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Levcromakalim-induced modulation of membrane potassium currents, intracellular calcium and mechanical activity in rat mesenteric artery

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Abstract. In freshly-dispersed cells from rat mesenteric artery, levcromakalim (1 and 10 μM) induced a non-inactivating potassium current (I_{KCO}), an event which was associated with increased current noise. I_{KCO} was fully inhibited in the presence of 10 μM glibenclamide. Stationary fluctuation analysis of the current noise associated with I_{KCO} induced by levcromakalim at a holding potential of −10 mV indicated that the unitary conductance of the underlying K-channels was 10.2 pS at 0 mV under the quasi-physiological conditions of the experiment.

In isolated arterioles both levcromakalim (10 nM–10 μM) and nifedipine (10 nM–10 μM) each elicted full, concentration-dependent, parallel reductions of the increases in $[Ca^{2+}]_i$ (assessed using fura-2) and tension induced by 10 μM noradrenaline. However, the effects of both drugs on KCl-induced increases in tension and in $[Ca^{2+}]_i$, did not follow a simple relationship. Levcromakalim relaxed KCl- and noradrenaline-induced sustained contractions with a similar potency. This was in contrast to nifedipine which was approximately 20 times more potent against KCl-induced contractions.

It is concluded that levcromakalim relaxes rat mesenteric arterioles primarily by the opening of a small conductance, glibenclamide-sensitive K-channel. An additional action of levcromakalim is suggested by its relative inability to suppress the increase in $[Ca^{2+}]_i$ produced by 30 mM K^+-PSS.

Key words: Levcromakalim – Whole-cell patch-clamp – Fura-2 – Mesenteric artery – Unitary conductance – K-channels – Nifedipine – Noradrenaline

Introduction

In spite of the many electrophysiological studies on the action of potassium (K) channel openers in vascular smooth muscle (Beech and Bolton 1989; Klöckner et al. 1989; Standen et al. 1989; Kajioka et al. 1990, 1991; Okabe et al. 1990; Clapp and Gurney 1991) there is no clear consensus regarding the nature of the channel responsible for mediating the vasorelaxant effects of these compounds. In veins, evidence is emerging that a relatively small conductance (10–30 pS under quasi-physiological conditions), glibenclamide- and ATP-sensitive K-channel (K_{ATP}) is the target for the K-channel openers (Kajioka et al. 1990, 1991; Noack et al. 1992a, b). However, in small arterioles, a K_{ATP} with a much higher unitary conductance (135 pS under asymmetrical K^+ gradient; $[K^+]_o = 60$ mM, $[K^+]_i = 120$ mM) has been implicated not only in the actions of K-channel openers, but also in the effects of several endogenous vasodilators (Standen et al. 1989; Nelson et al. 1990).

In addition to their reported electrophysiological actions, the K-channel openers can exert effects on the intracellular calcium concentration ($[Ca^{2+}]_i$) in smooth muscle. For example, levcromakalim inhibits the refilling of noradrenaline-sensitive Ca stores in the rabbit aorta (Bray et al. 1991) and trachea (Chopra et al. 1992). Furthermore, in rabbit mesenteric arteries levcromakalim also inhibits the increase in $[Ca^{2+}]_i$ generated by noradrenaline, an effect possibly mediated by an inhibition of agonist-induced inositol trisphosphate ($InsP_3$) synthesis (Ito et al. 1991b; Itoh et al. 1992).

The aim of the present study was to analyse in detail the actions of levcromakalim in small arterioles from the rat mesenteric arcade. A key objective was to determine whether the target K-channel of this agent exhibited the small unitary conductance previously described in veins (Kajioka et al. 1990, 1991; Noack et al. 1992a) or the much larger conductance previously reported in arterioles (Standen et al. 1989). Furthermore, any actions of levcromakalim on intracellular Ca-handling and tension were examined by comparing its effects with those of nifedipine on KCl- and noradrenaline-induce changes in these vessels.

A preliminary account of some of these studies has been presented (Criddle et al. 1992).

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Methods

Production of isolated cells. Male Sprague-Dawley rats (150–200 g) were killed by stunning and cervical dislocation and the mesenteric arterial arcade removed by careful dissection. The vessels were cleaned of fat and connective tissue while bathed in a nominally Ca-free physiological salt solution (PSS) (Klöckner and Isenberg 1985). The vessels were places in a collagenase solution (Sigma, Type VIII), agitated for 30 min at 37 °C, and single cells were then dispersed by trituration with a wide-bore pipette in Kraftbrühe (KB-medium; Klöckner and Isenberg 1985). Cells were stored at 4 °C in KB-medium and used within 8 h of separation.

Single-cell electrophysiology. The methodology used in the present study has previously been described in detail (Noack et al. 1992a). Briefly, experiments were performed using the whole cell configuration of the patch-clamp technique (Hamill et al. 1981). The settling time of the system was less than 500 µs. Patch pipettes were pulled from Pyrex glass (H 15/10, Jencons, UK) and had resistances of 3–4 MΩ when filled with internal (intracellular) solution.

Voltage commands, data acquisition and analysis were performed as described by Noack et al. (1992a). Leakage current was estimated at the end of each experiment by addition of 5 mM $CdCl_2$ to the extracellular solution (Noack et al. 1992a) and the leak resistance ranged from 3–5 GΩ. The cadmium-resistant current was not subtracted from the current traces or from the derived current-voltage relationships shown in this paper. In each experiment the currents evoked by voltage steps from the stated holding potential were measured at their peak level. The effects of levcromakalim were assessed by superfusion of the cells with a known concentration of this drug dissolved in the external solution at a constant flow rate of 1 ml min^{-1}. All experiments were performed at 26 °C.

Measurement of $[Ca^{2+}]_i$. Male Sprague-Dawley rats were killed by cervical dislocation and the mesenteric arcade was excised. The tissue was then pinned out in a Sylgaard-coated petri dish and after the removal of fat and connective tissue, the 3rd-4th branches of the mesenteric artery was removed. Alterations in cytosolic Ca were monitored by observing the change in fluorescence of a fura-2 loaded vessel which was excited at alternating wavelengths by light generated from a 75 W xenon lamp (Grynkiewicz et al. 1985).

