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Characterization of potassium currents modulated by BRL 38227 in rat portal vein

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1 Smooth muscle cells of the rat portal vein were dispersed by enzymatic treatment and recordings of whole-cell membrane potassium currents were made by the voltage-clamp technique. In isolated cells by use of combined voltage- and current-clamp the effect of BRL 38227 on membrane potential and ionic currents was also studied.

2 BRL 38227 (0.1 to 10 μM) induced a non-inactivating potassium current (I_{KCO}) which developed slowly (900 s to 300 s, respectively) to its full size. These effects of BRL 38227 were reversible.

3 In addition to its K-channel opening properties, BRL 38227 (1 to 10 μM) inhibited the amplitude and changed the activation and inactivation characteristics of a slowly-inactivating, calcium influx-independent, outward potassium current (I_{TO}).

4 Application of stationary fluctuation analysis to I_{KCO}, showed a mean single channel current of 0.65 pA at − 10 mV under a quasi-physiological potassium gradient.

5 In a combined voltage-clamp/current-clamp configuration, BRL 38227 (1 μM) induced a mean hyperpolarization of 22 mV.

6 The induction of I_{KCO} by BRL 38227 and the associated hyperpolarization were suppressed by glibenclamide (1 to 10 μM) in a concentration-dependent manner. Glibenclamide (1 μM) had no effect on the inhibition of I_{TO} by BRL 38227 (1 μM).

Keywords: BRL 38227; glibenclamide; voltage-clamp; current-clamp; rat portal vein; K-channel; K-current

Introduction

BRL 38227, the active enantiomer of the racemate cromakalim (BRL 34915), is a smooth muscle relaxant classified as a potassium channel opener (KCO) (Edwards & Weston, 1990). Although the effects of KCOs on potassium (K) currents in smooth muscle have been widely studied (Standen *et al.*, 1989; Beech & Bolton, 1989b; Klöckner *et al.*, 1989; Gelband *et al.*, 1990; Kajioka *et al.*, 1990; 1991; Okabe *et al.*, 1990; Clapp & Gurney, 1991; Lindeman *et al.*, 1991; Nakao & Bolton, 1991; Noack *et al.*, 1991; Pavenstädt *et al.*, 1991) the K-channel responsible for the characteristic hypotensive and vasorelaxant effects of these agents has not been unequivocally identified.

In rat mesenteric artery it was reported that KCOs opened a K-channel with a relatively large single channel conductance (Standen *et al.*,1989) and in human mesenteric artery an increased open probability of the large conductance calcium-activated K-channel (BK_{Ca}) was detected (Klöckner *et al.*, 1989). In contrast to these findings, a variety of studies in vascular smooth muscle has indicated that BK_{Ca} is not opened by KCOs (Beech & Bolton, 1989b; Okabe *et al.*, 1990; Lindeman *et al.*, 1991; Pavenstädt *et al.*, 1991). Furthermore, investigations on rat and rabbit portal vein have shown that the KCOs open a K-channel with a unitary conductance of approximately 10 pS (Kajioka *et al.*, 1990; Nakao & Bolton, 1991).

To date, the majority of studies on the detailed smooth muscle single cell electrophysiology of the KCOs have involved isolated membrane patches but no clear picture of the target K-channel has emerged. For this reason we decided to characterize the electrophysiological effects of the KCOs using BRL 38227 and whole-cell voltage-clamp techniques. In the present study in rat portal vein we now show that BRL 38227 induces a non-inactivating, calcium influx-independent K-current that is glibenclamide-sensitive. The results, some of which were previously presented to the German Physiological Society (Noack *et al.*, 1991) indicate that a small conductance K-channel is the site of action of the KCOs.

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Methods

All experiments were performed on single smooth muscle cells isolated from portal veins which were removed from male Spague-Dawley rats, previously killed by stunning and bleeding.

Production of isolated cells

Each portal vein (about 20 mm length) was carefully cleaned of fat and connective tissue with fine scissors in conjuction with a dissecting microscope. The vein was cut into small pieces and then incubated in a nominally Ca^{2+}-free physiological salt solution (PSS) for 30 min. The cell dispersion consisted of a consecutive treatment of the tissue with a high K^+ PSS (1 ml) containing purified collagenase (100 units ml^{-1}; Sigma) and a high K^+ PSS containing papain (20 units ml^{-1}; Sigma) and collagenase inhibitor (phosphoramidon, 200 μg ml^{-1}; Sigma) (for details see Lammel *et al.*, 1991). The cells were used for experiments within 12 h of separation, during which time they were stored at 4°C in Kraftbrühe (KB-medium; Klöckner & Isenberg, 1985). All experiments were performed at 26°C.

Single-cell electrophysiology

The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) using a clamp system designed in our laboratory which was functionally similar to the commercially-available EPC 7 (List, Germany), was used in

all experiments. The settling time of this system was less than 500 μs. Patch pipettes were pulled from Pyrex glass (H15/10, Jencons, UK) and had resistances of 3–4 MΩ when filled with the internal (intracellular) solution.

Voltage commands and data acquisition were performed on-line with an AT-compatible computer equipped with an appropriate interface (Axolab 1100 or Axon TL-1, Axon Instruments, U.S.A.). For cell stimulation and for recording and analyzing data the pCLAMP 5.5 programme was used (Axon Instruments, U.S.A.). A video recorder in conjunction with a pulse code modulator was used to store data. Voltage protocols and evoked membrane currents were monitored continuously on a chart pen recorder (Series 2400, Gould, U.S.A.). Currents were displayed on the computer monitor and hard copies were obtained using a plotter (Taxan, U.S.A.). Leakage current was estimated at the end of an experiment by addition of 5 mM $CdCl_2$ to the extracellular solution (Noack *et al.*, 1992). The leak resistance ranged from 3 to 5 GΩ and the experimental values were used to estimate errors of potential measurement under whole-cell current-clamp. The cadmium (5 mM) resistant current (leakage current) was not subtracted from either current traces or from current-voltage relationships in this paper. Recordings were evaluated from a total of 30 cells from which continuous measurements of at least 20 min were made. The effects of BRL 38227 were investigated by adding the appropriate amount of this agent to the main reservoir containing the external solution to ensure that responses were obtained under steady-state conditions. The bath (volume: 1 ml) was continuously perfused (1 ml min^{-1}) with fresh external solution using a pump (Microperpex, Pharmacia LKB, Freiburg, Germany); a second identical pump was used to remove excess solution from the recording chamber.

To determine the effects of BRL 38227 and glibenclamide on membrane potential, a series of measurements was performed with a voltage-clamp amplifier which permitted fast (< 1 ms) switching to current-clamp conditions. The properties of this combined voltage/current-clamp amplifier were similar to those described by Clark & Giles (1987).

