British Journal of Pharmacology (1996) 119, 691-701

Effects of cytochrome P450 inhibitors on potassium currents and mechanical activity in rat portal vein

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1 The effects of the cytochrome P450 inhibitors, proadifen, clotrimazole and 17-octadecynoic acid (17-ODYA) on K-currents in freshly-isolated single cells derived from rat portal vein and on mechanical activity in whole veins were studied.

2 When cells were stepped from −90 mV to a series of test potentials (from −80 to +50 mV), a delayed rectifier current ($I_{K(V)}$) and an A-type current ($I_{K(A)}$) could be identified. Proadifen (10 μM), clotrimazole (30 μM) and 17-ODYA (5 μM) each inhibited $I_{K(V)}$ but had little effect on $I_{K(A)}$.

3 When cells were held at −10 mV to inactivate the time-dependent K-currents, $I_{K(V)}$ and $I_{K(A)}$, levcromakalim (3 μM) induced a time-independent outward K-current ($I_{K(ATP)}$) which was totally inhibited by clotrimazole (30 μM) and almost fully inhibited by proadifen (10 μM). 17-ODYA (5 μM) had no effect on $I_{K(ATP)}$ and exerted only a minor inhibitory action on this current at 20 μM.

4 17-ODYA (5 μM) potentiated current flow through the large conductance, Ca-sensitive K-channel (BK_{Ca}). In contrast, proadifen (10 μM) had no effect on $I_{BK(Ca)}$ whereas clotrimazole (30 μM) exerted a small but significant inhibitory action.

5 Proadifen (10 μM) and clotrimazole (30 μM) each inhibited the magnitude but increased the frequency of spontaneous contractions in whole portal veins. 17-ODYA (5 μM) had no effect on spontaneous contractions but these were inhibited when the concentration of 17-ODYA was increased to 50 μM.

6 The spasmolytic effect of levcromakalim on spontaneous contractions was antagonized by proadifen (10-30 μM) in a concentration-dependent manner but 17-ODYA (up to 50 μM) was without effect.

7 These results in portal vein show that cytochrome P450 inhibitors exert profound effects on a variety of K-channel subtypes. This suggests that enzymes dependent on this cofactor may be important regulators of K-channel activity in smooth muscle. The relevance of these findings for the identification of the pathway involved in the synthesis of the endothelium-derived hyperpolarizing factor is discussed.

Keywords: EDHF; cytochrome P450; clotrimazole; proadifen; 17-ODYA; K-currents; rat portal vein; whole-cell voltage clamp; K_{ATP}; BK_{Ca}

Introduction

In blood vessels with an intact endothelium, tone can be influenced by the release of relaxing factors derived from the vascular endothelium. One such agent, nitric oxide (NO), is thought to act by stimulating the smooth muscle soluble guanylyl cyclase and thus increasing intracellular guanosine 3′:5′ cyclic-monophosphate (cyclic GMP) concentrations (Furchgott, 1993). In some tissues NO seems to cause the opening of Ca-sensitive potassium (K) channels while in others, the ATP-sensitive K-channel (K_{ATP}) may be involved (Garland & McPherson, 1992; Cohen & Vanhoutte, 1995). An additional endogenous substance, endothelium-derived hyperpolarizing factor (EDHF), is believed to relax vascular smooth muscle solely by stimulating the opening of K-channels (Taylor & Weston, 1988), the identity of which is still unknown (Garland *et al.*, 1995; Zygmunt & Högestätt, 1996).

Recent studies suggest that cytochrome P450-dependent enzymes in the vascular endothelial cells might be responsible for generating EDHF (see Harder *et al.*, 1995). This possibility is strengthened by the finding that the relaxant actions of EDHF in whole vessels are inhibited by cytochrome P450 inhibitors such as proadifen and clotrimazole (Bauersachs *et al.*, 1994; Hecker *et al.*, 1994; Lischke *et al.*, 1995; Zygmunt *et al.*, 1996a). However, both agents may also inhibit the smooth muscle K-channel opened by EDHF independently of any inhibitory effect which they may exert on the putative cytochrome P450-dependent pathway for EDHF synthesis in the endothelium (Zygmunt *et al.*, 1996a,b).

The aim of the present study was to clarify the effects of several cytochrome P450 inhibitors on K-channels and mechanical activity in rat portal vein. This tissue was chosen because the properties of its K-currents have been extensively studied (Noack *et al.*, 1992a; Edwards *et al.*, 1993; 1994). Furthermore, since EDHF release from this tissue has not been demonstrated, interpretation of the data obtained for the whole vein was simplified by the likely absence of (any possible) drug effects on EDHF synthesis. A preliminary account of some of these results has been presented to the British Pharmacological Society (Zygmunt *et al.*, 1996b).

Methods

Production of isolated cells

Tissues were placed in low-Ca^{2+} physiological salt solution (PSS) and carefully cleaned of fat and connective tissue with fine scissors in conjunction with a dissecting microscope. Cells were dispersed with a collagenase/pronase enzyme solution originally described by Klöckner & Isenberg (1985). Intact portal veins were agitated in 100% enzyme solution at 37°C for 22 min. They were then washed in the same solution free of enzyme and cut into four segments. These were subsequently triturated with a wide bore, smooth-tipped pipette before a further 5 min agitation at 37°C in (pre-warmed) 200% enzyme solution. The partially-digested (but essentially intact) tissue was then washed and triturated in Kraftbrühe (KB-medium; Klöckner & Isenberg, 1985). Cells were stored at 8°C in KB-medium and used within 9 h of separation.

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Single-cell electrophysiology

The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was employed using an Axopatch-1C amplifier (Axon Instruments). 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 a computer equipped with an appropriate interface, the sampling frequency of which was 15 kHz (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). Data were stored on a digital audio tape recorder (Sony; cut-off frequency 20 kHz) and the evoked membrane currents were monitored on a Gould Windograf recorder (−3 dB cut-off frequency 460 Hz).

The effects of the compounds were investigated by adding the appropriate amount of each agent to the 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 KLB, Freiburg, Germany); a second identical pump was used to

