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Bradyzide, a potent non-peptide B_2 bradykinin receptor antagonist with long-lasting oral activity in animal models of inflammatory hyperalgesia

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1 Bradyzide is from a novel class of rodent-selective non-peptide B_2 bradykinin antagonists (1-(2-Nitrophenyl)thiosemicarbazides).

2 Bradyzide has high affinity for the rodent B_2 receptor, displacing [^{3}H]-bradykinin binding in NG108-15 cells and in Cos-7 cells expressing the rat receptor with K_I values of 0.51 ± 0.18 nM ($n=3$) and 0.89 ± 0.27 nM ($n=3$), respectively.

3 Bradyzide is a competitive antagonist, inhibiting B_2 receptor-induced ^{45}Ca efflux from NG108-15 cells with a pK_B of 8.0 ± 0.16 ($n=5$) and a Schild slope of 1.05.

4 In the rat spinal cord and tail preparation, bradyzide inhibits bradykinin-induced ventral root depolarizations (IC_{50} value; 1.6 ± 0.05 nM ($n=3$)).

5 Bradyzide is much less potent at the human than at the rodent B_2 receptor, displacing [^{3}H]-bradykinin binding in human fibroblasts and in Cos-7 cells expressing the human B_2 receptor with K_I values of 393 ± 90 nM ($n=3$) and 772 ± 144 nM ($n=3$), respectively. Bradyzide inhibits bradykinin-induced [^{3}H]-inositol trisphosphate (IP_3) formation with IC_{50} values of 11.6 ± 1.4 nM ($n=3$) at the rat and 2.4 ± 0.3 μM ($n=3$) at the human receptor.

6 Bradyzide does not interact with a range of other receptors, including human and rat B_1 bradykinin receptors.

7 Bradyzide is orally available and blocks bradykinin-induced hypotension and plasma extravasation.

8 Bradyzide shows long-lasting oral activity in rodent models of inflammatory hyperalgesia, reversing Freund's complete adjuvant (FCA)-induced mechanical hyperalgesia in the rat knee joint (ED_{50}, 0.84 μmol kg^{-1}; duration of action >4 h). It is equipotent with morphine and diclofenac, and 1000 times more potent than paracetamol, its maximal effect exceeding that of the non-steroidal anti-inflammatory drugs (NSAIDs). Bradyzide does not exhibit tolerance when administered over 6 days.

9 In summary, bradyzide is a potent, orally active, antagonist of the B_2 bradykinin receptor, with selectivity for the rodent over the human receptor.

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; EGTA, ethyleneglycolbis(aminoethylether)tetraacetate; FCA, Freund's complete adjuvant; IP_3, inositol trisphosphate; NSAIDs, non steroidal anti-inflammatory drugs; TES, N-tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid; TRIS, Tris[hydroxymethyl]amino-methane

Introduction

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and the related peptide kallidin (Lys-bradykinin) are formed locally from kininogen precursors following the activation of tissue or plasma kallikreins by pathophysiological stimuli such as inflammation, tissue damage or anoxia (Bhoola *et al*., 1992; Bathon & Proud, 1991). Considerable evidence suggests that kinins contribute to the pathophysiological processes accompanying both acute and chronic inflammation. The activity of kinins is terminated by several degradative enzymes: kininase I liberates the biologically active metabolites desArg^{9}bradykinin and desArg^{10}kallidin, whilst kininase II and endopeptidases form inactive metabolites.

Two distinct receptors for kinins have been defined, based initially on pharmacological criteria (Farmer & Burch, 1992; Hall, 1992) and confirmed by molecular cloning techniques (Hess *et al*., 1992; Menke *et al*., 1994). B_2 bradykinin receptors are expressed constitutively and have higher affinity for the ligands bradykinin and kallidin. In contrast, B_1 receptors

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which are induced followed inflammation have higher affinity for the metabolites desArg^{9}bradykinin and desArg^{10}kallidin.

Bradykinin has a very short life-time in the circulation and acts close to the site of production on a wide variety of cell types, both neuronal and non-neuronal, with effects that include smooth muscle contraction, vasodilatation and increased vascular permeability, glandular secretion, immune cell stimulation, and sensitization and activation of sensory neurones. Bradykinin is one of the most algogenic substances known and it has been demonstrated that B_2 receptors are expressed on nociceptive neurones (Steranka *et al*., 1988) and are activated by bradykinin (Dray *et al*., 1992; Lang *et al*., 1990; Messlinger *et al*., 1994). As well as causing activation of the polymodal nociceptors, bradykinin sensitizes these neurones to other stimuli (Koltzenburg *et al*., 1992; Neugebauer *et al*., 1989; Rueff & Dray, 1993). There is good evidence that bradykinin is implicated in the etiology of a number of pain conditions associated with trauma or inflammation (Bathon & Proud, 1991; Farmer & Burch, 1992; Meller & Gebhart, 1992) and peptide B_2 antagonists can reverse inflammatory hyperalgesia (Dray & Perkins, 1993; Perkins & Kelly, 1993; 1994). Thus, in addition to their possible use as anti-inflammatory agents or inhibitors of the vascular effects of kinins, B_2 bradykinin receptor antagonists could be developed as drugs for the treatment of the hyperalgesia associated with a variety of inflammatory conditions.

Most B_2 bradykinin antagonists such as HOE-140 (Icatibant) (Hock *et al*., 1991) are peptides and thus not suitable for oral administration. To date, only two classes of non-peptide B_2 antagonists have been identified: the phosphonium-derived WIN 64338 (Salvino *et al*., 1993) and the heteroaryl benzyl ethers FR173657 and FR193517 (Asano *et al*., 1997; Abe *et al*., 1998). Although FR193157 has been shown to inhibit acute nociceptive responses in animal models (Griesbacher *et al*., 1998), its ability to reverse chronic inflammatory hyperalgesia has not been investigated. Here we report the discovery of a third, structurally distinct class of B_2 bradykinin antagonist based on a 1-(2-nitrophenyl)-4-benzyl thiosemicarbazide core (Figure 1). Bradyzide is a potent, orally active, rat-selective B_2 receptor antagonist that causes a long-lasting reversal of inflammatory hyperalgesia.

Methods

B_2 bradykinin receptor binding studies

NG108-15 membrane preparation Undifferentiated NG108-15 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum at 37°C. The cells were harvested by vigorous shaking of the culture vessel. All subsequent procedures were performed at 4°C. The cells were homogenized for 30 s with a Kinematica polytron homogeniser set at 10,000 r.p.m. in 25 mM potassium phosphate, pH 6.5. The homogenate was centrifuged at $40{,}000 \times g$ for 30 min and the resulting pellet washed twice with intermediate rehomogenization. The final pellet was resuspended at 7 mg of membrane protein ml^{-1} in the phosphate buffer and stored in aliquots at −70°C. The protein concentration was determined with a Bio-Rad kit based on the method of Bradford (1976).

