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Antagonistic effects of novel non-peptide chlorobenzhydryl piperazine compounds on contractile response to bradykinin in the guinea-pig ileum

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Abstract

Two novel compounds, *N*-phenylacetyl-*N*′-(4-methoxybenzyl)-*N*″-1-(4-chlorobenzhydryl)piperazine iminodiacetic acid triamide (compound **I**) and *N*-phenylacetyl-*N*′-(4-methylbenzyl)-*N*″-1-(4-chlorobenzhydryl)piperazine iminodiacetic acid triamide (compound **II**), designed and synthesized as novel non-peptide bradykinin B_2 receptor antagonists, were studied for their functional activities in isolated guinea-pig ileum smooth muscle. These compounds were compared with the conventional peptide bradykinin B_2 receptor antagonist, icatibant (H–DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DTic-Oic-Arg–OH) for their in vitro functional activities. Compounds **I** and **II** showed highly potent, time-dependent insurmountable antagonism against contractile responses to bradykinin (pK_B 8.80 and 8.57, respectively) with progressive reduction of maximum effect maintaining the concentration producing half maximal-response unchanged. Otherwise, icatibant, known as a non-competitive antagonist, showed a rightward displacement of cumulative concentration–response curves to bradykinin with decrease of its maximum effect (pK_B 8.73). The IC_{50} values of compounds **I** and **II** were 3.56×10^{-8} and 6.30×10^{-8} M, respectively, while that of icatibant was 5.02×10^{-8} M. The profile of action of compounds **I** and **II** varied when contact time was prolonged from 5 to 60 min, whereas that of icatibant did not. The inhibitory effects of the newly synthesized compounds and icatibant on the contractile response to bradykinin were differently reverted by washout (icatibant <100 min, compounds **I** and **II** >100 min). This class of compounds containing the chlorobenzhydryl piperazine moiety is expected to be a novel non-peptide bradykinin B_2 receptor antagonists.

Keywords: Bradykinin receptor antagonist; Non-peptide; Guinea-pig ileum smooth muscle contraction; Chlorobenzhydryl piperazine

1. Introduction

Bradykinin (H–Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg–OH), an endogenous nonapeptide generated by limited proteolysis of kininogen in tissues and body fluids, elicits numerous responses including vasodilation, edema, smooth muscle contraction, inflammation, trauma, burns, shock, and allergy (Regoli and Barabé, 1980; Bhoola et al., 1992; Hall, 1992, for review). The biological effects of bradykinin are mediated by two different types of G protein-coupled receptors, namely B_1 and B_2, which have been cloned from various species including humans (Eggerickx et al., 1992; Hess et al., 1992; Menke et al., 1994). Before their molecular identification, the existence of B_1 and B_2 receptors was postulated based on pharmacological studies (Regoli and Barabé, 1980). The B_2 receptors are constitutively expressed on most cell types, whereas the B_1 receptors are not present in tissues under 'normal' conditions but are induced during inflammatory insults (Marceau, 1995, for review; Regoli et al., 1998).

The representative "second generation" peptide antagonist, icatibant (H–DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DTic-Oic-Arg–OH) (Fig. 1), has shown highly potent and long-acting bradykinin antagonism both in vitro and in vivo (Hock et al., 1991; Wirth et al., 1991), but its therapeutic use is limited because of its poor oral bioavailability. The first non-peptide antagonist WIN 64338 ([[4-[[2-[[bis(cyclohexylamino) methylene]amino]-3-(2-naphthyl)-1-oxopropyl]amino]phenyl] methyl]tributylphosphonium chloride monohydrochloride) provides competitive (Sawutz et al., 1994) or non-competitive

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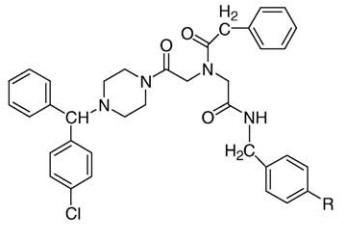

DArg[1]-Arg[2]-Pro[3]-Hyp[4]-Gly[5]-Thi[6]-Ser[7]-DTic[8]-Oic[9]-Arg[10]

Icatibant

Fig. 1. Chemical structures of compounds **I**, **II**, and icatibant.

