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Inhibition of bradykinin-evoked trigeminal nerve stimulation by the non-peptide bradykinin B_2 receptor antagonist WIN 64338 *in vivo* and *in vitro*

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1 This study investigated the effect of the recently described non-peptide bradykinin B_2 receptor antagonist, WIN 64338 ([[4-[[2-[[bis(cyclohexylamino)methylene]amino]-3-(2-naphthalenyl)-1-oxopropyl] amino]phenyl]methyl]tributylphosphoniumchloride monohydrochloride), in experimental models of bradykinin-evoked sensory nerve stimulation.

2 In the rabbit isolated iris sphincter *in vitro*, bradykinin-evoked contractile responses are mediated via tachykinins released from peripheral endings of the trigeminal sensory nerve. WIN 64338 (1-10 μM) competitively antagonised contractile responses to bradykinin with a pK_B estimate of 6.6 ± 0.1 ($n=11$). The antagonism was selective since WIN 64338 (10 μM) did not significantly inhibit submaximal contractile responses to the direct-acting spasmogens substance P (10 nM), neurokinin A (3 nM), substance P methyl ester (10 nM) or senktide (100 nM); nor by sensory non-adrenergic non-cholinergic nerve stimulation evoked by capsaicin (10 μM), or electrical field-stimulation (3, 10, 30 Hz) ($P>0.05$; $n=3-11$).

3 Topical application of bradykinin to the conjunctiva and to the nasal mucosa of the guinea-pig *in vivo* causes plasma extravasation predominantly via the release of tachykinins from peripheral endings of the trigeminal nerve. The increases in plasma extravasation (measured by extravasation of Evans blue dye) induced by bradykinin in the guinea-pig conjunctiva (20 nmol) and nasal mucosa (50 nmol) were markedly reduced (by $81 \pm 3\%$ and $69 \pm 5\%$, respectively) following pretreatment with WIN 64338 (30 nmol kg^{-1}, i.v.) ($n=5-6$; $P<0.05$), with almost complete inhibition at a higher dose of WIN 64338 (300 nmol kg^{-1}, i.v.; $n=5-6$). This inhibition was selective since at 300 nmol kg^{-1}, WIN 64338 did not inhibit plasma extravasation evoked by substance P in the conjunctiva (5 nmol; $P>0.05$; $n=6$) or in the nasal mucosa (50 nmol; $P>0.05$; $n=5$).

4 This study demonstrates that WIN 64338 is a selective and competitive bradykinin B_2 receptor antagonist and can be useful for analysing bradykinin-evoked trigeminal nerve stimulation both *in vitro* and *in vivo*.

Keywords: Bradykinin; kinin; sensory nerves; trigeminal; B_2-receptors; iris sphincter; plasma extravasation; WIN 64338; bradykinin antagonist

Introduction

Kinins, including bradykinin and kallidin, are a family of small endogenous peptide mediators formed *de novo* at sites of tissue damage where they have important actions that contribute to the acute inflammatory response (see Regoli & Barabé, 1980; Hall, 1992; Bhoola *et al.*, 1992; Geppetti, 1993). The classical proinflammatory effects of kinins include actions on the vascular endothelium and smooth muscle where they influence vascular permeability and vasomotor tone, and on primary afferent C- and Aδ-neurones where they stimulate or sensitize the neurones to elicit pain and hyperalgesia (Dray & Perkins, 1993; Fox *et al.*, 1993; see Hall, 1992). There is however, a growing appreciation that kinins can also contribute to the inflammatory response by stimulating the release of tachykinins and calcitonin gene-related peptide (CGRP) from peripheral endings of sensory nerves (neurogenic inflammation; see Geppetti, 1993). For example, plasma extravasation following antigen challenge in the airways of immunized guinea-pigs is inhibited both by bradykinin- and tachykinin-receptor antagonists (Bertrand *et al.*, 1993a,b).