Drugs and solutions

The nominally Ca^{2+}-free PSS used for the cell separation comprised (mM): NaCl 137, KCl 2.7, $MgCl_2$ 1.0, glucose 5.6, HEPES 4.2; buffered with NaOH to pH 7.3. High K^+ PSS contained (mM): KCl 140, $MgCl_2$ 1.0, glucose 5.6, HEPES 4.2, buffered with KOH to pH 7.3. The PSS in the bath 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. After buffering, the pipette solution contained a total of 152 mM K^+. Assuming a contaminant concentration of 10 μM calcium in the external and in the pipette solutions, the addition of 1 mM and 1.2 mM EGTA (ethylene glycol-*bis* (β-aminoethyl ether) tetra-acetic acid; Sigma) to the bath and pipette solutions, respectively, should have produced an average free calcium concentration in these solutions of less than 1 nM.

To minimize solvent effects on membrane currents, BRL 38227 (SmithKline Beecham, UK) was first dissolved in dimethyl sulphoxide (DMSO; Sigma) to produce a concentrated stock solution (250 mM). Glibenclamide (Hoechst, FRG) was prepared as 10 mM stock solution in DMSO. From such stocks, dilutions were prepared with distilled water immediately before they were required.

Results

Whole-cell currents in calcium-free PSS

When a rat portal vein cell was depolarized from a holding potential of − 90 mV to a test potential of − 30 mV in the calcium-free PSS (free calcium lower than 1 nM), a current with fast activation kinetics which inactivated to a steady current level within less than 20 ms was observed (Figure 1a(i), 6th and 7th trace from bottom). On depolarization to test potentials positive to − 30 mV (Figure 1a(i), 8th to 10th trace from bottom) an additional outward current component became activated. This component also inactivated but exhibited slower kinetics than the current previously described. Both of these time-dependent outward current components declined to a steady current level. The complete inactivation of the slower component is not shown in Figure 1a(i) because of the relatively short test pulse duration. The faster outward current showed some similarity to the A-like K-current which has previously been described in rabbit portal vein (Beech & Bolton, 1989a). The more slowly-inactivating current was comparable to I_{TO}, a K-current recently characterized in guinea-pig portal vein (Noack *et al.*, 1990) and the designation I_{TO} will also be used to describe the slowly-inactivating current in the present study. The steady current level observed after complete inactivation of I_{TO} (deviation from zero current; zero current indicated by arrows in Figure 1) could also be obtained when the membrane potential was stepped from a holding potential of − 10 mV to the same test potentials as those used when the holding potential was − 90 mV (Figure 1a(ii)). Thus the magnitude of I_{TO} is given by the difference between the peak outward current elicited from a holding potential of − 90 mV and that current elicited from a holding potential of − 10 mV. The difference between the non-inactivating current and zero current was designated I_{NI}.

Effects of BRL 38227 on whole-cell currents

When portal vein cells were held at a potential of − 10 mV and BRL 38227 (10 μM) was added to the bathing solution, the current slowly increased in an outward direction (Figure 2a). In contrast, when the cells were exposed to BRL 38227 (1 to 10 μM) at a holding potential of − 90 mV (close to the calculated potassium equilibrium potential, E_K = − 83 mV) BRL 38227 had no effect or induced a small inward current indicating that a K-current was induced by this substance (compare holding currents in Figure 1a(i) and b(i) and holding currents in Figure 1a(ii) and b(ii)). The time-course of action of BRL 38227 (10 μM) on membrane currents (I_{KCO}) was relatively slow and was essentially complete after 300 s. Such a slow effect cannot be explained by the time-course of the increase in the bath concentration of BRL 38227. For example, on perfusing with 10 μM BRL 38227, a concentration of 1 μM was achieved within the first 10 s. At a steady-state concentration of BRL 38227 (1 μM), a very marked enhancement of evoked membrane currents was observed at all test voltages (positive to E_K) on stepping from holding potentials of either − 90 mV or − 10 mV (Figure 1b). At a concentration of 1 μM, BRL 38227 produced near-maximal effects. Increasing the concentration of BRL 38227 to 10 μM did not produce significantly larger effects but the onset of I_{KCO} was somewhat faster. When BRL was used in a concentration of 0.1 μM, I_{KCO} reached approximately 20% of the magnitude produced by 1 μM BRL 38227. From this the EC_{50} value of BRL 38227 for generation of I_{KCO} can be estimated to be approximately 0.5 μM.

A more detailed analysis of the interaction of BRL 38227 with the different K-currents in rat portal vein was derived from the construction of the current-voltage relationships (I–V curves) under control conditions and in the presence of BRL 38227 with holding potentials of − 90 mV and − 10 mV (Figure 3a and b). In Figure 3a the peak

