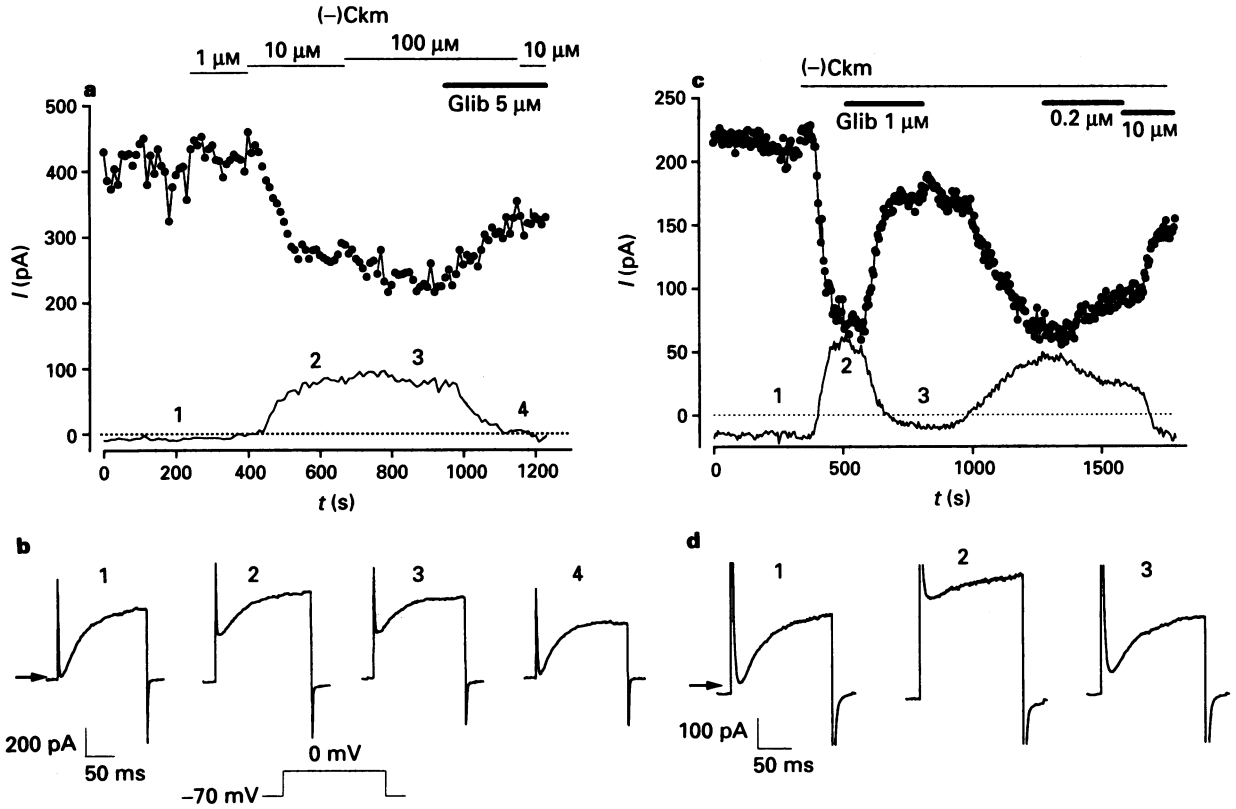

Figure 1 Induction of $I_{K(-Ckm)}$ and inhibition of $I_{K(DR)}$; whole-cell currents were recorded from cells bathed in solution A. (a) Plotted are the amplitudes of $I_{K(DR)}$ (●—●, upper record) and the absolute current value at − 40 mV (continuous record). Levcromakalim ((-)Ckm, 10 μM) induced $I_{K(-Ckm)}$ and inhibited $I_{K(DR)}$, and both effects were reduced by glibenclamide. $I_{K(DR)}$ was elicited by 150 ms depolarizing steps to 0 mV from a holding potential of − 70 mV every 10 s. $I_{K(-Ckm)}$ was the additional current occurring in response to (-)Ckm during 150 ms voltage steps to − 40 mV applied every 10 s. (b) Examples of actual currents (1–4). The arrow marks zero current. (c) Plotted are the amplitudes of $I_{K(DR)}$ (●—●, upper record) and the absolute current value at − 40 mV (continuous record) in the presence of (-)Ckm (10 μM) and various concentrations of glibenclamide (0.2–10 μM). Currents were measured as described in (a). (d) The single currents shown (1–3) are taken from the points marked in the plot. In this experiment 1 mM GDP was included in the pipette solution.

pipette solutions were measured using a 3 M KCl reference electrode and were $\leqslant 3$ mV; correction for these potentials was not made. Substances were applied using a bath-perfusion system. The bath volume was $\cong 150$ µl and the flow rate through it $\cong 4$ ml min^{-1}. The solutions in the bath were fully exchanged in <1 min.

Results

Inhibition of delayed rectifier K-current by (-)Ckm

In these experiments, delayed rectifier K-current ($I_{K(DR)}$) was defined as the time-dependent current that developed during a square depolarizing step from the holding potential (− 70 mV or − 80 mV) to − 30 mV or more positive. (-)-Ckm-induced K-current ($I_{K(-Ckm)}$) is not voltage-activated (Beech & Bolton, 1989a) and was measured (for example) during a step from − 70 mV to − 40 mV, or at a constant depolarized holding potential at which delayed rectifier K channels were inactivated (positive of − 20 mV). The bath solution contained Cd^{2+}, which abolished Ca^{2+}-influx through voltage-gated Ca channels (Beech, 1993), and the recording pipette solution included 10 mM EGTA to buffer $[Ca^{2+}]_i$ to a low and stable level.

