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BK_1 and BK_2 bradykinin receptors in the rat duodenum smooth muscle

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1 The dual action of bradykinin (relaxation and contraction) on the rat duodenum was investigated by studying its effect on adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in cultured duodenal smooth muscle cells, and the effects of apamin on the isolated muscle responses to agonists and antagonists of BK_1 and BK_2 receptors.

2 No change was observed in the cyclic AMP content of cultured cells incubated with up to 100 nM bradykinin.

3 Apamin (100–500 nM) inhibited the relaxant component and enhanced the contractile component of the responses to bradykinin and to the BK_2-specific analogue [$Thi^{5,8}$,D-Phe^7]-bradykinin.

4 Apamin (100–500 nM) did not affect the contractile response of stretched duodenum preparations to the BK_1-specific agonist des-Arg^9-bradykinin.

5 The BK_2 antagonist, [D-$Arg^0Hyp^3Thi^{5,8}$,D-Phe^7]-bradykinin, at a concentration which completely inhibited the relaxant response to bradykinin and to [$Thi^{5,8}$,D-Phe^7]-bradykinin, also prevented the contraction in response to either agonist in the presence of apamin.

6 Our results demonstrate two populations of bradykinin receptors in rat duodenum: a BK_2 subtype responsible for the biphasic response of the non-stretched duodenum, and a BK_1 subtype responsible for the contractile effect on the stretched tissue.

Keywords: Apamin; bradykinin; potassium channels; rat duodenum; receptors; smooth muscle

Introduction

Previous studies demonstrated that bradykinin produces hyperpolarization followed by depolarization in guinea-pig taenia caeci smooth muscle (Den Hertog *et al.*, 1988) and in neuroblastoma cultured cells (Brown & Higashida, 1988). The hyperpolarization is due to activation of a Ca^{2+}-dependent K^+ current by an increase in intracellular Ca^{2+}, mediated by the release of inositol 1,4,5-trisphosphate (Brown & Higashida, 1988; Lang *et al.*, 1991). The subsequent depolarization is associated with a fall in membrane conductance, primarily due to the inhibition of a voltage-dependent K^+ current (Brown & Higashida, 1988). As a consequence of these changes in membrane conductance, the response of isolated smooth muscles to bradykinin may have a biphasic character, consisting of a relaxation followed by contraction. However, the relaxant component of the response is observed only in smooth muscle preparations that maintain a naturally high tonus in the resting state, such as the guinea-pig taenia caeci and the rat duodenum (Carter *et al.*, 1986).

In the case of the rat duodenum, bradykinin has a predominantly relaxant effect but under conditions that decrease its resting tonus, such as low Ca^{2+} concentration in the medium or exposure to stretching, a distinct contractile component is also evident (Antonio, 1968; Boschcov *et al.*, 1984). Under these conditions, bradykinin concentrations ranging from 0.1 to 10 nM induce only relaxation, whereas higher concentrations induce relaxation followed by contraction. Two subtypes of bradykinin BK_1 and BK_2 receptors were proposed to mediate, respectively, the contractile and the relaxant components of the biphasic response (Boschcov *et al.*, 1984; Paiva *et al.*, 1989). The BK_2 receptor subtype was characterized by a distinct behaviour towards two bradykinin analogues that were shown to be BK_2 antagonists in other systems: [$Thi^{5,8}$,D-Phe^7]-bradykinin and [D-$Arg^0Hyp^3Thi^{5,8}$,D-Phe^7]-bradykinin (Stewart & Vavrek, 1986; Schachter *et al.*, 1987). In the rat duodenum the former is an agonist and the latter an antagonist for the relaxant effect. Furthermore, Hall & Morton (1991) showed competitive antagonism of relaxant responses to bradykinin with another BK_2 receptor antagonist, Lys, Lys-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin, with a pK_B estimate similar to that reported in other BK_2 receptor tissues. As for the BK_1 receptor subtype responsible for the contractile response of the duodenum, it was characterized (Paiva *et al.*, 1989) as being activated by both des-Arg^9-bradykinin (which is a BK_1 agonist in other systems) and des-Arg^9-[Leu^8]-bradykinin (which is a BK_1 antagonist in other systems) (Regoli & Barabe, 1980; Regoli *et al.*, 1986).

