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Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein

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1 The effects of the novel anti-hypertensive agent BRL 34915, (±) 6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol, have been compared with those of verapamil on rat isolated portal vein.

2 BRL 34915 produced a concentration-dependent reduction in mechanical responses to noradrenaline but had relatively little inhibitory effect on K^+-induced contractions. Verapamil reduced the magnitude of both noradrenaline and K^+-induced mechanical responses.

3 BRL 34915 delayed the appearance of the reduced noradrenaline contractions, a property not shared by verapamil.

4 BRL 34915 abolished spontaneous electrical and mechanical discharges and hyperpolarized the portal vein cells close to their calculated potassium equilibrium potential. Verapamil inhibited spontaneous electrical and mechanical discharges, effects associated with a small depolarization.

5 BRL 34915 produced a significant increase in the ^{86}Rb efflux rate coefficient whilst verapamil was without effect on this parameter.

6 The inhibitory effects of BRL 34915 were rapid in onset and readily reversible by washing, whilst those of verapamil were slower in onset and only slowly reversible.

7 It is concluded that the inhibitory effects of BRL 34915 in rat portal vein are produced by the opening of potassium channels in the smooth muscle cells. This inhibits spike activity and in sufficient concentration holds the membrane potential at or close to the potassium equilibrium potential, thereby reducing the effects of excitatory agents.

Introduction

BRL 34915, (±) 6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol, is one member of a novel series of benzopyran derivatives with anti-hypertensive properties in conscious animals (Ashwood *et al.*, 1984; Buckingham *et al.*, 1984b; 1986). In rat isolated portal vein, both BRL 34915 and nifedipine inhibit spontaneous mechanical activity, yet in contrast to nifedipine, BRL 34915 has been reported to exhibit little inhibitory activity against either noradrenaline or K^+-induced contractions (Buckingham *et al.*, 1984a).

The objective of the present study was to investigate further the mechanism of action of BRL 34915 on rat portal vein by comparing its actions with those of the calcium entry blocker, verapamil. A preliminary account of some of these results has been presented to the British Pharmacological Society (Hamilton *et al.*, 1985).

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Methods

All tissues were obtained from male Wistar rats (300–400 g) supplied by the Manchester University Animal Unit.

Tissue bath studies

Portal veins each approximately 2 cm in length, were mounted for isometric recording of tension changes as previously described (Jetley & Weston, 1980). After the initial equilibration period, responses of the veins

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to noradrenaline were investigated by constructing sequential concentration-effect curves. For KCl, cumulative concentration-effect curves were constructed, a 5 min contact time being used for each concentration. Noradrenaline-induced mechanical activity was quantified using integrators (Grass 7P10) by subtraction of the integrated mechanical activity obtained in the 2 min before exposure to noradrenaline from the value obtained during the first 2 min of exposure to noradrenaline. A similar procedure was used to assess the mechanical effects of KCl except that 5 min integration periods were used.

Modifying drugs (BRL 34915, phentolamine and verapamil) were allowed 30 min equilibration with the tissues, after which responses to the spasmogen were re-examined in the continuing presence of the modifying agent. Concurrent time-matched control tissues were exposed to the appropriate vehicle.

Electrical recording

For extracellular recording, the perfused capillary method described by Jetley & Weston (1980) was used. The electrical and mechanical signals were each quantified by use of integrators (Grass 7P10) and the effect of noradrenaline was measured using 2 min integration periods as described above. The intracellular recording method used was that described by Small & Weston (1980) with the addition of simultaneous calculation of electrical dV/dt using a differentiator (Grass 7P20). Noradrenaline and the modifying drugs verapamil and BRL 34915 were added to calibrated reservoirs supplying physiological salt solution (PSS) to the tissue to achieve the desired concentration.

^{86}Rb efflux

In these experiments ^{86}Rb was used as a K^+ marker (Imaizumi & Watanabe, 1981; Bolton & Clapp, 1984). The portal veins were removed from four rats and each assigned to one of four experimental groups:- (1) BRL 34915; (2) verapamil; (3) solvent (ethanol) control; (4) PSS control. Each vein was impaled on a syringe needle attached to a perspex gassing manifold and then inserted into a test-tube containing 5 ml PSS at 37°C bubbled with 100% 0_2 via the needle. Portal veins from a further 12 rats were similarly assigned to the four experimental groups so that sixteen veins were handled simultaneously.

