Br. J. Pharmacol. (1992), **106**, 9–16

Comparison of the actions of acetylcholine and BRL 38227 in the guinea-pig coronary artery

D.M. Eckman, J.D. Frankovich & [1]K.D. Keef

Department of Physiology, School of Medicine, University of Nevada, Reno, Nevada 89557, U.S.A.

1 The contractile and electrical responses to acetylcholine (ACh) in isolated segments of guinea-pig and rabbit coronary arteries were compared to those of the putative adenosine 5'-triphosphate (ATP)-dependent K^+ channel opener, BRL 38227.

2 Both ACh and BRL 38227 produced concentration-dependent relaxation of vessel segments contracted with the H_1-receptor agonist, 2-(2-aminoethyl)pyridine.

3 An IC_{90} of either vasodilator also produced 17–20 mV of hyperpolarization of the guinea-pig coronary artery.

4 Glibenclamide (1–35 μM) depolarized the guinea-pig coronary artery by 8–12 mV and antagonized BRL 38227- but not ACh-induced relaxation and hyperpolarization.

5 In the guinea-pig coronary artery, the K^+ channel blockers phencyclidine (PCP, 100 μM), tetraethylammonium (TEA, 10 mM) and scorpion venom (8.7 $\mu g\,ml^{-1}$) all significantly reduced ACh-induced relaxation and hyperpolarization whereas only PCP was an effective antagonist of both relaxation and hyperpolarization with BRL 38227.

6 Similar effects of glibenclamide and scorpion venom on ACh- and BRL 38227-induced relaxation were observed in the rabbit coronary artery.

7 Apamin (3.5 μM) was without effect on either the ACh- or BRL 38227-induced relaxation in the guinea-pig coronary artery.

8 In conclusion, the actions of BRL 38227 in coronary artery are compatible with its proposed effects on ATP-dependent K^+ channels. In contrast, the results with ACh suggest that some step between the initial binding of ACh to endothelial muscarinic receptors and the final relaxation of the smooth muscle depends upon the opening of Ca^{2+}-activated K^+ channels.

Keywords: Vascular smooth muscle; electrophysiology; potassium channel openers; membrane potential; endothelium; vasodilators; cromakalim; acetylcholine; scorpion venom; phencyclidine; apamin; tetraethylammonium; glibenclamide; BRL 38227

Introduction

Acetylcholine (ACh) releases a factor from the endothelium of some blood vessels which hyperpolarizes the smooth muscle. This has given rise to the acronym EDHF or 'endothelium-derived hyperpolarizing factor' (Chen *et al.*, 1988). There is evidence that this factor is independent of the more well known and widely studied relaxing factor EDRF (Chen *et al.*, 1988; Feletou *et al.*, 1988; Huang *et al.*, 1988; Komori *et al.*, 1988). EDRF is widely believed to be nitric oxide (see Furchgott & VanHoutte, 1989). The separation between these two factors has become more complicated, however, with the recent reports of nitric oxide-induced hyperpolarization in some blood vessels (Tare *et al.*, 1990; 1991).

Studies of the rabbit middle cerebral artery have suggested that endothelium-dependent hyperpolarization may involve the opening of an adenosine 5'-triphosphate (ATP)-dependent K^+ channel since the hyperpolarization produced with ACh is reversed by glibenclamide (Standen *et al.*, 1989; Brayden, 1990). This agent blocks ATP-dependent K^+ channels in pancreatic β cells (Sturgess *et al.*, 1985; Schmid-Antomarchi *et al.*, 1987a,b) and cardiac muscle (Fosset *et al.*, 1988). On the other hand, studies of the rat mesenteric artery suggest that the ACh-induced hyperpolarization and relaxation in this vessel are unaffected by glibenclamide so that different hyperpolarizing factors may be involved in the actions of ACh in different blood vessels (McPherson & Angus, 1991).

Another agent which hyperpolarizes vascular smooth muscle by stimulation of K^+ channels is cromakalim (Hamilton *et al.*, 1986; Weir & Weston, 1986b). Unlike ACh which requires release of EDHF, hyperpolarization with cromakalim occurs by direct stimulation of K^+ channels in the smooth muscle membrane e.g., in isolated patches of membrane from the rabbit mesenteric artery, cromakalim has been shown to open K^+ channels (Standen *et al.*, 1989) and in recordings from isolated cells of the rabbit portal vein, addition of cromakalim leads to hyperpolarization (Beech & Bolton, 1989). The K^+ channels stimulated by cromakalim appear to be modulated by ATP since channel activity is reduced in the presence of 1 mM ATP and enhanced in its absence (Standen *et al.*, 1989). In addition it has been reported that glibenclamide: (1) blocks cromakalim-stimulated K^+ currents in isolated patches (Standen *et al.*, 1989); (2) blocks whole-cell cromakalim currents in single portal vein cells (Beech & Bolton, 1989) and (3) blocks the cromakalim-induced reduction in coronary perfusion pressure in guinea-pig isolated heart (Daut *et al.*, 1990) and fall in blood pressure in the rat (Buckingham *et al.*, 1989). Recent studies suggest that the channel opened by cromakalim may also be modulated by calcium (Gelband *et al.*, 1989; Klöckner *et al.*, 1989; Hu *et al.*, 1990; Okabe *et al.*, 1990). We studied the relaxant and hyperpolarizing properties of BRL 38227 which is the active enantiomer present in the racemic mixture known as cromakalim (see Buckingham *et al.*, 1986; Weston, 1989).

In the present study the nature of the ACh-induced hyperpolarization in the guinea-pig coronary artery has been investigated. In this vessel, ACh initiates a large hyper-

[1] Author for correspondence.

polarization (Kitamura & Kuriyama, 1979; Keef & Kreulen, 1988) which is blocked by removal of the endothelium (Keef & Bowen, 1989; Chen *et al.*, 1991). Since there is evidence from studies of the cerebral artery that cromakalim and ACh produce hyperpolarization by activation of the same smooth muscle K^+ channel (Standen *et al.*, 1989; Brayden, 1990) we have compared the relaxant and hyperpolarizing actions of ACh and BRL 38227 in the presence and absence of glibenclamide. To investigate further the mechanism by which these agonists produce hyperpolarization and relaxation we also tested the effects of several other K^+ channel blockers including phencyclidine (PCP), tetraethylammonium (TEA), apamin and scorpion venom. These data have been published in part in abstract form (Eckman & Keef, 1991).

Methods

Contractile experiments

Male guinea-pigs (400–600 g) were killed by CO_2 inhalation followed by exsanguination and the heart removed and placed in cold (10 to 15°C) oxygenated Krebs solution. A 1 to 1.5 cm segment of the left circumflex coronary artery was carefully dissected out and cleaned of all adhering fat and cardiac tissue. Ring segments (3 mm long) were mounted onto two triangular tungsten wires (89 μm diameter). The preparation was mounted vertically in an isolated tissue bath. The upper triangle was attached to a Gould strain gauge and the lower to a stable mount. The tissue bath was maintained at 37°C in Krebs solution of the following composition mM: NaCl 118.5, KCl 4.7, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, KH_2PO_4 1.2, dextrose 11, $CaCl_2$ 2.5 aerated with 95% O_2/5% CO_2.

