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Functional characterization of bradykinin analogues on recombinant human bradykinin B_1 and B_2 receptors

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Abstract

We have examined the activity of a range of kinins on recombinant human bradykinin receptors, using a high throughput functional assay which measures intracellular Ca^{2+} responses. The most potent agonist for Chinese hamster ovary (CHO) cells stably expressing recombinant human bradykinin B_1 receptors were Des-Arg^9-bradykinin (EC_{50} = 7.9 nM) and Des-Arg^{10}-kallidin (EC_{50} = 8.6 nM), while the most potent agonist for CHO cells expressing human bradykinin B_2 receptors was bradykinin (EC_{50} = 2.0 nM). These findings confirm the validity of the recombinant system and the microtitre plate imaging-based characterization system when compared to known agonist properties of the native receptors. The concentration–response relationship for bradykinin at bradykinin B_2 receptors was potently inhibited by [D-Arg^0,Hyp^3,β-(2-thienyl)-Ala^5,D-Tic^7,Oic^8]-bradykinin (Hoe140) (IC_{50} = 71 nM), which was 500-fold more potent against the B_2-expressing cells than the B_1 cells. Bradykinin B_1 receptor-mediated responses activated by Des-Arg^{10}-kallidin were fully antagonized by Des-Arg^9-[Leu^8]bradykinin (IC_{50} = 59 nM), Des-Arg^{10}-Hoe140 (IC_{50} = 211 nM) and most potently by Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic (B9858) (IC_{50} = 14 nM), none of which displayed any activity against the bradykinin B_2 receptor cell line up to 3 μM. None of the antagonists displayed partial agonism activity in these cell lines. All bradykinin B_1 and B_2 receptor antagonists tested acted in an apparently non-competitive manner that is likely to be due in part to their kinetics and to the nature of the functional assay used.

Keywords: Des-Arg^{10}-kallidin; FLIPR (fluorometric imaging plate reader); Bradykinin; Ca^{2+}; Non-competitive antagonism; CHO cells

1. Introduction

Kinin receptors have been classified into two distinct subtypes, bradykinin receptors B_1 and B_2, initially on the basis of distinct induction and agonist activation profiles (Regoli and Barabe, 1980). Subsequently separate gene products for B_1 and B_2-mediated responses were identified and cloned from a number of tissues and species, and subtype-specific antagonists have been developed (Hess et al., 1992; Wirth et al., 1992; Menke et al., 1994; Marceau et al., 1998). Both have been suggested to play important roles in the inflammatory response to tissue injury, with the constitutively expressed bradykinin B_2 receptor likely to be important in the early stages of the response, and the inducible bradykinin B_1 receptor thought to be involved in the chronic period of inflammation and pain transduction (Dray and Perkins, 1993; Marceau et al., 1998). The involvement of kinin receptors in inflammation and pain transduction has been supported by the results of studies on mice lacking bradykinin B_2 receptor expression (Borkowski et al., 1995; Rupniak et al., 1997; Seabrook et al., 1997).

The nature of the bradykinin receptor modulation evoked by some kinins is not clearly established from literature reports. Distinct differences in agonist and antagonist potency and selectivity have been reported for the native kinin receptors found in different species and tissues (Wirth et al., 1992; Hall, 1997; MacNeil et al., 1997; Wohlfart et al., 1997). Also, compounds with subtype selectivity in one tissue have been reported to affect both subtypes in other preparations (Wirth et al., 1992). However, to date, no genetic evidence has been reported for subtypes of either the B_1 or B_2 bradykinin receptor. It is currently

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unclear whether these discrepancies are due to differences in assay format, equilibration period, or receptor reserve, such that compounds with low intrinsic activity may be detected as antagonists if the physiological response requires stimulation of a large proportion of the expressed receptors (Kenakin, 1993).

The study of antagonism using functional efficacy measurement is preferable to radioligand binding for understanding the nature and consequences of the ligand–receptor interaction. As both human subtypes have been cloned, it is possible to study receptor modulation in cell lines stably expressing one or other subtype. Luminescence studies in an aequorin-human embryonic kidney cell line (aequorin-HEK-293 cells) transiently transfected with bradykinin B_1 receptors have shown that Ca^{2+}-based screening is a viable approach to bradykinin receptor ligand characterization (MacNeil et al., 1997). We have, therefore, developed a Ca^{2+}-based high throughput screen for bradykinin receptor activity using Chinese hamster ovary (CHO) cells expressing recombinant human kinin receptors in a 96-well real-time fluorometric imaging plate reader (FLIPR). In the present study, we have reexamined the functional effects of a range of known kinin receptor ligands for both agonist and antagonist activity.

