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Bradykinin Analogs Antagonize Bradykinin-Induced Second Messenger Production in a Sensory Neuron Cell Line

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SUMMARY

Bradykinin is the prime initiator of pain and the key initial activator of the inflammatory response at the site of tissue injury. The subsequent transfer of nociceptive information (pain sensation) into the central nervous system is then mediated via afferent type C dorsal root ganglion neurons. A recently developed hybrid cell line, F-11, shows many qualities characteristic of these pain-sensitive cells. In these neuronal hybrids, we have found that bradykinin induces sequential elevation in the concentrations of several second messengers involved in neuronal activation, including inositol trisphosphate (6.5-fold), intracellular calcium (2.7-fold), and cyclic GMP (20.5-fold). Importantly, the production of these second messengers is potently inhibited by several novel bradykinin antagonists that possess no intrinsic agonist activity. The same relative rank order of potency of inhibition of bradykinin-induced second messenger production was achieved in the inositol trisphosphate, calcium, and cyclic GMP assay systems, suggesting strongly that all three messenger systems are being activated by the same bradykinin receptor. The most potent antagonist was D-Arg^0-Hyp^3-Thi^{5,8}-D-Phe^7-bradykinin, which inhibited in a competitive manner, with pA_2 values, upon Schild plot analysis, in the nanomolar range. These potent bradykinin antagonists may be useful in the characterization of bradykinin receptors and in the clinical management of pain and inflammation.

Bradykinin is recognized as the most potent stimulator of pain known and is generated any time active cellular injury occurs (1). Subsequently, bradykinin acts to initiate the cardinal manifestations of inflammation, in particular its pain component. Pain sensation itself is the interpretation by the central nervous system of direct stimulation of nociceptive nerve endings located in the periphery.

Bradykinin appears to stimulate pain sensation via activation of type C polymodal DRG cells, which contain substance P. Direct studies of this particular class of neurons *in vivo* is limited, however, because type C neurons constitute less than 20% of the sensory DRG subpopulation of cells (2). This heterogeneity makes it difficult to correlate the action of bradykinin with a particular population of cells. A recently developed hybrid cell line, F-11 (a DRG × N18TG2 neuroblastoma hybrid), shows many qualities characteristic of pain-sensitive DRG cells. For example, F-11 cells, when activated, generate action potentials in which calcium ions are a major component (3). In addition, these cells show biochemical responses to bradykinin, opiate agonists, and prostaglandins, all of which have important roles in neuronal mechanisms related to pain (3–6). F-11 cells contain substance P, a neurotransmitter contained in nociceptive DRG cells (7, 8), and release it upon depolarization (3, 4, 6). Finally, these cells depolarize in response to the agent capsaicin, a marker specific for nociceptive sensory neurons (6). The F-11 cell line, therefore, is a useful model system for studying the mechanisms underlying activation of nociceptive nerve endings and for studying the inhibition of these mechanisms.

We and others have been interested in the biochemical and biophysical reaction mechanisms that underlie the activation of pain-sensitive neurons by bradykinin. If bradykinin analogs block bradykinin-induced reactions in neurons (in particular the bradykinin-induced generation of second messenger molecules) with no intrinsic stimulating activities of their own, then these analogs could prove to be potent anti-inflammatory and anti-pain therapeutic agents.

In this study, we analyzed the inhibition of bradykinin-induced generation of three second messengers; IP_3, intracellular calcium, and cyclic GMP. These second messengers have important roles in the regulation of excitability of nociceptive nerve endings (9). We found that several bradykinin analogs act as potent antagonists of bradykinin-induced second messenger production.

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ABBREVIATIONS: DRG, dorsal root ganglion; IP_3, inositol trisphosphate; MOPS, 3-[*N*-morpholino]propanesulfonic acid; EGTA, [ethylenebis(oxyethylene nitrilo)]tetraacetic acid.

Materials and Methods

Chemicals. Analogs of bradykinin were a generous gift of J. Stewart and his colleagues (University of Colorado, School of Medicine, Denver, CO) (10, 11). All other reagents and supplies were purchased from commercial sources as listed below.

Culture of F-11 cells. F-11 cells (a mouse N18TG2 neuroblastoma × DRG sensory neuron cell line) were a generous gift of Dr. M. C. Fishman (Harvard Medical School, Boston, MA). The cells were cultured to near confluency in 60 mm plastic dishes (Falcon, Oxnard, CA) in Ham's F-12 media (GIBCO, Grand Island, NY) supplemented with 20% fetal bovine serum and HAT medium supplement (Sigma Chemical Co., St. Louis, MO) as previously described (4).

