Characteristics of the contractile response of rabbit aorta produced by cromakalim in calcium-free solution

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1 The effect of potassium channel opening compounds has been investigated in the smooth muscle of rabbit aorta under Ca-free conditions. Examination of the characteristics of the response has been performed using cromakalim as the prototype compound.

2 In order of potency, Ro 31-6930, cromakalim, minoxidil sulphate and pinacidil each produced a contraction in rabbit aortic strips bathed in Ca-free MOPS-buffered physiological salt solution (PSS). In contrast, forskolin, glyceryl trinitrate and nifedipine each failed to increase tension under identical conditions. Cromakalim also evoked contraction of bovine trachealis muscle bathed in Ca-free PSS.

3 The contractile response to cromakalim in rabbit aortic strips was of delayed onset (15–20 min) and reached a plateau after approximately 120 min (1.8 g maximum with 1 μ M cromakalim). No cromakalim-induced tension changes were observed in either 1 mM or 2.5 mM Ca-containing PSS.

4 Raising the [KCl] of the Ca-free PSS to 65.9 mM fully inhibited the cromakalim-induced contraction in rabbit aortic strips. In addition, pretreatment of aortic strips with the sulphonylurea glibenclamide antagonized the subsequent mechanical response to cromakalim.

5 In Ca-free PSS, cromakalim (1 μ M) stimulated ⁴²K-efflux with a time-course corresponding to the contractile event. Glibenclamide (1 μ M) inhibited this cromakalim-induced ⁴²K-efflux.

6 In sharp microelectrode studies in bovine trachealis, cromakalim (10 μ M) produced a sustained membrane hyperpolarization in normal PSS. In contrast, the cromakalim-induced hyperpolarization in Ca-free PSS was not sustained. The fading of the hyperpolarization was temporally correlated with the increase in tension under these experimental conditions.

7 It is concluded that the K-channel opener-induced smooth muscle contractile response revealed in Ca-free PSS is the consequence of K-channel opening. The nature of the detailed mechanism which underlies this contractile phenomenon remains to be determined.

Keywords: Cromakalim; Ro 31-6930; minoxidil sulphate; pinacidil; rabbit aorta; bovine trachealis; K-channels; glibenclamide; ⁴²K-efflux; calcium

Introduction

Potassium (K) channel openers such as cromakalim are well-recognised as potent smooth muscle relaxants (Hamilton & Weston, 1989). These mechano-inhibitory agents exert their effects on a variety of pre-contracted smooth muscles and inhibit the development not only of spontaneous tension changes but also of a variety of agonist-induced contractions (Newgreen *et al.*, 1990; Bray *et al.*, 1991). One consequence of K-channel opening is electrical hyperpolarization which indirectly restricts Ca entry through potential-dependent plasmalemmal Ca-channels (Hamilton *et al.*, 1986). In addition, recent studies with fura-2 fluorescent microscopy have more directly shown that K-channel openers reduce both Ca entry through receptor-operated routes and intracellular Ca release in smooth muscle preparations (Yanagisawa *et al.*, 1990; Duty & Weston, 1991a; Ito *et al.*, 1991).

In complete contrast to these numerous results regarding the smooth muscle relaxant properties of the K-channel openers, the present paper describes the contractile responses to these agents which are revealed under Ca-free conditions. A preliminary account of some of these findings has already been published (Bray *et al.*, 1989; Duty & Weston, 1991b).

Methods

Tissue bath experiments

Preparation of tissues Most of the experiments were performed on strips of rabbit aorta isolated from male Half-Lop rabbits (2.5–3.0 kg; supplied by the University of Manchester Animal Unit) which were killed by stunning and exsanguination. The thoracic aorta was removed and carefully cleaned of fat and connective tissue. The aorta was cut into segments of approximately 5 mm in length. Each of these was opened along the longitudinal axis to form a flat sheet. The vascular endothelium was then removed by gentle rubbing of the intimal surface with a cotton bud moistened with physiological salt solution (PSS). A fine pin with thread attached was inserted into the mid-part of each of the longitudinally-cut edges of the segment to enable isometric tension recording. Other investigations were performed in small strips of smooth muscle (approximately 5 mm $\times$ 10 mm), which were dissected from bovine trachealis muscle obtained freshly from the Manchester Abattoir on the morning of the experiment.

