Br. J. Pharmacol. (1992), **107**, 152–155

Effects of cromakalim on the membrane potassium permeability of frog skeletal muscle *in vitro*

D.C. Benton & [1]D.G. Haylett

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT

1 The effects of the potassium channel opener, cromakalim, and its active enantiomer, lemakalim, have been investigated in frog skeletal muscle.

2 Cromakalim (30–300 μM) increased ^{86}Rb efflux from muscles loaded with the isotope, hyperpolarized the fibres and reduced membrane resistance.

3 These effects were inhibited by the sulphonylureas, glibenclamide and tolbutamide. The IC_{50} for glibenclamide inhibition of ^{86}Rb efflux was ca. 8 nM.

4 Phentolamine (300 μM) (which blocks responses to cromakalim in smooth muscle and inhibits ATP-sensitive K^+ channels in pancreatic β-cells) had no effect on the reduction in membrane resistance caused by 100 μM lemakalim.

5 Diazoxide (600 μM) had no effect on ^{86}Rb efflux.

6 The similarities of the K^+ channel activated by cromakalim in frog skeletal muscle to the channel acted on in smooth muscle and to the ATP-sensitive K^+ channel of β-cells are discussed.

Keywords: Cromakalim; skeletal muscle; glibenclamide; potassium permeability

Introduction

Cromakalim is a smooth muscle relaxant belonging to the class of agents known as 'potassium channel openers' which are of interest for potential use in hypertension and asthma. Its ability to relax smooth muscle follows from its action in increasing the potassium permeability of the cell membrane and so reduce electrical excitability (Hamilton & Weston, 1989). Since the effects of cromakalim in smooth muscle are inhibited by sulphonylureas (e.g. glibenclamide) (Quast & Cook, 1989) which appear to be selective inhibitors of adenosine 5'-triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels (Sturgess *et al.*, 1985; Ashcroft, 1988) it has been proposed that the actions of cromakalim are on this type of channel. Cromakalim has indeed now been shown to activate identified K_{ATP} channels in excised membrane patches of ventricular myocytes (Escande *et al.*, 1988) and the important requirement for this hypothesis that smooth muscle possesses K_{ATP} channels has now been demonstrated (Standen *et al.*, 1989; Kajioka *et al.*, 1991).

Skeletal muscle is also known to possess K_{ATP} channels (Spruce *et al.*, 1985) which show sulphonylurea-sensitive opening under conditions of ATP depletion (Castle & Haylett, 1987). This suggested that it would be valuable to examine the actions of cromakalim in this tissue. Additional experiments were conducted to explore the actions of phentolamine, which has been reported to block the actions of cromakalim in smooth muscle (McPherson & Angus, 1989) and to inhibit K_{ATP} channels in pancreatic β-cells (Plant & Henquin, 1990), and diazoxide which is a well known activator of K_{ATP} channels in β-cells (e.g. Trube *et al.*, 1986; Garrino *et al.*, 1989).

In concurrent studies, Spuler *et al.* (1989) have shown cromakalim to increase K^+ conductance in human skeletal muscle and Weik & Neumcke (1990), using patch recording, have shown that cromakalim can increase the open probability of single K_{ATP} channels of mouse skeletal muscle.

Some of these results have already appeared in abstract form (Benton & Haylett, 1990).

[1] Author for correspondence.

Methods

Experiments were carried out on sartorius muscles from adult *Rana temporaria*. The frogs were killed by a blow to the head followed by destruction of the brain and spinal cord. Except for electrophysiological recordings, the tissue was bathed in a Ringer solution of the following composition (mM): NaCl 116, KCl 2.5, $CaCl_2$ 1.8, NaH_2PO_4 0.25 and Na_2HPO_4 1.75 (pH 7.2). All experiments were conducted at room temperature.

