Mechanisms of bradykinin-induced contraction of the guinea-pig gallbladder *in vitro*

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1 The mechanisms underlying bradykinin (BK)-mediated contractions in strips of guinea-pig gallbladder (GPG) were examined by use of selective bradykinin (BK) receptor agonists and antagonists.

2 Addition of BK and related kinins (0.1 pM–10 µM) after 2 h of equilibration of the preparation caused graded contractions characterized by two distinct phases: high affinity (0.1 pM–1 nM) and low affinity (3 nM–10 µM). The rank order of potency for the first phase (mean EC₅₀, pM) was: BK (1.36) = Hyp³-BK (1.44) = Lys-BK (1.54) > Tyr⁸-BK (2.72) > Met-Lys-BK (4.30). The rank order of potency for the second phase (mean EC₅₀, nM, at concentration producing 50% of the contraction caused by 80 mM KCl) was: Hyp³-BK (8.95) > Met-Lys-BK (12.78) > Tyr⁸-BK (33.75) > Lys-BK (60.92) > BK (77.35). The contractile responses (g of tension) to 3 µM of BK (the highest concentration tested) were: Hyp³-BK, 1.76 ± 0.09; BK, 1.65 ± 0.12; Lys-BK, 1.45 ± 0.13; Tyr⁸-BK, 1.36 ± 0.15 and Met-Lys-BK, 1.36 ± 0.15. The selective B₁ agonist, des-Arg⁹-BK, caused only a weak contraction with maximal response (0.21 ± 0.05 g), which corresponded to approximately 10% of that induced by BK.

3 BK-induced contraction in GPG was inhibited by indomethacin (3 µM) or ibuprofen (30 µM), and was partially reduced by phenidone (30 µM), but was not affected by atropine (1 µM), nicardipine (1 µM), Ca²⁺-free medium plus EGTA, dazoxiben (30 nM), L-655,240 (10 nM, a selective receptor antagonist of thromboxane A₂), MK-571 (0.1 µM, a selective leukotriene D₄ receptor antagonist), tetrodotoxin (0.3 µM), CP 96,345 (0.3 µM, a NK₁ receptor antagonist), mepyramine (1 µM), glibenclamide (1 µM), H-7 (3 µM), staurosporine (100 nM), or phorbol 12-myristate 13-acetate (1 µM). However, BK-induced contractions in GPG maintained in Ca²⁺-free medium were markedly attenuated by ryanodine (10 µM).

4 Prostaglandin E₂, prostaglandin F_{2α} or U46619 (0.1 nM to 100 µM), caused concentration-dependent contractions in GPG with mean EC₅₀s of 3.1 µM; 1.7 µM and 0.47 nM and maximal responses of 1.36 ± 0.15; 1.32 ± 0.20 and 0.96 ± 0.09 g, respectively.

5 The selective B₂ receptor antagonists, Hoe 140, NPC 17731 and NPC 17761 (0.01–1 µM), caused concentration-dependent displacements to the right of the contractile concentration-response curve for BK. The selective B₁ receptor antagonist, des-Arg⁹-[Leu⁸]-BK (1 µM), did not affect BK-induced GPG contraction.

6 These data suggest that both high and low affinity BK responses in GPG are mediated by activation of B₂ receptors, and that BK-mediated contraction in GPG depends on the release of intracellular Ca²⁺ sources sensitive to ryanodine. In addition, BK-induced contraction in GPG is mediated by release of proinflammatory eicosanoid(s) derived from the cyclo-oxygenase pathway from arachidonic acid metabolism unrelated to thromboxane A₂, and seems not to be coupled to activation of a protein kinase C-dependent mechanism.

