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Effects of a novel nonpeptide bradykinin B_2 receptor antagonist on intestinal and airway smooth muscle: further evidence for the tracheal B_3 receptor

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1 We examined the effects of phosphonium, [[4-[[2-[[*bis*(cyclohexylamino)methylene] amino]-3-(2-naphthalenyl) 1-oxopropyl]amino]-phenyl]-tributyl, chloride, monohydrochloride (WIN 64338), a novel, nonpeptide bradykinin B_2 receptor antagonist, on bradykinin-induced contractions of guinea-pig isolated ileum, and guinea-pig and ferret trachea.

2 WIN 64338 potently and competitively antagonized ileal contractions, in response to bradykinin, exhibiting a pA_2 value of 7.97 ± 0.10. The compound was without effect on contractions elicited by methacholine, a muscarinic receptor antagonist. Thus, WIN 64338 is a competitive and selective antagonist of ileal B_2 receptors.

3 In contrast, WIN 64338 was completely without effect on bradykinin-induced contractions of guinea-pig or ferret trachea. Thus, even at a concentration of 1 μM, which was sufficient to cause a 100 fold decrease in ileal sensitivity to bradykinin, WIN 64338 failed to shift the bradykinin log concentration-response curves in trachea isolated from either species.

4 These data confirm that WIN 64338 represents the first reported nonpeptide antagonist of guinea-pig ileal B_2 receptors. They also provide additional evidence for heterogeneity of bradykinin receptors within the same species (guinea-pig) and, furthermore, indicate that the tracheal bradykinin receptor (B_3?) is different from that in ileal tissue (B_2).

Keywords: Bradykinin; bradykinin receptors; B_2 receptor; B_3 receptor; nonpeptide; antagonist; WIN 64338; ileum; trachea; smooth muscle

Introduction

Bradykinin receptors are typically subdivided into B_1 and B_2 subtypes, based on the rank orders of potencies of agonists and antagonists (reviewed in Farmer & Burch, 1992; Hall, 1992). For example, B_2 receptors are preferentially activated by bradykinin, [Tyr(Me)[8]]-bradykinin and kallidin, and are antagonized by analogues of [D-Phe[7]]-bradykinin (Burch *et al.*, 1990). Conversely, desArg[9]-bradykinin is a specific B_1 receptor agonist and desArg[9]-[Leu[8]]-bradykinin, a B_1 receptor antagonist (Farmer & Burch, 1992; Hall, 1992).

A few years ago, it was reported that bradykinin-induced contraction of guinea-pig trachea was very weakly inhibited by B_1 or B_2 receptor antagonists (Farmer *et al.*, 1989b). Furthermore, we found that these antagonists did not displace [^{3}H]-bradykinin binding from guinea-pig and sheep tracheal membranes, and did not inhibit bradykinin-induced $^{45}Ca^{2+}$ efflux from tracheal smooth muscle (Farmer *et al.*, 1991b). This led to the proposal that guinea-pig tracheal smooth muscle cells may express a novel B_3 receptor (Farmer *et al.*, 1989b; 1991b). Interestingly, Field and colleagues (1992) noted that several analogues of [D-Phe[7]]-bradykinin did inhibit [^{3}H]-bradykinin binding in guinea-pig trachea. The reason for these contrasting binding results is unknown.

More recently, Pyne & Pyne (1993) reported that bradykinin-stimulated phospholipase C activity in guinea-pig trachea was inhibited by D-Arg-[Hyp[3],D-Phe[7]]-bradykinin (NPC 567), a B_2 receptor antagonist, whereas activation of phospholipase D was unaffected. These investigators proposed that guinea-pig tracheal smooth muscle cells, maintained in tissue culture, express both B_2 and B_3 receptors. We also reported that bradykinin-induced prostaglandin biosynthesis in the same cell type was inhibited by NPC 567 (Farmer *et al.*, 1991b).