Kinins mediate the majority of their biological effects via stimulation of B_1 and B_2 receptors (see Regoli & Barabé, 1980; Hall, 1992) and, in general, the acute proinflammatory effects of kinins are mediated via stimulation of the B_2 receptor type (Hall, 1992; Geppetti, 1993). Recently, Salvino and colleagues (Salvino *et al.*, 1993; Sawutz *et al.*, 1994) announced the development of the first relatively high affinity non-peptide bradykinin B_2 receptor antagonist, WIN 64338, ([[4-[[2-[[bis(cyclohexylamino)methylene]amino]-3-(2-naphthalenyl)-1-oxopropyl]amino]phenyl]methyl]tributylphosphoniumchloride monohydrochloride). In this present study, we used WIN 64338 to investigate bradykinin-evoked stimulation of trigeminal sensory nerves in three animal models: contraction of the rabbit iris sphincter *in vitro*, and plasma extravasation in the guinea-pig conjunctiva and nasal mucosa *in vivo*. The aim of our study was to determine the nature of the bradykinin receptors involved in trigeminal nerve stimulation, and to investigate the usefulness of WIN 64338 in studying the role of kinins *in vitro* and *in vivo*.

Methods

Rabbit iris sphincter

Male New Zealand albino rabbits (2.5-3.0 kg) were killed by an i.v. overdose of pentobarbitone sodium. The eyes were enucleated immediately after death and opened by a midline incision 2-3 mm dorsal to the limbus, followed by excision of

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the iris from the ciliarly margin. The sphincter pupillae muscles were dissected free of dilator muscle and mounted intact on stainless-steel tissue holders in 2.5 ml silanised glass organ-baths at 37°C. The preparations were attached to isometric Grass FT03B force-displacement transducers under an initial resting tension of 150 mg. Mechanical activity was recorded on Grass model 7E polygraphs. For experiments involving electrical field-stimulation, preparations were set up between parallel platinum electrodes, and electrical stimulation was carried out using Scientific Research Instruments 6051 stimulators. All experiments were carried out in Krebs solution composition (mM): NaCl 118, KH_2PO_4 1.17, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.95, $CaCl_2$ 2.5 and glucose 11. The Krebs solution was maintained at pH 7.4 by constant bubbling with 95%O_2:5%CO_2. The Krebs solution contained mepyramine, cimetidine, ibuprofen, guanethidine, atropine (all 1 μM) and hexamethonium (10 μM).

Protocols

In all experiments, one iris sphincter acted as a test preparation, whilst the second iris from the same animal acted as a control preparation. This procedure was necessary as it is only possible to obtain one concentration-response curve to bradykinin in a single preparation (Ueda *et al.*, 1984). Preparations were allowed to equilibrate for 60 min before determination of a standardising contractile response to carbachol (1 mM, in view of the presence of atropine in the Krebs solution). The test preparation was then incubated with WIN 64338 (1, 3 or 10 μM) for 30 min (see Farmer & DeSaito, 1994) followed by determination of a cumulative concentration-response curve to bradykinin (0.1 nM – 100 μM). The antagonist was re-applied for 30 min, followed by determination of a cumulative concentration-response curve to substance P methyl ester (1 nM – 10 μM), and this procedure was repeated to obtain responses to: neurokinin A (0.1 nM – 10 μM), senktide (100 nM), carbachol (1 mM), and finally substance P (10 nM). An identical protocol was followed in the control preparations, but using an appropriate volume of dimethylsulphoxide (DMSO; solvent for WIN 64338) instead of WIN 64338. In a separate series of experiments, a similar protocol was used to determine the effect of WIN 64338 on contractile responses to electrical field-stimulation (3, 10, 30 Hz; 0.5 ms duration, 70 V) and capsaicin (10 μM).

Guinea-pig conjunctiva and nasal mucosa

Guinea-pigs (350–450 g) were anaesthetized with pentobarbitone sodium (45 mg kg^{-1}, i.p.) and the jugular vein was cannulated. Deep anaesthesia was monitored throughout the experiment and additional anaesthetic administered as required. Evans blue dye (3% solution in 0.9% NaCl) was used to measure plasma extravasation, which was injected (30 mg kg^{-1}, i.v. over 5 s) via the jugular vein. Immediately after Evans blue administration, agents (dissolved in 10 μl of saline) were given by local instillation into the conjunctival sac by means of a micropipette. The solution was applied uniformly over the conjunctival surface. One conjunctiva, chosen at random, was treated with the stimulus in each animal. Local application of drugs or agents to the conjunctiva did not cause any adverse reaction or any aversive behavioural response. The solution was applied bilaterally by local installation (10 μl/nostril) into the nose with a micropipette.

