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Effect of apamin on responses to BRL 34915, nicorandil and other relaxants in the guinea-pig taenia caeci

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1 BRL 34915 ($4-64 \times 10^{-7}$ M), isoprenaline ($0.5-32 \times 10^{-8}$ M) and nicorandil ($4-64 \times 10^{-6}$ M) produced a slowly-developing relaxation of spontaneous tone of the guinea-pig taenia caeci; with no after-contraction on washout. These inhibitory responses were unaffected by apamin (10^{-7} M).

2 Adenosine triphosphate ($0.06-2 \times 10^{-3}$ M) and noradrenaline ($1-16 \times 10^{-7}$ M) produced a rapid inhibition of spontaneous tone with a prominent after-contraction, especially on washout. Both the inhibitory effect and the rebound contraction were abolished by apamin (10^{-7} M).

3 Exposure to both BRL 34915 (64×10^{-7} M) and to nicorandil (64×10^{-6} M) produced an increase in the ^{86}Rb efflux rate coefficient which was unaffected by apamin (10^{-7} M).

4 Exposure to isoprenaline (32×10^{-8} M) had no effect on the ^{86}Rb efflux rate coefficient.

5 Exposure to noradrenaline (16×10^{-7} M) produced an increase in the ^{86}Rb efflux rate coefficient which was abolished by apamin (10^{-7} M).

6 The results confirm that both BRL 34915 and nicorandil are capable of opening potassium channels in smooth muscle but show that the channel is not apamin-sensitive.

Introduction

In rat isolated blood vessels, BRL 34915, a novel benzopyran derivative (Ashwood *et al.*, 1984) produces inhibitory effects by opening potassium channels in the vascular smooth muscle cells thereby holding their membrane potential at or close to the potassium equilibrium potential (Hamilton *et al.*, 1986; Weir & Weston, 1986). This action is almost certainly responsible for the *in vivo* hypotensive properties of BRL 34915 described by Buckingham *et al.* (1984, 1986). Nicorandil (Sakai *et al.*, 1983) is another recently-developed vasodilator drug capable of opening smooth muscle potassium channels (Inoue *et al.*, 1983; 1984; Kajiwara *et al.*, 1984) and a comparative study of the actions of BRL 34915 and nicorandil on rat blood vessels has shown that there are qualitative similarities between these two vasodilators (Weir & Weston, 1986).

In mammalian smooth muscle, at least three types of potassium channel have been identified. Two of these are activated by depolarization and/or calcium entry and are responsible for repolarization (Bolton *et al.*, 1985). A third potassium channel, associated with hyperpolarization processes, has been identified in guinea-pig taenia caeci. It is activated by noradrenaline, adenosine triphosphate and the non-adrenergic, non-cholinergic inhibitory transmitter and can be blocked by the bee venom toxin, apamin (Banks *et al.*, 1979; Maas *et al.*, 1980; den Hertog, 1981; 1982).

The objective of the present experiments was to determine whether BRL 34915 and nicorandil had any actions on potassium channels in guinea-pig taenia caeci and to study the ability of apamin to modify these effects. In this way it was hoped to be able to clarify the actions of these novel potassium channel-opening drugs on both intestinal and vascular smooth muscle.

Methods

Guinea-pigs of either sex and weighing 250–350 g were supplied by the University of Manchester Animal Unit. The animals were killed by stunning and bleeding, the abdomen was opened and the caecum exposed.

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Tissue bath studies

Six segments of the taenia caeci, each approximately 2 cm long, were removed and mounted in 20 ml tissue baths containing a bicarbonate- and sulphate-free physiological salt solution (PSS) in which the buffer was 3-(N-morpholino)-propane sulphonic acid (MOPS) (Hamilton *et al.*, 1986), bubbled with 100% O_2 at 37°C. Tissue length changes were measured under a 1 g load using an isotonic transducer (Harvard) and potentiometric recorder (Rikadenki).

After a 45 min equilibration period, each tissue developed a variable degree of spontaneous tone. It was quickly observed that following exposure of tissues to noradrenaline and subsequent washout, the level of spontaneous tone increased markedly and was well-maintained. Thus to optimize the degree of resting tone, all tissues were treated with noradrenaline 8×10^{-7} M for 1 min after the initial 45 min equilibration period and subsequently washed with PSS several times during the next 106 min. After such treatment, most tissues maintained an adequate degree of tone and baseline stability which allowed the effects of relaxant drugs to be examined. Preparations which failed to do this were rejected.