2. Materials and methods

2.1. Preparation of experimental buffer and drug solutions

Balanced salt solution containing either 2.5 or 1.25 mM $CaCl_2$ was prepared daily in deionized water. Probenecid was prepared in 1 M sodium hydroxide solution giving a 500 mM stock solution, and the buffer made up to 2.5 mM probenecid concentration before being adjusted to pH 7.4 with sodium hydroxide solution. Des-Arg^{10}-kallidin and bradykinin were stored in aliquots at 50 mM at −20°C. Antagonists and agonists were made up in two separate plates at ×5 final desired concentration, 250 μl per well.

2.2. Preparation of cells

CHO cells stably expressing recombinant human B_2 receptors were produced by lipofectamine transfection as has been previously described (Hess et al., 1992). B_2 cDNA was subcloned into the pcDNA I-Neo expression vector. CHO cells stably expressing recombinant human B_1 receptors were produced by calcium phosphate precipitation using a previously published technique (Whiting et al., 1991; Hadingham et al., 1995). B_1 cDNA was subcloned into the pcDNA3 vector. Cells were grown in 2 mg ml^{-1} Geneticin (G418). Geneticin-resistant cell colonies were isolated using cloning rings and assayed for receptor expression using radioligand binding. The cell populations expressing the highest level of [^{3}H]Des-Arg^{10}-[Leu^9]kallidin binding (B_1) or [^{3}H]Bradykinin binding (B_2) were recloned by limiting dilution. Human B_1- or B_2-expressing CHO cell lines were maintained in Iscoves media supplemented with 10% Fetalclone II serum and G418 (1 mg ml^{-1}). Cells were split weekly, typically at a ratio of 1:40. In radioligand binding experiments in the B_1-CHO cells, [^{3}H]Des-Arg^{10}-[Leu^9]kallidin was found to bind to a single saturable site on bradykinin B_1 receptors with $B_{max} = 173 \pm 53$ fmol/mg, $K_d = 1.18$ (0.93, 1.50) nM and nH = 0.88 ± 0.08 ($n = 5$). [^{3}H]Bradykinin bound to a single saturable site on bradykinin B_2 receptors with $B_{max} = 747 \pm 349$ fmol/mg, $K_d = 2.08$ (1.43, 3.05) nM and nH = 1.26 ± 0.89 ($n = 3$) (Grimwood, Richards, Kennedy and Hutson, personal communication). For the imaging assay, cells were plated into 96-well blacksided cell plates (Corning Costar) at a density of 25,000 cells/well, and assayed 24 h later. Media used for plating into wells was as above except that it lacked G418. Experiments were performed 24–72 h after replating.

2.3. Fluorescence methodology

Cells in blacksided 96-well plates were removed from the incubator and washed two times with buffer using a Labsystems Cellwash, leaving 100 μl of buffer in each well. One hundred-microliter fluo 3-acetoxymethyl ester (fluo 3-AM) stock solution (200 μl of 1 mM stock in 25 ml assay buffer from aliquots prepared previously and stored at −20°C in the dark) was added to each well to give a final concentration of 4 μM. Two wells in each plate were left unloaded with dye so that the background fluorescence per well in a given plate could be recorded and later subtracted. The cells were then incubated in dye for 60 min in the dark. At this point, the wells were washed four times to remove excess dye, leaving 100 μl of buffer in each well. CHO cells export anionic forms of fluorescent dyes from their cytosol via anion exchangers in the plasma membrane, resulting in reduced dye loading and a decline in emission over the course of an experiment. The anion exchange inhibitor probenecid was therefore routinely present throughout loading and experiment to maintain dye levels within cells.

Cells were excited with a laser at 488 nm, and emission was measured through a 510–570 nm bandwidth emission filter. For each experiment, a solution of test compound was prepared from the stock solution that was five times the desired final concentration. Twenty-five microliters of this were added to 100 μl of buffer in each well to give the appropriate final bath concentration of test compound. Two-minute pretreatment with antagonist was allowed for equilibration and for measurement of any direct effects of compounds on Ca^{2+} levels. This stage is important because several test compounds have previously been found to directly mobilize Ca^{2+} via a non-bradykinin receptor mechanism; such compounds would appear to be func-

