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Levcromakalim may induce a voltage-independent K-current in rat portal veins by modifying the gating properties of the delayed rectifier

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1 Smooth muscle cells of the rat portal vein were dispersed by enzymatic treatment and recordings of whole-cell currents under calcium-free conditions were made by the voltage-clamp technique. The effects of the potassium (K)-channel opener, levcromakalim, on K-currents were compared with those of agents which modify protein phosphorylation.

2 Levcromakalim (1–10 μM) added to the extracellular (bath) fluid caused the development of a non-inactivating current ($I_{K(ATP)}$) and simultaneously inhibited the delayed rectifier current ($I_{K(V)}$) in a concentration-dependent manner. On prolonged exposure to levcromakalim (10 μM), $I_{K(ATP)}$ declined and $I_{K(V)}$ was further diminished.

3 Addition to the pipette (intracellular) solution of the selective inhibitor of protein kinase C, calphostin C, itself had no effect on K-currents and did not modify the induction of $I_{K(ATP)}$ or the simultaneous inhibition of $I_{K(V)}$ produced by 1 μM levcromakalim.

4 Addition of the protein kinase inhibitor (PKI(6-22)amide, 1 μM) to the pipette solution caused the production of a glibenclamide-sensitive, non-inactivating current and inhibited $I_{K(V)}$.

5 In an assay system, levcromakalim (10 μM) did not inhibit the activity of purified protein kinase A (Type 1 or Type 2).

6 Addition to the pipette solution of the phosphatase inhibitor, okadaic acid (1 μM), did not itself modify K-currents and had little effect on the simultaneous induction of $I_{K(ATP)}$ and inhibition of $I_{K(V)}$ by levcromakalim (1 μM).

7 When the pipette solution contained 1 mM MgATP (but was depleted of substrates for ATP production), a non-inactivating, glibenclamide-sensitive K-current developed spontaneously in 5 out of 11 cells with the simultaneous reduction of $I_{K(V)}$. In 3 of the 6 remaining cells, addition of the dephosphorylating agent, butanedione monoxime (5 mM) to the bath inhibited $I_{K(V)}$ and stimulated a glibenclamide-sensitive non-inactivating current.

8 Depletion of intracellular Mg^{2+} slightly enhanced $I_{K(V)}$. Under these conditions, levcromakalim (1 μM and 10 μM) did not significantly induce $I_{K(ATP)}$ or inhibit $I_{K(V)}$.

9 It is concluded that the effects of levcromakalim on K-currents can be mimicked by procedures designed to reduce channel phosphorylation. The results are consistent with the view that levcromakalim dephosphorylates the delayed rectifier channel, K_V, which becomes converted into a voltage-independent, non-inactivating form known as K_{ATP}. The possible mechanisms which underlie this interconversion are discussed.

Keywords: Levcromakalim; protein kinase C; protein kinase A; whole-cell voltage clamp; smooth muscle; butanedione monoxime; calphostin C; PKI(6-22)amide; delayed rectifier (K_V); ATP-sensitive K-channel (K_{ATP})

Introduction

K-channel openers such as levcromakalim, nicorandil, pinacidil and P1060 induce a voltage-independent, non-inactivating K-current in vascular smooth muscle. This current is thought to be carried by ATP-sensitive K-channels (K_{ATP}) (Clapp & Gurney, 1992; Noack *et al.*, 1992c; Russell *et al.*, 1992; Silberberg & van Breemen, 1992; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994) with an underlying unitary conductance in the range 10–30 pS (quasi-physiological conditions, Kajioka *et al.*, 1990; 1991; Noack *et al.*, 1992a; Bolton *et al.*, 1993; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994). Reducing the ability of the cell to synthesize ATP induces a K-current ($I_{K(ATP)}$; Noack *et al.*, 1992c; Silberberg & van Breemen, 1992) with characteristics identical to those of the current induced by the K-channel openers (Noack *et al.*, 1992c; Ibbotson *et al.*, 1993a). In smooth muscle, such results collectively suggest that these agents could compete with ATP for access to the inhibitory ATP binding site on an ATP-sensitive K-channel (K_{ATP}; see Edwards & Weston, 1993), a mechanism originally proposed to account for the actions of the K-channel opener RP49356 in cardiac myocytes (Thuringer & Escande, 1989). In support of this view is the recent finding in cultured fibroblasts that K-channel openers reduce the current through an ATP-sensitive chloride channel (Cl_{ATP}) which closes as ATP binding to the channel regulatory site *decreases* (Sheppard & Welsh, 1992).

In parallel with the induction of $I_{K(ATP)}$ by the K-channel openers in vascular smooth muscle is the simultaneous inhibition of the delayed rectifier current ($I_{K(V)}$; Beech & Bolton, 1989a; Noack *et al.*, 1992a; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994). Furthermore, removal of substrates for ATP synthesis, and the presumed reduction in the intracellular ATP concentration ($[ATP]_i$), also inhibits $I_{K(V)}$ (Noack *et al.*, 1992c). Since in other tissues the channels (K_V) which carry $I_{K(V)}$ are sensitive to phosphorylation (Perozo & Bezanilla, 1990; Duchatelle-Gourdon *et al.*, 1991), the K-channel openers could reduce the phosphorylation of K_V by competing with ATP for access to sites on enzymes like protein kinases (see Scott, 1991).

The objective of the present study was to obtain more

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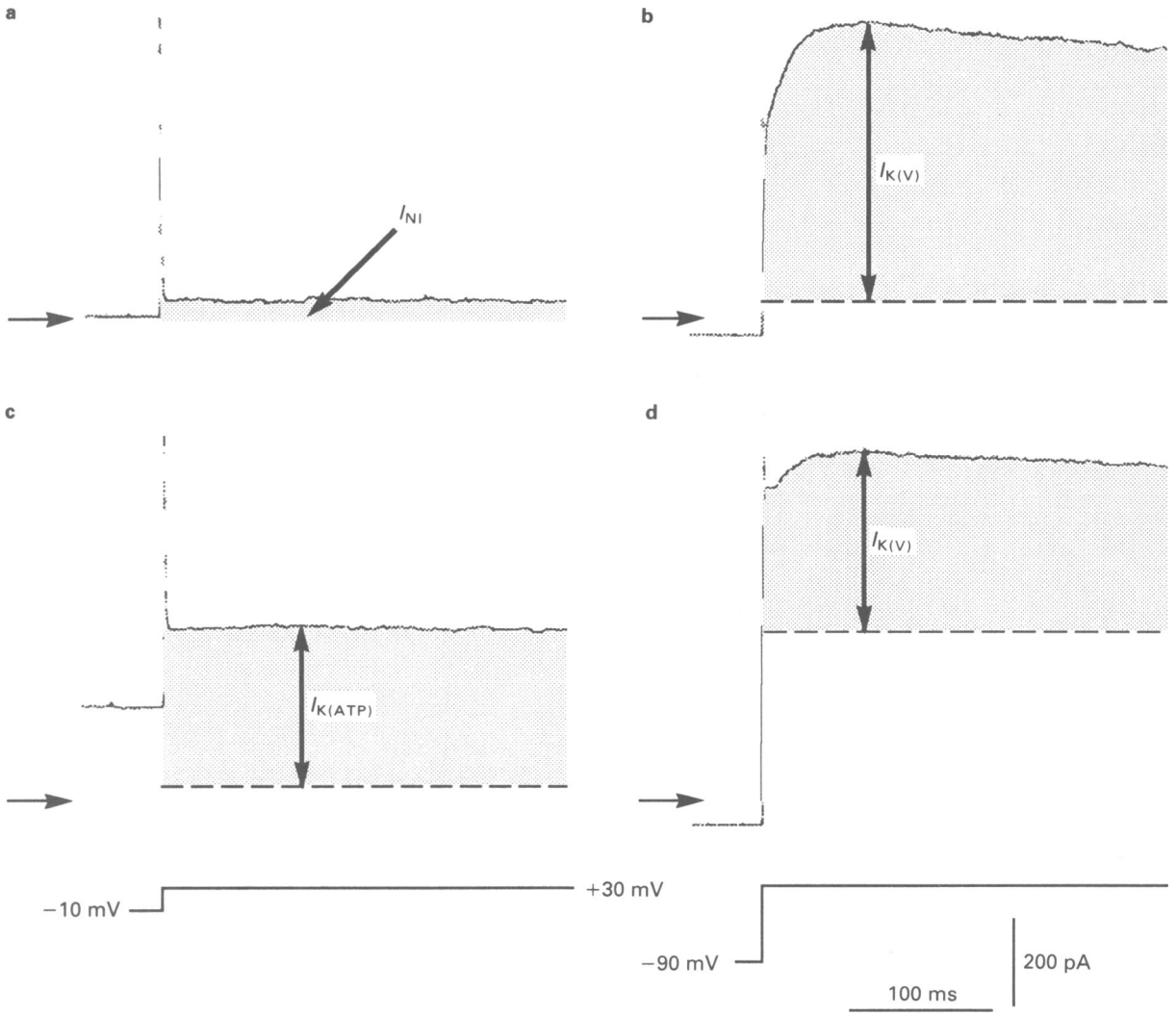

