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# Effect of the $K^+$ efflux stimulating vasodilator BRL 34915 on $^{86}Rb^+$ efflux and spontaneous activity in guinea-pig portal vein

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**1** The effect of BRL 34915 on $^{86}Rb^+$ efflux and myogenic activity was studied simultaneously in guinea-pig portal vein. $^{86}Rb^+$ was used as a tracer ion for $K^+$.

**2** BRL 34915 inhibited myogenic activity with an $IC_{50}$ value of 12 ± 2 nM by reducing primarily the frequency of spontaneous contractions. Washout of the substance was followed by hyperreactivity of the vessel.

**3** $^{86}Rb^+$ efflux was slightly reduced by concentrations of BRL 34915 below 100 nM; above 300 nM efflux was increased in a concentration-dependent manner.

**4** Above 10 μM BRL 34915, a slow desensitization of the effect on flux was observed during the 10 min application period of the agonist.

**5** The $Ca^{2+}$ entry blocker, isradipine (PN 200-110, 200–500 nM) did not modify BRL 34915-stimulated $^{86}Rb^+$ efflux at any BRL 34915 concentration tested, indicating that the influx of extracellular $Ca^{2+}$ through dihydropyridine-sensitive $Ca^{2+}$ channels is not necessary for this effect. However, by abolishing spontaneous activity, it allowed the $^{86}Rb^+$ efflux promoting effect of BRL 34915 to be observed at a concentration of 60 nM.

**6** The $K^+$ channel blockers tetraethylammonium and 3,4 diaminopyridine inhibited the BRL 34915-induced $^{86}Rb^+$ efflux with $IC_{50}$ values of 13 and 3 mM, respectively.

**7** Cell permeable derivatives of cyclic AMP and cyclic GMP had no major effect on BRL 34915-induced $^{86}Rb^+$ flux, indicating that cyclic nucleotide-induced phosphorylation does not play an important modulatory role here.

**8** In conclusion, there is an at least 5 fold difference between the concentrations of BRL 34915 necessary to inhibit myogenic activity and those needed to stimulate $^{86}Rb^+$ efflux. This may be explained by a primary effect of BRL 34915 on the pacemaker cells of the portal vein.

## Introduction

The benzopyran derivative BRL 34915 ((±) 6-cyano-3,4-dihydro-2,2 dimethyl-*trans*-4-(2-oxo-1-pyrollidyl) -2H-benzo[b]pyran-3-ol) is a novel vasorelaxant agent now in clinical trials as an antihypertensive (Vanden-Burg *et al.*, 1986). An increase in the permeability of the smooth muscle cell membrane to $K^+$ has been proposed as its mechanism of action (Hamilton *et al.*, 1986; Weir & Weston, 1986 a,b). This is based on experiments in rat portal vein where BRL 34915, at concentrations of 0.5 μM and higher, hyperpolarized the smooth muscle membrane shifting the membrane potential in a concentration-dependent manner towards the Nernst equilibrium potential for $K^+$ (Hamilton *et al.*, 1986; Weir & Weston, 1986b). In the same concentration range the agent increased the $^{86}Rb^+$ efflux in the portal vein (Weir & Weston, 1986b; Hamilton *et al.*, 1986). In these experiments $^{86}Rb^+$ was used as a convenient substitute for $^{42}K^+$ which is an established procedure for measuring $K^+$ fluxes (see e.g. Imaizumi & Watanabe, 1981; Martin & Gordon, 1983; Bolton & Clapp, 1984). It was thus demonstrated that the observed hyperpolarization was due to a drug-induced increase in membrane permeability to $K^+$ and $^{86}Rb^+$.

The portal vein of rabbits, guinea-pigs and rats (for reviews see Ljung, 1970; Johansson & Somlyo, 1980; Jones, 1981) is a spontaneously active vessel where some smooth muscle cells (pacemaker cells) spontaneously generate bundles of action potentials which propagate over large parts of the vein. This is accom-

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panied by contractile spikes. Depolarization is induced by the influx of $Ca^{2+}$ and $Na^+$ and is insensitive to tetrodotoxin. The ionic event terminating electrical excitation (and thereby contraction) is a $K^+$ outward current through voltage and/or $Ca^{2+}$ dependent $K^+$ channels (for references see reviews by Johansson & Somlyo (1980) and Jones (1981)).

Simultaneous measurements were made of $^{86}Rb^+$ efflux and spontaneous activity in guinea-pig portal vein in order to study the concentration-dependence of the effects of BRL 34915 on either phenomenon under identical conditions. This seemed of interest as Hamilton *et al.* (1986) had observed inhibition of spontaneous activity with concentrations of BRL 34915 which did not appear to induce hyperpolarization.

The pharmacological profile of BRL 34915 was characterized by studying its effect in the presence of isradipine (PN 200-110), a potent $Ca^{2+}$ entry blocking dihydropyridine (Hof *et al.*, 1984 a,b), and in the presence of the known $K^+$ channel inhibitors tetraethylammonium (TEA) and 3,4-diaminopyridine (DAP). Finally, as it is known that many ion channels are modulated by phosphorylation (see Levitan, 1985 for a review), the influence of dibutyryl adenosine 3':5'-cyclic monophosphate (dibutyryl-cyclic AMP) and 8Br-cyclic GMP (membrane permeating derivatives of cyclic AMP and cyclic GMP respectively) on the effect of BRL 34915 was examined.