A resting force of 0.3 g was applied to the guinea-pig coronary artery. In preliminary experiments this was found to stretch vessels to near the optimal length for tension development (i.e. L_0). Vessels were initially equilibrated for 2 h with alternating 4 min exposures to the histamine H_1-receptor agonist, 2-(2-aminoethyl)pyridine (AEP 10^{-3} M, Durant *et al.*, 1975) and to 55 mM KCl (3 M KCl stock solution). The stimulants were added at 10–15 min intervals. Maximum contraction was determined in some experiments by exposing vessel segments to a combination of 10^{-3} M AEP and 100 mM KCl.

Rabbits (1.5–2.5 kg) were killed with an overdose of pentobarbitone injected into the ear vein. The rabbit does not have a true circumflex coronary artery but rather the coronary artery courses down the middle of the left ventricle. This was the vessel used for these studies. A resting force of 0.5 g was applied to 3 mm long segments of vessel. Artery segments were otherwise treated as described above for the guinea-pig. In a few experiments the endothelium of vessel segments was removed to study the direct contractile effects of ACh on the smooth muscle. This was accomplished by gentle scraping of the lumen with a stainless steel rod. The successful removal of endothelium was judged by a lack of relaxant response to application of ACh between 0.1 and 10 μM when tissues were contracted with AEP (10^{-4} M).

Intracellular measurements

For experiments measuring membrane potential, a 0.5 to 0.7 cm segment of the guinea-pig circumflex coronary artery or the rabbit left ventricular artery was used. Vessels were carefully cut open along their longitudinal axis and pinned down in the bath, adventitial side up. Microelectrodes filled with 3 M KCl and having resistances between 70 and 100 MΩ were used. All intracellular measurements were made through the adventitia into the media. Intracellular measurements were made in the absence of the contractile stimulus AEP since it is easier to obtain long term impalements under these conditions. In a previous study we have shown that the magnitude of hyperpolarization with ACh is greater in the presence of AEP than in its absence presumably since the depolarization which occurs upon exposure to AEP brings the membrane farther away from E_K (Keef & Bowen, 1989). Impalements were judged on the basis of a rapid drop in potential upon entering the cell, a low noise level and minimal change in electrode resistance and zero potential before and after impalement. Signals were viewed on a digital oscilloscope (Nicolet) and stored on tape with a Vetter PCM Recording Adapter attached to a Panasonic Video Cassette Recorder.

Statistics

Statistical significance was determined by two-tailed paired or unpaired Student's t test and where appropriate analysis of variance was performed. Changes were considered significant at $P < 0.05$. Data are expressed as mean ± s.e.mean. IC_{50} values were calculated by use of a computer programme entitled 'Analysis of the Regression Line' which is based upon *Manual of Pharmacologic Calculations with Computer Programs* (Tallarida & Murray, 1987).

Drugs used

Acetylcholine HCl (ACh, Sigma), 2-(2-aminoethyl)pyridine, (AEP, Aldrich), glibenclamide (Sigma), scorpion venom (*Leiurus quinquestriatus hebraeus*, Sigma), phencyclidine (PCP, Sigma). ACh, AEP and PCP were made up in concentrated stock solution in distilled water. Scorpion venom was made fresh daily in distilled water. Glibenclamide was dissolved in polyethylene glycol and BRL 38227 (levrocromakalim) was dissolved in either 50% polyethylene glycol and 50% distilled water or dimethyl sulphoxide. All stock solutions were kept in the dark at 2° C during use and stored between days in the freezer. Neither polyethylene glycol nor dimethyl sulphoxide produced direct effects in the concentrations used in this study.

Results

Comparison of acetylcholine- and BRL 38227-induced relaxation

The time course and magnitude of relaxation with various concentrations of acetylcholine (ACh, 10 nM–1 μM) and BRL 38227 (10 nM–1 μM) were compared. Segments of guinea-pig coronary artery were contracted with 2-(2-aminoethyl)pyridine (AEP, 1 mM). This concentration of AEP produced a contraction which was 48.6 ± 2.9% of that produced by combined AEP and 100 mM KCl ($n = 11$). The various concentrations of ACh or BRL 38227 were added sequentially so that the time course of response could be measured. Relaxation with each concentration of ACh was more rapid in onset and reached a maximum faster than with BRL 38227. For example, relaxation with a 90% maximal inhibitory concentration of ACh (i.e., IC_{90}, 0.35 μM) relaxed the tissue in 0.64 ± 0.07 min ($n = 15$) whereas with an IC_{90} of BRL 38227 (1 μM) maximal relaxation was reached after 2.47 ± 0.27 min ($n = 17$). An example of the time course of relaxation with various concentrations of ACh and BRL 38227 is shown in Figure 1a. The IC_{50} for ACh-induced relaxation was 0.022 μM ($n = 17–24$, Figure 1b) whereas for BRL 38227 it was 0.029 μM ($n = 13–22$, Figure 1b).

Effect of glibenclamide on acetylcholine- and BRL 38227-induced relaxation

The concentration-response relationship for ACh was measured in the presence and absence of 1 and 35 μM glibenclamide in segments of guinea-pig coronary artery. Vessels were contracted with AEP and increasing concentrations of ACh (0.01 μM to 1 μM) were subsequently added in a cumulative manner. In 6 of the 17 tissues, addition of 35 μM

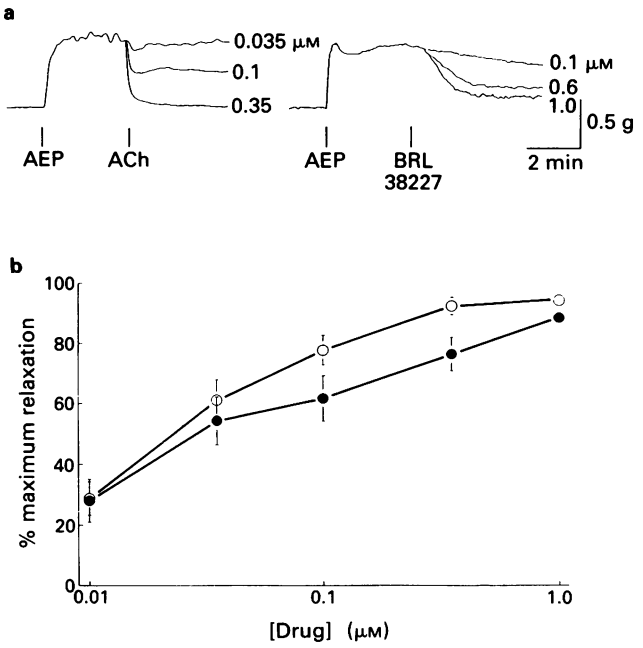

Figure 1 Comparison of the vasodilator properties of acetylcholine (ACh) and BRL 38227 in coronary artery segments contracted with 2-(2-aminoethyl)pyridine (AEP). (a) Typical examples of the relaxations observed with various concentrations of ACh (top left) and BRL 38227 (top right) in two vessel segments from the same animal. The entire contractile record for the lowest concentration of either vasodilator is shown. The relaxations obtained at higher concentrations of vasodilators have been superimposed at the arrows so that the time course of relaxations can be compared. The concentrations of vasodilator used are noted to the right of both contractile records. Note that both the onset of relaxation and the time to peak relaxation are slower for BRL 38227 than for ACh. (b) Concentration-response relationships for acetylcholine (○, $n = 8-10$)- and BRL 38227 (●, $n = 5-15$)-induced relaxations in the guinea-pig coronary artery in segments contracted with AEP. Shown are mean values with s.e. mean indicated by vertical bars.