Tension recordings were performed in both types of tissue. In addition, radiolabelled ion flux experiments were carried out in the aortic preparations and microelectrode experiments were performed with the trachealis preparations. In all of these studies tissues were bathed in either a Ca-containing MOPS-buffered PSS or in a Ca-free MOPS-buffered physiological salt solution containing EGTA (Ca-free PSS).

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Isometric tension recording Segments of rabbit aorta denuded of endothelium were mounted for isometric tension recording in PSS at 37°C, bubbled with 100% oxygen. Tissues were equilibrated for at least 60 min under a resting tension of 2 g. Where appropriate, tissues were subsequently transferred for equilibration in Ca-free PSS, with three changes of bathing solution at 5 min intervals, before experiments commenced in the continuing presence of Ca-free PSS. In the experiments with bovine trachealis muscle, these tissues were mounted under a resting tension of 1 g but were otherwise treated as described for the rabbit aorta denuded of endothelium.

Contractile effects of K-channel openers in Ca-free PSS Segments of aorta bathed in Ca-free PSS were exposed to the following K-channel openers by use of a cumulative protocol; cromakalim (0.1–1 μM), BRL 38226 (the inactive (+)- enantiomeric component of cromakalim: 1–100 μM), minoxidil sulphate (0.1–1 μM), pinacidil (0.1–10 μM) and Ro 31-6930 (0.01–1 μM). A contact time of at least 30 min was allowed for each concentration of agonist and the resulting increase in tension was allowed to develop fully before increasing the agonist concentration. Time-matched vehicle controls were performed and the EC_{50} values were determined for each agent following correction for vehicle effects. The effect of cromakalim was also determined in solutions containing different concentrations of CaCl_2 (0–2.5 mM) to ascertain the conditions necessary for expression of the contractile response. In addition, the effect of cromakalim was examined in bovine trachealis muscle strips in order to determine whether the mechanical changes obtained in the rabbit aorta were species- or tissue-specific.

Effects of glyceryl trinitrate, forskolin and nifedipine in Ca-free PSS In these studies we examined the ability of other smooth muscle relaxants to produce contraction in Ca-free conditions. Segments of aorta bathed in Ca-free PSS were exposed to either the guanylate cyclase stimulator, glyceryl trinitrate (0.3–10 μM), the adenylate cyclase stimulator, forskolin (0.01–1 μM), the Ca entry blocker nifedipine (300 nM) or appropriate vehicle by use of protocol described above. Changes in smooth muscle tension were measured.

Inhibitory effects of glibenclamide and high K-PSS Two characteristic effects of K-channel openers in normal PSS are their inability to relax contractions either produced by high K-PSS or by other agonists in the presence of high K-PSS and the ability of glibenclamide to antagonize K-channel opener-induced relaxations. Thus the effects of raising the $[\text{KCl}]$ of a Ca-free PSS or the addition of glibenclamide to Ca-free PSS on the contractions produced by cromakalim were examined.

Following equilibration in Ca-free PSS, segments of rabbit aorta were pre-incubated in Ca-free PSS containing glibenclamide (0.1 μM , 0.3 μM or 1 μM), 60 mM additional KCl (ie total $[\text{KCl}]$ 65.9 mM) or appropriate vehicle for a period of 20 min. Cumulative concentration-effect curves to cromakalim (1–30 μM) were then constructed in the continuing presence of the modifying condition. Only the upper part of the concentration-effect curve was determined in these experiments owing to the lengthy contact time (> 30 min) which was allowed for each concentration of cromakalim.