Two different mechanisms have been proposed for the relaxant response in the rat duodenum. The finding that apamin, a toxin that specifically blocks Ca^{2+}-dependent K^+ channels (Hugues *et al.*, 1982), inhibited both the relaxation and the opening of K^+ channels evoked by bradykinin in smooth muscle preparations indicates that the relaxation is caused by hyperpolarization due to activation of these channels (Hall & Morton, 1991). However, Liebmann *et al.* (1987) detected increased adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in duodenum strips treated with bradykinin and proposed that the relaxant response is due to stimulation of adenylyl cyclase activity.

The aim of this work was to investigate further the mechanism of the dual action of bradykinin (relaxation and contraction) on the rat duodenum by studying its effect on the cyclic AMP levels in cultured duodenal smooth muscle cells, and by examining the effects of apamin on the responses to agonists or antagonists of BK_1 and BK_2 receptors in the stretched and non-stretched rat duodenum.

Methods

Cyclic AMP determination

Duodenal smooth muscle cells were obtained from Wistar rats by enzymic dispersion and primary cultures were grown

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as previously described (Shimuta et al., 1990). The culture medium of confluent cells (10^6 cells) was removed and the cells were rinsed at 37°C with assay buffer (composition, mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.49, NaH_2PO_4 0.36, $NaHCO_3$ 11.9 and glucose 5.0) and then 1 ml of the assay buffer was added in the presence or in the absence of 1.0 mM theophylline. After equilibration for 30 min, the cells were incubated for the desired times with various test reagents. The reaction was terminated by aspirating off the assay medium and the cells in culture dishes were immediately frozen in an acetone-dry ice bath. The cyclic AMP formed was extracted with 0.3% ice-cold perchloric acid and the extract was neutralized with 30% $KHCO_3$. The cyclic AMP in the extract was measured by radioimmunossay, as described by Gilman (1970) and Tovey et al. (1974).

Isolated duodenum preparation

Wistar rats of either sex, weighing between 190 and 220 g, were killed by a blow to the head. After bleeding, the abdomen was opened and the duodenum removed. The preparation was suspended in a 5 ml chamber containing Tyrode solution kept at 37°C and bubbled with a mixture of CO_2 (5%) and O_2 (95%). The composition of the Tyrode solution was (mM): NaCl 137, KCl 12.7, $CaCl_2$ 1.36, $MgCl_2$ 0.49, NaH_2PO_4 0.36, $NaHCO_3$ 11.9, glucose 5.0. Isotonic recordings were made, under 1 g load, on smoked drums with frontal levers having a 6 fold amplification, after a 60 min equilibration period.

The concentration-response curves to bradykinin were obtained within the first 90 min after the end of the equilibration period. The drugs, in volumes not exceeding 0.2 ml, were added directly to the organ bath and the preparation was washed after 90 s contact. The interval between additions was 15 min for the lower bradykinin concentrations (which produced only relaxation), and 30 min when higher concentrations were used to elicit contractile responses (to avoid the tachyphylaxis observed for this component of the response). The relaxant component of the response was measured from the baseline to the lowest point, and the contractile component from the baseline to the peak of the recorded response. The dose-response curves were analyzed by linear regression of their double reciprocal plots. Maximum responses and EC_{50} values were estimated from the ordinate intercept and slope of the straight lines obtained. For the experiments with des-Arg^9-bradykinin, the duodenum preparations were left under 1 g load for 4 h before the responses were recorded. This stretching of the preparation is important to enhance the contraction produced by that analogue (Boschcov et al., 1984).