(Pruneau et al., 1995) antagonism towards bradykinin in several species, however, WIN 64338 shows low specificity and a species-dependent variable affinity for bradykinin B_2 receptors (Wirth et al., 1995; Regoli et al., 1996, for review).

To enhance receptor specificity and affinity to bradykinin B_2 receptors, a number of potent and selective non-peptide antagonists for bradykinin B_2 receptors have been identified in recent years, such as FR 173657 ((*E*)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxy-methyl]phenyl]-*N*-methylaminocarbonylmethyl]acrylamide), LF 16-0687 (1-[[2,4-dichloro-3-[[(2,4-dimethylquinolin-8-yl) oxy]methyl]phenyl]sulfonyl]-*N*-[3-[[4-(aminoiminomethyl)-phenyl]carbonylamino]propyl]-2(*S*)-pyrrolidinecarboxamide), and bradyzide ((2*S*)-1-[4-(4-benzhydrylthiosemicarbazido)-3-nitrobenzenesulfonyl]-pyrrolidine-2-carboxylic acid [2-[(2-dimethylaminoethyl)methylamino]ethyl]amide) (Altamura et al., 1999, for review; Bock and Longmore, 2000).

The quinoline derivatives synthesized by Fujisawa showed antagonist activity at the bradykinin B_2 receptors. In particular, FR 173657 was a potent, orally available bradykinin B_2 receptor antagonist whose biological activity has been extensively studied (Aramori et al., 1997; Asano et al., 1997, 1999; Inamura et al., 1997; Griesbacher et al., 1997; Abe et al., 1998a,b; Gobeil et al., 1999; Kayakiri et al., 1999; Meini et al., 2000a, 2004). In isolated guinea-pig ileum, both icatibant and FR 173657 exerted non-competitive antagonism (pK_B 9.5 and 9.2, respectively) to contractile response to bradykinin (Meini et al., 2000b).

On the other hand, Fournier compound LF 16-0687, which is structurally related to the Fujisawa compounds, behaves as a competitive antagonist of bradykinin-mediated contractions with pA_2 values of 9.1 in human umbilical vein, 7.7 in the rat uterus, and 9.1 in the guinea-pig ileum (Pruneau et al., 1999). Moreover, the structurally distinct bradykinin B_2 receptor antagonist bradyzide is orally bioavailable and selective for B_2 over B_1 receptors (Burgess et al., 2000).