Keywords: Guinea-pig gallbladder; bradykinin; kinin; B₁ and B₂ bradykinin agonists and antagonists; prostanoids; calcium

Introduction

Bradykinin (BK) is a nonapeptide generated in plasma by the action of plasma and glandular kallikreins on both high and low molecular weight kininogens (Regoli & Barabé, 1980; Bhoola *et al.*, 1992). Kinins are widely recognized as participants in many physiological and pathological states. Thus, kinins are known to promote contraction or relaxation responses in vascular or non-vascular smooth muscles. Although most BK responses are indirectly mediated by release of eicosanoid products derived from the arachidonic acid pathway (Gaginella & Kachur, 1989; Hall, 1992), studies on the structure-activity relationship of several analogues, and the use of potent and selective BK receptor antagonists, have led to the characterization of two classes of BK membrane receptors, denoted as B₁ and B₂ (Regoli & Barabé, 1980; Burch *et al.*, 1990; Marceau & Regoli, 1991; Bhoola *et al.*, 1992). The B₁ receptors exhibit higher affinity for kinin metabolites, des-Arg⁹-BK and des-Arg¹⁰-kallidin (Lys-BK), and are selectively and competitively antagonized by the B₁ receptor antagonists, des-Arg⁹-[Leu⁸]-BK or des-Arg¹⁰-[Leu⁸]-

LBK. However, most of the physiological and pathological actions of BK are mediated by activation of B₂ receptors which seem to be widely distributed throughout the peripheral and central nervous system. In contrast to B₁ receptors, B₂ receptors exhibit high affinity for BK and kallidin (Lys-bradykinin), but a low affinity for the B₁ agonist des-Arg⁹-BK (Regoli & Barabé, 1980; Burch *et al.*, 1990; Bathon & Proud, 1991).

In the present study we have attempted to investigate the mechanisms underlying BK-mediated contractile responses in the guinea-pig gallbladder (GPG) *in vitro* by using kinin-selective receptor agonists and antagonists. Some of these results were presented at the Meeting of KININ 93 (Silva *et al.*, 1993).

Methods

Tissue preparation

Guinea-pigs of either sex (300–400 g) were anaesthetized with ether and killed with a blow on the neck followed by

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cervical dislocation. Usually, 4 strips of each guinea-pig gallbladder (10–15 mm long, 3–4 mm wide) were isolated and each preparation was set up in a 5 ml glass organ bath containing Krebs-Henseleit solution of the following composition (mM): NaCl 118.0, KCl 4.4, MgSO₄ 1.1, CaCl₂ 2.5, NaHCO₃ 25.0, KH₂PO₄ 1.2 and glucose 11.0, maintained at 37°C and pH of 7.4, gassed with 95% O₂ and 5% CO₂. Isometric tension changes were recorded by means of an F-60 force transducer (Narco-Biosystem or Letica 6006) under a continuous basal tension of 0.5 g. Preparations were allowed to equilibrate for at least 2 h before drug addition, during which the bath solution was changed every 20 min. Usually, four to six baths were used in parallel.

Concentration-response curves (CRCs) for bradykinin and related peptides

Following the equilibration period of 2 h, and in order to confirm the viability of the tissues, preparations were exposed to high potassium concentration (KCl 80 mM, prepared by equimolar substitution of 74.4 mM NaCl by KCl) as a standard stimulus. After washout, replacement with normal medium and return to the original baseline (about 30 min), CRCs were obtained for BK and related peptides. The CRCs for all studied agonists were performed by means of the cumulative method (Van Rossum, 1963). Each concentration of the agonist was added when the effect of the preceding addition had reached its maximum. To ascertain whether BK-induced contraction in GPG underwent desensitization, in preliminary experiments ($n = 5$), several CRCs for BK (1 pM to 10 μ M) were carried out on the same preparation at 60 min intervals between curves, following 30, 60 or 120 min of equilibration period. No significant desensitization was observed for at least two consecutive CRCs for BK, when experiments were performed 120 min after setting up the preparation. Accordingly, no more than two CRCs for BK and related peptides were carried out on the same strip. Each preparation was exposed to only one agonist. All experiments with BK and analogues were carried out in the presence of captopril (3 μ M), to prevent the action of kininase II.

Influence of calcium on bradykinin-induced contraction

To confirm the possible involvement of extracellular Ca²⁺ in BK-induced contractile responses to GPG, after 2 h of equilibration, preparations were transferred to Krebs solution without Ca²⁺, containing 1 mM EGTA, for 20 min, during which the bath solution was renewed every 5 min. The tissues then remained in Ca²⁺-free solution plus EGTA for 10 min, and responses to BK were subsequently obtained (Calixto & Medeiros, 1991). In another set of experiments, GPG preparations were allowed to equilibrate for 2 h in normal medium and a CRC was obtained for BK (0.1 pM–10 μ M) either in the absence or presence of nifedipine (1 μ M, incubated 20 min beforehand). After obtaining a CRC for BK in Ca²⁺-free medium, some preparations were incubated for 20 min with ryanodine (10 μ M, a neutral alkaloid which affects either opening or closing of the sarcoplasmic reticulum Ca²⁺ channels) (Jenden & Fairbairn, 1969; Meissner, 1986), and another CRC for BK was subsequently obtained in Ca²⁺-free Krebs solution.