Each vessel was loaded with fura-2 by incubating the tissue in normal PSS containing 5 µM fura-2 AM and 0.02% cremophor EL at 4 °C for 2 h. After this time the vessel was fixed between two supports in a culture dish (Nunclon, Inter Med) and positioned on the stage of a Nikon inverted microscope. The tissue was washed with normal PSS for 10 min and then excited through a Nikon x10 objective with light alternating at a frequency of 1 Hz between the wavelengths of 340 nm and 380 nm. Fluorescence from the vessel was directed via a band-pass dichroic mirror (Nikon) to a photomultiplier tube (Thorn EMI). The changes in excitation wavelength and subsequent collection of data were controlled by a software system developed by Newcastle Photometric Systems (version 3.2).

Fluctuations in $[Ca^{2+}]_i$ produced by noradrenaline, KCl, levcromakalim and nifedipine were assessed in two ways. In one of these, no attempt was made to calibrate the changes in fura-2 fluorescence. The raw fluorescence ratio was taken as the indicator of $[Ca^{2+}]_i$ and the baseline fluorescence was taken as zero percent for the calculation of percentage changes (Sato et al. 1988; Roe et al. 1990). Additionally, however, the system was calibrated by defining maximum and minimum fluorescence ratios. To accomplish this, vessels were initially superfused with normal PSS to determine a basal fluorescence signal and they were then challenged with a high potassium PSS containing 90 mM K^+ to obtain a fluorescence signal which was designated the maximum (R_{max}). The minimum signal (R_{min}) was defined as 75% of the basal ratio. Values for $[Ca^{2+}]_i$ following various procedures were then calculated (equation 5: Grynkiewicz et al. 1985) assuming that the K_d for fura-2 was 224 nM (Grynkiewicz et al. 1985).

To determine the effects of drugs, the vessel was initially challenged with 90 mM K^+ and after a 10 min washout period, the tissue was then exposed to either a raised extracellular potassium concentration ($[K^+]_o$) or to noradrenaline (10 µM) to elevate $[Ca^{2+}]_i$. The effects of these agents in the presence and absence of levcromakalim or nifedipine were then assessed using a 5 min contact time per concentration of relaxant to minimize the extent of possible photochemical damage to the fura-2.

Tension measurements. Mesenteric arteries were removed as detailed above and mounted in an isometric myograph (JP Trading, Aarhus) using fine, 40 µm diameter, mounting wire. The vessels were set to a normalized internal circumference corresponding to 0.9 times the value that would be obtained if the vessel were relaxed and under a transmural pressure of 100 mmHg. Vessels were given three standardizing challenges of 90 mM K^+-PSS and then exposed to either 30 mM K^+-PSS or to increasing concentrations of noradrenaline. The effects of levcromakalim or nifedipine on the tension developed by 30 mM K^+-PSS or 10 µM noradrenaline were then assessed.

Drugs and solutions. In the electrophysiological experiments, the normally Ca^{2+}-free PSS in the bath (external solution) had the following composition (mM); NaCl 125, KCl 4.8, $MgCl_2$ 3.7, KH_2PO_4 1.2, glucose 11, HEPES 10, EGTA 1.0 buffered with NaOH to pH 7.3; aerated with O_2. The pipette (internal) solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, glucose 11, HEPES 10, EGTA 1.2, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, buffered with KOH to pH 7.3.

In the tension and epifluorescence studies, the normal PSS contained the following (mM): NaCl 117, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.6, glucose 11, $NaHCO_3$ 25; bubbled with 95% O_2 5% CO_2 at 37 °C. Whenever high K^+ solutions were used the $[Na^+]$ was lowered to ensure that the modified solution was isosmotic with normal PSS.

Levcromakalim (SmithKline Beecham, UK) and glibenclamide (Hoechst, Germany) were dissolved as a stock solutions in 70% ethanol and dilutions were made each day. Noradrenaline (Sigma) was prepared as a stock solution in 0.1 N HCl with a small amount of ascorbic acid. Nifedipine (Sigma) was prepared as a stock solution in 70% ethanol whilst illuminated by sodium lamp and all dilutions and experiments were performed under the same illumination. Fura-2 AM was obtained from Molecular Probes courtesy of Cambridge Biochemicals. Cremophor EL was a gift from BASF.

Results

Whole-cell currents in calcium-free PSS

In voltage-clamp mode, sequential depolarising steps from holding potentials of −90 mV and −10 mV elicited outward currents (Fig. 1a, b, respectively) within which several components could be distinguished. On stepping to potentials more positive than −30 mV from a holding potential of −90 mV, a transient, voltage-dependent current was apparent. This slowly-inactivating current was qualitatively comparable to the delayed rectifier current ($I_{K(V)}$) recently described in the rat portal vein (designated I_{TO}; Noack et al. 1992a) and more fully described in guinea pig portal vein cells by Pfründer and Kreye (1992). In the latter study, the authors used test pulses of 10 s duration and observed complete inactivation of $I_{K(V)}$ whereas in the present experiments a shorter test period was used and therefore only partial inactivation of $I_{K(V)}$ was obtained.