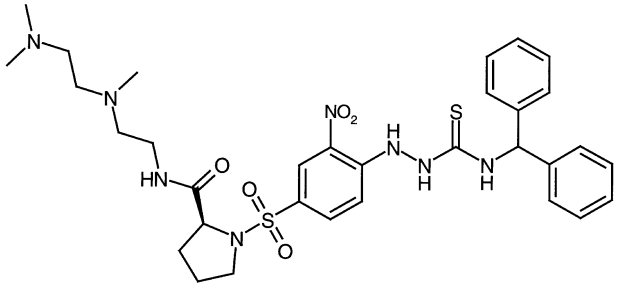

Figure 1 Structure of Bradyzide. Bradyzide, ((2S)-1-[4-(4-Benzhydrylthiosemicarbazido) - 3 - nitrobenzenesulfonyl] - pyrrolidine - 2 - carboxylic acid {2-(2-dimethylaminoethyl)methylamino]ethyl}amide) (MW 682.97).

WI-38 membrane preparation WI-38 cells were grown and membranes prepared as described in Phagoo *et al*. (1996).

Cos-7 cells expressing the cloned human and rat B_2 receptors Cloning of the human and rat B_2 bradykinin receptors, the transient transfection of DNA for the two receptors into Cos-7 cells and membrane preparation for [^{3}H]-bradykinin binding studies has been described in McIntyre *et al*. (1993).

[^{3}H]-bradykinin binding assays NG108-15 cell membranes were thawed, homogenized and diluted with binding buffer (composition (mM): potassium phosphate 25 pH 6.5, ethyleneglycolbis(aminoethylether)tetraacetate (EGTA) 1, bacitracin 0.1, 54 mg ml^{-1} chymostatin and 2 mg ml^{-1} bovine serum albumin (BSA)) to give a membrane protein concentration of about 0.3 mg ml^{-1}. The assay mixture comprised 100 μl of [^{3}H]-bradykinin (specific activity 65 Ci mmol^{-1} Amersham), 50 μl dimethyl sulphoxide (DMSO) (total binding), or 50 μl of the compound to be investigated in DMSO and 100 μl of binding buffer. The assay was started by the addition of 750 μl membrane suspension.

WI-38 membranes were thawed, homogenized and diluted with binding buffer with composition: 10 mM N- tris [Hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES), pH 7.4, 0.14 g l^{-1} bacitracin, 0.2 g l^{-1} phenanthroline and 1 mg ml^{-1} BSA. Membranes were added to give a membrane protein concentration of approximately 30 μg ml^{-1}. The assay was started by the addition of 100 μl membrane suspension to 850 μl binding buffer containing [^{3}H]-bradykinin and either 50 μl DMSO (total binding) or 50 μl of the compound to be tested dissolved in DMSO.

Cos cell membranes were thawed, homogenized, and diluted with binding buffer with composition: 10 mM TES, pH 7.4, 1 mM EGTA, 0.14 g l^{-1} bacitracin, 54 μg ml^{-1} chymostatin and 0.2% BSA. The assay mixture comprised 100 μl [^{3}H]-bradykinin in binding buffer, 50 μl DMSO (total binding) or 50 μl of the test compound in DMSO. The assay was started by addition of 750 μl membrane suspension to give a membrane protein concentration of 5 μg ml^{-1}.

For all binding assays, non-specific binding was determined in the presence of 10 μM bradykinin and the concentration of [^{3}H]-bradykinin in displacement assays was 1 nM. Samples were incubated for 60 min at 4°C, then filtered through GF/B filters, pre-soaked in polyethylenimine (6 g l^{-1}), using a Brandel Harvester. The filters were washed with ice cold wash buffer (25 mM potassium phosphate, pH 6.5 for the NG108-15 cells and 50 mM Tris[hydroxymethyl]-amino-methane (TRIS), pH 7,4 for WI-38 cells and the Cos-7 cells), placed in scintillation vials and counted in 'Ready-Micro' liquid scintillation fluid. Binding parameters were calculated by the method of Munson & Rodbard (1980) using LIGAND.

B_1 bradykinin receptor binding studies

Binding of the B_1-selective ligand, [^{3}H]-desArg^{10}kallidin, to membranes prepared from Cos-7 cells expressing either the human or the rat B_1 bradykinin receptor was carried out as described in Jones *et al.* (1999).

Broad receptor screen

Binding to broad range receptors was performed by Panlabs Taiwan Ltd., Taipei, Taiwan R.O.C.

Measurement of ^{45}Ca efflux

Undifferentiated NG108-15 cells were maintained as a monolayer in 80 cm^2 flasks in DMEM containing foetal calf serum (Myoclone Plus), 15%; penicillin, 100 IU ml^{-1}; streptomycin, 100 μg ml^{-1} and L-glutamine, 2 mM (supplemented DMEM). For measurement of ^{45}Ca efflux the cells were plated on Terasaki plates in DMEM supplemented as described above but containing only 2% foetal calf serum and with 1 mM dibutyryl cyclic AMP to differentiate the cells. After 5 days the cells stopped dividing and the differentiated NG108-15 cells were incubated at 37°C in DMEM containing 30 μCi ml^{-1} of ^{45}Ca for 2 h. They were then washed at 37°C in DMEM until the rate of ^{45}Ca efflux was stable (approximately 20 min). Agonist-induced changes in the rate of ^{45}Ca efflux were measured as described in Smith *et al.* (1995). EC_{50} and IC_{50} (concentrations producing half-maximal stimulation and inhibition respectively) were estimated by computer-assisted curve-fitting (MicroCal ORIGIN).

Measurement of phospholipase C activation in H4 rat hepatomas transfected with either rat or human B_2 bradykinin receptor-encoding cDNA

Rat H4 hepatoma cells, which do not express the B_2 bradykinin receptor, were grown and maintained in DMEM supplemented with 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM L-glutamine and 10% foetal calf serum. For cells transfected with the bradykinin receptors the medium was supplemented with 600 μg ml^{-1} geneticin. The cells were split 1 in 10 every 3–4 days, trypsin-EDTA solution (0.5 g l^{-1} trypsin, 0.2 g l^{-1} EDTA in modified Puck's saline A) being used to detach the cells from the culture flasks.

Rat and human B_2 bradykinin receptor cDNAs were cloned as described by McIntyre *et al.* (1993). The rat bradykinin receptor cDNA was introduced into H4 cells by the calcium phosphate precipitation method described by Sambrook *et al.* (1989) and stable recombinants were selected in DMEM containing 10% FCS and 550 μg ml^{-1} geneticin for 3 weeks, after which clones were obtained by plating out at limiting dilution. The clone expressing the highest level of rat B_2 receptor was used for further studies. The human bradykinin receptor cDNA was introduced into H4 cells by electroporation with a BioRad Genepulser as described by the manufacturer. Clones were taken directly from well isolated colonies after 3 weeks of selection in DMEM containing 10% FCS and 550 μg ml^{-1} geneticin. The clone expressing the highest level of human B_2 receptor was used for further studies. Bradykinin-induced phospholipase C activation was followed by measuring the formation of [^{3}H]-IP_3 in cells loaded with [^{3}H]-inositol as described by Horstman *et al.* (1986).