Each tissue was exposed only to a single relaxant drug and a cumulative dose-response protocol was used. In some experiments tissues were subsequently treated with apamin in varying concentrations for 30 min before repeating the exposure to the relaxant in the continuing presence of apamin. When these experiments had been completed, tissues were exposed to papaverine 10^{-4} M and the point to which they relaxed was defined as '100% relaxation'. Previous relaxations were then expressed with reference to this relaxation.

^{86}Rb efflux

In these experiments ^{86}Rb was used as a K^+ marker (Hamilton *et al.*, 1986). Eight segments of the taenia caeci, each 1.5–2 cm long, were removed from a single animal and each was assigned randomly to one of eight experimental groups: (1) nicorandil; (2) BRL 34915; (3) noradrenaline; (4) isoprenaline; (5) BRL 34915 + apamin; (6) noradrenaline + apamin; (7) control; (8) apamin. Each segment 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% O_2 via the needle. The design of the manifold and of the test-tube holder was such that 32 segments (from 4 animals) could be handled simultaneously and transfers from one group of 32 tubes to another similar group could be accomplished within a few seconds.

After a 45 min equilibration period, the tissues were transferred to identical tubes containing PSS + noradrenaline 8×10^{-7} M for 1 min. The noradrenaline was then removed during two successive 5 min periods in tubes containing PSS after which the tissues were loaded with ^{86}Rb, 1 μCi ml^{-1}, for the next 66 min. The tissues were then transferred to tubes containing either 5 ml PSS + ^{86}Rb, 1 μCi ml^{-1} (groups 1, 2, 3, 4 and 7) or 5 ml PSS + ^{86}Rb, 1 μCi ml^{-1}, + apamin 10^{-7} M (groups 5, 6 and 8) for the next 14 min.

After this total loading period of 80 min, the ^{86}Rb was allowed to efflux from the tissues by transferring them to tubes containing 5 ml PSS alone (groups 1, 2, 3, 4 and 7) or 5 ml PSS + apamin (groups 5, 6 and 8) for 13 successive 2 min periods. After 8 such 2 min periods (16 min into the efflux), the PSS into which the tissues were transferred contained the following additions for the times indicated: group (1) nicorandil, 6.4×10^{-5} M, 4 min; groups (2) and (5) BRL 34915 6.4×10^{-6} M, 4 min; groups (3) and (6) noradrenaline, 16×10^{-7} M, 2 min; group (4) isoprenaline, 3.2×10^{-7} M, 4 min. The PSS for groups (7) and (8) contained no addition. For the remaining 2 min periods, the tubes contained either 5 ml PSS alone (groups 1, 2, 3, 4 or 7) or 5 ml PSS + apamin, 10^{-7} M (groups 5, 6 and 8).

Aliquots of PSS and taenia segments were prepared for scintillation counting as described by Hamilton *et al.* (1986). 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

The following drugs were used: adenosine triphos-

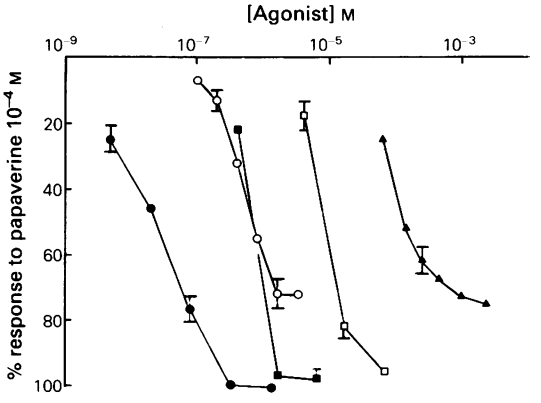

Figure 1 The effect of various relaxants on spontaneous tone in guinea-pig taenia caeci mounted under isotonic conditions. Relaxations are expressed as a percentage of the relaxation produced by papaverine 10^{-4} M which was arbitrarily defined as '100% relaxation'. (●) = Isoprenaline; (○) = noradraline; (■) = BRL 34915; (□) = nocorandil; (▲) = adenosine triphosphate. Each point is the mean derived from 6 experiments; vertical bars show sample s.e.mean values.

phate (Sigma); apamin (Sigma); (±)-BRL 34915 (±)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol) (Beecham); (±)-isoprenaline (Sigma); (−)-noradrenaline bitartrate (Sigma); nicorandil (Chugai); papaverine (Sigma).