## Methods

### *Preparation of portal veins*

Guinea-pigs of either sex, weighing 400–600 g were stunned by a blow on the head and exsanguinated. The portal vein was exposed and attached at either end to a cotton thread. After removal of surrounding connective tissue it was cut along the length axis. The lumen was washed free of blood, the vessel excised and a tension of 500 mg was applied. The vein was then incubated for 30 min in a HEPES buffered physiological salt solution (PSS) gassed with 95% $O_2$ and 5% $CO_2$ at 37°C. The PSS contained (in mM): NaCl 120, KCl 5, $NaHCO_3$ 15, $NaH_2PO_4$ 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11, HEPES 20; pH was 7.2 at 37°C. For loading with $^{86}Rb^+$ the vein was incubated for an additional 80 min in PSS to which $^{86}Rb^+$ 5 μCi $ml^{-1}$ had been added.

### *Simultaneous measurement of $^{86}Rb^+$ efflux and of myogenic activity*

After loading with $^{86}Rb^+$ the vein was shortly dipped into PSS to remove excess radioactivity and was mounted in a temperature-controlled (32°C) perfusion chamber similar to that described by Bolton & Clark (1981). A preload of 500 mg was applied and the chamber perfused with PSS at a rate of 2.5 ml $min^{-1}$. The upper cotton thread was attached to an isometric force transducer (Gould cell, Statham) which was connected to a home-built amplifier, from which the signal was given to a recorder and to an integrator for quantitation of myogenic activity.

For measurement of $^{86}Rb^+$ efflux the perfusate was collected at a sampling rate of 2 min (or 1 min at concentrations of BRL 34915 > 10 μM) and counted for radioactivity in the Čerenkov mode at 50% efficiency. The radioactivity remaining in the portal vein at the end of the assay, $C_f$, was determined by dissolving the vessel in 500 μl Lumasolve (Lumac) at 50°C overnight. The sample was then supplemented with 500 μl 1 N HCl and 10 ml Optifluor (Packard) and counted in the $^{32}P$ channel at 100% efficiency.

### *Concentration-response curves*

After about 30 min equilibration time, the first concentration of BRL 34915 was superfused for 10 min, followed by a washout phase of 20 to 30 min ([BRL] < 1 μM) or > 40 min (1 < [BRL] < 10 μM). In the low concentration range (< 1 μM), this procedure could be repeated up to 4 times with the same vein without signs of tachyphylaxis in the $^{86}Rb^+$ flux response. In the case of high concentrations of BRL 34915 (> 10 μM), however, tachyphylaxis was evident and BRL 34915 was applied only once (10 min application time, sampling rate 1 min).

### *Calculations and evaluation of data*

The rate constant $k_i$ of $^{86}Rb^+$ efflux at time $t = t_i$ was calculated as $k_i = \Delta C_i/[\Delta t \times C_i]$ where $\Delta C_i$ is the radioactivity released from the vessel in the time interval from $t_i$ to $t_i + \Delta t$; in general $\Delta t$ was 2 min. $C_i$, the radioactivity remaining in the vessel at time $t_i$, was calculated backwards as the sum of the radioactivity remaining in the vessel at the end, $C_f$, plus all radioactivity released from the end to time $t_i$ (see Durbin & Jenkinson, 1961 or Bolton & Clark, 1981). If the efflux follows a single exponential, the flux rate will be constant, i.e. independent of time. Drug effects on the efflux rate constant k were generally calculated as the peak value of k obtained in the presence of the drug divided by the basal value of k averaged over 6–10 min before drug application. However, at high concentrations of BRL 34915 the effect faded during application (see Figure 2) and the area under the curve (AUC) of the k vs time plot was chosen as a better measure of the drug effect. AUCs were determined by weighing and are given in mg where 100 mg corresponds to an increase in k by $1.6 \times 10^{-2}$ $min^{-1}$ during the 10 min application time. Concentration-effect curves were

fitted to the Law of Mass Action or to the Hill equation by weighted non linear least squares analysis with statistical weights proportional to (s.e.mean)$^{-2}$. Errors in the parameters of the fit were estimated by the univariate approximation (Draper & Smith, 1981).

*Drugs and solutions*

BRL 34915 was a generous gift from Beecham Pharmaceuticals, UK. Stock solutions were prepared in dimethylsulphoxide. The final concentration of solvent was $<0.3\%$ and had no obvious effect on spontaneous activity or $^{86}Rb^+$ efflux. Isradipine (PN 200-110, Sandoz Ltd, Basle) was dissolved in a similar manner to BRL 34915. Tetraethylammonium chloride (TEA) and 3,4-diaminopyridine (DAP) were from Janssen (Beerse, Belgium), 8Br-cyclic GMP and N$^6$,2'-O-dibutyryladenosine 3':5' cyclic monophosphate (db-cyclic AMP) were from Sigma. Concentrated stock solutions of these substances were prepared in $H_2O$ and further diluted in PSS. In the case of high TEA concentrations ($>10$ mM), the NaCl content of PSS was decreased correspondingly so as to keep ionic strength constant.