Figure 1 Currents obtained in isolated, single portal vein cells of the rat under calcium-free conditions in the absence (a, b) or presence (c, d) of levcromakalim. (a) On stepping to a test potential of + 30 mV from a holding potential of − 10 mV only a non-inactivating current (I_{NI}) was obtained (represented by shaded area above the zero current level). However, on stepping to the same test potential from a holding potential of − 90 mV (b) both I_{NI} and the rapidly activating and relatively slowly inactivating delayed rectifier K-current ($I_{K(V)}$) were observed. $I_{K(V)}$ (shaded area) is the portion of the total current remaining after subtraction of I_{NI} (magnitude indicated by dashed line). In the presence of 10 μM levcromakalim (c) a second non-inactivating current component was present on stepping from − 10 mV to + 30 mV. This component ($I_{K(ATP)}$, shaded area) is obtained by subtraction of I_{NI} (magnitude indicated by dashed line). On stepping to + 30 mV from a holding potential of − 90 mV in the presence of 10 μM levcromakalim, $I_{K(V)}$ was also present (d). Note that after subtraction of the non-inactivating currents (I_{NI} and $I_{K(ATP)}$; magnitude indicated by the dashed line), $I_{K(V)}$ (shaded area) was reduced (compare b and d). Each trace was derived by averaging the currents obtained by these protocols in 4 separate cells from different animals. For each trace, the zero current level is indicated by the horizontal arrow.

information on the modulation of K-currents by levcromakalim in vascular smooth muscle using whole-cell voltage-clamp techniques. The effects of this K-channel opener were examined and compared with those of protein kinase inhibitors, a phosphatase inhibitor and a dephosphorylating agent. Using this approach, it was hoped to clarify how levcromakalim could induce $I_{K(ATP)}$ and simultaneously inhibit $I_{K(V)}$. A preliminary account of some of these findings has been given (Edwards *et al.*, 1993).

Methods

Protein kinase A assay

Unless otherwise indicated, protein kinase A activity was determined by incubating 2 μM adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase (Type 1 or Type 2, derived from rabbit muscle; Sigma) with 100 μM ATP (magnesium salt), 10 μM cyclic AMP and substrate for protein kinase A, 50 μM Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) in a 50 mM Tris HCl buffer solution containing 10 mM $MgCl_2$ and 0.25 mg ml^{-1} bovine serum albumin (pH 7.5) for 15 min at room temperature in the presence or absence of the potential modifying agents or of a protein kinase A inhibitor (PKI(2-22)amide). After addition of [γ-^{32}P]-ATP (ICN Biomedicals, 167 TBq $mmol^{-1}$; final concentration 55 nM) the tubes were incubated at 30°C for 6 min. The reaction was stopped by spotting 20 μl of the reaction mixture from each tube onto phosphocellulose paper discs which were washed firstly with 1% (v/v) phosphoric acid and then with water before being placed in scintillation vials containing 4 ml Ecoscint A (National Diagnostics). The radioactivity associated with the peptide-incorporated ^{32}P was determined by liquid scintillation counting.

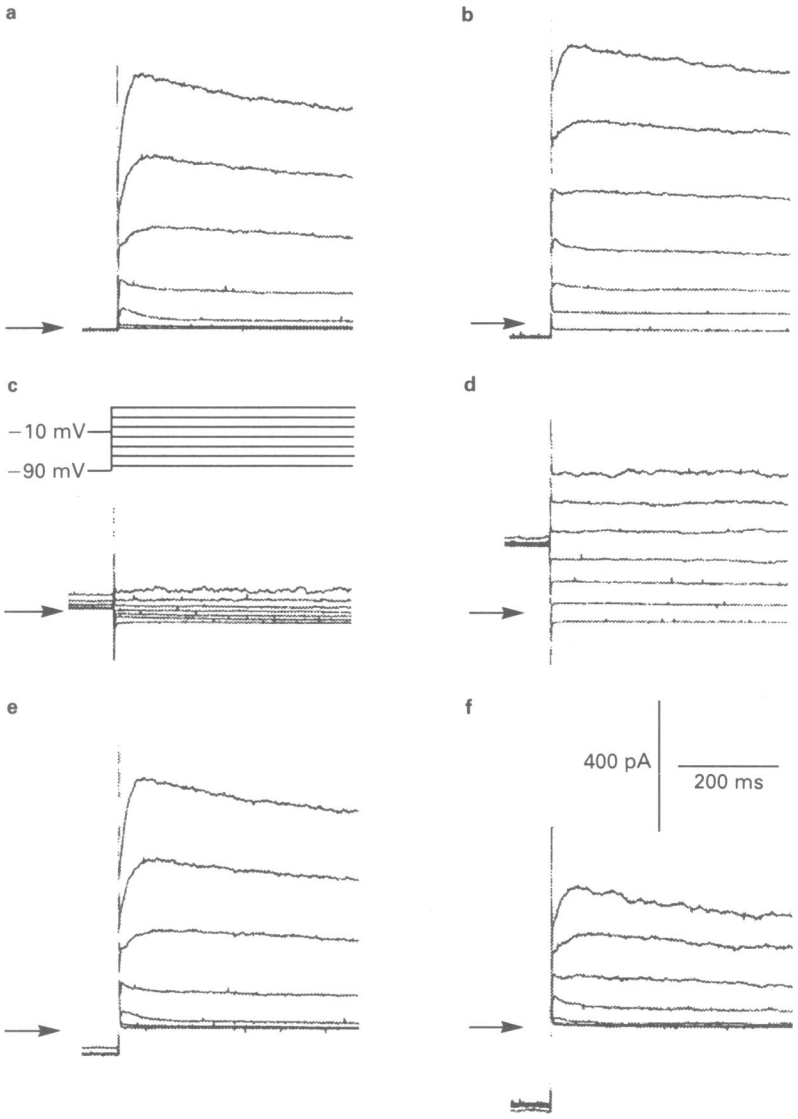

Figure 2 Families of currents obtained in isolated single portal vein cells of rat under calcium-free conditions in the absence (a, c, e) or presence (b, d, f) of levcromakalim. Cells were clamped at − 90 mV (a, b) or − 10 mV (c, d) and then stepped in 20 mV increments to a series of test potentials ranging from − 80 mV to + 40 mV (protocol illustrated in upper part of (c)). (a) Under control conditions, on stepping to the test potentials from a holding potential of − 90 mV, both I_{NI} and $I_{K(V)}$ were present. However, on stepping to the same test potentials from a holding potential of − 10 mV only I_{NI} was obtained (c). Digital subtraction of I_{NI} (c) from the peak total currents (a) for each test potential produced the trace shown in (e) which represents the component due to $I_{K(V)}$. In the presence of 10 μM levcromakalim (b) the total current at each test potential was slightly greater than in controls (a). However, the non-inactivating current component (which now comprises I_{NI} + the levcromakalim-induced $I_{K(ATP)}$) (d) was markedly enhanced (compare (d) and (c)). Subtraction of the non-inactivating currents from the total currents showed that $I_{K(V)}$ (f) was inhibited in the presence of 10 μM levcromakalim (compare (f) and (e)). Each trace was derived by averaging the currents obtained using these protocols in 4 separate cells from different animals. For each trace, the zero current level is indicated by the horizontal arrow. See Figure 1 for further explanation of currents.

