Action of cromakalim on potassium membrane conductance in isolated heart myocytes of frog

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- 1 The effects of cromakalim on membrane currents were studied at 20°C in frog atrial and ventricular cells in patch clamp recording using the whole cell configuration.
- 2 When cromakalim (1 μ M) was applied in the external medium, a time-independent current was activated in a few minutes. Cromakalim induced a weak increase of inward membrane currents recorded during hyperpolarization and a large increase of outward membrane currents recorded during depolarization.
- 3 The current voltage relationship of the cromakalim-induced current reversed near E_K and rectified in the outward direction.
- 4 The cromakalim-activated current was inhibited by external application of cesium (20 mM), barium (1.8 mM), tolbutamide (1 mM) and glibenclamide (1 μ M).
- 5 The effects of cromakalim were insensitive to a cytosolic increase in adenosine 5'-triphosphate (ATP, 3–5 mM). Cromakalim had no effects when applied in the cell.
- 6 Our results confirm that cromakalim activates an $I_{K(ATP)}$ -like conductance and suggest that the effects of the drug are due to an action on the external side of the membrane and are independent of the ATP cell content.

Introduction

Cromakalim (BRL 34915, ($\pm$)-6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrrolidyl)-2H-benzo(b)-pyran, 3-ol) is a smooth muscle relaxing agent which opens $^{86}Rb^+$ and $^{42}K^+$ permeable K^+ channels resulting in a membrane hyperpolarization in vasculature (Hamilton *et al.*, 1986; Weir & Weston, 1986b; Coldwell & Howlett, 1986; Quast, 1987; Shety & Weiss, 1987; Quast & Baulin, 1988), intestine (Weir & Weston, 1986a), uterus (Hollingsworth *et al.*, 1987), trachea (Allen *et al.*, 1986) and urinary bladder (Fujii, 1987; Foster & Brading, 1987). Numerous studies have tested the way in which cromakalim acts on membrane potential. This drug activates Ca^{2+} -activated K^+ channels in aortic smooth cells (Kusano *et al.*, 1987) but is insensitive to apamin, a blocker of low conductance Ca^{2+} -activated K^+ channels (Weir & Weston, 1986a; Allen *et al.*, 1986; Cook & Hof, 1988). Its action does not require Ca^{2+} entry through dihydropyridine Ca^{2+} channels in the portal vein (Quast, 1987; Southerton & Weston, 1987). Tetraethylammonium chloride, 3,4-dihydropyridine and procaine inhibit the cromakalim-stimulated Rb^+ efflux in the portal vein (Quast, 1987; Southerton & Weston, 1987) and trachea (Allen *et al.*, 1986) as does the venom of the scorpion *Leiurus quinquestriatus Hebraeus* in portal vein (Quast & Cook, 1988). Cromakalim relaxation of the vascular smooth muscle is reversed by the sulphonylurea drug, glibenclamide (Quast & Cook, 1988; Winquist *et al.*, 1988).

In cardiac muscle, cromakalim shortens the action potential duration (Cain & Metzler, 1985; Grosset & Hicks, 1986; Scholtysik, 1987; Osterrieder, 1988; Sanguinetti *et al.*, 1988). Cromakalim increases the K^+ conductance in the ventricular cell membrane of guinea-pig (Osterrieder, 1988; Sanguinetti *et al.*, 1988; Escande *et al.*, 1988). On these cells $BaCl_2$ and tetrahydroaminoacridine block in a potential-dependent manner the cromakalim-induced conductance (Osterrieder, 1988). Recently on guinea-pig ventricular cells Sanguinetti *et al.* (1988) have shown that glibenclamide, an inhibitor of adenosine 5'-triphosphate (ATP)-sensitive K^+ channels (Schmid-Antomarchi *et al.*, 1987) prevented or reversed the shortening of the effective refractory period and action potential duration produced by cromakalim. These results support the hypothesis that cromakalim activates the ATP-sensitive K^+ channels in the heart. The ATP-sensitive K^+ channels have been dis-

covered from single channel recordings by Noma (1983). The physiological significance of these channels in cardiac cells is still under debate. K^+ channel openers like cromakalim and sulphonylurea drugs like glibenclamide are useful tools for studying the role of ATP-sensitive K^+ channels in the heart.