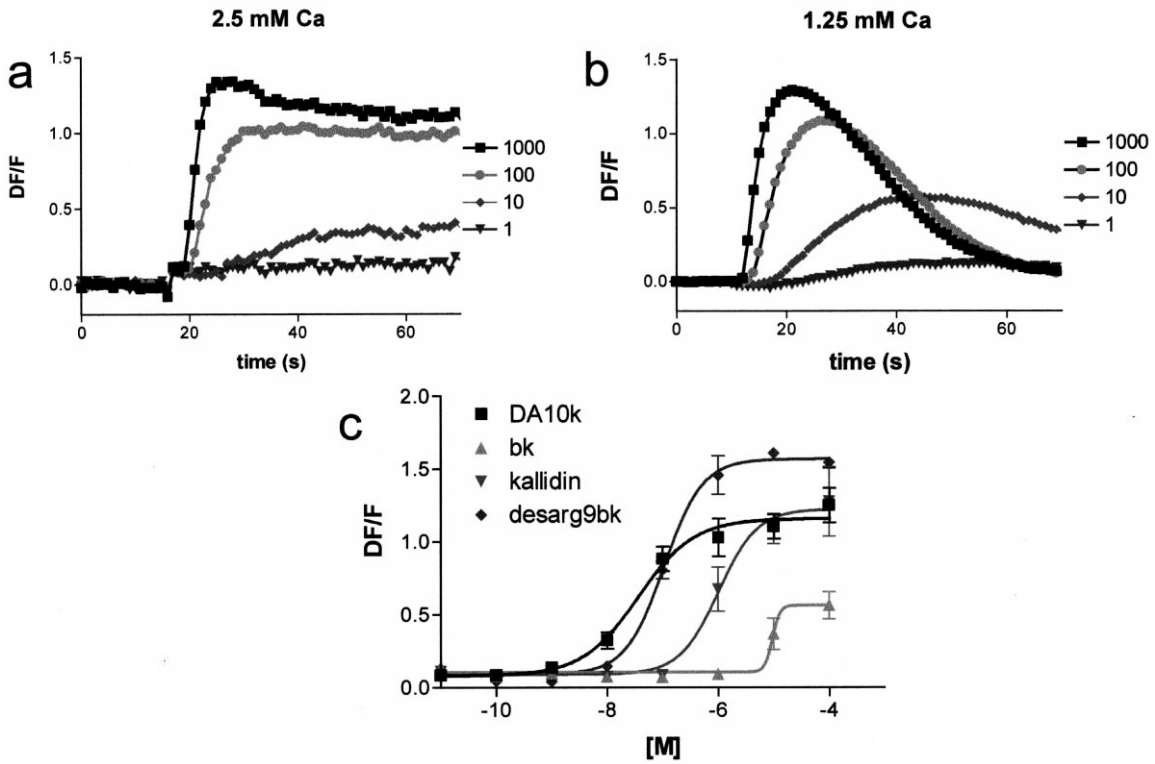

Fig. 1. Responses to Des-Arg^{10}-kallidin in B_1-expressing CHO cells. (a,b) Increasing concentrations of Des-Arg^{10}-kallidin (applied at $t = 16$s) (in nM in legend) evoked increasing Ca^{2+} responses. In 2.5 mM Ca^{2+}, these responses consisted of a rapid onset peak followed by a large sustained Ca^{2+} elevation that persisted throughout the timecourse of the experiment. In 1.25 mM Ca^{2+}-containing buffer, the concentrations of Des-Arg^{10}-kallidin evoked very similar peak Ca^{2+} elevations, but no persisting plateau phase. The peak Ca^{2+} elevation returned towards basal within 30 s of agonist application. (c) Des-Arg^{10}-kallidin, kallidin and Des-Arg^{9}-bradykinin were full agonists at bradykinin B_1 receptors, whereas bradykinin evoked only a modest response at high concentrations.

tional bradykinin receptor antagonists as they deplete the store mobilized by bradykinin receptor stimulation, if the direct effect on $[Ca^{2+}]$ was not measured. A 31.8 μl agonist solution (×5) was then pipetted by FLIPR into the 125 μl of buffer (containing the test compound) in each well.

To characterize kinin receptor agonists, concentration–response curves were obtained by addition of compounds at half-log unit increments, and the peak increase over basal measured to generate EC_{80} values of agonist at a given subtype (see Figs. 1 and 2). To generate an antagonist concentration–effect relationship, antagonists were ap-

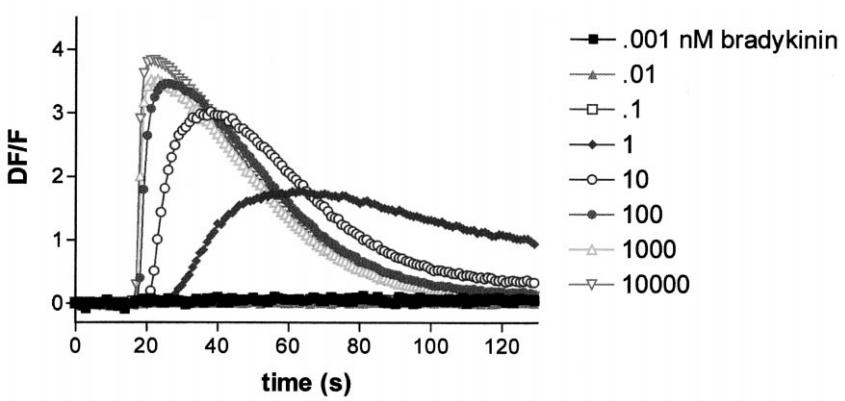

Fig. 2. Responses to bradykinin in B_2-expressing CHO cells. Increasing concentrations of bradykinin evoked a concentration-dependent elevation of Ca^{2+} within CHO cells expressing bradykinin B_2 receptors. The response consisted of a rapid onset peak elevation which in 1.25 mM Ca^{2+} returned towards basal without a substantial plateau phase.

