Br. J. Pharmacol. (1988), **94**, 1189–1197

The lack of involvement of cyclic nucleotides in the smooth muscle relaxant action of BRL 34915

John S. Gillespie & Hong Sheng

Department of Pharmacology, University of Glasgow, Glasgow, G12 8QQ, Scotland

1 The relaxant effect of BRL 34915 has been examined on four isolated preparations, the bovine retractor penis (BRP), guinea-pig taenia coli, guinea-pig trachea and rabbit aortic strip contracted by either histamine, carbachol, noradrenaline or 10 mM KCl. Even though the probability of the involvement of external calcium entering through voltage-operated channels in these tissues varied, there was little corresponding variation in sensitivity to BRL 34915.

2 The relaxant effect of BRL 34915 on the BRP and guinea-pig taenia coli was unaffected by haemoglobin 3.3 μM or Apamin 0.5 μM, concentrations which blocked completely the relaxant effect of non-adrenergic, non-cholinergic (NANC) nerve stimulation in these tissues.

3 BRL 34915 in doses causing maximum relaxation did not increase the levels of either cyclic AMP or cyclic GMP in the BRP, although the appropriate enzymes were present and could be stimulated by forskolin or sodium nitroprusside.

4 In the BRP isoprenaline 30 μM acting through β-receptors caused maximum relaxation but did not raise the levels of cyclic AMP, even though lower doses of 2 μM did raise the levels of this nucleotide in the rabbit uterus.

5 These results provide some indirect evidence that membrane hyperpolarization may not be the only cause of the smooth muscle relaxation induced by BRL 34915. However, neither a rise in cyclic AMP nor cyclic GMP are satisfactory alternative mechanisms.

Introduction

BRL 34915 ((±)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo(b) pyran-3-ol), a smooth muscle relaxant, is believed to act through a novel mechanism, the opening of potassium ion channels which hyperpolarizes the membrane, closes voltage-sensitive calcium channels and reduces the influx of calcium, itself the immediate cause of contraction (Hamilton *et al.*, 1986; Weir & Weston, 1986b; Allen *et al.*, 1986). The evidence in support of this explanation is that in the rat portal vein, a spontaneously active tissue, BRL 34915 abolishes spontaneous activity, inhibits the contractions due to noradrenaline (NA) and small increases in potassium ion and hyperpolarizes the membrane potential to values close to the potassium equilibrium potential (Hamilton *et al.*, 1986). BRL 34915 also increases ^{86}Rb efflux from the taenia coli (Weir & Weston, 1986a), rat aorta (Weir & Weston, 1986b) and guinea-pig trachealis (Allen *et al.*, 1986). Blocking potassium channels with tetraethylammonium (TEA), 4 amino-pyridine or procaine reduces the relaxant effect of the drug (Allen *et al.*, 1986). Finally, BRL 34915 had no effect on calcium contractures in 'skinned' trachealis muscle suggesting that it lacks any action on the final contractile machinery (Allen *et al.*, 1986). Such evidence indicates not only that BRL 34915 opens potassium channels but that this is responsible for the mechanical relaxation.

There are, however, some features of the action of the drug not entirely consistent with such a mechanism. For example, BRL 34915 is effective in smooth muscles in which contraction is accompanied by little or no depolarization, e.g. vascular smooth muscle contracted by noradrenaline (Hirst & Neild, 1981) and against agonists believed to utilize mainly intracellular calcium. At moderate doses the relaxant effect may be unaccompanied by any hyperpolarization or increase in ^{86}Rb efflux (Hamilton *et al.*, 1986; Weir & Weston, 1986a,b). It is possible that while the drug does open potassium channels and hyperpolarizes the membrane, this is not the only or even the main mechanism responsible for relaxation. If hyperpolarization and closure of voltage-sensitive calcium channels is responsible for relaxation, then

contractions initiated by depolarization and entry of external calcium should be more sensitive to BRL 34915 than contractions due mainly to the release of intracellular calcium. In this paper we describe the relaxant effect of BRL 34915 on four tissues, the bovine retractor penis (BRP) muscle, the rabbit aortic strip, the guinea-pig tracheal smooth muscle and the guinea-pig taenia coli. Since different receptors utilize extracellular and intracellular calcium to different extents we have used four agonists, histamine acting on H_1-receptors, noradrenaline acting on α-receptors, carbachol acting on muscarinic receptors and a 10 mM rise in potassium concentration. The latter, by depolarizing the muscle will open voltage-sensitive calcium channels and, so long as the increase in external potassium is small, opening membrane potassium channels can still hyperpolarize the muscle. If hyperpolarization underlies relaxation then the taenia coli stimulated by small rises in external potassium should be the most sensitive to the action of BRL 34915 with the others varying in sensitivity in line with the extent to which contraction is dependent on membrane depolarization rather than second messenger formation through, for example, increased phosphoinositol (PI) metabolism.

In all of these muscles isoprenaline is a powerful relaxant presumably by the production of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and in the BRP and aortic strip a rise in cyclic GMP will also cause relaxation (Rapoport & Murad, 1983; Bowman & Drummond, 1984). Methylene blue, a guanylate cyclase inhibitor, has been shown to reduce the increase in potassium permeability produced by BRL 34915 (Caldwell & Howlett, 1986). We have, therefore, measured each cyclic nucleotide to see if BRL 34915 causes a rise in level as an alternative mechanism for its relaxant effect. Finally, haemoglobin and methylene blue are known to block relaxation mediated by guanylate cyclase stimulation in the BRP and the bee venom Apamin blocks inhibition mediated by calcium-operated potassium channels in gut smooth muscle. We have, therefore, examined the effect of these drugs on the relaxant action of BRL 34915 on the BRP and the guinea-pig taenia coli.