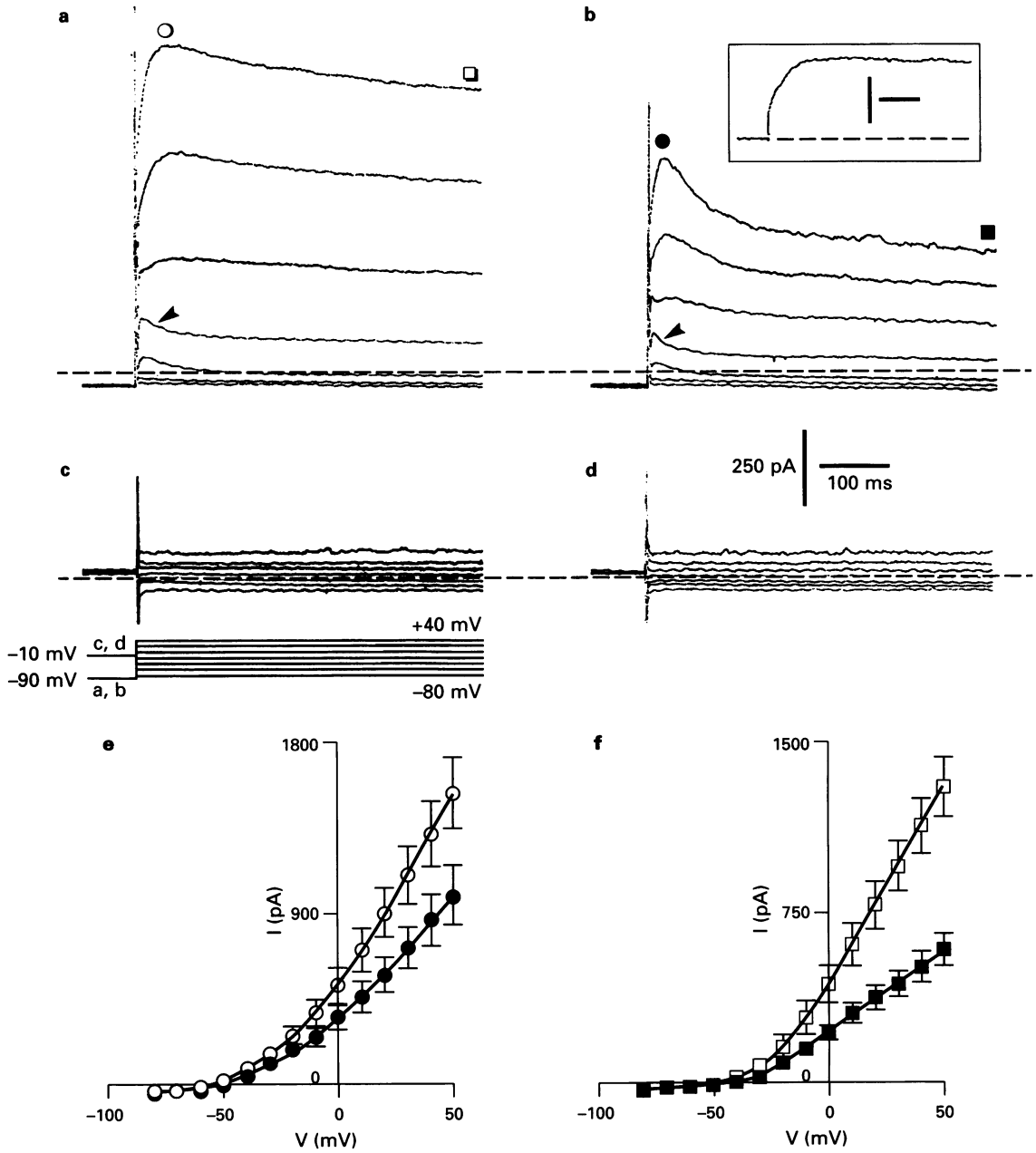

Figure 1 Whole-cell currents in rat portal vein cells under calcium-free conditions (a,c) before or (b,d) after 10 min exposure to 10 μM proadifen. (a,b) Currents at each test potential (from −80 to +40 mV in 20 mV increments) were elicited by stepping from a holding potential of −90 mV. The 'A' current (I_A) is clearly distinguished at −20 mV (indicated by arrowhead). The insert in (b) shows the difference current obtained by subtracting the current induced by stepping to +30 mV in the presence of proadifen from the control current at the same test potential (calibrations as in main figure). (c,d) Currents at each test potential on stepping from a holding potential of −10 mV to inactivate I_A and $I_{K(V)}$. Each trace is a computer-derived mean obtained from 5 cells from different animals; dashed line indicates the zero current level. (e,f) Effect of proadifen on current (I)-voltage (V) relationships determined in rat portal vein cells under calcium-free conditions before (open symbols) or after (closed symbols) 10 min exposure to 10 μM proadifen. Currents at each test potential were elicited by stepping from a holding potential of −90 mV and were measured at either at their peak (see a,b: ○, control; ●, proadifen test) or at the end of the 500 ms step to each test potential (see a,b: □, control; ■, proadifen test). Each point represents the mean ± s.e.mean, $n = 6$.

remove excess solution from the recording chamber. All experiments were performed at room temperature (23°C–24°C).

Tissue bath experiments

Intact portal veins were mounted under 10 mN tension for isometric recordings. The tissues were allowed to equilibrate in Krebs solution (see Drugs and solutions) for 1 h at 37°C before they were exposed at 6 min intervals to increasing concentrations of levcromakalim added cumulatively. Possible antagonism of the effects of levcromakalim by proadifen or 17-octadecynoic acid (17-ODYA) was studied by first incubating the tissues for 20 min with the appropriate concentration of each agent before the subsequent concentration-effect curve to levcromakalim was constructed in the continuing presence of proadifen or 17-ODYA. Mechanical responses of the tissues were recorded using an Apple Macintosh computer in conjunction with MacLab hardware (MacLab 8) and software (Chart, version 2.5) (Analog Digital Instruments). Basal tension was defined as the minimum tension (between contractions) and was measured at the end of the experiment. Spontaneous mechanical activity (above the basal level) was integrated with respect to time. The control activity was determined for the 5 min period immediately before drug addition and for each drug, effects were measured during the final 5 min period of exposure to each concentration and expressed as a percentage of the control activity.

Drugs and solutions

The low-Ca^{2+} PSS used for tissue dissection comprised (mM): KOH 130, $CaCl_2$ 0.05, taurine 20, pyruvate 5, creatine 5, HEPES 10, buffered with methanesulphonic acid to pH 7.4. The 100% enzyme solution for separation of portal vein cells had the same composition but also contained collagenase (Type VIII) 1 mg ml^{-1}, pronase (Calbiochem) 0.2 mg ml^{-1} and fatty acid-free albumin 1 mg ml^{-1}. The 200% enzyme solution was similar but contained 2 mg ml^{-1} collagenase and 0.4 mg ml^{-1} pronase. KB-medium 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 7.20 at 6°C with KOH.

The composition of the calcium-free bath (external) solution (Ca-free PSS) was (mM): NaCl 125, KCl 4.8, $MgCl_2$ 3.7, KH_2PO_4 1.2, (+)-glucose 11, HEPES 10, EGTA 1.0. The bath solution containing calcium (CaPSS) consisted of (mM): NaCl 125, KCl 4.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, (+)-glucose 11, HEPES 10. The bath solutions were buffered with NaOH to pH 7.30 and aerated with O_2. The Ca-free pipette (internal) solution comprised (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.1, (+)-glucose 11, HEPES 10, EGTA 1.2, ox-

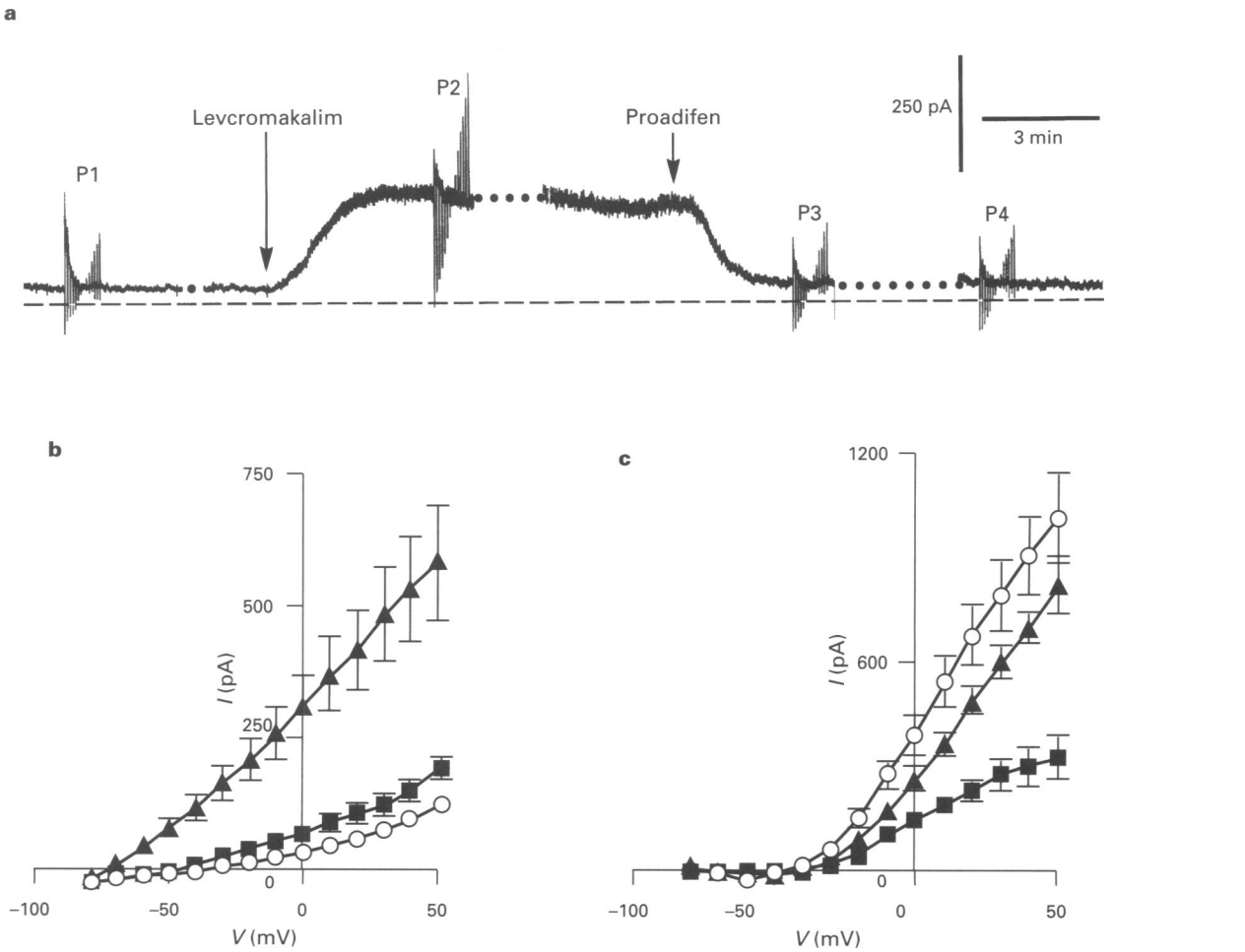

Figure 2 Inhibition by proadifen of $I_{K(ATP)}$ induced by levcromakalim in rat portal vein cells under essentially calcium-free conditions. (a) Typical trace showing the increase in outward current induced by levcromakalim (3 μM) in a cell clamped at −10 mV. Proadifen (10 μM) rapidly antagonized the effect of levcromakalim. During the experiment the cell was stepped four times (P1–P4) to a series of test potentials (ranging from −80 to +50 mV; 10 mV increments) from the −10 mV holding potential. The dashed line indicates the zero current level. During the periods marked by a dotted line (to scale) the cell was held at −90 mV. Mean current (I)-voltage (V) relationships derived from 6 cells by stepping to test potentials from −10 mV before exposure to drugs (○), after exposure to 3 μM levcromakalim (▲) and after exposure to 10 μM proadifen in the continued presence of levcromakalim (■) are shown in (b). The delayed rectifier currents obtained in the same cells by stepping from −90 mV are shown in (c).

alacetic acid 5, sodium pyruvate 2, sodium succinate 5, buffered with KOH to pH 7.30 at 24°C. The free calcium concentration in the 'Ca-free' solutions was calculated to be less than 1 nM (Edwards *et al.*, 1994). When the bath solution contained calcium (CaPSS), EGTA was omitted from the pipette solution.