When $I_{K(-Ckm)}$ and $I_{K(DR)}$ were measured simultaneously a pronounced inhibition of $I_{K(DR)}$ was often observed to occur concomitantly with the induction of $I_{K(-Ckm)}$. Two of the clearest examples are shown in Figure 1. In the first experiment, increasing concentrations of (-)Ckm were bath-applied (Figure 1a,b): 10 µM (-)Ckm induced a $I_{K(-Ckm)}$ of 100 pA and a simultaneous reduction in $I_{K(DR)}$; 100 µM (-)Ckm caused no further change in either current. Glibenclamide (5 µM) abolished $I_{K(-Ckm)}$, and $I_{K(DR)}$ returned towards pre-(-)Ckm levels. The ability of glibenclamide to reverse both effects of (-)Ckm is seen better in Figure 1c,d. (-)Ckm (10 µM) induced 60 pA of $I_{K(-Ckm)}$ and a 145 pA reduction of $I_{K(DR)}$. Glibenclamide (1 µM) almost abolished $I_{K(-Ckm)}$ and $I_{K(DR)}$ increased by 100 pA, and after wash-out of glibenclamide $I_{K(-Ckm)}$ returned and $I_{K(DR)}$ declined again. A lower concentration of glibenclamide (200 nM) partially inhibited $I_{K(-Ckm)}$ and caused a small increase in $I_{K(DR)}$. Scaling and superimposing the amplitudes of $I_{K(-Ckm)}$ and $I_{K(DR)}$ showed that the two K-currents changed with exactly the same time-course (not shown). It has been shown previously that glibenclamide ($\leqslant 10$ µM) has no effect on holding current at − 40 mV or on $I_{K(DR)}$ in the absence of (-)Ckm (Beech *et al.*, 1993a,b). It appears from Figure 1 that glibenclamide did not completely reverse the effect of (-)Ckm on $I_{K(DR)}$. We cannot, however, exclude the possibility that this small glibenclamide-insensitive effect was explained by run-down of $I_{K(DR)}$, which occurred gradually in several experiments.

In some recordings $I_{K(-Ckm)}$ was induced and yet there was no change in $I_{K(DR)}$ (e.g. Figure 2a,b). To express the variability of the effect of (-)Ckm and to look for a correlation between its two effects the amplitude of the reduction of $I_{K(DR)}$ was plotted against the amplitude of $I_{K(-Ckm)}$ for cells where the two currents were recorded simultaneously (Figure 2c). There was no correlation between the two effects and $I_{K(DR)}$ was never inhibited unless $I_{K(-Ckm)}$ occurred.

Effects of Ba^{2+}

Ba^{2+} inhibits (-)Ckm-induced $^{86}Rb^+$-flux with an IC_{50} of 30 µM (Quast, 1993). We compared the effects of bath-applied Ba^{2+} on $I_{K(-Ckm)}$ and $I_{K(DR)}$ (Figure 3). In Figure 3a the absolute amplitude of current at − 40 mV is plotted and $I_{K(-Ckm)}$ was about 70 pA. The addition of 0.02 mM Ba^{2+} had little effect but 0.2 mM Ba^{2+} caused about a 25 pA decline in outward current and 2 mM Ba^{2+} a further 35 pA reduction; 5 µM glibenclamide had only a slight additional effect. In control experiments Ba^{2+} was shown to have no effect on holding current in the absence of (-)Ckm (note also Figure 3c,d). The IC_{50} for the effect of Ba^{2+} on $I_{K(-Ckm)}$ at − 40 mV was 0.2 mM (Figure 3b). The effect of Ba^{2+} on $I_{K(-Ckm)}$ was voltage-dependent, as might be predicted from work on ATP-sensitive K channels in skeletal muscle (Quayle *et al.*, 1988). At a constant holding potential of 0 mV, 2 mM Ba^{2+} inhibited $I_{K(-Ckm)}$ by 26 ± 7% ($n = 5$). To allow a direct comparison with $I_{K(DR)}$ (which is elicited by depolarizations positive of − 40 mV) we also determined the sensitivity of $I_{K(-Ckm)}$ to block of 2 mM Ba^{2+} at − 30 mV and found 71 ± 6% inhibition ($n = 4$). Figure 3c,d shows examples of $I_{K(DR)}$ elicited by depolarizing to − 30 mV or 0 mV in the absence and presence of 2 mM Ba^{2+}. Ba^{2+} had little effect, slightly increasing the peak amplitude of $I_{K(DR)}$ elicited by depolarizing to − 30 mV (3 ± 3% increase, $n = 6$) or 0 mV (6 ± 3% increase, $n = 5$). In some of these cells, Ba^{2+} slowed the rate of activation of $I_{K(DR)}$ but the mean time constant of a single exponential fitted to the activation time-course at 0 mV was not significantly affected (37 ± 6 ms compared with 60 ± 15 ms, $P > 0.05$, $n = 6$). Thus, Ba^{2+} can strongly inhibit $I_{K(-Ckm)}$ while having little effect on $I_{K(DR)}$.