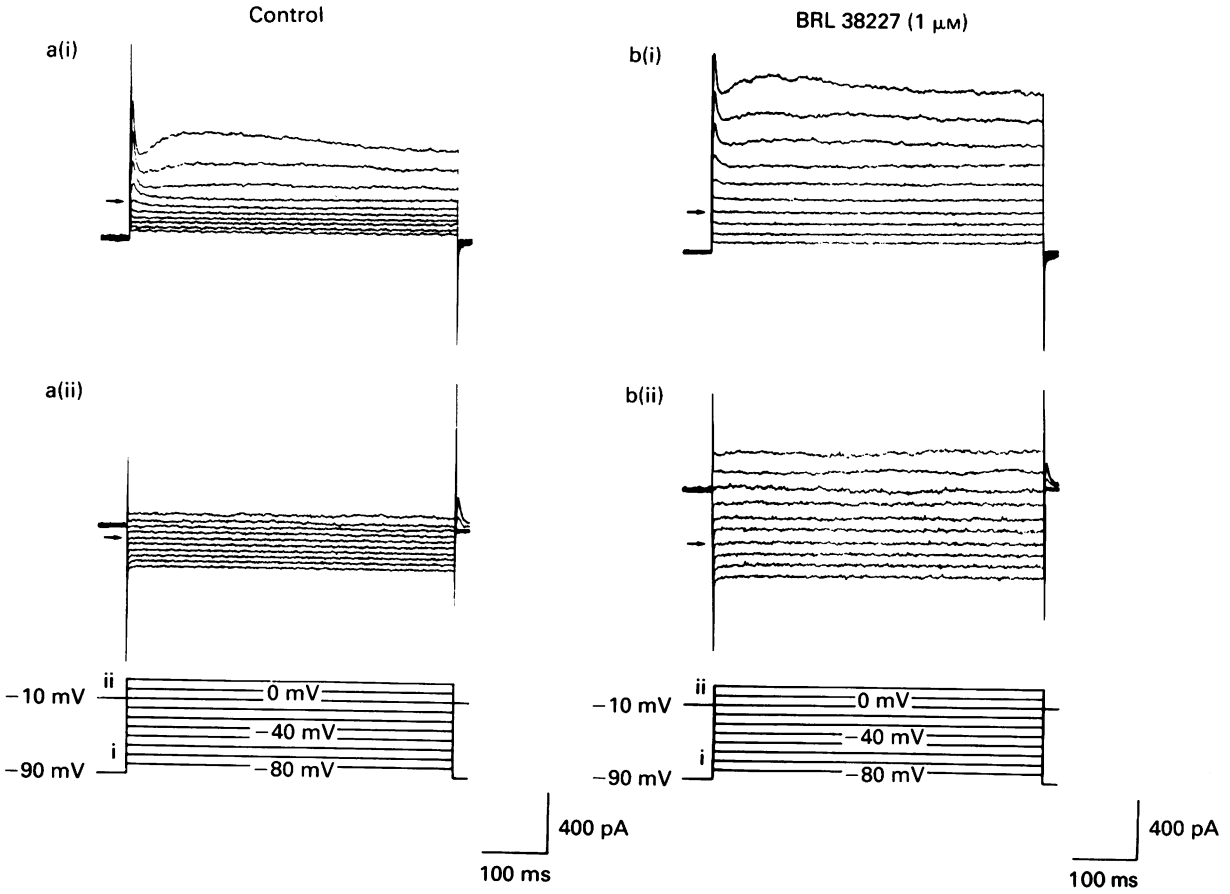

Figure 1 Effect of BRL 38227 on whole-cell currents in a smooth muscle cell from rat portal vein bathed in a calcium-free physiological salt solution (containing 1 mM EGTA). (a) The cell was held either at − 90 mV (i) or − 10 mV (ii) and stepped for 500 ms to the different test potentials as indicated. Note the fast inactivating outward current shortly after the capacitive transient in (i). (b) Conditions as in (a) but after exposure to BRL 38227 (1 μM) for 5 min. The arrows indicate the zero current. Note that the current associated with a holding potential of − 90 mV was virtually unchanged in the presence of BRL 38227.

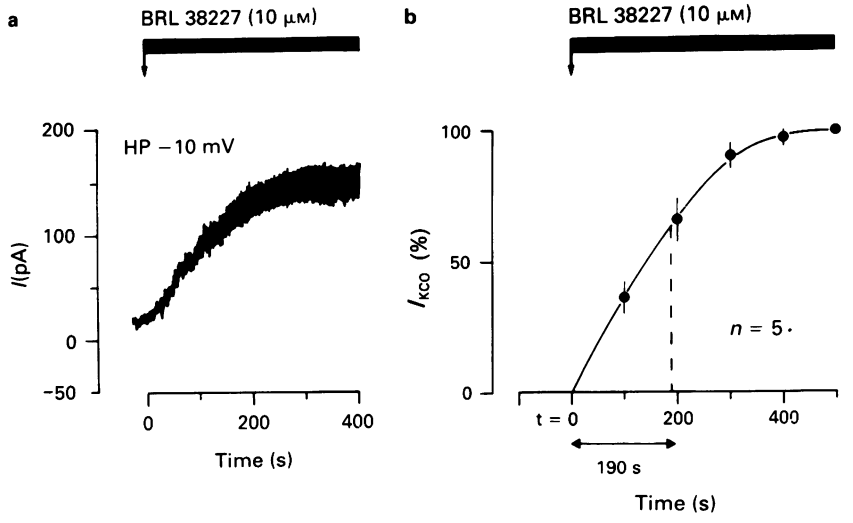

Figure 2 Effect of BRL 38227 (10 μM) on the current associated with a holding potential of − 10 mV. (a) The onset of the BRL 38227-induced current I_{KCO} was accompanied by increased current noise. (b) Time-course of the onset of I_{KCO} (curve fitted by eye). The results are expressed as a percentage of the maximum current induced by BRL 38227 (10 μM) in each cell ($n = 5$ cells). The vertical bars indicate s.e.mean. Assuming an exponential time-course for the development of I_{KCO}, the mean activation time constant (time taken to achieve 63% of the maximum current produced by BRL 38227) was 190 s (dashed line in b).

outwardly-directed current was measured between 100 ms and 300 ms after the start of the depolarizing pulse from a holding potential of − 90 mV. This peak current in the absence of BRL 38227 represents the sum of the components I_{TO} and I_{NI}. On exposure to BRL 38227 (1 μM) the current reflects the sum of I_{TO} and I_{NI}, together with the effects of BRL 38227 on one or both of these components, plus any new current (I_{KCO}) induced by BRL 38227 and which was not present under control conditions.

From a holding potential of − 90 mV, the I–V curve in

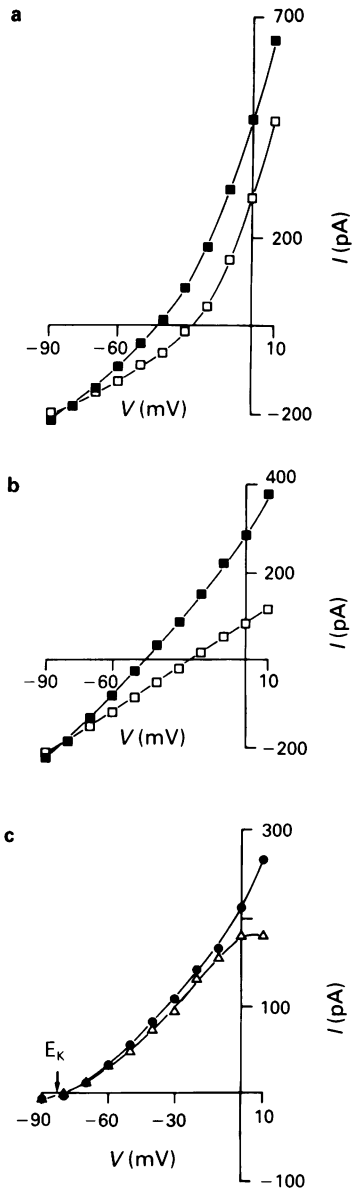

Figure 3 Current-voltage relationships of the currents in rat portal vein and the effect of BRL 38227. (a) Current-voltage relationship of the peak total current (peak of I_{TO}) under control conditions (□) and in the presence of BRL 38227 (1 μM) (■). The holding potential was − 90 mV to ensure full activation of I_{TO}. (b) Current-voltage relationship of the non-inactivating currents under control conditions (□) and in the presence of 1 μM BRL 38227 (■) 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) Current-voltage relationships of the net current induced by BRL (difference between currents in the presence and absence of BRL 38227: $I_{BRL} - I_{control}$) for the two holding potentials (Δ, − 90 mV; ●, − 10 mV). The data were obtained from three different cells. In all cells, the current induced by BRL 38227 had a reversal potential of − 80 mV and showed a significant flattening at potentials positive to − 10 mV when the holding potential was − 90 mV.

the presence of BRL 38227 lies above the control curve and crosses it at approximately − 80 mV. The difference between the control and test curves, which represents the net effect of BRL 38227 (1 μM) on I_{TO} and I_{NI} is shown in Figure 3c and exhibits a conspicuous flattening at potentials positive to − 10 mV. The current-voltage relationship for I_{NI} alone was constructed by plotting the steady current under control conditions against the test potentials at which it was activated from a holding potential of − 10 mV (Figure 3b). Compared with the control $I-V$ curve obtained at the same test potentials elicited from a holding potential of − 90 mV, the curve derived from a holding potential of − 10 mV exhibited very little or no outward rectification (Figure 3b). In the presence of BRL 38227 (1 μM) the steady current-voltage relationship ran above the control curve, crossed it near − 80 mV and showed outward rectification (Figure 3b).