When the membrane potential was stepped from a holding potential of −10 mV to the same test potentials as those employed when the holding potential was −90 mV (Fig. 1b: control), $I_{K(V)}$ became inactivated and a complex of non-inactivating, voltage-independent background currents was observed. The difference be-

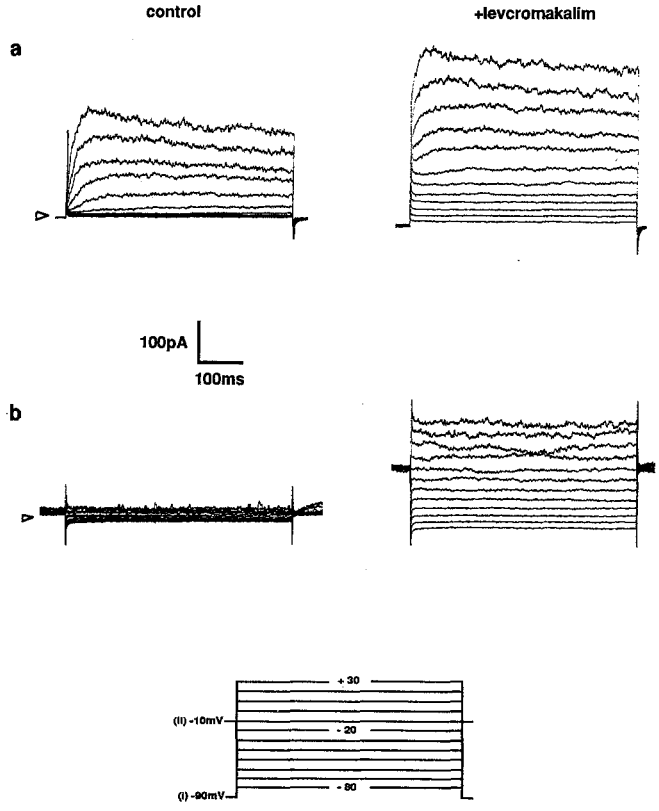

Fig. 1a, b. Representative traces showing the effects of levcromakalim on whole cell currents in a single mesenteric arterial smooth muscle cell bathed in a calcium-free PSS (containing 1 mM EGTA). The cell was held at either **a** −90 mV or **b** −10 mV and stepped for 500 ms from −80 mV to +30 mV in 10 mV increments in the absence or presence of levcromakalim (10 μM). The *arrows* indicate zero current

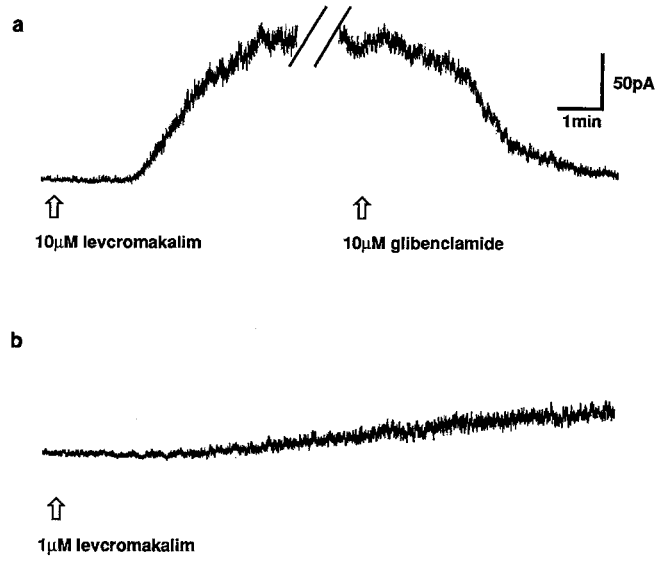

Fig. 2a, b. Typical traces showing the effects of **a** 10 μM and **b** 1 μM levcromakalim on the current associated with a holding potential of −10 mV. Note the onset of the levcromakalim-induced current (I_{KCO}) was associated with an increased current noise and that the rate of onset was concentration-dependent. This current was fully reversed by glibenclamide (10 μM) (**a**)

tween the non-inactivating current and zero current was designated I_{NI} (Noack et al. 1992a). Cells from the rat mesenteric artery appeared not to possess the very fast activating and inactivating, voltage-dependent, A-like current described in portal veins of the rabbit (Beech and Bolton 1989) and rat (Noack et al. 1992a). However, it is possible that such a current may have been obscured by the decay of capacitative transients and therefore it could not be clearly defined under the present experimental conditions (Fig. 1a).

Effects of levcromakalim on whole-cell currents

In rat mesenteric arterial cells held at a potential of −10 mV, levcromakalim (1 and 10 μM) elicited a slowly-developing, outward current (I_{KCO}; mean amplitude 52.5±14.6 pA and 85.8±13.2 pA, respectively ($n = 5$)), associated with an increased current noise (Fig. 2a, b). This current was fully reversed by glibenclamide (10 μM) (Fig. 2a). The onset of I_{KCO} was dependent on the concentration of levcromakalim used and required 3.2±0.2 min ($n = 5$) to achieve its full magnitude when induced by 10 μM levcromakalim compared with 7.8±0.9 min ($n = 5$) in the presence of 1 μM levcromakalim (Fig. 2a, b). In contrast, when the cells were clamped at a holding potential of −90 mV (close to the calculated potassium equilibrium potential of −83 mV), the same concentrations of levcromakalim produced no such effect or induced a small inward current, indicating that I_{KCO} was K-current (Fig. 1a, b: compare control and levcromakalim traces).

In order to examine the effects of levcromakalim on whole-cell K-currents in the mesenteric artery in more detail, current-voltage relationships (I–V curves) were investigated under control conditions and in the presence of levcromakalim at holding potentials of −90 mV (Fig. 3a, b). The outwardly-directed current was measured at its peak which occured between 100 ms and 300 ms after the start of the depolarising pulse from a holding potential of −90 mV (Fig. 3a). In the absence of levcromakalim under these conditions, this peak current comprised I_{NI} and the delayed rectifier current $I_{K(V)}$. On exposure to levcromakalim, the current reflected the sum of $I_{K(V)}$ and I_{NI} together with the effects of levcromakalim on one or both of these components and any new current (I_{KCO}) induced by levcromakalim and which was not present under control conditions. In the presence of levcromakalim (10 μM) the I–V relationship, derived from a holding potential of −90 mV, lay above the control curve, crossed it at approximately −80 mV, and exhibited outward rectification (Fig. 3a).