Until recently, B_2 receptor antagonists, including those utilized in the studies discussed above, were peptides that exhibited weak affinity (pK_b values of 6–7) for B_2 receptors and, often, displayed agonist-like activity in isolated smooth muscle preparations (Burch *et al.*, 1990). The discovery of a series represented by D-Arg-[Hyp[3],Thi[5],D-Tic[7],Oic[8]]-bradykinin (Hoe 140) (Hock *et al.*, 1991; Lembeck *et al.*, 1991) and D-Arg-[Hyp[3], D-HypS(transphenyl)[7],Oic[8]]-bradykinin (NPC 17761) (Kyle *et al.*, 1991; Burch & Kyle, 1992) yielded B_2 receptor antagonists with affinities two to three orders of magnitude greater than earlier agents. Several of these novel peptides, including Hoe 140 (Field *et al.*, 1992; Trifilieff *et al.*, 1992), NPC 17761 (Trifilieff *et al.*, 1993) and D-Arg-[Hyp[3], Thi[5],D-Tic[7],Tic[8]]-bradykinin (NPC 16731) (Farmer *et al.*, 1991a), potently antagonize bradykinin-induced responses of guinea-pig trachea. Thus, these novel agents may be antagonists of both B_2 and the putative B_3 receptors (Farmer *et al.*, 1991a; Perkins *et al.*, 1991; Pyne & Pyne, 1993).

WIN 64338 (phosphonium, [[4-[[2-[[*bis*(cyclohexylamino) methylene]amino]- 3- (2-naphthalenyl) 1-oxopropyl]amino]-phenyl]-methyl]-tributyl, chloride, monohydrochloride) is a recently discovered, nonpeptide, competitive antagonist of B_2 receptors in human lung fibroblasts and guinea-pig ileum (Sawutz *et al.*, 1993; Salvino *et al.*, 1993). In the present study, we examined the effects of WIN 64338 on the sensitivity of guinea-pig isolated ileum and trachea to bradykinin. Since ferret trachea contracts in response to bradykinin, we also investigated the effect of WIN 64338 in this tissue.

Methods

Tissue preparation

Male Dunkin-Hartley guinea-pigs (250–350 g) (Hazelton, PA, U.S.A) were killed by stunning and exsanguination.

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Transverse strips of epithelium-denuded trachea, and strips of terminal ileal longitudinal smooth muscle were prepared essentially as described previously (Farmer *et al.*, 1986; 1989a). Male ferrets (1.5–2.5 kg; Marshall Farms, PA), were killed with sodium pentobarbitone, (80 mg kg^{-1}, i.p.). The chest was opened along its midline, and the trachea was removed, cleared of surrounding fat and connective tissue, cut longitudinally along its ventral surface and divided into transverse strips containing 2–3 cartilaginous rings. The epithelium was not removed from ferret trachea as this procedure has been reported not to alter sensitivity to bradykinin (Dusser *et al.*, 1988).

Each tissue was suspended, under a resting tension of 1.5 g (guinea-pig trachea and ileum) or 3 g (ferret trachea), in a 4 ml water-jacketed tissue bath containing modified Krebs-Henseleit solution (mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 10.0) maintained at 37°C, and gassed with 5% CO_2 in O_2. Experiments with ileum and ferret trachea were conducted in buffer containing indomethacin (3 μM), tetrodotoxin (1 μM) and (+)-chlorpheniramine (1 μM). Indomethacin, which inhibits bradykinin-induced contraction of guinea-pig trachea (Farmer *et al.*, 1989b), was omitted from experiments with this tissue. Tissues were equilibrated for at least 60 min and the buffer replaced every 15 min. After this period, each preparation was exposed to methacholine (MCh, 10 μM) to ascertain tissue viability and obtain a reference contraction. After washout and relaxation to baseline tension, experiments were started.

Experimental protocols

For guinea-pig tissues, log concentration-response curves to bradykinin (10^{-10}–10^{-3} M) were obtained following its addition in a noncumulative fashion. After each agonist response was obtained, tissues were washed at least three times with fresh buffer until initial resting tension was recovered. Log concentration-response curves to bradykinin in ferret trachea were constructed after cumulative addition of the peptide to the bath. To obviate possibly confounding effects of tachyphylaxis (Farmer *et al.*, 1989a), only one curve was obtained in each ileal or tracheal preparation. Where appropriate, WIN 64338 was added to the bath 20 min prior to exposure to agonists. Although only one antagonist concentration was tested in a single tissue, several concentrations of WIN 64338 could be examined in tissues from the same animal. In order to test the selectivity of WIN 64338, its effect on ileal sensitivity to MCh was tested. At the end of each experiment, MCh (10 mM) was added to the bath, and all tissue responses expressed as a percentage of this maximum contraction.