Measurement of ^{42}K -efflux

The ability of cromakalim to increase the rate of ^{42}K -efflux under Ca-free conditions was investigated to characterize further the changes induced by cromakalim under Ca-free conditions. Strips of rabbit aorta with endothelium removed were impaled on syringe needles, attached to a perspex gassing manifold and placed over collection vials containing PSS bubbled with 100% O_2 . The rack of vials was positioned in a thermostatically-controlled water bath at 37°C. Following

30–60 min equilibration, tissues were loaded with ^{42}K (1.57 $\mu\text{Ci ml}^{-1}$) for 3.5 h. The ^{42}K was allowed to efflux from the tissues for fourteen 4-min collection periods (the first two of which were discarded). During these periods tissues were exposed either to PSS or Ca-free PSS alone, or to cromakalim (1 μM), glibenclamide (1 μM) plus cromakalim (1 μM) or vehicle for cromakalim (0.007% ethanol) each in the continuing presence of Ca-free PSS. At the end of the final collection period the tissues were blotted for 10 s between filter pads underneath a 1 kg weight and finally placed in vials. These, together with the tubes containing the collected incubation samples, were counted for gamma-emissions over a 2-min period by use of a Packard gamma counter. Correction factors for both the background radiation and short half-life of ^{42}K (12.4 h) were automatically made. The efflux of ^{42}K is expressed as an efflux rate coefficient which indicates the efflux per min as a percentage of the radiolabel remaining in the tissue at the beginning of each particular efflux period (% per min).

Microelectrode recordings

Muscle strips obtained from rabbit aorta contain only a relatively small proportion of smooth muscle and impalements with sharp microelectrodes are made difficult by the large amount of elastin and connective tissue present. However, bovine trachealis strips are comparably rich in smooth muscle and are thus much easier to study electrophysiologically. For this reason the present microelectrode studies were performed in the trachealis strips.

Strips of isolated bovine trachealis muscle, prepared as described above, were placed in a recording chamber through which oxygenated PSS was flowing at a rate of 5 ml min^{-1} . Each strip was secured onto the Sylgard floor of the chamber by fine pins and allowed to equilibrate for 30 min. Glass microelectrodes (tip resistance approximately 50 $\text{M}\Omega$) were inserted into the smooth muscle layer and the resting membrane potential was determined by sampling the potential of a number of different cells within the muscle strip. In some experiments the strips remained in PSS, whilst in others the tissues were then exposed to Ca-free PSS and the new resting membrane potential determined after 10 min equilibration. During subsequent exposure to cromakalim, most notably in the tissues bathed in Ca-free PSS, it was difficult to maintain electrode impalements for more than 10 min. Therefore, in order to sample the membrane potential over a longer period of 120 min (corresponding with the time course of the contractile response), cell-sampling techniques were employed. The electrode was thus deliberately withdrawn from the cell following attainment of the maximum initial change in potential produced by cromakalim. The membrane potential of the muscle strip was then monitored by impaling cells at varying times over the remaining experimental period.

In one series of experiments performed in tissues equilibrated in Ca-free PSS, two strips of trachealis muscle were placed in parallel in the recording chamber. The first strip was used for membrane potential recordings as above. The second strip, which was attached to a force transducer at one end and secured to the Sylgard base of the chamber at the other end, was used to monitor the simultaneous changes in tension.

Chemicals and solutions

The following chemicals were used in this study: cromakalim and BRL 38226 (+)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol; SmithKline Beecham), EGTA and forskolin (Sigma), glibenclamide (Hoechst), glyceryl trinitrate (ICI), minoxidil sulphate and Ro 31-6930 (2-(6-cyano-2,2-dimethyl-2H-1-benzopyran-4-yl)pyridine 1-oxide; Roche Products), pinacidil (Leo) and $^{42}\text{K}_2\text{CO}_3$ (University of Manchester, Risley reactor).

The normal composition of the MOPS-buffered PSS (PSS)

was (mM): NaCl 129.7, KCl 5.9, CaCl₂ 2.5, MgCl₂ 1.2, glucose 11.1, MOPS 10. Ca-free MOPS-buffered PSS (Ca-free PSS) was prepared as above but CaCl₂ was omitted and additional MgCl₂ (8.8 mM) and EGTA (2 mM) included. The total MgCl₂ content of the solution was thus 10 mM. In one series of experiments (as indicated in the text) both EGTA (2 mM) and MgCl₂ (8.8 mM) were omitted from the Ca-free PSS. When tissues were loaded with ⁴²K, KCl was excluded from the loading PSS and replaced with ⁴²K₂CO₃ to give a final [K] of 5.9 mM. All of the solutions were adjusted to pH 7.4 with 4 M NaOH.