Production of isolated cells

All whole-cell voltage-clamp experiments were performed on single smooth muscle cells isolated from portal veins which were removed from male Sprague-Dawley rats (100–125 g body weight), previously killed by stunning and bleeding. Each portal vein (about 15 mm length) was carefully cleaned of fat and connective tissue with fine scissors in conjunction with a dissecting microscope. For cell dispersal, intact portal veins were incubated in a low-Ca^{2+} physiological salt solution (PSS) containing collagenase and pronase (see Solutions) for 25 min. They were then cut into 4 segments and triturated in Kraftbrühe (Klöckner & Isenberg, 1985) in a wide bore, smooth-tipped pipette. The cells were used for experiments within 8 h of separation, during which time they were stored at 6°C in Kraftbrühe. All experiments were performed at 23°C.

Single-cell electrophysiology

The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used in all experiments. Patch pipettes were pulled from Pyrex glass (H 15/10, Jencons, UK) and had resistances of 3–4 MΩ when filled with the internal (intracellular) solution. Voltage commands and data acquisition were performed as described by Noack *et al.* (1992b). For cell stimulation and for recording and analysing data the pCLAMP 5.5 programme was used (Axon Instruments, U.S.A.). Data acquisition and storage were as described by Ibbotson *et al.* (1993a). In each experiment the currents evoked by voltage steps from the stated holding potential were measured at their peak level.

We have previously demonstrated the ability of K-channel openers to simulate $I_{K(ATP)}$ under nominally calcium-free conditions (Noack *et al.*, 1992a; Ibbotson *et al.*, 1993a). On the

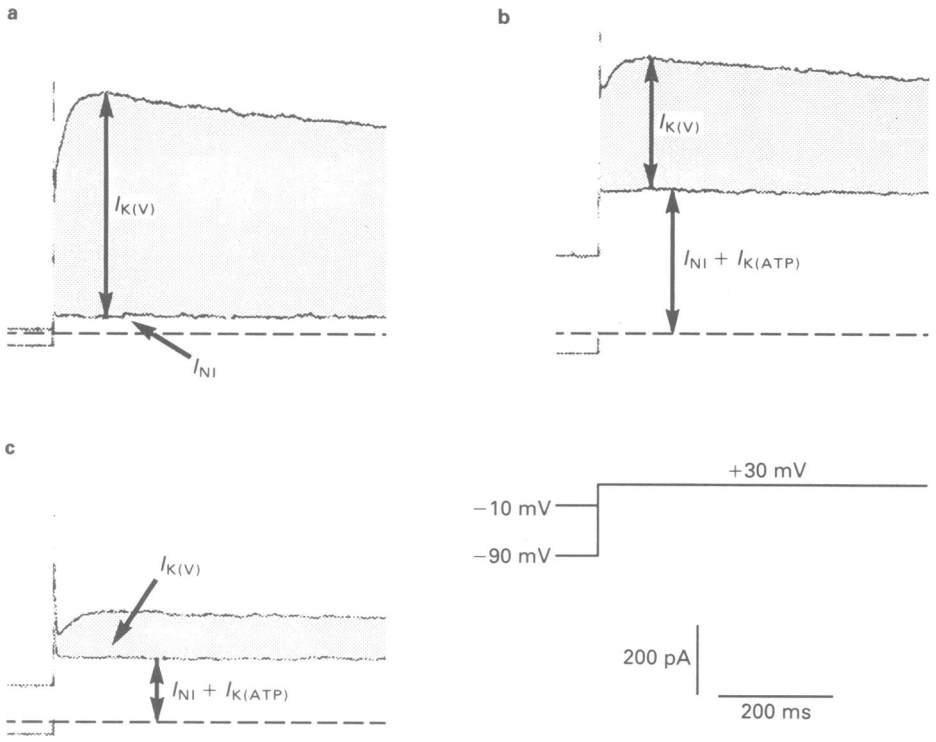

Figure 3 Currents obtained in isolated, portal vein cells of rat under calcium-free conditions in the absence (a) or presence (b, c) of levcromakalim. (a) On stepping to a test potential of + 30 mV from a holding potential of − 10 mV only a non-inactivating current (I_{NI}) was obtained. On stepping to the same test potential from a holding potential of − 90 mV both $I_{K(V)}$ (shaded area) and I_{NI} were observed. After 15 min superfusion with 10 μM levcromakalim (b) $I_{K(ATP)}$ and I_{NI} were present on stepping from − 10 mV to + 30 mV. On stepping to + 30 mV from − 90 mV, the total evoked current was slightly increased but $I_{K(V)}$ (shaded area) was markedly reduced (b). After exposure to levcromakalim for 30 min both $I_{K(V)}$ and $I_{K(ATP)}$, and thus the total current, were greatly reduced. Each trace was derived by averaging the currents obtained by these protocols in 4 separate cells from different animals. For each trace, the zero current level is indicated by a dashed line.

assumption that the bath and pipette solutions used in the present study had a contaminant calcium concentration of 10 μM (based on analysis by Petersen & Maruyama, 1983), the free calcium in these solutions was calculated (Fabiato, 1988) to be less than 1 nM due to the presence of EGTA. These conditions simplified the interpretation of data by effectively eliminating the involvement of K-currents carried by the large-conductance calcium-sensitive K-channel (BK_{Ca}). In whole portal veins, responses to levcromakalim are insensitive to selective inhibitors of BK_{Ca} (Winquist *et al.*, 1989; Wickenden *et al.*, 1991; Garcia & Kaczorowski, 1992).

The effects of the protein kinase A inhibitor, PKI(6-22)-amide, the protein kinase C inhibitor, calphostin C, and the phosphatase inhibitor, okadaic acid, were determined by inclusion of these agents in the pipette solution. The effects of butanedione monoxime (BDM), levcromakalim and glibenclamide were investigated by adding the appropriate amount(s) of these agents 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 (0.7 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.

Drugs and solutions

The low-Ca^{2+} PSS used for the cell separation comprised (mM): KCl 130, $CaCl_2$ 0.05, taurine 20, pyruvate 5, creatine 5, HEPES 10, collagenase (Type VIII, Sigma) 1 mg ml^{-1}, pronase (Calbiochem) 0.2 mg ml^{-1}, fatty acid free albumin 1 mg ml^{-1}, buffered with methanesulphonic acid to pH 7.4. Kraftbrühe comprised (mM): KCl 85, KH_2PO_4 30, $MgSO_4$ 5, Na_2ATP 5, K-pyruvate 5, creatine 5, taurine 20, β-OH-butyrate 5, fatty acid free albumin 1 mg ml^{-1}, pH adjusted to 7.2 with KOH. 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 (ethylene glycol-*bis* β-aminoethyl ether tetraacetic acid) 1.0, buffered with NaOH to pH 7.30; aerated with O_2. The pipette (internal) solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, HEPES 10, EGTA 1.2, glucose 11, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, buffered to pH 7.30 with KOH. Magnesium-free pipette solution was prepared similarly, but with exclusion of $MgCl_2$. The 1 mM MgATP pipette (internal) solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, HEPES 10, EGTA 1.2, MgATP 1. After the addition of MgATP, the pipette solution was buffered to pH 7.30 with KOH and used immediately.

Levcromakalim (Pfizer Central Research) and glibenclamide were first each dissolved in dimethyl sulphoxide (DMSO) to produce a concentrated stock solution (20 mM) from which dilutions were prepared with distilled water immediately before they were required. Calphostin C (Calbiochem) was dissolved in DMSO to give an 800 μM stock solution and diluted immediately before use. 2,3-Butanedione monoxime (BDM) and PKI(6-22)amide were dissolved directly in bath or pipette solutions, respectively, and were used immediately. Unless otherwise stated, all reagents and compounds were obtained from Sigma.

Data analysis

Treatment effects were analysed by 2-way within subject (repeated measures) ANOVA (Statistica v.3.0a:Statsoft). *P* values less than 0.05 were considered to be significant.