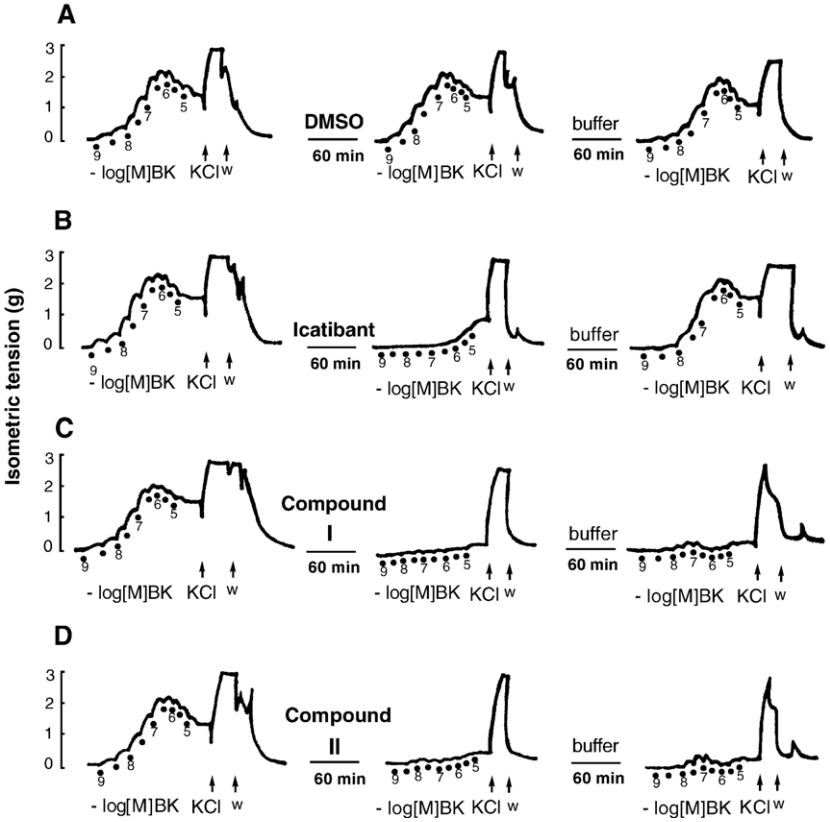

Fig. 2. Changes of isometric tension in grams (g) induced by bradykinin (BK) in guinea-pig isolated ileum. Contractile effect of increasing concentrations (1 nM–10 μM) of bradykinin tested in the absence (on the left) and presence (on the middle) of antagonists at 1 μM: (A) vehicle; (B) icatibant; (C) compound **I** or (D) compound **II** preincubated for 60 min. At the end of each curve, the maximal contractile response of the preparation was evaluated by administering KCl (80 mM) and washing out (w) with a fresh Tyrode solution. The reversibility of bradykinin B_2 receptor blockade (on the right) produced by (A) vehicle, (B) icatibant, (C) compound **I** or (D) compound **II** 60 min after washout of the antagonist.

In our search for new bradykinin B_2 receptor antagonists, we previously reported the bradykinin antagonistic effects of piperazine compounds derived from the anti-histamine drug, cetirizine (Choo et al., 1999). Furthermore, we have designed and synthesized new compounds with piperazine containing three amide bonds and a lipophilic ring system in each molecule (Kam et al., 2004).

Here, we describe the pharmacological characterizations of two novel compounds, compounds **I** [*N*-phenylacetyl-*N*′-(4-methoxybenzyl)-*N*″-1-(4-chlorobenzhydryl)piperazine iminodiacetic acid triamide] and **II** [*N*-phenylacetyl-*N*′-(4-methylbenzyl)-*N*″-1-(4-chlorobenzhydryl)piperazine iminodiacetic acid triamide] (Fig. 1), which were obtained by solution-phase combinatorial synthesis for non-peptide bradykinin B_2 receptor antagonists. Since the guinea-pig ileum assay has been the object of several studies on bradykinin B_2 receptor pharmacology (Hall, 1992, for review), we compared the functional antibradykinin activities of compounds **I** and **II** with that of icatibant in the guinea-pig ileum smooth muscle.

2. Materials and methods

2.1. Chemicals and preparation of drugs

Bradykinin, captopril, indomethacin, dithiothreitol, atropine and icatibant were obtained from Sigma (St. Louis, MO, USA). Dibenamine was obtained from TCI (Tokyo, Japan). The synthetic routes from iminodiacetic anhydride as template to the fifty new compounds, including compounds **I** and **II**, are reported by Kam et al. (2004). Bradykinin and icatibant were dissolved and diluted in Tyrode solution and dithiothreitol was dissolved in dimethyl sulfoxide (DMSO) and diluted in Tyrode solution. Captopril, indomethacin, atropine, dibenamine, and compounds **I** and **II** were dissolved and diluted in DMSO. The composition of the Tyrode solution was as follows (in mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.15, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, and glucose 5.6. The final DMSO concentration in the bath was less than 0.01% and it had no discernible effect on the tissue responsiveness to bradykinin.

2.2. Preparation and functional recording of guinea-pig ileal smooth muscle

Male Hartley guinea-pigs weighing 260–500 g (Jeil, Korea) were fasted overnight and then decapitated. A section of ileum approximately 40 cm in length was removed at a level 2 cm above the ileocecal junction and placed in warm (37 °C) Tyrode solution. Strips of intestinal muscle with mucosa, 1.5–2 cm in length, were then mounted in a 50 ml bath containing Tyrode solution (37 °C) and aerated with 95% O_2/5% CO_2. Tissue contractions were recorded isometrically on a Grass model 76E polygraph.