Terminal anaesthesia (pentobarbitone, 40 mg kg^{-1}, i.v.) was always carried out 30 s before ending the experiment. At this time it is assumed that the increase in vascular permeability is complete. Experiments were terminated 5 min after injection of the tracer by opening the chest, inserting a cannula into the ascending aorta through the left ventricle, and perfusing the circulation for 2 min with phosphate buffer (pH 5) at the pressure of 130 mmHg.

The guinea-pig was decapitated behind the orbit, and the bony nose was freed from the skin. The respiratory nasal mucosa was taken according to the methods described previously (Petersson *et al.*, 1993; Ricciardolo *et al.*, 1994). The conjunctiva adherent to either the palpebra or the sclera was removed. Tissues were blotted, weighed and incubated in 1 ml of formamide at 60°C for 18 h to extract the extravasated dye (Saria *et al.*, 1983). The extravasation of Evans blue dye-labelled macromolecules from the microcirculation was quantified by measurement of the optical density of the formamide extracts using an optical spectrophotometer (Model UV1204, Shimatzu Scientific Instruments, Inc., Japan) at 620 nm, and the concentration calculated by reference to a standard curve.

WIN 64338 or the vehicle of the highest dose of WIN 64338 (1% DMSO in 0.9% saline, 1 ml kg^{-1}) were injected (i.v.) 15 min before the administration of the stimulus. The doses of bradykinin and substance P were selected in order to give submaximal effects (Ricciardolo *et al*, 1994; Figini *et al.*, 1995).

Source of agents

Agents were obtained as follows: pentobarbitone sodium (Sagatal; RMB Animal Health Ltd, U.K.), carbachol (carbamylcholine chloride), capsaicin, hexamethonium bromide, ibuprofen (Sigma, U.K.), mepyramine maleate (May & Baker, U.K.), guanethidine (Ciba, U.K.), senktide (succ-[Asp^6,Me-Phe^8]-SP(6-11)), neurokinin A (NKA), substance P, substance P methyl ester and bradykinin (Bachem, U.K. or Peninsula Laboratories Europe), Evans blue dye, phosphate buffer (Sigma, U.S.A.), formamide (Merck, Germany). All salts were of analytical grade and were obtained from B.D.H., U.K. WIN 64338 was a gift from Dr J.M. Salvino, Sterling Winthrop, PA, U.S.A. Stock solutions of senktide and WIN 64338 were made up in DMSO, ibuprofen was dissolved in 5% Na_2CO_3. All other agents and all dilutions were made in distilled water or saline (0.9%), and peptides were stored at −20°C.

Data analysis

Contractile responses in rabbit iris sphincter were expressed as % response to carbachol (1 mM) in individual preparations determined at the start of the experiment. Data from individual experiments were combined, and are shown as mean ± s.e.mean. Analysis of Schild plots and tests for slope of Schild regressions were according to Mackay (1978), testing regression of pK_B vs. [A] against zero, using conventional regression analysis (MINITAB 8.2, P.A., U.S.A.). Since this showed no evidence of departure from simple competitive antagonism, a mean pK_B estimate were determined from dose-ratios estimated from shifts of individual curves compared to matched DMSO controls, using the Gaddum-Schild equation $pK_B = -\log K_B = \log (DR-1) - \log[A]$, where [A] is the applied antagonist concentration. Consequently, graphical display of the fitted Schild regression was with unit slope imposed. Tests for significant differences were carried out by use of paired or unpaired Student's t tests, as appropriate. Mean values of spectrophotometric measurements of Evans blue dye extravasation were analysed by one-way analysis of variance. Comparisons between means in each condition were performed by the Dunnett's multiple range test. Student's t test for paired or paired data was used when applicable. Differences of $P<0.05$ were considered significant. In all cases n refers to the number of animals.