Data analysis

Where appropriate, data were analysed by Analysis of Variance with Student's *t* test or unpaired *t* test. Probability levels (*P*) < 0.05 were taken to indicate a significant difference between mean responses.

Results

Isometric tension studies

Effects of K-channel openers in Ca-free PSS Cromakalim (0.1–10 μ M) produced a concentration-dependent contraction of both rabbit aortic and bovine trachealis smooth muscle strips bathed in Ca-free PSS. The contraction was delayed in onset by 15–20 min as shown in the original tension recording from rabbit aorta (Figure 1a) and reached a plateau contraction after approximately 120 min. The mean size of contraction produced by 1 μ M cromakalim was 1.8 ± 0.2 g (*n* = 5) in aortic segments and 1.1 ± 0.1 g (*n* = 4) in bovine trachealis strips. In rabbit aortic strips bathed in Ca-free PSS, significant contractions were produced by all the K-channel openers tested (Figure 1b). Taking into consideration the failure to attain maximal contractions with both pinacidil and BRL 38266, approximate EC₅₀ values were obtained by

Figure 1 (a) Typical contractile response to cromakalim (CK, 1 μ M) and vehicle (Veh) in two individual strips of rabbit aorta bathed in Ca-free PSS. (b) The contractile responses following exposure to increasing concentrations of Ro 31-6930 (○), cromakalim (□), pinacidil (Δ), minoxidil sulphate (●) and BRL 38226 (■) in rabbit aortic strips bathed in Ca-free PSS. These data have been corrected for changes occurring in the equivalent vehicle controls. The points show the mean values (*n* = 5 or 6) $\pm$ s.e.mean (vertical bars).

assigning an arbitrary value of 100% to the maximum effect produced in this study. In order of potency, the estimated EC₅₀ values were as follows: Ro 31-6930 (0.08–0.01 μ M, *n* = 6), cromakalim (0.22 $\pm$ 0.09 μ M, *n* = 6), minoxidil sulphate (1.47 ± 0.37 μ M, *n* = 5), pinacidil (1.77 ± 0.37 μ M, *n* = 5) and BRL 38226 (54.00 ± 7.44 μ M, *n* = 5).

Cromakalim-induced contractions were not dependent on the presence of EGTA or of a raised [MgCl₂] in the Ca-free PSS. Thus, cromakalim still produced contraction of rabbit aortic strips bathed in a nominally Ca-free PSS containing neither elevated MgCl₂ levels nor EGTA. However, no contraction was produced in vehicle-treated control tissues or following cromakalim addition to strips which were bathed in solutions containing 1 mM or 2.5 mM Ca (Figure 2).

Effects of other smooth muscle relaxants in Ca-free PSS In contrast to the K-channel openers, smooth muscle relaxants believed to act through mechanisms which do not involve K-channels failed to produce any contraction of rabbit aortic segments bathed in Ca-free PSS (data not shown). No tension increments were seen with either forskolin (0.01–1 μ M), glyceryl trinitrate (0.3–10 μ M) or a maximally-effective relaxant concentration of nifedipine (300 nM).

Inhibition of responses to cromakalim by high [KCl] and by glibenclamide The effect of pre-incubating rabbit aortic strips in Ca-free PSS containing an elevated [KCl] is shown in the pair of original tension recordings (Figure 3a,b). Addition of 60 mM KCl produced a small sustained contraction in Ca-free PSS (total [KCl] = 65.9 mM). No such tension increase was evoked following exposure of control tissues to the vehicle for KCl (Ca-free PSS containing 5.9 mM KCl). Subsequent addition of 1 μ M cromakalim produced a contraction in the control tissue pretreated with vehicle. In contrast, no contraction was produced by cromakalim in the tissue pre-treated with additional 60 mM KCl. Maximum tension increments produced by cromakalim in the two groups of tissues were 2.1 ± 0.4 g, *n* = 6 (3 μ M cromakalim; [KCl] = 5.9 mM) and 0.0 ± 0.1 g, *n* = 6 (10 μ M cromakalim; [KCl] = 65.9 mM).

When compared to vehicle (maximum of 0.01% ethanol), glibenclamide (0.1–1 μ M) produced a significant concentration-dependent inhibition of the contractile response to cromakalim in Ca-free PSS (Figure 3c). The response to cromakalim (1 μ M), which usually produced a near-maximal contraction, was fully inhibited. However, the maximum contraction produced by raising the concentration of cromakalim remained unchanged.