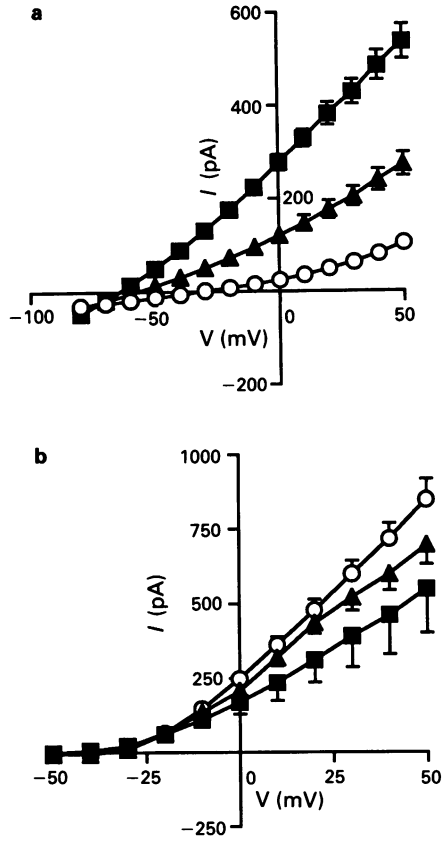

Figure 4 Effects of levcromakalim on current (I) – voltage (V) relationships determined in isolated single portal vein cells of rat under calcium-free conditions. Levcromakalim 1 μM (▲; $n = 10$–21) and 10 μM (■; $n = 4$–6) stimulated $I_{K(ATP)}$ ($P < 0.05$, a) and inhibited $I_{K(V)}$ ($P < 0.05$, b) in a concentration-dependent manner. (○) represents control non-inactivating (I_{NI}; a, $n = 26$) or inactivating ($I_{K(V)}$; b, $n = 14$) currents. Each point represents the mean ± s.e.mean value obtained.

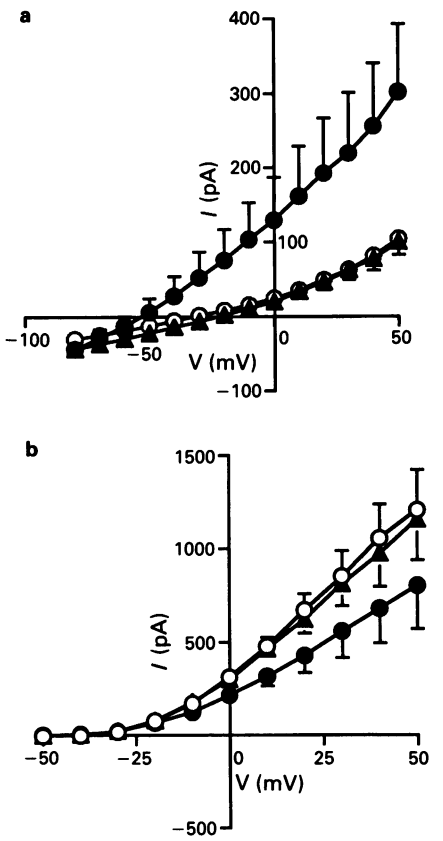

Figure 5 Effects of levcromakalim in the presence of a selective protein kinase C inhibitor, calphostin C (500 nM in the pipette solution) on (a) non-inactivating current and (b) the delayed rectifier K-current, $I_{K(V)}$: (○) represents control non-inactivating (I_{NI}; a) or inactivating ($I_{K(V)}$; b) current on breakthrough (i.e. immediately after obtaining the whole-cell recording configuration). Calphostin C (▲) had not modified either I_{NI} or $I_{K(V)}$ 18 ± 2 min after breakthrough. The induction of the non-inactivating current ($I_{K(ATP)}$) ($P < 0.05$) and the inhibition of $I_{K(V)}$ ($P < 0.05$) by 1 μM levcromakalim was similar in either the presence (●) or absence of calphostin C (see Figure 4). Each point represents the mean value obtained ± s.e.mean, $n = 4$.

Results

Effects of levcromakalim

In freshly-isolated rat portal vein cells under nominally calcium-free conditions, three types of current were evident. On stepping from a holding potential of − 90 mV to more positive test potentials, a rapidly-activating and -inactivating current developed (I_A). Although in some cells this could be observed over the full range of test potentials used (− 80 to + 50 mV), it was masked in most cells at potentials positive to approximately − 20 mV by the much larger delayed rectifier K-current, $I_{K(V)}$, which developed relatively slowly on stepping to potentials more positive than − 30 mV. $I_{K(V)}$ decayed fully over a period of several seconds leaving only the non-inactivating current (I_{NI}) which had a reversal potential of approximately − 30 mV. I_{NI} was thus not a pure K-current and was probably carried by cation channels.

To allow quantification of both the non-activating and inactivating currents, cells were clamped at a holding potential of either − 10 mV or − 90 mV (Figure 1). On holding at − 10 mV, the voltage-sensitive K-currents (I_A and $I_{K(V)}$) inactivated, and only the non-inactivating current component (I_{NI}) was present at each of the test potentials. $I_{K(V)}$ alone was thus determined by subtracting the non-inactivating current (holding potential − 10 mV) from the peak total current ($I_{NI} + I_{K(V)}$; holding potential − 90 mV) at each test potential (Noack *et al.*, 1992b).

Figure 2 shows the current obtained on stepping to a series of test potentials from a holding potential of − 90 mV (total currents) or a holding potential of − 10 mV (only non-inactivating currents available). Although the *total* current was slightly increased by 10 μM levcromakalim (Figures 2a and 2b), this agent produced a large increase in the non-inactivating current component by inducing $I_{K(ATP)}$ (Figures 2c and 2d). Under the conditions of the present study, subtraction of the non-inactivating current components ($I_{NI} + I_{K(ATP)}$) from the peak total current at each test potential revealed the current due to $I_{K(V)}$. It is evident from the traces shown in Figure 2 (e and f) that levcromakalim inhibited $I_{K(V)}$.

The non-inactivating K-current induced by levcromakalim ($I_{K(ATP)}$) is inhibited by glibenclamide and phentolamine and is thus easily distinguished from I_{NI} which is unaffected by these agents (Noack *et al.*, 1992a; Ibbotson *et al.*, 1993a). Note that $I_{K(ATP)}$ was never present under control, nominally calcium-free conditions. We have only observed this current in the presence of K-channel openers or under conditions in which protein dephosphorylation might occur (present study; Noack *et al.*, 1992c; Ibbotson *et al.*, 1993a).

Under control conditions $I_{K(V)}$ remained constant for at least 40 min (the duration of a typical experiment). However, in the presence of 10 μM levcromakalim (Figure 3) both $I_{K(V)}$ and $I_{K(ATP)}$ declined with time. Thus, 15 min after bath exposure to levcromakalim there was a large increase in the non-inactivating current component due to the induction of $I_{K(ATP)}$. However, $I_{K(V)}$ was simultaneously reduced, so that the total current in the presence of levcromakalim ($I_{K(V)} + I_{NI}$

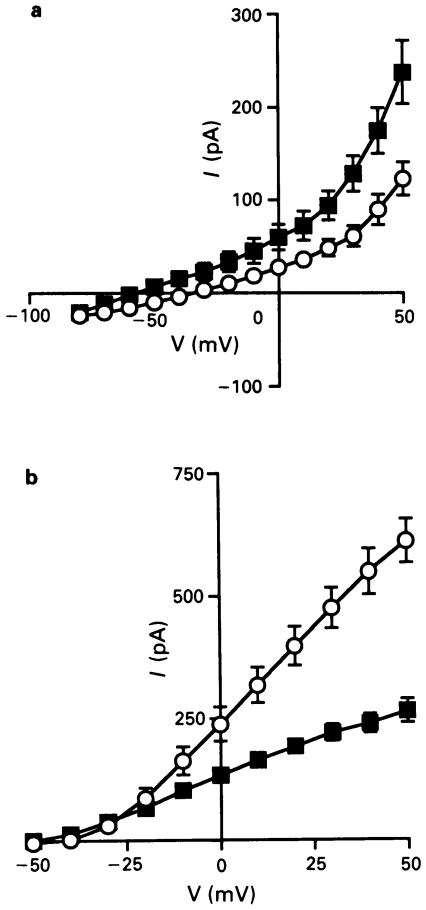

Figure 6 Induction of a non-inactivating K-current (a) and inhibition of the delayed rectifier K-current (b) by a protein kinase A inhibitor, PKI(6-22)amide (1 μM) in the pipette solution: (○) represents control non-inactivating (a) or inactivating ($I_{K(V)}$; b) currents on breakthrough (i.e. immediately after formation of the whole-cell recording configuration). (■) shows these currents at the peak of the development of the non-inactivating current (23 ± 2 min after breakthrough, $n = 5$). PKI(6-22)amide significantly enhanced the non-inactivating current and inhibited $I_{K(V)}$ ($P < 0.05$). Each point represents the mean ± s.e.mean value obtained, $n = 5$.