Methods

BRP muscles were obtained from the abattoir and normally used within three hours of death. The muscles were cleaned and 2–3 cm long strips 2–3 mm in diameter were dissected out and suspended under 1 g tension from hooks in a 10 ml bath containing Krebs saline at 37°C and gassed with a mixture of 95% O_2 + 5% CO_2. Guinea-pigs were killed with CO_2 gas, the trachea removed and paired rings of tracheal cartilage prepared. The cartilage was cut through in the midline and the preparation suspended by its cartilaginous ends from a hook in a 10 ml bath and under a resting tension of 1 g. Indomethacin 10^{-6} M was added to the Krebs solution for these preparations only, so as to reduce the background tone. Two to three cm lengths of taenia coli were dissected from the guinea-pig caecum and similarly suspended in 10 ml baths under 1 g tension. Finally, aortic strips were prepared from rabbits killed by exposure to CO_2. The abdominal aorta was removed, cleaned and cut into spiral strips 4 mm wide and about 2 cm long. The angle of the cut was approximately 30° to the long axis of the artery. These strips were also suspended under a resting tension of 1 g. In all experiments the composition of the Krebs solution (in mM) was: NaCl 118, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, dextrose 11 and, where noradrenaline was the agonist, 0.23 mM ascorbic acid and 0.06 mM EDTA were added. Drugs were made up from 10^{-3} M stock solutions in distilled water; final dilutions were in normal saline. The maximum volumes added to the bath were 0.3 ml and all final concentrations are the molar concentration of the active drug. In each tissue cumulative dose-response curves to each agonist were constructed and from these a dose selected which produced between 60% and 80% of the maximum contraction. This dose was then used for the spasmogen against which dose-response curves to relaxant drugs were constructed. In the BRP guanethidine 10^{-6} M was used as an indirect sympathomimetic to induce tone. Both cumulative and discrete dose-response curves were used. Inhibition was calculated as the percentage relaxation of spasmogen tone. In each tissue controls were run to demonstrate the persistence of a plateau of contraction for the duration of time needed to construct the relaxant dose-response curve.

Cyclic AMP and cyclic GMP were measured by radioimmunoassay. Tissues were removed at various times after exposure to relaxant drugs, but always after relaxation had reached a maxium, frozen in liquid nitrogen then extracted with 5% trichloracetic acid. Aliquots of the acid-soluble fraction were freed of trichloracetic acid by extracting the samples four times with three volumes of water-saturated diethyl ether. Cyclic AMP in the samples was measured after acetylation with acetic anhydride followed by exposure to goat antiserum to cyclic AMP. Radioactive iodine-labelled cyclic AMP was then added to bind to sites not already occupied by cyclic AMP from the sample and the excess unbound radioactivity adsorbed on activated charcoal. The radioactivity remaining in the supernatant was then measured on a gamma counter and compared with a

Table 1 ID_{50} values (in μM) for relaxation of agonist-induced spasm by BRL 34915

Agonist	*BRP*	*Aorta*	*Trachea*	*Taenia*
NA	0.27 ± 0.04 (15)	0.52 ± 0.11 (19)	—	—
Hist	0.32 ± 0.05 (11)	0.69 ± 0.38 (6)	3.32 ± 1.64 (6)	—
CCh	—	—	9.72 ± 5.16 (6)	1.51 ± 0.38 (8)
KCl	—	—	—	0.65 ± 0.07 (12)

Four tissues, the bovine retractor penis (BRP), rabbit aortic strip, guinea-pig trachea and taenia coli, were contracted with either noradrenaline (NA), histamine (Hist), carbachol (CCh) or potassium chloride. The values are the means ± s.e. of the number of preparations indicated by the number in parentheses. Within any tissue there was no significant difference in the ID_{50} values between agonists, with the exception of the taenia coli where $P < 0.05$.

control curve for known concentrations of cyclic AMP. Cyclic GMP was similarly measured by radioimmunoassay with a kit supplied by Amersham. In this tritium labelled cyclic GMP is used as a competitor for the cyclic GMP in the extracts; the amount of radioactivity bound to the antibody is measured by precipitating the antibody complex with ammonium sulphate, separating the precipitate by centrifugation in a refrigerated centrifuge at 4°C, redissolving the precipitate in water and measuring its radioactivity in a β counter. Statistical comparisons were by Student's t test with a probability of <0.05 considered significant.

Drugs

Haemoglobin solution was made by haemolysing guinea-pig whole blood (Bowman & Gillespie, 1982). The concentration of haemoglobin was measured spectrophotometrically against standard haemoglobin solutions (Sigma) after conversion to methaemoglobin.

The drugs used were Apamin (Sigma), atropine (Sigma), BRL 34915 (Beecham Pharmaceuticals), carbachol (Sigma), forskolin dissolved in dimethylsulphoxide (Sigma), guanethidine sulphate (CIBA), histamine acid phosphate (BDH), indomethacin (Sigma), isoprenaline sulphate (Burroughs Wellcome), noradrenaline bitartrate (Koch-Light), prazosin (Pfizer), sodium nitroprusside (BDH).