## Results

*Concentration-dependence of the effect of BRL 34915*

The effect of increasing concentrations of BRL 34915 on spontaneous activity and on $^{86}Rb^+$ efflux in guinea-pig portal vein is shown in Figure 1. As seen in the upper trace, 10 nM BRL 34915 decreased the frequency of myogenic spikes leading to a reduction in (integrated) spontaneous activity by 40%. At 30 nM, mechanical activity was inhibited by 75%, which was again essentially due to a reduction in the frequency of the contractile spikes with relatively little effect on their height. From 100 nM upwards, spontaneous activity was virtually abolished. The effect of BRL 34915 was not only readily reversed by washing out but even followed by a rebound increase in myogenic activity, i.e. a higher activity than before application of the substance. This rebound phase lasted more than 30 min and was quite often observed; sometimes myogenic activity was increased up to 3 times the control level. The concentration-dependence of the inhibition of myogenic activity by BRL 34915, as determined from 4 experiments, is shown in Figure 3a. Fitting the data to the Hill equation yielded an $EC_{50}$ value of $12 \pm 2$ nM and a Hill coefficient of $1.7 \pm 0.3$. The latter reflects the steepness of the curve as the effect increased from 10% to 90% with only a 13 fold increase in concentration.

Returning to the experiment displayed in Figure 1, the lower trace was examined, which presents the rate constant of $^{86}Rb^+$ efflux, k. At 10 and 30 nM BRL 34915 there was a small ($\approx 5\%$) but significant decrease in k which went in parallel with the reduction of spontaneous activity. At 100 nM BRL 34915 there was no significant effect on flux whereas at 300 nM an increase in k resulted. Higher concentrations of BRL 34915 increased the rate of $^{86}Rb^+$ flux in a concentration-dependent manner, as shown in Figure 2. From 10 $\mu$M upwards the flux rate reached its peak and started to decrease again during the application period of the drug, i.e. 10 min. This decrease in k may be interpreted as inactivation (desensitization) of the BRL 34915-induced $^{86}Rb^+$ efflux, due to a very high concentration of the agonist. In the case of 10 $\mu$M BRL 34915, desensitization appeared to be transient as the

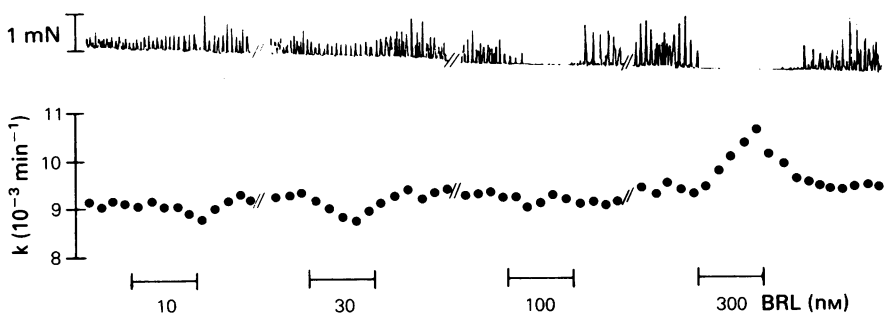


**Figure 1** Effect of BRL 34915 on the guinea-pig portal vein. The indicated concentrations of BRL 34915 were applied for 10 min; intermediate recovery times were 20 min after 10 nM BRL 34915 and 30 min after 30,100 and 300 nM BRL 34915 and are not shown completely. Temperature was 32°C. Upper trace: spontaneous activity. Note the rebound effect after washing out BRL 34915. Control level before addition of substance was 0.16 mN min$^{-1}$. Lower trace: rate constant of $^{86}Rb^+$ efflux, k, in $10^{-3}$ min$^{-1}$.

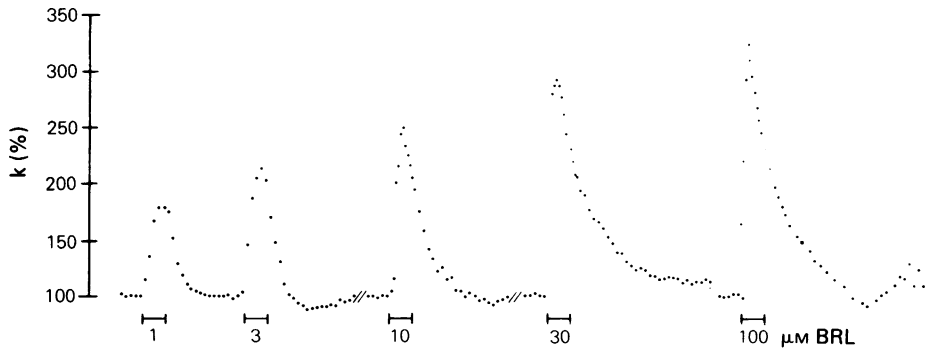


**Figure 2** Effect of higher concentrations of BRL 34915 on $^{86}Rb^+$ efflux. Application time 10 min, recovery time 30–50 min. Four different veins were used. The rate coefficient, k, is therefore given in a normalized form where $100\% = (9 \pm 1) \times 10^{-3}\,min^{-1}$.