Rat uterus preparation

Female Sprague-Dawley rats were injected with 0.5 mg kg^{-1} s.c. oestradiol benzoate 20 h before use. The rats were sacrificed by stunning and exsanguination and the uterus was removed and one horn cut into two strips, 1.5 cm in length. The strips were suspended under 0.5 g tension, at 30°C in 5 ml organ baths containing modified DeJalon's solution with composition (mM): NaCl 54, KCl 5.6, $NaHCO_3$ 5.6, $CaCl_2$ 0.54, glucose, 11.7 and continuously aerated with 95% O_2/5% CO_2. Contractions were recorded using a tension transducer and after 30–60 min control responses to an approximate EC_{50} value concentration of bradykinin (usually 1 nM, applied for 90 s) were obtained. Responses to bradykinin were then obtained every 30 min in the presence of increasing concentrations of antagonist applied to the tissue 1 min before the next addition of bradykinin. The response to bradykinin at each antagonist concentration was expressed as a percentage of the control response to bradykinin in individual tissues.

Neonatal spinal cord tail preparation

Sprague-Dawley rats (0–2 days old) were decapitated and the spinal cord exposed by laminectomy and dissected with the pelvic bone and the tail attached (Dray *et al.*, 1992). After removal of the tail skin, the preparation was placed in a recording chamber that allowed the spinal cord and tail to be superfused separately (rate of superfusion 2.5 ml min^{-1}) with synthetic cerebrospinal fluid of composition in (mM): NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.16, $NaHCO_3$ 21.0, NaH_2PO_4 0.58, glucose 10; and gassed with 95% O_2/5% CO_2 at 24°C. Peripheral nociceptive terminals were activated by application of algogenic chemicals, including bradykinin and the selective C-fibre stimulant, capsaicin, to the tail. Submaximal concentrations of bradykinin (100–350 nM) and capsaicin (100–300 nM) were applied for 10 s with at least a 60 min interval between applications to avoid tachyphylaxis. Antagonists were applied to the tail for at least 10 min before bradykinin or capsaicin were retested. The activation of peripheral fibres was assessed by measuring the amplitude of the ventral root potential (VRP) in the lumbar region using a low impedance, saline-filled glass pipette. Signals were amplified (Grass DC amplifier Model P16) and displayed on a Graphtec WR3107 chart-recorder. This preparation allows the study of the effect of antagonists on the B_2 receptors involved in nociception.

Blood pressure measurements

Female Sprague-Dawley rats (150–200 g) were anaesthetized with sodium pentobarbitone (Sagatal) 50 mg kg^{-1} intraperitoneally, supplemented as required, and the femoral artery cannulated for measurement of mean arterial blood pressure. Bolus injections of submaximal doses of bradykinin (0.1 nmole in 0.1 ml heparinized saline) were made *via* the carotid artery at 10 min intervals. Antagonists were infused (50 μl min^{-1} in saline) *via* the jugular vein for the 5 min period preceding bradykinin administration and for the duration of the response. Recovery of the response to bradykinin was followed for up to 45 min.

Measurement of plasma extravasation

Bradykinin-induced plasma extravasation from the bladder was measured by a modification of the method described by Lembeck *et al.* (1991). Female Sprague-Dawley rats (150–

200 g) were anaesthetized with urethane (1.5 g kg^{-1} i.p.). The jugular vein was cannulated and Evans blue dye (50 mg kg^{-1}) injected. Five minutes later the bradykinin antagonist was administered i.v. followed immediately by bradykinin (200 nmol kg^{-1} i.v.). Five minutes later the animals were perfused with saline and the bladder was then dissected and weighed. The Evans blue was extracted by incubation in formamide and measured photometrically. Plasma protein extravasation was expressed as mg Evans blue 100 mg $tissue^{-1}$.

Freund's adjuvant-induced mechanical hyperalgesia in the rat knee

Female Sprague-Dawley rats (100–120 g) were lightly anaesthetized with Enflurane and 100 μl of Freund's complete adjuvant was injected into one knee. Three to 6 days later, the animal was placed with each hind paw on a pressure transducer and a downward force was exerted until the uninjected leg was bearing 100 g. The force the animal would bear on the injected leg was determined and the reduction in the load tolerated indicated the degree of hyperalgesia (see Davis & Perkins, 1994). Half-hourly measurements were taken and drugs were administered intravenously, subcutaneously or orally after three control readings. The increase in load tolerated by the injected leg resulting from a drug was expressed as a percentage maximal reversal of the hyperalgesia (with 100% reversal representing an equal load tolerated by the control and injected leg). The ED_{50} value (defined as the dose that reversed the hyperalgesia by 50%) for groups of 8–10 animals was calculated from the peak response. Statistical analysis was performed by two-way analysis of variance (ANOVA) to compare pre- and post-treatment values.

All experiments were carried out on Sprague Dawley rats that were bred in-house. Animals were fed a normal diet and received water *ad libitum*. All procedures reported were subject to Home Office approval and were carried out under the Animal (Scientific Procedures) Act, 1986.

Materials

[^{3}H]-bradykinin (specific activity 65 Ci $mmol^{-1}$) [^{3}H]-Inositol (specific activity 17.1 Ci $mmol^{-1}$) and ^{45}Ca specific activity 5–50 mCi mg^{-1}) were from Amersham International plc (Amersham UK, Bucks, U.K.) and [^{3}H]-desArg^{10}-kallidin (specific activity 75–100 Ci $mmol^{-1}$) was provided by DuPont NEN (Hertfordshire, U.K.). Bradykinin and HOE-140 were obtained from Peninsula Laboratories Europe Ltd. (Merseyside, U.K.). The following materials were obtained from the sources indicated: bacitracin, EDTA, EGTA, TES, HEPES, BSA, 1,10 phenanthrolene, polyethylenimine, diclofenac, paracetamol (4-acetamidophenol) (Sigma Chemical Company Ltd. Poole Dorset, U.K.), chymostatin (Peptide Products Ltd., Wiltshire, U.K.), Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd., Hertfordshire, U.K.), GF/B glass fibre filtermats (Semat Technical Ltd., Hertfordshire, U.K.), DMEM and other culture reagents (Gibco Ltd., Scotland, U.K.). Bradyzide was synthesized at the Novartis Institute for Medical Sciences.

Results

In vitro *pharmacology*

The effect of bradyzide in NG108-15 cells NG108-15 mouse neuroblastoma x rat glioma hybrid cells possess B_2 bradykinin receptors on their plasma membranes and specific binding sites for the B_2-selective ligand [^{3}H]-bradykinin can be detected in membranes prepared from these cells (Snell *et al*., 1990). The K_D and B_{max} values for [^{3}H]-bradykinin, calculated from a one site model, were 0.24 ± 0.11 nM ($n=3$) and 30 ± 8 fmol mg^{-1} ($n=3$) respectively. Bradyzide displaced the specific binding of [^{3}H]-bradykinin from NG108-15 membranes with a K_I value of 0.51 ± 0.18 nM ($n=3$) (Figure 2 and Table 1). The K_I for the potent peptide B_2 antagonist, HOE-140 was 0.08 ± 0.03 nM ($n=3$).