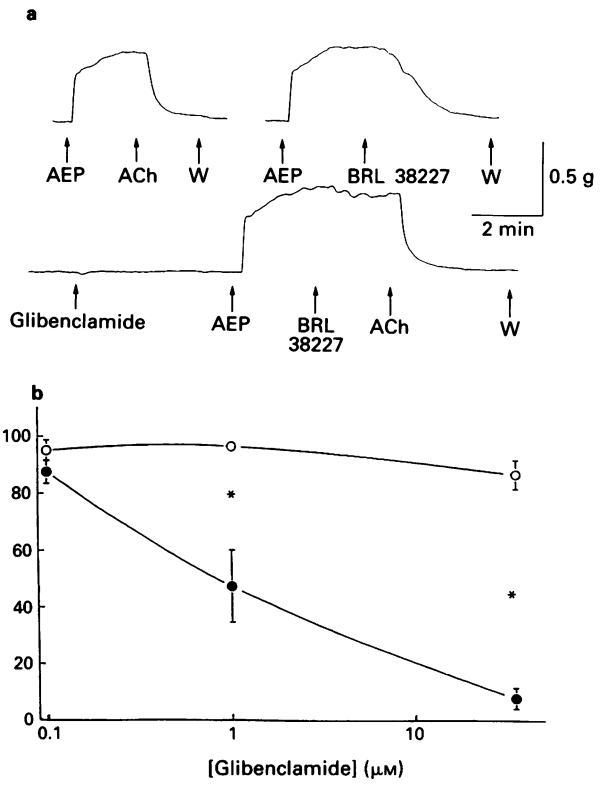

Figure 2 Comparison of the effects of glibenclamide on the relaxation initiated with either acetylcholine (ACh) or BRL 38227 in vessel segments contracted with 2-(2-aminoethyl)pyridine (AEP). (a) Example of the selective antagonism of the BRL 38227 (1 μM)- but not the ACh (0.35 μM)-induced relaxation with glibenclamide (35 μM) in a single vessel segment. Removal of drug from the bath is indicated with a W. Upper contractile recordings; control responses to application of either ACh or BRL 38227. Lower contractile record; in the presence of glibenclamide, BRL 38227 no longer relaxed the vessel whereas the response to ACh was still intact. (Note: the mean values shown in (b) of this figure for ACh-induced relaxations in the presence of glibenclamide were obtained in the absence of BRL 38227). (b) Concentration-response relationships for the effects of glibenclamide (0.1–35 μM) on relaxations induced with either ACh (○, 0.35 μM, $n = 3-8$) or BRL 38227 (●, 1 μM, $n = 6-8$). Note that 1 to 35 μM glibenclamide led to significant ($^*P < 0.05$) inhibition of the response to BRL 38227 whereas the responses to ACh were unaffected. Shown are mean values with s.e.mean indicated by vertical bars.

glibenclamide produced a contraction which was 1.6 ± 0.7% of that elicited with AEP. Neither 1 nor 35 μM glibenclamide significantly shifted the concentration-response relationship for ACh-induced relaxation (IC_{50} for ACh in control solution = 0.022 μM, $n = 8-10$; in 1 μM glibenclamide = 0.07 μM, $n = 5-9$; in 35 μM glibenclamide = 0.054 μM, $n = 5-10$). The effects of 0.1, 1 and 35 μM glibenclamide were also tested on relaxations produced with an IC_{90} of either BRL 38227 (1 μM) or ACh (0.35 μM). Glibenclamide addition produced a concentration-dependent inhibition of BRL 38227-induced relaxations whereas responses to ACh were unchanged. These data are summarized in Figure 2.

Comparison of acetylcholine- and BRL 38227-induced hyperpolarization

The time course and magnitude of changes in membrane potential with a 6–7 min exposure to ACh (0.35 μM) and BRL 38227 (1 μM) were compared in segments of guinea-pig coronary artery. The mean resting membrane potential in this preparation was − 52.4 ± 1.5 mV ($n = 31$). There was no significant difference between the magnitude of the hyperpolarization produced to ACh (17.7 ± 1.1, $n = 28$) or BRL 38227 (19.2 ± 1.6, $n = 21$). The time to peak hyperpolarization with ACh (1.64 ± 0.13 min, $n = 8$) was significantly faster than with BRL 38227 (3.28 ± 0.41 min, $n = 8$). Examples of the hyperpolarization produced with these agents are included in Figure 3.

Effect of glibenclamide on acetylcholine- and BRL 38227-induced hyperpolarization

In these experiments we examined the effects of glibenclamide on the hyperpolarization produced with both ACh and BRL 38227 in segments of guinea-pig coronary artery. Addition of glibenclamide alone led to a concentration-dependent depolarization of the membrane (10 μM glibenclamide: 8.8 ± 1.8 mV, $n = 10$; 35 μM glibenclamide: 12.3 ± 1.8 mV, $n = 8$; Figure 3a). In one experiment, an 8 mV depolarization was also observed upon exposure to 1 μM glibenclamide. In the presence of this agent the peak hyperpolarization elicited with ACh (0.35 μM) was not sigificantly reduced (control: 17.7 ± 1.1 mV, $n = 28$; 10 μM glibenclamide: 17.2 ± 2.6 mV, $n = 4$; 35 μM glibenclamide: 17.3 ± 1.7, $n = 4$). Glibenclamide (10 μM) prevented the BRL 38227 (1 μM)-induced hyperpolarization ($n = 4$). An example of the differential effects of glibenclamide on hyperpolarization with BRL 38227 versus ACh is shown in Figure 3b.