IP_3 measurement. IP_3 production was measured as previously described (5, 12). Briefly, cells were labeled with 15 μCi of myo-[2-^{3}H] inositol (12.5 Ci/mmol; Amersham, Arlington Heights, IL) in growth medium for 20 hr. Cells were rinsed twice with serum-free medium, and allowed to equilibrate in serum-free medium for 1 hr at 37°, in 5% CO_2. Drugs were added directly into the medium, and metabolic activity was terminated by aspiration of media followed by addition of 1 ml of 6% trichloroacetic acid. The cells were allowed to extract for at least 1 hr at 4° and were then centrifuged at 750 × g for 15 min. The supernatant was washed six times with 2 ml of water-saturated diethyl ether and neutralized to pH 7 with 50 mM disodium tetraborate. The [2-^{3}H]inositol-labeled metabolites were separated by anion-exchange chromatography on Dowex 1 × 8 200–400 mesh (formate form) columns as previously described (13).

Intracellular calcium. Calcium was measured with the calcium-chelating fluorescent indicator fura-2 (Molecular Probes, Inc., Eugene, OR) using a slight modification of the procedure of Osugi *et al.* (14). Briefly, cells were incubated with 4 μM fura-2-acetoxymethyl ester (cell membrane-permeable form of fura-2) for 60 min at 37°, under 5% CO_2 in growth medium. Medium was removed and cells were harvested in MOPS-buffered saline (140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 0.3% bovine serum albumin, 10 mM MOPS, pH 7.4). Cells were washed twice and then resuspended at 10^6 cells/ml in the same saline for 30 min at 25°. Cell fluorescence was measured at 37° at an excitation wavelength of 336 nm (5-nm slit) and an emission wavelength of 500 nm (10-nm slit) using an MPF-44P Perkin-Elmer fluorescence spectrometer. Cells were placed on ice to prevent diffusion of fura-2, centrifuged immediately before use, resuspended in 2 ml of 37° MOPS-saline, and placed in the cuvette until the baseline fluorescence stabilized. Drugs were added in 20-μl aliquots and mixed using a pipette, and the fluorescence output was measured as a function of time. After the maximum experimental calcium response was recorded, the maximum fluorescence (F_{max}) was determined by adding 10 μl of 10 mM digitonin in dimethyl sulfoxide. EGTA (60 μl of a 100 mM solution) in 1 M Tris base was subsequently added to determine F_{min}. The calibration of fura-2 fluorescence as a function of intracellular calcium concentration was determined as described by Grynkiewicz *et al.* (15) using the formula $[Ca^{2+}] = 224\ nM \times (F-F_{min})/(F_{max}-F)$ where F is the fluorescence and 224 nM is the equilibration constant.

Cyclic GMP measurement. cGMP was measured by radioimmunoassay, in a procedure similar to the measurement of cAMP by Francel *et al.* (4). Briefly, cells were grown to near confluence in 35-mm wells in six-well plates (Costar, Cambridge, MA) and washed twice with serum-free medium, followed by incubation in medium for 1 hr at 37°. Drugs were added directly to the medium, and metabolic activity was terminated by aspiration and addition of 0.5 ml of trichloroacetic acid. Supernatants were washed six times with 2 ml of diethyl ether, dried using air, and then resuspended in distilled water. Cyclic GMP levels were determined by radioimmunoassay, via competition with ^{125}I-2′-*O*-succinyl cyclic GMP for cyclic GMP antisera (Meloy Laboratories, Springfield, VA) in 50 mM acetate buffer, pH 4.75.

Protein was determined for all assays using the method of Lowry *et al.* (16) with bovine serum albumin as the standard. All points were performed in triplicate and all experiments were performed at least three times. Appropriate controls were also performed.