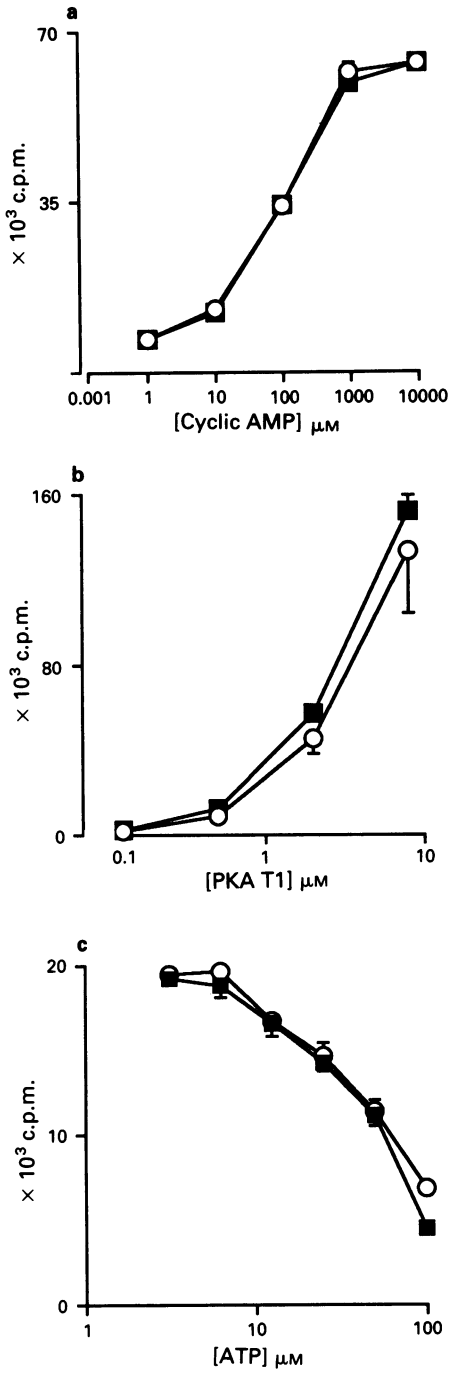

Figure 7 Effect of levcromakalim on Type 1 protein kinase A activity in the presence of varying concentrations of cyclic AMP (a) or type 1 protein kinase A (PKA T1; b). Incorporation of ^{32}P into the peptide substrate (counts per minute, c.p.m.) is an indication of the relative activity of protein kinase A in the presence of levcromakalim (■, 10 μM) or its vehicle (○, 0.1% dimethylsulphoxide). In (c), increasing the concentration of ATP reduced the incorporation of ^{32}P (○, vehicle control), an effect which was not modified by levcromakalim (■, 10 μM). Each point represents the mean value ± s.e.mean obtained ($n = 4$).

$+ I_{K(ATP)}$) was only slightly larger than that before superfusion with the drug ($I_{K(V)} + I_{NI}$). After 30 min exposure to levcromakalim the total current was markedly reduced and both $I_{K(V)}$ and $I_{K(ATP)}$ components had declined.

Figure 4a shows the concentration-dependent induction of $I_{K(ATP)}$ by levcromakalim. The current-voltage curves in the presence and absence of levcromakalim intersected between − 70 and − 80 mV, indicating that the induced current was K^+-selective. In every cell studied, the induction of $I_{K(ATP)}$ by levcromakalim was always accompanied by inhibition of $I_{K(V)}$ (Figure 4b). In the absence of K-channel openers the magnitude of both I_{NI} and $I_{K(V)}$ in the rat portal vein cells remained constant for up to 40 min under our control conditions.

Effects of protein kinase inhibitors

Addition of calphostin C (500 nM), a potent and highly selective inhibitor of protein kinase C (Kobayashi *et al.*, 1989), to the pipette solution had no effect on control currents and did not affect the induction of $I_{K(ATP)}$ or the inhibition of $I_{K(V)}$ by levcromakalim (1 μM) (Figure 5). In contrast, the inclusion of a potent inhibitor of protein kinase A, PKI(6-22)amide (1 μM; Glass *et al.*, 1989) in the pipette solution stimulated a non-inactivating K-current (Figure 6a). This current developed slowly, reaching a plateau at 23 ± 2 min ($n = 5$). Simultaneously, $I_{K(V)}$ was markedly reduced by this inhibitor in every cell tested ($P < 0.05$; Figure 6b). When the PKI(6-22)amide-induced K-current had reached its maximum it was inhibited by glibenclamide (1 μM; data not shown).

Several workers have concluded that K-channel openers might stimulate the opening of K_{ATP} by interfering with the binding of ATP to an inhibitory site on the channel (Thur-

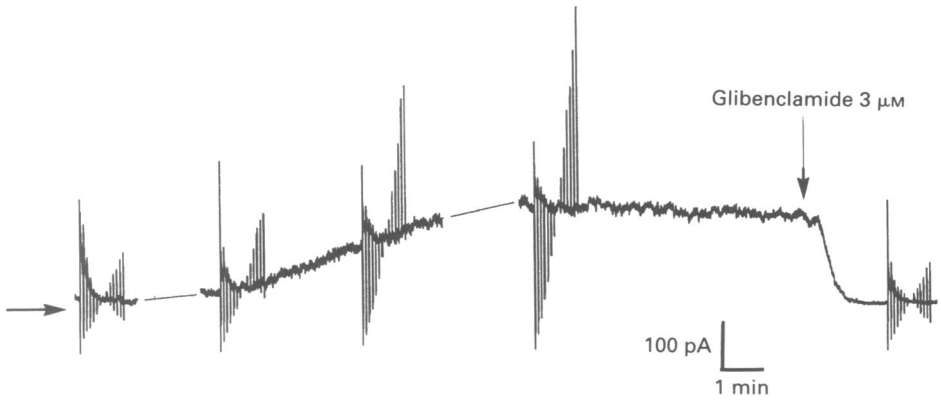

Figure 8 Slow development of a glibenclamide-sensitive outward current with time in a rat portal vein cell clamped at a holding potential of − 10 mV. The pipette solution contained 1 mM MgATP (see Drugs and Solutions). The trace commences immediately after breakthrough of the membrane within the pipette (i.e. formation of the whole-cell recording configuration), and zero current is indicated by the horizontal arrow. Deflections in the trace are the currents evoked on stepping from the holding potential to test potentials (− 80 to + 50 mV) in 10 mV increments. During gaps in the trace, the cell was clamped at − 90 mV to determine the changes in $I_{K(V)}$.