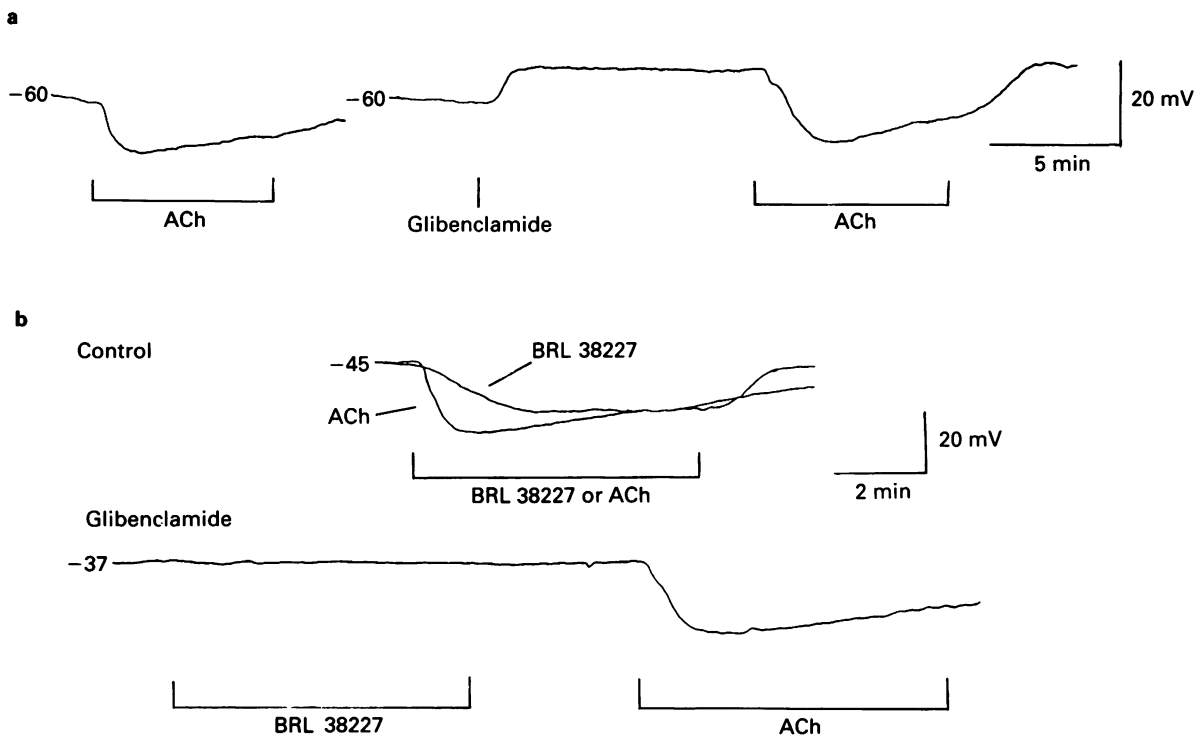

Figure 3 Effect of glibenclamide (35 μM) on resting membrane potential and the hyperpolarization produced with acetylcholine (ACh) or BRL 38227. (a) Effect of glibenclamide on resting membrane potential and the response to ACh (1 μM). Intracellular recording obtained from a single cell. Initial exposure to ACh resulted in a 15 mV hyperpolarization. Thirteen min after removal of ACh, glibenclamide was added to the bath leading to an 11 mV depolarization. Subsequent addition of ACh 10 min later induced a hyperpolarization of 27 mV. (b) Comparison of the effects of glibenclamide on the hyperpolarization produced with BRL 38227 (1 μM) versus ACh (0.35 μM). Control responses to these two agonists in the same cell are superimposed in the upper trace. Note that the onset of hyperpolarization as well as the rate of repolarization are more rapid for ACh than for BRL 38227. In the lower trace a later portion of the membrane potential recording in the same cell is shown following 10 min of exposure to glibenclamide. (Not shown: glibenclamide depolarized the tissue by 8 mV). Subsequent addition of BRL 38227 in the continued presence of glibenclamide did not hyperpolarize the tissue whereas hyperpolarization with ACh was still obtained.

Comparison of the effects of potassium channel blockers on the BRL 38227- and ACh-induced relaxation

In these experiments we examined the effects of PCP, TEA, scorpion venom and apamin to provide additional evidence for the involvement of K^+ channels in the actions of ACh and to differentiate further the BRL 38227- and ACh-induced hyperpolarization-relaxation pathways.

The concentration-response relationships for ACh- and BRL 38227-induced relaxation were measured in the presence and absence of either 100 μM PCP or 10 mM TEA in segments of guinea-pig coronary artery. Vessels were contracted with AEP and increasing concentrations (0.01 μM to 1 μM) of either ACh or BRL 38227 were added in a cumulative manner. The modifying agents were then added 10 min before addition of AEP and the concentration-response relationship for ACh or BRL 38227 repeated. In all tissues, PCP addition alone caused a contraction (12 ± 3% of the AEP response, $n = 6$). Likewise, in all tisssues, TEA addition alone caused contraction (5 ± 2% of the AEP response, $n = 5$). Both PCP (100 μM) and TEA (10 mM) significantly shifted the concentration-response relationship for ACh to the right whereas with BRL 38227 only PCP produced a significant shift (Figure 4). The actions of PCP on the shape of the concentration-response relationship for BRL 38227 and ACh differed, i.e., PCP produced a greater decrease in the steepness of the concentration-response relationship for BRL 38227 than for ACh. Thus, in the absence of PCP, both 1 μM ACh and 1 μM BRL 38227 produced near maximal relaxation. However, in the presence of PCP, ACh (1 μM) still elicited approximately 68% ($n = 6$) maximum relaxation whereas BRL 38227 (1 μM) elicited only 30% ($n = 6$) maximum relaxation (Figure 4).

In contrast to the effects of PCP and TEA on ACh-induced responses, apamin (3.5 μM, $n = 7$) did not significantly reduce relaxations with either ACh ($n = 4$) or BRL 38227 ($n = 3$).

Crude scorpion venom is known to contain several different compounds which block K^+ channels (Strong, 1990) and thus the effects of scorpion venom (8.7 μl ml^{-1}) on the relaxant responses to ACh (0.35 μM) and BRL 38227 (1 μM) were compared. Application of scorpion venom 30 min before AEP elicited a transient contraction (i.e., 3.4 ± 0.8% of the AEP response, $n = 8$). Scorpion venom significantly reduced the ACh relaxation to 24 ± 6% ($n = 4$) of control whereas BRL 38227 relaxations were unchanged ($n = 4$). Thus whereas glibenclamide preferentially blocked BRL 38227 responses, scorpion venom preferentially reduced ACh-induced relaxations (see Figure 5).