Levcromakalim (1 and 10 μM) increased the complex of non-inactivating currents (I_{NI} and I_{KCO}; derived from a holding potential of −10 mV) in a concentration-dependent manner (Fig. 3b), an effect which was fully reversible by glibenclamide (10 μM) (data not shown). In contrast to the total outward current elicited from a holding potential of −90 mV, the current-voltage relationships derived from a holding potential of −10 mV showed only slight rectification.

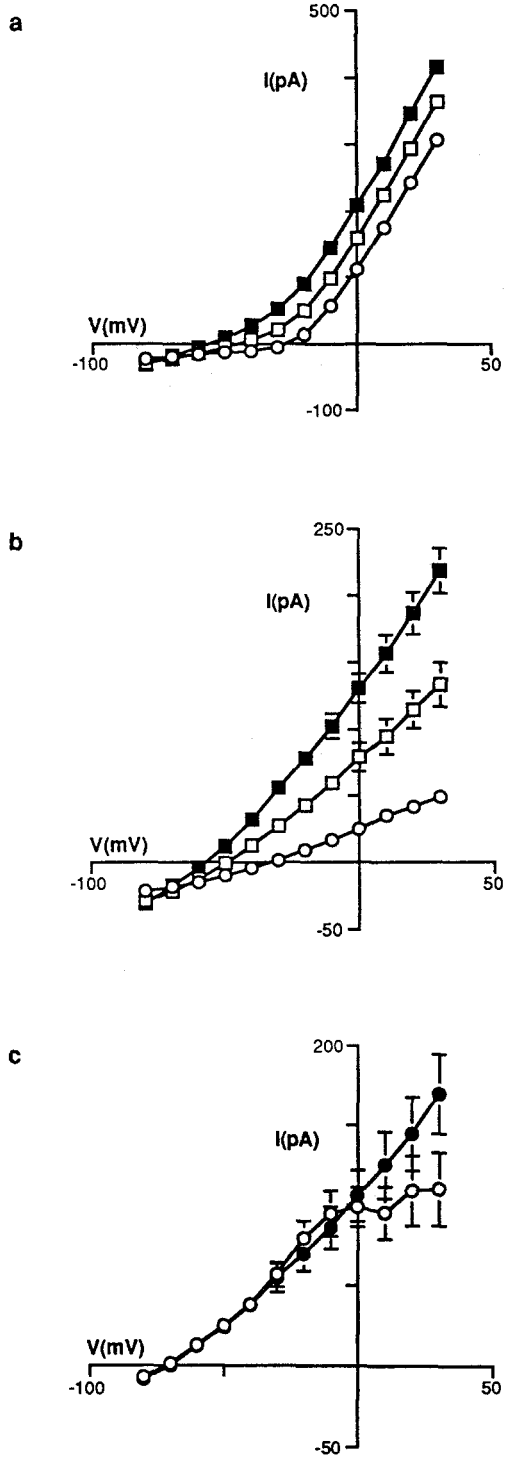

Fig. 3a–c. Current-voltage (I–V) relationships in rat mesenteric arterial cells and the effects of levcromakalim. **a** I–V relationship of the peak total current under control conditions (*open circles*) and in the presence of 1 μM (*open squares*) and 10 μM (*filled squares*) levcromakalim. The holding potential was −90 mV to ensure full activation of $I_{K(V)}$. **b** I–V relationship of the non-inactivating currents under control conditions (*open circles*) and in the presence of 1 μM (*open squares*) and 10 μM (*filled squares*) levcromakalim obtained from the same cells as in **a**. The holding potential was −10 mV which ensured complete inactivation of the time-dependent current components. **c** I–V relationships of the net current induced by levcromakalim (10 μM) (difference between currents in the presence and absence of levcromakalim) for the two holding potentials (*open symbols*, −90 mV; *filled symbols*, −10 mV). Each *point* represents the mean value derived from 5 observations ± s.e.mean

The net currents induced by levcromakalim (10 μM) from the two holding potentials of −90 mV and −10 mV, are shown in Fig. 3c. At potentials negative to −10 mV the curves were identical. However, on stepping from a holding potential of −10 mV to potentials more positive than −10 mV, the currents induced by levcromakalim were greater than those obtained from a holding potential of −90 mV. These data suggest an inhibition of the delayed rectifier current $I_{K(V)}$ by levcromakalim, since this current was only available on stepping to potentials positive to −30 mV from a holding potential of −90 mV. In all cells tested, levcromakalim reduced $I_{K(V)}$ in parallel with the increase in I_{KCO} (data not shown). The relatively large standard errors of the mean current values in Figure 3c reflect the variations in absolute current magnitude between different cells, rather than any variation in the inhibition of $I_{K(V)}$.

Single channel conductance underlying I_{KCO}

Application of levcromakalim to mesenteric arterial cells, held at a potential of −10 mV, produced the outward current I_{KCO}, the development of which was associated with an increase in the magnitude of current noise (Fig. 2). By examing segments of noise produced during the generation of I_{KCO} using stationary fluctuation analysis (Neher and Stevens 1977), the underlying single channel current was calculated to be 0.39±0.16 pA ($n = 4$). This value was determined by constructing amplitude histograms of the level of total current produced at holding potential of −10 mV. These were fitted with Gaussian normal distributions to provide a value for the mean current together with the variance. The unitary conductance underlying I_{KCO} was calculated as described in detail by Noack et al. (1992a, c). It was assumed that the channel opened by levcromakalim existed in either a conducting or a non-conducting state and that the gating of each channel was statistically independent of other channels. It was further assumed that the channel population was homogenous within the membrane. When the unitary conductance was calculated with these assumptions and according to the Goldman-Hodgkin-Katz equation, a value of 10.2±4.3 pS ($n = 4$; at 0 mV and under the quasi-physiological potassium gradient of the experiments) was obtained.

Effects of levcromakalim on $[Ca^{2+}]_i$ and tension

Mesenteric resistance vessels excited with alternating U–V light of wavelengths 340 nm and 380 nm produced a steady fluorescence with a baseline fluorescence ratio between 0.5 and 0.9. This level has been designated as 0% when % values are quoted. The mean internal diameter of mesenteric resistance vessels was 312±23 μm ($n = 15$).