In NG108-15 cells, B_2 bradykinin receptors are linked to phospholipase C and activation by bradykinin leads to a rise in $[Ca^{2+}]_i$ which stimulates the plasma membrane Ca^{2+} pump and increases the rate of calcium efflux from the cells. In cells that have been pre-equilibrated with ^{45}Ca, this increase in Ca^{2+} efflux can be detected by monitoring the radioactivity released from the cells (see e.g., Smith *et al*., 1995). The basal rate of ^{45}Ca efflux was 0.05 ± 0.004 min^{-1} ($n=9$) and this rose to 0.448 ± 0.036 min^{-1} ($n=6$) in the presence of a maximal (30 nM) concentration of bradykinin. The EC_{50} value for bradykinin-induced ^{45}Ca efflux was 2.0 ± 0.3 nM ($n=12$). Bradyzide, applied up to a concentration of 3 μM, did not increase the rate of ^{45}Ca efflux from the cells implying that it was not an agonist.

In the presence of increasing concentrations of bradyzide, there was a rightward shift of the log-concentration response curve for bradykinin with no drop in the maximum response (Figure 3). Figure 3 also shows a Schild plot for bradyzide in NG108-15 cells. The data could be fitted with a straight line with a slope of 1.05 ± 0.07 ($n=5$) which was not significantly different from 1 ($P<0.05$ Student t-test) which indicates competitive antagonism. The pK_B value for bradyzide in the NG108-15 cells was 8.0 ± 0.16 ($n=5$).

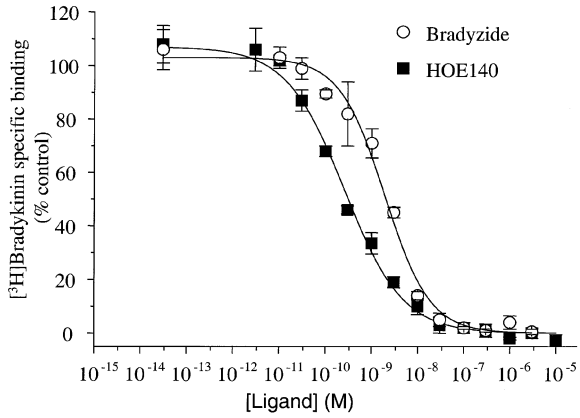

Figure 2 Displacement of [^{3}H]-bradykinin binding to NG108-15 membranes by HOE-140 and bradyzide. [^{3}H]-bradykinin (1 nM) and membranes (0.3 mg protein) were incubated with increasing concentrations of HOE-140 or bradyzide. The points represent the mean ± s.e.mean of least three separate determinations in triplicate.

Table 1 K_I values for bradyzide and HOE-140 in WI-38 human fibroblasts and NG108-15 cells

Compound	*WI-38 cells (human)* K_I *value* (nM)	*NG108-15 (rodent)* K_I *value* (nM)
HOE-140	0.022 ± 0.008	0.08 ± 0.03
bradyzide	393 ± 90	0.51 ± 0.18

The K_I values were calculated from a 1 binding site model using LIGAND. The values are means ± s.e.mean from three independent observations.

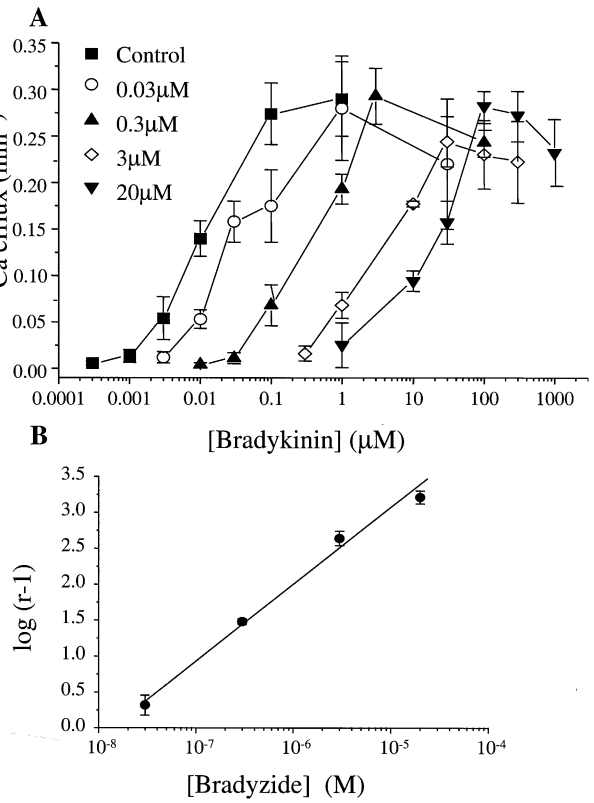

Figure 3 Bradyzide is a competitive B_2 antagonist in NG108-15 cells. (A) Increasing concentrations of bradyzide caused parallel, rightward shifts of the log-concentration response curve for bradykinin-induced ^{45}Ca efflux from differentiated NG108-15 cells. (B) Schild plot for bradyzide in NG108-15 cells. The data shown are mean ± s.e.mean from five separate experiments.

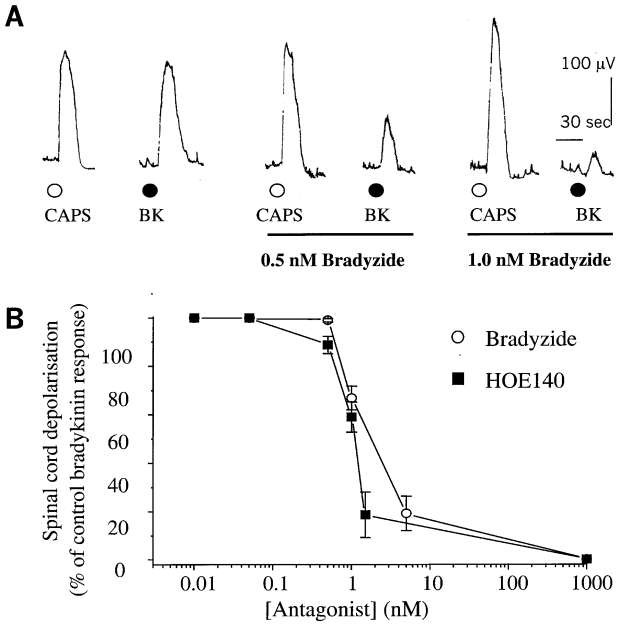

Figure 4 The effect of bradyzide and HOE-140 on bradykinin-induced ventral root depolarization in the *in vitro* neonatal rat tail and spinal cord preparation. (A) capsaicin (CAPS, 700 nM) and bradykinin (Bk 350 nM) was administered by perfusion (10 s) to the tail and depolarizations were recorded in the spinal cord. Antagonists were applied to the tail for 10 min before and during the application of bradykinin. (B) Log-concentration inhibition curves for bradyzide, and HOE-140. The data shown are mean ± s.e.mean of three independent experiments.