After a 30 min equilibration period in PSS, tissues were loaded with ^{86}Rb, 1μCi ml^{-1} for 80 min after which the ^{86}Rb was allowed to efflux from the tissues by transferring them to tubes containing 5 ml PSS alone for 13 successive 2 min periods. After 7 such periods (14 min into the efflux), the PSS into which the tissues were transferred contained the following additions for the next four collection periods:- group (1) BRL 34915, 5×10^{-6} M; group (2) verapamil, 10^{-6} M; group (3) 0.035 μl 7% v/v ethanol; group (4) no addition. For the remaining 2 min periods, the tubes contained PSS alone.

At the end of the efflux experiment, 1 ml aliquots of PSS were added to 4 ml Rialuma (Lumac) scintillation mixture and counted for radioactivity. Each vein was blotted and weighed (range 6–12 mg) and digested overnight in 0.5 ml Soluene (Packard): 4 ml Lumagel scintillation mixture (LKB) and 0.5 ml 0.5 N hydrochloric acid was then added to the resulting solution and the mixture counted for radioactivity. The efflux data were expressed in terms of the rate coefficient (fractional loss of ^{86}Rb from the tissue standardized for a 1 min period, expressed as a percentage).

Drugs and solutions/statistical analysis of results

Where KCl was used as a spasmogen the stated concentration excludes the KCl (5.9 mM) present in the physiological salt solution. The following drugs were used:- (±)-BRL 34915 (Beecham); MOPS (3-(N-morpholino)-propane sulphonic acid (Calbiochem), (−)-noradrenaline bitartrate monohydrate (Sigma); potassium chloride (Hopkin and Williams), phentolamine (Ciba) and verapamil (Knoll). The stock solution of BRL 34915 was prepared in 70% v/v ethanol:distilled water, noradrenaline was prepared in N/10 hydrochloric acid, other drugs in double distilled water. The MOPS PSS had the following composition ($\times 10^{-3}$ M): NaCl 129.7, KCl 5.9, $CaCl_2$ 2.54, $MgCl_2$ 1.19, MOPS 10, glucose 11.1. The pH was adjusted to 7.4 with NaOH.

The significance of differences between means was assessed by means of a two-tailed unpaired t test.

Results

Tissue bath experiments

BRL 34915 ($0.1-5 \times 10^{-6}$ M) abolished the characteristic spontaneous mechanical activity in portal vein within about 1–2 min and in this concentration range produced a significant, concentration-dependent reduction in mechanical responses to noradrenaline (Figure 1). In these concentration-effect experiments there was evidence that BRL 34915 delayed the onset of the noradrenaline contraction. However, this was difficult to quantify and the phenomenon is described later in Results. The responses to noradrenaline were mediated by α-adrenoceptors, the pA_2 value of phentolamine against noradrenaline being 8 ± 0.08 (mean ± s.e.mean, $n = 4$).

BRL 34915 also produced a significant concentration-dependent reduction of responses to added K^+ (Figure 1). However, the characteristics of the inhibi-