We studied the effects of cromakalim by whole cell voltage clamp techniques in single frog heart myocytes. Our results indicate that, as on mammalian cardiac cells, cromakalim activates potassium conductance. In contrast with mammalian cells, cromakalim had no effects when applied directly to the internal face of the cell. We propose that cromakalim activates an $I_{K(ATP)}$ -like current. Some of the results described here have appeared in abstract form (Pilsudski *et al.*, 1989a).

Methods

Preparation of isolated heart cells

Single atrial and ventricular cells from *Rana esculenta* were obtained by an enzymatic dissociation method as described by Bonvallet (1987). The complete auricular tissue and small chunks of ventricular tissue were bathed in nominally calcium-free solution (see the composition of solutions) for 30 min and then transferred to calcium-free solution + collagenase (2 mg ml⁻¹) + protease (0.3 mg ml⁻¹) for 60 min at room temperature. Tissues were kept overnight in calcium-free solution at 6°C. Cells were obtained by shaking the tissues in the superfusing experimental chamber containing calcium-free solution; the latter was then replaced by Ringer modified solution.

Whole cell/experiments/recordings/analysis

Experiments were carried out with the patch clamp technique in whole cell clamp mode at room temperature (20–22°C). Bath solution was continuously perfused and an internal perfusion device allowed fast exchange of the pipette dialysing medium. Patch pipettes were made from borosilicate glass (Clark Electromed., Reading, U.K.). Their resistance was between 2–5 megaohms when filled with 'intracellular-like' medium and the seals ranged from 1 to 10 gigaoohms. Whole-cell currents were recorded with a RK 300 patch-clamp amplifier (Biologic, Echirrolles France) and stored on a PC

microcomputer using the pCLAMP software (Axon Instr., Burlingame, CH, U.S.A.). Computer programmes for measurements, computations and plots from pCLAMP files were developed in our laboratory.

Pulses protocols

After the establishment of the whole cell recording configuration, a standard double step voltage-clamp protocol was applied at a frequency of 0.1 Hz: from a holding potential of -70 mV, symmetric pulses of ± 40 mV were applied to the cell, setting the membrane potential to -30 mV for 100 ms, then to -110 mV for 100 ms. The membrane currents were measured at the end of each step: -70 mV (triangle), -30 mV (lozenges) and -110 mV (squares) (as shown in the figures).

The current voltage relationships were obtained by the application of voltage ramps (1V/s) from -140 to $+20$ mV every 10 s and smoothed for display.

Drugs and solutions

Chemicals were purchased from Boehringer Mannheim, France (Meylan, France) except pronase (Sigma, St Louis, MO, U.S.A.). Cromakalim was obtained from Beechams Research Laboratories.

The composition of the modified Ringer solution was (mM): NaCl 110, KCl 2.5, CaCl_2 1.8, MgCl_2 2, glucose 10, pyruvic acid 5, HEPES buffer 10, CoCl_2 3 and tetrodotoxin 0.001, pH adjusted to 7.2 with NaOH. CsCl (20 mM), BaCl_2 (1.8 mM), tolbutamide (1 mM), glibenclamide (1 μM) and cromakalim (1 μM) were eventually added to this solution. The calcium-free solution was the same as modified Ringer solution with MgCl_2 (2 mM) replacing CaCl_2 (1.8 mM).

Patch pipettes were filled with an 'intracellular-like' medium containing (in mM): KCl 20, K aspartate 130, KH_2PO_4 4, MgCl_2 1, K-EGTA 5, HEPES buffer 10; pH adjusted to 7.3 with KOH. CsCl (20 mM), K_2ATP (3–5 mM) and cromakalim (1 μM) were eventually added to this solution.

Results

Control experiments

All the experiments were carried out in voltage clamp on frog atrial and ventricular cells in the presence of cobalt and tetrodotoxin to block the inward currents.