Results

Tissue sensitivity to BRL 34915

BRL 34915 relaxed all four tissues. Dose-response curves are shown in Figure 1 and ID_{50} values for 50% inhibition in Table 1. In the BRP the curves for relaxation of histamine- and guanethidine-

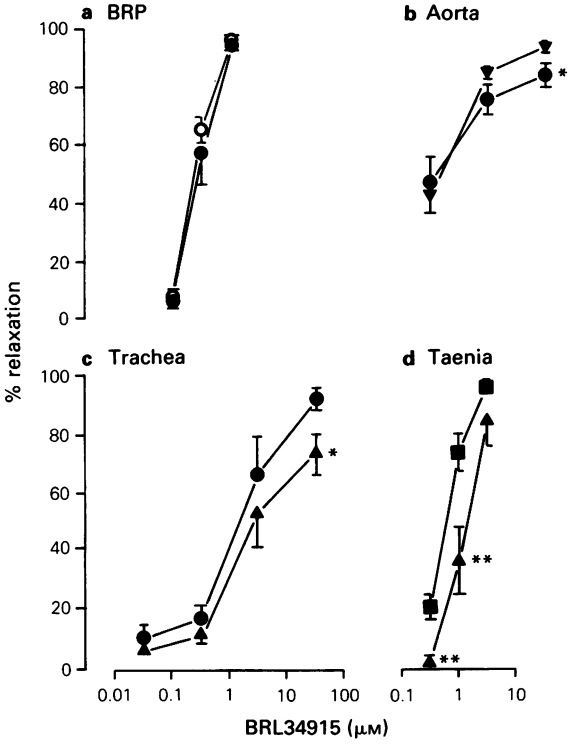

Figure 1 Log dose-response curves for the relaxant effect of BRL 34915 on (a) the bovine retractor penis (BRP) contracted indirectly by noradrenaline (○; guanethidine 1 μM) or histamine (●; 3 μM), (b) the rabbit aortic strip contracted by either noradrenaline (▼; 50 nM) or histamine (●; 3 μM), (c) the guinea-pig tracheal smooth muscle contracted by histamine (●; 3 μM) or carbachol (▲; 0.1 μM) and (d) the guinea-pig taenia coli contracted by KCl (■; 10 mM) or carbachol (▲; 30 nM). Each point is the mean of at least six preparations and the vertical lines indicate s.e. * $P < 0.05$, ** $P < 0.01$.

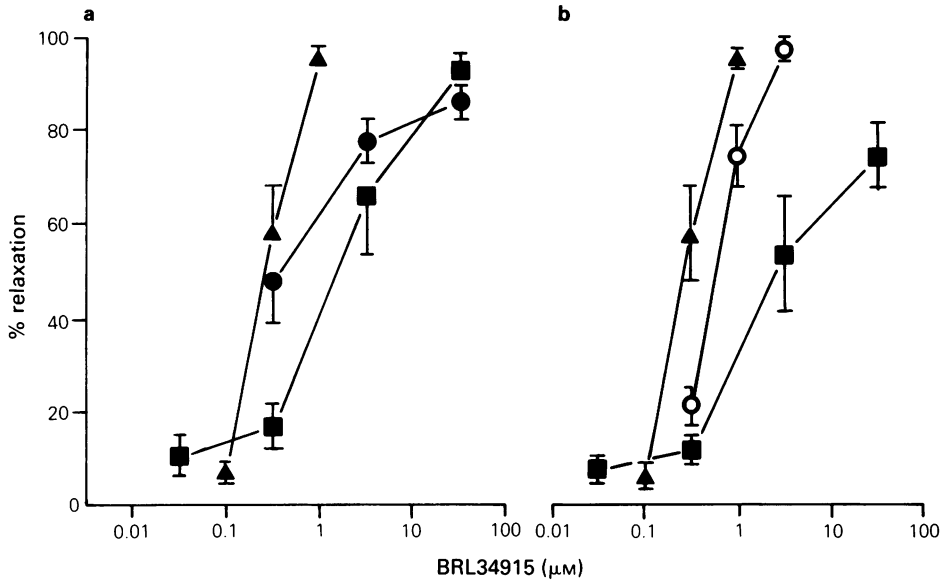

Figure 2 In (a) the dose-response curves for the relaxant action of BRL 34915 have been compared in three tissues, the bovine retractor penis (▲; BRP), guinea-pig trachea (■) and rabbit aortic strip (●), all of them contracted by the same dose of agonist, histamine 3 μM. There was a tissue difference in sensitivity. In (b) the dose-response curve for relaxation in the guinea-pig taenia coli contracted by 10 mM KCl (○) has been compared with the most sensitive preparation, the BRP contracted by noradrenaline (▲), and the least sensitive, the guinea-pig trachea contracted by carbachol (■). Low potassium contractures in the taenia coli are intermediate in sensitivity between the other two tissues. Each point represents the mean and vertical lines indicate s.e.mean.

(noradrenaline)-induced spasm are almost identical, as are the ID_{50} values. In the aortic strip and the guinea-pig trachea there was also no significant difference in the ID_{50} values and at only the highest concentration in the dose-response curve was there a small significant difference. In the taenia coli there was a significant difference between the ID_{50} value for relaxation of potassium-induced tone compared with that for carbachol-induced tone as well as a significant difference between the relaxation at the two lower concentrations in the dose-response curve. Although there was only a limited difference in the sensitivity of different spasmogens to relaxation in any given tissue, there was a greater difference between tissues as measured by the ID_{50} (Table 1).