Effect of prostanoid and protein kinase C in bradykinin-induced contraction

To assess whether prostanoids were involved in the contractile response elicited by BK, following 2 h equilibration, CRCs were obtained for BK (0.1 pM–10 μ M) in the absence or in the presence of the following drugs added 20 min before addition of BK: indomethacin (3 μ M), ibuprofen (30 μ M) (both cyclo-oxygenase inhibitors), phenidone (30 μ M, a lipoxygenase inhibitor), dazoxiben (30 nM, an inhibitor of thromboxane A₂ (TxA₂)-synthase), or MK-571 (100 nM, a

leukotriene D₄ selective receptor antagonist). In separate experiments, CRCs for PGE₂ (0.1 nM–100 μ M), PGF_{2 α} (0.1 nM–100 μ M) or for TxA₂, prostaglandin H₂-mimetic U46619 (0.1 nM–1 μ M) were also carried out by means of the cumulative method. To avoid desensitization, only one CRC was obtained in each preparation.

Another three sets of experiments were designed to assess the possible role of protein kinase C-dependent mechanisms in mediating responses to BK in GPG, by analysing the effects of staurosporine (0.1 μ M), H-7 (3 μ M) (two protein kinase C antagonists) or phorbol 12-myristate 13-acetate (1 μ M, an activator of protein kinase C). Drugs were added to the preparations 30 min before construction of the CRC for BK, except for phorbol 12-myristate 13-acetate which was added 60 min prior to BK.

Effect of B₁ and B₂ bradykinin receptor antagonists on bradykinin-induced contraction

Following 2 h of equilibration, CRCs were obtained for BK (0.1 pM–10 μ M), in the absence or presence of Hoe-140, NPC 17761 or NPC 17731 (three selective B₂ receptor antagonists, 0.01–1 μ M), or for the selective B₁ receptor antagonist, des-Arg⁹-[Leu⁸]-BK (1 μ M). All antagonists were added to the preparations for at least 10 min before challenge with the agonist. In order to achieve the best period of equilibration for antagonist with the receptor, we evaluated the effect of different preincubation periods (e.g. 5 to 30 min). Longer periods of incubation with the antagonist (up to 30 min) did not afford further inhibition compared to that achieved after 10 min (data not shown). Thus, a 10 min antagonist preincubation period was used in the present study. Control experiments for BK were always carried out in parallel in the presence of phosphate-buffered solution (PBS), (concentration: NaCl 137 mM; KCl 2.7 mM, and phosphate buffer 10 mM), in order to correct for any time-dependent changes in responsiveness to BK.

To evaluate further the mechanism of action of BK, following 2 h of equilibration, single or cumulative CRCs for BK were obtained in the absence or in the presence of each of the following drugs, added to the bath 10 to 30 min prior to BK: mepyramine (1 μ M, an H₁-histamine receptor antagonist); atropine (1 μ M, a muscarinic receptor antagonist); prazosin (0.1 μ M, α_1 -adrenoceptor-blocker); tetrodotoxin (0.3 μ M, a Na⁺ channel blocker); glibenclamide (1.0 μ M, an ATP-sensitive K⁺-channel blocker), CP 96,345 (0.3 μ M, an NK₁ tachykinin receptor antagonist) or WEB 2086 (100 nM, a selective PAF receptor antagonist).

Statistical analysis

Most data are presented as mean $\pm$ s.e.mean. The EC₅₀ values are given as geometric means accompanied by their respective 95% confidence limits. Tests for statistical significance were performed either by using Student's paired or unpaired *t* test or by analysis of variance followed by Dunnett's multicomparison test when appropriate. $P < 0.05$ or less was considered as indicative of significance. The corresponding tests used are indicated below each figure or table. The contractile response for kinin and other agonists are expressed in absolute tension (g) developed per preparation.