Agonist pD_2 values were obtained from regression analyses of logit-transformed log concentration-response curves (InPlot, GraphPad Software, San Diego, CA). EC_{50} ratios for bradykinin in the absence and presence of WIN 64338 were calculated, and pA_2 values for the antagonist were obtained from Schild plots. All data are expressed as mean ± s.e.mean, and data between different experimental groups were compared by analysis of variance (InStat, GraphPad Software, San Diego, CA, U.S.A.).

Drugs

Bradykinin was purchased from Bachem California (Torrance, CA, U.S.A.), and acetyl-β-methylcholine chloride (methacholine, MCh), indomethacin, (+)-chlorpheniramine maleate, and tetrodotoxin were obtained from Sigma (St. Louis, MO, U.S.A.). WIN 64338, which was a kind gift from Dr David G. Sawutz, Sterling-Winthrop Pharmaceuticals (Collegeville, PA, U.S.A.), was prepared as a 30 mM stock solution in dimethyl sulphoxide (DMSO). Indomethacin was prepared as a 30 mM stock solution in 100 mM Na_2CO_3 and stored at −20°C until required. All other drugs were dissolved extemporaneously in 0.9% w/v NaCl solution (saline), and all drug dilutions were made in saline.

Results

Guinea-pig ileum

Bradykinin elicited concentration-dependent contractions in this tissue, demonstrating a pD_2 value of 8.51 ± 0.13 (n = 13). WIN 64338 caused concentration-dependent right shifts in bradykinin concentration-response curves with no significant depression in maximum response (Figure 1a). The antagonist had no spasmogenic effects at any concentration tested. WIN 64338 was a competitive B_2 receptor antagonist in guinea-pig ileum, yielding a straight line Schild plot whose slope was 1.09 ± 0.19, and not significantly different from unity (Figure 1b). The pA_2 value for WIN 64338, determined from the intercepts of regression lines of individual Schild plots through the abscissa scale, was 7.97 ± 0.10 (n = 4–7) (Figure 1b). Even at a concentration of 1 μM, WIN 64388 was without effect on ileal sensitivity to MCh (Figure 2).

Guinea-pig and ferret trachea

At a concentration of 1 μM, which was sufficient to decrease ileal sensitivity to bradykinin by more than two log units, WIN 64338 was without effect on guinea-pig tracheal sensitivity to the kinin (Figure 3a). Similarly, WIN 64338 did

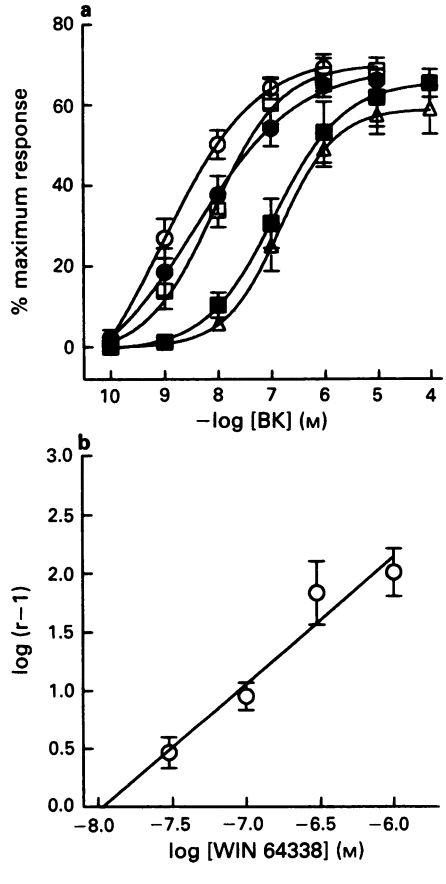

Figure 1 (a) Log concentration-response curves for bradykinin (BK) in guinea-pig isolated ileum in the absence (○) and presence of the B_2 receptor antagonist, WIN 64338: (●) 30 nM; (□) 100 nM; (■) 300 nM; (△) 1 μM. (b) Schild plot for antagonism of bradykinin-induced ileal contractions by WIN 64338. Slope was 1.09 ± 0.19, and the intercept of the abscissa was 7.97. Data are expressed as the mean ± s.e.mean of 4–13 observations in tissues from different animals.