plied and their direct effect measured for 2 min before the response to an EC_{80} concentration of Des-Arg^{10}-kallidin or bradykinin was measured. The peak response to agonist in the presence of varying concentrations of test compound was used to construct concentration–inhibition curves for each bradykinin analogue under investigation. As the agonist concentration–effect relationship may vary modestly from one experimental day to the next, the EC_{80} concentration of agonist was determined by performing an agonist concentration–effect curve on the day of antagonist testing.

2.4. Analysis

The fluorescence value in wells not loaded with fluo 3-AM was averaged at each time point then subtracted from the values for all other wells. This left only fluorescence generated by the dye, not fluorescence due to plate or cellular fluorescence. For each fluo 3-loaded well, the values over the basal time range (10 s before addition) were averaged to generate a value F. A DF/F value was generated for each timepoint (i.e., the fractional change in fluorescence over basal). The DF/F values for wells to which only buffer was added from both the test compound plate and the agonist plate were averaged across the wells for each timepoint. These averaged values were then subtracted from the corresponding values in all other wells. This compensated for fluorescence changes due to dye bleaching, injection artifacts etc. In each experimental well, the maximum peak increase in fluorescence over a defined time period beginning after agonist addition was measured. The mean of the value was then determined across all wells to which the same treatment was applied.

The Ca^{2+} response was normalized to that in control wells of the same plate to which only buffer had been previously applied. The effect of antagonist in reducing this peak response was measured and analyzed. Curve fitting within GraphPad Prism generated an IC_{50} value for the compound. IC_{50} generation in this way was the most appropriate way to examine compound activity at bradykinin receptor-expressing CHO cells.

2.5. Materials

Bradykinin, Des-Arg^{10}-kallidin, Des-Arg^{9}-bradykinin, Des-Arg^{9}-[Leu^{8}]bradykinin, and kallidin were obtained from Bachem. [D-Arg^{0},Hyp^{3},β-(2-thienyl)-Ala^{5},D-Tic^{7},Oic^{8}]-bradykinin (Hoe140) and Des-Arg^{10}-Hoe140 were obtained from Research Biochemicals International. Fluo 3-AM was obtained from Molecular Probes. Probenecid was obtained from Sigma. G418 and cell culture media were from Life Technologies and Fetalclone was from Hyclone. B9858 (Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic; Stewart et al., 1996, 1997) was custom-synthesized by Phoenix Pharmaceuticals. All other reagents were of the highest analytical grade available, and all solutions were made from double distilled deionized water. Drugs were dissolved in dimethyl sulphoxide (DMSO) where necessary, and applied at a final concentration of $< 0.3\%$ DMSO in buffer. DMSO had no discernible direct effect on fluorescence at up to 0.5% concentration.

3. Results

3.1. B_1 and B_2 agonists

Initial experiments were performed to characterize the responses activated in either human B_1 or bradykinin B_2 receptor-expressing CHO cells by a range of kinins. The peak increase in fluo 3 fluorescence measured by FLIPR within the CHO cells after stimulation with agonist was used to assess the agonist properties of a given compound. Several kinins were found to act as agonists on bradykinin B_1 receptors, with both Des-Arg^{9}-bradykinin and Des-Arg^{10}-kallidin being full agonists with $EC_{50} \sim 10$ nM (Table 1, Fig. 1a–c). Kallidin was also found in this study to be a full agonist at B_1 receptors, and bradykinin had a modest and variable agonist effect. The other kinins tested were found to have no agonist activity in this assay (Table 1). The same range of compounds were then tested on CHO cells stably transfected with the B_2 subtype of bradykinin receptors. Significant Ca^{2+} responses were ac-

Table 1
Agonism at human kinin receptors
All EC_{50} data are from curve fitting within GraphPad Prism; $n > 3$ experiments in all cases. pEC_{50} values are shown with 95% confidence limits.