The apparent EC₅₀ values for the second phase of BK and related kinin-induced responses, i.e. the concentration of agonists required to elicit 50% response relative to that caused by 80 mM KCl, were determined from individual experiments from agonist CRCs using the least-squares methods (Guimarães *et al.*, 1992). The EC₅₀s for the first phase were calculated as a function of their own respective maximal response. The contractile responses induced by kinins (10 μ M), the highest concentration used, were also compared.

Drugs

Drugs used were: phorbol 12-myristate 13-acetate, H-7 (5 isoquinoliny sulphonyl-2 methyl piperazine), staurosporine, Hyp³-BK, BK, Lys-BK, Tyr⁸-BK, Met-Lys-BK, des-Arg⁹-BK, captopril, indomethacin, ibuprofen, phenidone, nicardipine, tetradotoxin, mepyrmine hydrochloride, atropine sulphate, prostaglandin E₂ and prostaglandin F_{2α} (all from Sigma Chemical Company, St. Louis, MO, U.S.A.), prazosin (Pfizer, New York, U.S.A.), glibenclamide (Hoechst, Somerville, NJ, U.S.A.), MK-571 (3-(3-(2-(7-chloro-2-quinolinyl) ethenyl)phenyl ((3-dimethyl amino-3-oxo-propyl)thio)methyl) propanoic acid; kindly provided by Merck Frosst, Canada), Hoe 140 (D-Arg⁰-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK; kindly supplied by Hoechst, Frankfurt, Main, Germany), NPC 17731 (D-Arg⁰ [Hyp³, D-HypE (transpropyl)⁷, Oic⁸]-BK) and NPC 17761 (D-Arg⁰ [Hyp³, D-HypE (trans thiophenyl)⁷, Oic⁸]-BK) (kindly supplied by Scios Nova, Baltimore, U.S.A.). WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4] triazolo-4, 3-a) (1, 4]-diazepin-2-yl)-1-(4-morpholinyl)-1-propanon) (Boehringer Ingelheim, Germany), U46619 (9,11-dideoxy-9,2, 11α-methanoepoxy prostaglandin F_{2α}, UpJohn Co, Kalamazoo, MI, U.S.A.), ryanodine and CP 96,345 ((25,35)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2,2,2]octan-3-amine) (Research Biochemicals Incorporated RBI, Natick, MA, U.S.A.), des-Arg⁹-[Leu⁸]-BK and L-655,240 (3-[1-(4-chlorobenzyl)-5-fluoro-3-methylindol-2yl]2,2-dimethylpropanoic acid) (kind gifts from Dr P. D'Orleans-Juste and Dr Pierre Sirois, Department of Pharmacology, University of Sherbrooke, Sherbrooke, Canada), dazoxiben (Pfizer, Kent, UK). The stock solution for all peptides used was prepared in PBS (1–10 mM) and kept in siliconized plastic tubes, maintained in a freezer at –18°C. Stock solutions of prazosin, indomethacin, nicardipine, glibenclamide, phorbol 12-myristate 13-acetate, staurosporine, L-655,240 and U46619 were made in absolute ethanol. All other drugs were dissolved in PBS just before use at the desired concentration. The final bath concentration of ethanol did not exceed 0.05% and did not affect the CRC for BK.

Results

Concentration-response relationships of bradykinin and related peptides

The results in Figure 1 show that BK (0.1 pM–10 μM) caused a concentration-dependent contractile response in GPG characterized by two distinct phases: the first phase, plateauing at 1 nM, and a second phase above 10 μM. The results in Figure 1a also show the CRCs for BK (0.1 pM–10 μM) in GPG performed at 2, 3, 4 and 5 h after the equilibration period. As may be observed, the first phase of the contractile response elicited by BK increased significantly when preparations remained 4 h in equilibration. As shown in Figure 1b, all the kinins that were studied (0.1 pM–10 μM) when added to GPG after 2 h of equilibration caused reproducible and concentration-dependent contractile responses. The relative rank order of potency for the first phase was: BK = Hyp³-BK = Lys-BK > Tyr⁸-BK > Met-Lys-BK, and for the second phase was: Hyp³-BK > Met-Lys-BK > Tyr⁸-BK > Lys-BK > BK. The contractions (g of tension) caused by 10 μM of these peptides (the maximal tested concentration) in GPG were also similar (Table 1). The BK-mediated contraction of GPG appears to be mediated by the B₂ receptor subtype, because des-Arg⁹-BK, a selective B₁ agonist, up to 1 μM, caused only a very weak contraction in this preparation (0.21 ± 0.05 g) which corresponds approximately to 10% of that caused by BK (Figure 1b).