The activity of bradyzide at B_2 receptors in the rat uterus The contraction of the isolated oestrogen-primed rat uterus preparation *in vitro* is a standard bioassay for bradykinin B_2 receptors (see Regoli & Barabe 1988). Both bradyzide and HOE-140 antagonized the contractions evoked by bradykinin. Bradyzide caused a shift to the right of the log-concentration response curve with a pA2 of 8.60 ± 0.13. It did not reduce the maximum response to bradykinin but the slope of the Schild plot was 1.54 ± 0.11 ($n=4$) which was significantly greater than 1 ($P<0.05$, Student's *t*-test). The same analysis of the peptide antagonist HOE-140 gave a pA2 value of 8.96 ± 0.1 ($n=4$) with a slope of the Schild plot of 1.37 ± 0.5 which was also significantly greater than 1 ($P<0.05$, Student's *t*-test).

The effect of bradyzide on B_2 receptors on the peripheral terminals of sensory neurones

Bradykinin and capsaicin applied for 10 s to the tail caused excitation of the peripheral terminals of sensory neurones leading to ventral root depolarization. Figure 4 shows that bradyzide applied at 0.5 and 1 nM, blocked the ventral root depolarizations induced by bradykinin without affecting the response to capsaicin. The IC_{50} values for bradyzide and HOE-140 were 1.6 ± 0.5 nM ($n=3$) and 0.98 ± 0.03 nM ($n=3$) respectively.

The effect of bradyzide at the human B_2 bradykinin receptor The species-selectivity of bradyzide was examined in WI-38 human fibroblasts, which express B_2 bradykinin receptors constitutively (Phagoo *et al.*, 1996) and membranes from WI-38 cells were used to examine the species-selectivity of bradyzide. In WI-38 membranes specific binding of [^{3}H]-bradykinin was a saturable function of radioligand ([^{3}H]-bradykinin) concentration and the K_D and B_{max} values, calculated from a one site model, were 0.16 ± 0.022 nM ($n=4$) and 753 ± 98 fmol mg^{-1} protein ($n=4$).

Although bradyzide displaced the specific binding of [^{3}H]-bradykinin, the K_I value for bradyzide at the human B_2 receptor was considerably greater than its K_I value at the rodent B_2 receptor in NG108-15 cells, whereas the K_I value for the peptide antagonist HOE-140 was similar in both cell types (Table 1).

Comparison of bradyzide at the cloned human and rat B_2 receptors The difference in the affinity of bradyzide for the rodent and the human B_2 receptor was examined further in binding and functional assays with the cloned rat and human B_2 bradykinin receptors. In Cos-7 cells expressing the cloned rat B_2 receptor, specific binding of [^{3}H]-bradykinin was a saturable function of [^{3}H]-bradykinin concentration and K_D and B_{max} values, calculated from a one site model, were 0.051 ± 15 nM ($n=3$) and 466 fmol mg^{-1} protein ($n=3$) respectively. In Cos = 7 cells expressing the cloned human B_2 receptor the K_D value for [^{3}H]-bradykinin, calculated from a one site model, was 0.163 ± 29 nM ($n=3$) with a B_{max} value of 666 ± 80 fmol mg^{-1} protein ($n=3$). The K_I value for bradyzide in Cos-7 cells expressing the rat B_2 receptor was 0.89 ± 0.27 nM ($n=3$), which was close to the K_I value of 0.51 ± 0.18 nM ($n=3$) obtained in the NG108-15 cells. In contrast, in Cos-7 cells expressing the human B_2 receptor, bradyzide was over 800 times less potent, displacing the binding of [^{3}H]-bradykinin with a K_I value of 772 ± 144 nM ($n=3$).

Both the human and the rat B_2 bradykinin receptor coupled efficiently to phospholipase C when expressed stably in H4 rat

hepatoma cells. Bradykinin increased [^{3}H]-inositol trisphosphate (IP_3) formation with EC_{50} values of 12 ± 4.9 nM ($n=3$) and 31 ± 7 nM ($n=3$) in the H4 cells expressing the rat and human B_2 receptors, respectively.

In cells transfected with the rat receptor, the increase in [^{3}H]-IP_3 was inhibited by bradyzide with an IC_{50} value of 11.8 ± 1.4 nM ($n=3$). In the cells expressing the human B_2 receptor, bradyzide was again much less potent, blocking the response to bradykinin with an IC_{50} value of 2.4 ± 0.3 μM (Figure 5).

Affinity of bradyzide for other receptor binding sites In order to determine whether bradyzide was selective for the B_2 bradykinin receptor, its activity for a range of receptors and enzymes was determined. At concentrations up to 10 μM, bradyzide failed to displace the specific binding of [^{3}H]-desArg^{10}-kallidin from membranes prepared from Cos-7 cells transfected with cDNA for either the human or the rat B_1 receptor (Jones *et al*., 1999). In contrast, in the same experiments, the B_1-selective peptide antagonist, des-Arg^{10}-HOE-140, displaced the binding of [^{3}H]-desArg^{10}-kallidin to the human and rat B_1 receptors with K_I values of 4.9 ± 1.3 nM ($n=3$) and 35 ± 7 nM ($n=3$) respectively.

In a broad screen (carried out by Panlabs Taiwan Ltd.), bradyzide showed very low or negligible affinity in binding assays for a wide variety of receptors for peptide and non-peptide neurotransmitters and hormones apart from the B_2 bradykinin receptor (see Table 2). It had an IC_{50} value of

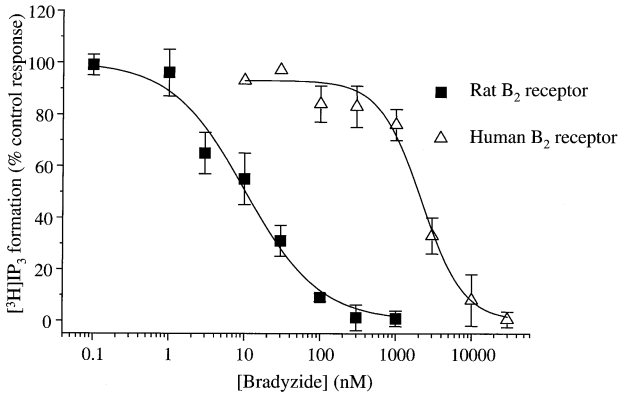

Figure 5 Effect of bradyzide on bradykinin-induced [^{3}H]-IP_3 formation in H4 cells expressing the rat and human B_2 receptor. The data has been expressed as a percentage of the control response, in each case using a concentration of bradykinin close to the EC_{50} value concentration (10 nM for the rat B_2 receptor H4 cells; and 40 nM for the human B_2 receptor H4 cells) and represents mean and s.e.mean from three experiments.