Krebs solution used for the tissue bath experiments had the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, (+)-glucose 11.1. This solution was gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C.

Levcromakalim (SmithKline Beecham), ciclazindol (Pfizer Central Research), 17-octadecynoic acid (17-ODYA; Tocris Cookson), NS 1619 (RBI Laboratories) and clotrimazole were each dissolved in dimethyl sulphoxide (DMSO) and proadifen (formerly SKF 525A) was dissolved in double-distilled water to produce concentrated stock solutions (20 mM). Dilutions of these were prepared in bath solution immediately before they were required. Unless otherwise stated, all reagents and compounds were obtained from Sigma.

Data analysis

Treament 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.

Results

Whole-cell currents under nominally Ca-free conditions

As previously described (Noack *et al.*, 1992a), when rat portal vein cells are voltage-clamped under essentially calcium-free conditions in the whole-cell configuration, the voltage-step protocols used elicit two main types of current which are usually reproducible for at least 1 h. Thus, on stepping from a holding potential of −90 mV to test potentials positive to −60 mV, a current with fast activation and inactivation kinetics, $I_{K(A)}$, was generated. At test potentials more positive than 0 mV this current was usually masked by the more prominent delayed rectifier current ($I_{K(V)}$) which had slower activation and inactivation characteristics (Figure 1a). Holding at −10 mV for several minutes inactivated $I_{K(A)}$ and $I_{K(V)}$. On stepping from this holding potential to a range of test potentials from −80 mV to +50 mV in 10 mV increments, a family of voltage-insensitive, non-inactivating background currents together with the leak current (designated in total I_{NI}. Noack *et al.*, 1992a) was produced (Figure 1c). This current complex had a reversal potential of approximately −35 mV and an essentially linear current-voltage relationship (Figure 2b).

Effects of proadifen on K-currents

$I_{K(A)}$ *and* $I_{K(V)}$ Under essentially calcium-free conditions, proadifen (10 μM) inhibited the outward K-current which was stimulated by stepping from −90 mV to potentials more positive than −40 mV (Figure 1). The magnitude of the inhibition was greater at the end of the 500 ms step than at the peak (Figure 1e,f). However, this is unlikely to indicate open-channel blockade since subtraction of the current which was induced by stepping to +30 mV in the presence of proadifen from that which was induced in the same cell prior to exposure to this agent, indicated that the magnitude of the inhibition did not increase with time over the whole period of the voltage step (inset in Figure 1b). Since the peak current comprises $I_{K(A)}+I_{K(V)}+I_{NI}$ whereas the current at 500 ms consists only of $I_{K(V)}+I_{NI}$, the data are consistent with preferential inhibition of $I_{K(V)}$ by proadifen. A comparison of the time-course of the current inhibited by proadifen (inset in Figure 1b) with that of the current remaining after exposure to proadifen (Figure 1b) suggests that $I_{K(A)}$ was revealed by inhibition of $I_{K(V)}$.

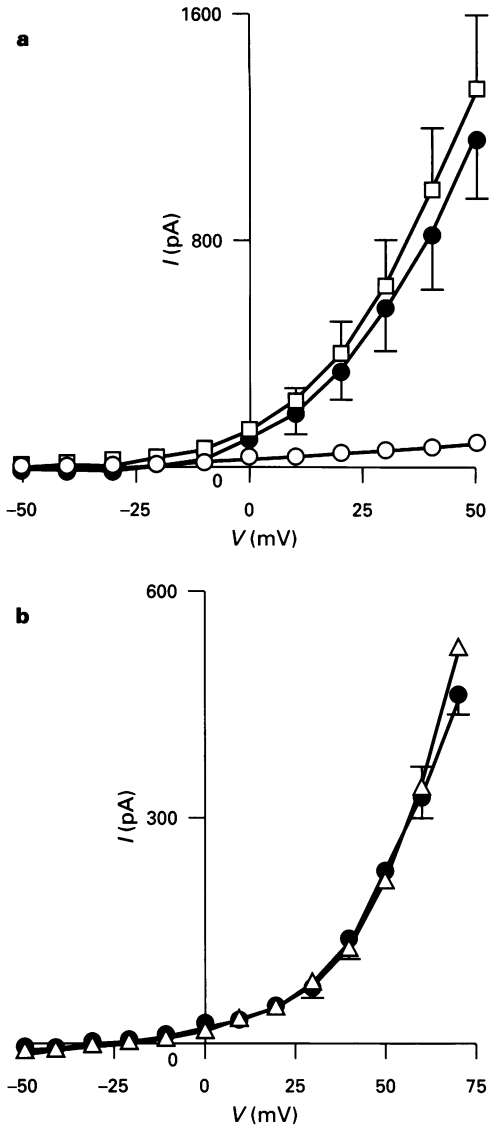

Figure 3 Effect of proadifen on $I_{BK(Ca)}$ in rat portal vein cells. $I_{BK(Ca)}$, induced by 33 μM NS1619 under essentially calcium-free conditions (○, a) or by inclusion of calcium in the bath solution (△, b) was not modified by exposure to 10 μM proadifen (●). All currents were measured at the indicated test potentials after stepping from a holding potential of −10 mV (to inactivate $I_{K(A)}$ and $I_{K(V)}$). In (a), ○ represents the current (*I*)-voltage (*V*) relationship obtained before exposure to NS1619. Each point represents the mean ± s.e.mean of 4 (a) or 3 (b) cells.

$I_{K(ATP)}$ *and* $I_{K(V)}$ When cells were voltage-clamped at −10 mV, levcromakalim (3 μM) stimulated an outward current ($I_{K(ATP)}$) which was essentially fully inhibited by subsequent exposure to proadifen (10 μM) (Figure 2a). The current-voltage relationships depicted in Figure 2b indicate the magnitude of $I_{K(ATP)}$ induced by levcromakalim over the whole range of test potentials and the inhibition of this current by proadifen (10 μM) in the continued presence of levcromakalim.

When levcromakalim-treated cells were stepped from a holding potential of −90 mV to test potentials more positive than −60 mV, the increase in peak total current was less than that which would have been expected if $I_{K(ATP)}$ (induced by levcromakalim) had summated with the currents ($I_{K(V)}+I_{K(A)}+I_{NI}$) which were induced by the same test potentials in control cells. This resulted from the previously-reported simultaneous inhibition of $I_{K(V)}$ by levcromakalim (Edwards *et al.*, 1993). To quantify both this phenomenon and the inhibitory effects of proadifen on $I_{K(V)}$, currents obtained in the presence of 3 μM levcromakalim on stepping from −90 mV and flowing at the end of each 500 ms voltage-step

