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Signal Transduction Pathways for B_1 and B_2 Bradykinin Receptors in Bovine Pulmonary Artery Endothelial Cells

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SUMMARY

Bovine pulmonary artery endothelial (CPAE) cells respond to bradykinin, and it has been suggested that the receptors on these cells do not fall into the normal B_1/B_2 classification of bradykinin receptors [*J. Pharmacol. Exp. Ther.* **244**:646–649 (1988)]. The present study describes a detailed characterization of the subtypes of bradykinin receptors on CPAE cells. The B_1-selective agonist des-Arg^9-bradykinin and the B_2-selective agonist bradykinin both activated polyphosphoinositide phospholipase C (PLC), caused an elevation in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), and increased the rate of $^{45}Ca^{2+}$ efflux in CPAE cells. The pharmacology of these responses was consistent with interactions with B_1 and B_2 receptors. The effects of maximal concentrations of bradykinin and des-Arg^9-bradykinin on the activity of PLC in populations of cells were not additive, suggesting that the two subtypes were expressed on the same cells. Indeed, des-Arg^9-bradykinin and bradykinin both evoked increases in $[Ca^{2+}]_i$ in 85% of single cells tested. The Ca^{2+} entry blocker $NiCl_2$ inhibited bradykinin-induced increases in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux. In contrast, $NiCl_2$ did not inhibit the increase in $^{45}Ca^{2+}$ efflux evoked by des-Arg^9-bradykinin and induced oscillatory increases in $[Ca^{2+}]_i$ in response to the B_1 agonist. $NiCl_2$ had no effect on [^{3}H]inositol trisphosphate generation by either agonist, indicating that its inhibitory effects on bradykinin-mediated Ca^{2+} responses were distal to B_2 receptor-induced activation of PLC. $LaCl_3$ did not differentiate between the $^{45}Ca^{2+}$ efflux responses evoked by bradykinin and des-Arg^9-bradykinin, attenuating both to a similar degree. Bradykinin-induced [^{3}H]inositol trisphosphate formation was desensitized after pretreatment with bradykinin, but the response to des-Arg^9-bradykinin was unchanged. Pretreatment with the B_1 agonist did not inhibit responses evoked by subsequent challenges with either des-Arg^9-bradykinin or bradykinin. These results provide pharmacological evidence for the existence of two distinct bradykinin receptor subtypes (B_1 and B_2) on CPAE cells, with no evidence for heterologous desensitization. Although both subtypes operated similar signal transduction pathways, the Ca^{2+} responses evoked by the two receptors could be differentiated by $NiCl_2$.

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is a nine-residue peptide that is formed locally, after inflammation or tissue damage, from kininogen precursors (1, 2). It is a potent mediator of a variety of responses, including smooth muscle contraction, vasodilation, and neurotransmitter release (3). Two subtypes of bradykinin receptor, B_1 and B_2, have been cloned (4–6). The deduced amino acid sequences of both receptors are consistent with their belonging to the G protein-coupled superfamily of receptors with seven transmembrane domains. The B_1 receptor has a higher affinity for the bradykinin metabolite des-Arg^9-bradykinin than for bradykinin itself and is blocked by the selective antagonist des-Arg^9,[Leu^8]-bradykinin (3, 7). The B_2 receptor has a higher affinity for bradykinin than for des-Arg^9-bradykinin and is blocked by the potent and selective B_2 receptor antagonist HOE140 (8). The B_1 receptor does not appear to be present to any significant extent in most normal tissues, although it is expressed constitutively in a number of cell lines (9–12). It can be induced during conditions of tissue injury and inflammation, and there is evidence that it may have a role in pathophysiological conditions such as septic shock and persistent inflammatory hyperalgesia (13). Unlike the B_1 receptor, the B_2 receptor is expressed in a wide variety of normal tissues (14).

One of the many important roles of bradykinin and des-Arg^9-bradykinin is in the modulation of vascular tone. Their vascular actions are mediated not only by direct effects on muscle cells but also by effects on vascular endothelial cells (see Refs. 14 and 15 for reviews), where they can stimulate nitric oxide release (10). CPAE cells have been used as a cellular model of vascular endothelial tissue. They possess B_2 bradykinin receptors that are coupled to PLC and elevate

ABBREVIATIONS: HOE140, D-Arg,[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; PLC, polyphosphoinositide phospholipase C; PI, phosphatidylinositol; CPAE, bovine pulmonary artery endothelial; IP_3, inositol trisphosphate; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NPC567, [D-Arg^0,Hyp^3,D-Phe^7]-bradykinin; ANOVA, analysis of variance; df, degrees of freedom; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

$[Ca^{2+}]_i$ (16). Bradykinin can stimulate prostaglandin synthesis in CPAE cells (17), and it has been suggested that these cells possess bradykinin receptors that are linked to phospholipase A_2 (18, 19). Evidence for the existence of B_1 receptors on CPAE cells and other bovine endothelial cells is less conclusive. Binding studies with [^{3}H]bradykinin pointed to the existence of two binding sites, one of which was thought to be the B_1 receptor (9). Des-Arg^9-bradykinin has also been shown to cause a small increase in $[Ca^{2+}]_i$ (20) and to increase prostaglandin synthesis (17, 21) in CPAE cells. However, in the latter study, anomalous results with antagonists suggested that the receptor or receptors mediating the response did not fit either the B_1 or B_2 receptor classification. Furthermore, in a related cell type, i.e., bovine aortic endothelial cells (10), although des-Arg^9-bradykinin increased cGMP, this response could be blocked by relatively low concentrations of the B_2-selective antagonist HOE140, as well as by the B_1-selective antagonist des-Arg^9,[Leu^8]-bradykinin.

In the present study we have performed a detailed pharmacological characterization of the effects of B_1 and B_2 agonists on PLC activity and $^{45}Ca^{2+}$ efflux in CPAE cells. In addition, agonist-evoked changes in $[Ca^{2+}]_i$ have been studied using the Ca^{2+}-sensitive dye fura-2. Because it was previously suggested that bradykinin operates a cation chanel through which Ca^{2+} can enter CPAE cells (22, 23), the effects of the Ca^{2+} entry blockers $NiCl_2$ and $LaCl_3$ (24, 25) on the responses evoked by these agonists were investigated. Interactions between B_1 and B_2 receptors on CPAE cells have been assessed by investigating whether B_1 and B_2 agonists show homologous or heterologous desensitization.

Experimental Procedures

Materials. CPAE cells were obtained from European Collection Animal Cell Cultures (Porton Down, UK). HOE140 was synthesized at the Sandoz Institute for Medical Research (London, UK). The following materials were obtained from the sources indicated: bradykinin, des-Arg^9-bradykinin, and des-Arg^9,[Leu^8]-bradykinin (Bachem, Bubendorf, Switzerland); NPC567 (Peninsula Laboratories Europe, St. Helens, UK); penicillin/streptomycin, fetal calf serum (Myoclone Plus), glutamine, HBSS, DMEM, and minimal essential medium (GIBCO-BRL, Life Technologies, Paisley, UK); fura-2/acetoxymethyl ester (Calbiochem, c/o Novabiochem, Nottingham, UK); pluronic acid (Molecular Probes, Eugene, OR); and $^{45}CaCl_2$ (5–50 mCi/mg of Ca^{2+}) and *myo*-[2-^{3}H]inositol (17.1 Ci/mmol) (Amersham International, Amersham, UK). All other chemicals and reagents were of analytical grade from BDH (Dagenham, UK).

Cell culture. CPAE cells were grown as monolayers in DMEM containing 20% fetal calf serum, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 2 mM glutamine (supplemented DMEM), in an atmosphere of 6% CO_2 at 37°. When confluent, the cells were removed from the flasks by treatment with 1 mM EDTA in Ca^{2+}/Mg^{2+}-free HBSS containing 10 mM HEPES, pH 7.4. For measurement of $^{45}Ca^{2+}$ efflux the cells were plated on Terasaki plates at a density of 500 cells/well and allowed to grow until confluent (1–2 days). For the measurement of [^{3}H]IP_3, cells were plated on polyornithine-coated coverslips (30,000 cells/coverslip) and allowed to grow until confluent (1–2 days). For measurement of $[Ca^{2+}]_i$ with fura-2, the cells were plated on coverslips (5000 cells/coverslip) and allowed to grow for 1–3 days.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured with the Ca^{2+} indicator fura-2, using a Deltascan D104B system (Photon Technology International). CPAE cells were incubated with 10 μM fura-2/acetoxymethyl ester in HBSS-HEPES, pH 7.4, containing 0.005% pluronic acid, for 45–60 min at room temperature. The coverslips were washed in HBSS-HEPES, transferred to a heated, laminar-flow chamber on the stage of a Nikon Diaphot microscope equipped with a 40× fluorescence objective, and perfused with HBSS-HEPES, pH 7.4, at 34° (flow rate, 3–4 ml/min). Drugs were applied in the perfusion buffer or through a U-tube positioned next to the cell (26).