Results

Rabbit iris sphincter

Bradykinin evoked concentration-related contractile responses in the rabbit iris sphincter (pD_2 7.51 ± 0.07; $n=14$). When determined in the presence of WIN 64338, the log concentration-response curve to bradykinin was shifted to the right in a concentration-related manner, and the slope of the Schild re-

gression did not differ significantly from unity (slope 0.77 ± 0.23; $P > 0.05$ *vs* unity; correlation 0.75; $n = 11$) which is compatible with simple competitive antagonism (Figure 1a), so the regression line is shown with imposed unit slope. The pK_B estimated from individual dose-ratios was 6.6 ± 0.1 ($n = 11$). At the highest concentration tested (10 μM), WIN 64338 did not significantly inhibit submaximal contractile responses to the direct-acting spasmogens carbachol (1 mM), substance P (10 nM), neurokinin A (0.1 nM – 10 μM), the NK_1 tachykinin receptor selective agonist substance P methyl ester (1 nM – 10 μM), or the NK_3 tachykinin receptor selective agonist senktide (100 nM) ($P > 0.05$, $n = 4-6$; Figure 1d), WIN 64338 inhibited neither contractile responses resulting from stimulation of sensory nerves by capsaicin (10 μM) ($P > 0.05$, $n = 3$; Figure 1b), nor those to electrical field-stimulation (3, 10 and 30 Hz) ($P > 0.05$, $n = 3$; Figure 1c).

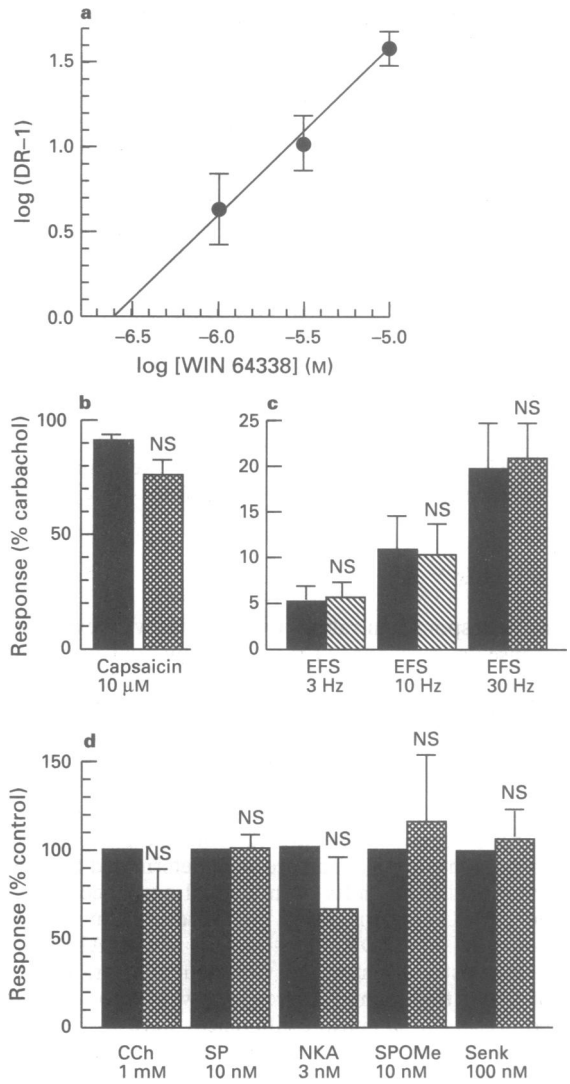

Figure 1 The effect of the bradykinin B_2 receptor antagonist WIN 64338 against contractile responses in the rabbit iris sphincter preparation *in vitro*. In (a) a Schild plot for WIN 64338 tested against bradykinin gives a regression compatible with competitive antagonism (slope 0.77 ± 0.23; $P > 0.05$ *vs* unity; correlation 0.75; $n = 11$) yielding a pK_B estimate from individual dose-ratios of 6.6 ± 0.1 ($n = 11$). Therefore, the Schild regression line fitted in the figure has unit slope. Tested against nerve-mediated responses, WIN 64338 (10 μM) was without effect (NS; ($P > 0.05$) on contractions evoked by capsaicin (b), or electrical field-stimulation at three frequencies (c). In tests for receptor-selectivity of antagonism by WIN 64338 (10 μM), it had no significant effect on submaximal contractile responses to carbachol (CCh; 1 mM), substance P (SP; 10 nM), neurokinin A (NKA; 3 nM), substance P methyl ester (SPOMe; 10 nM), or senktide (Senk; 100 nM) (d). In (b), (c) and (d): controls shown as solid columns and test as cross-hatched columns. Data are shown as mean ± s.e.mean ($n = 3-11$); NS denotes not significant ($P > 0.05$).