Figure 2 The response to cromakalim in rabbit aortic strips bathed in 2.5 mM Ca-PSS (○), 1 mM Ca-PSS (□), Ca-free PSS (ie containing 2 mM EGTA and a total [MgCl₂] of 10 mM) (●) and nominally Ca-free PSS (ie containing no EGTA and a total [MgCl₂] of 1.2 mM) (■). The points show the mean values (*n* = 4 or 5) $\pm$ s.e.mean (vertical bars). *Indicates a significant contractile effect of cromakalim in the Ca-free solutions compared to normal PSS (*P* < 0.05).

Figure 3 The contractile response produced by cromakalim (CK) in individual strips of rabbit aorta bathed in Ca-free PSS following pretreatment with (a) vehicle (Veh) for KCl (Ca-free PSS alone) or (b) 60 mM KCl. (c) The effect of pretreatment with glibenclamide, 0.1 μM (●), 0.3 μM (■) and 1 μM (▲) or vehicle (○) on the response to cromakalim in rabbit aortic strips bathed in Ca-free PSS. The points show the mean values ($n = 6$) $\pm$ s.e.mean (vertical bars). *Indicates a significant difference between the effects of glibenclamide and its vehicle.

⁴²K-efflux studies

The basal rate of ⁴²K-efflux from rabbit aortic strips was increased from a maximum of 0.38% per min to a maximum of 0.59% per min following incubation in Ca-free PSS. Despite this elevated baseline, cromakalim produced an approximate three fold increase in the ⁴²K efflux rate and this increase was fully inhibited by pretreatment with glibenclamide (1 μM for 15 min; Figure 4a). In tissues bathed in Ca-containing PSS, cromakalim (1 μM) produced a more modest two fold increase in ⁴²K-efflux rate which was likewise inhibited by pretreatment with glibenclamide (data not shown). Glibenclamide (1 μM) alone produced no change in the basal efflux for ⁴²K in tissues bathed either in PSS or in Ca-free PSS. In a separate experiment, the maximal cromakalim-induced increase in ⁴²K-efflux rate from aortic strips bathed in Ca-free PSS was found to persist over a time period consistent with that of the cromakalim-induced contraction (Figure 4b).

Microelectrode studies

The resting membrane potential of trachealis strips bathed in PSS was -62.5 ± 1.4 mV ($n = 20$ impalements from 4 tissues). Cromakalim (10 μM) produced a shift in membrane potential towards negative values (Figure 5a) and the maximum hyperpolarization obtained was 22.5 ± 2.6 mV ($n = 4$ impalements from 4 tissues). The membrane hyperpolarization was well-maintained throughout the period of exposure to cromakalim (approximately 160 min; Figure 5c). The mean resting membrane potential in the second group of trachealis strips was -63.7 ± 1.2 mV ($n = 39$ impalements from 7 tissues). Following equilibration of these strips in Ca-free PSS, the membrane potential decreased to a new

Figure 4 (a) The effect of cromakalim (1 μM) on the rate of ⁴²K-efflux from rabbit aortic strips bathed in Ca-free PSS in the absence (●) and presence (■) of glibenclamide (1 μM). The basal efflux rate is also shown (○). The points show the mean values ($n = 6$) $\pm$ s.e.mean (vertical lines). The thin and thick horizontal bars indicate the periods of exposure to glibenclamide (or vehicle) and glibenclamide (or vehicle) plus cromakalim, respectively. *Indicates a significant difference between cromakalim-induced efflux compared to both basal efflux and cromakalim-induced efflux in the presence of glibenclamide ($P < 0.05$). (b) The effect of prolonged exposure to cromakalim, 1 μM (●) or vehicle (○) on the rate of ⁴²K-efflux from strips of rabbit aorta bathed in Ca-free PSS. The points show the mean values ($n = 6$) $\pm$ s.e.mean (vertical lines). The horizontal bar represents the period of exposure to cromakalim.

resting level of -47 ± 2.0 mV ($n = 11$ impalements from 6 tissues). Subsequent exposure to cromakalim (10 μM) produced an initial membrane hyperpolarization (maximum hyperpolarization 24.4 ± 0.9 mV; $n = 3$ impalements from 3 tissues; Figure 5b). In contrast to the sustained hyperpolarization observed with cromakalim in PSS, the cromakalim-induced hyperpolarization in Ca-free PSS was not sustained. Over a period of approximately 80 min, the membrane potential returned to pre-cromakalim levels and after longer exposure, a mean depolarization of about 10 mV was observed (Figure 5c).