Records obtained under control conditions in an atrial cell are shown in Figure 1. The current appeared to be time-independent after the decay of the capacitive transient (Figure 1a). The membrane current was much larger on hyperpolarization than on depolarization, indicating a strong inward rectification. This current could be recorded for more than 30 min without any significant changes in amplitude and kinetics (Figure 1c); 30 min after the beginning of the experiment, the 'intracellular-like' medium was changed by addition of 20 mM CsCl. The inward membrane current recorded on hyperpolarization was then rapidly and completely suppressed (Figure 1b). The amplitude of the membrane current plotted as a function of time indicated the rapid action and reversal of internal perfusion with CsCl and showed the efficiency of the internal perfusion device (Figure 1c). Figure 1d presents the current voltage relationship. The current is maximum outward near -70 mV i.e. 10 to 15 mV positive to the resting potential. A clear negative slope conductance can be noticed for potentials more positive than -70 mV. This control current shares the common properties of the inward rectifying potassium current I_{K1} of amphibians (Hume & Giles, 1983; Simmons & Hartzell, 1987).

Experiments in the presence of cromakalim

The membrane current recorded in the presence of 1 μM cromakalim added to the bath control solution is shown in

Figure 1 Membrane current recorded from frog atrial myocytes in control conditions. (a) Individual current traces recorded in control solutions with a double voltage step stimulation protocol. The zero current level is indicated by the dots at the right and at the left ends of the traces. The potential was held at -70 mV (Δ), which is close to the potential at which the current flowing through the inward rectifying potassium channels is maximally outward. The first voltage step was to -30 mV ($\diamond$). The following step was to -110 mV ($\square$). The large inward current at this potential reflects the strong inward rectification of the resting cell current. (b) Current recorded from the same cell after 50 s of dialysing with 'intracellular-like' medium with 20 mM CsCl added. (c) Membrane current recorded at -70 mV (Δ), -30 mV ($\diamond$) and -110 mV ($\square$) during 37 min of continuous recording from one frog atrial muscle cell. The current is measured at the end of each step. The bar above the graph (Cs) indicates the period for which the cell was dialysed with CsCl. The arrows below the graph indicate when the traces in (a) and (b) were recorded from this cell. (d) Current voltage relationship from an atrial cell in control conditions. This relationship was obtained by the application of voltage ramps (1V/s) from -140 to $+20$ mV and has been smoothed for display.

Figure 2b. The membrane time independent current increased at all membrane potentials. The time course of the effect of external cromakalim indicates a relatively slow action of the molecule (Figure 2c). The membrane current activated by cro-

Figure 2 Membrane current recorded from frog atrial myocytes. Effects of cromakalim added in the bath solution upon membrane current. (a) Individual current trace recorded in control conditions with the same protocol as in Figure 1. (b) Current recorded from the same cell when it was superfused with cromakalim $1\ \mu\text{M}$ added in the bath. (c) Current recorded 6 min after the removal of cromakalim from bath solution. (d) Membrane current measured at the three membrane potentials during 17 min of continuous recording from one frog atrial muscle cell. The bar above the graph (Cromakalim) indicates the period for which the bath solution was changed for one which contained $1\ \mu\text{M}$ cromakalim. The arrows beneath the graph indicate when the traces in (a), (b) and (c) were recorded from this cell.

makalim was nearly completely suppressed when returned to the control (Figure 2c,d).

Figure 3a depicts the current-voltage relationships of the current in an atrial cell in control (filled circles) and in the presence of $1\ \mu\text{M}$ cromakalim (open circles).

In the presence of cromakalim, the current was larger than in control in the inward as well as in the outward direction. However, the increase of the outward membrane current was much larger than the increase of the inward membrane current. No negative slope conductance was recorded on this curve. The curves obtained in control and in the presence of cromakalim crossed each other near the potassium equilibrium potential. The difference current (Figure 3b) rectified strongly in the outward direction.

The effect of cromakalim ($1\ \mu\text{M}$) on membrane current was also examined in single isolated ventricular cells (Figures 4 and 5). As described above for atrial cells, the time-independent membrane current was increased at -70 and at -30 mV. A very small increase was seen at -110 mV (Figure 4). In Figure 5a the current voltage relationships obtained in the presence (open circles) and in the absence (filled circles) of cromakalim ($1\ \mu\text{M}$) are superimposed. In the presence of cromakalim the current was largely increased in the outward direction and not modified in the inward direction. The difference current (Figure 5b) rectified very strongly in the outward direction.

The current activated by cromakalim in atrial and ventricular cells was clearly distinct from the inward rectifying potassium current I_{K1} . It appears similar to the current recorded in mammalian ventricular cells (Osterrieder, 1988; Sanguinetti

et al., 1988). This current had no tendency to fade for tens of minutes (not shown).