The BRP was marginally the most sensitive tissue but there was little difference between it and the aorta and taenia coli. However, the trachea was about ten times less sensitive than the BRP. It was not possible to use all of the agonists on each tissue since they did not all produce a contraction. Histamine was generally the most effective agonist and Figure 2 illustrates the dose-response relationships for relaxation by BRL 34915 on three of the tissues, all contracted by 3×10^{-6} M histamine. The relatively low sensitivity of the trachea is clear. Figure 2 also illustrates the extremes of sensitivity to relaxation by BRL 34915. The most sensitive combination was the BRP contracted with noradrenaline and the least sensitive the trachea contracted by carbachol. For comparison the dose-response curve for the taenia coli contracted by 10 mM KCl has been superimposed and, as can be seen in Figure 2, there is no evidence that it is particularly sensitive to the relaxant effect of BRL 34915.

Effect of haemoglobin and Apamin on relaxation

In previous studies (Bowman & Gillespie, 1981; Bowman *et al.*, 1982) we have demonstrated the selective action of haemoglobin and Apamin in blocking relaxation in smooth muscle. In the BRP haemoglobin blocks the response to non-adrenergic, non-cholinergic (NANC) nerve stimulation and Apamin is without effect, whereas the reverse is true in the guinea-pig taenia coli. Haemoglobin inhibits guanylate cyclase and this is probably its mechanism of action in the BRP (Bowman & Drummond, 1984), whereas Apamin blocks calcium-operated potassium channels (Banks *et al.*, 1979). We examined the effect of both agents on the relaxant action of BRL 34915 on the BRP and taenia coli using the relaxant response to NANC nerve stimulation in each as a control. The results are illustrated in Figure 3. In the

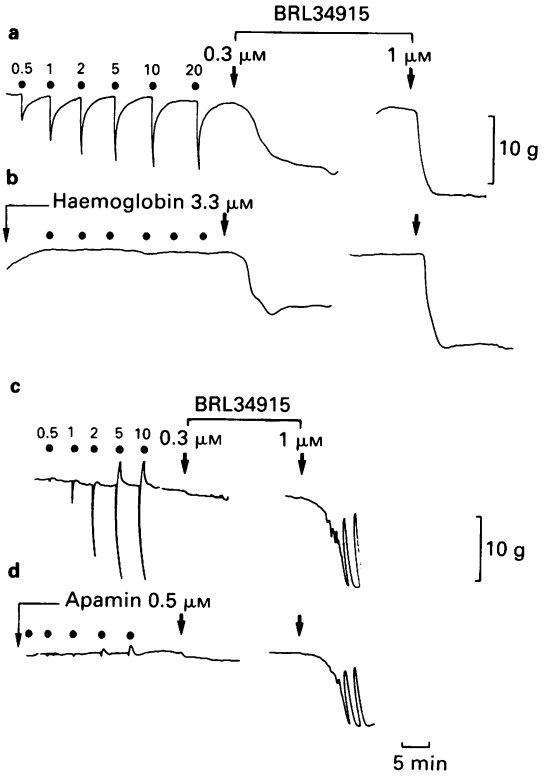

Figure 3 Representative records of the effect of haemoglobin 3.3 μM and Apamin 0.5 μM on the relaxant responses to NANC nerve stimulation and to two concentrations of BRL 34915 in the bovine retractor penis (BRP) and guinea-pig taenia coli. (a) and (b) are from the same experiment on the BRP. (a) Shows the control responses to field stimulation of the NANC nerves (the motor adrenergic nerves were blocked by guanethidine 1 μM). The frequency of stimulation in Hz is shown above each response. The relaxation produced by 0.3 μM and 1 μM BRL 34915 is also shown. Freshly prepared guinea-pig oxyhaemoglobin 3.3 μM was added and (b) shows that this completely blocked the response to nerve stimulation without affecting the response to BRL 34915. (c) and (d) Show a similar experiment but this time on the guinea-pig taenia coli and with Apamin 0.5 μM to block the NANC-mediated muscle relaxation. Again the drug in doses blocking completely the relaxation by nerve stimulation had no effect on the relaxation produced by BRL 34915. In the guinea-pig taenia, tone was raised by 10 mM KCl and the bath contained atropine 3×10^{-7} M and guanethidine 3×10^{-6} M.

BRP haemoglobin 3.3 μM completely abolished the response to NANC nerve stimulation with no effect on the relaxation to BRL 34915. In the taenia coli Apamin similarly abolished the relaxant nerve response again with no effect on the relaxant action of BRL 34915. These results suggested that BRL 34915 does not act either through guanylate cyclase or through calcium-operated potassium channels. The possible involvement of either cyclic GMP or cyclic AMP was investigated more directly by measuring the levels of these second messengers.

The effect on cyclic nucleotide levels

In the BRP, as in the guinea-pig trachealis, isoprenaline and sodium nitroprusside cause a powerful relaxation, the former presumably through β-adrenoceptors coupled to adenylate cyclase and the latter by direct stimulation of guanylate cyclase. In the guinea-pig trachea the proportion of muscle to other tissue elements is small and we have, therefore, used the BRP to see whether BRL 34915 raises the level of either cyclic nucleotide. As controls we used isoprenaline and forskolin to stimulate cyclic AMP production and sodium nitroprusside to stimulate cyclic GMP production. All three control drugs at the highest concentrations used caused complete relaxation of the BRP. The effect of increasing concentrations of BRL 34915 is shown in Figure 4. In concentrations between 3×10^{-7} M and 3×10^{-5} M there was no effect on either nucleotide. Sodium nitroprusside, by contrast, increased cyclic GMP about six fold and forskolin increased cyclic AMP about five fold showing that both synthetic enzymes were present. Surprisingly isoprenaline 3×10^{-5} M, in spite of relaxing the muscle completely, had no effect on the levels of cyclic AMP. These measurements were all made 2 min after adding the relaxant drug to the bath and at a time, at least with the higher concentrations, when relaxation was at its maximum. In case this was not the optimum time for cyclic AMP increase we repeated the experiments using different incubation times; measuring cyclic AMP levels at 4 and 6 min. The results are shown in Figure 5. Neither BRL 34915 nor isoprenaline had any effect on the levels of cyclic AMP. To check the technique we examined the effect of isoprenaline on the levels of cyclic AMP in the rabbit uterus, a tissue in which it is known to increase cyclic AMP; the results are shown in Figure 5. Isoprenaline, as expected, produced an increase in cyclic AMP which was maximal at 6 min.