Materials

The peptides used in this study were synthetic products made in this laboratory, with the exception of [Thi^{5,8},D-Phe^7]-bradykinin and [D-Arg^{0}Hyp^{3}Thi^{5,8}D-Phe^7]-bradykinin, which were kind gifts from Prof. J.M. Stewart and Dr R. Vavrek. The inorganic salts were products of the highest analytical grade from Merck Darmstadt. Apamin, phorbol-12,13-dibutyrate (PDBu) and isoprenaline were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The [^{3}H]-cyclic AMP assay kit was obtained from Diagnostic Products Corporation, Los Angeles, CA, U.S.A. Culture medium, supplements and foetal bovine serum were obtained from Gibco, N.Y., U.S.A.

All tracings presented in the figures are representative of at least 4 independent experiments and data are expressed as means ± standard errors and were analyzed by Student's t test.

Results

Cyclic AMP levels in cultured duodenal smooth muscle cells after bradykinin and isoprenaline stimulation

Liebmann et al. (1987), based on their finding of an increased cyclic AMP concentration in bradykinin-treated rat duodenum strips, proposed that this could be the mechanism of the relaxation produced by bradykinin in that tissue. However, these authors found that the bradykinin-evoked cyclic AMP production was increased in calcium-free medium, contrasting with the inhibition of the duodenum relaxation in this condition (Antonio, 1968). Since the results obtained with duodenum strips might be affected by the presence of the mucosa, we determined the effect of bradykinin on the cyclic AMP content of cultured duodenal smooth muscle cells.

Bradykinin, at a concentration which caused maximum relaxation in the rat isolated duodenum (10 nM), was added to the cultured cells, and the cyclic AMP content of the cells was determined after incubation times corresponding to the maximum amplitude of the relaxation observed in the tissue (30 s) and to the full return to the basal tonus (2 min). Figure 1a shows that no changes were observed in the cyclic AMP content with either incubation time. No changes in cyclic AMP content were also found when the cells were incubated for 2 min with a higher bradykinin concentration (100 nM, not shown), which in the isolated organ caused a biphasic response (relaxation followed by contraction).

As a control for the responsiveness of the cells to agents evoking cyclic AMP increases, we determined the effect of the β-adrenoceptor agonist, isoprenaline (2 μM), which caused a significant time-dependent increase in the level of cyclic AMP (Figure 1b). This increase was also observed in the presence of the phosphodiesterase inhibitor, theophylline (1 mM, not shown).

Since phorbol esters enhance agonist-induced cyclic AMP accumulation in other cells (Nabika et al., 1985), we determined the effect of bradykinin and isoprenaline on cyclic AMP levels of cells treated with 100 nM phorbol dibutyrate for 10 min. A significant increase in cyclic AMP accumu-

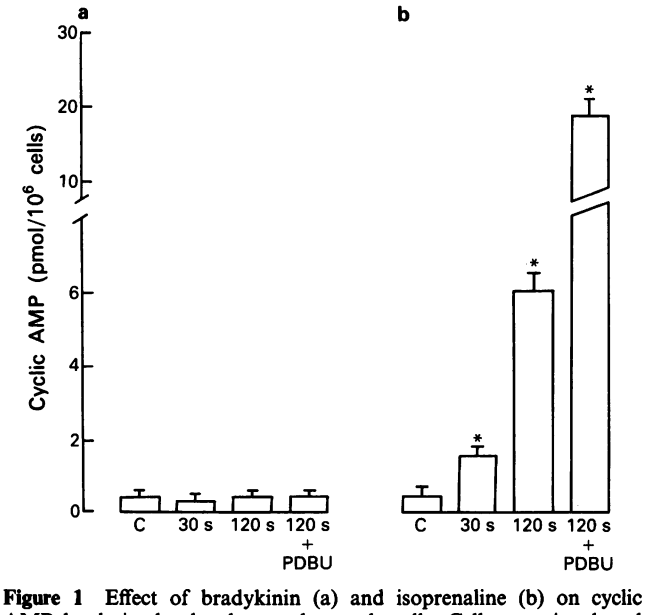

Figure 1 Effect of bradykinin (a) and isoprenaline (b) on cyclic AMP levels in duodenal smooth muscle cells. Cells were incubated with 10 nM bradykinin or 2 μM isoprenaline for 30 s or 120 s, in the presence or in the absence of 100 nM phorbol dibutyrate (PDBU) for 10 min. The values are mean (± s.e.mean, vertical bars) of four experiments done in triplicate. *Significantly different ($P<0.05$) from the respective controls.