After an equilibration period of about 60 min, a stable baseline tone was reached and two or three contractions were obtained in response to bradykinin (0.1 μM), at 20-min intervals, to assay the sensitivity and reproducibility of the contractile response. Only segments producing reproducible responses were used. Afterwards, a cumulative concentration–response curve to bradykinin (1 nM–10 μM) was constructed and repeated two or three times. At the end of each curve, the maximal contractile response of the preparation was evaluated by administration of KCl (80 mM). After washout and recovery of basal tone, each test compound (3 nM–1 μM) was pre-incubated for 60 min (when not stated otherwise) and the concentration–response curve to bradykinin was repeated in the presence of the test compound under study. To minimize degradation of bradykinin and to prevent responses due to neuronal activation or prostaglandin production, Tyrode solutions contained 1 μM each of captopril, atropine, dithiothreitol and indomethacin. Moreover, 1 μM of dibenamine, the irreversible histamine H_1 blocker, was also added in Tyrode solutions to prevent histaminergic responses.

The reversibility of B_2 receptor blockade produced by the antagonists under study was evaluated after construction of the

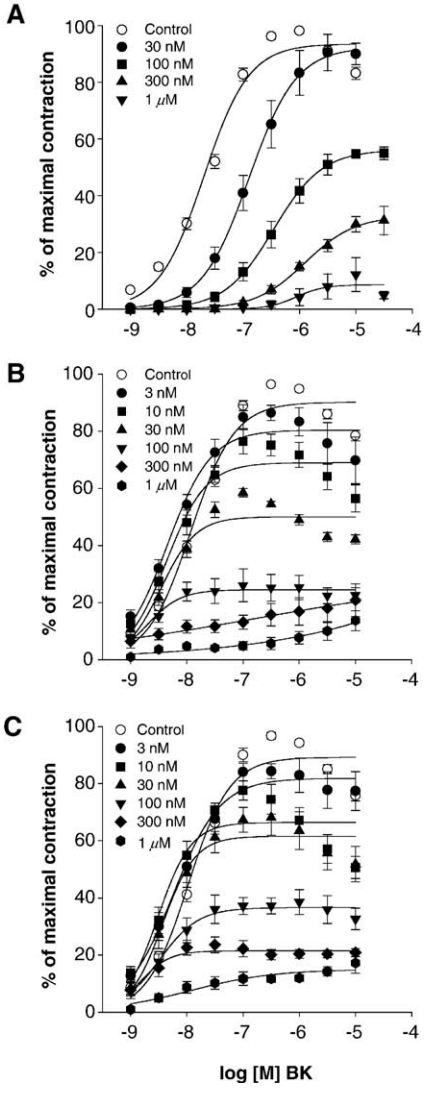

Fig. 3. Concentration–response curves to bradykinin in the guinea-pig ileum smooth muscle in the absence (control) and presence of various concentrations (3 nM–1 μM) of antagonists: (A) icatibant, (B) compound **I** and (C) compound **II**. Contact time of antagonists was 60 min. Values represent means ± S.E.M. of 5–8 experiments.

cumulative concentration–response curves to bradykinin (1 nM–10 μM) in the presence of antagonists (1 μM) or vehicle. The preparations were thoroughly washed with Tyrode solution, which was renewed every 20 min. Cumulative concentration–response curves to bradykinin were repeated at 20, 60, 100, 120, 160, and 200 min after washout of the antagonist, and the responses were compared to those obtained in control time-matched preparations. Bradykinin at 0.3 μM was used to describe its reversibility.

2.3. Data analysis

Contractions elicited by bradykinin were expressed as percentages of the maximum response obtained when the tissue was tested with cumulative increasing doses of bradykinin. The agonist concentration producing 50% of its maximal response was calculated as negative logarithm to base 10 and defined as pD_2. The 95% confidence limits (c.l.) were calculated by non-linear regression analysis of the concentration–response curve.