Results

In F-11 cells, bradykinin induces, in a time- (Fig. 1A) and dose- (Fig. 1B) dependent manner, increases in the intracellular levels of IP_3, calcium, and cyclic GMP. IP_3 in unstimulated controls was 104 ± 8 cpm/mg of protein. Maximal stimulation by 10^{-6} M bradykinin caused a 6.5 ± 0.3-fold increase. Similarly, intracellular calcium increased 2.7 ± 0.3-fold upon maximal stimulation, from a baseline of 220 ± 18 nM. Cyclic GMP levels increased 20.5 ± 1.4-fold, from 0.15 ± 0.03 pmol/mg of protein. The times of maximal increase of intracellular IP_3, calcium, and cyclic GMP were 5–10 sec, 15–20 sec, and 45–60 sec, respectively. These responses returned to control levels in 1, 1.5, and 2.5 min, respectively. In addition, bradykinin induces increases in other bioactive lipid metabolites (e.g., diacylglycerol and arachidonic acid; data not shown).

The structures of the bradykinin analogs we tested are presented in Table 1 and the ability of these various analogs to inhibit bradykinin-induced IP_3, calcium, and cyclic GMP production is shown in Table 2. Analogs [except for Lys-bradykinin (L)] are listed in order of increasing inhibitory potency as observed in the three second messenger assays. The rank order of potency for inhibition of bradykinin-induced IP_3, calcium, and cyclic GMP production was identical for all three tested assays, suggesting that the stimulation of these three second messengers is probably linked through the same or similar types of bradykinin receptor. None of the analogs presented in Table 1, except Lys-bradykinin (L) and D-Phe^6-D-Ala^7-bradykinin (analog **3**), showed any agonist activity [i.e., more than 5% of the activity of bradykinin in the antagonist concentration range of 10^{-10} to 10^{-5} M, when allowed to stimulate the cells in the range of 5 sec–10 min (data not shown)]. D-Phe^6-D-Ala^7-bradykinin and Lys-bradykinin showed agonist activity of approximately 20% and 55% of the activity of bradykinin, respectively, in the three assays.

The potency of the most potent bradykinin antagonist (analog **17**) in inhibiting bradykinin receptor-mediated second messenger production was measured. A representative tracing in the case of inhibition of bradykinin-induced Ca^{2+} activation is shown in Fig. 2. The maximal stimulation by 10^{-8} M bradykinin (Fig. 2A) is completely inhibited by preincubation with 10^{-6} M analog **17** (Fig. 2B). As shown in Fig. 3, analog **17** inhibited 10 nM bradykinin-induced intracellular calcium mobilization with a K_i of 18 ± 9 nM. Similar K_i values were determined for this analog in the IP_3 (14 ± 6 nM) and cyclic GMP (12 ± 6 nM) assay systems. Further studies of the most potent antagonist, analog **17**, were performed to determine whether or not the inhibition was competitive. Fig. 4 shows the Schild plot from the bradykinin-induced calcium experiments. A straight line was obtained (linear correlation coefficient = 0.992) with mean pA_2 for four experiments of 10.4 ± 4.6 nM and a slope of 0.97 ± 0.6, suggesting that analog **17** truly acts as a competitive antagonist at the bradykinin receptor. Likewise, in the IP_3 and cyclic GMP assays we achieved straight lines upon Schild plot analysis, with pA_2 = 12.2 nM and slope = 1.01 (linear correlation coefficient = 0.98) in the IP_3 assay and pA_2 = 10.1 nM and slope of 0.98 in the cyclic GMP assay (linear correlation coefficient = 0.99). In addition, this inhibition by analog **17** is reversible, inasmuch as thorough rinsing of the cells, followed by subsequent addition of bradykinin, results in the full bradykinin response. Finally, the inhibition by analog **17** was specific for the bradykinin receptor, because