Influence of extra- and intracellular calcium on bradykinin-mediated contraction

Preincubation of strips of GPG with nicardipine 20 min beforehand only partially attenuated the first (but not the

second) phase of the contractile response elicited for BK ($P < 0.05$, unpaired *t* test) (Figure 2a). The contractile response to BK of GPG was not significantly affected when the experiments were performed in Ca²⁺-free medium containing EGTA (1 mM) (Figure 2b). In contrast, pre-incubation of GPG maintained in Ca²⁺-free medium plus ryanodine (10 μM) markedly attenuated both phases of the BK-induced contraction (Figure 2c).

Influence of protein kinase C inhibitors and activator

Pre-incubation of GPG with the protein kinase C inhibitors, staurosporine (0.1 μM) or H-7 (3 μM) or with the protein kinase C activator, phorbol 12-myristate 13-acetate (1 μM), did not change the tonus of the preparation and also failed to affect BK-induced contraction significantly (Figure 3a, b and c).

Influence of prostanoids

The data in Figure 4a and b show that both phases of the BK-induced contraction in GPG were markedly antagonized

Figure 1 (a) Concentration-response curves for bradykinin in strips of the guinea-pig gallbladder performed in the same tissue at 2 (○); 3 (●), 4 (□) or 5 (■) h after setting up each preparation. Each preparation was exposed to consecutive increases in BK concentration and cumulative-response curves were constructed. The first curve was carried out at 2 h and the second curve was performed at 3, 4 or 5 h following the equilibration period of the preparation. (b) Mean concentration-response curves for bradykinin (○); Lys-BK (●); Met-Lys-BK (□); Tyr⁸-BK (■); [Hyp³]-BK (Δ) and des-Arg⁹-BK (▲) in the guinea-pig isolated gallbladder carried out 2 h after setting up the preparations. Only one concentration-response curve was performed for each peptide per preparation. Each point represents the mean with s.e.mean of 6 to 9 experiments. In some cases the error bars are hidden within the symbols. Significant differences from respective control values where * $P < 0.05$ (Student's unpaired *t* test).

by indomethacin (3 μM) or by ibuprofen (30 μM). However, the BK-induced contraction in GPG was only partially attenuated by phenidone (30 μM) and was not affected by dazoxiben (30 nM), L-655,240 (10 nM) or MK-571 (0.1 μM) (Figures 5 and 6). The results in Figure 7 (a, b and c) show that prostaglandin $\text{F}_{2\alpha}$, prostaglandin E_2 and U46619 (0.1 nM to 100 μM) all caused a concentration-dependent contraction of the GPG. The EC_{50} s (and 95% confidence limits) were: $\text{PGF}_{2\alpha}$ 1.7 (0.4–7.4) μM ; PGE_2 3.1 (0.9–10.7) μM and U46619 0.48 (0.1–1.5) nM. The maximal contractile responses in g of tension were 1.32 ± 0.20 for $\text{PGF}_{2\alpha}$; 1.36 ± 0.15 for PGE_2 and 0.96 ± 0.09 for U46619.

Analysis of the antagonism of Hoe 140, NPC 17761 or NPC 17731 to the BK-induced contraction

The selective B_2 receptor antagonists, Hoe 140, NPC 17761 and NPC 17731 (0.01–1 μM) did not affect the basal tone of the strips but caused a concentration-dependent displacement to the right of the BK-induced contraction in GPG (Figure 8a, b and c). Because BK concentrations higher than 10 μM were not tested, it was not possible to determine whether the actions of BK antagonists were surmountable, thus making difficult the estimation of the pA_2 values. Preincubation of

Table 1 Responsiveness of guinea-pig gallbladder to bradykinin and related kinins