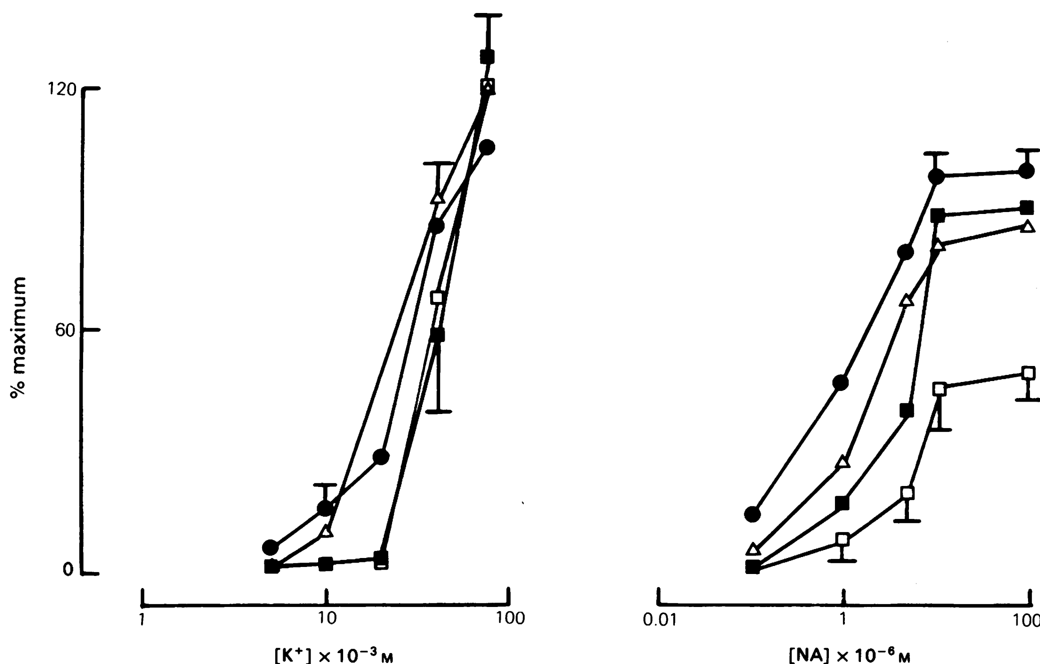

Figure 1 Effect of BRL 34915 on mechanical responses to K^+ and to noradrenaline (NA) measured by integrating the mechanical activity of rat portal vein. Control responses (●); responses in the presence of BRL 34915 0.1 × 10^{-6} M (△); 0.5 × 10^{-6} M (■); 5 × 10^{-6} M (□). Ordinate scale: % of the initial control maximum response. Each point is the mean derived from 4–6 experiments; vertical lines show sample s.e.mean values.

tion of K^+-induced responses were different from those seen when noradrenaline was the agonist. In portal vein, BRL 34915 was most effective against K^+ concentrations in the range 5–20 × 10^{-3} M, resulting in a characteristic flattening of part of the K^+ concentration-effect curve. There was no evidence of a BRL 34915-induced delay in the mechanical responses to K^+ which were themselves unaffected by phentolamine (up to 3 × 10^{-7} M). The inhibitory actions of BRL 34915 on both the noradrenaline and K^+ responses were readily reversible by washing.

Verapamil (0.01–1 × 10^{-6} M) reduced the amplitude but increased the frequency of spontaneous contractions in rat portal vein, effects which took up to 30 min to become fully established. Verapamil (0.01–1 × 10^{-6} M) also produced a significant, concentration-dependent reduction in mechanical responses to noradrenaline (Figure 2). In the same concentration-range, verapamil inhibited responses to added K^+ (Figure 2). The inhibitory effects of verapamil were impossible to reverse fully by washing.

Effects on electrical and mechanical activity

By use of extracellular recording, BRL 34915 (0.1–5 × 10^{-6}M) was found to produce a rapid and complete inhibition of spontaneous electrical and mechanical activity within 1–2 min. In this quiescent state, noradrenaline (10^{-6} M, an approximate EC_{50}) was able, after a time delay, to evoke fast electrical activity and accompanying mechanical changes (Figure 3). Quantification with integrators showed that BRL 34915 produced a parallel reduction in this noradrenaline-induced electrical and mechanical activity with no evidence of an uncoupling action. The delay in the appearance of the responses to noradrenaline, 10^{-6}M, was also measured and found to be dependent on the concentration of BRL 34915. In the presence of BRL 34915, 0.1 × 10^{-6} M, 0.5 × 10^{-6} M and 5 × 10^{-6}M, this delay was 0 s, 18.8 ± 3.5 s and 44.2 ± 2 s respectively (n = 6).

Verapamil (0.01–1 × 10^{-6} M) reduced, but did not abolish, the spontaneous electrical and mechanical discharges of portal vein. In this concentration-range it also produced a parallel reduction in the electrical and mechanical activity evoked by noradrenaline, 10^{-6} M, with no evidence of an uncoupling action (Figure 4). In the presence of verapamil there was no delay in the appearance of the reduced responses to noradrenaline.

The effects of BRL 34915 and verapamil were further examined by use of microelectrodes. The