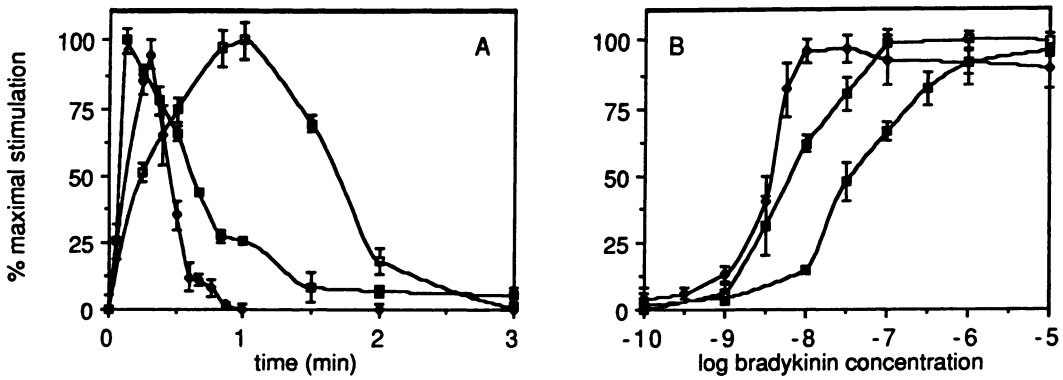

Fig. 1. Time course (A) and dose dependence (B) of bradykinin-induced elevations of IP_3 (■), intracellular calcium (♦), and cyclic GMP (□). Time courses were performed using 1 μM bradykinin (maximal stimulation). The dose dependence of production of each metabolite was measured at the time maximal elevation as determined in A. Cyclic GMP, calcium, and IP_3 values represent the mean of three experiments run in triplicate, with standard errors indicated by the *error bars*. All values are expressed as percentage of maximal stimulation relative to control. Maximal stimulated values were 676 cpm/mg of protein for IP_3, 594 nM for calcium, and 3.08 pmol/mg of protein for cyclic GMP. Control values were 104 ± 8 cpm/mg of protein for IP_3, 220 ± 18 nM for calcium, and 0.15 ± 0.03 pmol/mg of protein for cyclic GMP.

TABLE 1

Structure of bradykinin and related analogs

1 2 3 4 5 6 7 8 9

Structure of bradykinin: NH_2-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH.

Eac, ε-amino caproic acid; Hyp, L-4-hydroxyproline; Thi, β-(2-thienyl)-L-alanine.

Peptide analog	Peptide structure
L	Lys-bradykinin
1	des-Arg^9-bradykinin
2	des-Arg^9-Leu^8-bradykinin
3	D-Phe^6-D-Ala^7-bradykinin
4	Eac^0-Thi^{5,8}-D-Phe^7-bradykinin
5	D-Arg^0-D-Phe^7-bradykinin
6	Lys$^{-1,0}$-D-Phe^7-bradykinin
7	Hyp^3-D-Phe^7-bradykinin
8	Lys$^{-1,0}$-Hyp^2-D-Phe^7-bradykinin
9	Hyp^3-Thi^{5,8}-D-Phe^7-bradykinin
10	D-Arg^0-Thi^{5,8}-D-Phe^7-bradykinin
11	D-Arg^0-Hyp^2-D-Phe^7-bradykinin
12	Lys$^{-1,0}$-Thi^{5,8}-D-Phe^7-bradykinin
13	D-Arg^0-Hyp^2-Thi^{5,8}-D-Phe^7-bradykinin
14	Lys$^{-1,0}$-Hyp^3-D-Phe^7-bradykinin
15	D-Arg^0-Hyp^{2,3}-Thi^{5,8}-D-Phe^7-bradykinin
16	D-Arg^0-Hyp^3-D-Phe^7-bradykinin
17	D-Arg^0-Hyp^3-Thi^{5,8}-D-Phe^7-bradykinin

TABLE 2

IC_{50} Values for tested bradykinin analogs

The structures of the analogs are given in Table 1. IC_{50} values (18) are the means from at least three separate experiments of each analog in triplicate with [analog] varied from 10^{-10} to 10^{-5} M in the presence of 10^{-7} M bradykinin. Standard error was <20% for all tested drugs. AG, agonist.

Peptide analog	cGMP	Calcium	IP_3
		nM	
L	AG	AG	AG
1	>10,000	>10,000	>10,000
2	>10,000	>10,000	>10,000
3	AG	AG	AG
4	1180	1201	1251
5	748	790	794
6	687	645	602
7	532	570	562
8	521	561	547
9	507	532	508
10	500	522	501
11	497	520	479
12	491	505	451
13	365	500	351
14	225	350	294
15	186	239	177
16	165	140	155
17	60	136	100

analog **17** did not block angiotensin or substance P-induced IP_3 generation (data not shown).

Discussion

Bradykinin, as mentioned previously, is the prime initiator of pain and inflammation due to tissue injury. It induces production of the second messengers IP_3, calcium, and cyclic GMP, all of which play important roles in the regulation of the excitability of nociceptive nerve endings (9). We have shown that several bradykinin analogs, which possess no agonist activity, block the bradykinin-induced production of these second messengers. These antagonists may therefore block the activation of nociceptive neurons by bradykinin. This may, at the organismal level, abolish the sensation of pain. Such specific antagonists would not block activation of DRG neurons mediating other sensory modalities, e.g., touch, because bradykinin does not play a significant role in the activation of these other classes of sensory neurons (1).