Discussion

Smooth muscle may be induced to contract or relax through either voltage-dependent or voltage-independent mechanisms (Bolton & Large, 1986). In some smooth muscles, particularly the vas deferens and blood vessels, both mechanisms operate. The preparations in the present experiments range from

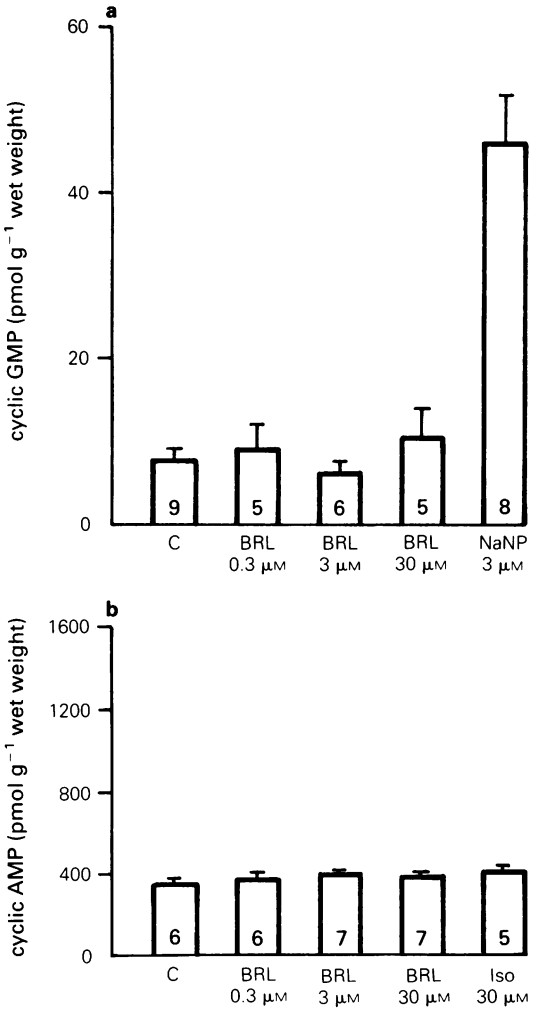

Figure 4 The effect of increasing concentrations of BRL 34915 (BRL) on the levels of cyclic GMP and cyclic AMP in the bovine retractor penis (BRP). (a) Shows the effects of cyclic GMP, (b) the effects on cyclic AMP. Doses of BRL 34915 between 0.3 and 30 μM had no effect on the levels of either nucleotide. The control levels of both cyclic AMP and cyclic GMP in untreated tissues (c) are also shown. By contrast sodium nitroprusside (NaNP) 3 μM increased cyclic GMP by about six times. Isoprenaline (Iso) 30 μM failed to increase cyclic AMP even though it produced complete relaxation in all preparations. The numbers in each column show the number of animals used and the vertical lines the s.e.mean. In the experiments with isoprenaline, prazosin 3×10^{-7} M was added to the bath to block α-adrenoceptors.

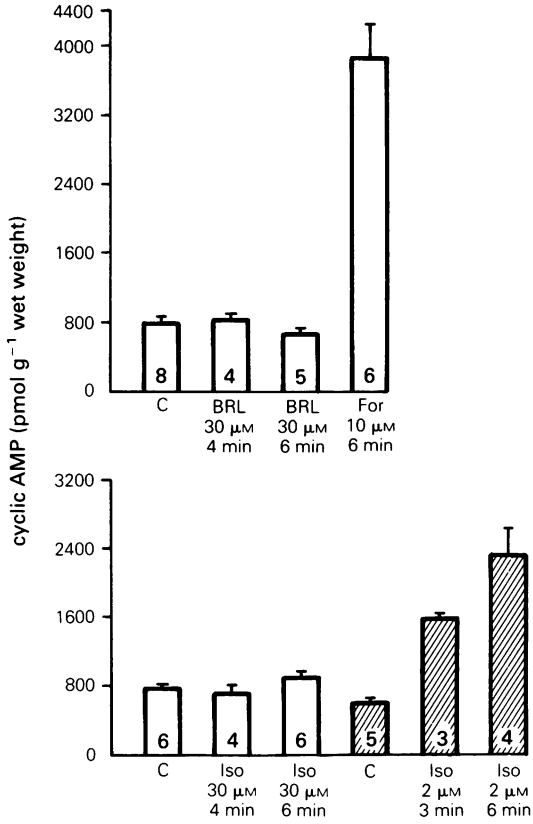

Figure 5 The effects of BRL 34915 (BRL), forskolin (For) and isoprenaline (Iso) in the doses shown on the levels of cyclic AMP in the bovine retractor penis (BRP; open columns). The effect of isoprenaline on the levels of cyclic AMP in the rabbit uterus is also shown (hatched columns). The control levels of cyclic AMP in untreated tissues (C) are also shown. The number of experiments is shown in each column and the vertical lines represent 1 s.e.mean. Neither BRL 34915 nor isoprenaline, each at a concentration of 30 μM, increased the levels of cyclic AMP either after 4 or 6 min exposure. In contrast forskolin at 6 min increased the levels of this nucleotide, approximately five times in the BRP, and isoprenaline in an even lower dose significantly increased the levels at both 3 and 6 min in rabbit uterus. In all experiments with isoprenaline, including controls, the bath contained prazosin 3×10^{-7} M to block α-adrenoceptors.