We also checked whether the addition of cromakalim ($1\ \mu\text{M}$) to the internal solution could activate the same membrane currents as the external application to the surface of the cell. When cromakalim was added in the pipette, the membrane current was unchanged for more than 30 min.

Ionic nature of the cromakalim-activated current

The experiments shown in Figure 6 were carried out on atrial cells to determine the effects of two potassium channel blockers on the increase in membrane current induced by cromakalim. Application of 20 mM CsCl (Figure 6a) or 1.8 mM BaCl₂ (Figure 6b) in the external medium after the activation of the cromakalim current dramatically reduced both inward and outward membrane currents.

Action of sulphonylurea drugs

The effects of sulphonylurea drugs, glibenclamide and tolbutamide, specific blockers of the ATP-sensitive K⁺ current (Schmid-Antomarchi *et al.*, 1987), were tested on the cromakalim-activated membrane current. The effects of glibenclamide on the atrial membrane current activated by cromakalim are shown in Figure 7. In control conditions (Figure 7a) membrane current showed the same characteristics as in Figure 1a. The effects of cromakalim are shown in Figure 7b. The addition of $1\ \mu\text{M}$ glibenclamide to the bath solution suppressed completely both the inward and outward

Figure 3 Current-voltage relationships from an atrial cell in control conditions and in the presence of cromakalim. These relationships were obtained as described in Figure 1(d) and measured at different times during the experiments. (a) Superimposed current traces recorded just after the start of whole cell recording (●) and after the superfusion of the cell with $1 \mu\text{M}$ cromakalim (○). Note the negative slope conductance in the depolarizing direction for the control current and its absence for the cromakalim current. (b) Cromakalim-induced current obtained by difference (membrane currents after addition of cromakalim, ○) – (control membrane currents, ●).

membrane current increase due to cromakalim. The effects of glibenclamide were rapidly reversed when the bath solution containing cromakalim was restored; these effects could be repeated. Similar results were obtained with glibenclamide ($1 \mu\text{M}$) in the internal medium or tolbutamide (1 mM) in the external solution. These results suggest that in frog heart myocytes cromakalim activates an ATP-sensitive K^+ -like current.

Action of intracellular ATP

In the previously described experiments the ATP nucleotide was not present in the 'intracellular-like' medium filling the patch pipette. The effects of internal ATP were examined upon the cromakalim-evoked cell current. Addition of 3 to 5 mM ATP to the intracellular medium, before or during the action of cromakalim did not modify the action of this compound.

Discussion

We describe here the effects of cromakalim on the whole-cell membrane current of atrial and ventricular frog myocytes. In the two types of cells, cromakalim activated a time-independent current; cromakalim had no effect when added to the internal medium. The activation of this current has been reported to be responsible for the decrease in the action potential duration in heart preparations (Osterrieder, 1988; Quast *et al.*, 1988; Sanguinetti *et al.*, 1988).

The reversal potential of the current activated by cromakalim was close to the potassium equilibrium potential, suggesting that this current is carried by potassium ions. We studied the effects of two potassium channel blockers upon the increase in membrane currents induced by cromakalim: 20 mM cesium and 1.8 mM barium rapidly suppressed the effect of cromakalim, confirming that the increase in conductance is due to an increase in potassium conductance as described in mammalian cardiac muscle (Scholtysik, 1987; Osterrieder, 1988; Quast *et al.*, 1988; Sanguinetti *et al.*, 1988; Escande *et al.*, 1988).

Many different types of K^+ channels have been identified in the heart (for review see Carmeliet *et al.*, 1988). The inwardly rectifying K^+ channel, the muscarinic receptor-operated K^+ channel, the Na^+ -activated K^+ channel and the delayed rectifier K^+ channel are undoubtedly not activated by cromakalim. Indeed the sulphonylurea drugs tolbutamide and glibenclamide are specific blockers of the ATP-regulated K^+ channel (Schmid-Antomarchi *et al.*, 1987). We have shown that $1 \mu\text{M}$ glibenclamide fully and selectively blocked the effect of cromakalim. We conclude that the current activated by cromakalim is the ATP-sensitive K^+ current. Such a result was first observed by Sanguinetti *et al.* (1988) and Escande *et al.* (1988) on cardiac myocytes from guinea-pig.