For the tissue bath experiments, the stock solutions of BRL 34915 and nicorandil were prepared in 70% v/v ethanol:distilled water; noradrenaline and isoprenaline were prepared in N/10 hydrochloric acid, other drugs in twice distilled water. Adenosine triphosphate solutions were prepared freshly each day; apamin solutions were stored at −20°C. In the ^{86}Rb efflux experiments, these stock solutions were also used with the exception of those containing BRL 34915 and nicorandil. Here, solid pure substances were added to the bulk PSS to avoid the necessity of ethanol controls.

The significance of differences between means was analysed using a two-tailed, unpaired *t* test.

Results

Tissue bath experiments

All experiments were carried out under isotonic conditions on taenia segments which had been 'primed' with noradrenaline (see Methods). In preliminary experiments, both BRL 34915 and nicorandil were found to lower the spontaneous tone of the taenia and the effects of these two substances were compared with those of ATP, isoprenaline and noradrenaline.

Effects of BRL 34915 and nicorandil

BRL 34915 ($4-64 \times 10^{-7}$ M) and nicorandil ($4-64 \times 10^{-6}$ M) produced a slowly-developing and concentration-dependent reduction of the spontaneous tone of segments of taenia caeci. The slopes of the concentration-effect curves of these two substances were quite steep and in the case of BRL 34915, the effect was almost all-or-none, the critical concentration being 16×10^{-7} M (Figures 1 and 2). There was no evidence of a rebound contraction in the continuing presence of either BRL 34915 or nicorandil and the inhibitory effects of both agents were reversible within approximately 30 min by washing. Both were capable of producing a full inhibition of sponaneous tone, using the inhibitory effect of papaverine 10^{-4} M as a standard (Figure 1).

Effect of isoprenaline

Isoprenaline ($0.5-32 \times 10^{-8}$ M) also produced a slowly-developing reduction in spontaneous tone which showed no secondary excitatory component in the continuing presence of this agonist. Isoprenaline was also capable of producing a full inhibitory response (Figures 1 and 2).

Effects of ATP and noradrenaline

ATP ($0.06-2 \times 10^{-3}$ M) and noradrenaline ($1-16 \times 10^{-7}$ M) each produced a very rapid reduction of the spontaneous tone of the taenia (Figures 1 and 2). There was much evidence to indicate that this inhibition was only the initial phase of a biphasic 'inhibitory-excitatory' response and conversion of the inhibition to an excitation was sometimes seen in the continuing presence of these agonists. A rebound increase in tone was always observed following the washout of ATP and noradrenaline in normal PSS and neither agent was capable of producing a full inhibition of spontaneous tone in comparison to that produced by papaverine 10^{-4} M (Figures 1 and 2).

Effect of apamin

Pretreatment of taenia segments with apamin (up to 10^{-7} M), had no effect on the inhibitory responses to

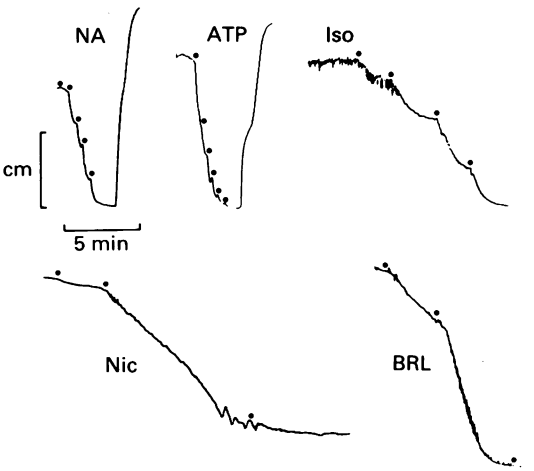

Figure 2 The effects of various relaxants on spontaneous tone in guinea-pig taenia caeci. Each panel shows a single cumulative concentration-effect experiment in a different segment of taenia. NA = noradrenaline ($1-16 \times 10^{-7}$ M); ATP = adenosine triphosphate ($0.06-2 \times 10^{-3}$ M); Iso = isoprenaline ($0.5-32 \times 10^{-8}$ M); Nic = nicorandil ($4-64 \times 10^{-6}$ M); BRL = BRL 34915 ($0.5-32 \times 10^{-8}$ M); the points at which successive additions of each agonist were made are indicated (●). Note the rapid relaxation produced by both NA and ATP and the rebound contraction on washout. In contrast, the relaxations produced by Iso, Nic and BRL were much slower and there was no rapid rebound contraction on washout (not shown).