^{86}Rb efflux experiments

Both sartorii were removed and tied to frames made of stainless steel tubing. The tissue was loaded with ^{86}Rb by incubation in Ringer solution containing the tracer at an activity of 0.1 MBq ml^{-1} for 90 min. At the end of the loading period the muscles were washed in a large volume of Ringer in order to remove superficial and much of the extracellular tracer. After 30 min (when the rate constant for ^{86}Rb efflux had become relatively steady) the tissue was transferred, at 2 min intervals, through a series of 15 test tubes. Each contained 5 ml of solution which was gassed via the tubular frame with 100% oxygen to provide mixing. The muscles were exposed to cromakalim or diazoxide for a 10 min period beginning 40 min after removal from the load solution. At the end of the experiment the muscles were dissolved in concentrated HNO_3 to allow the measurement of remaining radioactivity. The radioactivity lost into the washout solutions and in the muscle extract were determined by Cêrenkov counting in a scintillation counter (Beckman LS1801), a quench correction for the muscle extract being determined by internal standardization (spiking). The rate constant for ^{86}Rb efflux was expressed as the fractional loss of tracer per min. The increase in efflux stimulated by cromakalim was calculated by comparing the average rate constant during the 2nd, 3rd and 4th drug application periods with the average value for the three periods immediately preceding drug application. In experiments investigating the effect of glibenclamide one muscle from each pair was exposed to 100 μM cromakalim alone while the other was exposed to glibenclamide for 20 min before, and during exposure to the same concentration of cromakalim.

Intracellular recording

The effects of cromakalim and lemakalim on membrane potential and resistance were assessed by conventional electrophysiological techniques and employing microelectrodes filled with 3 M KCl. Fibres were impaled with two microelectrodes, one being used to measure membrane potential, the other to pass current. Changes in membrane resistance (in terms of input resistance) were detected by measuring the amplitude of electrotonic potentials caused by the injection of current pulses (400 ms, 10–20 nA). An Axoclamp 2A (Axon Instruments) was used for the measurement of membrane potential and for current passing. Trigger pulses were provided by a Digitimer D4030 (Digitimer Ltd) and recordings were made on a Devices chart recorder (MX4).

Muscles were mounted in a perspex recording chamber and superfused with Ringer solution at 4–5 ml min^{-1}. For these experiments a Cl^--free Ringer solution was used in the expectation that the removal of the large resting Cl^- conductance (Hodgkin & Nakajima, 1972) would allow changes in K^+ conductance to be observed more easily. The solution had the composition (mM): Na isethionate 116, K_2SO_4 1, $CaSO_4$ 2.4, NaH_2PO_4 0.25, Na_2HPO_4 0.75 (pH 7.2). In some experiments tetrodotoxin (10^{-7} M) was included to prevent the twitching which was sometimes induced in the Cl^--free Ringer solution.

Materials

^{86}Rubidium was supplied by New England Nuclear. Sodium isethionate was purchased from Fluka. Other reagents were of analytical grade. Tolbutamide was purchased from Sigma and the following were gifts: cromakalim and lemakalim (Beecham's Research Laboratories), glibenclamide (Hoechst), phentolamine (ICI) and diazoxide (Allen & Hanburys). Cromakalim, lemakalim, glibenclamide and tolbutamide were prepared as stock solutions in dimethylsulphoxide.

Statistics

Results are presented as means ± standard error of the mean. Differences were considered significant if P was less than 0.05 by Student's t test.

Results

^{86}Rb efflux

Cromakalim (30–300 μM) produced an increase in the rate constant for ^{86}Rb efflux. Figure 1 presents some results with 100 μM cromakalim and indicates that the response generally peaked between 2 and 6 min and showed some decline before the end of the drug exposure. Figure 2 gives information on the concentration-dependence of the response. The low potency of cromakalim, and our desire to avoid other effects of cromakalim at excessive concentrations (e.g. Ca^{2+}-channel block, Ito *et al.*, 1990) restricted our investigation of the upper part of the concentration-response curve. However, in those experiments ($n = 3$) where comparison was made within a single experiment, (and animal), 300 μM cromakalim did not produce a significantly greater increase in ^{86}Rb efflux than 100 μM. It is concluded that the maximal increase in ^{86}Rb efflux is unlikely to be much more than 200%. In three different muscles, diazoxide (600 μM) had no significant effect on ^{86}Rb efflux, the change in rate constant being − 5 ± 3%.