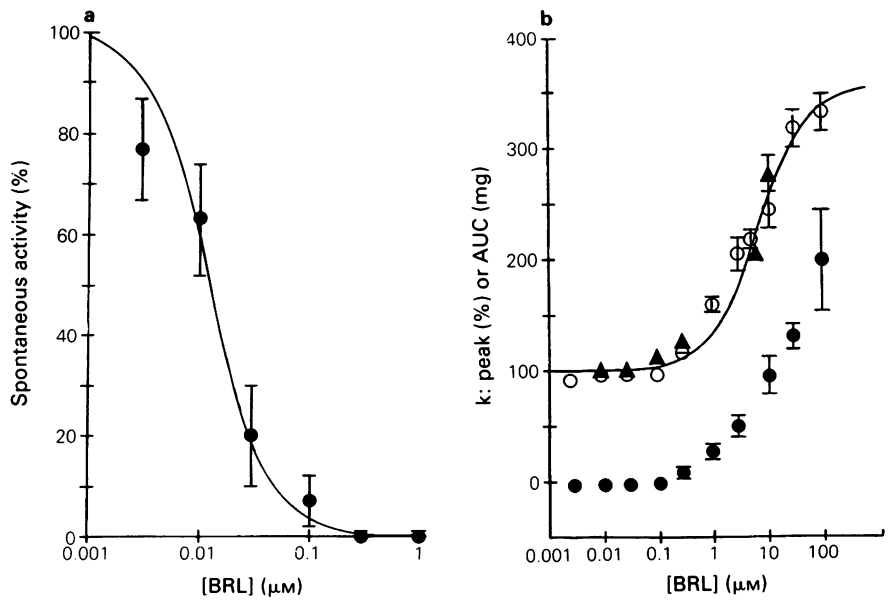


**Figure 3** Concentration-effect curves for BRL 34915 in guinea-pig portal vein. (a) Inhibition of spontaneous activity: mean values of 4 experiments are fitted to the Hill equation yielding an $IC_{50}$ value of 12 ± 2 nM and a Hill coefficient $n_H = 1.7 \pm 0.3$. Vertical lines show s.e.mean. (b) $^{86}Rb^+$ efflux: (○) peak value of rate constant expressed as % of pre-drug value ($n = 7$). The values below 100 nM are 95 or 96%, i.e. lower than the control value, in agreement with Figure 1. The solid curve is a weighted fit of the data at concentrations > 300 nM to the Law of Mass Action, yielding an apparent $EC_{50}$ value of 7.0 ± 3.8 μM and an effect at saturation of 354 ± 67% of control. (●) Area under the curve (AUC) in arbitrary units (mg) as a measure of flux promotion; 100 mg correspond to an increase in k by $1.6 \times 10^{-2}\,min^{-1}$ for 10 min. Below 100 nM BRL 34915 the AUCs were below the control baseline. They are counted as negative and their magnitude is − 1.5% of the AUC at 100 μM. (▲) Peak values of k (%) in the presence of 100 or 200 nM PN 200-110.

**Table 1** Effect of BRL 34915 on $^{86}Rb^+$ flux rate constant (k) in the absence and presence of other vasodilators

| | *Conditions* | | | |
|---|---|---|---|---|
| [BRL 34915] (μM) | *No other agent* | *PN 200-110* (200 nM) | *8Br-cyclic GMP* (100 μM) | *db-cyclicAMP* (300 μM) |
| 0 | 100% | 90 ± 4% (100) | 91 ± 5% (100) | 93 ± 3% (100) |
| 0.01 | 95 ± 1 | 100 ± 1 | 99 ± 2 | 99 ± 2 |
| 0.03 | 96 ± 1 | 102 ± 1 | 99 ± 1 | 99 ± 2 |
| 0.06 | | 104 ± 1 | | |
| 0.1 | 96 ± 1 | 109 ± 1 | 104 ± 1 | 112 ± 2 |
| 0.3 | 116 ± 3 | 123 ± 5 | 118 ± 1 | 138 ± 5 |
| 1.0 | 160 ± 6 | | 153 ± 8 | 208 ± 26 |
| 6.0 | 210 ± 6 | 204 ± 11 | 250 ± 9 | 223 ± 15 |
| 10.0 | 243 ± 18 | 276 ± 18 | | |

BRL 34915 was superfused for 10 min in the continuous presence of the other vasodilator indicated. Results given are means ± s.e.mean, $n = 7$ (2nd column), $n = 6$ (3rd column up to 300 nM BRL), $n = 3$ all other results. The results in the first row represent the basal efflux in the presence of either PN 200-110, 8Br-cyclic GMP or db-cyclic AMP alone when compared to the control efflux (no agent). This value is then set as 100% (number in parentheses) and is used as the reference value for the effect of the subsequent additions of BRL 34915. The values in the other rows represent the peak effect of BRL 34915 expressed as a % of k value before the addition of BRL 34915 (but in the presence of the other vasodilator).

response completely recovered after a period of 40–60 min; higher concentrations were not tried.

The concentration-dependence of the effect of BRL 34915 on $^{86}Rb^+$ efflux, as determined from 7 experiments, is shown in Figure 3b. The open circles represent the peak values of the flux rate constant expressed as % of control; some values are listed in Table 1 (column 1). Below 100 nM these values were around 95%. This reflects the slight depression of $^{86}Rb^+$ efflux seen with low concentrations of BRL 34915 as shown in Figure 1. From 300 nM upwards, k increased in a concentration-dependent manner up to 100 μM where the effect appeared to level off. The data fit reasonably well to the Law of Mass Action (solid curve in Figure 3b). However, saturation at high concentrations is only apparent as it is due to the desensitization process shown in Figure 2. As an alternative measure of the effect of BRL 34915 on the flux rate constant the area under the curve of the k vs time plot is also presented in Figure 3b. In this case it is seen that no saturation occurs at 100 μM.