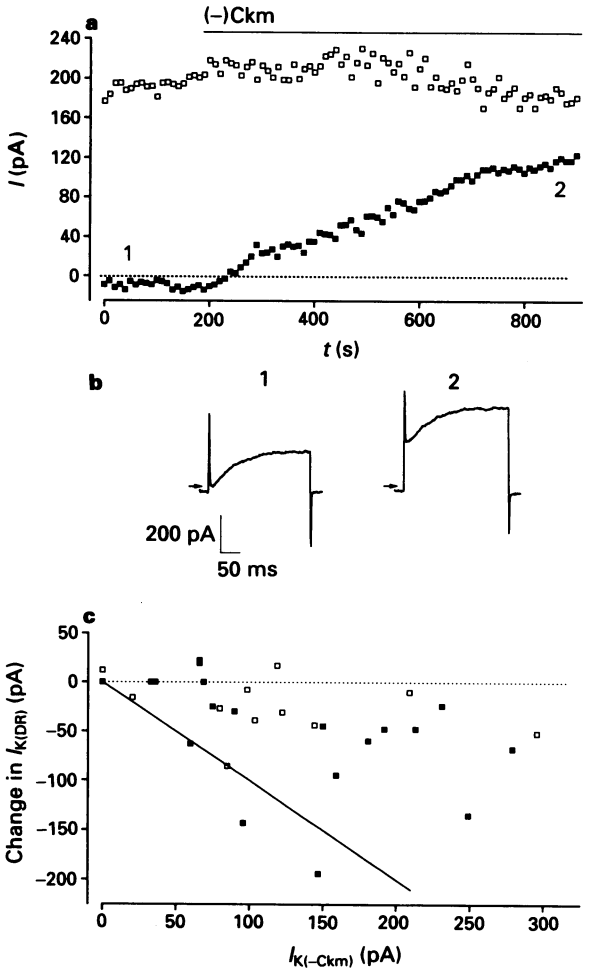

Figure 2 Relationship between the induction of $I_{K(-Ckm)}$ and the effects of levcromakalim ((-)Ckm) on $I_{K(DR)}$. Solution A was in the bath. (a) Example of a cell where $I_{K(-Ckm)}$ was induced by (-)Ckm (10 µM) but where there was no effect on $I_{K(DR)}$. $I_{K(DR)}$ was elicited by 250 ms depolarizing steps to 0 mV from a holding potential of − 70 mV every 10 s (□) and $I_{K(-Ckm)}$ (■) was assessed as in Figure 1(a). Actual currents are shown in (b). (c) Data points for 30 cells to which (-)Ckm (10 µM) was bath-applied. $I_{K(DR)}$ was elicited by 150 or 250 ms depolarizing steps applied every 10 s from a holding potential of − 70 mV to − 20 mV (□) or 0 mV (■). $I_{K(-Ckm)}$ was recorded at − 40 mV in all cases but the amplitude plotted has been multiplied by 3 for the 0 mV data or by 1.7 for the − 20 mV data (from Figure 2b in Beech & Bolton, 1989a). The line is the relationship if, for example, induction of 50 pA of $I_{K(-Ckm)}$ gave a 50 pA loss of $I_{K(DR)}$.

Effects of Zn^{2+}

Low concentrations of Zn^{2+} inhibit ATP-sensitive K-current but not delayed rectifier K-current in cardiac myocytes (Kwok & Kass, 1993). We have compared the effects of Zn^{2+} on $I_{K(-Ckm)}$ and $I_{K(DR)}$ (Figure 4). Kwok & Kass (1993) also showed that Cd^{2+} was a potent blocker of ATP-sensitive K channels in cardiac myocytes. However, we have found no effect of 100 μM Cd^{2+} on $I_{K(-Ckm)}$; in fact, all experiments were in the presence of 100 μM Cd^{2+} (Table 1). Zn^{2+} was a more potent blocker of $I_{K(-Ckm)}$ than Ba^{2+}, 40 μM Zn^{2+} caused an 85 ± 5% ($n = 4$) reduction of $I_{K(-Ckm)}$ at − 30 mV (e.g. Figure 4a). In contrast, 40 μM Zn^{2+} had only a small effect on $I_{K(DR)}$ elicited by depolarizing to − 30 mV (10 ± 3% reduction, $n = 4$) or 0 mV (5 ± 4% reduction, $n = 4$) (e.g. Figure 4b–d). Thus Zn^{2+}, like Ba^{2+}, has a more potent inhibitory effect on $I_{K(-Ckm)}$ than $I_{K(DR)}$.