the taenia coli, which is spontaneously active and responds to both acetylcholine and histamine by depolarization and an increase in spike potential frequency (Bülbring, 1955), through to the BRP and guinea-pig trachea neither of which show spike potentials but whose membrane potential is commonly at a level at which voltage-sensitive calcium channels are open and the tissues develop a steady maintained level of tone, to the rabbit aortic strip which in the absence of agonist has a steady high

membrane potential and no tone. The agonists chosen, noradrenaline, carbachol, histamine and potassium chloride, also vary in their coupling to contraction and the extent to which they utilize the movement of external calcium through voltage-dependent ion channels. Potassium contractions are entirely dependent on membrane depolarization and the opening of voltage-sensitive channels; consistent with this is the observation that a variety of organic calcium antagonists such as verapamil and nifedipine block potassium contractions in several smooth muscles including the guinea-pig and rat tracheal muscle (Cheng & Townley, 1983; Cerrina *et al.*, 1983; Foster *et al.*, 1984; Baba *et al.*, 1985; Nielsen-Kudsk *et al.*, 1986). Both acetylcholine and histamine depolarize guinea-pig tracheal muscle and cause a contraction but these contractions are resistant to the organic calcium antagonists and their magnitude is not dependent on the degree of depolarization (Ahmed *et al.*, 1984). Either entry of calcium through receptor-operated channels or release of intracellular calcium could be responsible for contraction in this tissue but, since removal of external calcium does not abolish contraction (Cerrina *et al.*, 1983; Cheng & Townley, 1983; Ito & Itoh, 1984; Foster *et al.*, 1984; Nielsen-Kudsk *et al.*, 1986), most authors conclude that carbachol and histamine act mainly by releasing intracellular calcium. A likely mechanism is the production of polyphosphoinositides such as inositol triphosphate (IP_3); an increase in IP_3 and phosphatidic acid in dog tracheal muscle stimulated with acetylcholine has been demonstrated (Hashimoto *et al.*, 1985). The mechanism of action of histamine is less clear. In the guinea-pig ileum it increases phosphotidyl-inositol turnover through H_1-receptor stimulation (Jafforji & Michell, 1976) but fails to do so in dog tracheal muscle (Hashimoto *et al.*, 1985). An additional complication is the presence in the guinea-pig lung of H_2-receptors mediating relaxation via activation of adenylate cyclase (Foreman *et al.*, 1986). The action of the fourth agonist, noradrenaline, either added to the bath for the aortic strip or released from nerve endings by gaunethidine in the BRP, is more complex. In arteries this transmitter, released by nerve stimulation in some blood vessels, gives rise to excitatory junction potentials (e.j.ps) and even spike potentials and this effect is insensitive to α-adrenoceptor blocking drugs. In others this fast e.j.p. is followed by a small slow depolarization which is sensitive to α-adrenoceptor blocking drugs. It is the latter effect which appears to be most closely related to contraction (for review see Bolton & Large, 1986). Two explanations for these results have been offered. Either the receptors in the neighbourhood of the nerve endings differ from the α-adrenoceptors elsewhere on the muscle in two respects; firstly, they can open ion channels and depolarize the membrane and, secondly, they are insensitive to α-adrenoceptor blocking agents. This view was first suggested by Hirst & Nield (1981). Alternatively, the nerves liberate a second transmitter, probably ATP which acts at the nerve endings to cause depolarization. Such a view is supported by the observation that desensitization by the stable ATP analogue α,β-methylene ATP reduces or abolishes the e.j.p. in the rat tail artery (Sneddon & Burnstock, 1984) and the rabbit ear artery (Allcorn *et al.*, 1985). In the BRP, noradrenaline liberated by nerve stimulation (Byrne & Muir, 1984) or indirectly by guanethidine, or noradrenaline added to the bath fluid causes small depolarizations and a contraction (Samuelson *et al*, 1983). There is no obvious difference between the effects of guanethidine and noradrenaline in the experiments of Samuelson *et al.* but Byrne & Muir (1984) found that the electrical effects of nerve stimulation were not blocked by prazosin but those of exogenous noradrenaline were.

Given these differences in the mode of action of different agonists and their variable dependence on voltage-operated calcium channels, one would have expected large differences in sensitivity to the relaxant effect of BRL 34915 if its mode of action is to close such voltage-operated channels by hyperpolarization. Little evidence of such variability was found. In particular, the sensitivity of the guinea-pig taenia to potassium sensitivity was neither particularly high nor particularly low. A recent study by Allen *et al.* (1986) found BRL 34915 to be much more effective on spontaneous tone than on tone due to acetylcholine or histamine. Indeed BRL 34915 10^{-5} M had little effect on the dose-response curve to these drugs. Our experiments were carried out differently in that we varied the dose of BRL 34915 rather than the agonist but, if comparable points were taken, then in our experiments BRL 34915 at a concentration of 10^{-5} M produced 60% relaxation whereas Allen *et al.* (1986) found this concentration relatively ineffective with a maximum inhibition of about 30%. A likely explanation for this discrepancy is that the effect of BRL 34915 produces marked desensitization to its own action in the trachea but not in other tissues, a difference we are now investigating.