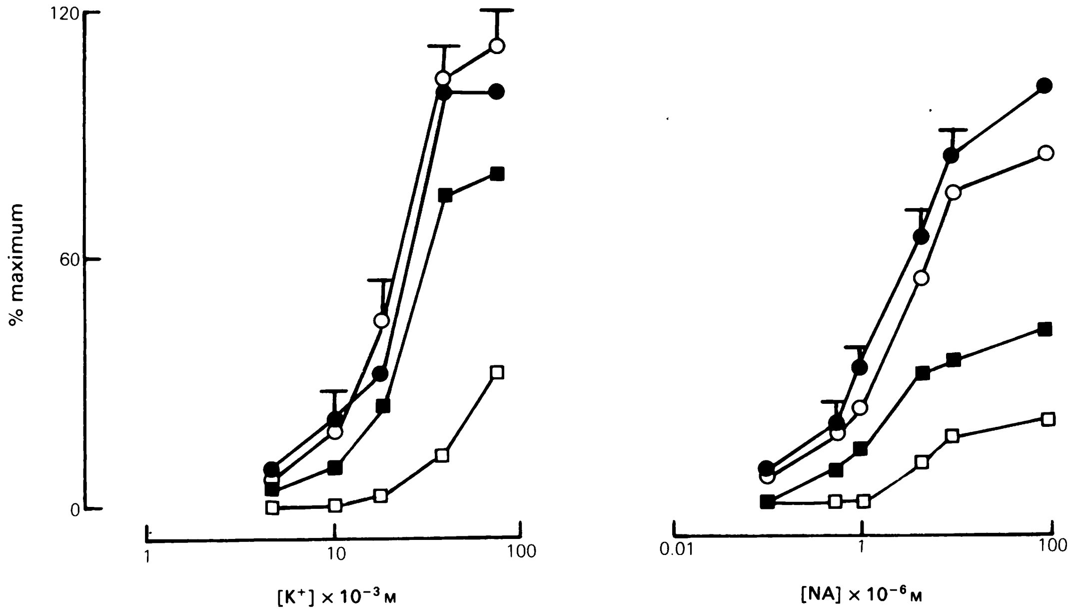

Figure 2 Effect of verapamil on mechanical responses to K^+ and to noradrenaline (NA) measured by integrating the mechanical activity of rat portal vein. Control responses (●); responses in the presence of verapamil 0.01×10^{-6} M (○); 0.1×10^{-6} M (■); 10^{-6} M (□). Ordinate scale: % of the initial control maximum response. Each point is the mean derived from 4–6 experiments; vertical lines show sample s.e.mean values.

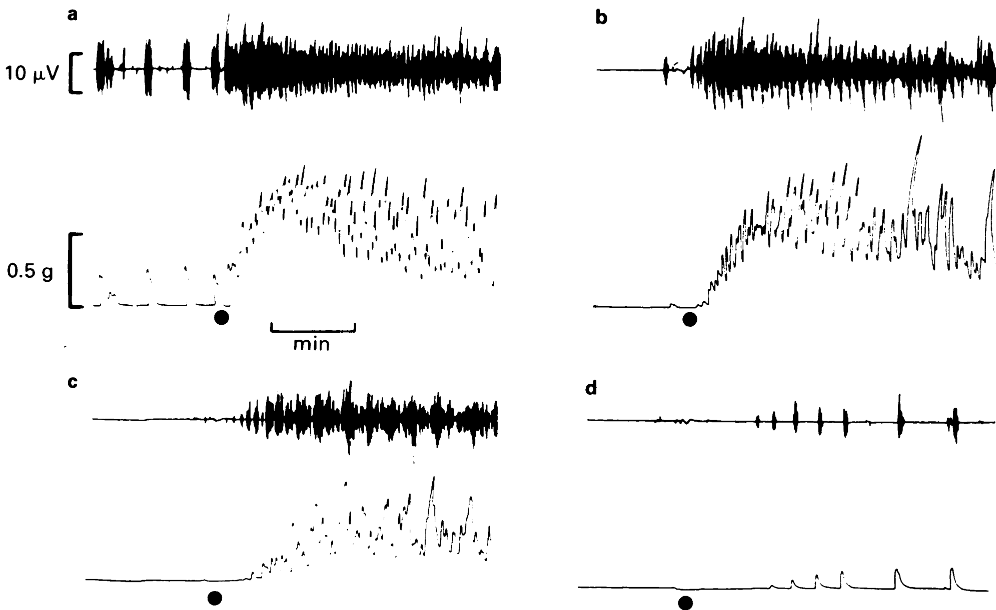

Figure 3 Effect of BRL 34915 on extracellular electrical activity (upper traces) and mechanical activity (lower traces) in a single experiment in rat portal vein. Responses to noradrenaline 10^{-6} M (●) in (a) control and after 30 min exposure to BRL 34915 (b) 0.1×10^{-6} M; (c) 0.5×10^{-6} M; (d) 5×10^{-6} M.