The effects of BRL 38227 can be seen more clearly in Figure 3c in which the current difference curves (obtained by subtracting control currents from currents in the presence of BRL 38227) for the two holding potentials of − 90 mV and − 10 mV are illustrated. In the potential range up to − 10 mV both curves are nearly identical. However, at potentials more positive than − 10 mV, the currents induced by BRL 38227 and observed on stepping from a holding potential of − 90 mV were smaller than those elicited from a holding potential of − 10 mV. Since the inactivating current I_{TO} was only available on stepping from − 90 mV and at potentials positive to − 30 mV, these data suggest that BRL 38227 inhibits I_{TO}.

To determine the effects of BRL 38227 on I_{TO} alone, this current was separated from total current by subtracting the current which was available on stepping to test potentials from a holding potential of − 10 mV from that current which was achieved at the same test potentials but by stepping from a holding potential of − 90 mV (i.e. $I_{TO} = I_{HP-90} - I_{HP-10}$). The fast inactivating A-like current was digitally subtracted from current traces and thus did not contaminate re-fitting to zero time.

Under control conditions the activation time constant for I_{TO} was about 20 ms and the inactivation could be described by a single exponential process with a time constant of 990 ms at + 10 mV (Figure 4). As indicated by the $I-V$ curves in Figure 3a and 3b and shown in Figure 3c, I_{TO} was reduced in magnitude (by 21%) by 1 μM BRL 38227. However, BRL 38227 reduced the activation and inactivation time constants of I_{TO} by approximately 50% (assuming a single exponential process for each), both of which would independently reduce the magnitude of I_{TO}. Thus the BRL 38227-induced reduction of I_{TO}, which was independent

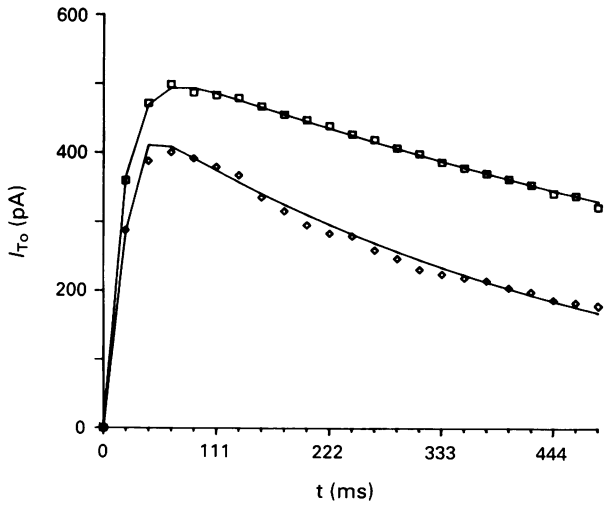

Figure 4 Time course of I_{TO} and effect of BRL 38227. The current values of I_{TO} were sampled in steps of 22 ms under control conditions (□) and in the presence of BRL 38227 (1 μM, ◇). Both sets of data were fitted with a single exponential process of activation and inactivation according to the equation: $I = I_o \times (1\text{-}e^{-t/\tau a}) \times e^{-t/\tau i}$, where I_o is the maximum current, t the time and τa and τi the activation and inactivation time constants, respectively. The continuous lines give the least square fits for control and BRL 38227 time courses. Note, that I_{TO} changed its inactivation in the presence of BRL 38227 so that it could no longer be described by a single exponential inactivation process. A second exponential power could not improve the fit.

of changes in the kinetics of I_{TO} activation and inactivation, was in the order of 14%. It should be noted that the inactivation phase of I_{TO} in the presence of BRL 38227 was not well-fitted by a single or double exponential process (Figure 4).

Single channel conductance underlying I_{KCO}

As can be seen from Figure 2b the increase of outward current induced by BRL 38227 was always accompanied by an increase in current noise. To derive information about the conductance of the channels associated with this effect of BRL 38227, we used the stationary fluctuation analysis technique (Neher & Stevens, 1977). Amplitude histograms of the level of total current at a constant holding potential were fitted by Gaussian normal distributions to provide the mean current (μ) and the standard deviation (σ) of the mean value. To determine single channel conductance from μ and σ, three general assumptions were made concerning the channels activated by BRL 38227. Firstly, they were assumed to exist only in either conducting or non-conducting states, with a probability p_o of being in the conducting state. Secondly, the gating of each channel was assumed to be statistically independent of other channels and thirdly, the population of channels was assumed to be homogeneous (for details see Noack *et al.*, 1992). Thus, the current (i) flowing through one channel is given by:

$$i = \sigma^2/(\mu \times (1\text{-}p_o)) \quad (1)$$

It can be seen from this equation that i is described by three variables of which only two (μ and σ) can be measured. However, for a low open probability, this expression can be simplified to $i = \sigma^2/\mu$ but the use of this simple equation leads to a significant error in the estimation of i if p_o is large.

As shown in Figures 2a and 2b, the increase of BRL 38227-induced current was very slow and required more than 300 s to become completely established. Thus the open probability for the channels influenced by BRL 38227 increased very slowly with time and this feature of I_{KCO} was used to determine the variable p_o. For this quasi-stationary fluctuation analysis, the time course of current was divided into segments, each of 2 s duration. Such a period was long enough to describe current fluctuation with a Gaussian normal distribution and short enough to ensure that the increase of mean current over this 2 s period was negligible compared with the current noise associated with I_{KCO}. Figure 5a shows amplitude histograms and fitted normal distributions obtained from such 2 s intervals of current recording at a holding potential of − 10 mV both under control conditions and in the presence of BRL 38227. In the case depicted, the mean control holding current was 6.62 pA with a standard deviation of 3.38 pA. The mean current had increased to 90.48 pA, with a standard deviation of 7.12 pA, 300 s after exposure to BRL 38227. As mentioned above, the current (I_{KCO}) generated by BRL 38227 showed outward rectification and was selective for K^+. Both these features of I_{KCO} are different from those of I_{NI} (see Figure 3b and 3c) and it thus seems likely that the current noise generated under control conditions and in the presence of BRL 38227 arose from independent sources (i.e. I_{NI} and I_{KCO}). Therefore, the mean current values (μ) quoted above and shown in Figure 5a and the associated variances (σ^2) are additive.

Thus the difference between the mean currents (μ) and the differences between the variances (σ^2) determined under control conditions and in the presence of BRL 38227 (10 μM) were calculated. These pairs of μ and σ^2 were grouped according to increasing values of μ. Observations where the difference between μ-values was smaller than 2 pA were summed. These values for mean current and variance were transferred to a graph (Figure 5b) and represent the pure membrane current and current noise associated with the presence of BRL 38227. The mean current can be expressed as a function of the number of channels involved (N), the current flowing through a single channel (i) and the channel open probability (p_o).

Thus:

$$\mu = N \times i \times p_o \quad (2)$$

or

$$p_o = \mu/(N \times i)$$

Substituting p_o in equation (1) gives the variance, σ^2 as a function of i, μ and N.

$$\sigma^2 = i \times \mu - \mu^2/N \quad (3)$$

This equation predicts a parabolic shape of the current variance - mean current plot; with increasing N the maximum of the curve increases and the right-hand intercept with the μ-axis shifts to the right. Fitting equation 3 to the experimental data points (criterion: weighted least squares) by variation of i and N produced a value of 500 for N with a corresponding single channel current (i) of 0.6 pA (Figure 5b). Data from six cells gave a mean single channel current of 0.65 pA ± 0.06 pA (mean ± s.e.mean). Although the experimental values in Figure 5b tend to deviate from a straight line, we never observed the opening of all the channels which carried I_{KCO} (which would have been indicated by a reduction of current noise to control values). The calculated number for N (500) gives the minimum number of channels involved in the observed BRL 38227-induced effects. From the rising phase of the parabola in Figure 5b the unitary current (i) could have been calculated from the simpler equation ($i = \sigma^2/\mu$). However, using this over the whole range of experimental points would have produced an error (a reduction of about 10%) in the calculated value of i.