At first it appeared that the effects of cromakalim were not external but internal. Cromakalim could for example depress ATP production and then activate the ATP- K^+ channel. The slow time course of action of cromakalim at 20°C (generally 5 min before any effect) strengthened this idea. In contrast, the rapid and complete reversal when the drug was washed out suggested that cromakalim acted on the external face of the cell membrane. It has been shown that ATP sensitive K^+ channels can be inhibited by intracellular nucleotides such as ADP, GTP, GDP, NAD but preferentially by ATP (for review see Ashcroft, 1988; Stanfield, 1987). The cromakalim-induced current can be recorded in the presence of ATP (3 or 5 mM) in the pipette solution. We observed that addition of ATP after the increase in conductance due to cromakalim did not modify the current. This cannot be due to a poor dialysis of the cell. In frog atrial myocytes we have shown that the exchange of soluble components such as ATP between the pipette and the cytoplasm is fast enough to stop and inhibit an ATP depletion phenomenon in less than 5 min (Pilsudski *et al.*, 1989b). Thus the potassium conductance evoked by cromakalim seems independent of the ATP cell content. The most surprising finding is that cromakalim is completely inactive when applied directly in the cytosol for 30 min. Escande *et al.* (1988) reported that application of cromakalim at the intracellular side of the membrane activated an ATP-sensitive K^+ channel. According to these authors, cromakalim as well as pinacidil or RP 49356 (Escande *et al.*, 1989) must penetrate the cell before acting on K^+ -ATP channels. We cannot explain this discrepancy. The difference between the two patch-clamp modes, whole cell in our conditions and inside out in those of Escande *et al.* could be one explanation.

Vascular muscle appears to be more sensitive to the relaxant properties of cromakalim than cardiac tissues (Quast & Cook, 1988; Winkler *et al.*, 1988). Our results showing that cromakalim acts at $1 \mu\text{M}$, indicate that frog heart tissues seem to be more sensitive than mammalian (Sanguinetti *et al.*, 1988; Osterrieder, 1988).

We conclude that cromakalim activates a potassium conductance in the cell membrane of isolated cardiac myocytes of

Figure 4 Membrane current recorded in frog ventricular myocytes. Effects of cromakalim added in the bath solution upon membrane currents. (a) Membrane current recorded shortly after establishing the whole cell recording configuration. (b) Membrane current recorded 10 min after superfusing the cell with cromakalim (1 μ M). (c) Membrane current recorded 7 min after the removal of cromakalim from bath solution. (d) Membrane current measured at the three membrane potentials during 21 min of continuous recording from one frog ventricular myocyte. Same symbols as in Figure 1.

Figure 5 Current-voltage relationships from a ventricular cell in control conditions and in the presence of cromakalim. These relationships were obtained as in Figure 1d. (a) Superimposed current traces recorded just after the start of whole cell recording (●) and after the superfusion of the cell with cromakalim 1 μ M (○). (b) Cromakalim-induced current obtained by difference (membrane currents after addition of cromakalim, ○) minus (control membrane currents, ●).

Figure 6 Effects of potassium channel blockers upon membrane current evoked by extracellular application of cromakalim. The format of each graph is the same as in Figure 1c. The bars above the graph indicate periods for which 20 mM cesium (a) and 1.8 mM barium (b) were applied to the external surface of the cell.

Figure 7 Inhibition of the effect of cromakalim on an atrial cell by addition of glibenclamide (1 μM) to the external solution. (a) Membrane current recorded shortly after establishing the whole cell recording configuration. (b) Membrane current recorded 11 min after the beginning of the superfusion of the cell with cromakalim 1 μM. (c) Membrane current recorded 17 min after the superfusion of the cell and 6 min after the superfusion of the cell with cromakalim and cromakalim + glibenclamide. The effects of cromakalim and glibenclamide can be repeated.

the frog as described previously in mammalian isolated cardiac myocytes. We suggest that cromakalim activates an ATP-sensitive potassium channel. This action is completely independent of the ATP cell content.

Frog isolated cardiac myocytes appear to be very appropri-

ate models for the study of cardiac potassium conductance and new pharmacological compounds acting as blockers or openers of K^+ channels.

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