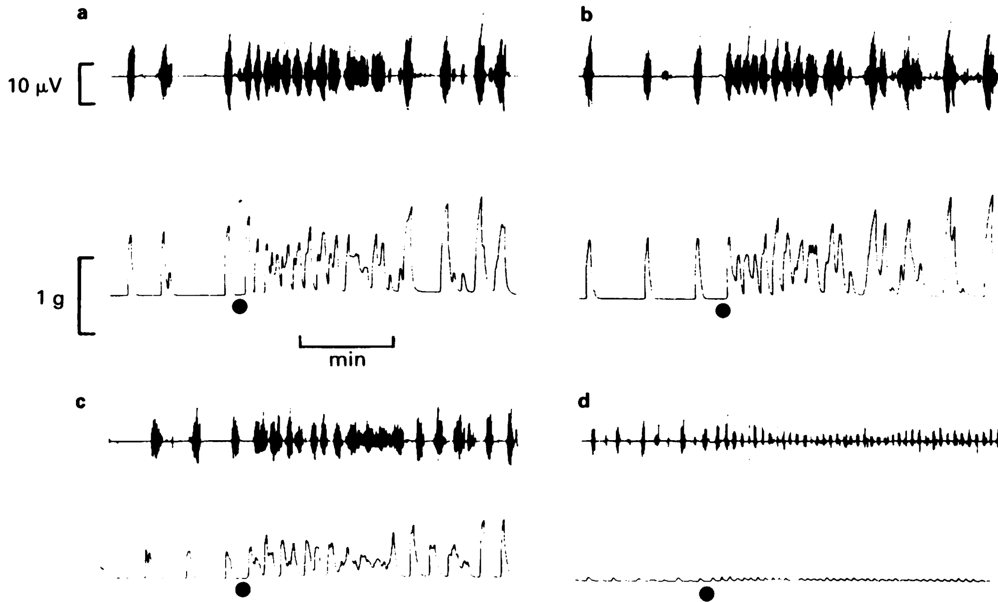

Figure 4 Effect of verapamil on extracellular electrical activity (upper traces) and mechanical activity (lower traces) in a single experiment in rat portal vein. Responses to noradrenaline 10^{-6} M (●) in (a) control and after 30 min exposure to verapamil (b) 0.01×10^{-6} M; (c) 0.1×10^{-6} M; (d) 10^{-6} M.

resting membrane potential of rat portal vein was -59 ± 0.3 mV ($n = 36$). In five tissues it was possible to carry out a concentration effect experiment with a microelectrode remaining in the cell during washout and re-exposure to BRL 34915. To achieve this, quite high degrees of stretch had to be used in the region of microelectrode impalement, often resulting in imperfect coupling between the electrical events recorded from a single cell and the mechanical changes developed by the whole vein. At 0.1×10^{-6}M, BRL 34915 abolished the generation of the multispike complexes characteristic of rat portal vein without detectable hyperpolarization. However, as the concentration of BRL 34915 was increased from 0.5×10^{-6}M to 5×10^{-6} M, a marked hyperpolarization was observed (Figure 5, Table 1). Although the speed of onset of the effect of BRL 34915 together with the discontinuous nature of the multispike complexes made it difficult to be certain, there appeared to be no effect of the drug on spike dV/dt.

Exposure to noradrenaline during a BRL 34915-induced hyperpolarization produced a slow reversal of the hyperpolarization to the firing threshold (Figure 6) and the time taken for this corresponded well with the delay times measured in the extracellular capillary experiments. Spikes evoked in the presence of both noradrenaline, 10^{-5} M, and BRL 34915, 0.5×10^{-6} M, had dV/dt values which were not different from those of spikes produced in the presence of noradrenaline, 10^{-5} M, alone. The overall depolarization produced by noradrenaline in the presence of BRL 34915 was less than in the absence of BRL 34915 (Figure 6).

Verapamil (10^{-6} M) produced a small depolarization of 4 ± 1 mV ($n = 4$) which took 20–30 min to develop fully. Spike dV/dt in the presence of verapamil was greatly reduced. In the presence of verapamil 10^{-6} M, noradrenaline, 10^{-5} M produced an initial burst of modified spikes, the dV/dt of which was much less than that produced by noradrenaline alone. The degree of depolarization produced by noradrenaline was, however, little affected by verapamil.

^{86}Rb efflux experiments

The average basal ^{86}Rb efflux rate coefficients measured between the 14th and 22nd min of the efflux period were $1.29 \pm 0.12\%$ min^{-1} and $1.33 \pm 0.09\%$ min^{-1} in PSS alone and PSS + ethanol respectively ($n = 8$; Figure 7). These values were not significantly different. BRL 34915 significantly increased the ^{86}Rb efflux rate coefficient to $2.91 \pm 0.17\%$ min^{-1} ($n = 8$) whilst in the presence of verapamil, 10^{-6} M, no sig-