Comparison of the effects of potassium channel blockers on the BRL 38227- and ACh-induced hyperpolarization

Membrane potential measurements were undertaken in segments of guinea-pig coronary artery to determine whether the relative ability of PCP (35 μM) and TEA (10 mM) to block hyperpolarization was similar to their ability to block relaxation. Addition of TEA led to a 10.5 ± 0.9 mV ($n = 6$) depolarization (Table 1). In the presence of TEA, ACh-induced hyperpolarization was reduced to 22.5% ($n = 3$) of control whereas the BRL 38227-induced hyperpolarization ($n = 3$) was greater than control, presumably because the

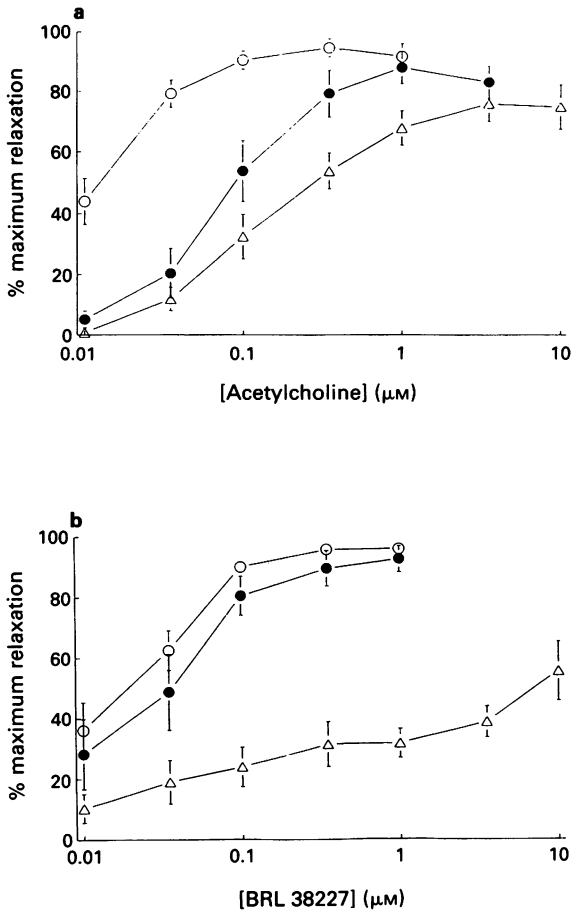

Figure 4 Comparison of the effects of control (○), phencyclidine (PCP, △, 100 μM, a, $n = 5-6$; b, $n = 3-6$) and tetraethylammonium (TEA, (●, 10 mM, a, $n = 3-5$; b, $n = 3-5$) on the concentration-dependent relaxation produced with either ACh (a) or BRL 38227 (b) in guinea-pig vessel segments (control responses, ○) contracted with 2-(2-aminoethyl) pyridine (AEP). PCP significantly shifted the concentration-response relationships for both ACh and BRL 38227 to the right. With TEA a significant shift was only observed for ACh responses. Shown are mean values with s.e.mean indicated by vertical bars.

membrane potential was further away from the potassium equilibrium potential (Table 1). Addition of PCP also depolarized the tissue (8.4 ± 1.7 mV, $n = 7$). Both the BRL 38227- ($n = 3$) and ACh ($n = 4$)-induced hyperpolarization were significantly reduced in the presence of 35 μM PCP (Table 1). These observations indicated that concentrations of PCP and TEA which reduced relaxation also significantly attenuated the associated hyperpolarization.

Scorpion venom elicited a transient depolarization of the tissue (5.5 ± 1.8 mV, $n = 4$). After 15 min exposure to scorpion venom the ACh-induced hyperpolarization was almost completely blocked (6.2% of control, $n = 4$) whereas that induced by BRL 38227 was still 53.6% of control amplitude ($n = 4$, Table 1).

Effects of glibenclamide and scorpion venom in the rabbit coronary artery

Since the effects of glibenclamide on membrane potential responses in the guinea-pig coronary artery (present study) differed from the actions of this drug in the cerebral artery (Brayden, 1990) we also performed several experiments in the rabbit coronary artery to determine whether the insensitivity to glibenclamide was a more general characteristic of ACh-induced responses in the coronary vasculature. Glibenclamide (35 μM) did not block either the ACh (10 μM)-induced relaxation ($n = 3$) or hyperpolarization ($n = 3$) in the rabbit coronary artery, suggesting that this may be a general characteristic of the actions of ACh in coronary vessels (Figure 5a). As observed in the guinea-pig coronary artery, scorpion venom (8.7 μg ml^{-1}) blocked the ACh (10 μM) but not the BRL 38227 (1 μM)-induced relaxation in the rabbit coronary vessel (Figure 5b, $n = 3$).

To ensure that the actions of scorpion venom on ACh-induced relaxation were not due to antagonism of muscarinic receptors we also tested the efffects of scorpion venom (8.7 μg ml^{-1}) on the ACh (10 μM)-induced contraction which can be evoked in this tissue. ACh (10 μM) initiated a 1.2 ± 1.19 g ($n = 3$) contraction when added to endothelium-denuded vessels in the absence of AEP. The response to ACh following a 30 min exposure to scorpion venom (8.7 μg ml^{-1}) was not reduced in magnitude ($n = 3$) suggesting that scorpion venom does not block muscarinic receptors at this concentration. In 1 of 3 vessels, scorpion venom elicited a transient contraction (0.2 g).

Table 1 Effect of K^+ channel blockers on the hyperpolarization induced by acetylcholine and BRL 38227

	Resting E_m (mV)	*Acetylcholine* (0.35 μM) *hyperpolarization* (mV)	*BRL 38227* (1 μM) *hyperpolarization* (mV)
Control	52.4 ± 1.5	17.7 ± 1.1	19.2 ± 1.6
	Amplitude depolarization (mV)	*Acetylcholine hyperpolarization* (mV) {% of control}	*BRL 38227 hyperpolarization* (mV) {% of control}
Phencyclidine (35 μM)	8.4 ± 1.7	5.7 ± 2.6 {32.2}	3.0 ± 1.0 {15.6}
Tetraethylammonium (10 mM)	10.5 ± 0.9	4.0 ± 0.5 {22.5}	20.7 ± 2.3 {107.8}
Glibenclamide (10 μM)	8.8 ± 1.8	17.2 ± 2.6 {97.1}	0 ± 0 {0}
Scorpion venom (8.7 μM)	5.5 ± 1.8	1.1 ± 0.8 {6.2}	10.3 ± 4.2 {53.6}

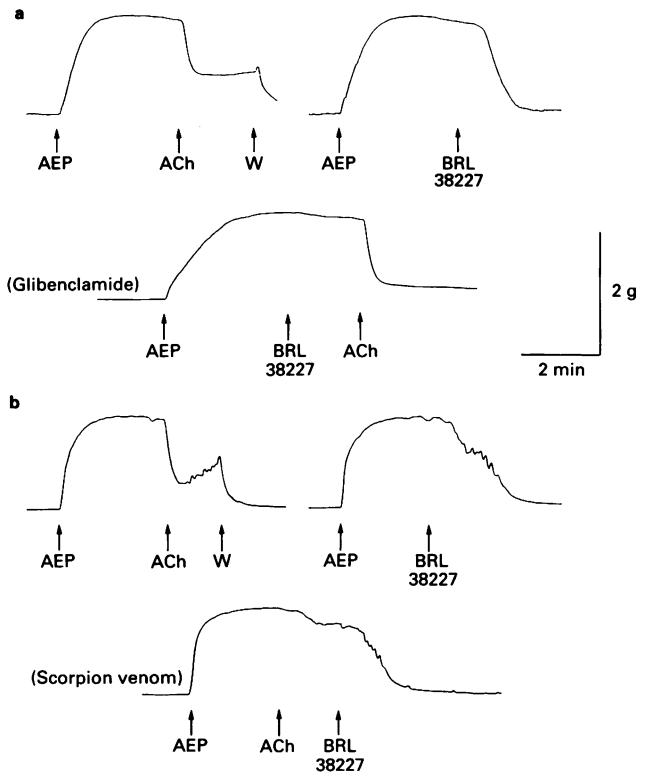

Figure 5 Comparison of the effects of (a) glibenclamide (35 μM) and scorpion venom (8.7 μg ml^{-1}) on either acetylcholine (ACh, 10 μM)- or BRL 38227 (1 μM)-induced relaxations in isolated segments of the rabbit coronary artery. Glibenclamide significantly reduced the BRL 38227-induced relaxation whereas the ACh-induced relaxation was unchanged. The opposite effect was observed with scorpion venom, i.e., scorpion venom inhibited the relaxation elicited with ACh, but was without significant effect on the response to BRL 38227.