either BRL 34915, nicorandil or isoprenaline (Figure 3). In contrast, responses to both ATP and to noradrenaline were reduced or completely inhibited by apamin (1.56×10^{-9} M–10^{-7} M) (Figure 4). This inhibition was observed not only for the relaxant actions of ATP and noradrenaline but also for the 'rebound' excitatory effects usually produced by these two agents. In the presence of 10^{-7} M apamin, very high concentrations of ATP ($> 4 \times 10^{-3}$ M) produced a slowly-developing inhibition of spontaneous tone, not dissimilar in its time course to the inhibition produced by either BRL 34915, nicorandil or isoprenaline. This phenomenon was not investigated further in view of the very high concentrations of ATP required.

These inhibitory effects of apamin developed rapidly (within 15 min) and could be reversed by washing, usually within 15–20 min.

^{86}Rb efflux experiments

In this study, the effects of BRL 34915, nicorandil, noradrenaline and isoprenaline on the ^{86}Rb efflux from taenia segments were investigated. The concentrations of inhibitory or modifying agents and their equilibration and exposure times were essentially identical to those used in the tissue bath experiments. Initially attempts were made to detect an increase in ^{86}Rb efflux using drug concentrations which produced

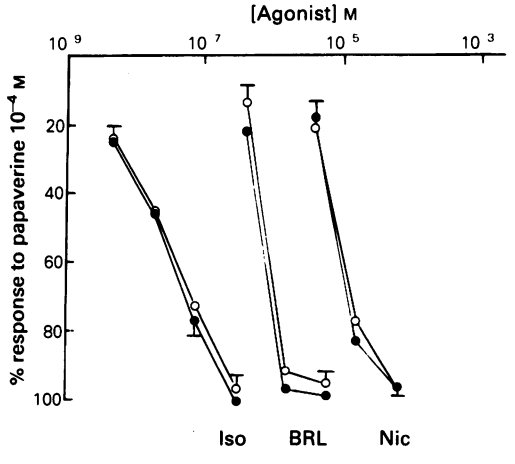

Figure 3 The effect of apamin 10^{-7} M (○) on the inhibition of spontaneous tone produced by isoprenaline (Iso), BRL 34915 (BRL) and nicorandil (Nic) in guinea-pig taenia caeci under isotonic conditions. Control relaxant responses (●). Relaxations are expressed as a percentage of the relaxation produced by papaverine, 10^{-4} M, which was arbitrarily defined as '100% relaxation'. Each point is the mean derived from 6 experiments; vertical bars show sample s.e.mean values.

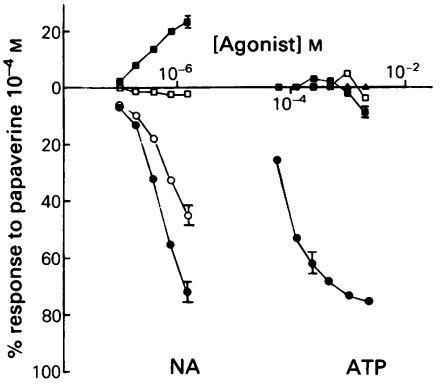

Figure 4 The effect of apamin 1.56×10^{-9} M (○); 6.25×10^{-9} M (■); 25×10^{-9} M (□); 100×10^{-9} M (▲) on the inhibition of spontaneous tone produced by adenosine triphosphate (ATP) and by noradrenaline (NA) in guinea-pig taenia caeci under isotonic conditions. Control relaxant responses (●). Relaxations are expressed as a percentage of the relaxation produced by papaverine, 10^{-4} M, which was arbitrarily defined as '100% relaxation'. Each point is the mean derived from 6 experiments; vertical bars show sample s.e.mean values.

an approximately 50% reduction in spontaneous tone. For BRL 34915, nicorandil, noradrenaline and isoprenaline these concentrations were 10^{-6} M, 10^{-5} M, 4×10^{-7} M and 2×10^{-8} M respectively. However, using the experimental design described in Methods, no significant changes in ^{86}Rb efflux rate coefficient were detected with any of the relaxants at these concentrations. Thus concentrations that produced maximum relaxations in the tissue bath experiments were finally employed.