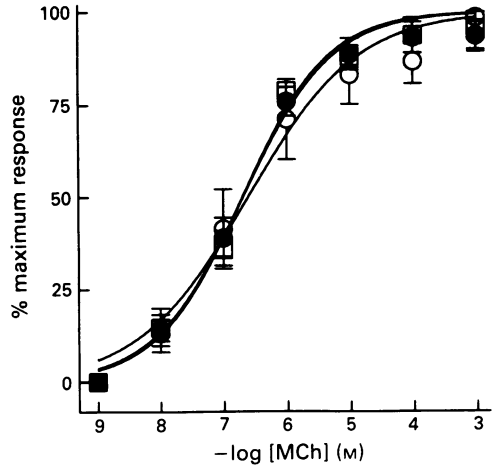

Figure 2 Log concentration-response curves for methacholine (MCh) in guinea-pig isolated ileum in the absence (○) and presence of the B_2 receptor antagonist, WIN 64338 100 nM (●); 1 μM (□). Data are expressed as the mean ± s.e.mean of four observations in tissues from different animals.

not significantly alter the sensitivity of ferret isolated trachea to bradykinin (Figure 3b).

Discussion

We examined the effects of WIN 64338, the first described nonpeptide antagonist of bradykinin B_2 receptor (Sawutz *et al.*, 1993; Salvino *et al.*, 1993), in guinea-pig ileum and guinea-pig and ferret trachea. This drug was reported recently to inhibit competitively bradykinin-induced contraction of guinea-pig ileum (Sawutz *et al.*, 1993). In the present study, we have confirmed that WIN 64338 is a competitive antagonist of bradykinin-induced contraction of guinea-pig ileum with a potency (pA_2 7.97) essentially identical to that reported by Sawutz and colleagues (1993) (pA_2 8.20). It has also been shown that WIN 64338 is selective for bradykinin receptors in that it had considerably lower activity at muscarinic receptors (Sawutz *et al.*, 1993; Salvino *et al.*, 1993). Furthermore, the drug appeared specific for B_2 receptors in that it was inactive as a B_1 receptor antagonist in rabbit aorta (Sawutz *et al.*, 1993). We also found that WIN 64338 was devoid of antagonist effects against methacholine-induced contractions of ileum.

In initial studies with WIN 64338, there appeared to be some species or, perhaps, tissue differences in its bradykinin receptor antagonist potencies (Sawutz *et al.*, 1993). For example, the antagonist was approximately ten times more potent against bradykinin-induced guinea-pig ileal contraction than against $^{45}Ca^{2+}$ efflux in foetal human lung fibroblasts (IMR 90 cells) (Sawutz *et al.*, 1993). In binding experiments in IMR 90 cells, WIN 64338 had a pK_i value of 6.19, and this is significantly less potent than its pK_i in guinea-pig ileum binding (8.10 ± 0.23, $n = 4$, unpublished data). Indeed, in preliminary experiments, we also found that WIN 64338 was around tenfold less potent in displacing [^{3}H]-bradykinin binding from human B_2 receptors expressed in murine erythroleukemia cells (pK_i 7.01 ± 0.15, $n = 4$, unpublished data) than in guinea-pig ileum. These data suggest that the guinea-pig ileal B_2 receptor has a higher affinity for WIN 64338 than human B_2 receptors. This may be indicative of species differences in B_2 receptors.

Alternatively, the different affinities for this novel antagonist may result from heterogeneity of bradykinin receptors in different tissues. In the present study, WIN 64338 displayed typical competitive antagonist activity in guinea-pig ileum.