Fura-2 was excited alternately at 340 and 380 nm (100 Hz), and the emitted light was monitored at 510 nm. For each cell background fluorescence was determined by exposure to 1 mM $MnCl_2$ in buffer containing ionomycin (10 μM). Basal $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescent intensities at the two excitation wavelenths (27). R_{max} and R_{min} were determined by exposing the cells to 10 μM ionomycin and 10 μM FCCP in buffer containing 2 mM $CaCl_2$ and to Ca^{2+}-free buffer containing 5 mM EGTA, respectively. The average basal ratio in the CPAE cells was 0.68 ± 0.01 (45 cells) and basal $[Ca^{2+}]_i$ was 38 ± 3 nM (40 cells).

Measurement of $^{45}Ca^{2+}$ efflux. CPAE cells on Terasaki plates were incubated at 37° in growth medium (see above) containing 45 μCi/ml $^{45}Ca^{2+}$. After 2–3 hr the $^{45}Ca^{2+}$ content of the cells reached a plateau and the cells were washed for 17 min with HBSS containing 10 mM HEPES, pH 7.4, at 37°, to remove excess extracellular $^{45}Ca^{2+}$. After this period the rate of efflux of $^{45}Ca^{2+}$ was stable. In some experiments the cells were incubated with $^{45}Ca^{2+}$ overnight. The results of these experiments were not significantly different from results of experiments in which the cells were incubated with the radioisotope for 2–3 hr, implying that all of the relevant pools were labeled during the first few hours. The cells were incubated with buffer (8 ml) for 1-min periods, and the $^{45}Ca^{2+}$ in each 1-min fraction was determined by liquid scintillation counting. Agonists were applied for 1 min unless otherwise indicated. Antagonists were applied for 2 min before, and during, the application of agonists. The $^{45}Ca^{2+}$ remaining in the cells at the end of each experiment was determined by lysing the cells with a solution of 0.2% sodium dodecyl sulfate. The increase in the rate of $^{45}Ca^{2+}$ efflux in response to agonists was defined as the rate of $^{45}Ca^{2+}$ efflux during the 1-min application of the agonist minus the average of the rate of efflux during the two collection periods before agonist application. The average basal rate of efflux for the CPAE cells was 0.038 ± 0.007 min^{-1} (five representative experiments). $^{45}Ca^{2+}$ efflux was also measured in Ca^{2+}-free HBSS-HEPES containing 200 μM EGTA, HBSS-HEPES containing 1 mM $NiCl_2$, and phosphate-free HBSS-HEPES containing 10 μM $LaCl_3$. The cells were incubated for 5 min in these buffers and then for an additional 8 min in the same buffer in the presence of agonist. The effect of removal of extracellular Ca^{2+} or addition of $NiCl_2$ or $LaCl_3$ on the response to the B_1 and B_2 agonists was determined by comparing the rate of $^{45}Ca^{2+}$ efflux in the control and test bufffers, using two-way ANOVA (Statistica). Analysis was performed using the fraction immediately before agonist administration (basal efflux) and all eight fractions containing the agonist. Individual *post hoc* comparisons between the rate of $^{45}Ca^{2+}$ efflux in control and test buffers at particular times were made using Newman-Keuls analysis (Statistica). In addition, within the control or test buffer group, *post hoc* comparison of basal $^{45}Ca^{2+}$ efflux with the rate of $^{45}Ca^{2+}$ efflux in the presence of agonist was made using Newman-Keuls analysis (Statistica).

[^{3}H]IP_3 formation. PLC activation was followed by measuring the formation of [^{3}H]IP_3 in cells that had been incubated with [^{3}H]inositol. CPAE cells, plated onto coverslips, were incubated overnight at 37° in Eagle's medium with Earle's salts, without L-methionine or L-glutamine (minimal essential medium), containing 20 μCi/ml *myo*-[2-^{3}H]inositol. At the end of this period the radioactive medium was removed, and the cells were washed and incubated in 25 mM HEPES-buffered DMEM at 37° for the duration of the experiment. Cells were exposed to agonists by dipping the coverslips into agonist-containing medium for 20 sec. Cells were exposed to antagonists for 2 min before, and during, exposure to the agonist. The reactions were terminated by plunging the coverslips into 1 ml of ice-cold 4.5% perchloric acid containing 1 mg/ml phytic acid. The [^{3}H]inositol phosphates were extracted into the acid for 20 min. The

perchloric acid was then removed and neutralized with 1 M KOH containing 9 mM $Na_2B_4O_7$. The samples were centrifuged at 1000 × *g* for 5 min to pellet the precipitate of potassium perchlorate. The [^{3}H]inositol phosphates in the supernatants were separated by anion exchange chromatography as described previously (28). The columns were washed with three 8-ml volumes of distilled water, followed by three 8-ml volumes of 0.4 M ammonium formate in 0.1 M formic acid. The [^{3}H]IP_3 was eluted from the columns with three 8-ml volumes of 0.8 M ammonium fomate in 0.1 M formic acid. The radioactivity in these [^{3}H]IP_3 fractions was expressed as a percentage of the total lipid radioactivity, almost all of which was associated with PI. The effect of the removal of extracellular Ca^{2+} or the addition of $NiCl_2$ or $LaCl_3$ on PLC activation was investigated by preincubating the cells in test medium (Ca^{2+}-free HEPES-buffered DMEM containing 200 μM EGTA, HEPES-buffered DMEM containing 1 mM $NiCl_2$, or phosphate-free HEPES-buffered DMEM containing 10 μM $LaCl_3$) for 5 min before, and during, incubation with agonist (20 sec). All determinations were made in triplicate. [^{3}H]IP_3 synthesis in the control and test buffers, in the presence and absence of agonists, was compared using two-way ANOVA (Statistica).

Data analysis. EC_{50} and IC_{50} values (concentrations producing half-maximal stimulation and inhibition, respectively), with their respective 95% confidence limits, were estimated by computer-assisted curve-fitting using MicrolCal ORIGIN.

Results

Effects of bradykinin and des-Arg^9-bradykinin on $[Ca^{2+}]_i$ in single CPAE cells. Exposure of single, fura-2-loaded, CPAE cells to bradykinin and des-Arg^9-bradykinin led to an increase in $[Ca^{2+}]_i$ (Fig. 1), with 85% of the cells tested (22 of 26 cells) responding to both B_1 and B_2 agonists. The shapes of the responses were different for the two agonists, varied between individual cells, and were dependent on agonist concentration.

A low concentration of bradykinin (0.3 nM) evoked small single spikes, which declined towards base-line in the presence of the agonist (five cells) or were followed by erratic oscillations (six cells) (Fig. 1A). With 1 nM bradykinin, the majority of cells (six of seven) responded with an initial spike, followed by a sustained increase in $[Ca^{2+}]_i$ that declined slowly to base-line only when the bradykinin was removed (Fig. 1B). A high concentration of bradykinin (30 nM) caused an initial transient response that declined toward base-line in the presence of the B_2 agonist (four cells) (Fig. 1C). Low concentrations of des-Arg^9-bradykinin (30 nM) tended to induce erratic oscillatory responses (10 cells) (Fig. 1D), although a minority of the cells tested (three cells) responded with a small sustained rise in $[Ca^{2+}]_i$. At higher concentrations of the B_1 agonist (100 nM and 1 μM), all of the cells tested (four at each concentration) responded with a spike followed by a sustained elevation in $[Ca^{2+}]_i$, which was maintained in the presence of agonist but declined rapidly to base-line upon agonist removal (Fig. 1, E and F). Higher concentrations of des-Arg^9-bradykinin (up to 10 μM) also caused a spike followed by a marked, prolonged, sustained phase (data not shown). Table 1 shows that there was a concentration-dependent decrease in response latency and a corresponding increase in the peak height of the initial transient responses evoked by both bradykinin and des-Arg^9-bradykinin.