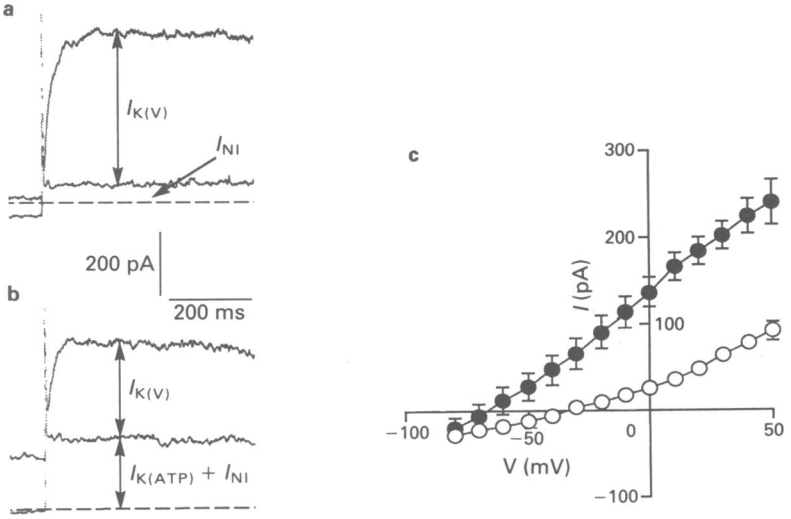

Figure 9 Induction of a non-inactivating current by butanedione monoxime (BDM). The trace in (a) shows the control non-inactivating current (I_{NI}) and the control delayed rectifier current ($I_{K(V)}$) obtained on stepping to a test potential of + 30 mV from holding potentials of − 10 mV or − 90 mV, respectively. (b) Shows the currents obtained in the same cell 6 min after superfusion with 5 mM BDM. Note that although the total current level (obtained on stepping from − 90 mV) was similar in the absence or presence of BDM, the non-inactivating current component ($I_{NI} + I_{K(ATP)}$, obtained on stepping from − 10 mV) was enhanced and the inactivating component ($I_{K(V)}$) was reduced in the presence of BDM (compare (a) and (b)). The graph in (c) represents the mean current (I) -voltage (V) relationship ($n = 3$) for the total non-inactivating currents ($I_{NI} + I_{K(ATP)}$) which were present at the peak of the response to bath application of BDM (●). Each point represents the mean values derived from those three (out of six) cells in which a non-inactivating current did not spontaneously develop when the pipette solution contained 1 mM MgATP (but was devoid of substrates for the tricarboxylic acid pathway) (see Drugs and Solutions). (○) represents the *I-V* relationship for the control non-inactivating current (I_{NI}) which was determined immediately before the addition of BDM. Each point represents the mean ± s.e.mean values obtained, ($n = 3$).

inger & Escande, 1989; Nakayama *et al.*, 1990). Inhibition by K-channel openers of the binding of ATP to a site on protein kinase A, and thus inhibition of protein kinase A activity, would be consistent with our finding that both the K-channel openers and the protein kinase A inhibitor stimulate a glibenclamide-sensitive, non-inactivating K-current and simultaneously inhibit $I_{K(V)}$. We therefore investigated the effect of a relatively high concentration of levcromakalim (10 μM) on protein kinase A activity in a biochemical assay. As shown in Figures 7a and 7b, levcromakalim had no effect on protein kinase A activity (measured as incorporation of ^{32}P into substrate, c.p.m.) over a range of concentrations of cycle AMP or of Type 1 protein kinase A (identical effects were produced with Type 2 protein kinase A, data not shown). Increasing the concentration of non-radiolabelled ATP reduced the transfer of the radiolabelled γ-phosphate by competing for binding sites on the kinase. Nevertheless, it is evident that levcromakalim had no effect on protein kinase A activity over a range of concentrations of ATP (Figure 7c).

Effects of butanedione monoxime

Channel phosphorylation is a dynamic process involving phosphorylation catalysed by kinases, and dephosphorylation stimulated by phosphatases. The dephosphorylating agent, butanedione monoxime (BDM), is known to inhibit $I_{K(V)}$ in human T-lymphocytes (Schlichter *et al.*, 1992). To avoid possible complications arising from effects of BDM on glycolysis and ATP production, we examined the effects of BDM on rat portal vein cells using a pipette solution con-

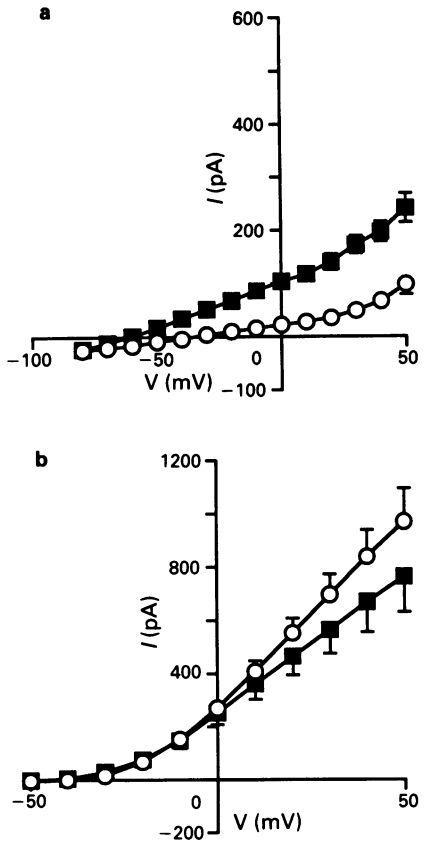

Figure 10 Effects of levcromakalim in the presence of a phosphatase inhibitor, okadaic acid (1 μM in the pipette solution). In the presence of okadaic acid, levcromakalim (1 μM) stimulated $I_{K(ATP)}$ ($P<0.05$; a) and inhibited the delayed rectifier K-current $I_{K(V)}$ ($P<0.05$; b): (○) represents control non-inactivating (I_{NI}; a) or inactivating ($I_{K(V)}$; b) currents before addition of levcromakalim and with 1 μM okadaic acid in the pipette; (■) shows the currents ($I_{NI} + I_{K(ATP)}$, a; $I_{K(V)}$, b) in the presence of 1 μM levcromakalim at the peak of the development of $I_{K(ATP)}$. Compare these effects with those produced by levcromakalim alone (Figure 4). Each point represents the mean ± s.e.mean value obtained, $n = 4$.

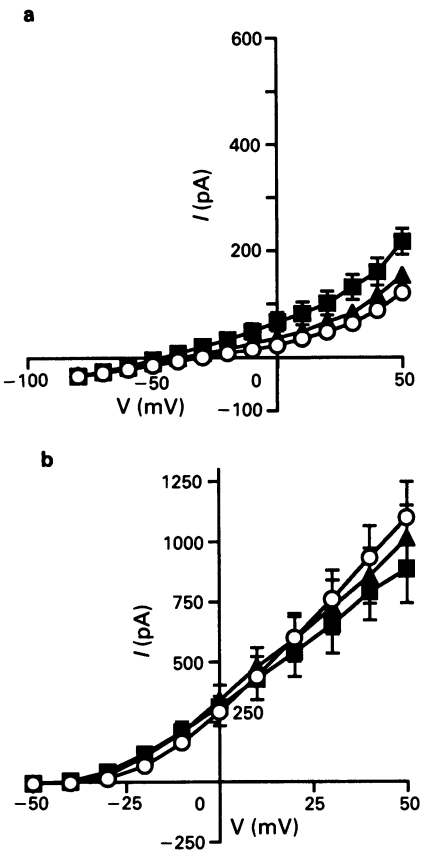

Figure 11 Failure of levcromakalim to induce $I_{K(ATP)}$ (a: $P>0.05$) and to inhibit $I_{K(V)}$ (b: $P>0.05$) when the pipette solution was essentially magnesium-free: (○) represents control non-inactivating (a) or inactivating ($I_{K(V)}$: b) currents on breakthrough ($n = 5$); (▲) and (■) show the currents after 15 min superfusion with 1 μM ($n = 4$) and subsequently 10 μM levcromakalim ($n = 3$), respectively. Compare this lack of effect with the changes produced by levcromakalim in the presence of magnesium (Figure 1). Each point represents the mean ± s.e.mean value obtained.

taining 1 mM MgATP (which was devoid of substrates for the tricarboxylic acid cycle, see *Drugs* and *Solutions*). Reducing the intracellular ATP concentration, by replacing the carboxylic acid substrates and glucose in the pipette solution with 1 mM MgATP and by omitting the extracellular glucose had an effect similar to the application of levcromakalim. In 5 out of 11 cells, $I_{K(V)}$ was reduced (data not shown) and a non-inactivating K-current was induced. Figure 8 shows the development of such a non-inactivating K-current with time and illustrates its glibenclamide-sensitivity. In 3 out of the 6 cells which did not spontaneously develop an outward current, bath application of BDM (5 mM) had no effect. However, in the remaining 3 cells, exposure to BDM immediately stimulated a non-inactivating, glibenclamide-sensitive K-current. Despite the induction of $I_{K(ATP)}$, the total whole-cell currents did not substantially increase (compare Figures 9a and 9b). Thus, as $I_{K(ATP)}$ appeared, $I_{K(V)}$ declined. The mean current-voltage relationship for the three cells in which BDM produced an effect is shown in Figure 9c.