Agonist	Bradykinin B_1 receptors				Bradykinin B_2 receptors			
	EC_{50} (nM)	pEC_{50}	% max	nH	EC_{50} (nM)	pEC_{50}	% max	nH
Des-Arg^{9}-bradykinin	8.6	8.06 (8.28, 7.85)	100	1.35	> 3000	–	–	–
Des-Arg^{10}-kallidin	7.9	8.10 (8.41, 7.79)	98	0.83	inact ≤ 10,000	–	–	–
Kallidin	243	6.61 (6.77, 6.46)	82	1.16	10	7.99 (8.14, 7.85)	110	1.27
Bradykinin	1000	–	< 30	–	2	8.64 (8.85, 8.44)	100	1.01
Des-Arg^{9}-[eu^{8}]bradykinin	inact ≤ 10000	–	–	–	inact ≤ 10,000	–	–	–
Hoe140	inact ≤ 3000	–	–	–	inact ≤ 3000	–	–	–
Des-Arg^{10}-Hoe140	inact ≤ 3000	–	–	–	inact ≤ 3000	–	–	–

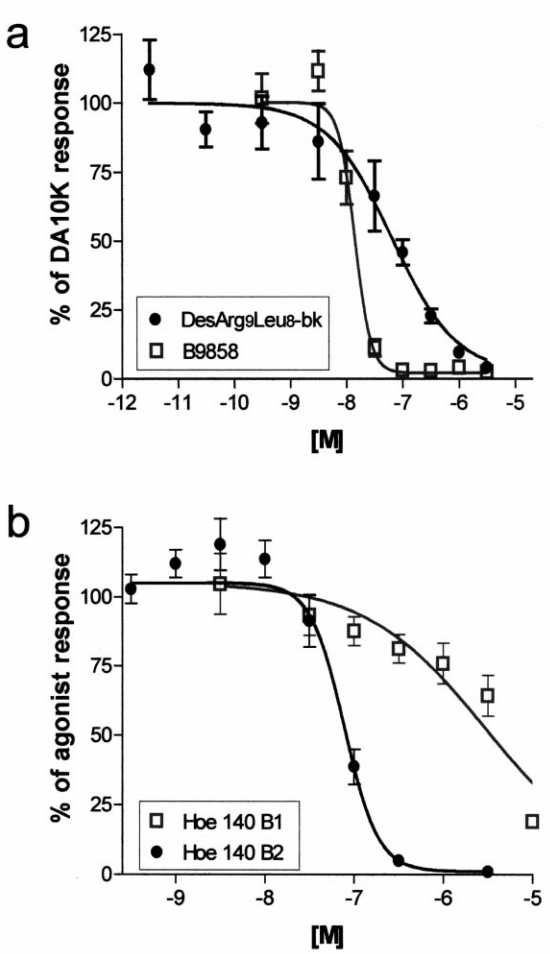

Fig. 3. (a) Concentration–inhibition relationships for bradykinin B_1 receptor antagonists. Antagonists were applied at a range of concentrations for 2 min before application of an EC_{80} concentration of Des-Arg^{10}-kallidin. B9858 and Des-Arg^9-[Leu^8]bradykinin were found to be potent full functional antagonists of bradykinin B_1 receptors. Both were able to inhibit the response to Des-Arg^{10}-kallidin without having any partial agonist properties, and were effective at submicromolar concentrations. (b) Concentration–inhibition relationships for Hoe140 at kinin receptors. Hoe140 was applied at a range of concentrations for 2 min on either B_1- or B_2-expressing CHO cells before application of an EC_{80} concentration of Des-Arg^{10}-kallidin (for B_1) or bradykinin (for B_2). Hoe140 abolished the response to bradykinin in B_2 cells at submicromolar concentrations, and caused inhibition in B_1 cells only at >100-fold higher concentrations.

tivated by bradykinin and by kallidin, with bradykinin being five-fold more potent than kallidin, whereas the other compounds tested exhibited little or no agonism for this receptor (Fig. 2, Table 1).

3.2. Characteristics of the Ca^{2+} response

The response characteristics of agonist-evoked Ca^{2+} elevations are dependent upon intra- and/or extracellular Ca^{2+} pools (Simpson et al., 1995). In the present study, bradykinin B_1 and B_2 receptor stimulation under the experimental conditions of this assay was found to result in a rapid-onset peak elevation of intracellular Ca^{2+}, followed by a gradual decline to a modest plateau level (Figs. 1 and 2). Kinin receptors are known to be coupled to inositol 1,4,5-trisphosphate generation and release of Ca^{2+} from intracellular stores, which often results in store-operated Ca^{2+} entry. To examine the dependence of the peak and plateau phases of the kinin receptor response upon extracellular Ca^{2+}, we compared the response characteristics in 2.5 mM and 1.25 mM extracellular Ca^{2+}. Changing extracellular [Ca^{2+}] had no effect upon peak Ca^{2+} elevations to a range of concentrations of agonist, but the plateau phase which was substantial in the presence of 2.5 mM Ca^{2+} was virtually absent in the lower Ca^{2+}-containing buffer (Fig. 1a,b). All further experiments were performed in 1.25 mM Ca^{2+}, such that the Ca^{2+} response being measured is due almost entirely to one event, i.e., the release of Ca^{2+} from intracellular stores.