For a more detailed analysis of the concentration dependency of the B_1 and B_2 agonist-evoked increases in $[Ca^{2+}]_i$, the responses were divided into an initial phase (the first 2 min) and a late phase (from 2 to 10 min) and the area under the curve was calculated for each period. Des-Arg^9-bradykinin caused a concentration-dependent increase in the initial and late phases of the increase in $[Ca^{2+}]_i$, both of which reached a plateau between 100 nM and 1 μM (Fig. 2). A similar pattern was obtained for the initial phase of the response to bradykinin, which reached a plateau between 1 nM and 30 nM. In contrast, there was a clear bell-shaped relationship between agonist concentration and the area under the curve for the late phase, reflecting suppression of the sustained increase in $[Ca^{2+}]_i$ at high concentrations of bradykinin.

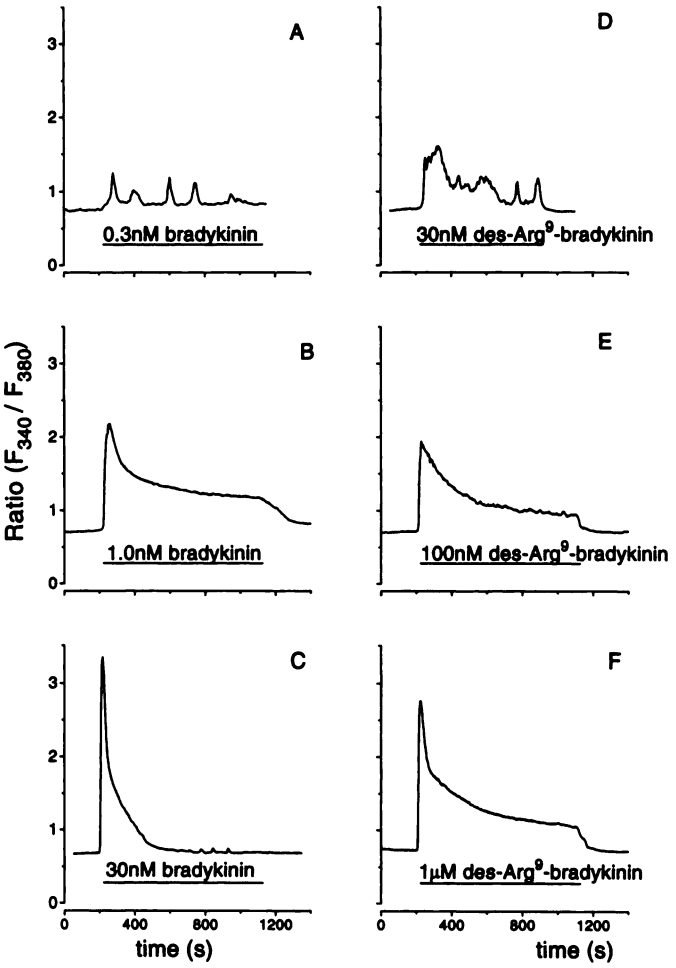

Fig. 1. Bradykinin- and des-Arg^9-bradykinin-induced increases in $[Ca^{2+}]_i$ in single CPAE cells. *Traces*, examples of $[Ca^{2+}]_i$ responses to bradykinin (0.3 nM, 1 nM, and 30 nM) and des-Arg^9-bradykinin (30 nM, 100 nM, and 1 μM). Agonists were applied at the concentrations indicated, through the perfusion system, for the period indicated (*horizontal lines*).

Pharmacology of bradykinin- and des-Arg^9-bradykinin-mediated responses in CPAE cells. Pharmacological analysis of the responses to bradykinin and des-Arg^9-bradykinin in single cells was limited to experiments with single concentrations of the B_1- and B_2-selective antagonists des-Arg^9,[Leu^8]-bradykinin and HOE140, respectively. In two of two cells the response to 100 nM bradykinin was unaffected by 3 μM des-Arg^9,[Leu^8]-bradykinin but was blocked by 3 nM HOE140. The response to 1 μM des-Arg^9-bradykinin was blocked by 3 μM des-Arg^9,[Leu^8]-bradykinin (four cells) but not by 3 nM HOE140 (six of seven cells). This implied that

TABLE 1

Concentration dependency of bradykinin- and des-Arg^9-bradykinin-evoked increases in $[Ca^{2+}]_i$ in single CPAE cells

Cells were incubated with agonist at the concentrations indicated, applied through the perfusion system. The magnitude of the initial spike response and the response latency were determined. Data represent means ± standard errors of responses from the number of cells (*n*) indicated.

Agonist	Concentration	Peak height ratio	Peak $[Ca^{2+}]_i$	Latency	*n*
	nM		*nM*	*sec*	
Bradykinin	0.3	1.2 ± 0.09	302 ± 44	18 ± 3	11
	1.0	1.9 ± 0.24	640 ± 141	10 ± 2	6
	30	2.7 ± 0.43	1100 ± 290	1	4
Des-Arg^9-bradykinin	30	1.1 ± 0.24	218 ± 8	18 ± 2	13
	100	1.66 ± 0.20	507 ± 98	7 ± 5	4
	1000	2.26 ± 0.19	825 ± 119	2 ± 1	4

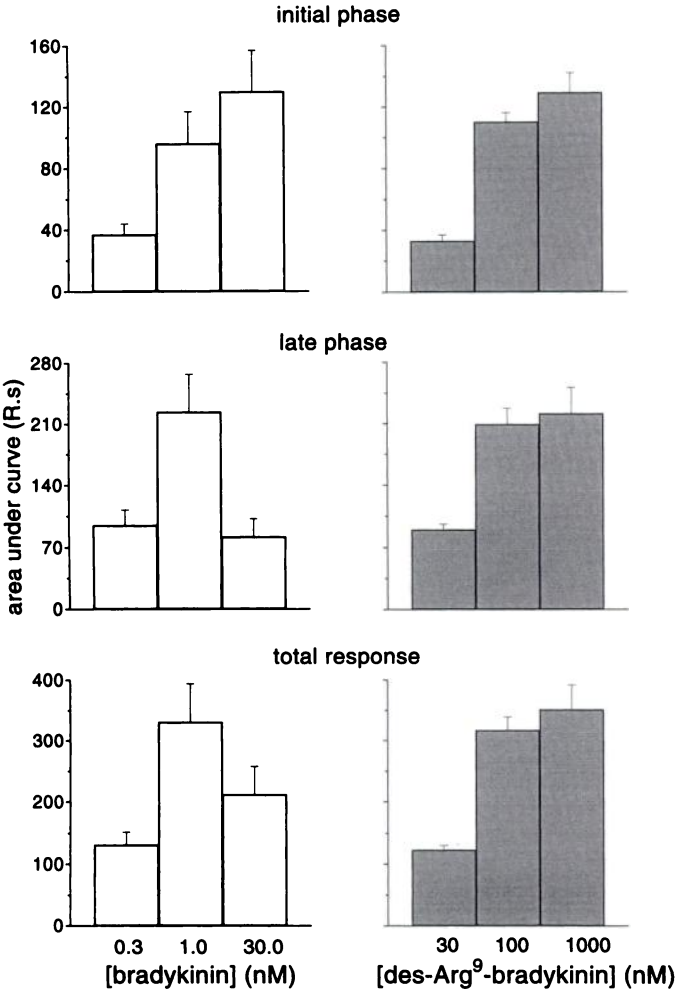

Fig. 2. Concentration dependency of bradykinin- and des-Arg^9-bradykinin-induced increases in $[Ca^{2+}]_i$ in single CPAE cells. The cells were exposed to either bradykinin (□) or des-Arg^9-bradykinin (■) for 10 min. The responses were divided into an intitial phase (the first 2 min) and a late phase (from 2 to 10 min), and the area under the curve (ratio·sec) was calculated. The area under the curve for the total response (10 min) is also shown. The values shown are means ± standard errors of four to 13 cells.

des-Arg^9-bradykinin and bradykinin were acting via B_1 and B_2 receptors, respectively, and, because the majority (85%) of the cells tested responded to both agonists (see above), that B_1 and B_2 receptors were expressed on the same cells.