The average basal ^{86}Rb efflux rate coefficient measured between the 16th and 22nd min of the efflux period was 1.31 ± 0.11 % min^{-1} (mean ± s.e.mean, $n = 8$). In the presence of apamin, 10^{-7} M, the basal rate coefficient measured over the same period was 1.50 ± 0.26 % min^{-1} ($n = 8$). These values were not significantly different (Figure 6). Both BRL 34915 6.4×10^{-6} M and nicorandil 6.4×10^{-5} M increased the ^{86}Rb efflux rate coefficient to 3.11 ± 0.46 % min^{-1} and 2.91 ± 0.37 % min^{-1}, respectively ($n = 8$). This increase was relatively slow in onset and the rate of ^{86}Rb efflux remained above the basal level for several minutes after washout of either substance (Figure 5). In the presence of apamin, 10^{-7} M, BRL 34915, 6.4×10^{-6} M, raised the ^{86}Rb efflux rate coefficient to 3.34 ± 0.30 % min^{-1} ($n = 8$) which was not significantly different from that seen in the absence of apamin (Figure 5).

Noradrenaline, 16×10^{-7} M, increased the ^{86}Rb efflux rate coefficient to 3.42 ± 0.48 % min^{-1} ($n = 8$). This effect was immediate and the ^{86}Rb efflux rate

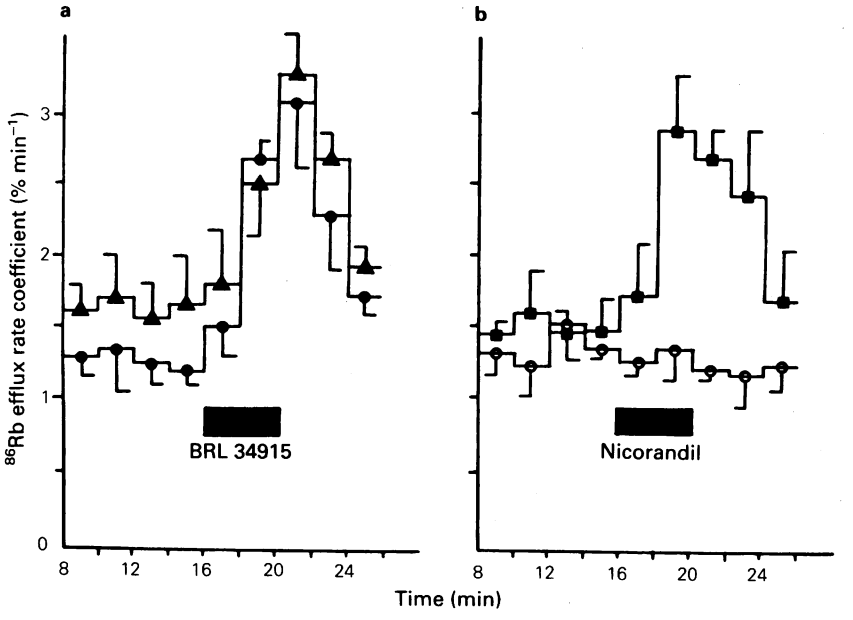

Figure 5 The effect of exposure to BRL 34915 and nicorandil (at solid rectangles) on the loss of ^{86}Rb from guinea-pig taenia caeci. (a) Effect of BRL 34915, 6.4×10^{-6} M in the absence (●) and presence (▲) of apamin 10^{-7} M; (b) effect of nicorandil, 6.4×10^{-5} M (■) compared with control basal ^{86}Rb loss (○). Ordinate scale: ^{86}Rb efflux rate coefficient expressed as a percentage min^{-1}. Abscissa scale: time (min) after start of the efflux period. Each point is the mean derived from 8 experiments; vertical bars show s.e.mean values.