During the contact period with cromakalim (10 μM) in Ca-free PSS the tension in trachealis strips, mounted in parallel to the strip used for electrical recording, increased to a maximum of 2.2 ± 0.1 g ($n = 3$). This period of increasing tension followed the time-course of the fade in membrane hyperpolarization (Figure 6). Correlation coefficients determined from the scatter plots related these two variables (ie fade in membrane hyperpolarization and increase in tension) with a mean correlation coefficient of 0.76 ± 0.01 which did not deviate significantly from linearity.

Discussion

In the present study, a contractile response was always evoked when segments of rabbit aorta bathed in Ca-free, MOPS-buffered PSS were exposed to K-channel openers. Segments of bovine trachealis muscle bathed in Ca-free PSS

Figure 5 The early hyperpolarizing response to cromakalim (CK, 10 μ M) in bovine trachealis strips bathed in PSS (a) or in Ca-free PSS (b). The effects of prolonged exposure to cromakalim on membrane potential in strips bathed in PSS (●) or in Ca-free PSS (○) are shown in (c). The points show the mean values ($n = 2$ to 6) $\pm$ s.e.mean (vertical bars).

Figure 6 The effect of cromakalim (10 μ M) on simultaneous recordings of membrane potential (●) and tension (○) in trachealis strips in Ca-free PSS. The points show the mean values ($n = 3$) $\pm$ s.e.mean (vertical bars).

produced a similar contraction in response to the prototype compound, cromakalim. Although identical Ca-free solutions were used in an earlier investigation of the modulatory role of K-channel openers in intracellular Ca storage and release experiments (Bray *et al.*, 1991), a K-channel opener-induced increase in tension has not been previously demonstrated. However, in the experiments of Bray *et al.* (1991), K-channel openers were in contact with tissues bathed in Ca-free PSS for a maximum period of 15 min, before the addition of potent spasmogens. Since there was at least a 15 min delay before the onset of the K-channel opener-induced contractions under these experimental conditions it is not surprising that such effects remained undetected in the previous study.

The cromakalim-induced contraction was dependent on the absence of Ca from the bathing solution. However, the response did not require the presence of either the elevated levels of $MgCl_2$ or of EGTA in the Ca-free PSS since the contractions were also obtained in a nominally Ca-free PSS which contained neither of these modifications. There was some decrease in the potency of cromakalim in the nominally Ca-free PSS compared with the Ca-free PSS, (containing EGTA and raised Mg). It thus seems likely that inclusion of EGTA which is known to produce smooth muscle membrane destabilization even in very low concentrations (50 μ M; Guan *et al.*, 1988), may in some way potentiate the contractile response to cromakalim.

The contractile response in Ca-free PSS was not a feature unique to cromakalim. Rather the response was observed with all the structurally-disparate K-channel openers tested and was elicited at concentrations similar to those which produce the relaxant responses in PSS. The rank order of potency of these agents (Ro 31-6930 > cromakalim > minoxidil sulphate > pinacidil > BRL 38226) was also similar to that found for the relaxant effects of the K-channel openers in a variety of other experimental systems (Duty *et al.*, 1990; Newgreen *et al.*, 1990; Paciorek *et al.*, 1990; Piper *et al.*, 1990). The observed low potency of BRL 38226 (the (+)-enantiomeric component of cromakalim) is consistent with previous findings that this enantiomer is the less potent relaxant and hypotensive component of cromakalim (Buckingham *et al.*, 1986; Hof *et al.*, 1988).