The rise in $[Ca^{2+}]_i$ that occurs in cells after agonist-induced activation of PLC leads to an increase in the rate of efflux of Ca^{2+} from cells as a result of Ca^{2+}-mediated stimulation of the Ca^{2+}-ATPase pump in the plasma membrane and, in some cells, the Na^+/Ca^{2+} exchanger (see Ref. 29 for a review). Non-steady state tracer studies with $^{45}Ca^{2+}$ can be used to monitor this change in the rate of Ca^{2+} flux from the cells. Therefore, $[^3H]IP_3$ formation and $^{45}Ca^{2+}$ efflux were measured in populations of CPAE cells to provide more rapid and convenient methods for the detailed characterization of the pharmacology of responses to bradykinin and des-Arg^9-bradykinin.

The log concentration-response curves for bradykinin- and des-Arg^9-bradykinin-mediated increases in $[^3H]IP_3$ formation and $^{45}Ca^{2+}$ efflux are shown in Fig. 3. The EC_{50} values (with 95% confidence limits) for bradykinin-mediated $[^3H]IP_3$ formation and $^{45}Ca^{2+}$ efflux were 9.4 (5.5–24.3) nM and 0.36 (0.33–0.42) nM (three experiments), respectively.

The EC_{50} values (with 95% confidence limits) for des-Arg^9-bradykinin-evoked $[^3H]IP_3$ formation and $^{45}Ca^{2+}$ efflux were 0.27 (0.05–1.17) μM and 0.07 (0.05–0.9) μM (three experiments), respectively. The B_1 agonist was considerably less potent than bradykinin; however, the $^{45}Ca^{2+}$ efflux responses (and increases in $[Ca^{2+}]_i$) evoked by maximally effective concentrations of des-Arg^9-bradykinin and bradykinin were of similar magnitudes. In contrast, the increase in $[^3H]IP_3$ formation in response to a maximal concentration of bradykinin was approximately twice the size of the response to des-Arg^9-bradykinin.

The effects of the B_2-selective antagonists HOE140 and NPC567 and the B_1 antagonist des-Arg^9,[Leu^8]-bradykinin on the responses to both agonists were examined. In all of the antagonist experiments described below, concentrations of the agonists corresponding to the EC_{50} values were used. Fig. 4A shows that HOE140 inhibited bradykinin-induced $^{45}Ca^{2+}$ efflux with an IC_{50} value (with 95% confidence limits) of 1.3 (1.1–1.9) nM (three experiments). In contrast, high concentrations of HOE140 had little effect on the response to des-Arg^9-bradykinin. NPC567 also inhibited bradykinin-induced $^{45}Ca^{2+}$ efflux, with an IC_{50} value of 60 ± 13 nM (three experiments), but had no effect on des-Arg^9-bradykinin-evoked $^{45}Ca^{2+}$ efflux, even at 10 μM.

The effects of the B_1-selective antagonist des-Arg^9,[Leu^8]-bradykinin on the $^{45}Ca^{2+}$ efflux responses to bradykinin and des-Arg^9-bradykinin are shown in Fig. 4B. Des-Arg^9,[Leu^8]-bradykinin inhibited des-Arg^9-bradykinin-mediated $^{45}Ca^{2+}$ efflux with an IC_{50} value (with 95% confidence limits) of 1.1 (0.43–3.1) μM (three experiments) but, even at high concentrations (10 μM and above), it had no effect on the responses to bradykinin.

Similar results were obtained when the pharmacology of

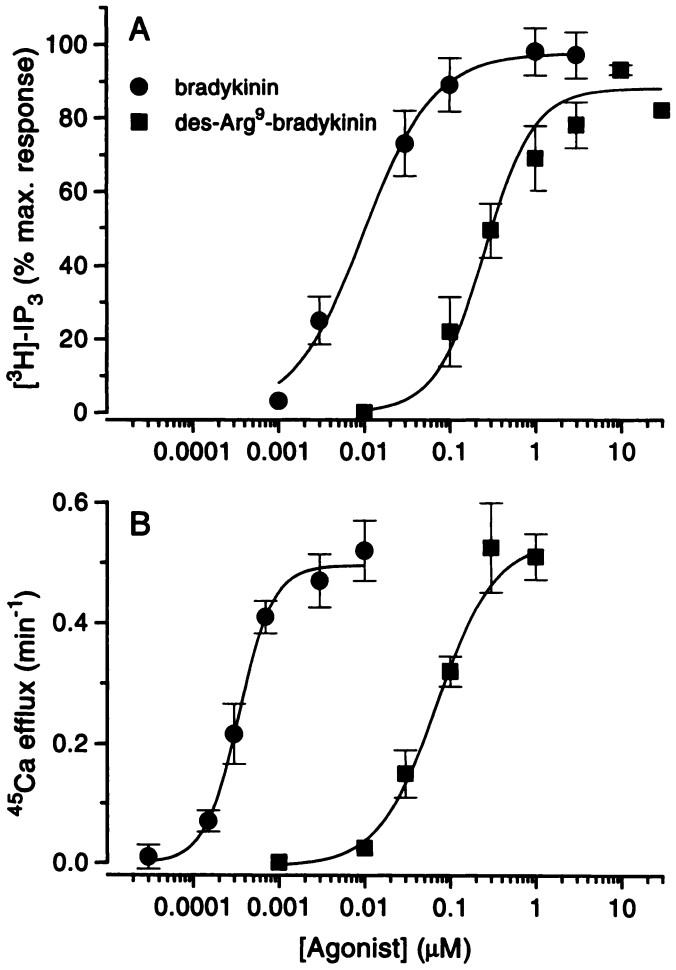

Fig. 3. Log concentration-response relationships for bradykinin- and des-Arg^9-bradykinin-mediated [^{3}H]IP$_3$ formation and $^{45}Ca^{2+}$ efflux in CPAE cells. A, [^{3}H]IP$_3$ formation was measured and the results were expressed as a percentage of the maximum response for each agonist; B, $^{45}Ca^{2+}$ efflux was measured. CPAE cells were incubated with agonist (bradykinin or des-Arg^9-bradykinin) for 20 sec in [^{3}H]IP$_3$ experiments and for 1 min (the duration of a collection period) in $^{45}Ca^{2+}$ efflux experiments. The average basal level of [^{3}H]IP$_3$ was 0.33 ± 0.04% of [^{3}H]PI (four experiments), and this increased to 1.1 ± 0.08% of [^{3}H]PI and 0.66 ± 0.09% of [^{3}H]PI in the presence of maximal concentrations of bradykinin and des-Arg^9-bradykinin, respectively. The results shown are means ± standard errors of three independent experiments.

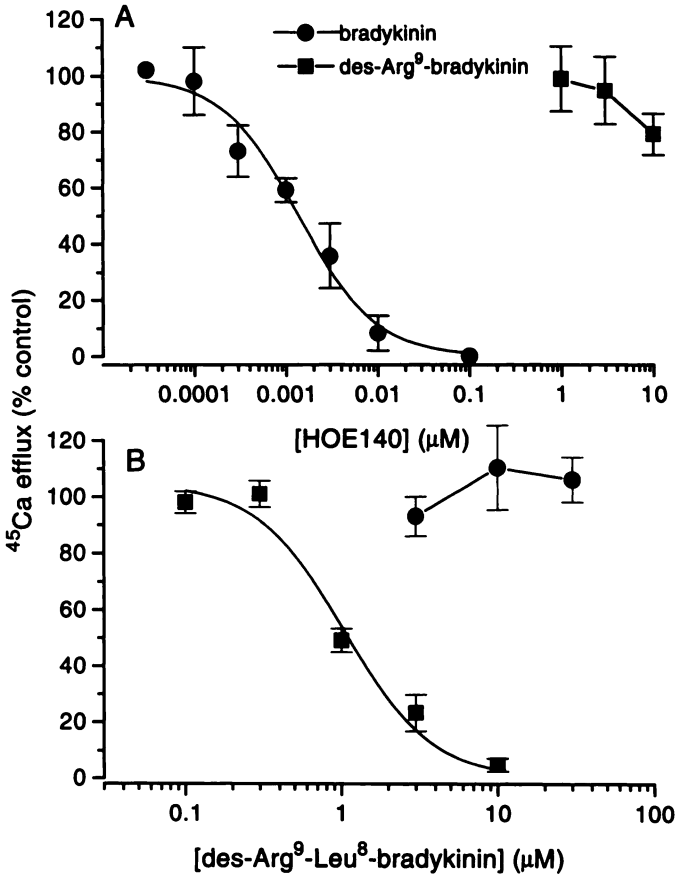

Fig. 4. Effects of HOE140 and des-Arg^9,[Leu^8]-bradykinin on bradykinin- and des-Arg^9-bradykinin-induced $^{45}Ca^{2+}$ efflux in CPAE cells. The effect of HOE140 (A) and the effect of des-Arg^9,[Leu^8]-bradykinin (B) on agonist-induced $^{45}Ca^{2+}$ efflux were measured. Bradykinin and des-Arg^9-bradykinin were applied as described in Fig. 3, at their EC_{50} value concentrations. The antagonists were applied 2 min before, and during, exposure to the agonists. The results have been expressed as a percentage of the control response to the EC_{50} value concentrations and are means ± standard errors of three or four independent experiments.