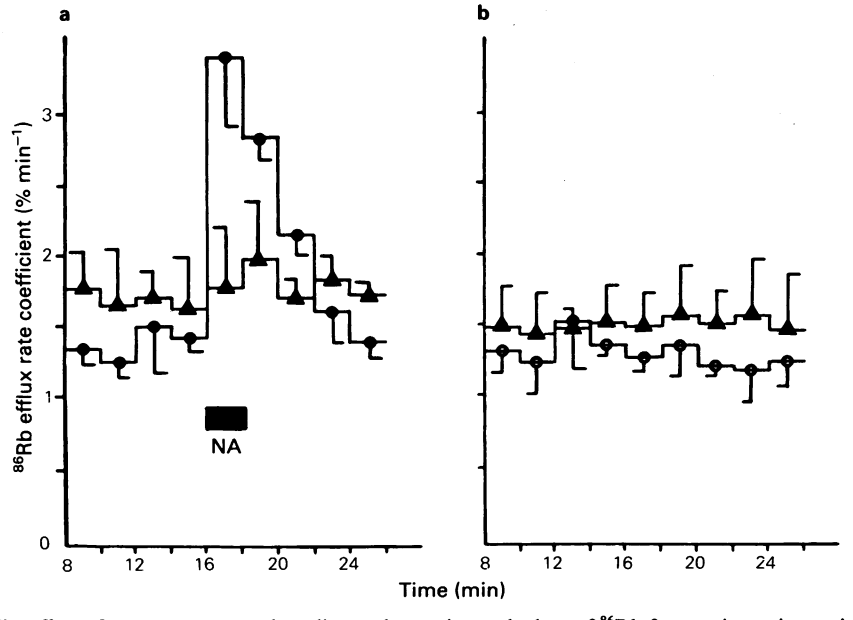

Figure 6 The effect of exposure to noradrenaline and apamin on the loss of ^{86}Rb from guinea-pig taenia caeci. (a) Effect of noradrenaline (NA) 16×10^{-7} M (at solid rectangle) in the absence (●) and presence (▲) of apamin, 10^{-7} M; (b) effect of apamin, 10^{-7} M (▲) compared with control basal ^{86}Rb loss (○). Ordinate scale: ^{86}Rb efflux rate coefficient expressed as a percentage min^{-1}. Abscissa scale: time (min) after start of the efflux period. Each point is the mean derived from 8 experiments; vertical bars show s.e. mean values.

coefficient was raised above the basal level for several collection periods after noradrenaline washout. Apamin, 10^{-7} M prevented this noradrenaline-induced increase in the ^{86}Rb efflux rate coefficient (Figure 6). Isoprenaline, 32×10^{-8} M, had no effect on the ^{86}Rb efflux rate coefficient.

Discussion

The results of the present experiments have allowed the relaxants employed in this study to be divided into three distinct groups.

Isoprenaline was the most potent inhibitory agent and when compared with the papaverine standard, it was capable of producing a full relaxation with a characteristically slow time course. The effect of isoprenaline was not modified by apamin, confirming the results reported by Jenkinson (1981) nor was it associated with an increase in ^{86}Rb efflux, in agreement with Bülbring & den Hertog (1980).

ATP and noradrenaline each produced a very rapid relaxation of spontaneous tone and this was accompanied by an after-contraction which was most marked on washout of these agonists. Although neither agent produced a relaxation as large as the papaverine standard, this could have been an artefact due to the occurrence of the rebound contraction and to the cumulative protocol employed in the tissue-bath experiments.

In the presence of apamin, the relaxant effects of both ATP and noradrenaline were abolished as were the characteristic after-contractions. Only those changes in ^{86}Rb efflux produced by noradrenaline were studied since the rebound contraction in the presence of ATP was so rapid that it would have been difficult to associate any efflux changes with either the inhibitory or the excitatory phase of the response to ATP. The increase in ^{86}Rb efflux produced by noradrenaline and its abolition by apamin support the view of Banks *et al.* (1979) that α-adrenoceptor-mediated inhibition in the guinea-pig taenia caeci (and by inference the inhibitory action of ATP) is mediated by apamin-sensitive potassium channels that are capable of opening at membrane potentials more negative than the resting membrane potential. However, some of the measured increase in ^{86}Rb efflux could have been associated with the after-contraction phase since contractile events in smooth muscle are also accompanied by an increase in ^{86}Rb efflux. This occurs through potassium channels which open at membrane potentials less negative than the resting membrane potential (Bolton & Clapp, 1984; Bolton *et al.*, 1985).

An interesting observation which was not pursued was the slowly-developing inhibitory response to very high concentrations of ATP observed in the presence of apamin. The mechanism underlying this relaxation awaits further study.