If these experiments raise some doubts as to whether the sole mode of action of BRL 34915 is through membrane hyperpolarization, our search for an alternative mechanism via either raised cyclic GMP or cyclic AMP levels failed. Concentrations of BRL 34915 producing complete relaxation of the BRP, the tissue most sensitive to the drug, failed to alter the level of either nucleotide, although the controls, with sodium nitroprusside and forskolin, showed the appropriate enzymes were present and capable of producing easily measured rises in the

corresponding cyclic nucleotides. Consistent with this was the failure of haemoglobin, in concentrations which completely blocked the response to NANC inhibitory nerve stimulation in the BRP, to reduce the relaxant action of BRL 34915. We also looked at the effects of methylene blue 10 μM and 30 μM because of an earlier finding that methylene blue 1–100 μM inhibited the increased ^{86}Rb efflux produced by BRL 34915 (Caldwell & Howlett, 1986). In our hands the methylene blue was unselective in its effects on the BRP reducing both inhibitory NANC and motor adrenergic responses almost equally. Cyclic GMP in the rabbit mesenteric artery and both cyclic AMP and cyclic GMP in the rat heart have recently been shown to be unaltered by BRL 34915 (Caldwell & Howlett, 1987).

An unexpected finding was the failure of isoprenaline, in doses producing complete relaxation in the BRP, to cause a rise in the levels of cyclic AMP. This failure is unlikely to be due to technical errors since the rise of this nucleotide with isoprenaline in the rabbit uterus was easily detected. Nor is the duration of exposure to isoprenaline a likely explanation since, in addition to the standard 2 min exposure, we used 4 min and 6 min without success in the BRP, whereas 3 min was satisfactory in the rabbit uterus. There are other accounts in the literature of discrepancies between the relaxant effect of isoprenaline and rises in cyclic AMP. In the bovine trachea and rat uterus propranolol blocks the relaxant effect of isoprenaline but not the rise in cyclic AMP (Palocek & Daniel, 1971; Lau & Lum, 1983). In the rabbit uterus the reverse has been obtained; propranolol blocked the rise in cyclic AMP but not the relaxant effect of isoprenaline (Nesheim *et al.*, 1975). In the rabbit uterus both isoprenaline and prostaglandin E_2 (PGE_2) raised cyclic AMP and activated cyclic AMP-dependent protein kinase but, while isoprenaline caused relaxation, PGE_2 contracted the tissue. During such a contraction the addition of isoprenaline reversed it to relaxation with little change in the cyclic AMP content (Do Khac *et al.*, 1986). The conclusion appears to be that isoprenaline acting through β-receptors can relax smooth muscle by a method other than cyclic AMP elevation. If so, then the BRP seems to be another example of this.

Finally, if BRL 34915 relaxes smooth muscle by opening potassium channels then, in the guinea-pig taenia coli, this is not an Apamin-sensitive channel. These findings confirm recent results of others (Allen *et al.*, 1986; Weir & Weston, 1986a).

In conclusion these results seem to exclude a role for cyclic AMP or cyclic GMP in the smooth muscle relaxant action of BRL 34915. If the relaxant action is due to hyperpolarization following the opening of potassium ion channels these are not Apamin-sensitive and paradoxically the drug appears to be as effective in relaxing spasm due to the internal release of calcium as it is against contractions induced by agonists dependent on external calcium and entry through voltage-sensitive channels. A recent preliminary study suggests hyperpolarization may inhibit such a nifedipine-insensitive contraction (Bray *et al.*, 1988).

H.S. is a University of Glasgow Postgraduate Scholar. The authors gratefully acknowledge support provided by the Medical Research Funds of the University of Glasgow and the ORS Award to H.S.

References

AHMED, F., FOSTER, R.W., SMALL, R.C. & WESTON, A.H. (1984). Some features of the spasmogenic actions of acetylcholine and histamine in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **83**, 227–233.

ALLCORN, R.J., CUNNANE, T.C., MUIR, T.C. & WARDLE, K.A. (1985). Does contraction in the rabbit ear artery require excitatory junction potentials (ejp's) and 'spikes'? *J. Physiol.*, **362**, 30P.

ALLEN, S.L., BOYLE, J.P., CORTIJO, J., FOSTER, R.W., MORGAN, G.P. & SMALL, R.C. (1986). Electrical and mechanical effects of BRL 34915 in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **89**, 395–405.

BABA, K., KAWANISHI, M., SATAKE, T. & TOMITA, T. (1985). Effects of verapamil on the contractions of guinea-pig tracheal muscle induced by Ca, Sr and Ba. *Br. J. Pharmacol.*, **84**, 203–211.

BANKS, B.E.C., BROWN, C., BURGESS, G.M., BURNSTOCK, G., CLARET, M., COCKS, T.M. & JENKINSON, D.H. (1979). Apamin blocks certain neurotransmitter-induced increases in potassium permeability. *Nature*, **282**, 415–417.

BOLTON, T.B. & LARGE, W.A. (1986). Are junction potentials essential? Dual mechanism of activation by transmitter released from autonomic nerves. *Q. J. Exp. Physiol.*, **71**, 1–28.

BOWMAN, A. & DRUMMOND, A.H. (1984). Cyclic GMP mediates neurogenic relaxation in the bovine retractor penis muscle. *Br. J. Pharmacol.*, **81**, 665–674.

BOWMAN, A. & GILLESPIE, J.S. (1981). Differential blockade of non-adrenergic inhibitory mechanisms in bovine retractor penis and guinea-pig taenia caeci. *J. Physiol.*, **317**, 92–93P.