agonist-induced PLC activation was examined. HOE-140 inhibited bradykinin-induced [^{3}H]IP$_3$ formation with an IC_{50} value (with 95% confidence limits) of 8.3 (3.5–12.7) nM (three experiments), but 10 μM HOE140 caused only weak inhibition (18 ± 7%, three experiments) of the response to des-Arg^9-bradykinin. Des-Arg^9,[Leu^8]-bradykinin inhibited des-Arg^9-bradykinin-evoked [^{3}H]IP$_3$ formation with an IC_{50} value (with 95% confidence limits) of 0.8 (0.45–1.2) μM (three experiments) but was ineffective against bradykinin at concentrations up to 30 μM. These data are consistent with the hypothesis that bradykinin and des-Arg^9-bradykinin act at separate receptors, which have the characteristics of classical B_2 and B_1 subtypes, on the CPAE cells.

Effects of extracellular Ca^{2+}, Ni^{2+}, and La^{3+}. To characterize the actions of the two agonists further, experiments were carried out in Ca^{2+}-free HBSS-HEPES containing 200 μM EGTA, HBSS-HEPES containing the Ca^{2+} blocker $NiCl_2$ (1 mM), or phosphate-free HBSS-HEPES containing the Ca^{2+} blocker $LaCl_3$ (10 μM). Fig. 5, A and B, shows the time course of the increases in $^{45}Ca^{2+}$ efflux induced by bradykinin (0.4 nM) and des-Arg^9-bradykinin (30 nM) in control HBSS-HEPES containing 1.3 mM $CaCl_2$. For both agonists the response consisted of a transient increase followed by a sustained elevation in the rate of $^{45}Ca^{2+}$ efflux. Removal of extracellular Ca^{2+} significantly reduced the responses to 0.4 nM bradykinin ($F = 35.2$, $p < 0.001$) and 30 nM des-Arg^9-bradykinin ($F = 53.9$, $p < 0.001$). *Post hoc* analysis indicated that in the absence of extracellular Ca^{2+} the initial transient phases of the responses (defined as the responses in the first 2 min after agonist application) to bradykinin and des-Arg^9-bradykinin were reduced by 48 and 56%, respectively, compared with controls ($p < 0.001$, Newman-Keuls). The later phases of the responses to both agonists (defined as the responses 3–4 min after agonist application) were completely abolished in Ca^{2+}-free medium ($p > 0.05$, compared with the basal rate of $^{45}Ca^{2+}$ efflux, Newman-Keuls).

The Ca^{2+} entry blocker $LaCl_3$ inhibited the transient and sustained phases of the response to 0.4 nM bradykinin ($p > 0.05$, compared with basal efflux, Newman-Keuls) (Fig. 5C). $LaCl_3$ abolished the sustained phase of the response to 30 nM des-Arg^9-bradykinin ($p > 0.05$, compared with basal efflux,

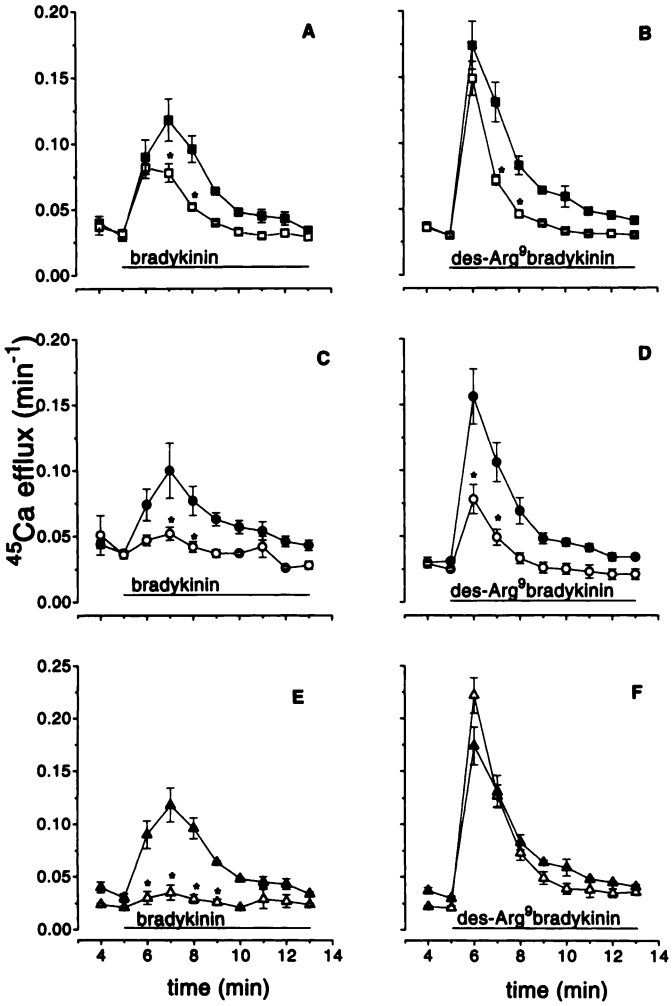

Fig. 5. Effects of bradykinin and des-Arg^9-bradykinin on the rate of $^{45}Ca^{2+}$ efflux from CPAE cells in control, Ca^{2+}-free, La^{3+}-containing, and Ni^{2+}-containing buffers. Cells were preincubated for 5 min in control HBSS-HEPES, pH 7.4 (■, ▲), Ca^{2+}-free buffer containing 200 μM EGTA (□), phosphate-free HBSS-HEPES (●), phosphate-free HBSS-HEPES containing 10 μM $LaCl_3$ (○), or HBSS-HEPES containing 1 mM $NiCl_2$ (△) before incubation for 8 min with 0.4 nM bradykinin (A, C, and E) or 30 nM des-Arg^9-bradykinin (B, D, and F) in the same buffer. Data represent means ± standard errors of three or four independent experiments, each performed in duplicate. The rate of $^{45}Ca^{2+}$ efflux in the control and test buffers was compared using two-way ANOVA. A, Two-way ANOVA, $F = 35.2$, df = 1, $p < 0.001$. *, $p < 0.001$, significantly different from control (Newman-Keuls). B, Two-way ANOVA, $F = 53.9$, df = 1, $p < 0.001$. *, $p < 0.01$, significantly different from control (Newman-Keuls). C, Two-way ANOVA, $F = 41.9$, df = 1, $p < 0.001$. *, $p < 0.05$, significantly different from control (Newman-Keuls). D, Two-way ANOVA, $F = 64.9$, df = 1, $p < 0.001$. *, $p < 0.05$, significantly different from control (Newman-Keuls). E, Two-way ANOVA, $F = 133.1$, df = 1, $p < 0.001$. *, $p < 0.01$, significantly different from control (Newman-Keuls). F, Two-way ANOVA, $F = 1.65$, df = 1, $p = 0.21$.