Guinea-pig conjunctiva and nasal mucosa

Baseline Evans blue dye extravasation in untreated conjunctiva of guinea-pigs was 10.2 ± 1.3 ng mg^{-1} ($n = 6$). Instillation of 10 µl of saline into the conjunctiva contralateral to the treated eye did not significantly increase plasma extravasation (11.7 ± 1.3 ng mg^{-1}, $n = 5$; $P > 0.05$). Administration of WIN 64338 (300 nmol kg^{-1}, i.v.) immediately after the Evans blue dye injection did not increase plasma extravasation (9.68 ± 1.11 ng mg^{-1}, $n = 4$; $P > 0.05$). Instillation of bradykinin (20 nmol) or substance P (5 nmol) increased the Evans blue dye extravasation to 111.5 ± 4.42 ng mg^{-1} ($n = 8$) and 71.11 ± 5.55 ng mg^{-1} ($n = 9$), respectively (Figure 2). Pretreatment with WIN 64338 (30 nmol kg^{-1}) inhibited the bradykinin-evoked plasma extravasation by $81.3 \pm 3\%$ ($P < 0.01$, $n = 9$, Figure 2). Bradykinin-evoked Evans blue dye extravasation was abolished by pretreatment with WIN 64338 (300 nmol kg^{-1}). WIN 64338 (30 and 300 nmol kg^{-1}), however, did not affect the substance P-induced plasma extravasation ($n = 6$; $P > 0.05$; Figure 2).

Baseline plasma extravasation in the guinea-pig nasal mucosa was 8.54 ± 0.93 ng ml^{-1} ($n = 5$). Local application of 10 µl of saline did not affect baseline plasma extravasation (8.97 ± 0.78 ng mg^{-1}, $n = 4$). Administration of WIN 64338 (300 nmol kg^{-1}, i.v.), immediately after the Evans blue dye injection, did not significantly increase plasma extravasation (10.14 ± 1.24 ng mg^{-1}, $n = 4$). Instillation of bradykinin (50 nmol) or substance P (50 nmol) increased extravasation of Evans blue dye to 93.5 ± 7.5 ng mg^{-1} ($n = 6$) and 64.7 ± 6.57 ng ml^{-1} ($n = 6$), respectively (Figure 3). Pretreatment with WIN 64338 (30 nmol kg^{-1}) significantly inhibited bradykinin-evoked plasma extravasation by $69 \pm 5\%$ ($P < 0.01$, $n = 5$), and, at a higher dose (300 nmol kg^{-1}) abolished bradykinin-evoked plasma extravasation. WIN 64338 (30 and

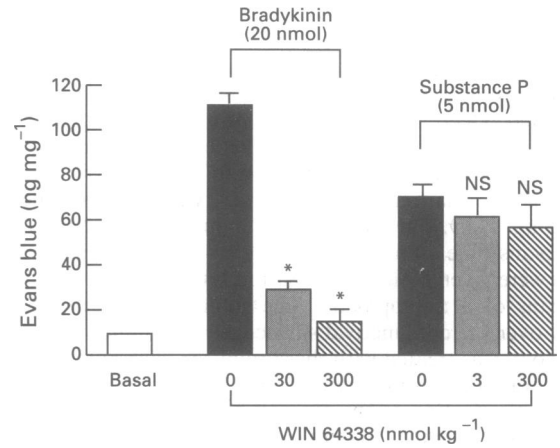

Figure 2 The effect of the bradykinin B_2 receptor antagonist WIN 64338 on bradykinin-evoked plasma extravasation in the guinea-pig conjunctiva. WIN 64338 (30 and 300 nmol kg^{-1}), significantly inhibited bradykinin-induced extravasation (bradykinin 20 nmol; $^*P < 0.05$), but was without effect on that induced by substance P (5 nmol; $P > 0.05$). Plasma extravasation was estimated in terms of Evans blue leakage. WIN 64338, or its vehicle (dimethylsulphoxide), was administered i.v. 15 min before bradykinin or substance P. Plasma extravasation in the absence of treatment (basal) is indicated by the open column. Mean data (± s.e.mean) are shown for at least 6 experiments.