lation was observed in the cultured cells incubated with isoprenaline, but not with bradykinin, either at 10 nM (Figure 1) or 100 nM (not shown).

Effect of apamin on the responses to bradykinin and analogue peptides

The relaxant effect of a low concentration of bradykinin (1 nM) on the rat duodenum was shown to be blocked by apamin (Hall & Morton, 1991). We have extended this observation to include higher bradykinin concentrations, in which the biphasic response becomes evident, in order to determine the effect of apamin on the contractile component of the response. In agreement with Hall & Morton's (1991) finding, we found that 100 nM apamin completely abolished the relaxation elicited by 1 nM bradykinin but at agonist concentrations of 10 nM or higher, the inhibition consisted mainly of a reduction of the amplitude and duration of the relaxation. This effect was dependent on the apamin concentration, being most pronounced at the highest concentration used, i.e. 500 nM (Figure 2). At this apamin concentration, the relaxation elicited by 100 nM bradykinin had its amplitude reduced from 31 ± 6 mm to 18 ± 3 mm and its duration reduced from 60 ± 4 s to 8 ± 1 s ($P<0.001$). The inhibition of the relaxation was accompanied by an increase of the concentration-dependent contractile component, the pD_2 for which was significantly greater (7.2 ± 0.05, $n = 8$) in the presence of 500 nM apamin than in its absence (6.6 ± 0.08, $n = 12$).

The response to the BK_1-specific agonist, des-Arg^9-bradykinin, which in the stretched rat duodenum causes only a contraction (Paiva *et al.*, 1989), was not affected by apamin in the concentration-rage, 100–500 nM (Figure 3). In contrast, the response to a maximally effective concentration (800 nM) of the BK_2-specific analogue, [Thi^{5,8},D-Phe^7]-bradykinin, which in the non-stretched rat duodenum produces only relaxation, was affected by apamin in a very similar way to that observed in the case of bradykinin (Figure 4): the relaxation was inhibited and a contractile component appeared which is absent in the normal response to that analogue.