Superfusion of these arterioles with 90 mM K^+-PSS for 5 min produces an initial, rapid increase in fluorescence signal, indicative of an elevation of $[Ca^{2+}]_i$. This signal peaked within about 30 s and was followed by a decline to a maintained plateau phase which was 76±2.5% ($n = 14$) of the initial, peak response. On subsequent washing of the tissue with normal PSS for 5 min, the fluorescence ratio returned to pre-stimulus levels. A

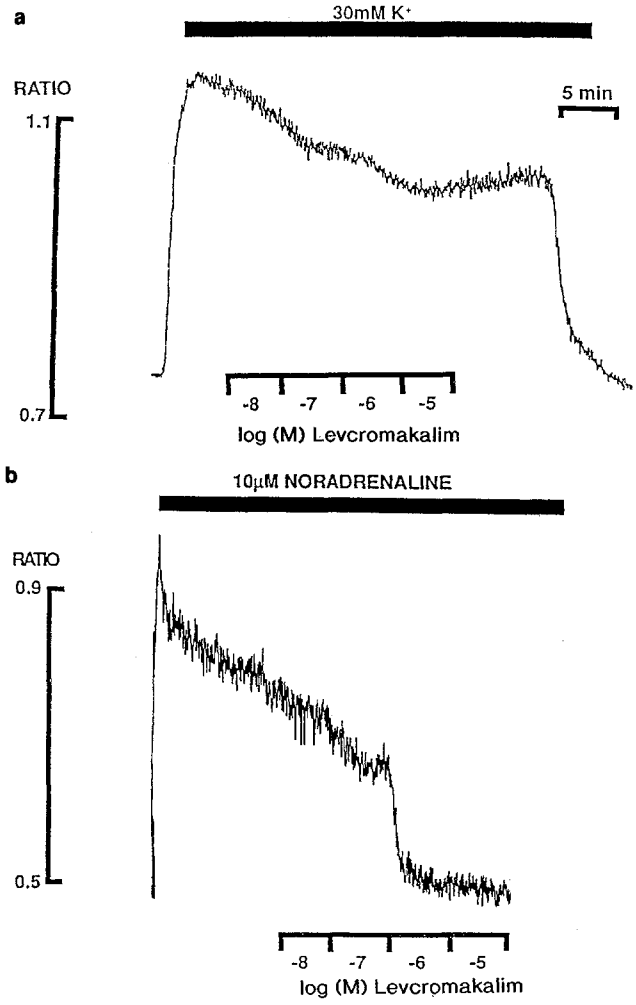

Fig. 4a, b. Representative traces showing the effects of cumulative concentrations of levcromakalim (10 nM – 10 μM) on the increases in 340 nm/380 nm signal ratio induced by **a** 30 mM K^+-PSS and **b** 10 μM noradrenaline in rat mesenteric resistance vessels

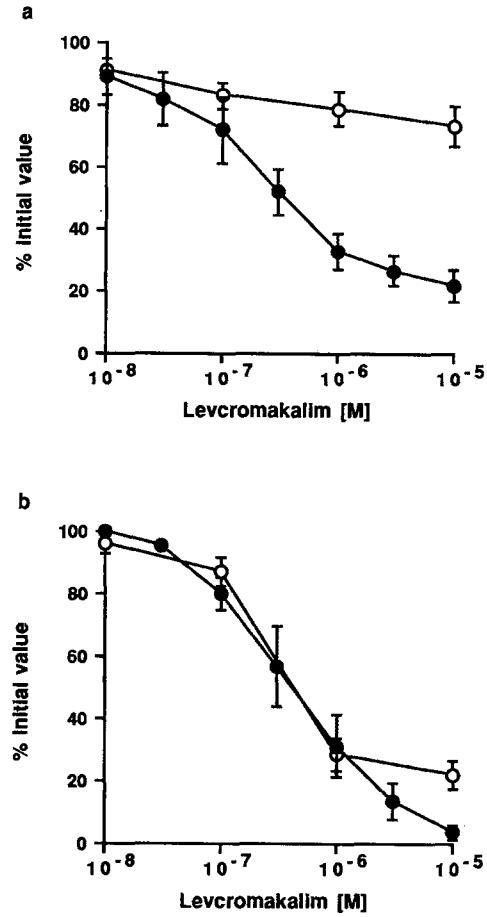

Fig. 5a, b. Effects of levcromakalim (10 nM – 10 μM) on the increase in $[Ca^{2+}]_i$ (*open symbols*) and tension (*filled symbols*) induced by **a** 30 mM K^+-PSS and **b** 10 μM noradrenaline. Each *point* represents the mean ± sem ($n \geq 4$)

5 min contact period in the fluorescence experiments was chosen as a compromise between the time-course of changes observed in the single-cell experiments (carried out at room temperature) and those of the mechanical events detected in the myograph at 37 °C. The increase in tension produced by mesenteric resistance vessels in response to 90 mM K^+-PSS comprised an initial, rapid phase (8.7 ± 1.1 mN, $n = 11$) followed by a plateau (9.1 ± 1.1 mN, $n = 11$). Superfusion of the arterioles with 30 mM K^+-PSS induced a monophasic increase in fluorescence ratio, the magnitude of which was 70 ± 5.6% ($n = 9$) of the 90 mM K^+-PSS-induced plateau (Fig. 4a). The increase in fluorescence ratio evoked by 30 mM K^+-PSS was well-maintained for 30 min (data not shown). The increase in tension induced by 30 mM K^+-PSS was also monophasic with a magnitude of 3.13 ± 0.5 mN ($n = 10$).