Effects of phosphatase inhibition or magnesium depletion

One possibility emerging from our results was that the effects of the K-channel openers could be explained by stimulation of a phosphatase and thus induction of channel dephosphorylation. In an attempt to gain further insight into the mechanism of action of the K-channel openers we examined the effect of okadaic acid, an inhibitor of protein phosphatases 1 and 2A (Cohen, 1989), on the responses to levcromakalim. Figure 10 shows that inclusion of okadaic acid (1 μM) in the pipette solution had no effect on the non-inactivating currents but slightly enhanced $I_{K(V)}$ (compare with Figure 4). Furthermore, okadaic acid only slightly reduced the induction of $I_{K(ATP)}$ and the inhibition of $I_{K(V)}$ by levcromakalim. However, levcromakalim (1 μM and 10 μM) had no effect on $I_{K(V)}$ and did not induce $I_{K(ATP)}$ ($P>0.05$) when magnesium was omitted from the pipette solution (compare Figures 4 and 11). Depletion of intracellular magnesium alone enhanced $I_{K(V)}$ (compare Figures 4 and 11).

Discussion

The K-channel openers, levcromakalim, P1060 and aprikalim, simultaneously induce $I_{K(ATP)}$ and reduce $I_{K(V)}$ in venous and arterial smooth muscle (Noack *et al.*, 1992a,b,c; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994). This modulation of K-currents could result from competition with intracellular ATP for access to the regulatory site on K_{ATP} (see

Edwards & Weston, 1993). Alternatively, or in addition, ATP binding to sites on protein kinases (Scott, 1991) could be modified by the K-channel openers resulting in inhibition of these enzymes. This would dephosphorylate channel proteins and modify channel gating properties (Perozo & Bezanilla, 1990; Duchatelle-Gourdon *et al.*, 1991). The primary objective of the present study was to assess the extent to which modification of channel phosphorylation could account for the actions of the K-channel openers.

Does levcromakalim inhibit a protein kinase?

If the changes in K-currents induced by levcromakalim resulted from channel dephosphorylation secondary to a reduction of kinase activity, then similar effects should be produced by protein kinase inhibitors. Under our essentially calcium-free conditions it seemed unlikely that K-channel openers could inhibit a calcium-dependent protein kinase C. However, the possibility existed that the divalent cation Mg^{2+} could substitute for Ca^{2+}, and in addition, calcium-independent forms of protein kinase C have been described (Andrea & Walsh, 1992). Thus the effects of calphostin C on whole cell K-currents in the presence and absence of levcromakalim were examined. Calphostin C, which is a potent, selective inhibitor of protein kinase C, was used since it inhibits the kinase by an effect on its regulatory subunits (Kobayashi *et al.*, 1989; Davis *et al.*, 1992). The use of less selective protein kinase C inhibitors such as staurosporine would have complicated interpretation of any resulting changes since these compounds inhibit several kinases by interacting with ATP binding sites (Davis *et al.*, 1992). Thus, any effects of staurosporine-like agents on K_{ATP} could have resulted from inhibition of the binding of ATP to its inhibitory site on this channel (site 2, see Edwards & Weston, 1993) rather than from inhibition of protein kinase C itself.

In the present study, calphostin C had virtually no effect in either the absence or presence of levcromakalim. It is nevertheless possible that inhibition of protein kinase C could contribute to the effects of the K-channel openers *in vivo* (i.e. in the presence of calcium). However, this is unlikely since the induction of $I_{K(ATP)}$ by these agents occurs in the absence of calcium (present study; Noack *et al.*, 1992a,b; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994). Furthermore, current-clamp studies (Noack *et al.*, 1992a) showed that under calcium-free conditions the associated membrane hyperpolarization was of similar magnitude to that observed with microelectrode recordings in whole tissues and in the presence of physiological concentrations of calcium (Hamilton *et al.*, 1986).

In contrast to the results with calphostin C, the inclusion in the pipette (intracellular) solution of a selective inhibitor of protein kinase A (PKI(6-22)amide; Glass *et al.*, 1989) did indeed reduce K_V and simultaneously stimulated a non-inactivating glibenclamide-sensitive K-current, suggesting that channel dephosphorylation following inhibition of this enzyme could be the mechanism which underlies the action of the K-channel openers. Although the unitary conductance of the channel underlying the induced non-inactivating current was not determined, the noise associated with the development of this current was typical of that associated with the current induced by the K-channel openers (Noack *et al.*, 1992a; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994) implying that the channel opened by the protein kinase A inhibitor had a small unitary conductance.

In the biochemical assays, however, a relatively high concentration of levcromakalim (10 μM) had no effect on protein kinase A activity irrespective of the concentration of protein kinase A, cyclic AMP or ATP employed. Thus, although the presumed channel dephosphorylation following inhibition of protein kinase A mimicked the effects of the K-channel openers, it seems unlikely that these agents exert their actions by inhibiting this enzyme.

Does levcromakalim stimulate a phosphatase?

Although the K-channel openers do not appear to dephosphorylate following *kinase* inhibition, the ability of PKI(6-22)amide to mimic the ability of the K-channel openers to inhibit $I_{K(V)}$ and to induce $I_{K(ATP)}$ suggested that dephosphorylation could underlie the effects of these agents. Thus the possibility that phosphatase stimulation was the basis of their action was tested.

Numerous types of protein phosphatase exist and these can be broadly characterized by the use of activators or inactivators (see Cohen (1989) and Shenolikar & Nairn (1991) for reviews). Of these, types 1 and 2A are sensitive to okadaic acid, with K_i values of 20 and 0.2 nM respectively (Bialojan & Takai, 1988; Cohen, 1989). In the present study, a relatively high concentration of okadaic acid (1 μM) in the pipette solution had only a small inhibitory effect on the actions of levcromakalim. This suggests that the magnesium-independent phosphatases 1 and 2A (Cohen, 1989) are not the site of action of the K-channel openers.

In the absence of magnesium the K-channel openers are unable to open K_{ATP} in insulinoma cells (Kozlowski *et al.*, 1989) or to induce $I_{K(ATP)}$ in smooth muscle (present study; Bolton *et al.*, 1993) a possible indication that a *magnesium-dependent* protein phosphatase is involved in the actions of the K-channel openers. Such an enzyme is the Type 2C protein phosphatase which is both magnesium-dependent (Cohen, 1989) and not inhibited by up to 10 μM okadaic acid (Bialojan & Takai, 1988). In the heart, such a magnesium-dependent phosphatase is thought to be involved in maintaining the normal degree of phosphorylation of K_V (Duchatelle-Gourdon *et al.*, 1991).

In the present study, removal of magnesium from the pipette had little effect on $I_{K(V)}$. However, under these Mg^{2+}-free conditions, levcromakalim was unable to induce $I_{K(ATP)}$ or to inhibit $I_{K(V)}$. Such results are consistent with the view that K-channel openers could exert a dephosphorylating action by stimulation of a Mg-dependent phosphatase. Furthermore, the so-called chemical phosphatase, butanedione monoxime (BDM), stimulated a non-inactivating, glibenclamide-sensitive K-current and inhibited $I_{K(V)}$, effects similar to those of the K-channel openers. The ability of this compound to inhibit K_V (in T-lymphocytes) has already been described (Schlichter *et al.*, 1992). Consistent with our findings that BDM enhanced a non-inactivating K-current in rat portal vein cells, this agent (0.1–3.75 mM) also stimulated K_{ATP} in insulinoma cells (under whole-cell recording conditions with the inclusion of 0.3 mM MgATP in the pipette solution; P.A. Smith, personal communication).

Collectively the results of the present study suggest that dephosphorylation via stimulation of a phosphatase is the mechanism which could underlie the effects of the K-channel openers on both $I_{K(V)}$ and $I_{K(ATP)}$. The involvement of such an action in ion channel modulation would not be novel since somatostatin is thought to enhance the opening of the large-conductance calcium-sensitive K-channel (BK_{Ca}) by stimulating a phosphatase (White *et al.*, 1991). Furthermore, somatostatin also activates K_{ATP} in insulinoma cells (de Weille *et al.*, 1989), and it is tempting to speculate that this could also involve phosphatase stimulation.