If the mean single channel current (0.65 pA) is simply divided by the driving force [holding potential E_K: (10 mV − (− 83 mV) = 73 mV)], a single channel conductance of 8.9 pS ± 0.82 pS (mean ± s.e.mean) results. When the single channel conductance was calculated according to the Goldman-Hodgkin-Katz equation a value of 16.6 pS at 0 mV was the result. Under symmetrical potassium concentrations $[K_i] = [K_o] = 152$ mM this value would be approximately 34 pS.

Effect of BRL 38227 on membrane potential

The above calculations suggest that in a single cell, BRL 38227 (1 μM) induces a current (I_{KCO}) carried by a few hundred channels each with a relatively small single channel conductance. To what extent this current can induce hyperpolarization can be approximately determined from the shift in the zero current potentials of the I–V curves in Figure 3b. A more precise way to determine the hyperpolarization induced by BRL 38227 was to measure the membrane potential and the ionic currents in the same cells. This was performed with an amplifier which provided fast switching between voltage-clamp and current-clamp. The upper traces of Figure 6 show the mean ionic currents generated by stepping to 0 mV from a holding potential of − 50 mV under control conditions and in the presence of BRL 38227 (1 μM). Data from three different cells, each subjected to 10 identical voltage/current-clamp protocols have been averaged. After 600 ms, the system was switched to current-clamp at 0 pA and the corresponding lower traces show the measured membrane potentials. BRL 38227 (1 μM) induced an average hyperpolarization of 22 mV in the investigated cells. An injection of a positive-going current pulse (10 pA) gave information about the input resistance of the cell under each condition. The marked deviation of membrane potential which was achieved immediately after switching into current-clamp from that potential achieved after 3000 ms at the end of the current-clamp was due to the deactivation of I_{TO}. The absolute value of membrane potential measured using whole-cell current-clamp is always influenced by the leakage current between membrane and pipette. Although the leakage current

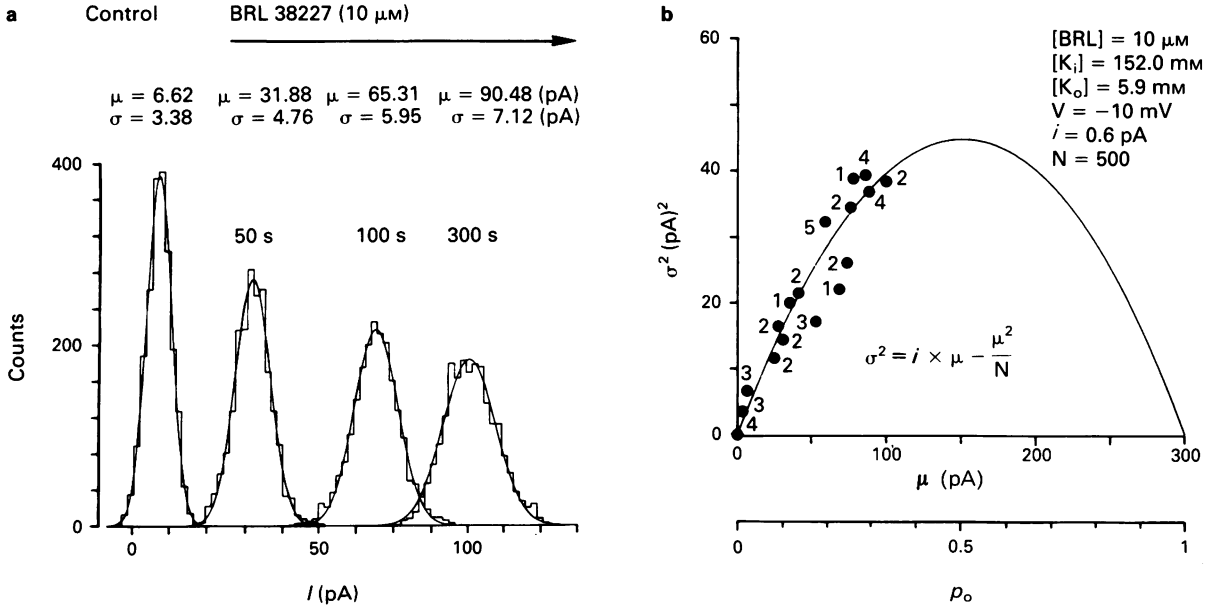

Figure 5 Determination using stationary fluctuation analysis of the single channel current associated with I_{KCO}. (a) Typical amplitude histograms of the current and accompanying current noise under control conditions (first peak) and in the presence of 10 μM BRL 38227 (peaks 2–4) in a single cell. The control reading was taken immediately prior to exposure to BRL 38227 and the effects of this agent were assessed at three different times, chosen to reflect the time-course of action of BRL 38227 in that particular cell. The numbers in (a) show the mean current (μ) after 50 s, 100 s and 300 s exposure to 10 μM BRL 38227 with the associated standard deviation (σ) of the current. Each histogram was constructed from 2048 data points. (b) Current variance (σ^2) — mean current (μ) plot for I_{KCO}. The numbers near each data point indicate the number of histograms and thus observations from which the points were derived. The data points were best fitted with a single channel current of 0.6 pA and with N (number of channels) = 500 channels. The open probability of the channels was calculated according to these values (lower scale in b).

was small because of the high leakage resistance (3 to 5 GΩ), the measured membrane potential was nevertheless shifted 10 to 20 mV in a positive direction (see Methods for an estimation of leakage current). However, the relative hyperpolarization measured with the present experimental configuration was little affected by such leakage.

Effect of glibenclamide on membrane currents and potential in the presence of BRL 38227

To study the effect of glibenclamide on the currents induced (I_{KCO}) and inhibited (I_{TO}) by BRL 38227, the same voltage/current-clamp protocol was used as described above. At a concentration of 1 μM, nearly 600 s was required to produce a steady-state response to BRL 38227, and thus the recording conditions of these long-lasting experiments (usually more than 45 min) had to be extremely stable. Figure 7 shows an experiment in which it was possible to determine the effects of glibenclamide at two different concentrations (1 and 10 μM). The results clearly demonstrate that the large hyperpolarizations induced by BRL 38227 (1 μM) were substantially reversed by glibenclamide (1 μM). Almost complete reversal of the BRL 38227-induced hyperpolarization was established at a concentration of 10 μM glibenclamide.