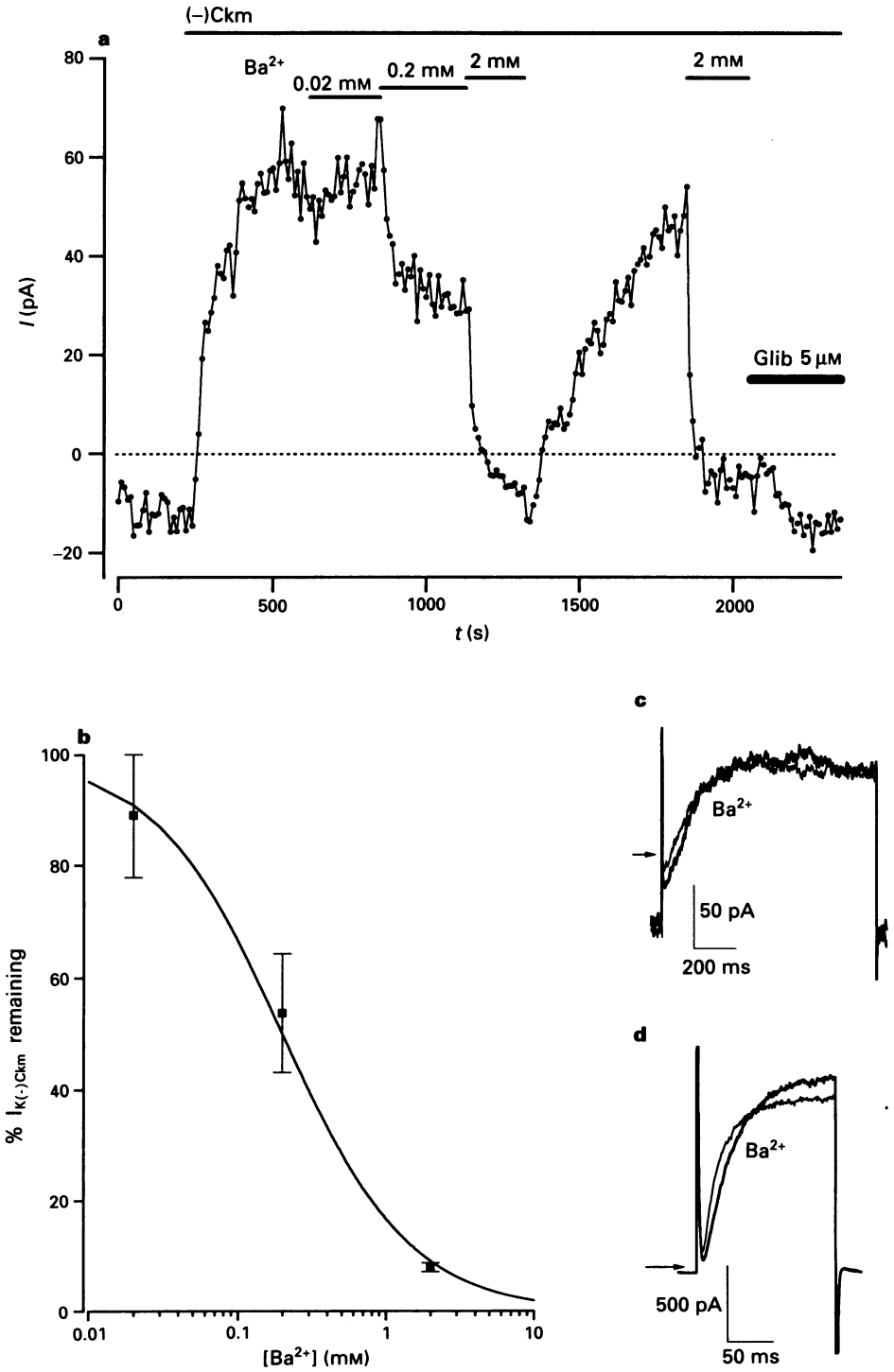

Figure 3 Effect of Ba^{2+}. Solution A was in the bath. (a) Plot of the amplitude of current at − 40 mV, showing the effect of several concentrations of Ba^{2+} on $I_{K(-Ckm)}$, which was induced by bath-applied levcromakalim ((-)Ckm, 10 μM). (b) Plotted is the mean percentage of $I_{K(-Ckm)}$ remaining in the presence of 0.02, 0.2 and 2 mM Ba^{2+} ($n = 4$–6). The curve is the Hill equation with a slope of 1, fitted by a least-squares method. The mid-point (IC_{50}) is at 0.2 mM. (c,d) The effect of Ba^{2+} on $I_{K(DR)}$. $I_{K(DR)}$ was elicited by: (c) 1 s steps to − 30 mV from a holding potential of − 80 mV; (d) 200 ms steps to 0 mV from − 70 mV. Example current records are shown for before and once 2 mM Ba^{2+} had been bath-applied.

Current carried by Rb^+

The amplitudes of inward K^+ currents were compared with inward Rb^+ currents through (-)Ckm-activated and delayed rectifier K channels. When the NaCl in the bath solution was replaced with KCl $I_{K(-Ckm)}$ was inward at voltages negative to 0 mV and averaged -298 ± 1 pA at -120 mV ($n = 3$). However, when K^+ was replaced by 135 mM Rb^+ in the same cells $I_{Rb(-Ckm)}$ averaged only -12 ± 1 pA ($n = 3$), giving $I_{Rb(-Ckm)}/I_{K(-Ckm)} \cong 0.04$ (e.g. Figure 5a). In contrast, delayed rectifier K channels allowed a large inward Rb^+-current (Figure 5b). Depolarizing from a holding potential of -70 mV to -20 mV in 135 mM K^+ gave an average $I_{K(DR)}$ of -212 ± 95 pA and changing to 135 mM Rb^+ gave an $I_{Rb(DR)}$ of -427 ± 129 pA ($n = 3$), leading to an $I_{Rb(DR)}/I_{K(DR)}$ of 2.0. In the same cells, depolarizing from a holding potential of -70 mV to -10 mV gave $I_{Rb(DR)}/I_{K(DR)}$ as 1.2. It also appeared that exchanging K^+ for Rb^+ may have caused the activation curve for delayed rectifier K channels to shift to more negative voltages (Figure 5b) but this phenomenon was not investigated further. From these experiments it was apparent that delayed rectifier K channels passed inward Rb^+-current much better than (-)Ckm-activated K channels.

No effect on $I_{K(DR)}$ when current through (-)Ckm-activated channels is blocked

As 2 mM Ba^{2+} almost abolished $I_{K(-Ckm)}$ and yet had little effect on $I_{K(DR)}$ we tried to investigate the effect of (-)Ckm on $I_{K(DR)}$ in the absence of contaminating $I_{K(-Ckm)}$ by working in the presence of 2 mM Ba^{2+}. However, under this condition a distinct effect of (-)Ckm (10 or 100 μM) on $I_{K(DR)}$ was never observed; a mean inhibition of $5 \pm 2\%$ was measured ($n = 7$). Similarly, if we measured inward delayed rectifier current in the presence of 135 mM extracellular Rb^+, (-)Ckm (10 or 100 μM) again had no distinct effect; a mean inhibition of $3 \pm 2\%$ was measured ($n = 4$). We, therefore, investigated if the effect of (-)Ckm on $I_{K(DR)}$ might occur simply because there was K^+-flux through (-)Ckm-activated K channels.

By experimenting with 25 mM RbCl extracellularly and 130 mM KCl in the pipette solution we were able to induce an outward current with (-)Ckm at voltages positive of -40 mV but little or no inward current at voltages negative of -40 mV. Outward and inward delayed rectifier currents were, however, observed. (We chose to record in the presence of 25 mM Rb^+ rather than 135 mM Rb^+ because the outward current induced by (-)Ckm was larger). Measurement of the amplitudes of outward and inward delayed rectifier currents before and during the response to (-)Ckm (Figure 6a,b) revealed that the outward delayed rectifier current (labelled 1 in Figure 6a) was reduced in 6 experiments by $19 \pm 5\%$ as outward (-)Ckm-induced current (labelled 2) occurred (96 ± 25 pA at -15 mV). In contrast, there was little effect on inward delayed rectifier current ($2.5 \pm 1\%$ reduction; labelled 4) and induced inward current was small (0 pA at -65 mV and -4 ± 2 pA at -80 mV, $n = 6$). When recording with 25 mM K^+ extracellularly, (-)Ckm (10 μM) inhibited inward delayed rectifier current ($34 \pm 9\%$ reduction; compare, for example, the current traces labelled 'control' and 'Ckm (DR only)' in Figure 6d) and induced inward current