3.3. Kinin receptor antagonists

From the above agonist experiments with a variety of kinin compounds, Des-Arg^{10}-kallidin was selected as the standard agonist for bradykinin B_1 receptors for all subsequent experiments, and bradykinin was selected as the standard agonist for bradykinin B_2 receptors. A range of putative kinin receptor antagonists were tested for their ability to inhibit the Ca^{2+} response activated by EC_{80} concentrations of Des-Arg^{10}-kallidin and bradykinin (3 nM bradykinin, 10 nM Des-Arg^{10}-kallidin in these experiments) at bradykinin B_1 and B_2 receptors, respectively. The kinin receptor antagonists B9858, Des-Arg^{10} Hoe140 and Des-Arg^9-[Leu^8]bradykinin inhibited B_1-CHO Ca^{2+} responses completely and specifically, having no significant effect on bradykinin B_2 receptors over the concentration range at which the Des-Arg^{10}-kallidin response in B_1-CHOs was inhibited (Fig. 3a,b; Table 2). Hoe140

Table 2
Antagonism at human kinin receptors
All IC_{50} data are from curve fitting within GraphPad Prism; $n > 3$ experiments in all cases. pEC_{50} values are shown with 95% confidence limits.

Antagonist	Bradykinin B_1 receptors				Bradykinin B_2 receptors			
	IC_{50} (nM)	pIC_{50}	%	nH	IC_{50} (nM)	pIC_{50}	% inhibition	nH
Des-Arg^9-[Leu^8]bradykinin	59	7.23 (7.48, 6.98)	100	0.8	inact ≤ 3000	–	–	–
Hoe140	3327	5.48 (5.81, 5.14)	–	1	71	7.15 (7.31, 6.99)	100	1.9
Des-Arg^{10}-Hoe140	211	6.68 (7.04, 6.39)	100	0.8	>3000	–	–	–
B9858	14	7.87 (8.00, 7.34)	100	1	>3000	–	–	–

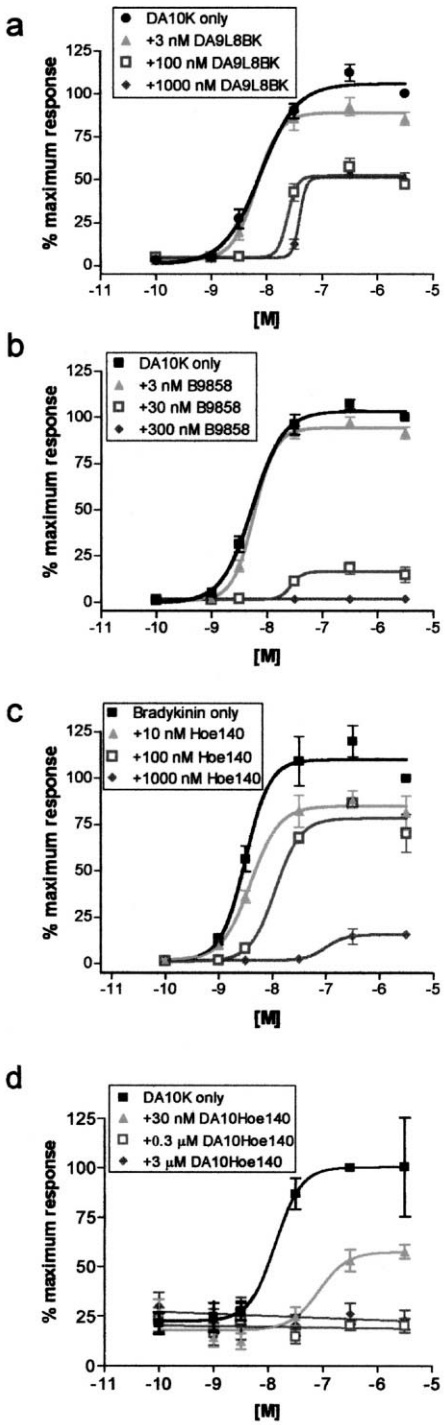

Fig. 4. Examination of the apparent mechanism of action of kinin receptor antagonists. Cells were pretreated with several different concentrations of antagonists (Des-Arg^9-[Leu^8]bradykinin (a) on B_1 cells, B9858 (b) or Des-Arg^{10}-Hoe140 (d) on B_1 cells, or Hoe140 (c) on B_2 cells), and then a range of concentrations of the standard receptor agonist was applied. The effect of the antagonists on the concentration–response relationship for the agonist was examined. In each case, little or no rightward shift in the concentration–response relationship was evident, but rather each antagonist caused a marked depression of the maximal agonist response.

inhibited bradykinin B_1 receptor-mediated responses at high concentrations and was a much more potent antagonist for bradykinin B_2 receptors (Fig. 3b). Hoe140 was the only effective bradykinin B_2 receptor antagonist of the compounds examined in this study (Table 2).