Protein phosphatases also seem to be involved in the regulation of calcium (Ca)-channels, since whole-cell Ca-currents are increased by okadaic acid (Hescheler *et al.*, 1988). Interestingly, inhibition of L-type Ca-channels, which was attributed to the stimulation of phosphatase 2A by atrial natriuretic peptide and somatostatin (White *et al.*, 1991; 1993), is also a feature of K-channel openers under certain conditions (Okabe *et al.*, 1990; Lodge *et al.*, 1992). The observations that these agents are capable of inhibiting L-type Ca-channels is consistent with the possibility that they reduce Ca-channel phosphorylation via phosphatase stimulation. In addition, the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, which is inhibited

by several K-channel openers (Sheppard & Welsh, 1992) is dephosphorylated and inactivated by protein phosphatase Type 2A (Berger *et al.*, 1993).

The K-channel openers convert $I_{K(V)}$ into $I_{K(ATP)}$ – a working hypothesis

In both the present and in previous studies, and using either phosphorylation modifiers or several chemically-distinct K-channel openers, we have never been able to induce $I_{K(ATP)}$ without simultaneously reducing $I_{K(V)}$ (Noack *et al.*, 1992a,b,c; Ibbotson *et al.*, 1993a,b). In addition, numerous agents which inhibit $I_{K(V)}$ (alinidine, 4-aminopyridine, antazoline, cibenzoline, ciclazindol, clonidine, guanabenz, phencyclidine, phentolamine, quinidine, tedisamil) also inhibit the effects of K-channel openers in similar concentrations (Beech & Bolton, 1989a; Bray & Quast, 1992; Noack *et al.*, 1992b; Pfründer & Kreye, 1992; Ibbotson *et al.*, 1993a,b). Thus, with the exception of glibenclamide (see below), it is not possible pharmacologically to distinguish between $I_{K(V)}$ and $I_{K(ATP)}$. Furthermore, when the induction of $I_{K(ATP)}$ was prevented (by inclusion of MgATP in the pipette solution), $I_{K(V)}$ was not inhibited by the K-channel opener, P1060 (Ibbotson *et al.*, 1993a).

These data lead to the conclusion that the non-inactivating, voltage-independent K-channel currently designated K_{ATP} may not be a separate entity but may simply be a *partially-dephosphorylated* state of K_V. This state can be induced by agents such as the K-channel openers and by others which directly or indirectly influence the degree of channel phosphorylation. Although there was usually some increase in the total evoked current as $I_{K(ATP)}$ developed, such an observation does not conflict with our view that K_V and K_{ATP} are different states of the same channel. Indeed, some increase in current would be predicted if the property of inactivation were removed from a fraction of the population of K_V (see Hille, 1992).

The so-called ATP-sensitive K-channel has been well characterized in a variety of tissues (see Edwards & Weston, 1993 for review). In isolated patches, this channel rapidly runs down, i.e. enters a state from which channel opening is not possible. Since run-down can be slowed or reversed by the presence of MgATP (but not by non-hydrolysable analogues of ATP: see Ashcroft, 1988), it has been suggested that run-down itself results from dephosphorylation of site 1 on K_{ATP} (see Edwards & Weston, 1993). If the K-channel openers were to induce $I_{K(ATP)}$ by dephosphorylating K_V, then such channel dephosphorylation could only be partial, since full dephosphorylation would also inhibit $I_{K(ATP)}$. However, under conditions unfavourable to phosphorylation such as in the absence of MgATP, the K-channel openers *only* exert an *inhibitory* effect on K_{ATP} (Kozlowski *et al.*, 1989; Dunne, 1990). Such K-channel opener-induced inhibition thus probably results from run-down of K_{ATP} *via* further dephosphorylation. Furthermore, in the present study, relatively long exposure to a high concentration of levcromakalim induced marked inhibition of both $I_{K(ATP)}$ and $I_{K(V)}$ even with the presence of carboxylic acid substrates and glucose in the pipette.

Thus, under normal conditions of phosphorylation, we propose that the target channel of the K-channel openers is not K_{ATP} but is instead K_V, which these agents convert via dephosphorylation into K_{ATP}. This putative interconversion is summarised in the following scheme.

$$K_V \underset{\text{phosphorylation}}{\overset{\text{levcromakalim dephosphorylation}}{\rightleftharpoons}} K_{ATP} \underset{\text{phosphorylation}}{\overset{\text{levcromakalim dephosphorylation}}{\rightleftharpoons}} K_{ATP}(\text{inactive})$$

Further support for the view that K_{ATP} is a substate of K_V can be obtained by comparing the unitary conductances of these channels in smooth muscle. Analysis of the current induced by the K-channel openers suggests that the underlying mean single channel conductance is approximately 11 pS in quasi-physiological conditions (Noack *et al.*, 1992a; Bolton *et al.*, 1993; Ibbotson *et al.*, 1993a; Langton *et al.*, 1993; Criddle *et al.*, 1994). This is essentially identical to the mean value of 9 pS obtained for the smooth muscle K_V (Beech & Bolton, 1989b; Boyle *et al.*, 1992; Volk & Shibata, 1993).

The suggestion that dephosphorylation can convert $I_{K(V)}$ into a non-rectifying, voltage-sensitive current is not new. To our knowledge, such a change in the gating properties of the delayed rectifier current (induced by reducing $[ATP]_i$ in frog skeletal muscle) was first convincingly demonstrated by Fink & Wettwer (1978). In addition, numerous agents are known to reduce or abolish inactivation in sodium channels (see Hille, 1992). Channel conversion was also proposed by Beech & Bolton (1989a) to explain the reduction in $I_{K(V)}$ which occurred concurrently with the induction of a non-activating K-current by cromakalim in rabbit portal vein. This proposal did not gain favour, largely because of the inability of glibenclamide to inhibit K_V, despite the fact that it was an inhibitor of K_{ATP}.

To overcome this difficulty it is merely necessary to propose that glibenclamide binds selectively to the partially-dephosphorylated state of K_V (i.e. K_{ATP}). This proposal is entirely consistent with the finding of Schwanstecher and coworkers (1991) in pancreatic β-cells that [^{3}H]-glibenclamide binding is indeed enhanced under non-phosphorylating conditions. If K_{ATP} is not a separate entity but is rather a configuration of K_V, then it is possible that K_V in pancreatic β-cells is normally in the partially-dephosphorylated form (i.e. K_{ATP}). This would then explain both the sensitivity of this channel in the β-cell to glibenclamide and in addition its relative insensitivity to the K-channel openers (Garrino *et al.*, 1989). Under the present working hypothesis these agents do not open K_{ATP} *per se* but induce the K_{ATP} 'configuration' by dephosphorylation of K_V. Because the opening of K_{ATP} is voltage-independent (see Edwards & Weston, 1993), the change from K_V to K_{ATP} would automatically generate K-current flow by converting the channel from a state in which it is voltage-dependent and inactivates (i.e. K_V) to one in which it is voltage-independent and non-inactivating (i.e. K_{ATP}). Under prolonged dephosphorylating conditions, K_{ATP} runs down and becomes K_{ATP}(inactive), a process enhanced by the presence of K-channel openers (Kozlowski *et al.*, 1989; Dunne, 1990). Such run-down also occurs after exposure to high concentrations of levcromakalim (present study).

Conclusion

The results of the present study do not support the view (Thuringer & Escande, 1989) that the (so-called) K-channel openers compete with ATP for access to the inhibitory ATP binding site on K_{ATP} (site 2, see Edwards & Weston, 1993). Instead, all the effects of these agents on K-, Ca- and Cl-channels could be explained by a basic dephosphorylating action. Together with the results of previous studies it now seems likely that the target channel for the K-channel openers is the delayed rectifier, K_V, which loses its voltage-dependence and inactivation properties by conversion into a partially-dephosphorylated form, currently known as K_{ATP}. Dephosphorylation does not seem to be associated with inhibition of protein kinase A (associated with site 1 on K_{ATP}, see Edwards & Weston, 1993) but is more likely to be exerted via stimulation of a magnesium-dependent phosphatase. The exact mechanism by which the K-channel openers reduce channel phosphorylation forms the basis of an on-going study.

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