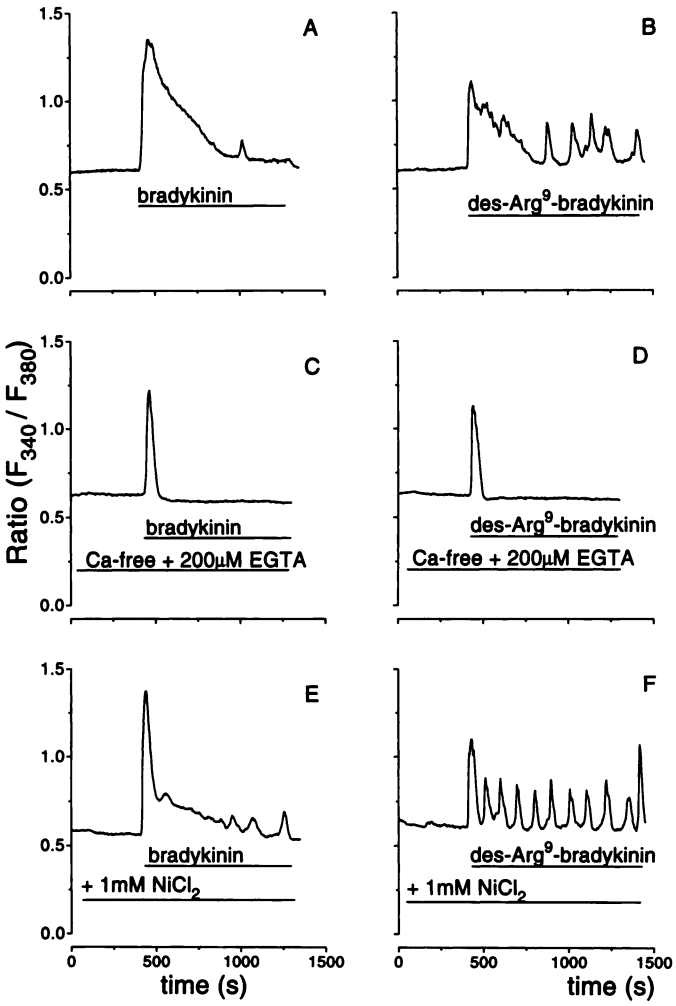

Fig. 6. Effects of Ca^{2+}-free medium and $NiCl_2$ on bradykinin- and des-Arg^9-bradykinin-mediated increases in $[Ca^{2+}]_i$ in single fura-2-loaded CPAE cells. Single CPAE cells were preincubated for 5 min in control HBSS-HEPES, pH 7.4 (A and B), Ca^{2+}-free buffer containing 200 μM EGTA (C and D), or HBSS-HEPES containing 1 mM $NiCl_2$ (E and F) before incubation with 0.3 nM bradykinin (A, C, and E) or 30 nM des-Arg^9-bradykinin (B, D, and F) in the same buffer for the period shown (*horizontal lines*).

Newman-Keuls) and reduced the transient phase by 58% ($p < 0.001$, Newman-Keuls) (Fig. 5D).

The Ca^{2+} channel blocker $NiCl_2$ (10 μM) abolished both the transient and sustained phases of the $^{45}Ca^{2+}$ efflux response to a low concentration of bradykinin (0.4 nM) ($p > 0.05$, compared with the basal rate of $^{45}Ca^{2+}$ efflux, Newman-Keuls) (Fig. 5E). In contrast, it did not inhibit either the transient or sustained phase of the response to a concentration of des-Arg^9-bradykinin (30 nM) that produced a response similar in magnitude to that to 0.4 nM bradykinin in control buffer ($F = 1.65$, $p = 0.21$) (Fig. 5F). The effect of $NiCl_2$ was also tested on the responses to higher concentrations of each agonist. Again, although it caused a significant reduction (47%) in the response to 1.0 nM bradykinin ($p < 0.001$, significantly different from control, Newman-Keuls), it did not cause any reduction of the response to 80 nM des-Arg^9-bradykinin. In fact, the responses to both high and low concentrations of des-Arg^9-bradykinin were slightly larger in the presence of $NiCl_2$ than in control buffer.

The effects of extracellular Ca^{2+} and 1 mM $NiCl_2$ on bradykinin- and des-Arg^9-bradykinin-evoked changes in $[Ca^{2+}]_i$ were also investigated in single fura-2-loaded CPAE cells (Fig. 6). In the absence of extracellular Ca^{2+}, bradykinin (0.3 nM) and des-Arg^9-bradykinin (30 nM) gave short spike responses with no sustained phase (four of four cells for each agonist) (Fig. 6, C and D). No oscillatory responses were seen with either agonist in the absence of extracellular Ca^{2+}. Higher concentrations of both bradykinin (1 nM and 30 nM) and des-Arg^9-bradykinin (300 nM and 1 μM) also gave short spikes, with no sustained phase, in the absence of extracellular Ca^{2+} (data not shown).

$NiCl_2$ attenuated the increases in $[Ca^{2+}]_i$ induced by bradykinin (eight cells). In contrast, it promoted rhythmic oscillations (five cells) or had no marked effect (four cells) on the responses to 30 nM des-Arg^9-bradykinin. The results of these experiments are summarized in Fig. 7, which shows the area under the curve for the responses to both agonists. Removal of extracellular Ca^{2+} caused a significant reduction in the responses evoked by both bradykinin and des-Arg^9-bradykinin, whereas $NiCl_2$ inhibited only the the response to bradykinin.

In parallel experiments, neither the removal of extracellular Ca^{2+} nor the addition of either $NiCl_2$ (1 mM) or $LaCl_3$ (10 μM) had any significant effect on bradykinin- or des-Arg^9-bradykinin-mediated [^{3}H]IP_3 production (Table 2). In two experiments, which gave similar results, 1 mM $NiCl_2$ had no effect on the [^{3}H]IP_3 formation evoked by a 90-sec incubation with 1 μM bradykinin or 10 μM des-Arg^9-bradykinin (data not shown). Similarly, $NiCl_2$ had no effect on [^{3}H]IP_3 formation after a 20-sec incubation with lower concentrations of des-Arg^9-bradykinin (100 nM) or bradykinin (3 nM) (data not shown).

Desensitization. The increase in [^{3}H]IP_3 formation in CPAE cells evoked by coincubation with bradykinin (1 μM) and des-Arg^9-bradykinin (10 μM) was not significantly greater than the response to bradykinin alone (basal level of [^{3}H]IP_3, 0.33 ± 0.04% of [^{3}H]PI; 20 sec after 1 μM bradykinin, 1.11 ± 0.08% of [^{3}H]PI; 20 sec after 10 μM des-Arg^9-bradykinin, 0.66 ± 0.09% of [^{3}H]PI; 20 sec after 1 μM bradykinin plus 10 μM des-Arg^9-bradykinin, 1.22 ± 0.34% of [^{3}H]PI) (one-way ANOVA, $p < 0.05$; $p > 0.05$ for des-Arg^9-bradykinin plus bradykinin, compared with bradykinin alone, Newman-Keuls). These data are consistent with the observation that 85% of single CPAE cells responded to both des-Arg^9-bradykinin and bradykinin, indicating that B_1 and B_2 receptors are coexpressed on the same cells.

To determine whether bradykinin or des-Arg^9-bradykinin induced either homologous or heterologous desensitization in CPAE cells, the relationship between the responses to the two agonists was examined. Pretreatment of CPAE cells with 1 μM bradykinin for 3 min reduced the increase in [^{3}H]IP_3 formation evoked by a subsequent challenge with 30 nM bradykinin by >70% (Fig. 8). The same pretreatment with 1 μM bradykinin had no effect, however, on the responsiveness of the cells to des-Arg^9-bradykinin (0.5 μM). Fig. 8 also shows that pretreatment of the cells with des-Arg^9-bradykinin (5 μM) did not reduce the ability of the cells to respond to subsequent challenges with either des-Arg^9-bradykinin or bradykinin. A longer pretreatment with des-Arg^9-bradykinin (5 min) (data not shown) also failed to reduce the size of responses evoked by challenges with either des-Arg^9-bradykinin or bradykinin.

TABLE 2

Effect of removal of extracellular Ca^{2+} or addition of $NiCl_2$ or $LaCl_3$ on agonist-induced [^{3}H]IP_3 formation

Cells were pretreated in the appropriate buffers for 5 min and the agonists were applied for 20 sec in the same buffer. There was no significant effect of any of the treatments on the responses to either bradykinin ($F = 1.39$, $p = 0.25$) or des-Arg^9-bradykinin ($F = 0.9$, $p = 0.47$). The data shown are the means ± standard errors of five or six independent experiments performed in triplicate.