To examine the nature of the inhibition caused by these compounds, we tested their ability to shift the concentration–response relationship to kinin receptor agonists. Des-Arg^9-[Leu^8]bradykinin at increasing concentrations caused a marked depression of the maximum response for Des-Arg^{10}-kallidin on bradykinin B_1 receptors (Fig. 4a). Non-competitive kinetics were also evident for the bradykinin B_1 receptor antagonists B9858 (Fig. 4b) and Des-Arg^{10}-Hoe140 (Fig. 4d) in this assay. Hoe140 was similarly tested for its ability to shift the concentration–response relationship for bradykinin on B_2-CHOs. As for the bradykinin B_1 receptor antagonists, Hoe140 was found to have non-competitive kinetics in the FLIPR assay, causing suppression of the maximal response without alteration in the apparent agonist EC_{50} (Fig. 4c).

4. Discussion

In this study, functional activation of recombinant human kinin receptor subtypes has been measured, and the potency, selectivity and efficacy of bradykinin receptor antagonists compared. The agonist profiles obtained from the stably transfected receptors closely reflects that obtained in native tissues, with Des-Arg^9-bradykinin and Des-Arg^{10}-kallidin being potent bradykinin B_1 receptor agonists, whereas kallidin and bradykinin were found to be potent B_2 receptor agonists. Previous binding studies have indicated that the K_i for Des-Arg^{10}-kallidin is $\sim$20-fold lower than that for Des-Arg^9-bradykinin, having binding affinity comparable to the functional IC_{50} value obtained in the present study (MacNeil et al., 1997; Meini et al., 1999). Hoe140 and Des-Arg^9-[Leu^8]bradykinin, which have been reported to have partial agonism effects in some previous studies (Wirth et al., 1992; Nakhostine et al., 1993; Allogho et al., 1995; MacNeil et al., 1997), were found to have no agonist activity in this assay (Table 1). All effective agonists activated a large elevation in intracellular Ca^{2+} consisting of a rapid peak elevation followed by a persisting plateau, the latter being largely absent in modest extracellular Ca^{2+} levels. These results are consistent with the peak Ca^{2+} elevation in response to kinin receptor stimulation being due to Ca^{2+} release and the plateau being due to Ca^{2+} entry. This effect of a modest change in extracellular Ca^{2+} was greater than would have been expected a priori, and likely indicates that the Ca^{2+} entry pathway activated downstream of kinin receptor activation requires a substantial downhill Ca^{2+} gradient to overcome extrusion mechanisms balancing its activity.

Bradykinin receptors of both known subtypes may represent viable targets for drug therapy in inflammatory diseases. B_2 receptor antagonists might be expected to ameliorate pain and inflammation in early stage disease, whereas B_1 antagonists could be useful in the prolonged chronic condition (Dray and Perkins, 1993; Marceau et al., 1998). Therapeutic utility is dependent upon the identification of potent and selective compounds with in vivo activity. Several compounds structurally related to bradykinin have been previously reported as antagonists of B_1 and/or bradykinin B_2 receptors (Hock et al., 1991; Wirth et al., 1992; Stewart et al., 1996). We have examined the nature and selectivity of several known bradykinin receptor antagonists whose specificity and partial agonism has been unclear on the basis of previous studies. None of the antagonists Hoe140, Des-Arg^{10}-Hoe140, B9858 or Des-Arg^9-[Leu^8]bradykinin exhibited any partial agonism in this study at either B_1 or bradykinin B_2 receptor subtypes, their effects in inhibiting agonist-evoked Ca^{2+} responses being apparently mediated by straightforward receptor antagonism. B9858 exhibited a very high Hill slope ($nH > 3$) for inhibition of B_1 Ca^{2+} responses, the reason for which is currently unclear. For each antagonist a substantial degree of subtype selectivity was found, with Hoe140 being >100-fold selective for bradykinin B_2 receptors, and the others being >100-fold selective for bradykinin B_1 receptors. The bradykinin B_1 receptor antagonist selectivity is consistent with previous reports (e.g., Wirth et al., 1992). The functional IC_{50} values obtained are not tightly correlated with reported values for binding affinities (e.g., Hock et al., 1991; MacNeil et al., 1997). It has been reported that buffer composition markedly affects the determination of bradykinin receptor ligand binding affinity (Paquet et al., 1999), and that binding affinity varies for bradykinin receptor antagonists across tissues expressing apparently the same bradykinin receptor subtype (Hall, 1997; Wohlfart et al., 1997; Paquet et al., 1999). This highlights the need for a better understanding of bradykinin receptor subtype distribution and of the mechanism of receptor antagonism utilized by these compounds.