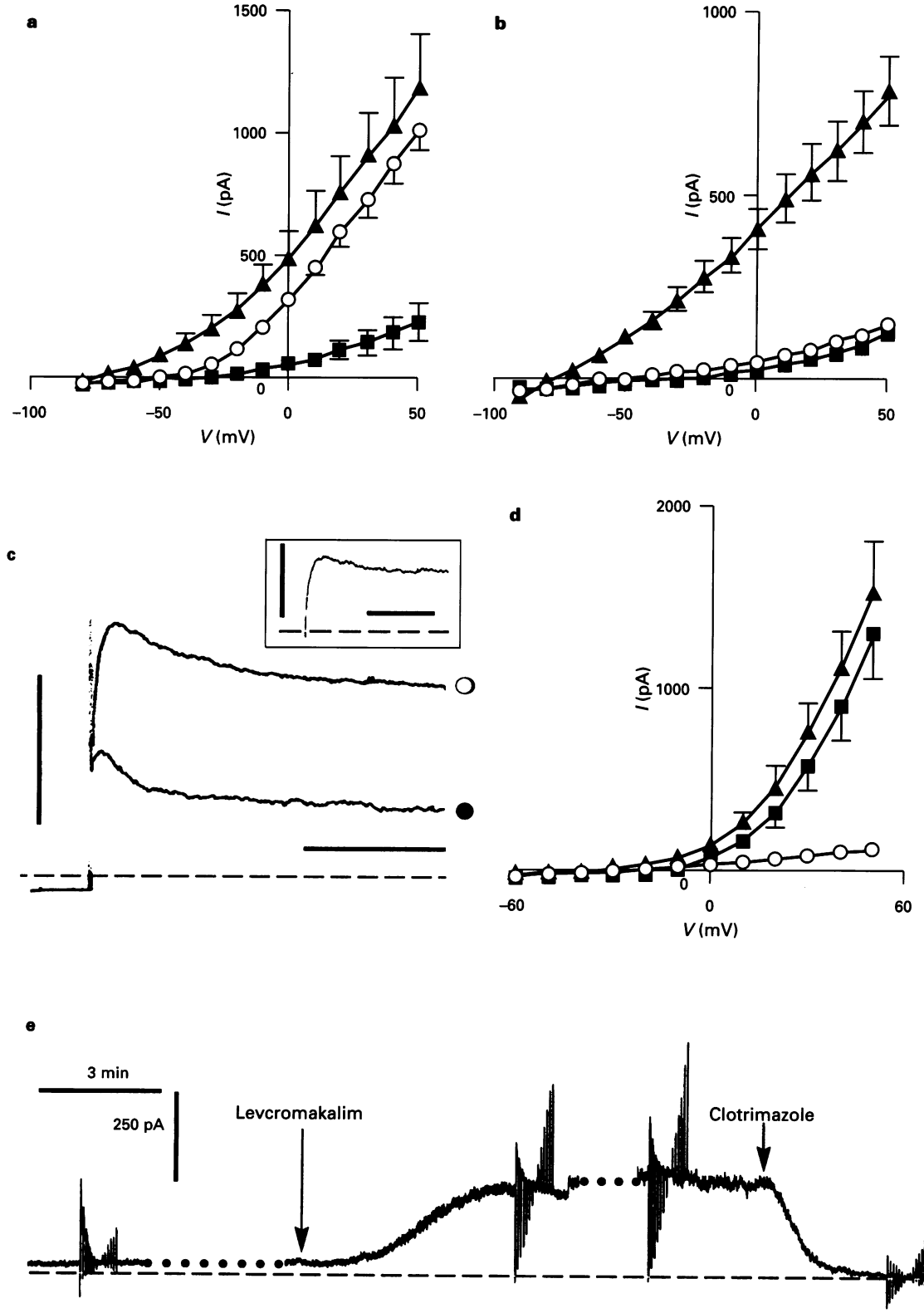

Figure 4 Effect of clotrimazole on whole-cell currents in rat portal vein cells under essentially calcium-free conditions. Current (I)-voltage (V) relationships were obtained on stepping from holding potentials of −90 mV (a: total currents, measured 500 ms after stepping to test potential) or −10 mV (b,d: non-inactivating currents) before (○) and after (▲) exposure to 3 μM levcromakalim (a,b) or 33 μM NS1619 (d) and after subsequent exposure to 30 μM clotrimazole for 10 min in the continued presence of these K-channel openers (■). Each point represents the mean ± s.e.mean, $n = 4-5$. The inhibition by clotrimazole of levcromakalim-induced $I_{K(ATP)}$ is evident in (a) and (b). The simultaneous inhibition of the delayed rectifier current ($I_{K(V)}$) by clotrimazole, suggested in (a) is confirmed in (c) which shows a current trace obtained on stepping the cell from −90 mV to +30 mV before (○) and after exposure to 30 μM clotrimazole (●). Inset is the computer-derived difference current which shows that clotrimazole had inhibited a current with activation and inactivation kinetics typical of $I_{K(V)}$. In (c) and (inset) the vertical and horizontal scale markers represent 500 pA and 200 ms, respectively. $I_{BK(Ca)}$, induced by NS1619 was only slightly inhibited by clotrimazole (d; $n = 4$). A typical trace demonstrating the induction of an outward current ($I_{K(ATP)}$) at −10 mV by levcromakalim (3 μM) and its inhibition by clotrimazole is shown in (e) (see Figure 3a legend for further details). The horizontal dashed lines in (c) and (e) represent the zero current level.

(to allow $I_{K(A)}$ to inactivate; i.e. $I_{K(V)}+I_{NI}+I_{K(ATP)}$) were measured. From these, the equivalent currents evoked from a holding potential of −10 mV (i.e. $I_{NI}+I_{K(ATP)}$) were subtracted to provide an estimate of the magnitude of $I_{K(V)}$. Using this procedure it can be seen (Figure 2c) that $I_{K(V)}$ was inhibited by 3 μM levcromakalim ($P<0.05$) and that 10 μM proadifen produced further inhibition of this delayed rectifier current ($P<0.05$).

$I_{BK(Ca)}$ The effects of proadifen on $I_{BK(Ca)}$ were assessed in two ways. This current was induced by NS1619 which opens BK_{Ca} even under the essentially calcium-free conditions employed in the present study (Edwards *et al.*, 1994). Thus, using a holding potential of −10 mV to inactivate $I_{K(A)}$ and $I_{K(V)}$, the outward current obtained on stepping to test potentials more positive than −10 mV was markedly increased in the presence of 33 μM NS1619 (Figure 3a). This current was not inhibited by subsequent exposure (for 20 min) to 10 μM proadifen in the continued presence of NS1619. Similarly, when $I_{BK(Ca)}$ was induced by voltage-steps in the presence of calcium (CaPSS bath solution and EGTA omitted from the pipette) this current was not modified by 10 μM proadifen (Figure 3b).

Effects of clotrimazole on K-currents

As already explained, the total current flowing at the end of each 500 ms voltage-step and elicited from a holding potential of −90 mV comprised $I_{K(V)}+I_{NI}$. After exposure to 3 μM levcromakalim this current increased due to the induction of $I_{K(ATP)}$ (Figure 4a). In the additional presence of 30 μM clotrimazole, the total current was inhibited by approximately 80%, suggesting that this agent had inhibited both $I_{K(V)}$ and $I_{K(ATP)}$ (Figure 4a). When cells were stepped to the same test potentials from a holding potential of −10 mV to inactivate $I_{K(V)}$, the marked inhibitory effects of clotrimazole on the levcromakalim-induced current could be observed more clearly