Glibenclamide inhibited the response to cromakalim, as shown in Figure 1. Concentrations of glibenclamide between 3 and 100 nM were tested for their ability to inhibit the response to 100 μM cromakalim, the concentration-inhibition curve being shown in Figure 3. The fitted curve provided an estimate of 8.0 ± 1.7 nM for the IC_{50}. Glibenclamide had no effect on the resting rate of efflux.

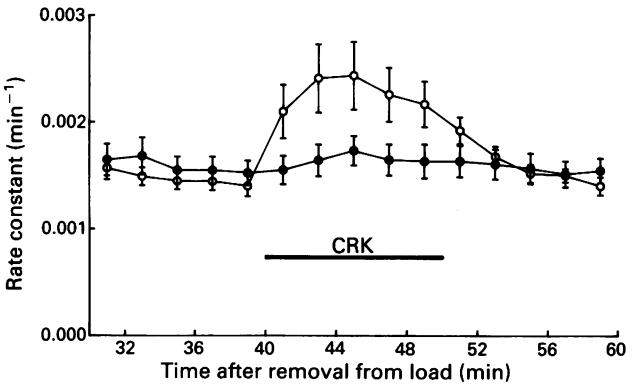

Figure 1 Effect of cromakalim (CRK) (100 μM), applied from 40 to 50 min after removal from the load solution, on ^{86}Rb efflux from frog sartorius muscle: (●) indicate the rate constants observed in experiments where glibenclamide (30 nM) was applied between 20 and 50 min; means with s.e.mean for 5 pairs of muscles are shown.

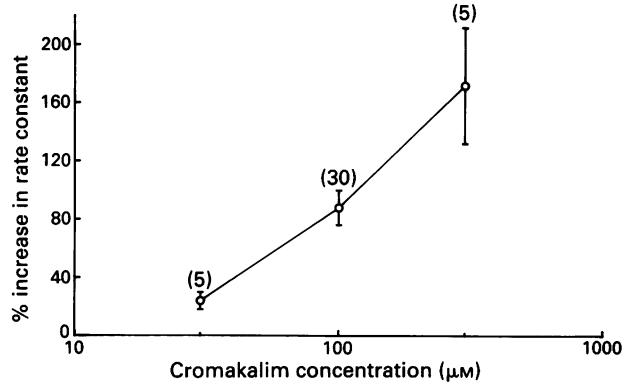

Figure 2 Concentration-response relationship for the increase in ^{86}Rb efflux rate constant produced by cromakalim (means with s.e.mean shown by vertical bars, n indicated in parentheses).

Intracellular recording

Cromakalim (100–300 μM) and lemakim (100 μM) caused cell membrane hyperpolarization and a reduction in input resistance which could be maintained for up to 10 minutes (Figure 4). Although (as previously noted) the results presented were obtained in Cl^--free Ringer these effects could be observed also in normal Ringer solution. Resting membrane potentials (uncorrected for any change in tip potential) averaged 91.5 mV (s.d. 6.1 mV, $n = 14$) and 200 μM cromakalim increased this by 7.9 ± 1.5 mV (s.e.mean, $n = 10$) and reduced 'input resistance' by 42 ± 4% ($n = 10$). In 5 fibres (from 3 frogs) 100 μM lemakalim caused a hyperpolarization of 6.6 ± 2.9 mV and reduced resistance by 49 ± 5%. It should be noted that the measurement of changes in input resistance is qualitative in that the current-passing and voltage-recording electrodes were usually 3–5 fibres diameters apart. The input resistance will therefore be underestimated but the measured percentage change in input resistance with cromakalim is likely to be increased as the space constant is reduced.

In 4 experiments, 300 μM tolbutamide completely blocked the actions of either 200 μM cromakalim or 100 μM lemakalim. Glibenclamide was more potent, 1 μM proving sufficient to abolish the response to cromakalim ($n = 3$).