When antagonists caused non-parallel shifts of the concentration–response curves to bradykinin and decreased the maximal response (E_{max}), the estimate of the affinity (K_B) was calculated by a method for non-competitive and/or pseudo-irreversible antagonists as described by Kenakin (1997b). In practice, a double-reciprocal plot of equieffective concentrations of agonist (A) in the absence ($1/A$) and in the presence ($1/A'$) of the antagonist (B) was constructed and K_B was derived

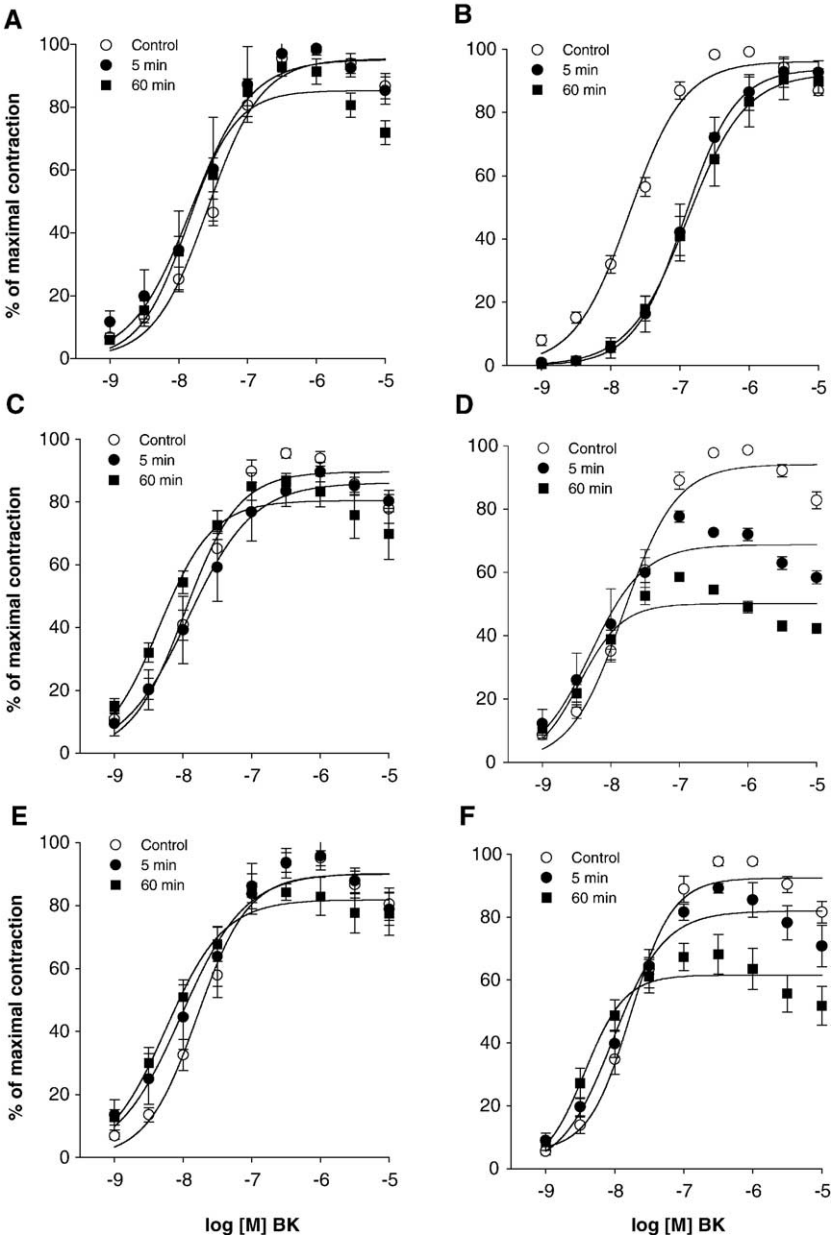

Fig. 4. Concentration–response curves to bradykinin in the guinea-pig ileum smooth muscle in the absence (control) and presence of antagonists, after 5 and 60 min contact time: (A) icatibant, 3 nM; (B) icatibant, 30 nM; (C) compound **I**, 3 nM; (D) compound **I**, 30 nM; (E) compound **II**, 3 nM and (F) compound **II**, 30 nM. Values represent means±S.E.M. of 4–8 experiments.

from the equation: $K_B=[B]/\text{slope}-1$. In order to obtain more accurate estimates of K_B, we selected the experiments in which E_{max} to the agonist was depressed to less than 50% of control by the antagonist.