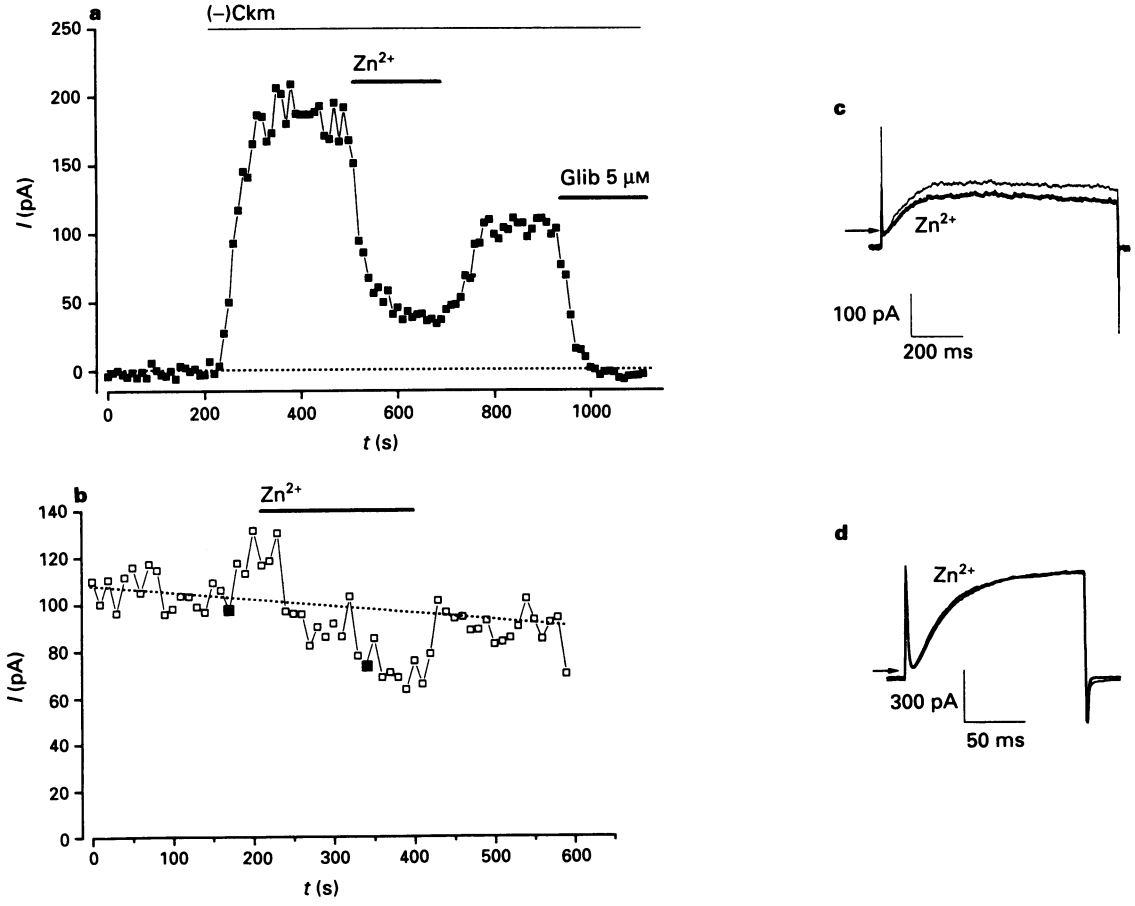

Figure 4 Effect of Zn^{2+}. Solution A was in the bath. (a) Plot of current sampled every 10 s at a holding potential of -30 mV showing inhibition by Zn^{2+} of $I_{K(-Ckm)}$. Levcromakalim ((-)Ckm) was bath-applied at 10 μM. (b) The effect of Zn^{2+} on $I_{K(DR)}$. Plotted is the amplitude of $I_{K(DR)}$ elicited by 1 s-steps to -30 mV from a holding potential of -80 mV in the absence and presence of Zn^{2+} (40 μM). The dotted line describes our estimate of the time-course of run-down of the delayed rectifier, and the filled symbols indicate the points at which sample traces were taken for (c). (d) In another cell, currents elicited by steps to 0 mV from a holding potential of -70 mV in the absence and presence of Zn^{2+} (40 μM).