Levcromakalim applied to the tissue in the continued presence of 30 mM K^+-PSS produced a concentration-dependent but incomplete reduction in the fluorescence signal (Figs. 4a, 5a). Thus, after exposure to the maximal concentration of levcromakalim (10 μM) in the continued presence of either 20 mM K^+-PSS or 30 mM K^+-PSS, the K^+-induced increase in fluorescence ratio was only reduced to 60 ± 5% ($n = 4$) and 73.4 ± 6.4% ($n = 4$), respectively, of the initial value obtained in the absence of levcromakalim. When the data involving 30 mM K^+-PSS were analysed using the designated R_{max} and R_{min} values as described in Methods, 35 ± 7.4% ($n = 4$) of the initial increase in fluorescence ratio still remained after application of 10 μM levcromakalim. In comparison, levcromakalim relaxed the tension induced by the application of 30 mM K^+-PSS in a concentration-dependent manner, with a maximal reduction of approximately 80% (Fig. 5a, $n = 4$). The mean IC_{50} value for the action of levcromakalim against 30 mM K^+-PSS-induced tension was 0.15 μM (range, 0.03 – 0.75 μM; $n = 4$).

Noradrenaline (10 μM) produced an initial and rapid increase in fluorescence signal which was equalivalent to 88 ± 5.4% of the 90 mM K^+-PSS-induced plateau ($n = 8$). After approximately 2 min, a plateau phase was established which was 64 ± 4.2% of the 90 mM K^+-PSS-induced plateau response (Fig. 5a, b). In some tissues, 10 μM noradrenaline evoked oscillatory changes in $[Ca^{2+}]_i$, with a frequency of approximately 1 oscillation every 5 s. Noradrenaline (0.1 – 30 μM) generated a concentration-dependent increase in tension in mesenteric re-

sistance vessels which was biphasic at concentrations of noradrenaline greater than 1 μM. Exposure of the arterioles to 10 μM noradrenaline always produced biphasic contractions which were variable in the extent to which the phasic component was larger than the tonic component. Thus, the mean initial, rapid increase measured after approximately 1 min exposure to noradrenaline was 11.4±1.7 mN which followed by a later, sustained phase with a mean value measured after 6 min of 10.8±1.7 mN ($n = 13$ each value). Levcromakalim (0.1–10 μM) applied cumulatively to the tissue in the continued presence of noradrenaline produced a concentration-dependent reduction in the fluorescence signal (Figs. 4b, 5b) with a mean IC_{50} value of 0.29 μM (range, 0.26–0.31 μM; $n = 3$). Similarly, levcromakalim (0.1–10 μM) also produced a full relaxation of the sustained tone induced by 10 μM noradrenaline (Fig. 5b), with a mean IC_{50} value of 0.46 μM (range, 0.21–1.0 μM; $n = 5$).

Effects of nifedipine on $[Ca^{2+}]_i$ and tension

Nifedipine (0.1–10 μM) elicited a concentration-dependent reduction of the increase in fluorescence signal induced by 30 mM K^+-PSS (Figs. 6a, 7a) with a mean IC_{50} value of 310 nM (range, 240–400 nM; $n = 4$). When the effect of nifedipine was calculated using values for the fluorescence signal calibrated to a R_{max} value determined by 90 mM K^+, the IC_{50} was increased to 63 nM (range, 17–234 nM). In comparison, nifedipine effectively relaxed the maintained increased in tension induced by 30 mM K^+-PSS with a mean IC_{50} of 2.54 nM (range, 2.13–3.02 nM; $n = 6$).

Nifedipine inhibited the increase in both fluorescence and tension induced by 10 μM noradrenaline in a concentration-dependent manner (Figs. 6b, 7b). The mean IC_{50} value for the reduction of the 340/380 nm fluorescence ratio by nifedipine was 0.24 μM (range, 0.22–0.26 μM; $n = 5$); compared with a mean relaxant IC_{50} for nifedipine of 0.04 μM (range, 0.02–0.08 μM; $n = 6$).

Discussion

General features of levcromakalim-induced I_{KCO}

In the present study in cells dispersed from rat small mesenteric arteries, levcromakalim induced a non-inactivat-

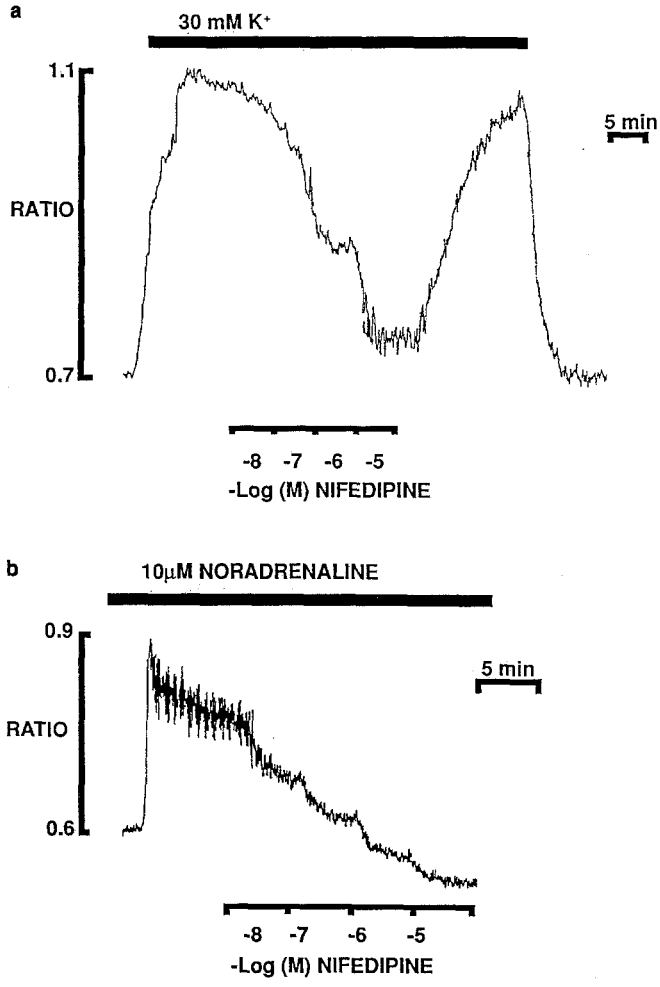

Fig. 6a, b. Representative traces showing the effects of cumulative concentrations of nifedipine (10 nM–10 μM) on the increases in 340/380 ratio induced by **a** 30 mM K^+-PSS and **b** 10 μM noradrenaline in rat mesenteric resistance vessels