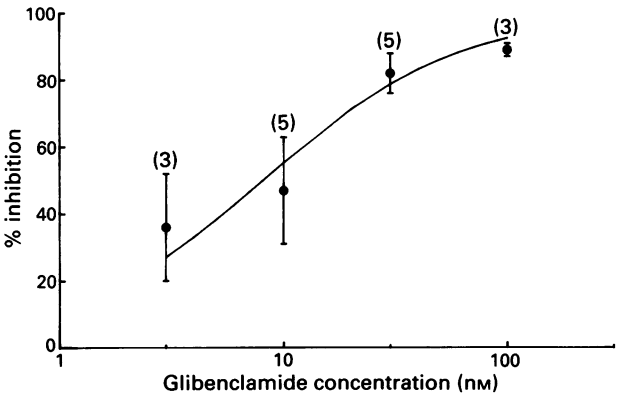

Figure 3 Relationship between glibenclamide concentration and inhibition of the ^{86}Rb efflux response to 100 μM cromakalim (means with s.e.mean shown by vertical bars, *n* indicated in parentheses). The fitted curve (non-linear least squares) is for simple competition with a Hill coefficient of unity.

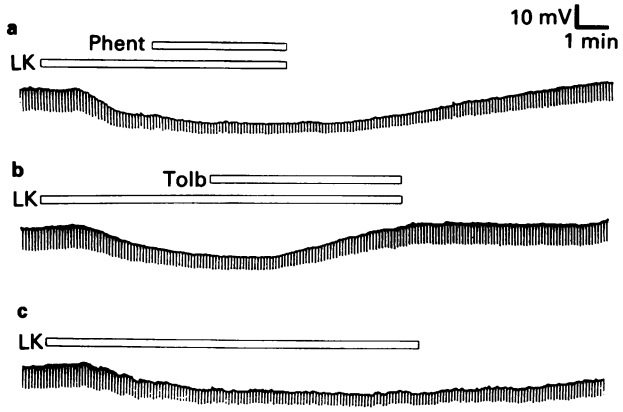

Figure 4 Effects of phentolamine and tolbutamide on the changes in membrane potential and input resistance produced by lemakalim (LK). Record (c) shows a control response to 100 μM lemakalim. In (a) 300 μM phentolamine (Phent) was added to the perfusate for the period indicated; (b) shows the reversal of the action of lemakalim by 300 μM tolbutamide (Tolb). These records are from the same fibre in the sequence (a), (b), (c). The records are broken for 17 min between (a) and (b) and for 15 min between (b) and (c). Note: the lag between the time at which drug application is indicated and any observed effect is largely attributed to the time taken for the drug to reach the muscle.

Phentolamine, tested in 3 fibres at 300 μM and 1 fibre at 100 μM, had no effect on the response to 100 μM lemakalim. Figure 4 demonstrates, in a continuous recording from a single fibre the lack of effect of phentolamine and complete block by tolbutamide.

Discussion

The increase in ^{86}Rb efflux, decrease in membrane resistance and hyperpolarization produced by cromakalim in frog skeletal muscle are each consistent with the opening of K^+ channels. As expected, lemakalim, which is the more active enantiomer of cromakalim (Hof *et al.*, 1988) reproduced these actions at a lower concentration. All of these effects could be inhibited by either glibenclamide or tolbutamide, which as described in the introduction provides *prima facie* evidence for involvement of K_{ATP} channels.

Although the electrophysiological experiments did not give a quantitative measurement of changes in specific membrane resistance, the magnitude of the change in input resistance is broadly in keeping with the 2–3 fold increase in ^{86}Rb efflux. It is worth noting that these changes in input resistance and ^{86}Rb efflux are much less than those produced by metabolic exhaustion (Fink & Luttgau, 1976; Castle & Haylett, 1987), and which are likely to involve the activation of K_{ATP} following ATP depletion. If it is supposed that K_{ATP} channel activation underlies both responses, what is the reason for the larger changes seen on poisoning? Firstly we can note that in exhausted muscle a component of the increase in ^{86}Rb efflux is not blocked by glibenclamide (Castle & Haylett, 1987), suggesting that K^+ channels other than the K_{ATP} type may be activated additionally under these conditions. Secondly, the gating of K_{ATP} channels by cromakalim is ATP-dependent. Thus Weik & Neumcke (1990) have demonstrated in mouse skeletal muscle that cromakalim will activate K_{ATP} channels that have been blocked by 0.1 mM ATP but has no effect on the open probability if ATP is raised to 1 mM. In non-poisoned frog muscle the intracellular concentration of ATP is of the order of 4 mM (Fink *et al.*, 1983) which may be sufficient to impair the effect of cromakalim.