Table 2 Broad screen evaluation of bradyzide (Panlabs Ltd)

Binding site	*Ligand*	*Full name of ligand*	*Source*	*Inhibition of specific binding at 10 μM bradyzide* (%)
Adenosine A_1	[^{3}H]-DPCPX	(8-Cyclopentyl-1,3-dipropylxanthine)	Rat striatum	0
Adenosine A_2	[^{3}H]-CGS-21680	2-p-(2-carboxyethyl-phenethylamino-5′-N-ethylcarboxamidoadenosine	Rat striatum	0
Angiotensin II	[^{3}H]-Angiotensin II		Rabbit adrenal gland	0
Bradykinin B_2	[^{3}H]-Bradykinin		Guinea-pig ileum	100
Cholecystokinin A	[^{3}H]-L-364718	Devazepide	Rat pancreas	0
Cholecystokinin B	[^{3}H]-CCK-8	Cholecystokininoctapeptide	Mouse brain	0
Galanin	[^{125}I]-Galanin		Rat whole brain	0
Histamine H_3	[^{3}H]-NAMH	N-alpha-methyl histamine	Rat whole brain	0
Insulin	[^{125}I]-Insulin		Rat liver	0
Interleukin 1α	[^{125}I]-IL-1α	Interleukin 1α	Mouse 3T3 cell line	0
Kainate	[^{3}H]-Kainate		Rat whole brain	0
Leukotriene B_4	[^{3}H]-LT B_4	Leukotriene B_4	Guinea-pig spleen	0
Muscarinic M_1	[^{3}H]-Pirenzepine		Rat brain cortex	85
Muscarinic M_2	[^{3}H]-NMS	N-methyl-scopolanine methyl cholride	Rat heart	20
Neurokinin NK_1	[^{3}H]-Substance P		Rat submaxillary gland	20
Neuropeptide Y	[^{3}H]-NPY	Neuropeptide Y	Rabbit kidney medulla	0
N-Methyl-D-Aspartic Acid (NMDA)	[^{3}H]-CGS-19755	cis-4-(Phosphonomethyl)piperidine-2-carboxylic acid	Rat brain cortex	0
Platelet Activating Factor (PAF)	[^{3}H]-PAF	Hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine	Rabbit platelet	0
Phencyclidine	[^{3}H]-TCP	1-[1-(2-Thienyl)cyclohexyl]piperidine	Rat cortex	0
Phorbol ester	[^{3}H]-PDBu	Phorbol 12, 13-dibutyrate	Mouse whole brain	0
Serotonin 5-HT_{1A}	[^{3}H]-8-OH-DPAT	8-Hydroxy-2(di-n-propylamino)-tetralin	Rat brain cortex	0
Serotonin 5-HT_3	[^{3}H]-GR-65630	3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone	Rabbit ileum	0
Sigma	[^{3}H]-DTG	1,3-Di(2-tolyl)guanidine	Guinea-pig brain	67
Sodium Channel	[^{3}H]-Batrachotoxin			0
Thromboxane A_2	[^{3}H]-SQ-29548	(1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(Phenylamino) carbonyl]hydrazion]methyl]-7-oxabicyclo [2.2.1]hept-2-yl]-5-heptanoic acid	Rabbit platelet	0
Thyrotropin Releasing Hormone	[^{3}H]-(Me) TRH	Thyrotropin releasing hormone	Rat whole brain	0
Tumor Necrosis Factor (TNFα)	[^{125}I]-TNF	Tumour necrosis factor α	Hela S_3 cell line	0
Vasoactive Intestinal Polypeptide	[^{125}I]-VIP	Vasoactive intestinal polypeptide	Guinea-pig lung	21

The values represent means of two experiments performed in triplicate. An internal control was included for each assay.

0.5 μM for displacement of [^{3}H]-pirenzepine binding from the M_1 muscarinic receptor and in functional studies in the rabbit vas deferens, it was a very weak M_1 antagonist with an IC_{50} value of $\sim$30 μM (Panlabs Ltd). Although high concentrations of bradyzide had activity in the sigma opioid binding assay ([^{3}H]-1,3-Di-(2-[5-^{3}H]tolyl)-guanidine binding, IC_{50} value 3 μM), it was neither an agonist nor antagonist in a functional assay for the sigma opiate receptor in the rabbit vas deferens (Panlabs Ltd). Thus bradyzide appeared to be highly selective for the B_2 bradykinin receptor.

In vivo *pharmacology*

Effect of bradyzide on bradykinin-induced hypotension in the rat Resting blood pressure in normotensive rats was 122 ± 7 mmHg ($n=7$). Intra-arterial injection of a submaximal concentration of bradykinin (0.1 nmol in 0.1 ml heparinized saline *via* the carotid artery) caused a transient fall in blood pressure of 50 ± 6 mmHg ($n=7$) which recovered within 1–2 min. This response was mediated *via* B_2 bradykinin receptors as it was blocked by infusion of the selective peptide B_2 antagonist HOE-140 into the jugular vein (50 μl min^{-1}), with an IC_{50} value of 0.2 ± 0.04 nmol min kg^{-1}. Bradyzide, also given by intravenous infusion, inhibited the bradykinin-mediated fall in blood pressure with an IC_{50} value of 13 ± 4 nmol min^{-1} kg^{-1} ($n=3$). The effect of bradyzide was long-lasting, such that bradykinin responses were significantly reduced to $55\pm8\%$ ($n=7$) of control 3 h following a bolus injection of bradyzide (10 μmol kg^{-1} i.v.; $P<0.05$ ANOVA followed by Tukey's HSD, see Figure 6). Bradyzide-evoked inhibition of bradykinin-induced hypotension appeared to show two phases. There was an initial inhibition of greater than 85% of the control response to bradykinin, which lasted for 30 min after administration of bradyzide. The response then recovered to 50–55% of the level in saline-injected animals, where it remained for the rest of the measurement period. A similar bolus injection of HOE-140 (0.5 μmol kg^{-1} i.v.) reduced responses to bradykinin by 80–90% for the duration of the measurement period (3 h) (Figure 6).

Bradykinin-induced plasma extravasation in the rat In untreated animals, following perfusion with Evans blue dye (50 mg kg^{-1}) 1.7 ± 0.54 μg Evans blue 100 mg^{-1} ($n=4$) of tissue could be extracted from the bladder. This rose to 10.4 ± 1.1 μg Evans blue 100 mg^{-1} tissue following administration of a maximal dose of bradykinin (500 nmol kg^{-1} i.v.). A sub-maximal dose of bradykinin of 200 nmol kg^{-1} i.v. was selected for use in experiments with antagonists, which were administered immediately prior to the bradykinin. Bradyzide (i.v.) produced a dose-related inhibition of bradykinin-induced plasma extravasation with an IC_{50} value of 0.10 ± 0.009 nmol kg^{-1} ($n=4$). The highest dose of bradyzide (1 nmol kg^{-1}) reduced the extravasation to basal levels. HOE-140 (i.v.) also reduced the plasma extravasation from the bladder with an IC_{50} value of 3.2 ± 0.53 nmol kg^{-1} ($n=4$).