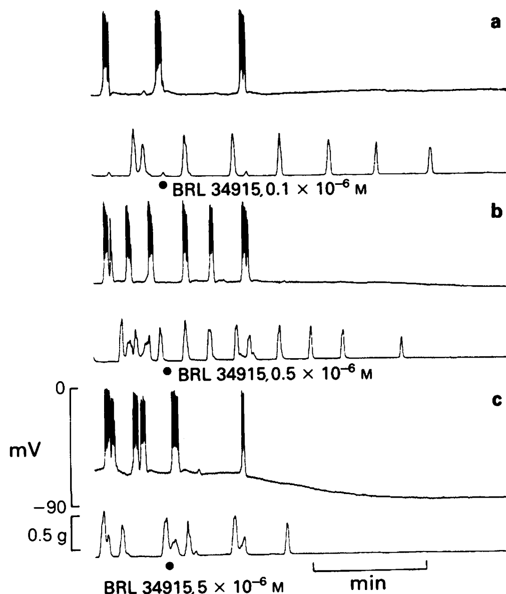

Figure 5 The effect of exposure to BRL 34915 (●) (a) 0.1×10^{-6} M; (b) 0.5×10^{-6} M; (c) 5×10^{-6} M on membrane potential (upper traces) and tension (lower traces) in a single experiment in rat portal vein. The traces are parts of a continuous impalement of a single cell with washout of BRL 34915 and recovery between segments (a) and (b), (b) and (c). For further details, see Table 1.

Table 1 Effect of BRL 34915 on resting membrane potential in rat portal vein

BRL 34915 ($\times 10^{-6}$ M)	*Hyperpolarization* (mV)	*Membrane potential* (mV)
0.1	0 ± 0.94	-59.2 ± 1.5
0.5	10.4 ± 1.2	-68.8 ± 1.2
5.0	28.1 ± 1.0	-88.2 ± 1.1

Mean values ± s.e.mean, $n = 5$ are shown. For further details, see text.

nificant changes in ^{86}Rb loss were detected (Figure 7).

Discussion

The results obtained in the present study suggest that the mechanism of the inhibitory action of BRL 34915 in rat portal vein is quite different from that of the calcium entry blocker, verapamil. In low concentrations, BRL 34915 was able to inhibit spontaneous electrical and mechanical activity both rapidly and reversibly. Noradrenaline-induced contractions were also inhibited and this action was characterized by a delay of many seconds in the appearance of the contraction, a property not shared by verapamil. The slope of the inhibitory concentration-response relationship to BRL 34915 against noradrenaline was much steeper than that to verapamil and furthermore, BRL 34915 was relatively ineffective at inhibiting the mechanical activity induced by K^+.

An important indication of the mode of action of BRL 34915 was obtained from the microelectrode studies in which the drug produced a concentration-dependent hyperpolarization of the cells of the portal vein. Using the value of either 163×10^{-3} M (Haljamaë *et al.*, 1970) or 198×10^{-3} M (Wahlström, 1973) for the intracellular K^+ concentration $[K^+]_i$ of rat portal vein, the equilibrium potential for K^+ (K_{eq}) at 37°C can be calculated using the Nernst equation to lie between −89 mV and −94 mV in the PSS used in the present study. Thus the ability of BRL 34915 to raise the membrane potential of the portal vein close to the K_{eq} suggests most strongly that the drug is able to open a group of potassium channels which are essentially closed at the resting membrane potential. Such a view is confirmed by the radioisotope experiments in which BRL 34915 produced a significant increase in the fractional loss of ^{86}Rb from the tissues. It must be remembered, however, that the spontaneous electrical discharges in the portal vein were inhibited by low concentrations of BRL 34915 without detectable hyperpolarization. This might suggest an initial action on potassium channels involved in spike repolarization with a further action at higher concentrations on additional potassium channels to produce the observed hyperpolarization. Alternatively only a single channel could be involved.

The microelectrode studies have also shown that the delay in the appearance of the noradrenaline contraction is a consequence of the time taken to reverse the hyperpolarizing effect of BRL 34915. Such a delay is surprising since the interaction of noradrenaline with the adrenoceptor would be expected to produce rapid opening of depolarizing ion channels. It is thus possible that the BRL 34915-induced hyperpolarization reduces the probability of the opening of these channels, although further work is necessary to test this suggestion.

The depolarization itself produced by noradrenaline probably results from the opening of α-adrenoceptor-operated sodium channels, since its magnitude was little affected by verapamil 10^{-6} M. This concentration of the voltage-operated calcium channel inhibitor abolished noradrenaline-induced calcium influx into the portal vein (Weir & Weston, unpublished observations), thus excluding a possible role of α-adrenoceptor-operated calcium channels.