The ability of glibenclamide to oppose BRL 38227-induced membrane hyperpolarization has been described above. However, this does not indicate which current components were influenced by glibenclamide. We therefore performed a series of voltage-clamp protocols in which the initial control run was followed by a second sequence in the presence of 1 μM BRL 38227 and a third in the presence of both 1 μM BRL 38227 and 1 μM glibenclamide. Current traces obtained on stepping to test potentials from holding potentials of − 90 mV and − 10 mV under each of the three conditions were analysed and current-voltage relationships were constructed (Figure 8). Stepping from a holding potential of − 90 mV under control conditions elicited I_{NI} and I_{TO} (Figure 8a). The addition of BRL 38227 induced I_{KCO} and inhibited a fraction (designated β) of I_{TO}. This dual effect of BRL 38227 (also evident in Figure 3) is clearly demonstrated in Figure 8c, in which the current induced by BRL 38227 was less under conditions in which I_{TO} was activated than when there was no current contribution from I_{TO}. The current-voltage relationship for the current induced by BRL 38227 (Figure 8c) was similar to that shown in Figure 3c which was obtained in a cell from a different animal. Glibenclamide (1 μM) added to the bathing fluid in the continuous presence of BRL 38227, inhibited I_{KCO} in a concentration-dependent manner. The fraction of I_{KCO} inhibited by glibenclamide is designated α in Figure 8. It is clear from Figure 8c that glibenclamide (1 μM) did not inhibit I_{TO} since the currents which were available from holding potentials of − 90 mV or − 10 mV were inhibited by glibenclamide to the same extent. Any inhibition of I_{TO} by glibenclamide would have produced a larger suppression of current when stepping from a holding potential of − 90 mV than when stepping from − 10 mV. Thus the current I_{KCO} was always reversed by glibenclamide (with an EC_{50} value of approximately 3 μM; $n = 5$) but no significant reduction of I_{TO} by glibenclamide (1 μM) was detected.

Discussion

The results described in this paper demonstrate that BRL 38227 (100 nM to 10 μM) opens K-channels in the plasma membrane of isolated smooth muscle cells from the rat portal vein. The time taken for K-channel opening to reach steady-state (several minutes) was dependent on the concentration of BRL 38227. In those experiments in which a low concentration of BRL 38227 (100 nM) was used, it took nearly 15 min to produce such a response. A slow onset of the action of cromakalim was also observed in cells from rat and rabbit portal veins (Beech & Bolton, 1989b; Okabe *et al.*, 1990).

The current induced by BRL 38227 was non-inactivating

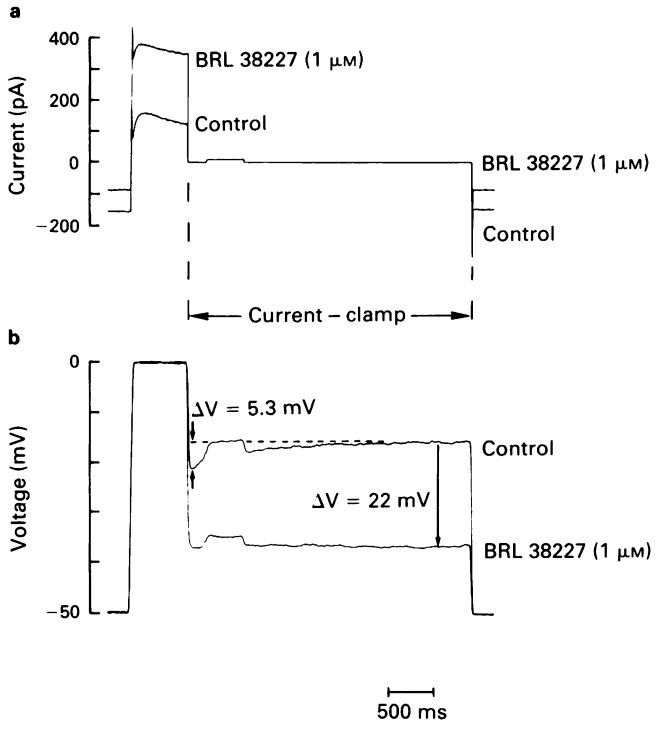

Figure 6 Mean effect of BRL 38227 (1 μM) on membrane currents and membrane potential using the voltage-clamp/current-clamp protocol. (a) The superimposed current traces show the effect of BRL 38227 on membrane currents when the potential was stepped from a holding potential of − 50 mV to 0 mV. Under current-clamp the membrane current was held at 0 pA apart from a current pulse of + 10 pA injected to give an indication of membrane input resistance. (b) Voltage protocol and membrane potential which corresponds to the current traces above. The size of the BRL 38227-induced hyperpolarization at the end of the 3000 ms current-clamp protocol (V = 22 mV) is given by the vertical arrow. The slow decay of membrane potential immediately after switching into current-clamp (V = 5.3 mV) is due to deactivation of I_{TO}. From the electrotonic potential, following current injection of 10 pA it can be deduced that I_{TO} contributed about 20 pA to the total current immediately after switching into current-clamp. Data are the mean of 10 identical successive voltage/current-clamp protocols in each of three different cells (i.e. a total of 30 episodes).

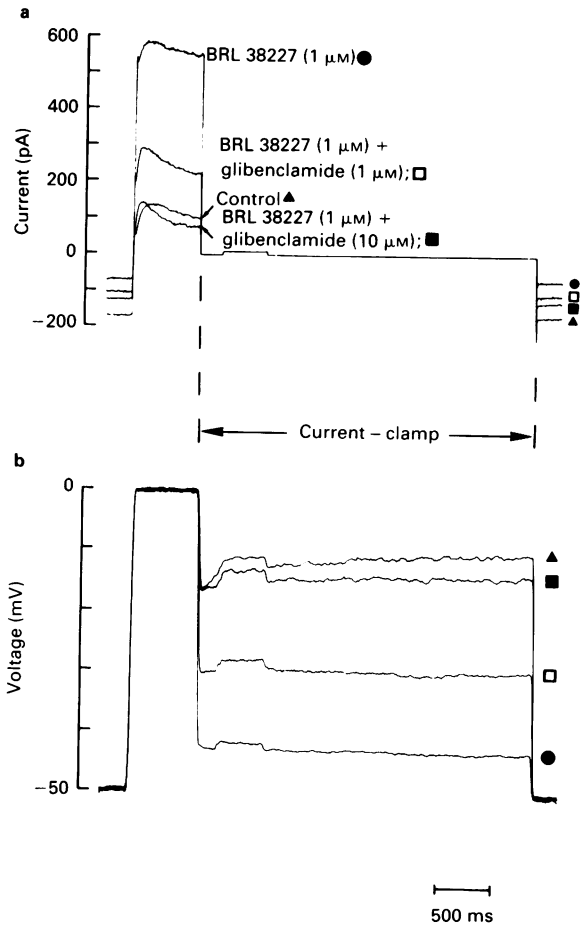

Figure 7 Effect of glibenclamide on membrane currents in the presence of BRL 38227 (1 μM). The experimental protocol was the same as that used in Figure 6. After the first trial (control, ▲) the cell was exposed to BRL 38227 (1 μM, ●). The outward current induced by depolarization to 0 mV was markedly increased by BRL application and the membrane potential hyperpolarized by more than 30 mV. The addition of glibenclamide (1 μM, □ or 10 μM, ■) in the continuous presence of 1 μM BRL reversed BRL 38227-induced current increase and shifted the membrane potential towards control values in a concentration-dependent manner. Each current and voltage trace is the average derived from 10 identical successive voltage/current-clamp protocols.