### *Modulation of BRL 34915-stimulated $^{86}Rb^+$ efflux by various agents*

These studies were undertaken in order to obtain a pharmacological characterization of the BRL 34915-stimulated $^{86}Rb^+$ flux. First the influence of the calcium entry blocking dihydropyridine PN 200-110 (isradipine; Hof *et al.*, 1984a,b) on the effect of BRL 34915 was investigated. Superfusion with the $Ca^{2+}$ antagonist (200 to 500 nM) led rapidly to complete quiescence of the vein (not shown) and this was accompanied by a 10% decrease in the $^{86}Rb^+$ flux rate constant (see Figure 4). Addition of 10 and 30 nM BRL 34915 did not decrease the flux rate constant further but left it unchanged, whereas from 100 nM on an increase was observed. At higher concentrations there was little difference between the values observed in the absence and presence of PN 200-110 (see Table 1). These experiments show that the $Ca^{2+}$ entry blocker does not interfere with the effect of BRL 34915 on flux. Instead, by suppressing spontaneous activity and the $^{86}Rb^+$ efflux component associated with it (i.e. ≈10%), the stimulating effect of BRL 34915 on flux could be observed at lower concentrations. The mean values of 6 experiments are presented in Table 1, column 3. At 60 nM BRL 34915, there was a small but significant increase in flux of 4 ± 1%; at 100 nM, the effect was 9 ± 1%.

The results obtained in the presence of 8Br-cyclic GMP, a cell permeable and smooth muscle relaxant derivative of cyclic GMP (see e.g. Schultz *et al.*, 1979), resemble those obtained with PN 200-110. Superfusion with 100 μM 8Br-cyclic GMP led to complete relaxation of the vein within 5 min (not shown) and, again, this was accompanied by a 9% decrease in the $^{86}Rb^+$ flux rate constant (see Table 1). The effect of subsequent additions of BRL 34915 on flux was also similar to that observed in the presence of PN 200-110. In order to study the effects of cyclic AMP, the cell permeable analogue dibutyryl-cyclic AMP (db-cyclic AMP) was used. Its action on spontaneous activity was slow to develop and relatively weak (estimated $IC_{50}$ ≈ 200 μM, data not shown). The interaction studies with BRL 34915 were performed in the

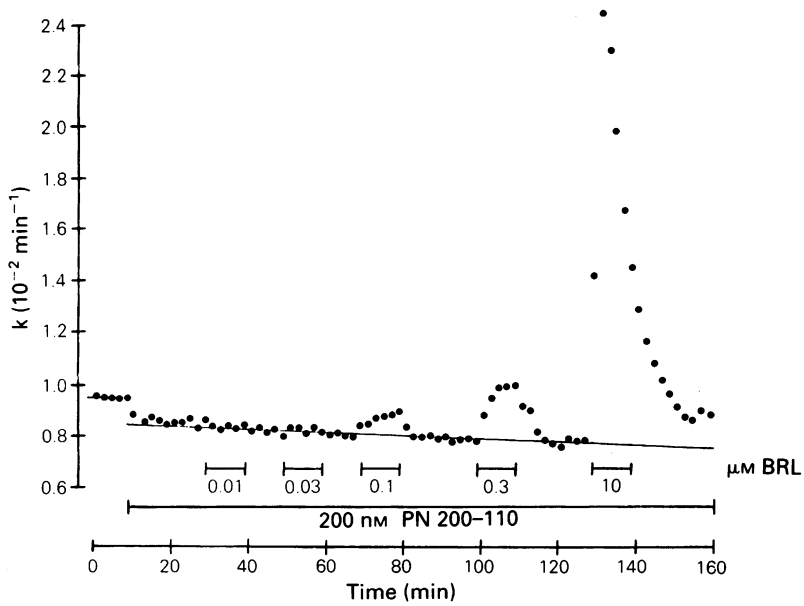


**Figure 4** BRL 34915-induced $^{86}Rb^+$ efflux in the presence of 200 nM PN 200-110. After 10 min, the portal vein was superfused with 200 nM PN 200-110. At the times indicated, the given concentrations of BRL 34915 were added for 10 min.

presence of 300 μM db-cyclic AMP, a concentration which inhibited spontaneous activity by 63% of control (not shown) and reduced the flux rate constant by 7% (Table 1). The effect of BRL 34915 on $^{86}Rb^+$ flux in the presence of db-cyclic AMP was again similar to that seen in the presence of 8Br-cyclic GMP or PN 200-110 (see Table 1).

Next, the effect of the $K^+$ channel blockers TEA and DAP on the BRL 34915-stimulated $^{86}Rb^+$ efflux was investigated. These experiments proved to be complex. Complications arose from the fact that TEA and DAP were excitatory on the guinea-pig portal vein and by themselves led to an increase in $^{86}Rb^+$ efflux (see below and Figure 6).

In order to avoid these difficulties, experiments were performed in the continued presence of 500 nM PN 200-110 which inhibited spontaneous activity and the excitatory effect of the $K^+$ channel blockers. A typical example of the effects on $^{86}Rb^+$ flux is shown in Figure 5. Application of 500 nM PN 200-110 led to a decrease in the flux rate constant (concomitant with cessation of spontaneous activity, not shown). Addition of 20 mM TEA (a) or 5 mM DAP (b) did not change the flux rate constant (or the mechanical activity, not shown). Superfusion of 6 μM BRL 34915 for 10 min (in the continued presence of PN 200-110 and the $K^+$ channel blocker) resulted in a relatively small increase in the flux rate constant. After 40 min the $K^+$ channel blocker was discontinued and, as a control, BRL 34915 was superfused again. As estimated from the AUCs in Figure 5, 20 mM TEA and 5 mM DAP inhibited the effect of BRL 34915 on $^{86}Rb^+$ flux by 63% and 47%, respectively.