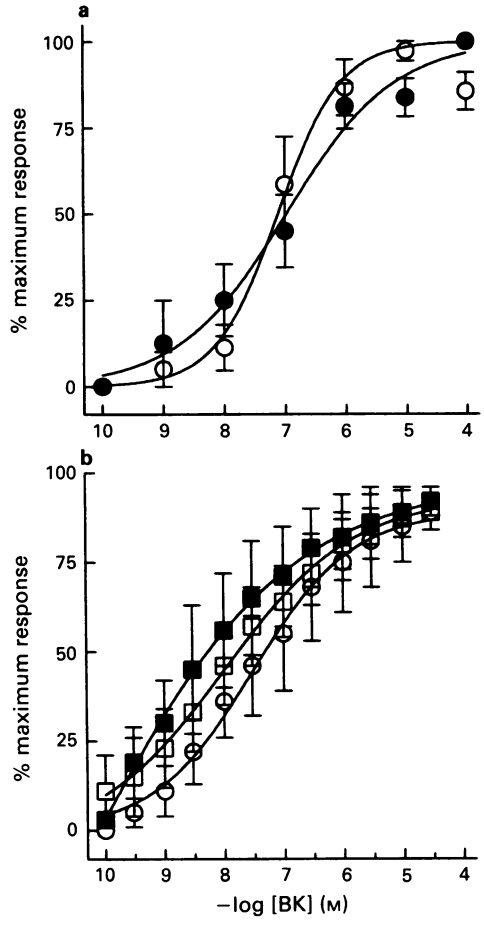

Figure 3 (a) Log concentration-response curves for bradykinin in guinea-pig isolated trachea in the absence (○) and presence of the B_2 receptor antagonist, WIN 64338 (●) 1 μM. (b) Log concentration-response curves for bradykinin in ferret isolated trachea in the absence (○) and presence of WIN 64338: (□) 1 μM; (■) 10 μM. Data are expressed as the mean ± s.e.mean of four observations in tissues from different animals.

The drug potently inhibited bradykinin-induced contractions, yielding a Schild slope of unity. Moreover, there was no decrease in the maximum contractile response to bradykinin, no partial agonist effects were evident even at high concentrations, and WIN 64338 had no effect on contractions in response to a muscarinic receptor agonist. Yet, even at a concentration (1 μM) that shifted the bradykinin log concentration-response curve 100 fold to the right in ileum, WIN 64338 was inactive as an antagonist of bradykinin-induced contractions in guinea-pig or ferret trachea.

Previous studies noted that several [D-Phe^7]-substituted analogues of bradykinin, which are B_2 receptor antagonists (Burch *et al.*, 1990), did not displace [^{3}H]-bradykinin binding in guinea-pig and sheep tracheal smooth muscle membranes (Farmer *et al.*, 1989b), and were very weak or inactive as inhibitors of bradykinin-induced tracheal contraction (Farmer *et al.*, 1989b; Perkins *et al.*, 1991). In addition, in guinea-pig tracheal smooth muscle cells, bradykinin-stimulated efflux of $^{45}Ca^{2+}$ (Farmer *et al.*, 1991b) and phospholipase D activity (Pyne & Pyne, 1993) are not affected by NPC 567, a peptide B_2 receptor antagonist. These data led to the proposal that tracheal smooth muscle may express B_3 receptors (reviewed by Farmer & Burch, 1991, 1992).

Newer, more potent bradykinin receptor antagonists such as NPC 16731 (Farmer *et al.*, 1991a), Hoe 140 (Field *et al.*, 1992; Trifilieff *et al.*, 1992), and NPC 17761 (Trifilieff *et al.*, 1993) potently antagonize bradykinin-induced responses of

guinea-pig trachea. Thus, these peptides may represent antagonists of B_2 and B_3 receptors. Conversely, it has been suggested that effects of bradykinin in guinea-pig trachea are not mediated by receptors at all, and this possibility was cited as the reason for the lack of effect of NPC 567 and other similar antagonists (Regoli & Nantel, 1990; Rhaleb *et al.*, 1992). This proposal is fallacious since, as noted above, Hoe 140, NPC 17761 and NPC 16731 do inhibit bradykinin-induced tracheal contraction. Because of the observation that Hoe 140 is much less potent as an antagonist of guinea-pig tracheal (and intestinal) responses to bradykinin than in tissues from other species (Rhaleb *et al.*, 1992; Field *et al.*, 1992), it has also been proposed that guinea-pig B_2 receptors are 'different' from B_2 receptors in other species (Regoli *et al.*, 1992; Rhaleb *et al.*, 1992). It has even been suggested that '. . . the proposed B_3 receptor might perhaps be regarded as a B_2 receptor from a guinea-pig' (Field *et al.*, 1992).

The present study may refute these arguments. WIN 64338 has been demonstrated to be a potent antagonist of B_2 receptors in human and guinea-pig tissues, albeit with approximately 10 fold differences in potency (Sawutz *et al.*, 1993; Salvino *et al.*, 1993; present study). Nevertheless, this agent was completely devoid of antagonist activity in tracheal preparations isolated from guinea-pigs or ferrets. These results indicate strongly that the bradykinin receptor subtypes expressed in tracheal smooth muscle from these two species differ from those expressed in guinea-pig ileum or human lung fibroblasts (B_2). Thus, like trachea from guinea-pigs, that from ferrets also appears to express a novel bradykinin receptor. In the absence of a drug that specifically inhibits bradykinin-induced tracheal contraction, however, the existence of the B_3 receptor remains to be proven.

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