Inflammatory mechanical hyperalgesia

Freund's adjuvant-induced hyperalgesia in the rat knee Following intra-articular injection of Freund's complete adjuvant into one knee joint of a rat, the load that the rat will tolerate on that leg decreases and remains depressed for up to 5 days (Perkins *et al.*, 1992). This reduction is indicative of a prolonged mechanical hyperalgesia and is responsive to NSAIDs and opiates. The ability of bradyzide to reverse established hyperalgesia was investigated in this model. Three days after injection of Freund's complete adjuvant (100 μl) into the knee joint, the load that the animal would tolerate on the inflamed leg fell from a maximum of 100 to 51 ± 1 g ($n=12$). Bradyzide reduced this hyperalgesia with an ED_{50}

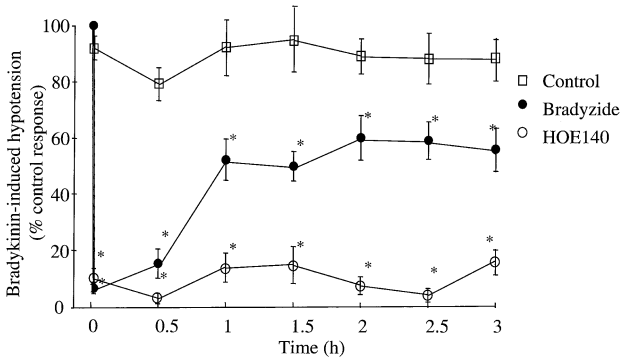

Figure 6 Duration of inhibition of hypotensive responses to bradykinin by bradyzide and HOE-140. Bradyzide (10 μmol kg^{-1}) and HOE-140 (0.5 μmol kg^{-1}) were given as bolus injections i.v. at $t=0$. *Significant different from the saline controls, $P<0.05$ ANOVA followed by Tukey's HSD. Data are means$\pm$s.e.mean of three independent experiments.

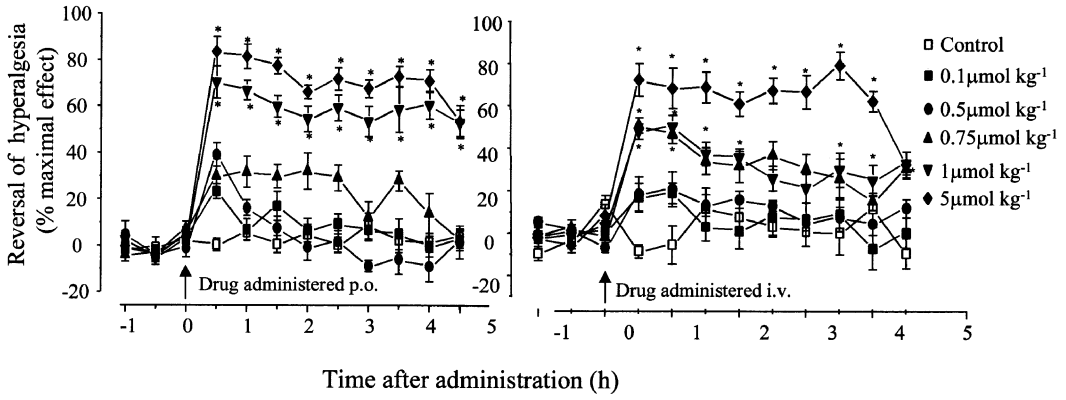

Figure 7 Bradyzide reverses Freund's-adjuvant-induced mechanical hyperalgesia in the rat knee joint. Bradyzide was given both by the i.v. and p.o. routes. The increase in load tolerance resulting from the drug was expressed as per cent maximal reversal of the hyperalgesia (equal load tolerated by control and injected leg). The control vehicles were tragacanth p.o., saline i.v. *Post-treatment values significantly different from pre-treatment values $P<0.05$ ANOVA followed by Tukey's HSD. The data shown are mean$\pm$s.e.mean ($n=8-10$).

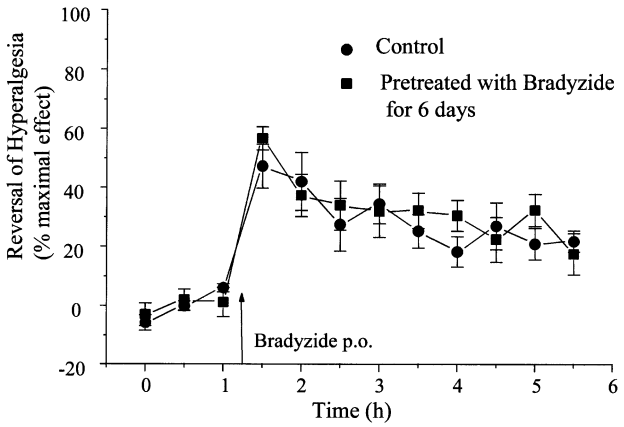

Figure 8 The effect of repeated treatment with bradyzide on its ability to reverse Freund's adjuvant-induced mechanical hyperalgesia. Bradyzide (0.75 μmol kg^{-1}) was given daily for 6 days with a test on day 7. On day 7 bradyzide (0.75 μmol kg^{-1}) was administered immediately subsequent to the reading at 1 h indicated by the arrow. Control animals were given the oral vehicle, tragancanth, for 6 days then tested on Day 7 with bradyzide (0.75 μmol kg^{-1}). Data are mean $\pm$ s.e.mean ($n=8$). There was no significant difference between the control and the treated groups $P>0.05$ ANOVA followed by Tukey's HSD.

Table 3 Comparison of bradyzide, morphine and NSAIDs in two models of chronic inflammatory hyperalgesia

		Freund's adjuvant-induced mechanical hyperalgesia		
Antagonists		*ED_{50}* (μmol kg^{-1})	*Maximal effect* (%)	*Duration* (h)
Bradyzide (non-peptide B_2 antagonist)	i.v.	0.9	79 $\pm$ 7	>4
	p.o	0.84	84 $\pm$ 6	>4
HOE-140 (peptide B_2 antagonist)	i.v. s.c.	50% reversal* not reached		
Diclofenac (NSAID)	p.o.	1.9	64 $\pm$ 5	6
Paracetamol (NSAID)	p.o.	1323	30 $\pm$ 9	4
	s.c.	3300	52 $\pm$ 7	3
Morphine (opiate)	i.v.	2.1	89 $\pm$ 5	1.5
	s.c.	9.5	99 $\pm$ 2	3

ED_{50} (μmol kg^{-1}) values were measured at the peak response and the duration of action (h) and maximal percentage reversal were measured at the maximally effective dose. Where appropriate data, are means $\pm$ s.e.mean. ($n=8$–10). ED_{50} values were obtained from the average dose-response curve for each compound.

value of 0.9 μmol kg^{-1} when given by the intravenous route and 0.84 μmol kg^{-1} when given orally (see Figure 7). The time to onset was rapid, with a maximum response observed within 30 min of both intravenous and oral administration, and the duration of the anti-hyperalgesic effect was in excess of 4 h at doses of 0.75 μmol kg^{-1} p.o. and above (see Figures 7 and 8).