The concentrations of cromakalim needed to increase P_K vary quite markedly between tissues, suggesting differences in the K^+ channels or their regulation. Cromakalim is most potent in smooth muscle where the EC_{50} for the increase in ^{86}Rb efflux is around 1 μM (Quast & Cook, 1989). In cardiac muscle the EC_{50} is in the range 5–30 μM (Osterrieder, 1988; Escande *et al.*, 1988; Sanguinetti *et al.*, 1988) whilst in insulin-secreting cells cromakalim is relatively ineffective (Garrino *et al.*, 1989; Dunne *et al.*, 1990). Our results in frog skeletal muscle, like those of Spuler *et al.* (1989) in human muscle suggest a potency between those in cardiac muscle and insulin-secreting cells. The relative activity of diazoxide to increase P_K in these tissues is quite different. In smooth muscle it is 100 × less potent than cromakalim (Quast & Cook, 1989) whereas in the β-cell it is more potent (Garrino *et al.*, 1989). In cardiac muscle diazoxide can *block* $P_{K(ATP)}$ (Faivre & Findlay, 1989). We have found diazoxide to have no effect on ^{86}Rb efflux in frog muscle which may thus resemble mouse skeletal muscle where diazoxide (400 μM) has no effect on K_{ATP} channel opening (Weik & Neumcke, 1990).

The complex pharmacology of cromakalim responses and K_{ATP} channels is further illustrated by our observations with phentolamine. Although phentolamine can abolish responses to cromakalim in vascular smooth muscle (McPherson & Angus, 1989) and inhibit the opening of K_{ATP} channels in β-cells (Plant & Henquin, 1990; Dunne, 1991) in the present study it was quite unable to block the change in input resistance and membrane potential produced by lemakalim.

From the results presented it is clear that cromakalim can cause the opening of K^+ channels in frog skeletal muscle. In view of the inhibition of this effect by glibenclamide and the positive identification of K_{ATP} channels in this tissue it seems likely that the ATP-sensitive K^+ channel is the target channel for cromakalim. It is also clear that the pharmacological properties of the channel differ from those of the channel acted on in other tissues.

This work was supported by a grant from the Wellcome Trust. We thank Professor D.H. Jenkinson for valuable discussions and those pharmaceutical companies mentioned in the materials section for their gift of drugs.

References

ASHCROFT, F.M. (1988). Adenosine 5'-triphosphate-sensitive potassium channels. *Annu. Rev. Neurosci.*, **11**, 97–118.

BENTON, D.G. & HAYLETT, D.G. (1990). Cromakalim increases the membrane permeability of frog skeletal muscle *in vitro*. *Br. J. Pharmacol.*, **99**, 196P.

CASTLE, N.A. & HAYLETT, D.G. (1987). Effect of channel blockers on potassium efflux from metabolically exhausted frog skeletal muscle. *J. Physiol.*, **383**, 31–43.

DUNNE, M.J. (1991). Block of ATP-regulated K^+ channels by phentolamine and other α-adrenoceptor antagonists. *Br. J. Pharmacol.*, **103**, 1847–1851.

DUNNE, M.J., ASPINALL, R.J. & PETERSEN, O.H. (1990). The effects of cromakalim on ATP-sensitive potassium channels in insulin secreting cells. *Br. J. Pharmacol.*, **99**, 169–175.

ESCANDE, D., THURINGER, D., LEGUERN, S. & CAVERO, I. (1988). The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent K^+ channels in isolated cardiac myocytes. *Biochem. Biophys. Res. Commun.*, **154**, 620–625.