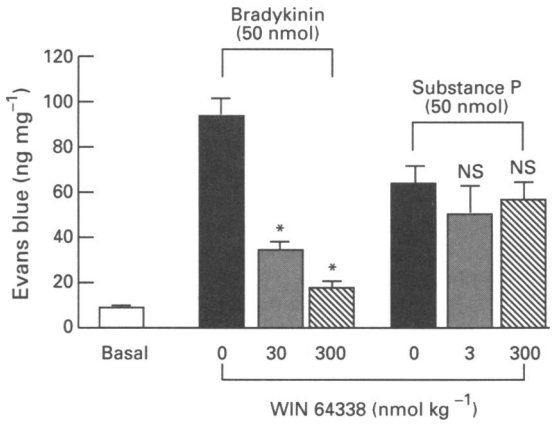

Figure 3 The effect of the bradykinin B_2 receptor antagonist WIN 64338 against bradykinin-evoked plasma extravasation in the guinea-pig nasal mucosa. WIN 64338 (30 and 300 nmol kg^{-1}), significantly inhibited bradykinin-induced extravasation (bradykinin 50 nmol; $*P<0.05$), but was without effect on that induced by substance P (50 nmol; $P>0.05$). Plasma extravasation was estimated in terms of Evans blue leakage. WIN 64338, or its vehicle (dimethylsulphoxide), was administered i.v. 15 min before bradykinin or substance P. Plasma extravasation in the absence of treatment (basal) is indicated by an open column. Mean data (±s.e.mean) are shown for at least 5 experiments.

300 nmol kg^{-1}) was without significant effect on plasma extravasation induced by substance P (50 nmol; $P>0.05$, $n=5$; Figure 3).

Discussion

This study demonstrates that the non-peptide bradykinin B_2 receptor antagonist WIN 64338, is a selective and relatively high affinity inhibitor of bradykinin-evoked stimulation of trigeminal sensory nerves both *in vitro* and *in vivo*. Our determinations in the rabbit isolated iris sphincter *in vitro* demonstrate that WIN 64338 inhibits bradykinin-evoked stimulation of sensory nerves via competitive B_2 receptor antagonism; and our studies in the guinea-pig conjunctiva and nasal mucosa demonstrate that WIN 64338 may be used *in vivo* to investigate the involvement of kinins and B_2 receptors in neurogenic inflammation.

Rabbit iris sphincter

Bradykinin contracts the rabbit isolated iris sphincter via the release of tachykinins from the peripheral terminals of primary afferent C-fibres (Ueda *et al.*, 1984; Hall *et al.*, 1993b; see Hall, 1994). In the present study, we demonstrated that the B_2 receptor antagonist WIN 64338 competitively antagonized responses to bradykinin in this preparation, showing that the bradykinin receptors involved are of the B_2 type. This conclusion is consistent with our results from a previous study where we showed that the peptide bradykinin B_2 receptor antagonist D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK (HOE140; icatibant) also competitively inhibited contractile responses to bradykinin in this preparation (Hall *et al.*, 1992). The inhibition by WIN 64338 was selective for bradykinin B_2 receptors. Notably, high concentrations of WIN 64338 (10 μM) did not inhibit contractile responses to exogenously applied tachykinins, including the NK_1 and NK_3 receptor-selective agonists substance P methyl ester and senktide, respectively; or to the sensory neurone tachykinins substance P and neurokinin A. This point is important since, in the rabbit iris sphincter, bradykinin releases tachykinins from the trigeminal nerve to mediate the contractile response, and both NK_1 and NK_3 tachykinin receptors are present postjunctionally in the iris smooth muscle in the rabbit (Hall *et al.*, 1991; 1993b). Additionally, unlike several recently described non-peptide tachykinin NK_1 receptor antagonists, including CP-96,345 (Wang *et al.*, 1994), the non-peptide bradykinin B_2 receptor antagonist WIN 64338 (10 μM), did not show non-specific depressant effects, and did not significantly inhibit contractile responses to sensory NANC nerve-stimulation evoked by electrical field-stimulation or capsaicin.