Verapamil was a potent inhibitor of the contrac-

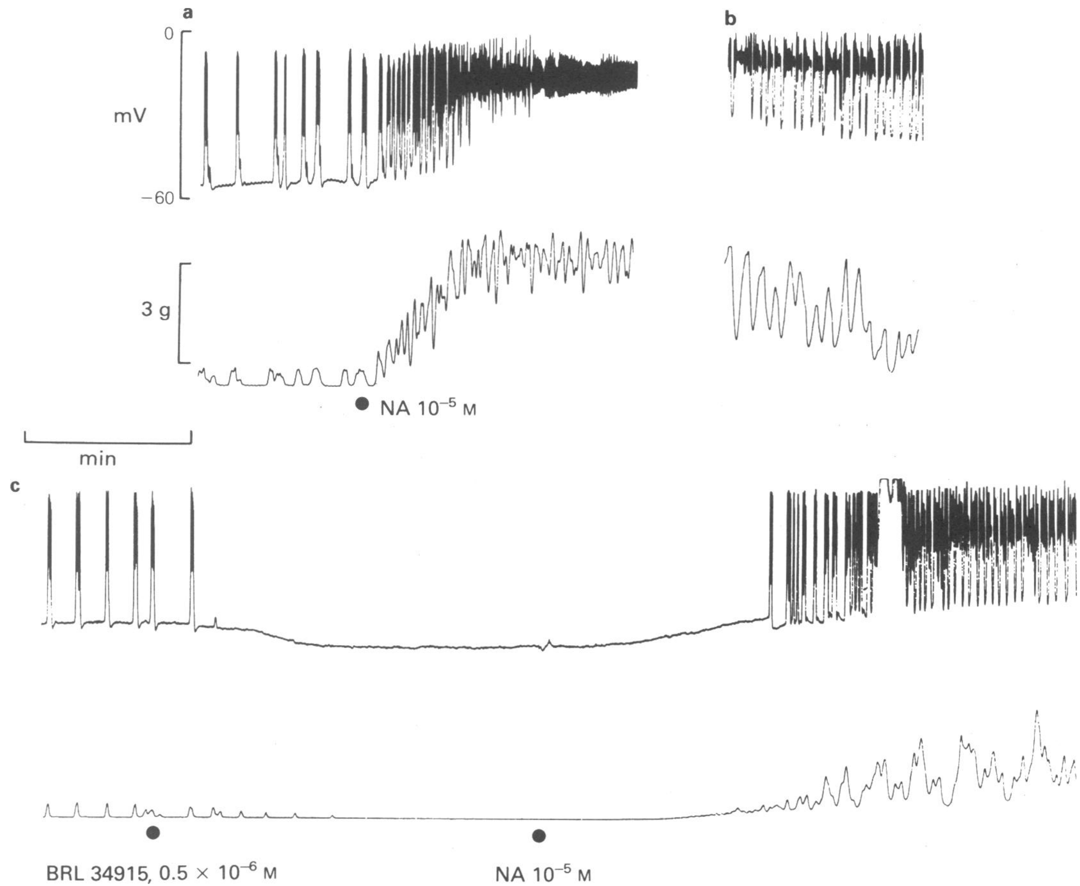

Figure 6 The effect of noradrenaline (NA) on membrane potential (upper traces) and tension (lower traces) in a single experiment in rat portal vein. (a) Effect of NA 10^{-5}M; (b) washout of NA starting approximately 1 min after (a); (c) effect of BRL 34915, 0.5×10^{-6} M and subsequent exposure to NA 10^{-5} M in the continuing presence of BRL 34915. Parts of a continuous intracellular recording up to the break in the record in (c) after which re-impalement was made.

tions produced by addition of K^+ to the PSS, an action reflecting its ability to block the voltage-operated calcium channels activated by the K^+-induced depolarization. In contrast BRL 34915 was relatively impotent in this respect, producing inhibition of responses to added 5, 10 and 20×10^{-3} M K^+, with little effect on added 40 and 80×10^{-3} M K^+. Using the Nernst equation and the values for $[K^+]_i$ given by Haljamaë *et al.* (1970) and Wahlström (1973), the K_{eq} values corresponding to the addition of 5, 10, 20, 40 and 80×10^{-3} M K^+ are −72/−77, −62/−67, −49/−54, −34/−39 and −17/−22 mV respectively. Thus if BRL 34915 is able to open a group of potassium channels, prior exposure to this drug followed by an increase in the extracellular K^+ concentration would tend to change the membrane potential to one of the K_{eq} ranges given above. The ability of BRL 34915 to inhibit responses to added 20×10^{-3} M K^+ (K_{eq}, −49/−54 mV) yet have little effect on 40×10^{-3} M K^+ (K_{eq}, −34/−39 mV) therefore suggests that the voltage-operated calcium channel in portal vein is activated at a membrane potential which lies somewhere between these ranges.