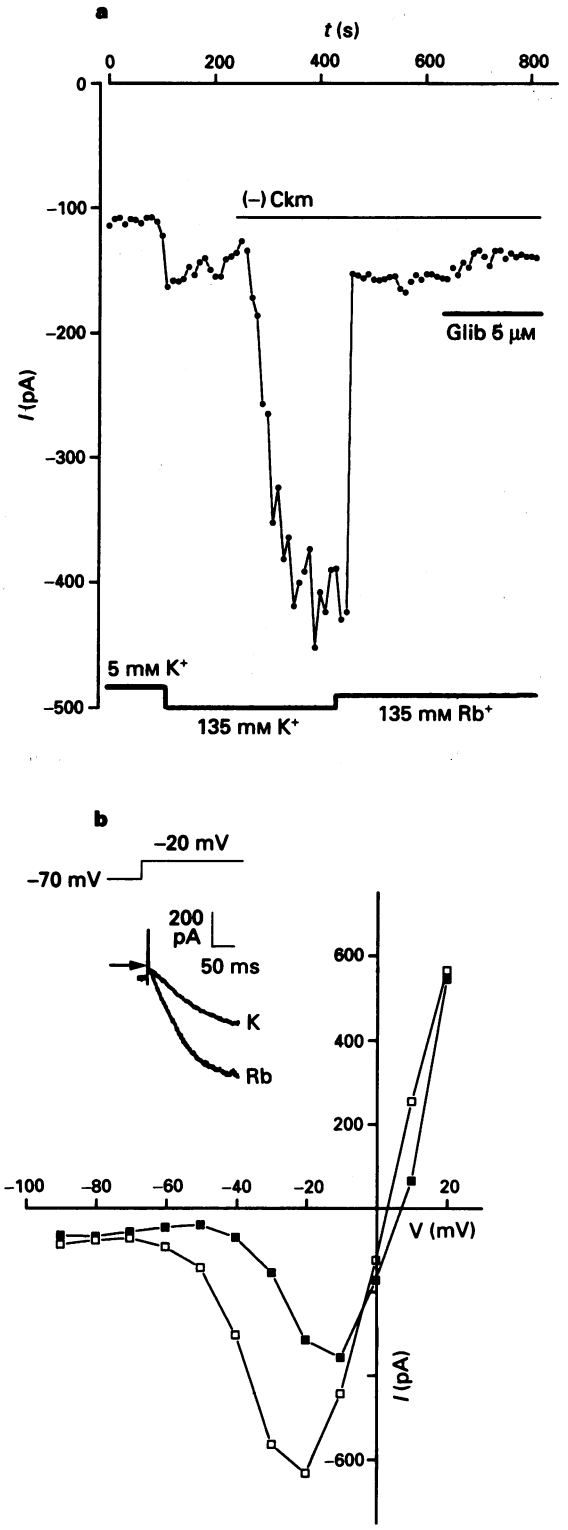

Figure 5 Inward K^+-current and Rb^+-current through levcromakalim ((-)Ckm)-activated K channels and delayed rectifier K channels. (a) Inward currents elicited by bath-applied (-)Ckm (10 μM) in the presence of 135 mM K^+ (solution D) or 135 mM Rb^+ (solution E). Ramp changes in potential from − 40 mV to − 120 mV (duration 400 ms) were applied every 10 s and the current amplitude at − 120 mV is plotted. (b) Plot of the amplitude of current at the end of a 200 ms test step from the holding potential of − 70 mV (without subtraction of leak current) in the presence of 135 mM K^+ (■) and then 135 mM Rb^+ (□). Actual currents are inset for the − 20 mV test potential (the arrow indicates zero current). The slightly positive reversal potential in (b) cannot be explained solely by a junction potential as all junction potentials were measured as ≤ 3 mV (see Methods). It is plausible, however, that a Donnan equilibrium potential could contribute to the effect.

(− 98 ± 16 pA at − 65 mV and − 140 ± 21 pA at − 80 mV, $n = 3$). An example is shown in Figure 6c,d. Thus, (-)Ckm inhibited delayed rectifier current only when (-)Ckm-induced current was flowing in the same direction.

Discussion

In these guinea-pig intestinal smooth muscle cells we have been able to demonstrate that, as in portal vein cells (Beech & Bolton, 1989a; Ibbotson *et al.*, 1993b), (-)Ckm does inhibit delayed rectifier K-current ($I_{K(DR)}$) via a glibenclamide-sensitive mechanism. A hypothesis which explains this effect is that (-)Ckm converts delayed rectifier K channels from a voltage-dependent to a voltage-independent gating mode. If this were true it might be expected that both $I_{K(-Ckm)}$ and $I_{K(DR)}$ would be similarly sensitive to inhibition by Ba^{2+} and Zn^{2+} and that both could be carried equally well by Rb^+ instead of K^+. However, in this study we have shown that $I_{K(-Ckm)}$ can be strongly inhibited by concentrations of Ba^{2+} or Zn^{2+} that have little or no effect on $I_{K(DR)}$, and although $I_{Rb(DR)}$ was larger than $I_{K(DR)}$, $I_{Rb(-Ckm)}$ was only 4% of $I_{K(-Ckm)}$. In addition, we have used these effects of cations to show that (-)Ckm inhibits $I_{K(DR)}$ only when there is K^+-flux through (-)Ckm-activated K channels and consequently suggest there is no direct effect of (-)Ckm or a (-)Ckm-binding protein on delayed rectifier K channels.

A number of observations indicate that (-)Ckm only inhibits $I_{K(DR)}$ when it can induce K^+-current. The strongest evidence for this was found when we observed outward and inward delayed rectifier currents but only outward (-)Ckm-induced current (Figure 6). (-)Ckm inhibited delayed rectifier current only when there was (-)Ckm-induced current in the same direction. Other observations are also consistent with the view that it was K^+-flux through (-)Ckm-activated K channels which caused the reduction in $I_{K(DR)}$. (-)Ckm had no effect on delayed rectifier current when (-)Ckm-induced current was prevented with Ba^{2+} or Rb^+, and $I_{K(DR)}$ was never inhibited unless $I_{K(-Ckm)}$ was induced. In an insulinoma cell line, margatoxin had no effect on $I_{K(DR)}$ but it did inhibit $I_{K(-Ckm)}$ and the ability of (-)Ckm to inhibit $I_{K(DR)}$ (Edwards & Weston, 1993b). When intracellular Mg^{2+} levels were very low, (-)Ckm did not induce K-current (Beech *et al.*, 1993b) or inhibit $I_{K(DR)}$ (Edwards *et al.*, 1993). Both $I_{K(DR)}$ and A-type K-current were inhibited by KCO drugs in rat portal vein smooth muscle cells (Ibbotson *et al.*, 1993a; Figure 1), suggesting activation of a non-specific mechanism. (-)Ckm 100 μM did not induce K^+-current or inhibit delayed rectifier K^+-current in defolliculated *Xenopus* oocytes expressing the delayed rectifier K channel protein *drk1* (D.J. Beech, unpublished observation). Lastly, the ability of glibenclamide to prevent the actions of (-)Ckm on $I_{K(DR)}$ also supports the hypothesis because it can be explained simply by closure of (-)Ckm-activated K channels.

A reasonable explanation for why K^+-flux through (-)Ckm-activated K channels reduced $I_{K(DR)}$ would seem to be that the flux caused the K^+ concentration in the vicinity of delayed rectifier K channels to deviate from that of the bulk solution so that the driving force on K^+ was reduced, and perhaps also so that the conductance of the channels was reduced. A rough estimate of what the change in local $[K^+]$ might have been can be gained from a mathematical reconstruction of $I_{K(DR)}$ and $I_{K(-Ckm)}$ (from Hodgkin & Huxley, 1952). By this approach we found that the (-)Ckm-induced reduction in outward delayed rectifier K-current at − 15 mV shown in Figure 6d could be described well if an effective K^+-equilibrium potential of − 25 mV was assumed, a value 18 mV depolarized of the calculated K^+-equilibrium potential for the bulk solutions. If local $[K^+]_o$ and $[K^+]_i$ changed equally then the effective $[K^+]_o/[K^+]_i$ would become 44.5 mM/120.5 mM, but if only $[K^+]_i$ was depleted (see below) then $[K^+]_o/[K^+]_i$ would become 25 mM/67 mM.