Discussion

The present study has shown that both ACh and BRL 38227 can produce complete relaxation of isolated segments of the guinea-pig coronary artery when contracted with the histamine H_1-receptor agonist, AEP and that both are capable of eliciting a large hyperpolarization. Although the responses to ACh and BRL 38227 superficially resemble one another, the differential inhibitory effects of several different K^+ channel blockers on these vasodilators suggests that the mechanism by which they elicit relaxation and hyperpolarization differs in important ways.

The results of the present study indicate that the ACh (0.35 μM)-induced hyperpolarization occurs by a glibenclamide (10 μM)-insensitive mechanism in both the guinea-pig and rabbit coronary artery. These data are in agreement with some studies (McPherson & Angus, 1991; Chen *et al.*, 1991) but differ from the results in rabbit middle cerebral artery (Standen *et al.*, 1989; Brayden, 1990). There are several possible reasons for the differences including: (1) a different hyperpolarizing factor released from the two kinds of blood vessels; (2) the same hyperpolarizing factor affecting different K^+ channels on the smooth muscle cells or (3) a glibenclamide-sensitive release mechanism in the endothelial cells of the cerebral artery. Further research is required to determine which of these possibilities is correct. In contrast to ACh responses, glibenclamide was very effective in blocking both the relaxation and hyperpolarization produced with BRL 38227. Such data indicate that glibenclamide-sensitive channels are present in coronary artery even though this pathway is not affected by factors which ACh releases from the endothelium.

Much evidence links the relaxant actions of ACh in other blood vessels to the release of endothelium-derived relaxing factor (EDRF) which stimulates guanylate cyclase activity leading to a reduction in free intracellular calcium concentration (see Furchgott & VanHoutte, 1989). However, in tissues in which ACh also initiates hyperpolarization, an additional mechanism which must be considered is a decrease in the open probability of voltage-dependent L-type Ca^{2+} channels. This mechanism has been proposed by others for a variety of different vasodilators (e.g., Weir & Weston, 1986b; Meisheri *et al.*, 1988; Standen *et al.*, 1989; Brayden *et al.*, 1991). L-type Ca^{2+} channels have recently been characterized in single cells of the guinea-pig and rabbit coronary arteries (Ganitkevich & Isenberg, 1990; Matsuda *et al.*, 1990). Relaxation with ACh may therefore involve two concurrent mechanisms i.e., stimulation of guanylate cyclase activity and a decrease in the open probability of voltage-dependent Ca^{2+} channels. Evidence for this hypothesis has recently been reported in studies of rat femoral veins (Nagao & VanHoutte, 1991).

Unlike ACh, BRL 38227 does not stimulate guanylate cyclase activity in vascular smooth muscle (Coldwell & Howlett, 1987; Taylor *et al.*, 1988). Thus the hyperpolarization produced by BRL 38227 suggests that the associated relaxation involves a reduction in the open probability of voltage-dependent Ca^{2+} channels. The fact that glibenclamide and PCP block not only the hyperpolarization but also the relaxation initiated with BRL 38227 also suggests that the former event is causally related to the latter one.

An interesting observation made in this study is that addition of glibenclamide alone (1–35 μM) led to depolarization (8–12 mV) of the guinea-pig coronary artery. This result is in agreement with studies of rat mesenteric arteries in which glibenclamide produced a concentration-dependent (0.1–3.5 μM) depolarization (1–9 mV) of the tissue (McPherson & Angus, 1991) but differs from the rabbit cerebral artery where depolarization was not observed with glibenclamide at concentrations up to 5 μM (Brayden, 1990). In the rat mesenteric arteries, glibenclamide-induced depolarization was obtained both in the presence and absence of the endothelium. Glibenclamide may depolarize the coronary and mesenteric vessels because K^+ channels sensitive to this agent are open at the resting membrane potential. In pancreatic β cells, much lower concentrations of glibenclamide (i.e., 0.1 to 10 nM) are required to antagonize ATP-dependent K^+ channels (Sturgess, 1985; Schmid-Antomarchi *et al.*, 1987a,b) than are required in vascular smooth muscle to block the actions of cromakalim (Beech & Bolton, 1989; Buckingham *et al.*, 1989; Winquist *et al.*, 1989; Standen *et al.*, 1989), BRL 38227 (Wilson *et al.*, 1989) or diazoxide (Winquist *et al.*, 1989). It is possible that the higher concentrations of glibenclamide needed to antagonize the BRL 38227 response in blood vessels might also affect other K^+ channels in some way. However, if such a mechanism were occurring it could not be entirely nonspecific because glibenclamide apparently does not affect the K^+ channels involved in ACh-induced hyperpolarization.

The ability of other K^+ channel blockers to inhibit relaxation and hyperpolarization with BRL 38227 and ACh also differed. TEA (10 mM), for example, significantly reduced the relaxation and hyperpolarization initiated with ACh in the coronary artery but was without effect on BRL 38227 responses. The relative insensitivity of BRL 38227 responses to TEA is compatible with the proposed actions of cromakalim on either ATP-dependent K^+ channels (Standen *et al.*, 1989; Nelson *et al.*, 1990) or delayed rectifier K^+ channels (Beech & Bolton, 1989) both of which are relatively insensitive to TEA as opposed to Ca^{2+} activated K^+ channels which are very sensitive to this agent (Beech & Bolton, 1989). The greater effectiveness of TEA in inhibiting the actions of ACh suggest that large conductance Ca^{2+} activated K^+ channels may be involved in the pathways by which ACh produces relaxation in this vessel. This hypothesis was also suggested by Chen *et al.* (1991).

While PCP antagonized both ACh and BRL 38227 responses, its action was greater on BRL 38227 than on ACh. The ability of PCP (100 μM) to almost completely block the BRL 38227-induced hyperpolarization is commensurate with its reported effects on outward currents in single cells of the portal vein (Beech & Bolton, 1989). In their study, 100 μM PCP produced complete abolition of the cromakalim currents but only a 25% reduction in Ca^{2+} activated K^+ channel activity. The effects of PCP on the ACh-induced relaxation and hyperpolarization on the other hand are more difficult to interpret since PCP has also been reported to act as a partial antagonist of muscarinic receptors (Albuquerque *et al.*, 1983). In spite of this additional action of PCP on muscarinic receptors, PCP produced greater antagonism of BRL 38227 responses than of ACh responses.