FAIVRE, J.-F. & FINDLAY, I. (1989). Effects of tolbutamide, glibenclamide and diazoxide upon action potentials recorded from rat ventricular muscle. *Biochim. Biophys. Acta*, **984**, 1–5.

FINK, R., HASE, S., LUTTGAU, H.C. & WETTWER, E. (1983). The effect of cellular energy reserves and internal calcium ions on the potassium conductance in skeletal muscle of the frog. *J. Physiol.*, **336**, 211–228.

FINK, R. & LUTTGAU, H.C. (1976). An evaluation of the membrane constants and the potassium conductance in metabolically exhausted muscle fibres. *J. Physiol.*, **263**, 215–238.

GARRINO, M.G., PLANT, T.D. & HENQUIN, J.C. (1989). Effects of putative activators of K^+ channels in mouse pancreatic β-cells. *Br. J. Pharmacol.*, **98**, 957–965.

HAMILTON, T.C. & WESTON, A.H. (1989). Cromakalim, nicorandil and pinacidil: Novel drugs which open potassium channels in smooth muscle. *Gen. Pharmacol.*, **20**, 1–9.

HODGKIN, A.L. & NAKAJIMA, S. (1972). The effect of diameter on the electrical constants of frog skeletal muscle fibres. *J. Physiol.*, **221**, 105–120.

HOF, R.P., QUAST, U., COOK, N.S. & BLARER, S. (1988). Mechanism of action and systemic and regional hemodynamics of the potassium channel activator BRL 34915 and its enantiomers. *Circ. Res.*, **62**, 679–686.

ITO, Y., MIYAMORE, I., MATSUBARA, T., TAKEDA, R. & HIGASHIDA, H. (1990). Cromakalim, a vasodilator, differentially inhibits Ca^{2+} currents in NG108-15 neuroblastoma × glioma hybrid cells. *FEBS Lett.*, **262**, 313–316.

KAJIOKA, S., KITAMURA, K. & KURIYAMA, H. (1991). Guanosine diphosphate activates an adenosine 5'-triphosphate-sensitive K^+ channel in the rabbit portal vein. *J. Physiol.*, **444**, 397–418.

MCPHERSON, G.A. & ANGUS, J.A. (1989). Phentolamine and structurally related compounds selectively antagonize the vascular actions of the K^+ channel opener, cromakalim. *Br. J. Pharmacol.*, **97**, 941–949.

OSTERRIEDER, W. (1988). Modification of K^+ conductance of heart cell membrane by BRL 34915. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, 93–97.

PLANT, T.D. & HENQUIN, J.C. (1990). Phentolamine and yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic β-cells. *Br. J. Pharmacol.*, **101**, 115–120.

QUAST, U. & COOK, N.S. (1989). *In vitro* and *in vivo* comparison of two K^+ channel openers, diazoxide and cromakalim, and their inhibition by glibenclamide. *J. Pharmacol. Exp. Ther.*, **250**, 261–271.

SANGUINETTI, M.C., SCOTT, A.L., ZINGARO, G.J. & SIEGL, P.K.S. (1988). BRL 34915 (cromakalim) activates ATP-sensitive K^+ current in cardiac muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8360–8364.

SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1985). Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature*, **316**, 736–738.

SPULER, A., LEHMANN-HORN, F. & GRAFE, P. (1989). Cromakalim (BRL 34915) restores *in vitro* the membrane potential of depolarized human skeletal muscle fibres. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **339**, 327–331.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

STURGESS, N.C., ASHFORD, M.L.J., COOK, D.L. & HALES, C.N. (1985). The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet*, **31**, 474–475.

TRUBE, G., RORSMAN, R. & OHNO-SHOSAKU, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic β-cells. *Pflügers Arch.*, **407**, 493–499.

WEIK, R. & NEUMCKE, B. (1990). Effects of potassium channel openers on single potassium channels in mouse skeletal muscle. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **342**, 258–263.

(Received March 24, 1992
Revised April 30, 1992
Accepted May 6, 1992)