Agonists	First phase (μM)	EC_{50}	Second phase (nM)	Contraction ^b (g)	n
KCl (80 mM)	—	—	—	1.50 ± 0.17	20
BK	1.35 (0.63–2.89) ^a		77.35 (26.23–228.07)	1.65 ± 0.12	10
Hyp ³ -BK	1.44 (0.41–5.08)		8.95 (4.04–19.86)	1.76 ± 0.09	5
L-BK	1.54 (0.25–9.26)		60.92 (21.69–171.07)	1.45 ± 0.13	9
Tyr ⁸ -BK	2.72 (0.45–16.33)		33.75 (5.85–194.82)	1.36 ± 0.15	8
Met-L-BK	4.30 (0.52–35.25)		12.78 (2.51–64.89)	1.36 ± 0.15	5

^a95% confidence limits; ^bResponses to 80 mM KCl or 3 μM of agonists (highest concentration tested) are reported as mean $\pm$ s.e.mean. Results were analysed by means of analysis of variance followed by Dunnett's multiple comparison test.

Figure 2 Concentration-response curves for bradykinin in the guinea-pig isolated gallbladder in the absence (○) or presence of (a) nicardipine (●, 0.1 μM), (b) in calcium-free Krebs plus EGTA (●, 1 mM), or (c) in the presence of calcium-free Krebs plus ryanodine (●, 10 μM). Each point represents the mean $\pm$ s.e.mean of 6 to 7 experiments. Significant differences from respective control values where $*P < 0.05$ (Student's unpaired t test).

Figure 3 Concentration-response curves for bradykinin in the guinea-pig isolated gallbladder in the absence (○) or in the presence of (a) staurosporine (●, 0.1 μM), (b) H-7 (●, 3 μM), or (c) phorbol 12-myristate 13-acetate (●, 1 μM). Each point represents the mean $\pm$ s.e.mean of 5 to 6 experiments.

GPG with the selective B₁ receptor antagonist, des-Arg⁹-[Leu⁸]-BK (1 μ M) did not affect the CRC for BK (Figure 9).

Effect of several classes of drugs on BK-induced contraction

Preincubation of GPG preparations with tetrodotoxin (0.3 μ M), glibenclamide (1.0 μ M), mepyramine (1 μ M), atropine (1 μ M), prazosin (0.1 μ M), CP 96,345 (0.3 μ M) and WEB 2086

(0.1 μ M) did not significantly affect the contractile responses induced by BK (Figure 6c for WEB 2086 alone, Table 2).

Discussion

In the present study we have demonstrated that BK and related kinins cause concentration-dependent contractions of GPG *in vitro*, characterized by two distinct phases, and that these effects are likely to be mediated by activation of B₂

Figure 4 Concentration-response curves for bradykinin in the guinea-pig isolated gallbladder obtained in the absence (○) or in the presence of (a) indomethacin (●, 3 μ M), (b) ibuprofen (●, 30 μ M). Each point represents the mean $\pm$ s.e.mean of 6 to 7 experiments. Significant differences from respective control values where * P < 0.05 (Student's unpaired t test).

Figure 5 Concentration-response curves for bradykinin in the guinea-pig isolated gallbladder obtained in the absence (○) or the presence of (a) dazoxiben (●, 30 μ M) or (b) L655,240 (●, 10 nM). Each point represents the mean $\pm$ s.e.mean of 5 to 6 experiments.

Figure 6 Concentration-response curves for bradykinin in the guinea-pig isolated gallbladder in the absence (○) or the presence of (a) phenidone (●, 30 μ M), (b) MK-571 (●, 0.1 μ M) or (c) WEB 2086 (●, 0.1 μ M). Each point represents the mean $\pm$ s.e.mean of 5 to 6 experiments. Significant differences from respective control values where * P < 0.05 (Student's unpaired t test).

Figure 7 Concentration-response curves for prostaglandin $F_{2\alpha}$ (a), prostaglandin E_2 (b) or U46619 (c) in the guinea-pig isolated gallbladder. Each point represents the mean $\pm$ s.e.mean of 6 experiments.

Figure 8 Concentration-response curves for bradykinin in the guinea-pig isolated gallbladder in the absence (○) or in the presence of increasing concentrations of (a) Hoe 140 (μ M): 0.01 (●), 0.1 (□) and 1 (■); (b) NPC 17761 (μ M): 0.01 (●), 0.1 (□) and 1 (■) or (c) NPC 17731 (in μ M): 0.01 (●), 0.1 (□) and 1 (■). Each point represents the mean $\pm$ s.e.mean of 5 to 6 experiments. In some cases the error bars are hidden within the symbols.