To verify that the contractile response was a specific feature related to K-channel opening, the ability of other smooth muscle relaxants, devoid of K-channel opening activity, to initiate a similar contractile response in Ca-free PSS was investigated. These studies revealed that neither forskolin, glyceryl trinitrate nor nifedipine produced any contraction under these experimental conditions. This indirectly suggests that the contractile response to cromakalim does not involve stimulation of either adenylate or guanylate cyclases or inhibition of Ca entry through dihydropyridine-sensitive Ca channels. From these findings it is concluded that the observed contraction is a unique feature of the K-channel openers and which is not shared by the other classes of smooth muscle relaxant tested.

Having established that this response was a characteristic of K-channel openers, we examined whether the contraction could be modified by procedures known to inhibit the relaxant effects of these agents. The first of these, which is a diagnostic feature of agents like cromakalim (Buckingham *et al.*, 1989; Murray *et al.*, 1989; Newgreen *et al.*, 1990; Piper *et al.*, 1990) was the ability of glibenclamide, a blocker of the ATP-sensitive K-channel in cardiac myocytes (Escande *et al.*, 1988) and pancreatic β cells (Zünkler *et al.*, 1988), to inhibit the response. Pretreatment with this agent produced a competitive-like inhibition of the K-channel opener induced contraction to cromakalim, consistent with an association between this event and the opening of glibenclamide-sensitive K-channels.

Relaxations induced by K-channel openers are also inhibited in PSS containing high concentrations of KCl (usually ≥ 40 mM). Elevation of extracellular K depolarizes the smooth muscle cells and shifts the potassium equilibrium potential (E_K) to a less negative value (Hamilton *et al.*, 1986). Under these experimental conditions the relaxant efficacy of cromakalim is markedly reduced or even abolished as has been widely reported for a variety of smooth muscles (Allen *et al.*, 1986; Hamilton *et al.*, 1986; Hollingsworth *et al.*, 1987; Foster *et al.*, 1989). Likewise, in the present study cromakalim failed to produce any contractile response in rabbit aortic strips following pre-incubation in Ca-free PSS containing an elevated [KCl] of 65.9 mM. This finding adds further to the similarities between the pharmacological characteristics of the contractile response and those of other K-channel opener-induced responses in normal PSS. The tension increment produced in the present study by elevating the [KCl] of

Ca-free PSS *per se* may result from depolarization-induced release of intracellular Ca (Kobayashi *et al.*, 1986).

The ^{42}K -efflux studies showed that cromakalim produced an approximately 3 fold increase in the rate of ^{42}K -exchange from strips of rabbit aorta bathed in Ca-free PSS. Consistent with the ability of glibenclamide to inhibit the cromakalim-induced contraction, this rise in ^{42}K -efflux was fully inhibited following pre-treatment with glibenclamide. Sulphonylurea-sensitive K-efflux in normal Ca-containing PSS has been observed both in the present study (data not shown) and on many previous occasions (Buckingham *et al.*, 1989; Quast & Cook, 1989; Newgreen *et al.*, 1990). In all these cases, the increase in K-efflux was lower than that produced by cromakalim under the present Ca-free conditions. In Ca-free solutions there was an elevated basal efflux rate which is thought to reflect destabilization of the plasmalemma. This elevated K-permeability is often experimentally restricted by the inclusion of raised levels of Mg in the bathing solution (Martin & Gordon, 1983; Coldwell & Howlett, 1988). Although this procedure was adopted in the present study, there still remained an increased basal level of ^{42}K efflux, in agreement with more recently published data (Post *et al.*, 1989; Cox, 1990). Clearly this destabilizing effect of Ca-free solution may explain why the increments in efflux produced by cromakalim were higher in Ca-free compared to Ca-containing PSS.