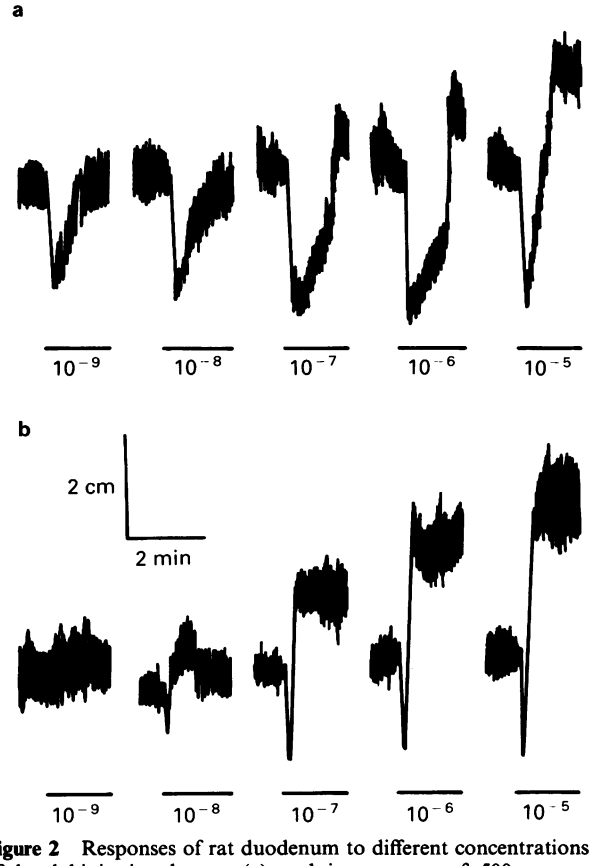

Figure 2 Responses of rat duodenum to different concentrations (M) of bradykinin in absence (a) and in presence of 500 nM apamin added 5 min before the addition of bradykinin (b). Each treatment with bradykinin lasted for 90 s (horizontal bars) and was followed by washing and a rest period (indicated by the interruptions in the tracings). The interval between bradykinin additions was 15 min for the concentrations 1 and 10 nM and 30 min for the higher doses. These results are representative of those obtained in 8 experiments.

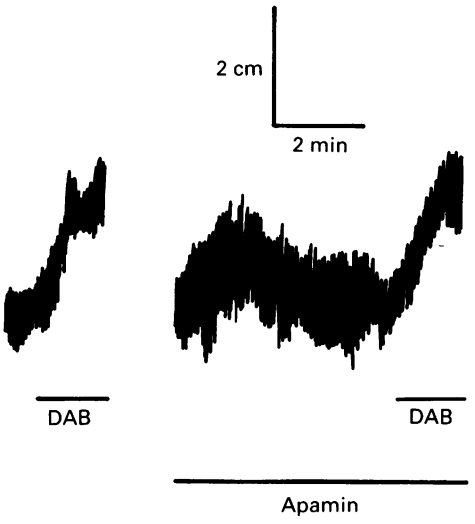

Figure 3 Responses to 20 nM des-Arg^9-bradykinin (DAB) in the absence and in the presence of 500 nM apamin, added 5 min before the addition of the agonist. The duodenum preparation was equilibrated for 4 h under 1 g load before the experiment. The tracing shown is representative of 4 experiments.

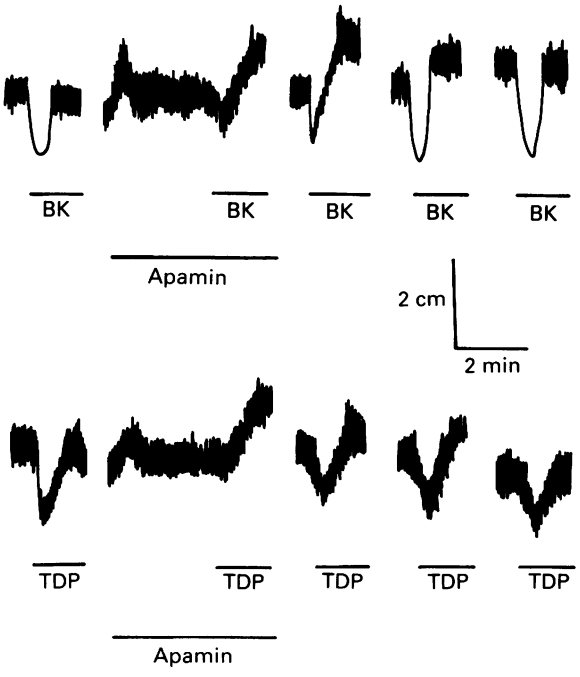

Figure 4 Effect of apamin (500 nM) on the relaxant responses (a) to 2 nM bradykinin (BK) and (b) to 800 nM BK_2 agonist [Thi^{5,8},D-Phe^7]-bradykinin (TDP). The interval between successive additions of the agonist was 30 min and the preparation was washed 90 s after each addition. Apamin was added (both panels) 3 min before the next addition of the agonists. These results are representative of 6 experiments.

After removal of apamin, the normal responses to new additions of bradykinin and to [Thi[5,8],D-Phe[7]]-bradykinin slowly recovered, the amplitude and duration of the relaxant component gradually increasing, and the contractile component disappearing after approx. 90 min (Figure 4).