Apamin has been reported to block small conductance Ca^{2+} activated K^+ channels. These channels are coupled to activation of both purinoceptors and adrenoceptors in the gastrointestinal tract of some species (Hugues *et al.*, 1982; Weir & Weston, 1986a; Strong, 1990). In our studies we found that a large concentration of apamin (i.e., 3.5 μM) was without effect on the relaxant actions of either ACh or BRL 38227 suggesting that this channel is not involved in the pathway by which either vasodilator produces relaxation.

Scorpion venom selectively antagonized ACh- but not BRL 38227-induced relaxations in both the guinea-pig and rabbit coronary artery. These data are similar to the differential effects of scorpion venom on ACh but not BRL 34915 in rat mesenteric arteries (Adeagbo & Malik, 1990). The actions of scorpion venom were not due to antagonism of muscarinic receptors since scorpion venom did not block ACh-induced contractions in segments of endothelium-denuded rabbit coronary artery. Charybdotoxin, a relatively specific blocker of large conductance Ca^{2+} activated K^+ channels (Miller *et al.*, 1985) is present in crude scorpion venom (see Strong, 1990). The actions of scorpion venom may therefore have been related to this component of the crude mixture. However, the concentration of charybdotoxin estimated to be present in our studies (i.e., 2–4 nM assuming 0.1–0.2% of total protein content, Strong, 1990) is somewhat less than the K_d reported for the inhibition of Ca^{2+} channels in patch clamp studies (e.g., 10 nM, Miller *et al.*, 1985). Since other K^+ channel blockers are also present in crude scorpion venom (see Strong, 1990), it is entirely possible that inhibition was due to a component of the crude extract other than charybdotoxin.

The present study has shown that BRL 38227 and ACh produce equivalent hyperpolarization at concentrations which elicit equivalent relaxation making it likely that membrane potential-dependent mechanisms contribute equally to the relaxations which are observed. In contrast to this similarity, the results suggest that these agonists initiate hyperpolarization by different mechanisms. Whereas the inhibitory effects of glibenclamide on the BRL 38227-induced hyperpolarization are compatible with the activation of ATP-dependent K^+ channels, the actions of ACh do not appear to include this channel. The effects of various K^+ channel blockers on responses to ACh suggest instead that some step in the sequence of events occurring between the initial binding of ACh to endothelial muscarinic receptors and the final relaxation of the smooth muscle depends upon the opening of Ca^{2+} activated K^+ channels.

Our appreciation to Drs J.R. Hume, C.H. Gelband and J.M. Post for reading this manuscript and offering helpful advice and to Dr J. Kenyon for many informative talks on K^+ channel blockers. We would also like to express our sincere gratitude to the Reno Eagles club for helping to make this study possible. This research was supported with NIH grant no. HL40399, a grant-in-aid from the AHA Nevada Affiliate and an award from the Reno Eagles club. Our appreciation to SmithKline Beecham for their generous donation of BRL 38227.

References

ADEAGBO, A.S.O. & MALIK, K.U. (1990). Endothelium-dependent and BRL 34915-induced vasodilatation in rat isolated perfused mesenteric arteries: role of G-proteins, K^+ and calcium channels. *Br. J. Pharmacol.*, **100**, 427–434.

ALBUQUERQUE, E.X., AGUAYO, L.G., WARNICK, J.E., ICKOWICZ, R.K. & BLAUSTEIN, M.P. (1983). Interactions of phencyclidine with ion channels of nerve and muscle: behavioral implications. *Fed. Proc.*, **42**, 2584–2589.

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.

BRAYDEN, J.E. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *Am. J. Physiol.*, **259** (*Heart. Circ. Physiol.* **28**), H668–H673.

BRAYDEN, J.E., QUALE, J.M., STANDEN, N.B. & NELSON, M.T. (1991). Role of potassium channels in the vascular response to endogenous and pharmacological vasodilators. *Blood Vessels*, **28**, 147–153.

BUCKINGHAM, R.E., CLAPHAM, J.C., COLDWELL, M.C., HAMILTON, T.C. & HOWLETT, D.R. (1986). Stereospecific mechanism of action of the novel antihypertensive agent, BRL 34915. *Br. J. Pharmacol.*, **87**, 78P.

BUCKINGHAM, R.E., HAMILTON, T.C., HOWLETT, F.R., MOOTOO, S. & WILSON, C. (1989). Inhibition by glibenclamide of the vasorelaxant action of cromakalim in the rat. *Br. J. Pharmacol.*, **97**, 57–64.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br.. J. Pharmacol.*, **95**, 1165–1174.

CHEN, G., YAMAMOTO, Y., MIWA, K. & SUZUKI, H. (1991). Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. *Am. J. Physiol.*, **260**, (*Heart Circ. Physiol*, **29**), H1888–H1892.

COLDWELL, M.C. & HOWLETT, D.R. (1987). Specificity of action of the novel anti-hypertensive agent, BRL 34915, as a potassium channel activator; comparison with nicorandil. *Biochem. Pharmacol.*, **36**, 3663–3669.

DAUT, J., MAIER-RUDOLPH, W., VON BECKERATH, N., MEHRKE, G., GUNTHER, K. & GOEDEL-MEINEN, L. (1990). Hypoxic dilation of coronary arteries is mediated by ATP-sensitive potassium channels. *Science*, **247**, 1341–1344.

DURANT, G.J., GANELLIN, C.R. & PARSONS, M.E. (1975). Chemical differentiation of histamine H_1 and H_2-receptor agonists. *J. Med. Chem.*, **18**, 905–909.

ECKMAN, D.M. & KEEF, K.D. (1991). Comparison of the actions of acetylcholine and BRL 38227 in the guinea pig coronary artery. *Biophys. J.*, **59** (2 part 2), 75a.

FELETOU, M. & VANHOUTTE, P.M. (1988). Nitric oxide, ACh and electrical and mechanical properties of canine arterial smooth muscle. *Am. J. Physiol.*, **255**, H207–H212.

FOSSET, M., DE WEILLE, J.R., GREEN, R.D., SCHMID-ANTOMARCHI, H. & LAZDUNSKI, M. (1988). Antidiabetic sulfonylureas control action potential properties in heart cells via high affinity receptors that are linked to ATP-dependent K^+ channels. *J. Biol. Chem.*, **263**, 7933–7936.

FURCHGOTT, R.F. & VANHOUTTE, P.M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.*, **3**, 2007–2018.

GANITKEVICH, V. & ISENBERG, G. (1990). Isolated guinea pig coronary smooth muscle cells: acetylcholine induces hyperpolarization due to sarcoplasmic reticulum calcium release activating potassium channels. *Circ. Res.*, **67**, 525–528.

GELBAND, C.H., LODGE, N.J. & VAN BREEMEN, C. (1989). A Ca^{++}-activated K^+ channel from rabbit aorta: modulation by. *Eur. J. Pharmacol.*, **167**, 201–210.