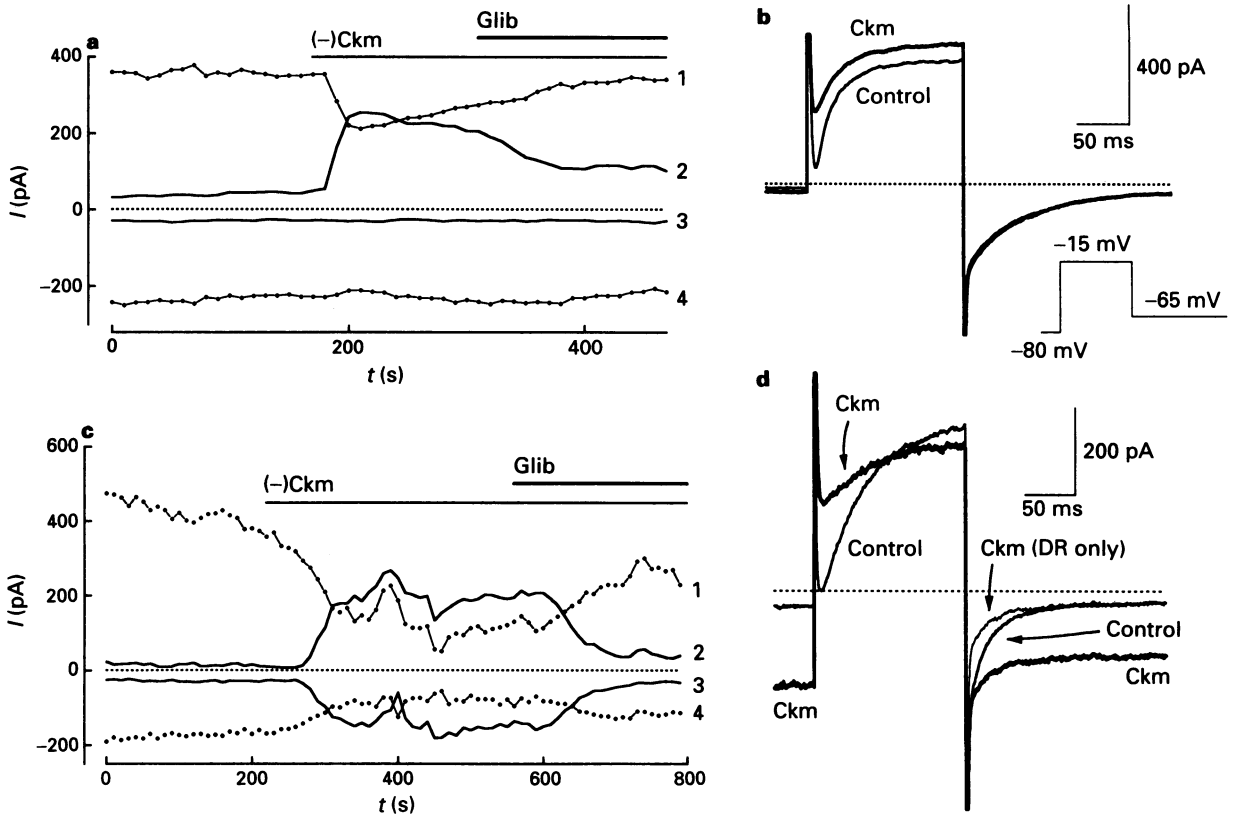

Figure 6 Dependence on K^+-flux. In the upper part (a, b) bath solution C (25 mM Rb^+) was used, and in the lower part (c, d) bath solution B (25 mM K^+) was used. In both (a) and (c) there are plots against time of the amplitude of delayed rectifier current at − 15 mV (outward current, 1) and − 65 mV (inward current, 4). Delayed rectifier current was defined as the difference between the current measured 10 ms after the start of the voltage step to − 15 mV (1) or − 65 mV (4) and that at the end of the voltage step. Also plotted is the amplitude of the quasi-instantaneous current on depolarization to − 15 mV (2), which was defined as the 'dip point' once the capacity current had decayed but before delayed rectifier current was appreciably activated (see b and d), and the steady-state current occurring on repolarization to − 65 mV (3). In all cases points were sampled every 10 s but for plots 2 and 3 the data points have been joined by lines so that a continuous record is apparent. Levcromakalim ((-)Ckm 10 μM) and glibenclamide (1 μM) were bath-applied to both cells as marked by the horizontal bars. (b) A diagram of the voltage protocol and examples of actual currents for the Rb^+ experiment shown in (a): before the application of (-)Ckm (thin line) and in the presence of (-)Ckm but not glibenclamide (thick line). (d) examples of actual currents for the K^+ experiment shown in (c): before the application of (-)Ckm (thin line) and in the presence of (-)Ckm but not glibenclamide (thick line). In (d) a modified version of the 'Ckm' current trace has been superimposed for the period at − 65 mV (but not − 80 mV and − 15 mV). This trace has been labelled 'Ckm (DR only)' because the steady-state current in the presence of (-)Ckm at − 65 mV has been subtracted, leaving only delayed rectifier (DR) current. Note especially from this figure that when the bath solution contained 25 mM Rb^+, (-)Ckm induced outward but not inward current (labelled 2 and 3 respectively in (a)) and inhibited outward but not inward delayed rectifier current (labelled 1 and 4 respectively in (a)). Both inward and outward currents were affected when the bath solution contained 25 mM K^+.