BOWMAN, A. & GILLESPIE, J.S. (1982). Block of some non-adrenergic inhibitory responses of smooth muscle by a substance from haemolysed erythrocytes. *J. Physiol.*, **328**, 11–25.

BOWMAN, A., GILLESPIE, J.S. & POLLOCK, D. (1982). Oxyhaemoglobin blocks non-adrenergic non-cholinergic inhibition in the bovine retractor penis muscle. *Eur. J. Pharmacol.*, **85**, 221–224.

BRAY, K.M., WESTON, A.H., MCHARG, A.D., NEWGREEN, D.T. & SOUTHERTON, J.S. (1988). Comparison of the

inhibitory effects of BRL 34915 and nifedipine on noradrenaline- and KCl-induced contractions in rabbit aorta. *Br. J. Pharmacol. Proc. Suppl.*, **93**, 205P.

BÜLBRING, E. (1985). Correlation between membrane potential, spike discharge and tension in smooth muscle. *J. Physiol.*, **128**, 200–221.

BYRNE, N.G. & MUIR, T.C. (1984). Electrical and mechanical responses of the bovine retractor penis to nerve stimulation and to drugs. *J. Auton. Pharmacol.*, **4**, 261–271.

CALDWELL, M.C. & HOWLETT, D.R. (1986). BRL 34915 induced potassium channel activation in rabbit isolated mesenteric artery is not calcium dependent. *Br. J. Pharmacol.*, **88**, 443P.

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

CERRINA, J., ADRENIER, P., RENIER, A., FLOCH, A. & DUROUX, P. (1983). Effects of diltiazem and other Ca^{2+} antagonists on guinea-pig tracheal muscle. *Eur. J. Pharmacol.*, **94**, 241–249.

CHENG, J.B. & TOWNLEY, R.G. (1983). Pharmacological characterisation of effects of nifedipine on isolated guinea-pig and rat tracheal smooth muscle. *Archs Int. Pharmacodyn.*, **263**, 228–244.

DO KHAC, L., MOKHTARI, A. & HARBON, S. (1986). A re-evaluated role for cyclic AMP in uterine relaxation. Differential effect of isoproterenol and forskolin. *J. Pharmacol. Exp. Ther.*, **239**, 236–242.

FOREMAN, J.C., NORRIS, D.B., RISING, T.J. & WEBBER, S.E. (1986). A study of the H_2 receptor for histamine stimulating adenylate cyclase in homogenates of guinea-pig lung parenchyma. *Br. J. Pharmacol.*, **87**, 37–44.

FOSTER, R.W., OKPALUGO, B.I. & SMALL, R.C. (1984). Antagonisms of Ca^{2+} and other actions of verapamil in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **81**, 499–507.

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.

HASHIMOTO, T., HIRATA, M. & ITO, Y. (1985). A role for inositol 1,4,5 triphosphate in the initiation of agonist-induced contractions of dog tracheal smooth muscle. *Br. J. Pharmacol.*, **86**, 191–199.

HIRST, G.D.S. & NIELD, T.O. (1981). Localisation of specialised noradrenaline receptors at neuromuscular junctions on arterioles of the guinea-pig. *J. Physiol.*, **313**, 343–350.

ITO, Y. & ITOH, T. (1984). The roles of stored calcium in contractions of cat tracheal smooth muscle produced by electrical stimulation, acetylcholine and high K^+. *Br. J. Pharmacol.*, **83**, 667–679.

JAFFORJI, S.S. & MICHELL, R.H. (1976). Stimulation of phosphatidylinositol turnover in various tissues by cholinergic and adrenergic agonists, by histamine and by caerulein. *Biochem. J.*, **182**, 669–676.

LAU, Y.S. & LUM, B.K.B. (1983). role of cyclic AMP in adrenergically-induced tracheal muscle relaxation. *Archs Int. Pharmacodyn.*, **261**, 36–50.

NESHEIM, B.-I., OSNES, J.-B. & OYE, I. (1975). Role of cyclic adenosine 3'5'-monophosphate in the isoprenaline-induced relaxation of the oestrogen dominated rabbit uterus. *Br. J. Pharmacol.*, **53**, 407.

NIELSEN-KUDSK, J.E., KARLSSON, J.A. & PERSSON, C.G.A. (1986). Relaxant effects of Xanthines, a β receptor agonist and Ca^{2+} antagonists in guinea-pig tracheal preparations contracted by potassium or carbachol. *Eur. J. Pharmacol.*, **128**, 33–40.

PALOCEK, I. & DANIEL, E.E. (1971). Effect of α- and β-adrenergic stimulation on the uterine motility and adenosine 3'5'-monophosphate level. *Can. J. Physiol. Pharmacol.*, **49**, 988–998.

RAPOPORT, R.M. & MURAD, F. (1983). Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: role of cyclic GMP. *J. Cyclic Nucleotide & Protein Phosphorylation Res.*, **9**, (4–5), 281–296.

SAMUELSON, U., SJÖSTRAND, N.O. & KLINGE, E. (1983). Correlation between electrical and mechanical activity in myogenic and neurogenic control of the bovine retractor penis muscle. *Acta Physiol. Scand.*, **119**, 335–345.

SNEDDON, P. & BURNSTOCK, G. (1984). ATP as co-transmitter in rat tail artery. *Eur. J. Pharmacol.*, **106**, 149–152.

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.

(Received August 1, 1987
Revised March 10, 1988
Accepted April 13, 1988)