When the effect of the $K^+$ channel blockers was examined in the absence of PN 200-110, their excitatory effect complicated matters. A typical example is shown in Figure 6 where superfusion with 10 mM TEA induced an intense spasm (increase in mechanical activity by more than 20 fold) accompanied by an increase in the flux rate constant to 140% of the control level. Addition of 6 μM BRL 34915 to the TEA-containing solution for 10 min lead to a fast relaxation of the vein together with a small increase in $^{86}Rb^+$ flux. During the subsequent washout of BRL 34915 over 40 min in the continued presence of TEA, the tissue first remained relaxed and $^{86}Rb^+$ efflux decreased, then mechanical activity and flux rate returned to the levels they were at before application of BRL 34915. TEA was then discontinued and 6 μM BRL 34915 was again superfused for 10 min, yielding the BRL 34915 effect in the absence of TEA as the control.

The concentration-dependent inhibition of BRL-34915-stimulated $^{86}Rb^+$ efflux by TEA and DAP is shown in Figure 7. Obviously, the inhibition is independent of the presence of PN 200-110 and inhibition constants of 3 mM for DAP and 13 mM for TEA were estimated from these data.

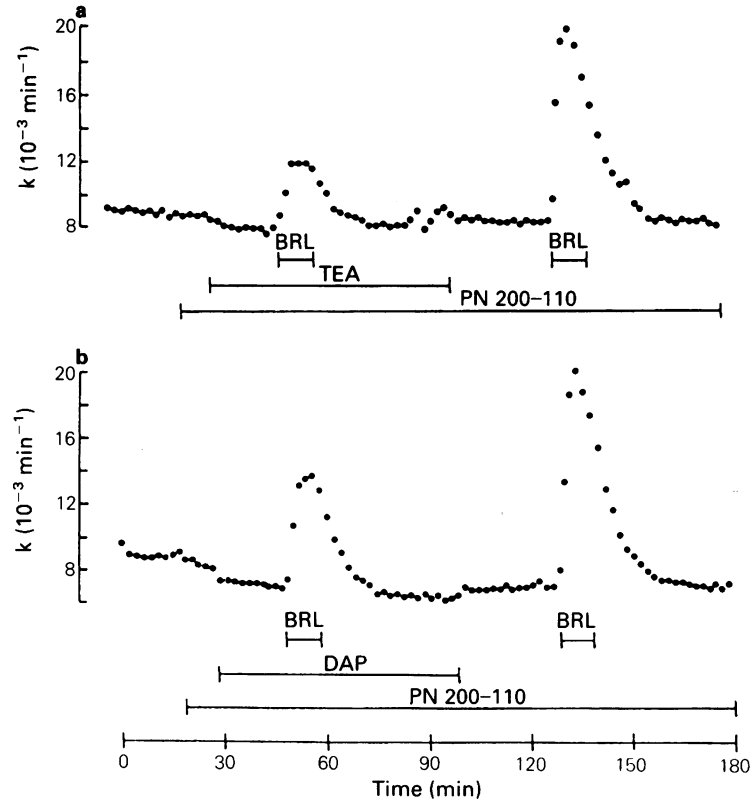


**Figure 5** Inhibition of the effect of BRL 34915 on $^{86}Rb^+$ efflux by (a) tetraethylammonium (TEA) 20 mM and (b) 3,4-diaminopyridine (DAP) 5 mM in the presence of PN 200-110. After 20 min, the two veins were superfused with 500 nM PN 200-110, then 20 mM TEA (a) or 5 mM DAP (b) was added. BRL 34915 6 mM was applied for 10 min as indicated. Comparison of the areas under the curves in the k vs time plot showed that 20 mM TEA inhibited the effect of BRL 34915 by 63%, 5 mM DAP by 47%.

## Discussion

This paper presents the detailed concentration-dependence of the effects of BRL 34915 on spontaneous activity and $^{86}Rb^+$ efflux in the guinea-pig portal vein. The salient feature of this study is the 20 fold difference between the concentrations necessary to inhibit spontaneous activity ($IC_{50} = 12$ nM) and to increase $^{86}Rb^+$ flux by an appreciable amount, e.g. 10% ($ED_{110} \approx 250$ nM, see Figure 3b and Table 1). If spontaneous activity and the $^{86}Rb^+$ efflux component associated with it were abolished (e.g. by application of the $Ca^{2+}$ channel blocker isradipine), then stimulation of $^{86}Rb^+$ flux could be detected at lower concentrations of BRL 34915 (4% at 60 nM, 9% at 100 nM) and the concentration gap between the two effects narrowed down to a factor of 5 to 8. This is still difficult to reconcile with the working hypothesis that the BRL-induced $^{86}Rb^+$ efflux is the cause of the inhibition of spontaneous activity; it rather suggests that the two effects are unrelated.

At first it may be thought that the $Rb^+$ efflux technique might not be sensitive enough to pick up small changes in $K^+$ permeability. This can be examined following the calculations of Wahlström (1973) (see also Brading (1981) for the pertinent equations). In the presence of PN 200-110, 60 nM BRL 34915 augmented k by $4 \pm 1\%$ (Table 1) which converts to an increase in potassium permeability of $\approx 1.5 \times 10^{-9}$ cm $s^{-1}$. Using the Goldman equation a resulting hyperpolarization of the membrane of $-0.7$ mV was calculated (Wahlström (1973), parameters for rat portal vein). This crude estimate shows that, in principle, the $^{86}Rb^+$ efflux method is of adequate sensitivity.