Treatment	[^{3}H]IP_3 formation	
	Bradykinin (1 μM)	Des-Arg^9-bradykinin (10 μM)
	% increase	
None	241 ± 27	127 ± 28
Ca^{2+}-free buffer plus 200 μM EGTA	240 ± 28	127 ± 24
$NiCl_2$ (1 mM)	294 ± 79	103 ± 19
$LaCl_3$ (10 μM)	240 ± 45	181 ± 39

Discussion

Pharmacological evidence for the existence of B_1 and B_2 receptors on CPAE cells. The pharmacological data presented in this study suggest that there are two distinct receptors on CPAE cells and that these have the characteristics of classical B_1 and B_2 bradykinin receptors. Activation of each subtype resulted in stimulation of PLC, elevation of $[Ca^{2+}]_i$, and an increase in the rate of $^{45}Ca^{2+}$ efflux from the cells. Although des-Arg^9-bradykinin was significantly less potent than bradykinin, the EC_{50} values ob-

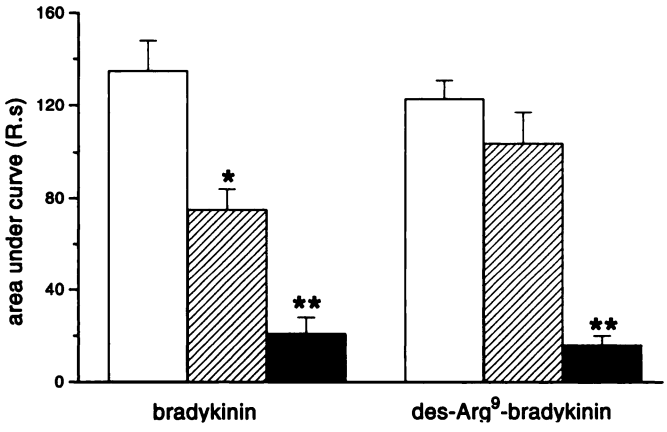

Fig. 7. Effects of Ca^{2+}-free medium and $NiCl_2$ on bradykinin- and des-Arg^9-bradykinin-mediated increases in $[Ca^{2+}]_i$. Single cells were incubated with bradykinin (0.3 nM) or des-Arg^9-bradykinin (30 nM) for 10 min in control HBSS-HEPES (□), HBSS-HEPES plus 1 mM $NiCl_2$ (▨), or Ca^{2+}-free buffer plus 200 μM EGTA (■). The area under the curve (ratio·sec) was then calculated. Data represent means ± standard errors for four to 13 cells. *, $p < 0.05$; **, $p < 0.01$, significantly different from the response in control buffer (Student's t test).

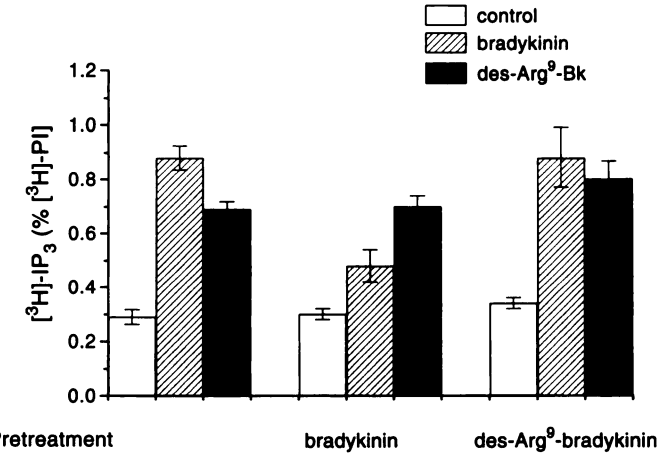

Fig. 8. Effects of pretreatment with bradykinin and des-Arg^9-bradykinin on B_1 and B_2 receptor-evoked [^{3}H]IP_3 formation in CPAE cells. The cells were pretreated with buffer, 1 μM bradykinin, or 5 μM des-Arg^9-bradykinin for 3 min. They were then washed and allowed to recover for 10 min before being challenged with either 30 nM bradykinin or 0.5 μM des-Arg^9-bradykinin (*des-Arg^9-Bk*) for 20 sec. The results shown are means ± standard errors of three independent experiments.

tained in the present study are similar to those reported for B_1-mediated responses in smooth muscle preparations and cell cultures (30–32). The efficacies of bradykinin and des-Arg^9-bradykinin were similar with respect to their abilities to increase $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux. However, the maximum increase in [^{3}H]IP_3 production induced by des-Arg^9-bradykinin was less than half of that evoked by the B_2 agonist, suggesting that the amount of IP_3 produced by bradykinin was in excess of the amount required to produce a maximum increase in $[Ca^{2+}]_i$. A similar observation has been made in tracheal smooth muscle cells, where maximal stimulation of PLC by des-Arg^9-bradykinin was only 16% of the response to bradykinin, although the two agonists induced increases in $[Ca^{2+}]_i$ of similar magnitudes (32).

The responses to bradykinin were blocked by low concentrations of the potent and selective B_2 antagonist HOE140, but not by high concentrations of the B_1-selective antagonist des-Arg^9,[Leu^8]-bradykinin. In addition, the IC_{50} value for inhibition of bradykinin-induced $^{45}Ca^{2+}$ efflux by the B_2 antagonist NPC567 was similar to that reported for inhibition of B_2 receptor-mediated responses in other tissues (33). These results are consistent with the hypothesis that the responses to bradykinin were mediated through B_2 and not B_1 bradykinin receptors. Conversely, responses to des-Arg^9-bradykinin were inhibited by des-Arg^9,[Leu^8]-bradykinin but HOE140, even at high concentrations (up to 10 μM), had a minimal effect on the responses to the B_1 agonist. Furthermore, NPC567 (up to 10 μM) failed to block the responses to des-Arg^9-bradykinin. These results suggest that des-Arg^9-bradykinin acted via B_1 rather than B_2 bradykinin receptors.

These observations are at odds with an earlier study by Conklin *et al.* (17) that examined bradykinin and des-Arg^9-bradykinin stimulation of prostaglandin production in CPAE cells. Those authors reported that the B_2 antagonist NPC567 was a poor inhibitor of the response induced by bradykinin (IC_{50} value, 370 nM) and that at high concentrations it antagonized the response to des-Arg^9-bradykinin. Based on these observations, they proposed that CPAE cells possess a novel bradykinin receptor subtype. One difference between the results presented here and those of Conklin *et al.* (17) is in the functional responses measured. Although it is possible that bradykinin-evoked prostaglandin synthesis is mediated by another subtype of receptor, binding studies (9) have indicated that there are only two sites for [^{3}H]bradykinin in CPAE cells. These consist of a high affinity site corresponding to the B_2 receptor and a low affinity site thought to correspond to the B_1 receptor. Indeed, the lack of potency of NPC567 reported by Conklin *et al.* (17) may simply reflect the very high concentration of bradykinin (10 nM), relative to its EC_{50} value for prostaglandin synthesis (0.2 nM), used in their antagonist experiments.

In a study (10) in a related cell type, i.e., bovine aortic endothelial cells, it was reported that a low concentration of HOE140 (0.1 μM) completely blocked des-Arg^9-bradykinin-mediated cGMP formation. Those authors proposed that there might be a novel, HOE140-sensitive, B_1 receptor subtype or, alternatively, that the B_1 receptors on these cells could 'de-differentiate' in culture. Although we have not measured cGMP directly, it is unlikely that this novel subtype occurs in CPAE cells. cGMP is formed as a consequence of the rise in $[Ca^{2+}]_i$ that occurs after stimulation of PLC and, in the present study, HOE140 had a negligible effect on des-Arg^9-bradykinin-evoked [^{3}H]IP_3 formation, increases in $[Ca^{2+}]_i$, or $^{45}Ca^{2+}$ efflux.

Characteristics of the B_1 and B_2 receptor-mediated responses. To characterize further the B_1 and B_2 receptors on CPAE cells, the effects of extracellular Ca^{2+} and of the Ca^{2+} channel blockers $NiCl_2$ and $LaCl_3$ on agonist-evoked responses were determined. Coupling of the B_1 and B_2 receptors to PLC did not appear to be sensitive to the extracellular Ca^{2+} concentration, because incubation of CPAE cells in Ca^{2+}-free medium containing EGTA had no effect on bradykinin- or des-Arg^9-bradykinin-mediated [^{3}H]IP_3 formation. These observations are consistent with those of earlier studies (16, 34) that reported that bradykinin-induced activation of PLC in endothelial cells was independent of extracellular Ca^{2+}. However, in a recent study in smooth muscle cells (11), des-Arg^9-bradykinin-induced activation of PLC was found to be partially dependent on extracellular Ca^{2+}, whereas bradykinin-evoked activation was essentially Ca^{2+} independent.