When considering a K^+ accumulation-depletion hypothesis it might be expected that a correlation should exist between the amplitude of $I_{K(\text{-Ckm})}$ and the amplitude of the inhibition of $I_{K(DR)}$. Although in an individual cell the inhibition of $I_{K(DR)}$ was proportional to the amplitude of $I_{K(\text{-Ckm})}$, comparing data from 30 different cells showed there was no correlation between the two effects (Figure 2c). If, however, the local $[K^+]$ change occurred because there was a structural feature in the cell that generated an 'unstirred layer' (see below) the volume and geometry of this layer could well vary between cells and so the degree of accumulation/depletion would vary. A second point to consider is that when the $[K^+]$ changes the reversal potential must also change. However, the change may not be readily detectable because any shift in reversal potential will be proportional to the amplitude of $I_{K(\text{-Ckm})}$ and as $I_{K(\text{-Ckm})}$ will be small in the region of the reversal potential little shift will occur. In addition, if the unstirred layers are small (see below), deviation from and re-equilibration with the bulk solution will be rapid as the amplitude of $I_{K(\text{-Ckm})}$ changes. We have not been able to detect a significant change in reversal potential as $I_{K(\text{-Ckm})}$ increases (unpublished observation; see also Beech *et al.*, 1993b), and the activation, deactivation and inactivation time-courses of $I_{K(DR)}$ are not altered when it is inhibited by (-)Ckm (Figures 1 and 6; analysis not shown; Beech & Bolton, 1989a).

Frankenhaeuser & Hodgkin (1956) considered the possibility that K^+ accumulated in an extracellular aqueous space between the membranes of the *Loligo* axon and the adjacent Schwann cells when repetitive action potentials were evoked. The accumulation and recovery rates were variable but they had time constants that ranged from 30–100 ms. It was predicted that the extracellular space had a thickness of 30 nm. A similar space may exist in single smooth muscle cells between the plasma and sarcoplasmic reticulum membranes. This intracellular space appears to have a thickness of 12–20 nm and it is fragmented (Somlyo & Franzini-Armstrong, 1985), in contrast to the continuous Frankenhaeuser-Hodgkin Space. The intracellular space in smooth muscle cells has previously been considered as a restriction to equilibration between ions in the bulk cytoplasm and those at the membrane by Chen *et al.* (1992) who have developed a 'superficial buffer barrier' hypothesis to explain intracellular Ca^{2+}-handling. If this intracellular space is also a barrier to K^+ movements the degree and rate at which accumulation occurs will depend on the complex geometry of the space and on the permeability of the sarcoplasmic reticulum membrane to K^+. Even without an intracellular space $[K^+]$ may change

sufficiently near an open (-)Ckm-activated K channel to affect current through a delayed rectifier K channel because ion channels can be very close to each other, within 8 nm (Gogol & Unwin, 1988). It has been proposed that this type of interaction occurs between voltage-gated Ca channels and Ca-activated K channels. Local changes in $[Ca^{2+}]$ are predicted to occur within 10–100 nm of open voltage-gated Ca channels and the changes develop and decline in the sub-millisecond time scale (Smith & Augustine, 1988; Roberts *et al.*, 1990). In smooth muscle cells, Ca^{2+}-activated K^+-current occurs in <20 ms when voltage-gated Ca channels open in response to a square depolarizing voltage step (Ohya *et al.*, 1987), and more detailed experiments on hair cells have shown that Ca^{2+}-influx through Ca channels can affect Ca-activated K channels within a millisecond (Roberts *et al.*, 1990).

The hypothesis that (-)Ckm acts by converting delayed rectifier K channels into a voltage-independent gating mode is based on several observations; (1) (-)Ckm simultaneously inhibits $I_{K(DR)}$ as it induces $I_{K(-Ckm)}$; (2) $I_{K(-Ckm)}$ and $I_{K(DR)}$ show similarities in their sensitivities to various K channel inhibitors, both are resistant to inhibition by charybdotoxin and apamin and both are inhibited by similar concentrations of 4-aminopyridine, quinidine, phencyclidine and nicardipine (Beech & Bolton, 1989a; S.G. Brown and D.J. Beech, unpublished observations); (3) (-)Ckm-activated channels and delayed rectifier channels have a similar unitary conductance, in the region of 5 to 20 pS with a near-physiological K^+-gradient (Beech & Bolton, 1989b; Boyle *et al.*, 1992; Volk & Shibata, 1993; Beech *et al.*, 1993b); (4) a protein kinase inhibitor and a dephosphorylating agent can induce K-current and inhibit $I_{K(DR)}$ (Edwards *et al.*, 1993). If our K^+-flux hypothesis is true, observations (1) and (4) can no longer be used to support the conversion hypothesis. Observation (2) is interesting but weakened by the findings that Ba^{2+}, Zn^{2+} and Rb^+ interact differently with (-)Ckm-activated K channels and delayed rectifier K channels. If the conversion hypothesis is not true the pharmacological similarities between $I_{K(-Ckm)}$ and $I_{K(DR)}$ may suggest structural similarities between the underlying channel proteins. If the conversion hypothesis is true (-)Ckm must not only modulate the gating of delayed rectifier channels but also increase their sensitivity to Ba^{2+} and Zn^{2+} and reduce their ability to pass Rb^+. The suggestion that the underlying channels have a similar unitary conductance (observation 3) requires further study because the conductances have not been compared under identical conditions. Finally, the conversion hypothesis is weakened by the data in Figure 2 because $I_{K(-Ckm)}$ can occur without loss of $I_{K(DR)}$ (see also Russell *et al.*, 1992). Thus, although initially attractive, the conversion hypothesis seems unlikely to be true.

(-)Ckm-like drugs act to open an ATP-sensitive K channel which, in smooth muscle cells, has been noted for its dependence on intracellular nucleotide diphosphates (Kajioka *et al.*, 1991; Beech *et al.*, 1993a,b; Kamouchi *et al.*, 1993; Zhang & Bolton, 1994). We suggest that this channel is distinct at a molecular level from K channels of the delayed rectifier type, and that (-)Ckm-like drugs (or a (-)Ckm-binding protein) do not interact directly with delayed rectifier K channels but instead, by inducing K^+-flux, cause the K^+ concentration in the vicinity of delayed rectifier K channels to deviate from that of the bulk solution in such a way that the driving force on K^+ ions is diminished. Primary sequence data for a rat heart K_{ATP} channel further establish a molecular distinction between delayed rectifier and K_{ATP} channels (Ashford *et al.*, 1994).

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