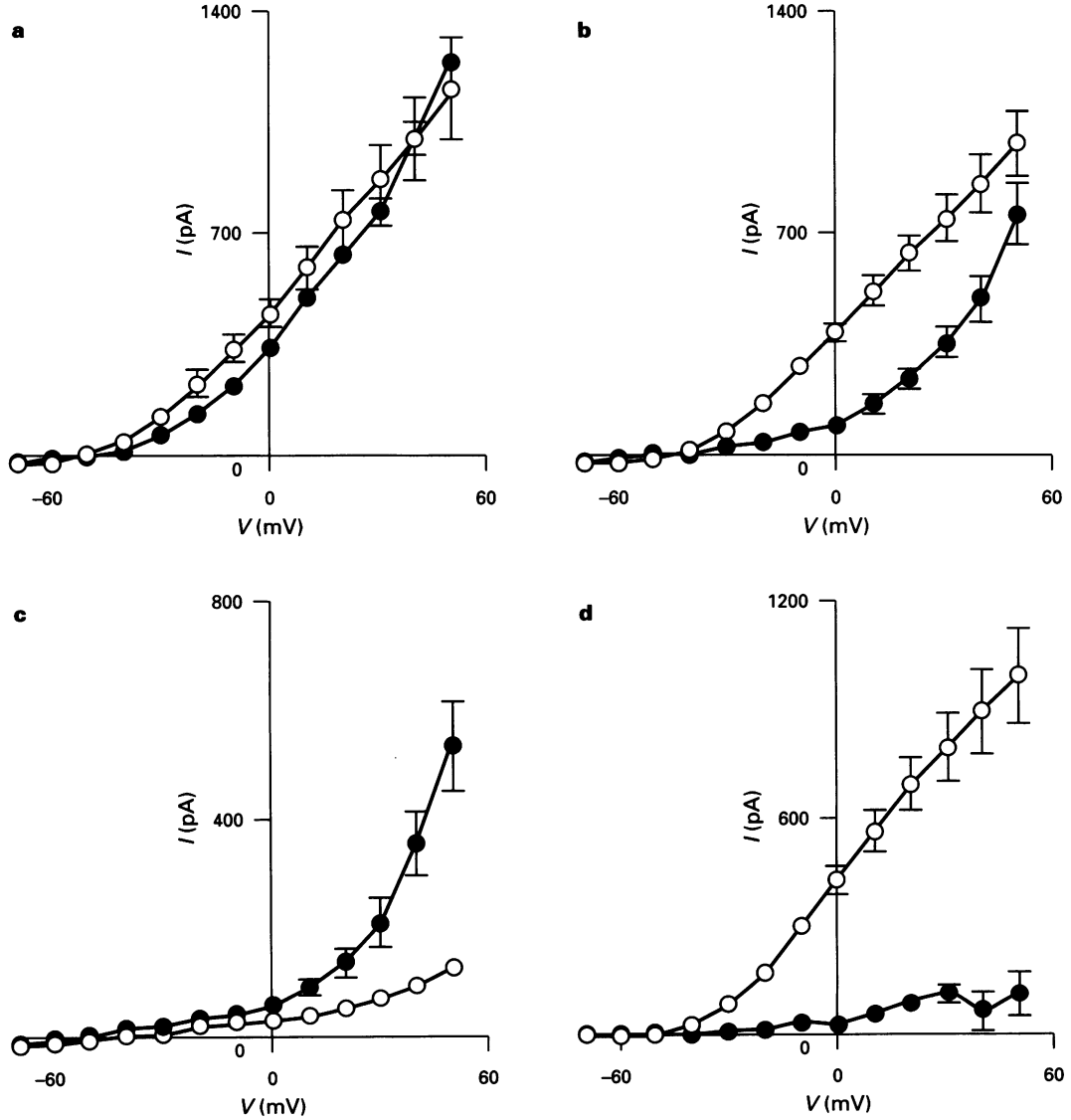

Figure 5 Effect of 17-ODYA on whole-cell currents in rat portal vein cells under calcium-free conditions. Current (I)-voltage (V) relationships were obtained after cells were stepped for 500 ms to the indicated test potentials from a holding potential of −90 mV (a,b) or −10 mV (c) before (○) or after (●) 20 min exposure to 17-ODYA (5 μM). 17-ODYA had little effect on the peak outward current (a) but inhibited the total current measured at the end of the 500 ms test pulse which comprises $I_{K(V)}$ + non-inactivating currents (b). When cells were held at −10 mV to inactivate the time-dependent currents ($I_{K(A)}$ and $I_{K(V)}$) there was initially a small outward current at potentials more positive than −30 mV (c, ○). However, after 20 min exposure to 17-ODYA (5 μM, ●) an additional outward, non-inactivating and voltage-sensitive current was observed. The non-inactivating currents, measured at each potential after stepping from −10 mV, were also present when cells were stepped from −90 mV. Subtraction of these non-inactivating currents from the total current measured at the end of the 500 ms test pulses indicated that $I_{K(V)}$ was almost fully inhibited by ODYA (d).

(Figure 4b,e). Confirmation that clotrimazole could also inhibit $I_{K(V)}$ was obtained in 2 cells in which the effects of this agent were examined in the absence of levcromakalim. The results from one of these cells are shown in Figure 4c. In the other cell, the percentage inhibition of $I_{K(V)}$ by clotrimazole was greater than that shown in Figure 4c and the effects of this

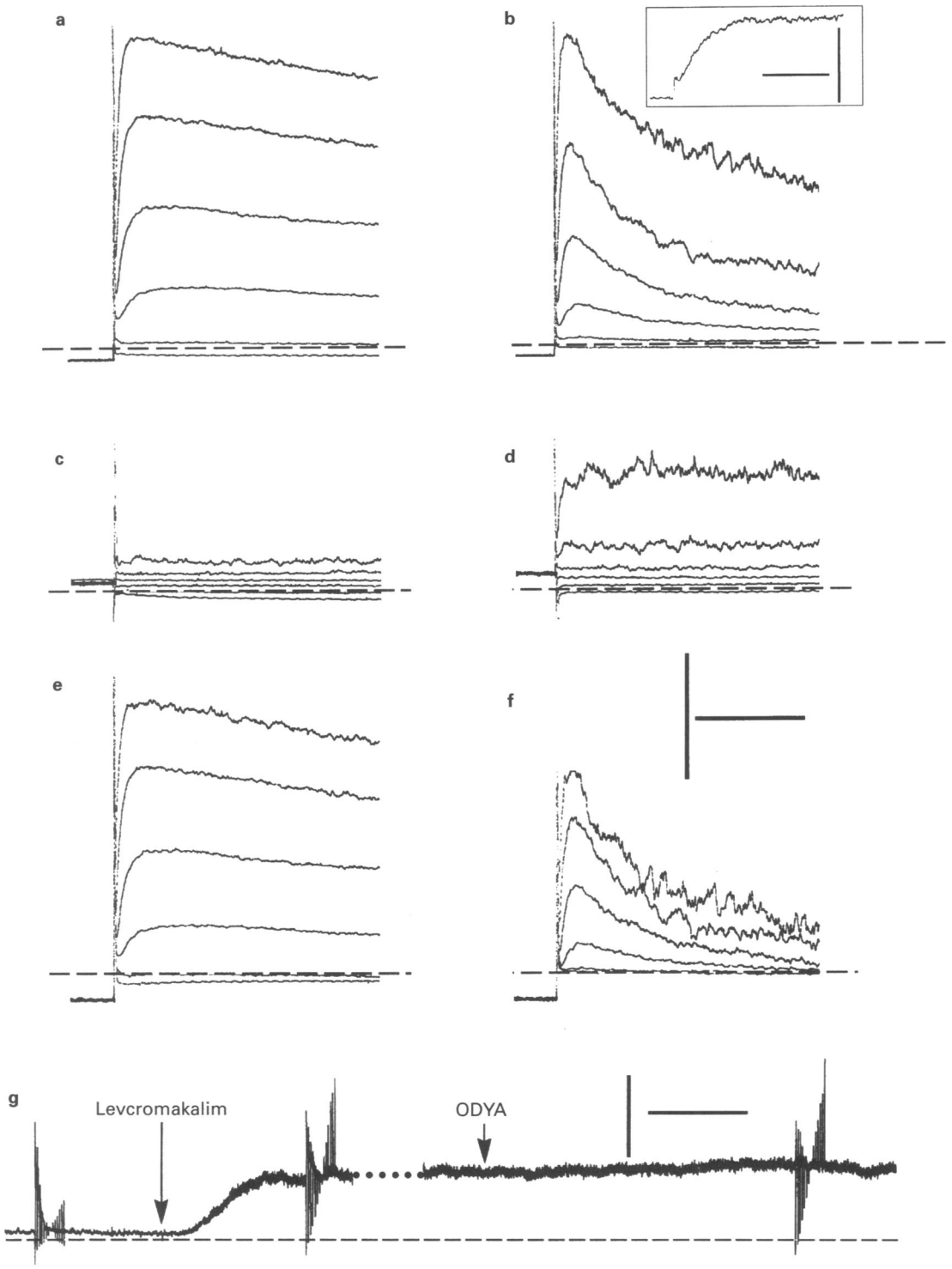

Figure 6 Effect of 17-ODYA on whole-cell currents in rat portal vein cells under essentially calcium-free conditions. (a,b) Total currents ($I_{NI}+I_{K(V)}+I_{K(A)}$) were obtained on stepping for 500 ms from −90 mV to a series of test potentials ranging from −60 to +40 mV (in 20 mV increments) before (a) or 20 min after (b) exposure to 5 μM 17-ODYA. After holding the cells at −10 mV to inactivate $I_{K(V)}$ and $I_{K(A)}$, stepping to the same test potentials elicited only non-inactivating currents (I_{NI}) which were again determined before (c) or 20 min after (d) exposure to 5 μM 17-ODYA. Subtraction of the non-inactivating currents (I_{NI}) from the total currents highlights the effect of 17-ODYA on the delayed rectifier current (f; compare with (e) which shows subtracted currents before exposure to 17-ODYA). The inset in (b) is the computer-derived difference current obtained by subtracting the total currents in the presence of 17-ODYA from those before exposure to the inhibitor. It indicates that 17-ODYA had inhibited a current with activation kinetics typical of $I_{K(V)}$ and had not modified $I_{K(A)}$. (g) A typical trace demonstrating that the outward current induced by levcromakalim (3 μM) at −10 mV ($I_{K(ATP)}$) was unaffected by 5 μM 17-ODYA (see Figure 3a legend for further details). In (a)–(g) the horizontal dashed lines represent the zero current level. The horizontal scale marker represents 200 ms (a–f) or 3 min (g). The vertical scale marker represents 400 pA (a–f) or 250 pA (g). Each trace in (a)–(f) is a computer-derived mean obtained from 5 cells from different animals.

agent were slowly reversible (approximately 50%) over a 20 min washout period. In contrast to its marked inhibitory effects on both $I_{K(V)}$ and $I_{K(ATP)}$, clotrimazole exerted only a small inhibitory action on $I_{BK(Ca)}$ induced by NS1619 (Figure 4d). However, this effect was produced in each cell ($n=4$) and was significant ($P<0.05$).

Effects of 17-ODYA on K-currents

$I_{K(A)}$ and $I_{K(V)}$ 17-ODYA (5 μM) had little effect on the peak outward K-current which was stimulated by stepping from a holding potential of −90 mV to more depolarized test potentials but produced a marked inhibition of the current flowing at the end of the 500 ms voltage-step (Figures 5 and 6). Subtraction of the current which was induced by such a step in the presence of 17-ODYA from that which was induced in the same cell prior to exposure to this agent over a range of test potentials from −60 mV to 0 mV strongly suggested that 17-ODYA had inhibited $I_{K(V)}$ with essentially no inhibitory effect on the more rapidly-inactivating $I_{K(A)}$ (Figure 6b).