In this model bradyzide (p.o.) was comparable in potency to morphine given i.v. and s.c. but had a longer duration of action. It was comparable in potency to diclofenac and considerably more potent than paracetamol and its maximal effect exceeded that of these NSAIDs (Table 3). HOE-140 had an unusual profile in Freund's adjuvant-induced hyperalgesia. In contrast to the effect of bradyzide, HOE-140 was poorly active, giving a maximal 30 $\pm$ 6% ($n=8$) reversal of hyperalgesia following administration of 0.1 nmol kg^{-1} (i.v.). Higher doses were inactive. The lack of effectiveness of HOE-140 in this model appears to be due to its ability (unrelated to the B_2 receptor) to cause mast cell degranulation which results in increased hyperalgesia (Davis & Perkins, 1994).

Bradyzide did not exhibit tolerance following repeated administration. Thus there was no reduction in the magnitude or duration of the reversal of the hyperalgesia when bradyzide was administered daily at a sub-maximal dose (0.75 μM kg^{-1} p.o.) for 6 days following administration of the Freund's adjuvant. Figure 8 shows that there was no significant difference in the response to 0.75 μmol kg^{-1} (p.o.) bradyzide on day 7 post Freund's adjuvant in animals that had been treated with bradyzide for 6 days and in animals that had been given the oral vehicle (tragacanth) for the same period.

Discussion

Bradykinin, acting *via* B_2 receptors, causes excitation and sensitization of primary afferent nociceptors leading to pain and hyperalgesia. As well as these direct effects, it causes the production of other inflammatory mediators which not only act directly on nociceptors, but are also involved in the generation and maintenance of inflammation. We have developed a potent, orally active non-peptide B_2 bradykinin antagonist (bradyzide) that reverses hyperalgesia in models of chronic inflammatory hyperalgesia and shows no evidence of tolerance. We believe that this compound will prove of value in the further evaluation of bradykinin in pain and inflammation as well as in other physiological and pathophysiological conditions.

Bradyzide, (2S)-1-[4-(4-Benzhydrylthiosemicarbazide)-3-nitrobenzenesulfonyl]-pyrrolidine-2-carboxylic acid {2-[(2-dimethylaminoethyl)methylamino]ethyl}amide, was developed from a high-throughput screening lead by appending appropriate binding determinants onto the nitrophenylthiosemicarbazide core. It is a potent and selective inhibitor of the rat B_2 bradykinin receptor with a molecular weight of 682.97. It is structurally distinct from existing non-peptide B_2 antagonists, the phosphonium-derived WIN64338 (Sawutz *et al*., 1994) and the heteroaryl benzyl ethers FR173657 and FR193517 (Asano *et al*., 1997; Abe *et al*., 1998). Unlike WIN64338, which is charged at both its terminals and FR173657 and FR193517 which are largely hydrophobic in nature, bradyzide is amphiphilic, with a diamine terminal and a hydrophobic diphenyl methyl moiety at the opposite terminal. Although it is unlikely that these three structural classes of B_2 antagonists will bind to the same determinants on the receptor, this will only be clarified with further biochemical and molecular biological experiments.

In vitro, bradyzide exhibits all the characteristics of a B_2 bradykinin receptor antagonist. It displaced the specific binding of [^{3}H]-bradykinin to NG108-15 cell membranes and it inhibited the functional effects of bradykinin in a number of assays, including a sensory fibre preparation. In the rat cells and tissues in which it was tested, including sensory neurones, it was a very potent antagonist and showed no signs of agonism at concentrations up to 3 μM. In NG108-15 cells bradyzide was a competitive antagonist, causing parallel, rightward shifts of the log-concentration response curves to bradykinin. The competitive nature of the antagonism was confirmed by Schild plot analysis of the data in these cells. In the rat uterus, neither bradyzide nor the peptide antagonist HOE-140 suppressed the size of the maximum response to

bradykinin, but the slopes of the Schild plots were significantly greater than 1 for both compounds, raising the possibility of a degree of non-competivity to the antagonism in this tissue. In contrast to HOE-140, bradyzide showed strong species selectivity. This was investigated in cells expressing the human and rat receptors constitutively and also using the cloned human and rat B_2 receptors expressed in Cos-7 cells and in H4 hepatomas, neither of which show constitutive expression of the B_2 receptor. At both the native receptors and the cloned receptors, bradyzide was up to 800 times more potent at the rat than at the human B_2 receptor. The regions of the receptors responsible for this striking difference are being investigated in studies with human and rat B_2 receptor chimeras and by point mutations. In addition to showing species-selectivity, high concentrations (up to 10 μM) of bradyzide failed to interact with either the B_1 bradykinin receptor or with receptors for a range of other hormones and neurotransmitters, indicating that it is highly selective for the B_2 bradykinin receptor.

Many inflammatory disorders involve the release of kinins, and bradykinin plays a key role in the pathogenesis of inflammatory pain. In support of this, studies with peptide B_2 antagonists have indicated that blocking the B_2 bradykinin receptor reduces chronic inflammatory hyperalgesia in animal models (Dray & Perkins, 1993; Perkins & Kelly, 1993; 1994). Although it has been shown that the non-peptide B_2 antagonist FR173657 can block acute pain, it has not yet been shown that an orally active B_2 antagonist can reverse established hyperalgesia in models of chronic inflammatory hyperalgesia. Present studies with bradyzide demonstrate that it is possible to reverse 80–85% of a well established mechanical hyperalgesia with a single oral dose of bradyzide in a model of chronic inflammatory hyperalgesia. Bradyzide was as potent as morphine with ED_{50} values of approximately to 0.8 μmol kg^{-1} (p.o.). The duration of the reversal was long lasting (up to 6 h) which was significantly longer than the duration of action of morphine (2–3 h). Moreover, bradyzide had similar potency to diclofenac and was over 1000 times more potent than paracetamol. Its maximal effect exceeded that of both NSAIDs tested. Strikingly, bradyzide showed no signs of tolerance when administered over a period of 6 days. Similar reversal of mechanical hyperalgesia was obtained in a model of turpentine-induced chronic inflammatory hyperalgesia in the rat paw (Perkins *et al*., 1992) (data not shown). Bradyzide also reversed UV-induced thermal hyperalgesia in the rat paw (Perkins & Kelly, 1993) (data not shown) indicating that B_2 receptors are involved in both mechanical and thermal hyperalgesia.

As well as reversing hyperalgesia B_2 antagonists reduce inflammation *per se* (Costello & Hargreaves, 1989; Wirth *et al*., 1991; Asano *et al*., 1997) and bradyzide was extremely potent and effective in reducing bradykinin-mediated plasma extravasation from the bladder.

These studies confirm that the B_2 bradykinin receptor is a good point of intervention for the treatment of inflammatory hyperalgesia. Bradyzide has a profile that includes potency *in vitro*, and oral bioavailability, efficacy, long duration of action and lack of tolerance in models of inflammatory hyperalgesia. This provides strong support for the concept that orally-active B_2 bradykinin receptor antagonists would provide excellent anti-inflammatory and anti-hyperalgesic therapy, for diseases, such as rheumatoid arthritis, where chronic treatment is necessary.

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