We tested a series of these analogs synthesized by Dr. J. Stewart, University of Colorado, and his colleagues. These investigators have synthesized analogs of bradykinin in which one or more of the constituent amino acids have been replaced by specific synthetic amino acids (10). The agonist activity of each analog was initially screened (in the concentration range of 10^{-10} to 10^{-5} M for times of 5 sec to 10 min) because, as we have previously shown (5), bradykinin receptor activation induces desensitization of the receptor, which can mask itself as antagonist activity in the three assays.

The bradykinin analogs showed a range of inhibitory potency, as evidenced by their IC_{50} values, presented in Table 2. Des-Arg^9-bradykinin and des-Arg^9-Leu^8-bradykinin had no agonist activity in the three assays and had little or no antagonist activity. D-Phe^6-D-Ala^7-bradykinin was a weak antagonist in the three assays, but this may be due to its partial agonist activity (see Results). The crucial substitution that converted bradykinin into compounds that have no agonist activity and some antagonist activity was the replacement of the proline at position 7 with the amino acid D-phenylalanine. Antagonist

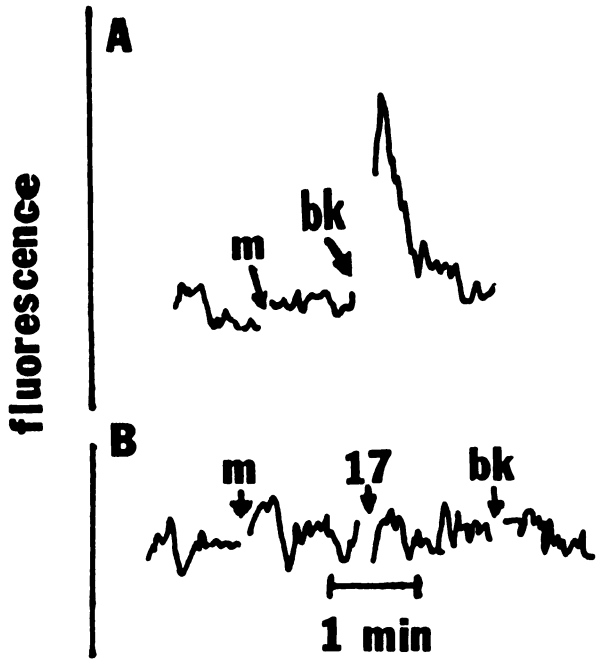

Fig. 2. Effect of bradykinin (A) and analog **17** plus bradykinin (B) upon intracellular calcium. Calcium is measured using the fluorescent dye fura-2 in F-11 neuroblastoma × DRG cells (see Materials and Methods). Cells are mixed, *m*, until a stable baseline is obtained. In A, baseline calcium is 220 nM and increases 2.4-fold in the presence of 10^{-8} M bradykinin (*bk*). B is similar to A except that 10^{-6} M analog **17** (*17*) is present 2 min before bradykinin is added.

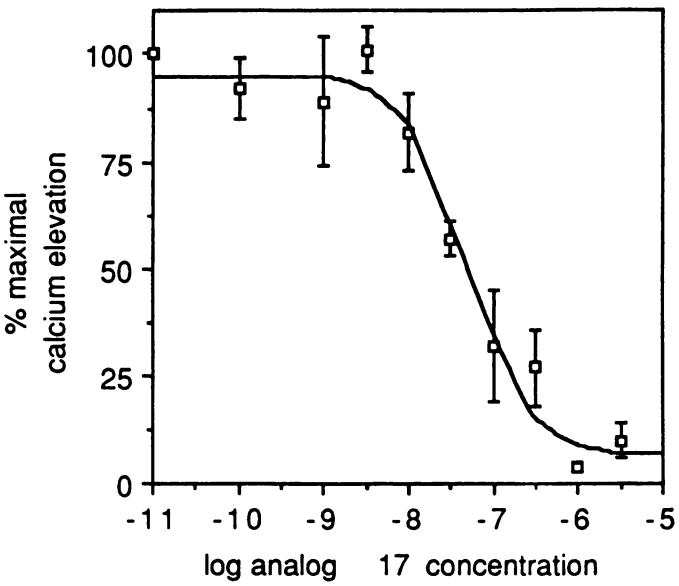

Fig. 3. Potency of inhibition of bradykinin-dependent calcium elevation by analog **17**. Calcium concentrations were determined as in Fig. 2 and are expressed as a percentage of maximal stimulation (10 nM bradykinin) relative to control. The concentration of inhibitor was varied from 100 pM to 10 μM. Values are the means of three experiments run in duplicate, with standard errors indicated by the *error bars*.