$I_{BK(Ca)}$ In addition to the inhibition of $I_{K(V)}$, the current at test potentials more depolarized than 0 mV became very noisy after exposure to 5 μM 17-ODYA (compare Figure 6a and b). To investigate this phenomenon further, cells were held at −10 mV to inactivate $I_{K(V)}$ and $I_{K(A)}$ and then subjected to a series of test voltage steps. Surprisingly, the current evoked at test potentials positive to 0 mV was potentiated in the presence of 17-ODYA (Figures 5c, 6c,d). The additional current was inhibited by 100 nM charybdotoxin, indicating that it was $I_{BK(Ca)}$ (data not shown). Thus, the true extent of inhibition of $I_{K(V)}$ by 5 μM 17-ODYA (Figure 6f) was estimated by subtraction of the currents shown in Figure 6d ($I_{NI}+I_{BK(Ca)}$) from those in Figure 6b ($I_{NI}+I_{BK(Ca)}+I_{K(V)}+I_{K(A)}$). When currents were measured at the end of the 500 ms voltage steps (after inactivation of $I_{K(A)}$) the resultant *I-V* relationship (Figure 5d) shows that 17-ODYA had almost abolished $I_{K(V)}$. The relatively smaller effect of 17-ODYA on the peak current is consistent with little or no inhibition of $I_{K(A)}$ by this compound.

$I_{K(ATP)}$ Over a time-course similar to that which allowed full inhibition of $I_{K(ATP)}$ by clotrimazole and proadifen, 5 μM 17-ODYA did not inhibit the levcromakalim-induced $I_{K(ATP)}$ (Figure 6g). However, after 20 min exposure to a higher concentration of 17-ODYA (20 μM), some inhibition of $I_{K(ATP)}$ was observed (Figure 7).

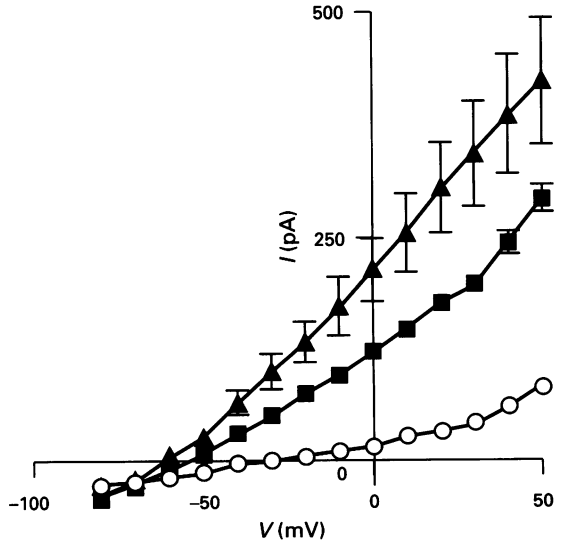

Figure 7 Effect of 20 μM 17-ODYA on $I_{K(ATP)}$ induced by levcromakalim in rat portal vein cells under essentially calcium-free conditions. Current (*I*)-voltage (*V*) relationships were obtained after cells were stepped for 500 ms to the indicated test potentials from a holding potential of −10 mV before (○), during exposure to 3 μM levcromakalim (▲) or after 20 min exposure to 17-ODYA (20 μM) in the continued presence of levcromakalim (■). Each point represents the mean ± s.e.mean derived from 4 cells from different animals.

Effect of the cytochrome P-450 inhibitors on spontaneous mechanical activity in intact veins

Clotrimazole (30 μM) and proadifen (10 μM) each inhibited the magnitude of the spontaneous contractions of whole portal veins and increased their frequency (Figure 8). This was somewhat surprising since in a previous study, inhibitors of $I_{K(V)}$, such as ciclazindol, increased the magnitude, prolonged the duration and disrupted the regular pattern of spontaneous contractions of rat portal veins (Noack *et al.*, 1992b; Figure 8). Despite its intrinsic relaxant activity, proadifen antagonized, rather than enhanced, the mechano-inhibitory effect of levcromakalim (Figure 9a). 17-ODYA (5 μM) had no effect either on the spontaneous activity of the portal vein or on the relaxant effect of levcromakalim on this tissue (Figure 8c). After 30 min exposure to a higher concentration of 17-ODYA (50 μM), the integrated spontaneous mechanical activity was 53 ± 6% ($n=4$) of the initial activity due to a reduction in the magnitude, rather than any change in the frequency, of the spontaneous contractions. The concentration-effect curve for inhibition of spontaneous activity by levcromakalim was not modified by 50 μM 17-ODYA (Figure 9b).

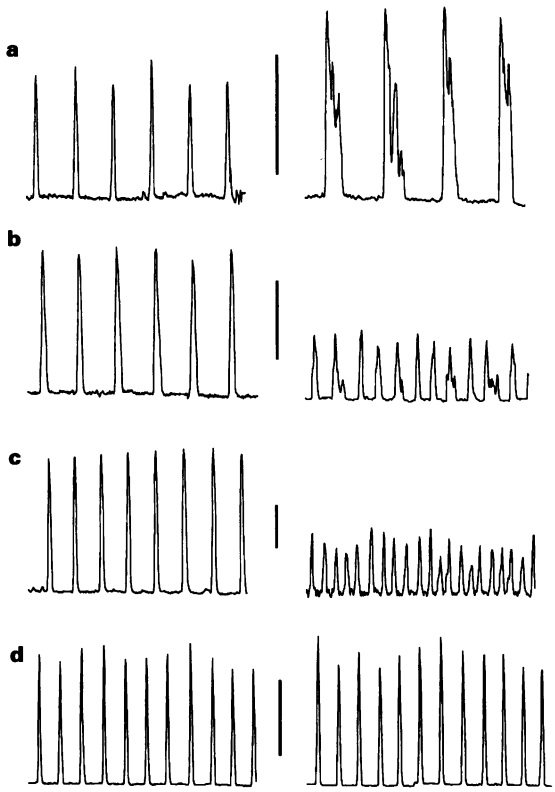

Figure 8 Comparison of the effects of three structurally-dissimilar cytochrome P450 inhibitors and those of ciclazindol on spontaneous contractions in rat portal vein. Each panel is derived from a different tissue and shows a typical trace obtained during a 5 min recording period which started before (left) or after 15 min exposure to the drug (right). Ciclazindol (30 μM; a), an inhibitor of K_V, increased the magnitude and disrupted the regular pattern of each contraction and reduced their frequency. In contrast, both proadifen (10 μM, b) and clotrimazole (30 μM, c) reduced the magnitude and increased the frequency of contractions. 17-ODYA (5 μM, d) did not modify the spontaneous mechanical activity of the vein. In each panel, the vertical scale marker represents 2 mN.

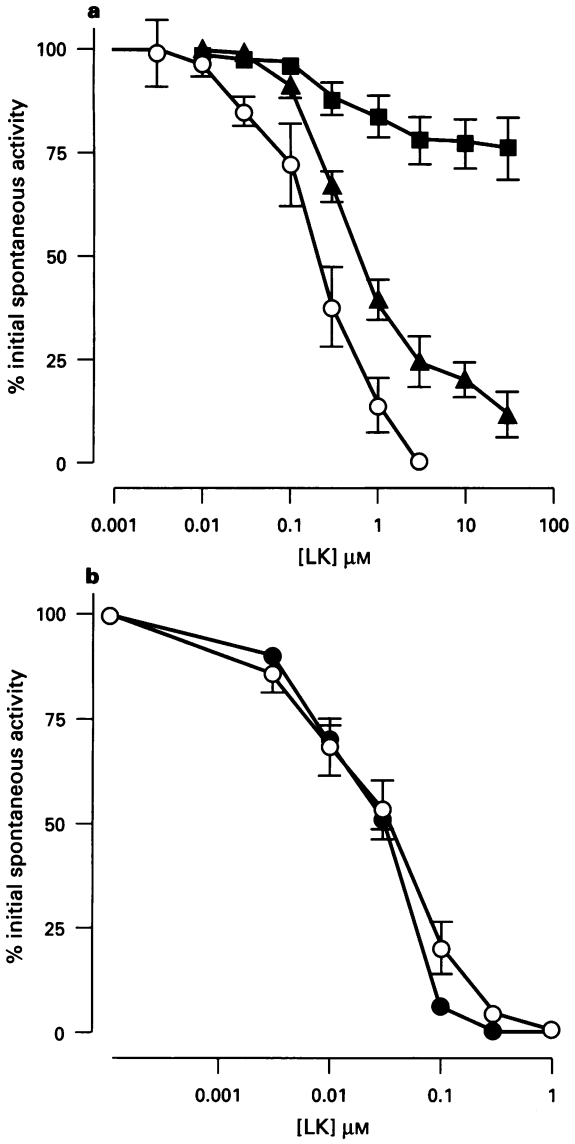

Figure 9 Effect of cytochrome P450 inhibitors on the mechano-inhibitory effect of levcromakalim (LK) in rat portal vein. (a) the concentration-effect curve to levcromakalim (○) was inhibited in a non-competitive manner by 10 μM (▲) or 30 μM (■) proadifen but (b) was not modified by 50 μM 17-ODYA (●). Each point represents the mean ± s.e.mean, $n = 4-6$.

Discussion

The present study was undertaken against a background of reports that cytochrome P450 inhibitors reduce the vasorelaxant and hyperpolarizing effects of EDHF in arterial preparations. From these investigations it has been concluded that EDHF may be a metabolite of arachidonic acid, such as an epoxyeicosatrienoic acid (EET), generated via a cytochrome P450-dependent enzymatic pathway (Bauersachs *et al.*, 1994; Hecker *et al.*, 1994; Lischke *et al.*, 1995; Campbell *et al.*, 1996).