HAMILTON, T.C., WEIR, S.W. & WESTON, A.H. (1986). Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. *Br. J. Pharmacol.*, **88**, 103–111.

HU, S., KIM, H.S., OKOLIE, P. & WEISS, G.B. (1990). Alterations by glyburide of effects of BRL 34915 and P 1060 on contraction, ^{86}Rb efflux and the maxi-K^+ channel in rat portal vein. *J. Pharmacol. Exp. Ther.*, **253**, 771–777.

HUANG, A.H., BUSSE, R. & BASSENGE, E. (1988). Endothelium-dependent hyperpolarization of smooth muscle cells in rabbit femoral arteries is not mediated by EDRF (nitric oxide). *Naunyn-Schmiedbergs Arch. Pharmacol.*, **338**, 438–442.

HUGUES, M., DUVAL, D., SCHMID, H., KITABGI, P., LAZDUNSKI, M. & VINCENT, J.P. (1982). Specific and pharmacological interactions of apamin, the neurotoxin from bee venom with guinea pig colon. *Life Sci.*, **31**, 437–443.

KEEF, K.D. & KREULEN, D.L. (1988). Electrical responses of the guinea pig coronary artery to transmural stimulation. *Circ. Res.*, **62**, 585–595.

KEEF, K.D. & BOWEN, S.M. (1989). Effect of ACh on electrical and mechanical activity in guinea pig coronary arteries. *Am. J. Physiol.*, **257** (*Heart Circ. Physiol.*, **26**), H1096–H1103.

KITAMURA, K. & KURIYAMA, H. (1979). Effect of acetylcholine on the smooth muscle cell of isolated main coronary artery of the guinea pig. *J. Physiol.*, **293**, 119–133.

KLÖCKNER, U., TRIESCHMANN, U. & ISENBERG, G. (1988). Pharmacological modulation of calcium and potassium channels in isolated vascular smooth muscle cells. *Drug Res.*, **39**, 120–126.

KOMORI, K.R., LORENZ, R.R. & VANHOUTTE, P.M. (1988). Nitric oxide, ACh and electrical and mechanical properties of canine arterial smooth muscle. *Am. J. Physiol.*, **255** (*Heart Circ. Physiol.*, **24**), H207–H212.

MATSUDA, J.J., VOLK, K.A. & SHIBATA, E.F. (1990). Calcium currents in isolated rabbit coronary arterial smooth muscle myocytes. *J. Physiol.*, **427**, 657–680.

MCPHERSON, G.A. & ANGUS, J.A. (1991). Evidence that acetylcholine-mediated hyperpolarization of the rat small mesenteric artery does not involve the K^+ channel opened by cromakalim. *Br. J. Pharmacol.*, **103**, 1184–1190.

MEISHERI, K.D., CIPKUS, L.A. & TAYLOR, C.J. (1988). Mechanism of action of minoxidil sulfate-induced vasodilation: a role for increased K^+ permeability. *J. Pharmacol. Exp. Ther.*, **245**, 751–760.

MILLER, C., MOCZYDLOWSKI, E., LATORRE, R. & PHILLIPS, M. (1985). Charybdotoxin, a protein inhibitor of single Ca^{2+} activated K^+ channels from mammalian skeletal muscle. *Nature*, **313**, 316–318.

NAGAO, T. & VANHOUTTE, P.M. (1991). Hyperpolarization contributes to endothelium-dependent relaxations to acetylcholine in femoral veins of rats. *Am. J. Physiol.*, **261** (*Heart Circ. Physiol.*, **30**), H1034–H1037.

NELSON, M.T., PATLAK, J.B., WORLSEY, J.F. & STANDEN, N.B. (1990). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol.*, **259** (*Cell Physiol.*, **28**), C3–C18.

OKABE, K., KAJIOKA, S., NAKAO, K., KITAMURA, K., KURIYAMA, H. & WESTON, A.H. (1990). Actions of cromakalim on ionic currents recorded from single smooth muscle cells of the rat portal vein. *J. Pharmacol. Exp. Ther.*, **252**, 832–839.

SCHMID-ANTOMARCHI, H., DE WEILLIE, J.R., FOSSET, M. & LAZDUNSKI, M. (1987a). The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channel in insulin-secreting cells. *J. Biol. Chem.*, **262**, 15840–15844.

SCHMID-ANTOMARCHI, H., DE WEILLE, J.R., FOSSET, M. & LAZDUNSKI, M. (1987b). The antidiabetic sulfonylurea glibenclamide is a potent blocker of the ATP-modulated K^+ channel in insulin secretin cells. *Biochem. Biophys. Res. Commun.*, **146**, 21–25.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

STRONG, P.N. (1990). Potassium channel toxins. *Pharmacol. Ther.*, **46**, 137–162.

STURGESS, N.C., ASHFORD, M.L.J., COOK, D.L. & HALES, C.N. (1985). The sulfonylurea receptor may be an ATP-sensitive potassium channel. *Lancet*, **ii**, 474–475.

TALLARIDA, R.J. & MURRAY, R.B. (1987). Procedure 5, Analysis of the regression line. In *Manual of of Pharmacologic Calculations with Computer Programs*. pp. 16–18. New York: Springer-Verlag.

TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NEILD, T.O. & DUSTING, G.J. (1990). Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature*, **346**, 69–71.

TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NEILD, T.O. & DUSTING, G.J. (1991). Nitric oxide hyperpolarization arterial smooth muscle. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 3), S108–S112.

TAYLOR, S.G., SOUTHERTON, J.S., WESTON, A.H. & BAKER, J.R.L. (1988). Endothelium-dependent effects of acetylcholine in rat aorta: a comparison with sodium nitroprusside and cromakalim. *Br. J. Pharmacol.*, **94**, 853–863.

WEIR, S.W. & WESTON, A.H. (1986a). Effect of apamin on responses to BRL 34915, nicorandil and other relaxants in the guinea pig taenia caeci. *Br. J. Pharmacol.*, **88**, 113–120.

WEIR, S.W. & WESTON, A.H. (1986b). The effects of BRL 34915 and nicorandil on electrical and mechanical activity and on ^{86}Rb efflux in rat blood vessels. *Br. J. Pharmacol.*, **88**, 121–128.

WESTON, A.H. (1989). Smooth muscle K^+ openers; their pharmacology and clinical potential. *Pflügers Arch.*, **414** (Suppl. 1), S99–S105.

WILSON, C., HAMILTON, T.C. & CAWTHORNE, M.A. (1989). Antagonism of vasorelaxation to BRL 38227 by non-sulfonylurea hypoglycemic agents. *Br. J. Pharmacol.*, **98**, 723P.

WINQUIST, R.J., HEANEY, L.A., WALLACE, A.A., BASKIN, E.P., STEIN, R.B., GARCIA, M.L. & KACZOROWSKI, G.J. (1989). Glyburide blocks the relaxation response to BRL 34915 (cromakalim), minoxidil sulfate and diazoxide in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **248**, 149–156.

(Received August 2, 1991
Revised December 12, 1991
Accepted January 7, 1992)