Hoe140 has been widely reported to be a potent, long-lasting and relatively specific bradykinin B_2 receptor antagonist in a range of in vitro and in vivo studies (Hock et al., 1991; Wirth et al., 1991, 1992; Lembeck et al., 1992; Santiago et al., 1993; Sakamoto et al., 1994; Andre et al., 1998; Li et al., 1998; Ricciardolo et al., 1998). However, its mechanism of action has been less clear: conflicting reports of competitive or non-competitive inhibition for bradykinin B_2 receptors in differing tissues and species have appeared (Cuthbert et al., 1992; Feletou et al., 1994; Seabrook et al., 1995; Li et al., 1998) and partial bradykinin B_2 receptor agonism has been reported in some studies (e.g., Hock et al., 1991; Feletou et al., 1994). In the present study, we have utilized cells with no native bradykinin receptors recombinantly expressing human bradykinin B_1 or B_2 receptors to characterize the effect of this compound. Under these conditions, it had clearly no agonism. The lack of agonist properties for Des-Arg^9-Hoe140, and for [Leu^8]bradykinin, in this fluo 3-based assay may be related to receptor expression level and receptor reserve in the stably expressed cell line compared to other preparations (Kenakin, 1993), or to the use of recombinant human receptors for this study. There have been reported to be marked species differences in affinity and in the ability of kinin antagonists to display partial agonism, such that Des-Arg^9-[Leu^8]bradykinin appears to be a partial agonist of the rodent, but not human, B_1 subtype (MacNeil et al., 1997). A recent report has suggested that the ability of kinin receptor ligands to act as partial agonists or antagonists in different assay systems depends on the basal unstimulated receptor activity (Fathy et al., 1999). In the three-state paradigm of kinin receptor binding proposed by these authors, Hoe140 is proposed to stabilize an intermediate receptor activity state, such that it would be expected to act as a partial agonist in systems where there was little spontaneous fully activated receptor formed, but as an antagonist where spontaneous activity was relatively high. The level of basal receptor activity in the stably expressed B_1 or B_2 CHO cell line has not been measured in the present study.

Schild analysis indicated that Hoe140 is among several kinin receptor antagonists characterized in this study which have a non-competitive appearance in FLIPR on bradykinin receptor-expressing CHO cells. The reasons for this latter observation however may not be entirely straightforward. The very high affinity of the antagonists Hoe140 and B9858 for their kinin receptor targets (Hock et al., 1991; MacNeil et al., 1997) may contribute to the non-competitive antagonist kinetics. High affinity ligands typically have very slow off rates (Kenakin, 1993) and this may preclude equilibrium being reached at low antagonist concentrations. Non-competitive inhibition by Hoe140 has been found in some intact preparations expressing bradykinin B_2 receptors (Feletou et al., 1994). Other possible explanations such as positive cooperativity in antagonist binding or non-specific binding are unlikely given the nature of the preparation used in this study and the reports of competitive antagonism at bradykinin B_2 receptors for Hoe140 in several other studies (cf. Cuthbert et al., 1992; Feletou et al., 1994; Li et al., 1998; Paquet et al., 1999). Apparently non-competitive antagonism may thus reflect the equilibrium characteristics of the FLIPR assay system being utilized, consistent with a previous report (see Miller et al., 1999). It is possible that more prolonged equilibration times might result in a different apparent antagonism for such compounds (Miller et al., 1999), although for very high affinity compounds, such as B9858 and Hoe140, the time periods required for this to occur might be expected to prove impractical for a functional assay.

This study demonstrates that the pharmacology of recombinant human bradykinin receptors stably transfected

in CHO cells can be readily characterized using a high throughput 96-well FLIPR and the Ca^{2+} sensitive dye fluo 3. Consistent with previous reports, bradykinin was found to be a selective agonist on bradykinin B_2 receptors, and its effects were selectively antagonized by Hoe140. Des-Arg^9-bradykinin, despite having relatively low binding affinity for human bradykinin B_1 receptors (Stewart et al., 1996, 1997; MacNeil, 1997; Meini et al., 1999), was equipotent to Des-Arg^{10}-kallidin as a bradykinin B_1 receptor agonist, and both were inactive on bradykinin B_2 receptors. The most potent bradykinin B_1 receptor antagonist tested was B9858, and this compound was highly selective for human bradykinin B_1 over B_2 receptors, upon which it was inactive up to micromolar concentrations. Hoe140 was a selective and potent bradykinin B_2 antagonist without agonism at either B_1 or B_2 receptor subtypes.

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