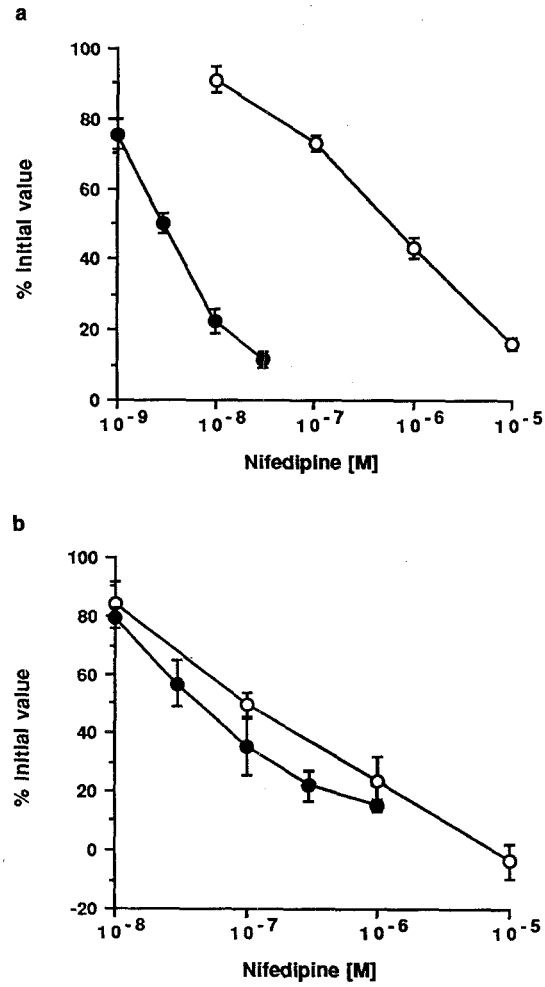

Fig. 7a, b. Effects of nifedipine (10 nM–10 μM) on the increase in $[Ca^{2+}]_i$ (*open symbols*) and tension (*filled symbols*) induced by **a** 30 mM K^+-PSS and **b** 10 μM noradrenaline. Each point represents the mean±sem ($n \geq 4$)

ing, glibenclamide-sensitive outward current (I_{KCO}), similar to the recently described in rat portal vein (Noack et al. 1992a) and in human mesenteric artery (Russell et al. 1992). The development of I_{KCO} was associated with an increased current noise and in common with other studies in vascular smooth muscle (Beech and Bolton 1989; Okabe et al. 1990; Noack et al. 1992a), several minutes elapsed before I_{KCO} attained its full magnitude. This delay is unlikely to have been an artefact due to mixing of the drug in the PSS since the cells were constantly superfused at a flow rate sufficient to exchange the bath contents within 1 min. In addition, the rate of onset of I_{KCO} was dependent on the concentration of levcromakalim used. Thus the relatively long time to achieve peak effect may reflect the involvement of an intracellular site of action and/or of a series of biochemical events prior to K-channel opening. Evidence which suggests that levcromakalim may be a dephosphorylating agent has recently been published (Edwards et al. 1993).

Target channel for levcromakalim in arterioles and its unitary conductance

Preliminary experiments showed that the electrophysiological effects of levcromakalim could be obtained in calcium-free PSS and with EGTA in the pipette and the present study was conducted in such conditions. It therefore appears unlikely that I_{KCO} was carried by the large conductance calcium-dependent K-channel (BK_{Ca}), a site of action for the K-channel openers proposed by Gelband et al. (1989) and Carl et al. (1992). In support of the lack of involvement of BK_{Ca}, other workers have shown that the vasorelaxant effects of cromakalim (and also minoxidil sulphate) are not inhibited by charybdotoxin, an inhibitor of BK_{Ca} (Winquist et al. 1989; Wickenden et al. 1991).

Stationary fluctuation analysis of the current noise generated by levcromakalim suggests that the channel opened by this agent exhibits the relatively small unitary conductance of approximately 10 pS under quasi-physiological conditions. Moreover, I_{KCO} was reduced by glibenclamide, an inhibitor of K_{ATP} in pancreatic β-cells, cardiac and smooth muscle (Escande et al. 1988; Sanguinetti et al. 1988; Noack et al. 1992a). Thus our present findings in smooth muscle cells from small resistance arterioles are in accord with previous studies in rat and rabbit portal veins, which have shown that a relatively small conductance (10–30 pS) K_{ATP} is indeed the target of the K-channel openers (Kajioka et al. 1990, 1991; Noack et al. 1992a, b; Beech et al. 1993; Ibbotson et al. 1993). Thus a discrepancy exists between the relatively small conductance K-channel detected both in these investigations and in the present study and the much larger conductance K-channel previously implicated in the action of K-channel openers in rat and rabbit mesenteric arteries (Standen et al. 1989; Nelson et al. 1990).

In the investigation by Standen et al. (1989), unitary conductance (135 pS at 0 mV) was measured under $[K^+]_i$ and $[K^+]_o$ conditions of 120 mM and 60 mM, respectively, in contrast to the quasi-physiological conditions ($[K^+]_i$, 160 mM; $[K^+]_o$, 6 mM) of the present study and of the earlier ones from this laboratory (Noack et al. 1992a; Ibbotson et al. 1993). If the values for unitary conductance are each recalculated for quasi-physiological conditions of 160 mM ($[K^+]_i$) and 6 mM ($[K^+]_o$), values in the region of 100 pS (Standen et al. 1989) and in the range 10–20 pS (present study; Noack et al. 1992a; Beech et al. 1993; Ibbotson et al. 1993) are obtained.