Inhibitory activity, expressed as IC_{50}, was calculated from the concentration-dependent inhibitory effect curves of antagonists.

Pair-wise comparisons were done by the one-tailed Student's t test. Probability values of <0.05 were considered to be statistically significant. All results are expressed as means±S.E.M. and the sample size, n, represents the number of individual strips of ileum assayed. The experiments were designed such that the sample size would also represent the same number of animals. For example, two to four strips from one guinea-pig were used for two to four different tests for an $n=1$ sample size and those from another guinea-pig were used for the same two to four tests to increase the sample size for a given test to $n=2$, and so on.

3. Results

Control experiments were performed to optimize the experimental conditions for contractions of the guinea-pig ileum to bradykinin. In isolated guinea-pig ileum, bradykinin (1 nM–10 μM) induced a concentration-dependent contraction with a pD_2 value of 7.9 (95% c.l. 7.7–8.1; $n=83$).

Bradykinin-induced contractile response was examined in the presence of antihistamine (dibenamine) and anticholinergic (atropine) drugs in the bath solution in order to block histaminergic- or cholinergic-mediated responses. Both dibenamine and atropine at 1 μM did not produce any influence on the bradykinin-induced contractile response (not shown).

Tracings showing cumulative concentration–response curves obtained with bradykinin in the absence and in the presence of compounds **I**, **II**, or icatibant are presented in Fig. 2. The reversibility at 60 min after washout of the antagonist is also shown. Compounds **I**, **II**, or icatibant did not reduce the maximal response to KCl (80 mM) and therefore they did not affect smooth muscle contractility. No partial agonist activity was observed for any compound at the concentrations used. All three antagonists decreased the E_{max} of bradykinin to a similar extent at the concentrations tested. However, the reversibility after washout of the novel compounds and icatibant showed different profiles.

To define more precisely the type of antagonism, concentration–response curves to bradykinin were obtained in the absence and presence of increasing concentrations of compounds **I**, **II**, or icatibant (Fig. 3). The E_{max} of bradykinin was reduced dose-dependently by icatibant and novel test compounds, indicating an insurmountable mode of antagonism.

Although compounds **I**, **II**, and icatibant all induced a progressive reduction in E_{max}, their cumulative concentration–response curves were differently characterized according to the concentration producing half maximal-response (EC_{50}) in the presence of increasing concentrations of antagonists. While a rightward shift of dose–response curves of bradykinin was clearly observed in the presence of icatibant, the novel compounds **I** and **II** produced no rightward shift of dose–response curves of bradykinin and the EC_{50} of bradykinin remained unchanged. In the presence of the novel compounds, the cumulative concentration–response curve to bradykinin reached a plateau at lower concentrations of bradykinin than that in the presence of icatibant.

In order to evaluate a possible time dependency of the interaction with bradykinin B_2 receptor, the effect of the three antagonists was examined after 5 and 60 min contact time (Fig. 4). Compounds **I**, **II**, and icatibant were tested at two concentrations: 3 and 30 nM. The effect for each antagonist at 3 nM was superimposable after 5 and 60 min contact time (Fig. 4A, C and E). Icatibant at 30 nM produced a rightward shift of the concentration–response curve to bradykinin and its effect was also superimposable after 5 and 60 min contact time (Fig. 4B). On the other hand, compounds **I** and **II** at 30 nM depressed the E_{max} of bradykinin more after 60 min contact time than 5 min (Fig. 4D and F).

The calculated apparent antagonistic affinity measured in functional experiments (pK_B) and in vitro IC_{50} values of the novel non-peptide compounds **I** and **II** were compared to those of the peptide bradykinin B_2 receptor antagonist, icatibant (Table 1). In the present study, icatibant antagonized the bradykinin-induced contraction of guinea-pig smooth muscle with a pK_B of 8.73 and an IC_{50} of 5.02×10^{-8} M, which are similar to those found previously (Hock et al., 1991; Pruneau et al., 1995; Meini et al., 2000b). The apparent pK_B values were 8.80±1.36 for compound **I** and 8.57±0.84 for compound **II**. The IC_{50} values were 3.56×10^{-8} M for compound **I** and 6.30×10^{-8} M for compound **II**.