WIN 64338 inhibited contractile responses to bradykinin in the rabbit iris sphincter with an affinity (pK_B 6.6) similar to its reported affinity in another rabbit preparation, the jugular vein (pA_2 6.14; Marceau *et al.*, 1994), and in a human smooth muscle preparation, the umbilical vein (pA_2 5.99, Marceau *et al.*, 1994) and also human IMR-90 fibroblast measuring $^{45}Ca^{2+}$ efflux (pA_2 7.1; Sawutz *et al.*, 1994). In contrast, in the guinea-pig WIN 64338 has higher affinity; in the guinea-pig ileum pA_2 estimates of 7.97 (Farmer & DeSaito, 1994) and 8.2 (Sawutz *et al.*, 1994) have been obtained. On the other hand, WIN 64338 (1 μM) showed no inhibition of kinin responses in guinea-pig or ferret trachea, at receptors Farmer and DeSaito (1994) have termed B_3 in view of their resistance to blockade with WIN 64338 and a number of other B_2 receptor antagonists. A number of 'first-generation' peptide bradykinin antagonists also showed differences in affinity for B_2 receptors in different isolated preparations (Hall *et al.*, 1992; 1993a). Thus these various data suggest that WIN 64388, as with peptide antagonists, can distinguish between species homologues or receptor subtypes of bradykinin B_2 receptors (see Hall *et al.*, 1993a).

Guinea-pig conjunctiva and nasal mucosa

Stimulation of trigeminal sensory nerves of the rat or guinea-pig conjunctiva (Lee *et al.*, 1994; Figini *et al.*, 1995) and nasal mucosa (Lundblad *et al.*, 1983) increases plasma protein leakage in these tissues. The plasma extravasation induced by local application of bradykinin to the guinea-pig conjunctiva (Figini *et al.*, 1995) and nasal mucosa (Ricciardolo *et al.*, 1994) is due largely to the stimulation of sensory nerves and the release of tachykinins. Since the bradykinin B_2 receptor antagonist, HOE140, blocked the plasma leakage produced by bradykinin in the conjunctiva (Figini *et al.*, 1995) and nasal mucosa (Ricciardolo *et al.*, 1994) it has been proposed that bradykinin stimulates trigeminal sensory nerves of these tissues by acting on B_2 receptors (Ricciardolo *et al.*, 1994; Figini *et al.*, 1995). The present findings, showing that the non-peptide bradykinin B_2 receptor antagonist, WIN 64338, blocked the bradykinin-induced increases in plasma extravasation, support this hypothesis.

WIN 64338 shows high affinity for B_2 receptors in certain different guinea-pig preparations (see above). These observations are in agreement with the fact that a relatively low dose of WIN 64338 (300 nmol kg^{-1}), similar to the dose of the high affinity peptide B_2 receptor antagonist HOE140 (100 nmol kg^{-1}) previously used to block the bradykinin-induced plasma extravasation in the conjunctiva and nasal mucosa (Ricciardolo *et al*, 1994; Figini *et al.*, 1995), was able to abolish bradykinin responses. Selectivity of WIN 64338 administered *in vivo* against bradykinin B_2 receptors is indicated by the absence of effect of WIN 64338 on the plasma extravasation induced by substance P both in the conjunctiva and the nasal mucosa.

This study demonstrates that WIN 64338 is a selective and competitive bradykinin B_2 receptor antagonist and can be useful for analysing bradykinin-evoked trigeminal nerve stimulation both *in vitro* and *in vivo*. If stimulation of bradykinin B_2 receptors on trigeminal sensory nerves is involved in the mechanisms of diseases in man, including migraine and neuralgias, WIN 64338, similar to other B_2 receptor antagonists may be of therapeutic value.

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