Figure 9 Concentration-response curves for bradykinin in the guinea-pig isolated gallbladder in the absence (○) or in the presence of des-Arg⁹-[Leu⁸]-bradykinin (●, 1 μ M). Each point represents the mean $\pm$ s.e.mean of 5 to 6 experiments. In some cases the error bars are hidden within the symbols.

receptors at the smooth muscle. This evidence is substantiated by the following observations. First, the order of potency of the kinins studied in GPG is compatible with the action of kinins acting at B_2 receptors (Regoli & Barabé, 1980). Secondly, the selective B_1 receptor agonist des-Arg⁹-BK, up to 1 μ M, had very weak agonist activity, and the selective B_1 receptor antagonist, des-Arg⁹-[Leu⁸]-BK, up to 1 μ M, failed to inhibit the contractile response induced by BK in GPG. In addition, the potent and selective B_2 receptor antagonists, Hoe 140, NPC 17731 and NPC 17761, caused a concentration-dependent displacement to the right of the BK-induced contraction. However, our results do not allow us to ascertain whether or not the interaction between BK with the B_2 receptors in GPG involves a competitive mechanism, because the maximal contractile response for BK in the presence of the B_2 antagonists was not obtained, due to the limited concentration of BK used.

Results of this study also demonstrated that BK-induced contraction in GPG is largely mediated indirectly by release of prostanoid metabolite(s) derived from the cyclo-oxygenase pathway as BK response was inhibited by indomethacin or ibuprofen. Similar results have been reported for kinin-induced contraction in the GPG (Woods & Baird, 1991) and for BK-stimulated electrogenic bicarbonate secretion in mucosal and serosal GPG (Baird & Margolius, 1989). The fact that BK-induced contraction in this preparation was not affected by dazoxiben, a selective inhibitor of TxA_2 synthesis (Randall *et al.*, 1981), or by L-655,240, a competitive receptor antagonist of TxA_2 (Hall *et al.*, 1987; Girard *et al.*, 1989), strongly suggests that the cyclo-oxygenase metabolites released by BK in GPG are probably unrelated to TxA_2 . We

Table 2 Effect of several classes of drugs on bradykinin (100 nM)-induced contraction in guinea-pig gallbladder

Agents	Concentration (μ M)	Contraction (g)	
		Control	Antagonist
Tetrodotoxin	0.3	0.69 $\pm$ 0.09	0.80 $\pm$ 0.01
Glibenclamide	1.0	0.55 $\pm$ 0.15	0.55 $\pm$ 0.12
Mepyramine	1.0	0.69 $\pm$ 0.10	0.71 $\pm$ 0.11
Atropine	1.0	0.65 $\pm$ 0.08	0.51 $\pm$ 0.03
CP 96,345	0.3	1.01 $\pm$ 0.06	1.03 $\pm$ 0.07
Prazosin	0.1	1.08 $\pm$ 0.08	1.08 $\pm$ 0.11

Each group represents the mean $\pm$ s.e.mean of 4 to 6 experiments.

Results were analysed by means of Student's paired *t* test.

and others have previously shown that B₂ receptors mediating relaxation in guinea-pig trachea, in circular muscle from guinea-pig ileum as well as the B₁ and B₂ receptor-mediated contraction for BK in rat portal vein rings, are also indirectly mediated by release of a cyclo-oxygenase product derived from the arachidonic acid pathway (Bramley *et al.*, 1990; Calixto & Medeiros, 1991; Schlemper & Calixto, 1994; Campos & Calixto, 1994). While the B₂ receptor mediates BK-induced relaxation in the trachea and gut which depends on the release of prostaglandin E₂, the contractile responses elicited by either BK or des-Arg⁹-BK in rat portal vein are mediated by release of TxA₂. At this stage of our study it is difficult to ascertain the nature of prostanoid released by BK in GPG. However, based on the potencies of the prostanoids tested, it is probable that the cyclo-oxygenase derived metabolite released by BK in the GPG is PGE₂. A similar conclusion was suggested for BK-stimulated electrogenic bicarbonate secretion in GPG (Baird & Margolius, 1989). In addition, BK-induced contraction in GPG was also partially attenuated by phenidone, suggesting some role of a lipo-oxygenase metabolite from arachidonic acid in its contractile responses. However, pre-incubation of GPG with MK-571, a selective leukotriene D₄ receptor antagonist (Jones *et al.*, 1989), which has been shown to antagonize competitively leukotriene D₄-induced contractions in GPG (Freedman *et al.*, 1993), failed to affect BK-mediated contraction in GPG. These results suggest that leukotriene D₄ is unable to mediate BK-induced contractions in this preparation. However, additional biochemical studies are required to clarify this point.