An implicit but important assumption made in this calculation is that all cells of the portal vein respond homogeneously to BRL 34915. Apart from the fact that only about 50% of all cells in portal vein are actually smooth muscle cells (Ljung, 1970), the

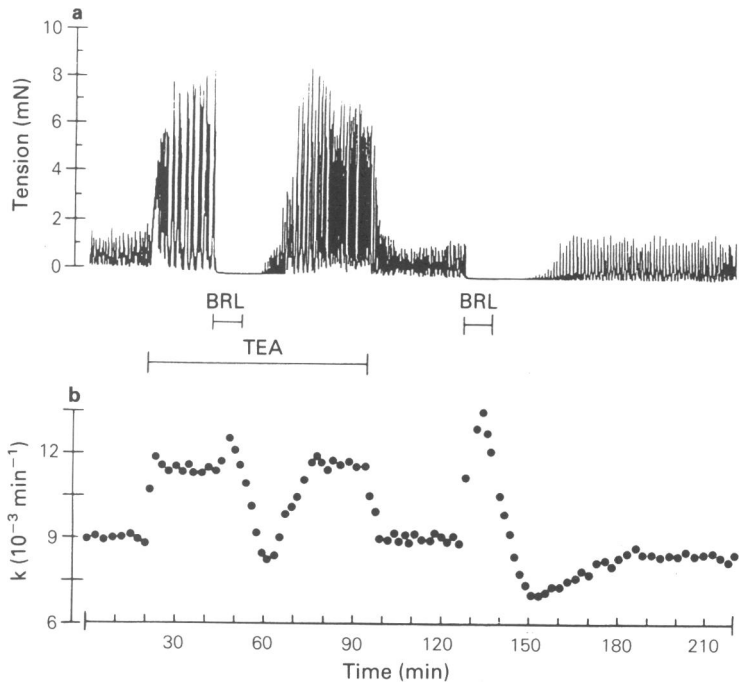


**Figure 6** Effect of 6 μM BRL 34915 in the presence of tetraethylammonium (TEA; 10 mM). (a) Spontaneous activity. Control level: 0.2 ± 0.02 mN $min^{-1}$. (b) Rate constant of $^{86}Rb^+$ efflux, k, in $10^{-3}$ $min^{-1}$. After 20 min TEA (10 mM) was superfused as indicated; application time of BRL 34915 (6 μM) was 10 min. The minimum value of k after addition of BRL 34915 was taken as baseline for the areas under the curves in the k vs time plot.

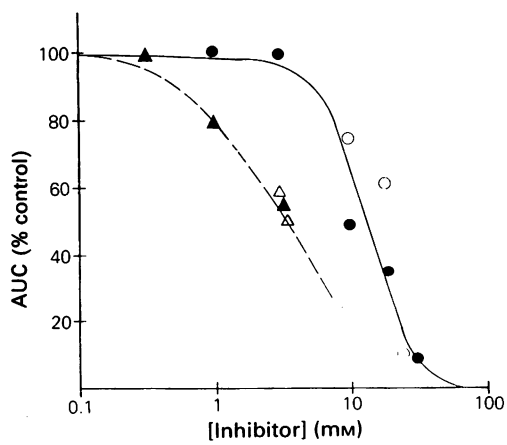


**Figure 7** Concentration-dependent inhibition of BRL 34915-stimulated $^{86}Rb^+$ efflux by (●, ○) tetraethylammonium (TEA) and (▲, △) 3,4-diaminopyridine in the absence (●, ▲) and presence (○, △) of 500 nM PN 200-110. Conditions were as in Figures 5 and 6. $IC_{50}$ values were estimated to be 3 mM (DAP) and 15 mM (TEA).

mechanical data in Figure 1 indicate that this assumption is probably not correct. The BRL 34915-induced inhibition of spontaneous activity results primarily from a decrease in the frequency of the contractions, their amplitude being affected only at higher concentrations of the agent. This decrease in frequency is due to an increased hyperpolarization of pacemaker cells between bursts of activity, probably by activation of $Ca^{2+}$-dependent $K^+$ channels (Hille, 1984). Three different types of $Ca^{2+}$-dependent $K^+$ channels have been identified in the rabbit portal vein (Inoue *et al.*, 1985; 1986). A preferential action of BRL 34915 on the pacemaker cells of the portal vein (see also Hamilton *et al.*, 1986) may be explained by the presence of a particular $K^+$ channel in these cells. Alternatively, this could be due to the particular voltage conditions there which 'sensitize' a $K^+$ channel, also present in other smooth muscle cells of the vein, to BRL 34915. The observation that any area of the portal vein can serve as a pacemaker for the entire preparation and that shifts of pacemaker area occur frequently during the spontaneous activity (Ljung, 1970) may favour the second alternative.

If the pacemaker hypothesis is true then spontaneous activity in portal vein should be more sensitive to BRL 34915 than tension of stimulated smooth muscle of tonic nature. Indeed, the $EC_{50}$ value of BRL 34915 in relaxing the rat aorta precontracted by noradrenaline or 20 mM KCl appears to be larger than 100 nM (Weir & Weston, 1986b).