Two of these groups of workers have further suggested that EETs relax smooth muscle by opening Ca-sensitive K-channels. Thus, Hecker *et al.* (1994) reported that the relaxant effects of EDHF (liberated by bradykinin) in porcine coronary artery were inhibited by apamin and by tetrabutylammonium (TBA). Somewhat surprisingly, however, these authors only showed that the relaxant actions of EETs were TBA-sensitive while no data using apamin, a more selective K-channel inhibitor than TBA, were presented. Similarly, Campbell *et al.* (1996) showed that EETs relaxed bovine coronary arteries in a charybdotoxin- and tetraethylammonium (TEA)-sensitive manner. However, these investigators showed only that the relaxant and hyperpolarizing actions of EDHF (liberated by methacholine) were inhibited by TEA while again no information on the sensitivity of these responses to the more selective inhibitor, charybdotoxin, was presented.

In the rat hepatic artery, the possibility that EDHF could be an EET was excluded by the failure of synthetic EETs to relax the tissue and by the inability of 17-ODYA (an inhibitor of the EET-generating pathway) to antagonize EDHF-induced relaxations (Zygmunt *et al.*, 1996a). Furthermore, proadifen and clotrimazole antagonized levcromakalim-induced relaxations in the rat hepatic artery (Zygmunt *et al.*, 1996a) and inhibited K-currents in rat portal vein (Zygmunt *et al.*, 1996b), a tissue in which there are no reports of EDHF as an endogenous factor. Collectively, therefore, these results suggest that any inhibition of EDHF by cytochrome P450 inhibitors could have resulted from inhibition of the smooth muscle K-channel(s) opened by this factor rather than of the pathways involved in the synthesis of EDHF in the vascular endothelium.

Effects of clotrimazole, proadifen and 17-ODYA on $I_{K(V)}$

In the present investigation, all three inhibitors of cytochrome P450 reduced $I_{K(V)}$. The inhibitory effects of clotrimazole and proadifen were rapid in onset and at least partially reversible on washout. In contrast, the effect of 17-ODYA was slow and irreversible. 17-ODYA is a 'suicide-substrate' which irreversibly inhibits both epoxygenases and ω-hydroxylases (Zou *et al.*, 1994). Thus the slow, irreversible effects of this compound would be consistent with the gradual loss of cytochrome P450-dependent enzymatic activity.

Inhibitors of K_V, such as ciclazindol (present study; see also Noack *et al.*, 1992b), increase the force and duration of contractions in the rat portal vein. Neither proadifen nor clotrimazole increased the magnitude of spontaneous tension waves in the intact vessel. The reason for this was not investigated, although it may have resulted from the inhibition of calcium currents by the cytochrome P450 inhibitors since in preliminary experiments using perforated patches such currents were inhibited by 10 μM proadifen ($n = 2$, data not shown). Nevertheless, in the intact vessel, the increase in the frequency of spontaneous contractions and the disruption of their regular pattern by clotrimazole and proadifen is consistent with inhibition of K_V, the opening of which normally contributes to the termination of action potentials. The lack of a similar effect by 5 μM 17-ODYA was perhaps surprising but may have been due to functional antagonism of any inhibitory effect on $I_{K(V)}$ by the observed potentiation of $I_{BK(Ca)}$ by this agent.

Effects of cytochrome P450 inhibitors on $I_{K(ATP)}$

In general, agents which inhibit $I_{K(V)}$ in smooth muscle also inhibit $I_{K(ATP)}$ (see Edwards *et al.*, 1993). Thus, it was perhaps not surprising that proadifen and clotrimazole (and also 17-ODYA under certain conditions) inhibited $I_{K(ATP)}$ induced by levcromakalim and that proadifen inhibited the mechano-inhibitory effect of levcromakalim in the intact portal vein. The failure of 17-ODYA to antagonize levcromakalim-induced relaxations was thus surprising but may have resulted from functional antagonism between the inhibitory effects of this agent on $I_{K(ATP)}$ and its potentiation of $I_{BK(Ca)}$.

Inhibition of $I_{K(ATP)}$ by clotrimazole could have been anticipated on the basis of its imidazoline nucleus, a structural feature shared by several other inhibitors of $I_{K(ATP)}$ (and $I_{K(V)}$; Ibbotson *et al.*, 1993). Proadifen is also a known inhibitor of $I_{K(ATP)}$ (Sakuta & Yoneda, 1994). However, the inhibition of $I_{K(ATP)}$ and $I_{K(V)}$ by 17-ODYA has not been previously reported.

Effects of cytochrome P-450 inhibitors on $I_{BK(Ca)}$

In human red cells, clotrimazole is a potent inhibitor of a large conductance calcium-sensitive K-channel (Gardos channel)

with an IC_{50} value of 0.05 μM (Alvarez *et al.*, 1992). However, in the present study 30 μM clotrimazole produced only a slight inhibition of NS1619-induced $I_{BK(Ca)}$ and proadifen was without effect on $I_{BK(Ca)}$ induced either by NS1619 or by the presence of calcium. This is consistent with the lack of effect of proadifen and miconazole on $I_{BK(Ca)}$ in isolated patches (Campbell *et al.*, 1996) and with the failure of proadifen and clotrimazole (present study) to increase portal vein mechanical activity in a manner typical of a BK_{Ca} inhibitor like charybdotoxin (see Edwards & Weston, 1995).

These results apparently contrast with the inhibitory effects of clotrimazole on $I_{BK(Ca)}$ reported by Hu & Kim (1993). However, in their study, BK_{Ca} activity was decreased by clotrimazole only under cell-attached recording conditions (where the intracellular calcium concentration would be increased by any opening of plasmalemmal calcium channels) but not when the inside-out patch-clamp configuration was used (and when both the intracellular and extracellular calcium concentrations were effectively clamped). This suggests that clotrimazole might inhibit BK_{Ca} indirectly by inhibiting Ca^{2+} influx, a property reported for both clotrimazole and proadifen in cultured cell lines (Villalobos *et al.*, 1992; Daly *et al.*, 1995).

The mechanism by which 17-ODYA stimulated $I_{BK(Ca)}$ in the present experiments is unknown, but the slow, gradual development of this current during exposure to 17-ODYA and the irreversibility of this effect would be consistent with inhibition of cytochrome P450 as the underlying cause. The stimulation of opening of a large-conductance K-channel (probably BK_{Ca}) by 17-ODYA in smooth muscle cells isolated from cat cerebral microvessels has been previously reported (Harder *et al.*, 1994). Antagonism of the 17-ODYA effect by 20-hydroxyeicosatrienoic acid was interpreted as an indication that the effect of 17-ODYA resulted from inhibition of cytochrome P450 (Harder *et al.*, 1994). In the present study, the lack of any effect of 17-ODYA on spontaneous contractions of the rat portal vein may have resulted from its opposing effects on BK_{Ca} and K_V. Thus, as shown in Figure 5, the inhibitory effect of 17-ODYA on $I_{K(V)}$ could have been negated to some extent by its stimulatory effect on $I_{BK(Ca)}$ which resulted in little change in the total outward peak K-currents. Nevertheless, 17-ODYA did have a substantial effect on the total curernt which was measured at the end of each 500 ms voltage step. This may be an indication that the termination of action potentials by K-channel opening depends more on the availability of conducting K-channels immediately after the depolarizing stimulus than on the prolonged opening of K-channels.

Does cytochrome P-450 inhibition modulate K-channels?

Three structurally- and mechanistically-unrelated inhibitors of cytochrome P450-dependent enzymes were employed in the present study in the hope that a consistent pattern of channel modulation would emerge and allow any role of cytochrome P450 to be clarified. Nitrogen heterocycles, such as clotrimazole, bind reversibly to both the haem moiety of the cytochrome and the lipophilic regions of the associated enzyme and thus interfere with substrate and oxygen binding (Ortiz de Montellano & Correia, 1995). Alkylamines such as proadifen are catalysis-dependent inhibitors which are metabolized to form intermediates which then reversibly bind and inhibit the P450-dependent enzymes. 17-ODYA is a 'suicide-substrate' which irreversibly inhibits cytochrome P450-dependent epoxygenases and ω-hydroxylases (Zou *et al.*, 1994; Ortiz de Montellano & Correia, 1995). Any irreversible modulation of channel activity by 17-ODYA could thus indicate an underlying role for these specific enzymes.

From the data obtained it is difficult to be certain whether the observed reduction of $I_{K(V)}$ and $I_{K(ATP)}$ resulted from the inhibition of cytochrome P450-dependent enzymes or whether the compounds used had inhibited these channels by a different mechanism. However, inhibition of these currents was a feature common to all three inhibitors employed and there are no reports of any actions of 17-ODYA which are unrelated to inhibition of cytochrome P450 enzymes. Furthermore, other known cytochrome P450 inhibitors such as quinacrine, quinidine and phencyclidine (Jacqz-Aigran *et al.*, 1991; Ortiz de Montellano & Correia, 1995) are also K-channel inhibitors (Beech & Bolton, 1989; Rubart *et al.*, 1993; Sakuta & Yoneda, 1994).