The reversibility of bradykinin B_2 receptor blockade produced by the antagonists was evaluated as the capacity of the ileum smooth muscle to recover to contraction produced by a cumulative dose of bradykinin (1 nM–10 μM) after a 60 min contact time with the antagonists. As shown in Fig. 5, all three antagonists at 1 μM produced comparable degrees of inhibition of the response to bradykinin (0.3 μM), which were 94±2% for compound **I**, 88±1% for compound **II**, and 98±1% for icatibant. The inhibition exerted by icatibant was slowly reversed by washout and a full recovery of the response to the agonist occurred within 100 min from removal of the antagonist. In contrast, compounds **I** and **II** produced a persistent antagonism at bradykinin B_2 receptors, which was

Table 1
Antagonistic affinity and in vitro IC_{50} of compounds **I**, **II**, and icatibant in the guinea-pig ileum

	pK_B	In vitro IC_{50} (M)
Compound **I**	8.80±1.36	3.56×10^{-8}
Compound **II**	8.57±0.84	6.30×10^{-8}
Icatibant	8.73±0.60	5.02×10^{-8}

Antagonistic activity is expressed as apparent pK_B calculated from the equation $K_B=[B]/\text{slope}-1$, where a double-reciprocal plot of equieffective concentrations of bradykinin (A) in the absence ($1/A$) and in the presence ($1/A'$) of the antagonist (B) is constructed. Inhibitory activity, expressed as IC_{50}, was calculated from the concentration-dependent inhibitory effect curves of antagonists. pK_B values represent means±S.E.M. of 3–4 experiments.

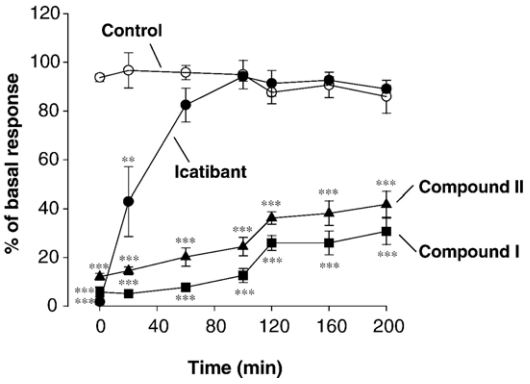

Fig. 5. The reversibility of bradykinin B_2 receptor blockade produced by icatibant and compounds **I** and **II** in the guinea-pig ileum smooth muscle preparation. The contractile response to bradykinin (0.3 μM) is shown in the presence (first contraction at time 0 min) or in the absence (all other contractions) of icatibant, compound **I** or **II** at 1 μM, after 20, 60, 100, 120, 160 and 200 min from washout of the antagonists. Antagonist contact time was 60 min. Values represent means±S.E.M. of five experiments. $^{**}P<0.01$, $^{***}P<0.001$ vs. matched vehicle responses.

not fully reversed up to 200 min from removal of the antagonist from the bath solution.

4. Discussion

This study investigates the activity of two new bradykinin receptor antagonists in a standard in vitro test for bradykinin B_2 receptor ligands. The compounds belong to a new class of B_2 receptor antagonists and this is the first time their pharmacological activity has been characterized.

Regarding the previously reported piperazine compounds with antibradykinin effects (Choo et al., 1999), we have designed and synthesized new compounds containing piperazine, three amide bonds, and a lipophilic ring system in each molecule by solution-phase combinatorial synthesis. About fifty non-peptide bradykinin receptor antagonists have been synthesized by using iminodiacetic anhydride as a template and their antibradykinin activities were screened at 0.1 μM (Kam et al., 2004). In the present study, two novel non-peptide compounds, **I** and **II** (Fig. 1), were selected among fifty compounds to characterize their pharmacological profiles for the bradykinin B_2 receptor in the guinea-pig ileum smooth muscle.