There are two additional points in favour of BRL 34915 acting to increase the membrane permeability of the smooth muscle cell for $K^+$. Firstly, both vasorelaxation and $^{86}Rb^+$ efflux stimulation are stereospecific effects, residing in the 3 **S**, 4 **R** diastereomer (Buckingham *et al.*, 1986; Quast, unpublished observation). Secondly, there are other vasodilators of a different chemical nature (pyridine derivatives) which also increase $^{86}Rb^+$ efflux at higher concentrations, i.e. pinacidil (Southerton *et al.*, 1987; Quast & Cook, 1987) and nicorandil (Weir & Weston, 1986b). These compounds also inhibit spontaneous activity in the rat portal vein by reducing the frequency of the contractions. The $IC_{50}$ values for pinacidil and nicorandil are 0.06 and 2.1 $\mu$M, respectively, and again their $ED_{110}$ values for stimulation of $^{86}Rb^+$ efflux are higher by a factor of about 20, i.e. 1.4 and 50 $\mu$M (Quast & Cook, 1987; Quast, unpublished).

The curve showing the concentration-dependent inhibitory effect of BRL 34915 on spontaneous activity in guinea-pig portal vein was found to be steep. The Hill coefficient of 1.7 does not necessarily indicate a cooperativity in the binding of BRL 34915. It may be explained as well by nonlinear links between the different events which, according to Weston and coworkers (Hamilton *et al.*, 1986; Weir & Weston, 1986b), constitute the antivasoconstrictor mechanism of BRL 34915, i.e. increase in $K^+$ efflux $\rightarrow$ membrane hyperpolarization $\rightarrow$ inhibition of $Ca^{2+}$ entry $\rightarrow$ inhibition of contraction.

Turning now to the effects of BRL 34915 on $^{86}Rb^+$ efflux, it was first noted that the 5% decrease in the $^{86}Rb^+$ flux rate constant observed at 10 or 30 nM BRL was due to the partial inhibition of electrical activity of the vessel. Total inhibition decreased k by $\approx$10% (Table 1, values for PN 200-110 or db-cyclic GMP). With BRL 34915 this level was not reached. At 100 nM, when inhibition was complete, BRL stimulated $^{86}Rb^+$ flux (Table 1, data in presence of PN 200-110), and both effects cancelled each other out (Figure 1, Table 1). Prolonged application of high concentrations ($>10\,\mu$M) of BRL 34915 led to a decrease in the flux response over the time course of about 5 min. This may be due to various causes; an appealing possibility is that it is the result of a slow inactivation (desensitization) of the $Rb^+$ transporting protein; this is known to occur with many ion channels (see e.g. Hille, 1984).

The experiments performed in the presence of the $Ca^{2+}$ antagonist PN 200-110 show that the effect of BRL 34915 on $K^+$ conductance does not require the entry of extracellular $Ca^{2+}$ through dihydropyridine-sensitive $Ca^{2+}$ channels (but see Kreye & Weston, 1986). However, this does not preclude the possibility that BRL 34915 acts on a $Ca^{2+}$-dependent $K^+$ channel, as binding of the agent could shift the $Ca^{2+}$ dependence so much to the left that the channel opens at resting intracellular $Ca^{2+}$ concentrations. Nevertheless, an action of BRL 34915 on the apamin-sensitive $Ca^{2+}$ dependent $K^+$ channel can be excluded, as it has been shown that apamin (0.1 $\mu$M) does not inhibit the effects of BRL 34915 in either the guinea-pig taenia caeci (Weir & Weston, 1986a) or in the guinea-pig isolated trachealis (Allen *et al.*, 1986).

It was found in this study, that the (rather non-specific) $K^+$ channel blockers TEA and DAP inhibited the flux response to 6 $\mu$M BRL 34915 with $IC_{50}$ values of 13 mM (TEA) and 3 mM (DAP). Thus, DAP is about 4 times more potent than TEA. This is in qualitative agreement with the observation of Allen *et al.* (1986) that 4-aminopyridine (5 mM) inhibited the relaxant action of BRL 34915 in guinea-pig trachealis more strongly than 8 mM TEA.

8Br-cyclic GMP and db-cyclicAMP showed no significant effect on the BRL 34915-induced flux response (except for removing the interference of the $Rb^+$ flux associated with spontaneous activity). As these compounds are known to act via activation of their respective kinases it appears that cyclic nucleotide-dependent phosphorylation, if it occurs at all along the pathway leading to BRL 34915-induced $K^+$ flux, has no important modulatory function there.

In conclusion, an attempt has been made to characterize simultaneously the effect of BRL 34915 on spontaneous activity and $^{86}Rb^+$ efflux in the guinea-pig portal vein. The at least 5 fold concentration shift between the two effects prevents an unambiguous confirmation of the proposed mechanism of action, although this may be explained by a preferential action of BRL 34915 on the pacemaker region of the portal vein. In addition, pharmacological properties of BRL 34915-stimulated $^{86}Rb^+$ efflux were determined. However, at present it is not known whether this $^{86}Rb^+$ efflux occurs via a $Rb^+$ permeable $K^+$ channel or via an, as yet unidentified, electrogenic ion exchanger. Together with the known multiplicity of $K^+$ channels, the inhibition of the BRL 34915-induced $Rb^+$ flux by TEA and DAP and the apparent desensitization of the system at high agonist concentrations may favour the former (channel) hypothesis. A definite answer needs to be determined by use of patch clamp analysis. Finally, elucidation of the mode of action of BRL 34915 at its target protein (direct activation by binding or indirect modification by a 'second messenger') also requires further study.

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