Several assumptions must be made to allow channel unitary conductance to be determined from whole-cell current noise (see Noack et al. 1992a, c) and this contrasts with the direct calculation which is possible from the isolated patch measurements made by Standen et al. (1989). However, both in the present study and in the three earlier investigations in rat portal vein (Noack et al. 1992a, b; Ibbotson et al. 1993), the amplitude of current noise generated by levcromakalim, and by the two chemically-distinct K-channel openers, P1060 and aprikalim, was much too small to have been generated via a large conductance K-channel such as that reported by Standen et al. (1989). Furthermore, switching to current-clamp conditions showed that the low-noise I_{KCO} produced by levcromakalim was associated with a large hyperpolarisation typical of the membrane potential changes produced by K-channel openers in whole tissues (Noack et al. 1992a, b).

Similar low-amplitude current noise was generated by K-channel openers in two other whole-cell clamp studies (rabbit portal vein; Beech and Bolton 1989; human mesenteric artery; Russell et al. 1992) and the recent study in rabbit portal vein by Beech et al. (1993) involved the direct estimation of unitary conductance from isolated patch recordings. Thus data from many laboratories now strongly indicate that a relatively small conductance K-channel is the target for the K-channel openers in vascular smooth muscle.

Inhibition of noradrenaline- and KCl-induced changes in $[Ca^{2+}]_i$ and tension

Levcromakalim was equipotent at reducing the contraction and increase in $[Ca^{2+}]_i$ induced by noradrenaline. Both these inhibitory effects could have been mediated via the indirect closure of voltage-dependent Ca-channels following the generation of I_{KCO} and the ensuing tissue hyperpolarisation. In some experiments we thus compared the effects of levcromakalim with those of the Ca-channel blocking drug, nifedipine. In these, levcromakalim relaxed established contractions to noradrenaline and KCl with a similar potency, whereas nifedipine was approximately 20 times more potent at inhibiting KCl-induced contractions than those produced by noradrenaline. Such a discrepancy suggests that the effects of noradrenaline are not simply the result of Ca-influx but may involve additional processes such as the release of Ca from internal stores (Ito et al. 1991b). Moreover, noradrenaline might increase the influx of Ca through T-type, voltage-sensitive Ca-channels which are less sensitive to dihydropyridine Ca-channel antagonists than L-type Ca-channel. However, the existence of these channels in smooth muscle cells has not been unequivocally demonstrated.

In comparison to its effects on noradrenaline-induced increases in $[Ca^{2+}]_i$, levcromakalim was *relatively* ineffective at reducing the increase in $[Ca^{2+}]_i$ induced by K^+-rich PSS whereas the associated increase in tension was more markedly inhibited. This discrepancy existed not only when the fluorescence ratio was taken as the indicator of $[Ca^{2+}]_i$, but also when the ratios were transformed after determination of R_{max} and R_{min} values. Furthermore, nifedipine was almost 100 times more potent as an inhibitor of KCl-induced tension than of the associated fura-2 fluorescence signal. These data collectively suggest that levcromakalim can relax developed tension by an action at locations additional to the smooth muscle plasmalemma (see Greenwood and Weston 1993; Quast 1993). Indeed, the possibility that the K-channel openers exert dephosphorylating effects at both plasmalemmal ion channels and at intracellular sites has recently been discussed (Edwards et al. 1993).

The comparatively small levcromakalim-induced inhibition of the raised $[Ca^{2+}]_i$ levels induced by 30 mM K^+ has been previously reported in canine coronary arteries (Yanagisawa et al. 1990). This study, using fura-2, showed that cromakalim, pinacidil and nicorandil could only reduce the rise in $[Ca^{2+}]_i$ induced by 30 mM K^+-PSS by approximately 40%, an effect also shared by KRN 2391, a K-channel opener structurally-related to nicorandil (Okada et al. 1991). A similar effect of levcromakalim was also observed in canine coronary artery preparations and this was considered to the consequence of a reduction in the Ca^{2+}-sensitivity of the contractile proteins by this K-channel opener (Okada et al. 1993).

An alternative explanation is that the discrepancies reported in the present study could be related to the use of fura-2 as a marker for $[Ca^{2+}]_i$. At present there is no information regarding the intracellular distribution within mesenteric arteriolar smooth muscle of fura-2, following its cleavage from the methoxy ester. However, studies in endothelial (Steinberg et al. 1987) and renal tubular cells (Giligorski et al. 1986) have indicated that this dye can localise in subcellular compartments, and potential problems associated with the use of fura-2 as a marker for intracellular calcium have recently been reviewed (Karaki 1989; Roe et al. 1990). Thus in the presence of raised $[K^+]_o$, fura-2 could gain access to more intracellular compartments than when noradrenaline is used as a contractile agent. If so, the ensuing calcium signal might not be well-correlated with the associated contraction. Recent studies, however, do not support this view since Jensen et al. (1993) have reported that the $[Ca^{2+}]_i$ indicated by fura-2 is very similar to that detected by a Ca-sensitive microelectrode.

Another possibility is that the KCl-induced Ca-influx into mesenteric resistance vessels may comprise two distinct components. One of these, which is inhibited by levcromakalim and by nifedipine, may be intimately concerned with contraction probably via calcium-induced calcium release (see Ito et al. 1991a; Ganitkevich and Isenberg 1992). The second and apparently major component may involve a more generalised calcium influx into the cell which is resistant to both levcromakalim and nifedipine. Thus the fura-2 signal associated with exposure to KCl would not be subject to substantial reduction by either levcromakalim or nifedipine in their relaxant concentration ranges. These possibilities are the subject of an on-going investigation.

Conclusions

Collectively, the data obtained in the present study suggest that levcromakalim induces the generation of I_{KCO} via the opening of a relatively small conductance, glibenclamide-sensitive K-channel. The induction of this current is sufficient to account for most of the mechano-inhibitory actions of this agent in mesenteric resistance vessels. Unexpected anomalies in the KCl-induced fura-2 signal which were not due to saturation of the fura-2 – Ca^{2+} interaction, preclude a more definitive conclusion. However, the use of this dye suggests that the mechanisms which determine tension development may be more complex than previously described.

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