and was associated with an increase of current noise density. Analysis of this noise indicated that the channel involved in this response had a quite small single channel conductance and the single channel current associated with I_{KCO} was 0.65 pA at − 10 mV. The macroscopic current induced by BRL 38227 was well-described by the Goldman-Hodgkin-Katz equation and using this, the unitary conductance of the channel which underlies I_{KCO} was calculated to be close to 17 pS. Importantly, this current was inhibited in a concentration-dependent manner by glibenclamide, a compound which blocks all the effects of the KCOs both *in vivo* and *in vitro* (Buckingham *et al.*, 1989; Newgreen *et al.*, 1990). In experiments using isolated patches, the KCOs have been demonstrated to increase the open probability of a large variety of K-channels, ranging from small conductance (7.5 pS) K-channels (Nakao & Bolton, 1991) to very large (251 pS) K-channels (BK_{Ca}; Hu *et al.*, 1990) and from small conductance (30 pS) K-channels sensitive to extracellular calcium (Inoue *et al.*, 1989) to small conductance calcium-dependent K-channels sensitive to intracellular ATP (Kajioka *et al.*, 1990). In the present study, the whole-cell current induced by BRL 38227 was associated with a small (17 pS) K-channel. The involvement of a small conductance K-channel in the whole-cell current was also reported by Beech & Bolton (1989b).

This contrasts with data obtained from isolated patches, or from planar lipid bilayers into which several KCOs were found to increase the open probability of a large conductance Ca-sensitive K-channel which was identified as BK_{Ca} by its sensitivity to charybdotoxin (Gelband *et al.*, 1990; Hu *et al.*, 1990). However, purified charybdotoxin (which blocks BK_{Ca}) has no effect *in vitro* on the mechano-inhibitory response to either cromakalim or the structurally dissimilar KCO, minoxidil sulphate, in rat portal vein or rabbit aorta (Winquist *et al.*, 1989; Wickenden *et al.*, 1991). In addition, charybdotoxin has no effect on cromakalim-induced $^{86}Rb^+$ efflux from rabbit aorta (Strong *et al.*, 1989).

Furthermore, the whole-cell current induced by cromakalim in the rabbit portal vein was unaffected by charybdotoxin (Beech & Bolton, 1989b). Thus, the observations that KCOs are capable of increasing the open probability of large conductance Ca-sensitive K-channels in isolated patches or after incorporation into lipid bilayers (Gelband *et al.*, 1990; Hu *et al.*, 1990) may have little physiological relevance.

Since the effects of the KCOs are inhibited by glibenclamide (Buckingham *et al.*, 1989; Newgreen *et al.*, 1990), a selective blocker of the ATP-sensitive K-channel (K_{ATP}) in insulin-secreting cells (Sturgess *et al.*, 1988) and cardiac cells

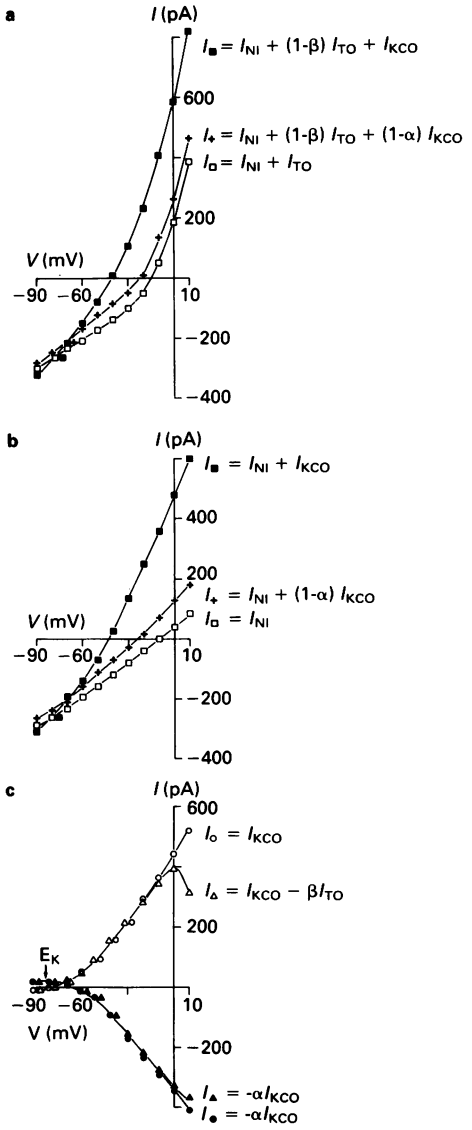

Figure 8 Current-voltage relationships of the currents in rat portal vein and effects of BRL 38227 and glibenclamide. (a) Current-voltage relationship of the peak of total current (peak of I_{TO}) under control conditions (□), in the presence of BRL 38227 (1 μM) (■) and in the presence of both 1 μM BRL 38227 and 1 μM glibenclamide (+). The holding potential was − 90 mV to ensure full activation of I_{TO}. (b) Current-voltage relationship of the non-inactivating currents under control conditions (□) and in the presence of 1 μM BRL (■), obtained from the same cell as in (a). The holding potential was − 10 mV which ensured complete inactivation of the time-dependent current components. (c) Current-voltage relationships of the net current induced by BRL 38227 (difference between currents in the presence and absence of BRL 38227) for the two holding potentials (△, − 90 mV; ○, − 10 mV). Also shown is the effect of glibenclamide (difference between currents in the presence of BRL 38227 and those in the presence of both BRL 38227 and glibenclamide) for the two holding potentials (▲, − 90 mV; ●, − 10 mV).

(Escande *et al.*, 1988) it has been suggested that this channel might be involved in the effects of the KCOs in smooth muscle (Quast & Cook, 1989). However, the relative sensitivity of the heart and pancreatic β-cell to KCOs and glibenclamide differs from that of smooth muscle (see Weston & Edwards, 1991), suggesting that the channel activated by the KCOs in smooth muscle differs from that at which they exert their effect in the heart or pancreas. Indeed, a channel similar to K_{ATP} in the pancreatic β-cell or the heart has not been found in smooth muscle. A brief report has described an ATP-sensitive K-channel in rat and rabbit mesenteric artery which was opened by cromakalim and inhibited by glibenclamide (Standen *et al.*, 1989). Whether ATP *per se* or whether magnesium released from the ATP salt was responsible for the channel inhibition (Kajioka *et al.*, 1991) is not certain. Further evidence that an ATP-sensitive K-channel exists in vascular smooth muscle is still awaited. Nevertheless, the suggestion (Standen *et al.*, 1989) that this relatively large conductance channel (135 pS under asymmetrical K^+ conditions, 60 mM extracellular K^+; 120 mM intracellular K^+) might be the main carrier of the K-current induced by BRL 38227 would be inconsistent with the findings of the present study.