Support for the suggestion that BRL 34915 can open potassium-channels can be derived from the preliminary observations of Cain & Metzler (1985) in guinea-pig papillary muscle. In this tissue, BRL 34915 produced a marked shortening of the duration of the cardiac action potential, an effect consistent with an action on cardiac potassium channels.

The results of the present experiments clearly suggest that the blood pressure-lowering action of

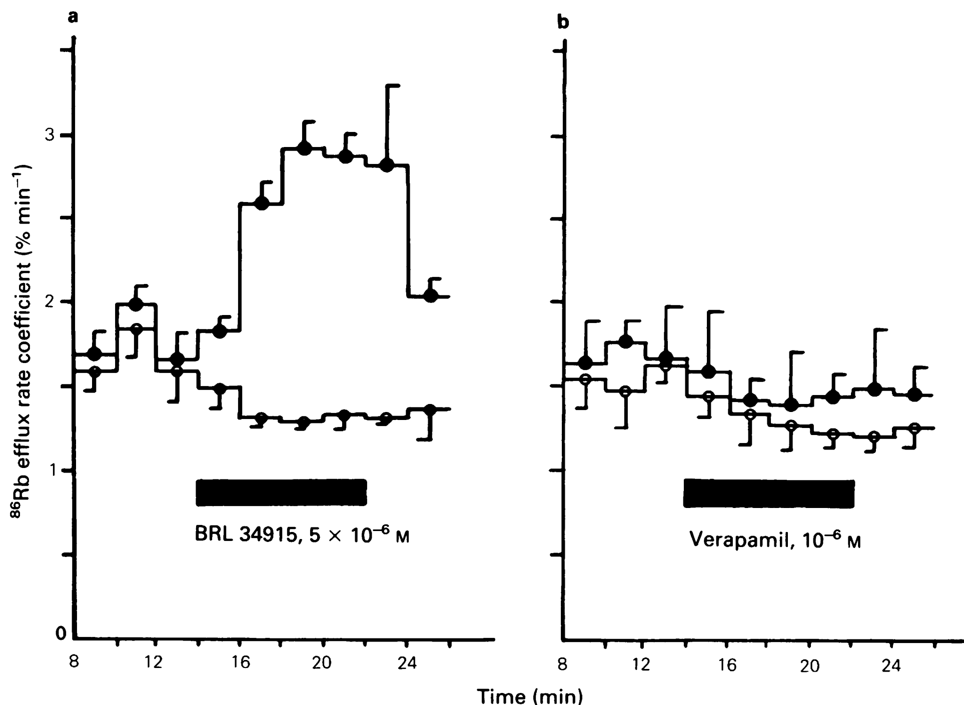

Figure 7 Effect of (a) BRL 34915, 5 × 10^{-6} M (●) and 0.035% v/v ethanol (○); (b) verapamil, 10^{-6} M (●) and PSS alone (○) on the loss of ^{86}Rb from rat portal vein. Ordinate Scale; ^{86}Rb efflux rate coefficient expressed as a percentage min^{-1}. Abscissal scale: time (min) after start of the efflux period. Tissues were exposed to modifying conditions between the 14th and 22nd min of the efflux period (■). Each point is the mean derived from 8 experiments; vertical bars show s.e.mean values.

BRL 34915 is a consequence of its ability to hold the membrane potential of vascular smooth muscle cells close to their K_{eq}. The subsequent inhibition of responses to pressor agents is thus the result of physiological antagonism, the depolarizing action of a pressor substance being effectively short-circuited by the potassium channel-opening properties of BRL 34915.

From this analysis, it might appear that BRL 34915 should be capable of inhibiting all types of smooth muscle cell since even if the resting membrane potential lies at the K_{eq}, the membrane shunt produced by the opening of potassium-channels should inhibit the effects of stimulatory agents. However, our knowledge of smooth muscle potassium-channels is relatively limited and already several types have been described (Bolton *et al.*, 1985). Their distribution is not homogeneous and some may be insensitive to BRL 34915. Thus, the inhibitory actions of this drug may be restricted to those tissues in which membrane depolarization plays a significant role in excitation processes. However, the powerful anti-hypertensive action of BRL 34915 suggests that this possible limitation may be of little practical significance and its ability to reduce the resistance of renal vascular beds but not that of hind limbs (Buckingham *et al.*, 1984b; 1986) may in fact confer haemodynamic advantages on BRL 34915 in comparison with other anti-hypertensive drugs.

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