In order to determine whether the enhancement of the contractile responses by apamin is a consequence of the inhibition of the relaxant component of the response, we studied the effect of apamin when the responses to bradykinin and [Thi[5,8],D-Phe[7]]-bradykinin were inhibited by an antangonist of the BK_2 receptor. It was previously shown that the specific BK_2 antagonist, [D-Arg[0]Hyp[3]Thi[5,8],D-Phe[7]]-bradykinin, inhibits the relaxant but not the contractile component of the response of the duodenum to bradykinin (Pereira & Paiva, 1989). Figure 5 shows that this antagonist, at a concentration (150 nM) which completely inhibited the relaxant response to bradykinin and to [Thi[5,8],D-Phe[7]]-bradykinin, also prevented the contraction in response to either agonist in the presence of apamin. After washout of the preparation, new additions of either of the two agonists yielded responses consisting of a contractile component that gradually disappeared during the 1 h recovery period. This indicates that, whereas [Thi[5,8],D-Phe[7]]-bradykinin is quickly washed out, the effect of apamin is only slowly reversed, as also seen in the experiment depicted in Figure 4.

Discussion

Since the classification of bradykinin receptors into BK_1 and BK_2 types (Regoli & Barabé, 1980), the great majority of the responses to that peptide have been attributed to BK_2 receptors, BK_1 receptors having been found in few tissues and under special conditions. An interesting system is the rat duodenum, in which the two components of the biphasic response were attributed to subtypes of the BK_1 and BK_2 receptors (Paiva *et al.*, 1989).

The relaxant component of the response of the duodenum to bradykinin has been ascribed to two different mechanisms. Whereas Liebmann *et al.* (1987) postulate the mediation by adenylyl cyclase stimulation, based on their finding of increased cyclic AMP in duodenum strips treated with bradykinin, Hall & Morton (1991) interpret the inhibition of the relaxation by apamin as evidence that it is due to activation of Ca^{2+}-dependent K^+ channels, as also postulated for the taenia caeci (Carter *et al.*, 1986; Den Hertog *et al.*, 1988).

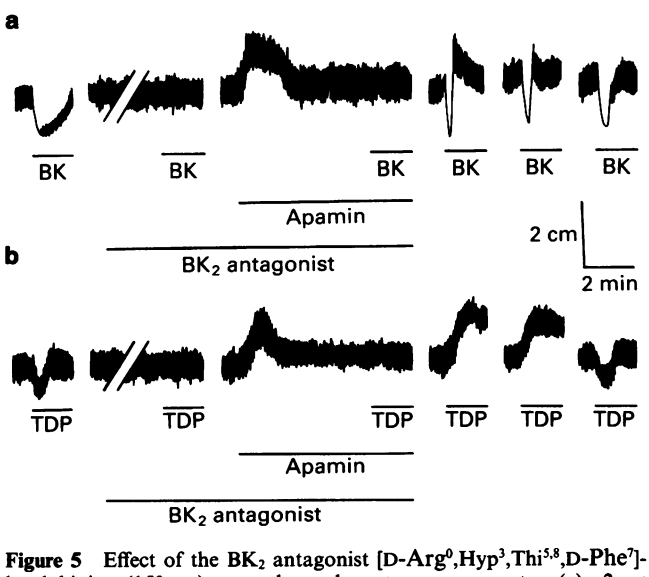

Figure 5 Effect of the BK_2 antagonist [D-Arg[0],Hyp[3],Thi[5,8],D-Phe[7]]-bradykinin (150 nM) on the relaxant responses to (a) 2 nM bradykinin (BK), and (b) 800 nM [Thi[5,8],D-Phe[7]]-bradykinin (TDP) in the absence and in the presence of 500 nM apamin. The BK_2 antagonist and the apamin were added 10 and 5 min respectively before the next addition of the agonists. The intervals between additions were 30 min, and the preparation was washed 90 s after each addition. These results are representative of 6 experiments.