Concerning the sources of Ca²⁺ necessary for BK-mediated contraction in GPG, although dihydropyridine L-type Ca²⁺ channels are present in GPG (Renzetti *et al.*, 1990) our data indicate that the contractile response elicited by this peptide is dependent on the release of Ca²⁺ from intracellular stores. This conclusion is based on the fact that even at higher concentrations nifedipine (a voltage-sensitive L-type Ca²⁺ channel antagonist) failed to affect BK-induced contraction in GPG. Furthermore, the contraction in response to BK in GPG was not modified in Ca²⁺-free medium plus EGTA. The contractile response elicited by BK in GPG maintained in Ca²⁺-free solution was markedly attenuated by ryanodine. In contrast with the results reported for BK-induced contraction in circular muscle from the guinea-pig ileum (Calixto & Medeiros, 1991), rabbit aorta and jugular vein (Calixto &

Medeiros, 1992) and rat portal vein (Campos & Calixto, 1994) which are largely dependent on the activation of protein kinase C-dependent mechanisms, BK-induced contractions in GPG were not affected by high concentrations of either staurosporine or H-7, two putative protein kinase C antagonists (for review see Hidaka & Kobayashi, 1992).

The contractile responses induced by BK in GPG were not affected by tetrodotoxin, glibenclamide, mepyramine, atropine, CP 96,345, WEB 2086 or prazosin, indicating that BK has a direct myogenic effect on GPG, excluding the participation of action potential-dependent neural release of histamine, acetylcholine, noradrenaline, tachykinins (NK₁ receptor), or PAF or by activation of ATP-sensitive K⁺ channels.

Falcone *et al.* (1993) reported that [³H]-BK and related kinins bind with high affinity (K_i 0.45 nM) to a B₂ receptor in GPG. In addition, the authors also demonstrated that BK caused a graded increase of polyphosphoinositide hydrolysis in GPG cells, and that the BK responses were competitively and noncompetitively antagonized by NPC 17731 and Hoe 140, respectively. Also, the B₂ receptor antagonists, NPC 567, NPC 17731, NPC 17761 and Hoe 140 behaved as pure competitive antagonists against BK-induced contraction in GPG, yielding pK_i values of 5.0; 7.2; 7.7 and 8.5, respectively. Similar values of pK_i for these antagonists were observed in the presence of indomethacin, which as reported here, significantly decreased both the potency and the efficacy of BK in GPG. Interestingly, Falcone *et al.* (1993) did not find the higher affinity site for BK reported in the present study. However, there are marked methodological differences between these studies, such as the duration of equilibrium period, the basal tension of the preparations, the presence of different drugs in bath medium, and the frequent use of capsaicin to reduce the release of calcitonin gene-related peptide. Such differences make it difficult to compare studies.

In summary, we present functional evidence that both high and low affinity contractions induced by BK in GPG are mediated by activation of B₂ receptors, and that this contraction depends on the release of Ca²⁺ from intracellular stores sensitive to ryanodine. In addition, part of this BK-induced contraction is probably mediated indirectly by release of a cyclo-oxygenase derived product from the arachidonic acid pathway unrelated to TxA₂, and that the contractions are not coupled to activation of a protein kinase C-dependent mechanism. Considering the cyclo-oxygenase metabolites play an important role in inflammatory processes affecting the gallbladder and the biliary systems (Kaminski & Deshpande, 1980; Thornell *et al.*, 1981; Kaminski *et al.*, 1985; Kaminski, 1989; Myers & Bartula, 1992), and also the fact that BK has an important proinflammatory action (Marceau *et al.*, 1983; Proud & Kaplan, 1988) which can release eicosanoids and stimulate electrogenic bicarbonate secretion in GPG (Baird & Margolius, 1989) it seems probable that kinins have a physiopathological role in the GPG.

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