activity was increased further by the addition of D-arginine at position 0 or lysine at positions −1 and 0, by the substitution of the prolines at positions 2 and/or 3 with hydroxyproline, and by the substitution of the synthetic amino acid, β-2-thienyl-L-alanine at positions 5 and 8. In particular, analog **17** showed potent competitive inhibition at the bradykinin receptor. Although studies of the kind reported in this paper have not been

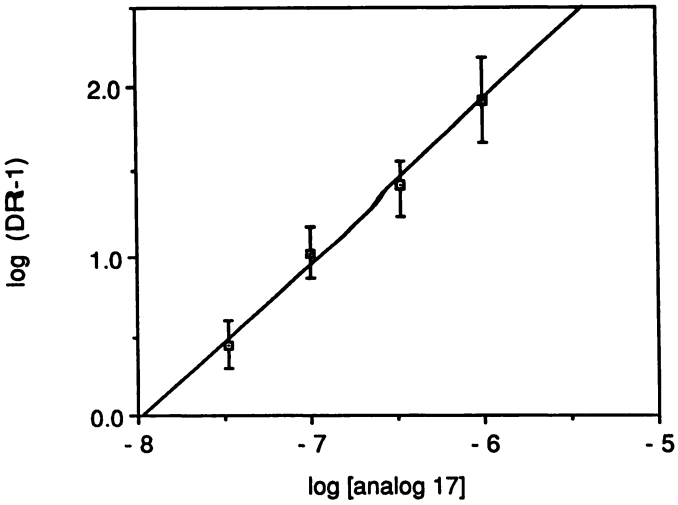

Fig. 4. Schild plot analysis of bradykinin-induced calcium production in the presence of analog **17**. *DR*, dose ratio, which is the EC_{50} for bradykinin in the presence of antagonist divided by the EC_{50} for bradykinin. The values in the plot were determined as previously defined in Arunlakshana *et al.* (17) and were pA_2 = 10.4 ± 4.6, slope = 0.97 ± 0.5, correlation coefficient = 0.992. Similar values were achieved in the IP_3 and cyclic GMP assays (see Results). The Results are the mean of four experiments done in triplicate, with standard error indicated by the *error bars*.

previously described, recently analogs **16** and **17** were found to be potent specific antagonists of bradykinin activity in both the guinea pig ileum and rat uterus smooth muscle assays and in the rat blood pressure kinin assay (10, 11), suggesting their physiological significances.

These results suggest that the bradykinin receptors present in the F-11 cells are similar to those present in ileal, uterine, and vascular smooth muscle, designated by Regoli and Barabe (1) as the B-2 receptor. Interestingly, the bradykinin receptors in F-11 cells show another property similar to the bradykinin receptors present in the various smooth muscle cell types, namely increased potency of bradykinin relative to Lys-bradykinin and des-Arg^9-bradykinin in activating target cells. Whether the bradykinin receptors in F-11 cells and smooth muscle cells are similar or identical, structurally as well as functionally, remains to be determined.

The Schild plot analysis (Fig. 4) of analog **17** in all three second messenger systems yielded pA_2 values in the nanomolar range. The slopes of the Schild plots were linear, as expected, with a correlation coefficient approximately 1.0 in all three second messenger systems. Interestingly, the bradykinin dose response for IP_3 release is shifted slightly to the right of that for calcium and cyclic GMP. The working hypothesis from our data is that a submaximal release of the 1,4,5-isomer of IP_3 is sufficient for maximal release of intracellular calcium and maximal cyclic GMP production.

In conclusion, we have found that a particular bradykinin analog, **17**, is a specific, competitive, high affinity (nanomolar range) antagonist of the bradykinin receptor in F-11 cells and possibly of neuronal bradykinin receptors *in vivo*. In addition, we observed that several other bradykinin analogs showed a range of inhibitory potency. These various analogs may prove useful not only in the precise pharmacological characterization of bradykinin receptors in different tissues but also in the clinical control of pain and inflammation.

Acknowledgments

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