Although single channel recordings are useful to indicate the properties of identified channels, it is impossible to extrapolate to what extent changes in single channel parameters influence membrane potential. The experimental conditions used in the present study (whole-cell voltage-clamp; whole-cell current-clamp; stationary fluctuation analysis) provide information about whole-cell currents, single channel current and membrane potential in the same cell. Using this approach the observed single channel responses and associated whole-cell currents are the determinants of the change in membrane potential. Having concluded that, under our experimental conditions, the main channel responsible for the actions of BRL 38227 is a non-inactivating channel with a conductance of 17 pS, future single channel experiments can now be performed in which patches which do not possess this channel will be excluded. In the present experiments I_{KCO} was recorded under conditions of very low intra- and extracellular calcium (free calcium lower than 1 nM). This suggests that the calcium-dependency of the channel is very low, especially in comparison to BK_{Ca}. However, it is not possible to conclude that the current is totally Ca-independent since the intracellular Ca^{2+} distribution might not be homogeneous. Under similar experimental voltage-clamp conditions, I_{KCO} was described as a non-inactivating current with a small single channel conductance by Okabe *et al.* (1990) and Kajioka *et al.* (1990). However, these workers concluded that I_{KCO} was calcium-dependent from the observation that I_{KCO} decreased when manganese was substituted for extracellular calcium. In our experiments extracellular calcium was replaced with magnesium and EGTA was added (see Methods) which should have provided a much lower concentration of extracellular calcium in our experiments than in those of Okabe *et al.* (1990) and Kajioka *et al.* (1990). The most likely explanation for the discrepancy is that manganese can act as a K-channel blocker.

In the present study, BRL 38227 inhibited a transient outward current which activated and inactivated relatively slowly (I_{TO}) and which is probably identical to the delayed rectifier current (I_{dK}) described by Beech & Bolton (1989b). Although we have already briefly described this effect (Noack *et al.*, 1991), the finding that a K-channel opener can also inhibit a K-current is essentially novel. A similar inhibition of K-current by nicorandil or cromakalim was not described in the papers by Kajioka *et al.* (1990) or Okabe *et al.* (1990), respectively, although this inhibitory effect appears to be evident in many of the figures which illustrate current-voltage relationships in the presence of these KCOs. A suppression of I_{TO} and an induction of I_{KCO} by the KCOs can lead to a net current which exhibits two reversal potentials. Such $I-V$ relationships were usually obtained when positive test potentials (up to + 50 mV) were applied. One reversal potential corresponded to the potassium equilibrium potential, indicating that both I_{KCO} and I_{TO} were K^+ currents. The other reversal potential varied between + 10 mV and + 50 mV in different cells and was dependent on the magnitude of I_{KCO} and the degree of suppression of I_{TO}. The variability in this second reversal potential was evidence that the induction of I_{KCO} and the inhibition of I_{TO} were independent processes. The suppression of I_{TO} by BRL 38227 led to a reduction in the outward current which was quite marked at

very depolarized potentials. However, such potentials (+ 10 mV to + 50 mV) are unlikely to be achieved physiologically and it is unlikely that inhibition of I_{TO} would diminish the hyperpolarizing effect of BRL 38227 *in vivo*. Nevertheless, it will be interesting to determine whether such an effect on I_{TO} is shared by other structurally-dissimilar KCOs.

Based on the relative potencies of a variety of compounds to inhibit the K-channels present in the rabbit portal vein, Beech & Bolton (1989b) concluded that cromakalim exerted a stimulatory effect on the delayed rectifier current, I_{dK} (I_{TO}). Although cromakalim apparently induced a non-inactivating current (in contrast to I_{dK} itself), Beech & Bolton (1989b) concluded that the voltage sensitivity of the channel underlying I_{dK} had been modified by cromakalim. In our study, the total K-current (compare Figures 3c and 8c) in the presence of BRL 38227 showed a pronounced point of inflection at potentials over which I_{TO} was strongly activated. This cannot be explained by modification of the voltage-dependent activation and inactivation parameters of I_{TO} (I_{dK}) by BRL 38227, such that I_{TO} is always activated but that the underlying single channel conductance is unchanged. Moreover, noxiustoxin, a blocker of the delayed rectifier current (Carbone *et al.*, 1982), did not inhibit the mechano-inhibitory response to either cromakalim or minoxidil sulphate in rabbit aorta (Wickenden *et al.*, 1991). In the study performed by Beech & Bolton (1989b), glibenclamide (50 μM) blocked only the current induced by cromakalim but apparently not I_{dK} which was of a similar magnitude in the presence of glibenclamide and cromakalim as before exposure to these two agents (which would mean that cromakalim both changes the voltage-dependency of I_{dK}-channels and induces glibenclamide-sensitivity to the channels). In the present study, glibenclamide inhibited the current induced by BRL 38227 but had no relevant effect on the inhibition of I_{TO} by BRL 38227. This is illustrated in Figure 8c but is clearly evident in Figure 7, in which the current suppressed by glibenclamide was not influenced by changing the holding potential from − 90 mV to − 10mV. Since glibenclamide neither modified the effect of BRL 38227 on I_{TO} significantly nor reduced the non-inactivating current component which could not be attributed to BRL 38227, it appears that glibenclamide is a selective blocker of I_{KCO}. This is consistent with the finding *in vitro* that glibenclamide only inhibits BRL 38227-induced efflux of $^{42}K^+$ but has no effect on the basal $^{42}K^+$ efflux from rat portal vein (Edwards *et al.*, 1991).

Using the fast-switching voltage-clamp/current-clamp amplifier, a very large hyperpolarization was induced by 1 μM BRL 38227. Such a large hyperpolarization, produced by activation of I_{KCO} alone, would be sufficient to reduce calcium influx through voltage-operated calcium channels and to account for the observed effects of BRL 38227 in whole tissues. This observation, together with the inability of charybdotoxin to modify the response to BRL 38227 *in vitro* (see earlier) questions the possible role of other K-channel types (e.g. BK_{Ca}; Gelband *et al.*, 1989; Hu *et al.*, 1989) in the action of the KCOs.

The present study strongly suggests that the effects of BRL 38227 in whole tissues can be fully explained by the generation of a K-current which we have designated I_{KCO}. Based on stationary fluctuation analysis, the channel underlying I_{KCO} has a unitary conductance of 17 pS. Some of the characteristics of I_{KCO} are apparently different from those of the KCO-induced current and KCO-activated K-channel described by Kajioka *et al.* (1990, nicorandil; 1991, pinacidil), Xiong *et al.* (1991, pinacidil) and Nakao & Bolton (1991, cromakalim). However, it seems possible that all these recent investigations (present study; Nakao & Bolton,1991; Kajioka *et al.*, 1990; 1991; Xiong *et al.*, 1991) describe an identical channel, the properties of which are modified by the different cell separation procedures and/or detailed recording conditions in the various laboratories. In particular there are close similarities between our raw data and those of Beech & Bolton (1989b); the more detailed analysis in the present study strongly suggests that the K-current activated by KCOs is indeed a separate current (I_{KCO}) rather than a modified I_{dK} (I_{TO}) as suggested by Beech & Bolton (1989b). Studies are now in progress to characterize I_{KCO} further and to establish its ATP-sensitivity and unitary conductance in isolated patches.

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