The potentiation of $I_{BK(Ca)}$ by 17-ODYA was a property not shared by proadifen or clotrimazole. Whether this indicates that cytochrome P450-dependent epoxygenases or ω-hydroxylases are involved in the gating of BK_{Ca} awaits further study.

Conclusions

The present study has clearly demonstrated that K-currents in vascular smooth muscle can be modulated by agents classified as cytochrome P450 inhibitors. Whether cytochrome P450-dependent enzymes necessarily play a role in modulating K-channel activity cannot be stated with certainty, although inhibition of K_V and K_{ATP} by all three tested inhibitors suggests that this is a possibility. In view of the general K-channel inhibitory properties of these agents, the ability of cytochrome P450 inhibitors to antagonize EDHF-induced relaxations is not a firm basis upon which to conclude that EDHF is the product of a cytochrome P450-dependent pathway.

G.E. was supported by the Medical Research Council and P.M.Z. by a Swedish Medical Research Council – Wellcome Trust Travelling Fellowship (Grant Nos. 11524 and 11525). E.D.H. was funded by the Swedish Medical Research Council (Grant No. 11582).

References

ALVAREZ, J., MONTERO, M. & GARCIA-SANCHO, J. (1992). High affinity inhibition of Ca^{2+}-dependent K^+ channels by cytochrome P-450 inhibitors. *J. Biol. Chem.*, **267**, 11789–11793.

BAUERSACHS, J., HECKER, M. & BUSSE, R. (1994). Display of the characteristics of endothelium-derived hyperpolarising factor by a cytochrome P450-derived arachidonic acid metabolite on the coronary microcirculation. *Br. J. Pharmacol.*, **113**, 1548–1553.

BEECH, D.J. & BOLTON, T.B. (1989). Properties of the cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. *Br. J. Pharmacol.*, **98**, 851–864.

CAMPBELL, W.B., GEBREMEDHIN, D., PRATT, P.F. & HARDER, D.R. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ. Res.*, **78**, 415–423.

COHEN, R.A. & VANHOUTTE, P.M. (1995). Endothelium-dependent hyperpolarization: beyond nitric oxide and cyclic GMP. *Circulation*, **92**, 3337–3349.

DALY, J.W., LUEDERS, J., PADGETT, W.L., SHIN, Y. & GUSOVSKY, F. (1995). Maitotoxin-elicited calcium influx in cultured cells – effect of calcium-channel blockers. *Biochem. Pharmacol.*, **50**, 1187–1197.

EDWARDS, G., IBBOTSON, T. & WESTON, A.H. (1993). Levcromakalim may induce a voltage-independent K-current in rat portal veins by modifying the gating properties of the delayed rectifier. *Br. J. Pharmacol.*, **110**, 1037–1048.

EDWARDS, G., NIEDERSTE-HOLLENBERG, A., SCHNEIDER, J., NOACK, TH. & WESTON, A.H. (1994). Ion channel modulation by NS 1619, the putative BK_{Ca} channel opener, in rat portal vein. *Br. J. Pharmacol.*, **113**, 1538–1547.

EDWARDS, G. & WESTON, A.H. (1995). Potassium channels in the regulation of vascular smooth muscle tone. In *Pharmacological Control of Calcium and Potassium Homeostasis: Biological, Therapeutic and Clinical Aspects*. ed. Godfraind, T., Mancia, G., Abbracchio, M.P., Aguilar-Bryan, L. & Govoni, S. pp. 85–93. London: Kluwer Academic Publishers.

FURCHGOTT, R.F. (1993). The discovery of endothelium-dependent relaxation. *Circulation*, **87**, 3–8.

GARLAND, C.J. & MCPHERSON, G.A. (1992). Evidence that nitric oxide does not mediate the hyperpolarisation and relaxation to acetylcholine in the rat small mesenteric artery. *Br. J. Pharmacol.*, **105**, 429–435.

GARLAND, C.J., PLANE, F., KEMP, B.K. & COCKS, T.M. (1995). Endothelium-dependent hyperpolarization: a role in the control of vascular tone. *Trends Pharmacol. Sci.*, **16**, 23–30.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HARDER, D.R., CAMPBELL, W.B. & ROMAN, R.J. (1995). Role of cytochrome P-450 enzymes and metabolites of arachidonic acid in the control of vascular tone. *J. Vasc. Res.*, **32**, 79–92.

HARDER, D.R., GEBREMEDHIN, D., NARAYANAN, J., JEFCOAT, C., FALCK, J.R., CAMPBELL, W.B. & ROMAN, R. (1994). Formation and action of a P-450 4A metabolite of arachidonic acid in cat cerebral microvessels. *Am. J. Physiol.*, **266**, H2098–H2107.

HECKER, M., BARA, A.T., BAUERSACHS, J. & BUSSE, R. (1994). Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J. Physiol.*, **481**, 407–414.

HU, S. & KIM, H.S. (1993). Activation of K^+ channel in vascular smooth muscles by cytochrome P450 metabolites of arachidonic acid. *Eur. J. Pharmacol.*, **230**, 215–221.

IBBOTSON, T., EDWARDS, G. & WESTON, A.H. (1993). Antagonism of levcromakalim by imidazoline-derivatives and guanidine-derivatives in rat portal vein – involvement of the delayed rectifier. *Br. J. Pharmacol.*, **110**, 1556–1564.

JACQZ-AIGRAIN, E., GUEGUEN, M., ZANGER, U.M., ROBIEUX, I. & ALVAREZ, F. (1991). Cytochrome P450IID in non-human primates. Catalytical and immunological characterization. *Biochem. J.*, **41**, 1657–1663.

KLÖCKNER, U. & ISENBERG, G. (1985). Action potentials and net membrane currents of isolated smooth muscle cells (urinary bladder of the guinea-pig). *Pflügers Arch.*, **405**, 329–339.

LISCHKE, V., BUSSE, R. & HECKER, M. (1995). Selective inhibition by barbiturates of the synthesis of endothelium-derived hyperpolarizing factor in the rabbit carotid artery. *Br. J. Pharmacol.*, **115**, 969–974.

NOACK, TH., DEITMER, P,M EDWARDS, G. & WESTON, A.H. (1992a). Characterization of potassium currents modulated by BRL38227 in rat portal vein. *Br. J. Pharmacol.*, **106**, 717–726.

NOACK, TH., EDWARDS, G., DEITMER, P., GREENGRASS, P., MORITA, T., ANDERSSON, P.O., CRIDDLE, D., WYLLIE, M.G. & WESTON, A.H. (1992b). The involvement of potassium channels in the action of ciclazindol in rat portal vein. *Br. J. Pharmacol.*, **106**, 17–24.

ORTIZ DE MONTELLANO, P.R. & CORREIA, M.A. (1995). Inhibition of cytochrome P450 enzymes. In *Cytochrome P450. Structure, Mechanism and Biochemistry*. ed. Ortiz de Montellano, P.R. pp. 305–364. New York: Plenum Press.

RUBART, M., PRESSLER, M.L., PRIDE, H.P. & ZIPES, D.P. (1993). Electrophysiological mechanisms in a canine model of erythromycin-associated long QT syndrome. *Circulation*, **88**, 1832–1844.

SAKUTA, H. & YONEDA, I. (1994). Inhibition by SKF525A and quinacrine of endogenous glibenclamide-sensitive K^+ channels in follicle-enclosed Xenopus oocytes. *Eur. J. Pharmacol.*, **251**, 117–121.

TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol. Sci.*, **9**, 272–274.

VILLALOBOS, C., FONTERIZ, R., LOPEZ, M.G., GARCIA, A.G. & GARCIA-SANCHO, J. (1992). Inhibition of voltage-gated Ca^{2+} entry into GH3 and chromaffin cells by imidazole antimycotics and other cytochrome P450 blockers. *FASEB J.*, **6**, 2742–2747.

ZOU, A.-P., IMIG, J.D., KALDUNSKI, M., ORTIZ DE MONTELLANO, P.R., SUI, Z. & ROMAN, R.J. (1994). Inhibition of renal vascular 20-HETE production impairs autoregulation of renal blood flow. *Am. J. Physiol.*, **266**, F275–F282.

ZYGMUNT, P.M., EDWARDS, G., WESTON, A.H., DAVIS, S.C. & HÖGESTÄTT, E.D. (1996a). Effects of cytochrome P450 inhibitors on EDHF-mediated relaxation in the rat hepatic artery. *Br. J. Pharmacol.*, **118**, 1147–1152.

ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1966). Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br. J. Pharmacol.*, **117**, 1600–1606.

ZYGMUNT, P.M., HÖGESTÄTT, E.D., EDWARDS, G., ORTIZ DE MONTELLANO, P.R. & WESTON, A.H. (1996b). Effects of cytochrome P450 inhibitors on endothelium-dependent and levcromakalim-induced responses in rat blood vessels. *Br. J. Pharmacol.*, **117**, 120P.

(Received April 26, 1996
Revised July 10, 1996
Accepted July 19, 1996)