Both BRL 34915 and nicorandil each produced a slowly-developing relaxation, not dissimilar in time course to that produced by isoprenaline. No rebound contraction was observed and BRL 34915 was approximately ten times more potent than nicorandil. Both agents were capable of producing a relaxation identical to the papaverine standard and in contrast to isoprenaline, such concentrations produced a significant increase in ^{86}Rb efflux. The relaxations produced by both BRL 34915 and nicorandil and the increase in ^{86}Rb efflux produced by BRL 34915 were unaffected by apamin, excluding the involvement of an apamin-sensitive potassium channel in the inhibitory actions of these two drugs.

The effects of apamin on inhibitory responses in the guinea-pig taenia caeci have been studied by others and the present results are in general agreement with the brief report of Banks *et al.* (1979). A more comprehensive study has been made by the Dutch workers Maas & den Hertog (1979), Maas *et al.* (1980) and den Hertog *et al.* (1984). However, since these groups carried out their experiments at room temperature, in low potassium (2.8×10^{-3} M) PSS and additionally on occasion, in low sodium (24×10^{-3} M) PSS, it is difficult to know how seriously to view any agreement or otherwise between these results and those obtained in the present experiments at 37°C under normal ionic conditions. However, the results of the present study are in agreement with those of the Dutch workers with one exception. At 37°C both the inhibitory responses to ATP and noradrenaline *and* the rebound contractions were blocked by apamin whilst Maas & den Hertog (1979) concluded that these two events were independent phenomena.

Although the rebound contraction did not form a major part of this study, the results obtained may provide some evidence for its mechanism of generation. The phenomenon was not simply associated with relaxation *per se* or with the general opening of potassium channels since it did not accompany the relaxation produced by isoprenaline or the inhibitory responses to BRL 34915 or nicorandil. The rebound contraction was, however, associated with the inhibitory actions of ATP and noradrenaline and is also known to accompany the stimulation of non-adrenergic, non-cholinergic inhibitory nerves (see Small & Weston, 1979). Since these three diversely-mediated events are blocked by apamin, a possible conclusion is that the rebound phenomenon is preceded by the opening of the apamin-sensitive potassium channel. Further work is needed to clarify this matter.

The results obtained with BRL 34915 and nicorandil are in agreement with those recently reported with BRL 34915 in vascular tissues (Hamilton *et al.*, 1986; Weir & Weston, 1986) and with nicorandil in a variety of smooth muscles (Karashima *et al.*, 1982; Inoue *et*

al., 1983; 1984; Kajiwara *et al.*, 1984). They have confirmed that both agents have qualitatively similar actions and that BRL 34915 is more potent than nicorandil. Both drugs cause the opening of potassium channels although an effect at the apamin-sensitive potassium channel has been excluded.

The actions of BRL 34915 and nicorandil are not restricted to smooth muscle; both agents shorten the duration of the cardiac action potential, an effect which has been attributed to the opening of potassium channels (Yanagisawa & Taira, 1980; Imanishi *et al.*, 1983; Cain & Metzler, 1985). Analysis of the channels involved has suggested that nicorandil at least modifies the time-dependent and time-independent potassium channels (Imanishi *et al.*, 1983) which have important repolarizing functions.

Thus it is possible that the relatively sparse electrophysiological results to date offer pointers towards the type of potassium channel affected by these two drugs. Since in cardiac muscle, action potential duration is reduced (Cain & Metzler, 1985; Imanishi *et al.*, 1983) and in smooth muscle, spike potentials are shortened until they disappear (Karashima *et al.*, 1982; Hamilton *et al.*, 1986), a general action of these drugs on repolarizing potassium channels might be indicated. However, the smooth muscle repolarizing potassium channels described by Bolton *et al.* (1985) are normally closed at resting and more negative membrane potentials. Thus the opening of such channels would not explain the hyperpolarizing actions of these drugs in portal veins (Karashima *et al.*, 1982; Hamilton *et al.*, 1986), and by inference in rat aorta (Weir & Weston, 1986).

Such diverse actions could be explained by a dual action of BRL 34915 and nicorandil on both a repolarizing and on a hyperpolarizing potassium channel, effects which, in the case of the taenia caeci, are apamin-insensitive. However, an alternative hypothesis is that these drugs act on the gating mechanism of a single potassium channel to influence the voltage and/or other conditions under which the channel opens (see Siegelbaum & Tsien, 1983). In this way, a repolarizing channel (say) might remain open at resting and more negative potentials giving rise to both spike cessation and variable degrees of hyperpolarization. Experiments are currently under way to test these alternatives.

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