Stimulation of the CPAE cells with a low concentration of bradykinin (0.3 nM) caused an initial transient increase in $[Ca^{2+}]_i$ that, in some cells, was followed by erratic oscillations. The majority of cells responded to a low concentration of des-Arg^9-bradykinin (30 nM) with small erratic oscillations. For both agonists there was a concentration-dependent increase in the size of the initial transient phase of the $[Ca^{2+}]_i$ response. The B_1 agonist evoked a concentration-dependent increase in the later sustained phase of the $[Ca^{2+}]_i$ response but, in contrast, this late phase was suppressed at high concentrations of the B_2 agonist. In the absence of extracellular Ca^{2+}, the oscillations evoked by low agonist concentrations and the sustained phases apparent at higher agonist concentrations were abolished, indicating that they were dependent on Ca^{2+} entry. Therefore, the loss of the sustained phase of the response at high concentrations of bradykinin may be a function of inhibition of Ca^{2+} entry, as has been reported previously in bovine aortic endothelial cells (35). In a number of cell types it has been suggested that an unknown component of the Ca^{2+} entry mechanism can be inhibited by high $[Ca^{2+}]_i$ (36–38). However, des-Arg^9-bradykinin- and bradykinin-evoked increases in $[Ca^{2+}]_i$ were of similar magnitude, and it is likely that the differences in the shape of the responses, at higher concentrations, may be a feature of B_2 receptor activation, related perhaps to its propensity to desensitize.

The biphasic nature of the B_1 and B_2 agonist-evoked responses was confirmed in $^{45}Ca^{2+}$ efflux experiments. In the absence of extracellular Ca^{2+} the sustained phase of the $^{45}Ca^{2+}$ efflux response was abolished, consistent with the hypothesis that it was dependent upon Ca^{2+} entry. Furthermore, the Ca^{2+} channel blocker $LaCl_3$ attenuated both bradykinin- and des-Arg^9-bradykinin-evoked $^{45}Ca^{2+}$ efflux responses, to similar extents. However, $NiCl_2$ was able to differentiate between B_1 and B_2 agonist-evoked responses. $NiCl_2$ abolished the increase in $^{45}Ca^{2+}$ efflux induced by a low concentration of bradykinin and caused a substantial reduction in the response to a higher concentration of the B_2 agonist. In marked contrast, $NiCl_2$ did not inhibit des-Arg^9-bradykinin-evoked $^{45}Ca^{2+}$ efflux at either low or high agonist concentrations. $NiCl_2$ also reduced the increase in $[Ca^{2+}]_i$ induced by bradykinin, whereas it appeared to promote rhythmic oscillations in $[Ca^{2+}]_i$ in response to des-Arg^9-bradykinin. It is not clear why $NiCl_2$ was able to discriminate

between the responses to the two agonists or why this effect was opposite to that recently reported in mesangial cells (12), where $NiCl_2$ reduced the response to des-Arg^9-bradykinin but had little effect on the response to bradykinin. $NiCl_2$ has been reported to have both direct (39) and indirect (40) inhibitory effects on PLC activity. In the latter study, $NiCl_2$ reduced the late phase (from 30 sec onwards) of muscarinic receptor-induced [^{3}H]IP_3 formation in human neuroblastoma cells by inhibiting the Ca^{2+} entry responsible for feed-forward activation of PLC. However, the results of the present study suggest that $NiCl_2$-induced inhibition of bradykinin-evoked $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux responses was mediated at a point distal to PLC. Firstly, both bradykinin and des-Arg^9-bradykinin induced Ca^{2+} entry, but removal of extracellular Ca^{2+} had no effect on either bradykinin- or des-Arg^9-bradykinin-mediated [^{3}H]IP_3 formation; secondly, and more importantly, B_1 and B_2 agonist-mediated stimulation of [^{3}H]IP_3 formation was unaltered in the presence of $NiCl_2$ at either 20 sec or 90 sec after agonist application.

$NiCl_2$ is known to block the Na^+/Ca^{2+} exchanger (41), which may contribute, together with the plasma membrane Ca^{2+}/Mg^{2+}-ATPase, to agonist-induced increases in $^{45}Ca^{2+}$ efflux (29). It is, therefore, conceivable that the inhibitory effect of $NiCl_2$ on bradykinin-induced $^{45}Ca^{2+}$ efflux is mediated at the level of this exchanger. However, preliminary experiments demonstrated that exchange of sodium for choline had no effect on B_1 or B_2 agonist-evoked $^{45}Ca^{2+}$ efflux, indicating that the Na^+/Ca^{2+} exchanger had a minimal role in mediating this response in CPAE cells (data not shown).

A number of groups (22, 23) have reported that bradykinin evokes a La^{3+}-sensitive inward current in vascular endothelial cells that is carried by both Na^+ and Ca^{2+}. This pathway permits Ca^{2+} to enter the cells and is likely to be at least partly responsible for the reported bradykinin-induced rise in $[Ca^{2+}]_i$. One interpretation of the present findings is that activation of this cation channel contributes to the rise in $[Ca^{2+}]_i$ evoked by bradykinin and, thereby, to the increase in $^{45}Ca^{2+}$ efflux in CPAE cells. If this channel were sensitive to $NiCl_2$, then $NiCl_2$ would inhibit the bradykinin-mediated increase in $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ efflux. In this context, it is of interest to note that bradykinin has been reported to evoke Ca^{2+} entry into fibroblasts by two mechanisms, only one of which is sensitive to $NiCl_2$ (42). The presence of multiple Ca^{2+} entry pathways that differ in their sensitivity to $NiCl_2$ may explain the observation that removal of extracellular Ca^{2+} was more effective than $NiCl_2$ in reducing the size of the $[Ca^{2+}]_i$ response evoked by bradykinin. Although $NiCl_2$ had no overall effect on the increase in $[Ca^{2+}]_i$ induced by des-Arg^9-bradykinin, it did appear to change the erratic oscillatory increases in $[Ca^{2+}]_i$ induced by activation of the B_1 agonist into more pronounced rhythmic oscillations. The mechanism for this effect is unclear but it is possible that, by blocking a minor Ca^{2+} entry pathway for des-Arg^9-bradykinin, $NiCl_2$ unmasked oscillatory activity due to Ca^{2+} entry via a different route. To elucidate the precise mechanism by which des-Arg^9-bradykinin and bradykinin evoke Ca^{2+} influx, it would be useful to measure the currents evoked by the two agonists, along with their ionic sensitivities.

Desensitization. One of the most striking aspects of the response to bradykinin in many tissues is that it undergoes a rapid and profound desensitization (for review, see Ref. 14). This is particularly apparent in neuronal cells, where even very short (5-sec) exposures to bradykinin can lead to long-lasting desensitization (43, 44). In the present study the responses to bradykinin and des-Arg^9-bradykinin in the CPAE cells were distinguished further by the fact that it was relatively easy to demonstrate homologous desensitization to bradykinin but not to des-Arg^9-bradykinin. There was a clear bell-shaped relationship between the concentration of the B_2 agonist and the sustained phase of the increase in $[Ca^{2+}]_i$. This was reflected in the time course for bradykinin-induced [^{3}H]IP_3 formation, which at high concentrations (1 μM) peaked and then declined rapidly towards base-line (data not shown), suggesting that desensitization had occurred. In contrast, at high concentrations of des-Arg^9-bradykinin there was no suppression of the sustained phase of the increase in $[Ca^{2+}]_i$, and the increase in [^{3}H]IP_3 induced by a high concentration of the B_1 agonist (10 μM) was maintained at a relatively high level for up to 5 min (data not shown). In support of these observations, it was possible to cause a substantial reduction in the response to a test challenge with bradykinin by prior exposure of the cells to 1 μM bradykinin, whereas pretreatment with a high concentration of des-Arg^9-bradykinin (5 μM) did not reduce the response to a subsequent test pulse of the B_1 agonist. However, pretreatment of the cells with bradykinin did not cause any reduction in the response of the cells to a subsequent challenge with des-Arg^9-bradykinin. This differed from results obtained in mesangial cells, where concentrations of des-Arg^9-bradykinin as low as 1 μM greatly reduced the response to a subsequent challenge with des-Arg^9-bradykinin (12). Our observations are consistent with the hypothesis that bradykinin and des-Arg^9-bradykinin do not exhibit heterologous desensitization (see also Ref. 12) and they provide additional evidence for the existence of distinct subtypes of bradykinin receptors on the CPAE cells.

In conclusion, the pharmacological data presented provide evidence for distinct B_1 and B_2 receptors on CPAE cells, with no evidence to support the presence of a novel bradykinin receptor subtype. The observation that bradykinin did not induce desensitization of des-Arg^9-bradykinin-mediated responses is also consistent with the proposal that these cells express more than one receptor subtype. Although both receptor subtypes activated PLC and increased $[Ca^{2+}]_i$, the differential sensitivity to $NiCl_2$ suggests that there may be some diversity in their signaling pathways.

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