In duodenal cultured smooth muscle cells, we did not detect cyclic AMP accumulation in response to bradykinin concentrations above those known to cause maximum relaxant responses of the duodenum. An increase in cyclic AMP levels was not observed even in the presence of phorbol ester, under conditions which enhance agonist-induced cyclic AMP accumulation in other tissues, including vascular smooth muscle cells (Nabika *et al.*, 1985). The difference between our results and those of Liebmann *et al.* (1987) may be due to the fact that we used only smooth muscle cells, whereas these authors used whole duodenum strips, where cells other than smooth muscle are present. The mucosa was shown to accumulate cyclic AMP under other stimuli (Karlstrom, 1986), and the presence of these cells might influence the results obtained with the strips.

Our results are in agreement with those of Hall & Morton (1991), who found that the relaxant effect induced by 1 nM bradykinin in the rat isolated duodenum was totally inhibited by apamin. In addition, we further observed that, in the presence of higher bradykinin concentrations (but within a range where only relaxant responses are normally observed), apamin significantly modified these responses: the relaxant component was present, but with smaller amplitude and very short duration, and a dose-dependent contraction became evident, with a pD_2 value (7.2 ± 0.05) significantly higher than that seen in the absence of apamin (6.6 ± 0.05).

These results support the conclusion that the relaxant response of the duodenum to bradykinin is due to the activation of Ca^{2+}-dependent K^+ channels and they further indicate that the biphasic response reflects a balance between hyperpolarization (relaxation) and depolarization (contraction). The appearance of the contractile component at low bradykinin concentrations in the presence of apamin would, therefore, result from the inhibition of hyperpolarization, allowing the predominance of the contractile effect due to membrane depolarization. A similar balance between the two components of the biphasic response of the duodenum was described in spontaneously hypertensive rats (Miasiro *et al.*, 1985), in which the predominantly contractile response was ascribed to a reduced relaxant component, probably because low calmodulin levels impair Ca^{2+}-dependent K^+ channels in these animals (Feres *et al.*, 1992).

The finding that the contractile component of the response may be present at low bradykinin concentrations, being masked by the relaxant component, and that this also occurs with [Thi[5,8],D-Phe[7]]-bradykinin, previously thought to be a pure BK_2 relaxant agonist in the rat duodenum (Paiva *et al.*, 1989), suggests a revision of the proposal that different receptor subtypes are responsible for the two components of the response of the non-stretched duodenum. This is also suggested by the finding that BK_2 antagonist [D-Arg[0],Hyp[3],Thi[5,8],D-Phe[7]]-bradykinin, which was previously shown to inhibit the relaxation, also inhibits the contractions induced by bradykinin and [Thi[5,8],D-Phe[7]]-bradykinin in the presence of apamin. It is possible that stimulation of the same population of BK_2 receptors by bradykinin could activate the phosphoinositide pathway, as has been shown in other tissues, such as the guinea-pig ileum (Ransom *et al.*, 1992). This could give rise to a dual effect, as proposed for other systems (Brown & Higashida, 1988): mobilization of calcium ions through inositol trisphosphate could produce hyperpolarization through Ca^{2+}-dependent K^+ channels, and diacylglycerol could cause depolarization through inhibition of voltage-dependent K^+ channels.

Our finding that the contractile response of the stretched duodenum to des-Arg[9]-bradykinin is not affected by apamin is in line with previous results showing that this response is due to activation of a subtype of BK_1 receptor that is distinct from the BK_2 subtype involved in the biphasic response of the non-stretched tissue.

In conclusion, our results, indicate that the rat duodenum

possesses two distinct populations of bradykinin receptors: (1) a BK_2 subtype, which is activated by [Thi^{5,8},D-Phe^7]-bradykinin and inhibited by [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, and is responsible for the dual effect resulting in the biphasic response of the non-stretched duodenum; and (2) a BK_1 subtype, activated by both des-Arg^9-bradykinin and des-Arg^9-[Leu^8]-bradykinin, which is responsible for the contractile response of the stretched duodenum.

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