Effects of compounds **I** and **II** were compared with those of the currently most widely used peptide B_2 receptor antagonist, icatibant (Fig. 1). Icatibant has consistently shown a high affinity for the human bradykinin B_2 receptor sites as well as for the rabbit and guinea-pig sites (Regoli and Barabé, 1980). Icatibant is known to act as a competitive antagonist for the human bradykinin B_2 receptors (Marceau et al., 1994; Félétou et al., 1995; Gobeil et al., 1996) but is non-competitive for other bradykinin B_2 receptor systems (e.g., rabbit and guinea-pig). In these latter preparations, icatibant is considered to be a non-equilibrium antagonist which interacts with the same receptor sites as bradykinin but dissociates slowly and prevents full occupation of receptors by the agonist (Regoli et al., 1993).

The pharmacological profiles of both novel non-peptide compounds (**I** and **II**) and peptide icatibant were clearly insurmountable. The insurmountable interaction of those compounds at guinea-pig ileum bradykinin B_2 receptors was demonstrated by producing non-parallel shifts in the bradykinin contractility concentration–response curves with a progressive reduction in maximum response (Fig. 3). Unlike surmountable antagonism, in which excess concentrations of agonist allow maximal response to the agonist even in the presence of antagonist, insurmountable antagonism produces depression of maximal response to the agonist, whether or not it is accompanied by a shift of the dose–response curve to the right (Kenakin, 1997a).

Despite the similarity in their insurmountable antagonism, a striking difference in the antagonistic profile of compounds **I** and **II** was observed when compared to that of icatibant. The rightward shift of the dose–contractile response curves of bradykinin in guinea-pig ileum was produced in the presence of icatibant with increase of EC_{50} of bradykinin, which concurs with observations made during the previous studies (Griesbacher and Lembeck, 1992; Meini et al., 2000b) (Fig. 3A). If the antagonist decreases the potency of the agonist for the receptor, a more pronounced shift to the right of the dose–response curves is observed with depression of maximal response (Kenakin, 1997b). On the other hand, the EC_{50} of the agonist in the presence of increasing concentrations of compounds **I** or **II** was not increased, indicating no decrease of the agonist potency for contractile response to bradykinin by these compounds, even though the compounds inhibited the E_{max} of bradykinin (Fig. 3B and C). It can be also considered that the difference between the novel compounds (**I** and **II**) and icatibant is that compounds **I** and **II** are full insurmountable antagonists while the results with icatibant rather shows the profile of a full insurmountable antagonist with a nonlinear stimulus–response relationship due to the occurrence of spare receptors.

According to the non-equilibrium conditions, a difference in the incubation time existed between the novel compounds and icatibant. The inhibitory effect of icatibant was independent of the contact time, whereas compounds **I** and **II** showed time-dependent insurmountable antagonism. When the contact time was extended from 5 to 60 min, the inhibitory effect of icatibant did not modify, but the effect of compounds **I** or **II** was further increased after 60 min of contact time (Fig. 4). Also, the difference in the type of antagonism produced by novel compounds and icatibant can be possibly explained, at least in part, by the results of reversibility studies which showed that receptor blockade produced by compounds **I** and **II** was reversed more slowly than that produced by icatibant (Fig. 5). What can be further considered is allosteric interaction (Kenakin, 1997b). The binding of these novel antagonists is possibly at a site distinct from the binding domain for bradykinin, but the interference to bradykinin binding is achieved by a protein conformational change induced by the binding of antagonists. Receptor binding studies will be necessary to clarify the mode of interaction with the bradykinin receptor.

In conclusion, the novel compounds, **I** and **II**, were synthesized very easily by solution-phase combinatorial synthesis containing a chlorobenzhydryl piperazine moiety

and iminodiacetic anhydride as a template and these novel non-peptide compounds produced highly potent anti-bradykinin effect with an insurmountable antagonistic profile to bradykinin B_2 receptor. The present study introduces new non-peptide bradykinin B_2 receptor antagonists, which can be developed as a novel non-peptide B_2 receptor antagonist drug.

Acknowledgements

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