Complex electrical changes occurred when bovine trachealis strips bathed in Ca-free PSS were exposed to cromakalim. Initially, a significant membrane hyperpolarization was observed in both Ca-containing and Ca-free PSS on exposure to cromakalim. Such an increase in membrane potential is a well-documented effect of cromakalim in Ca-containing PSS (Allen *et al.*, 1986; Hamilton *et al.*, 1986; Foster *et al.*, 1989; Bray *et al.*, 1991). However, in contrast to the well-maintained hyperpolarization observed in Ca-containing PSS, the cromakalim-induced hyperpolarization in Ca-free PSS was found to fade gradually leading eventually to a depolarization relative to pre-cromakalim levels. The reason for this phenomenon is not understood although a similar effect has been described for adrenaline-induced hyperpolarization of guinea-pig taenia caeci. Like the response to cromakalim in trachealis, these adrenaline-induced hyperpolarizations of taenia caeci are well-maintained in Ca-containing PSS yet exhibit fading in Ca-free PSS. Intracellular Ca release and the subsequent opening of Ca-dependent K channels are thought to be responsible for the adrenaline-induced hyperpolarization and the non-sustained nature of the response in Ca-free PSS may reflect depletion of internal Ca stores (Den Hertog & Van Den Akker, 1987). Whether the present fade also reflects such a depletion of Ca stores cannot be determined from the present data. Indeed, the Ca-dependency of the cromakalim-sensitive K-channel is still subject to controversy (e.g. Beech & Bolton, 1989; Gelband *et al.*, 1989; Noack *et al.*, 1992) and the state of the intracellular Ca stores during the K-channel opener-induced contractile response remains unknown. Clearly further work is needed to clarify this matter.

Whatever the cause of the non-sustained hyperpolarization, it is arguably an important event considering the temporal correlation between this response and the gradual rise in tension observed with cromakalim in Ca-free PSS. The possibility remains, therefore, that this reduction in the hyperpolarization is of paramount significance in the expres-

sion of the contractile response. Such a fall in membrane potential may indeed initiate some secondary intracellular event associated with the resultant tension generation. However, as yet no causal relationship has been demonstrated between these two parameters. Furthermore, the lack of Ca together with the presence of EGTA in the PSS makes it unlikely that residual extracellular Ca entering through voltage-dependent Ca-channels is responsible for the contraction.

Although the time course of the cromakalim-induced contraction differs markedly from typical intracellular Ca release phenomena, the absence of extracellular Ca during the response argues in favour of a contributory role of such Ca release. For some time links have been proposed between intracellular Ca release and K-channel modulation in the sarcoplasmic reticular membrane of skeletal muscle (Somlyo *et al.*, 1981). In functional studies in a diversity of preparations, Ca release from internal stores such as those in the sarcoplasmic and endoplasmic reticulum of skeletal muscle has been shown to require a countercurrent flow of K^+ in order to maintain electroneutrality across the membrane of the store (Muallem *et al.*, 1985; Fink & Stephenson, 1987; Shah & Pant, 1988). In the study of Fink & Stephenson (1987), K-channel blockade in skeletal muscle increased the loading and subsequent agonist-induced release of Ca from internal stores. Consistent with this effect, K-channel opening (induced by cromakalim) reduced subsequent release and/or refilling of intracellular Ca stores in rabbit renal artery and aorta (Wilson & Cooper, 1989; Bray *et al.*, 1991). Since K-channel blockade was shown to inhibit leakage of Ca from the internal store in skeletal muscle by reducing transmembrane K-conductance, it follows that K-channel opening may induce leakage from the Ca store in smooth muscle by enhancing the K-conductance of this membrane. Such an increased K-movement across the membrane could in turn induce an enhanced leak of Ca from the store leading to its eventual depletion. The outcome of such a response could have produced the effects described by Bray *et al.* (1991) and Wilson & Cooper (1989). In direct connection to the present study, this speculative Ca leak may feasibly account for both the non-maintained hyperpolarization and the tension generation observed with cromakalim in Ca-free solution. However, recently-published data in smooth muscle cells isolated from rabbit trachea suggest that BRL 38227 does not release intracellular calcium *per se* although it does inhibit release and re-uptake of this calcium source (Chopra *et al.*, 1992). Clearly further work is required to clarify these issues and to determine the potential significance of these findings with respect to the present contractile response.

Smooth muscle contractions associated with the release of Ca from intracellular stores generally exhibit a much faster time-course than that of the cromakalim-induced increase in tension (Iino, 1990). In contrast, the time-dependency of the response to cromakalim was similar to that of the smooth muscle contractions produced by phorbol esters (under both Ca-containing and Ca-free conditions) via activation of protein kinase C (Sybertz *et al.*, 1986; Khalil & van Breemen, 1988). Whether a similar mechanism involving activation of this enzyme accounts for the cromakalim-induced contraction is the subject of current investigations.

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