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Effects of dexamethasone and protein kinase C inhibitors on the induction of bradykinin B_1 mRNA and the bradykinin B_1 receptor-mediated contractile response in isolated rat ileum

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Abstract

We detected the expression of inducible bradykinin (BK) B_1 receptor mRNA in the rat ileum by the reverse transcriptase–polymerase chain reaction (RT–PCR) method, when the isolated ileum was suspended for at least 1 hr in an aerated Tyrode's solution at 37°. The induction of this mRNA was both time- and temperature-dependent, and was followed by a contractile response to des-Arg^9-BK at around 3 hr of incubation; this response increased in magnitude with time and was maximal at 6 hr. In contrast, the contraction in response to BK and the expression of B_2 receptor mRNA were constant throughout this 6-hr incubation period. The contraction due to des-Arg^9-BK was selectively suppressed by B_1 receptor antagonists, i.e. des-Arg^9[Leu^8]-BK and des-Arg^{10}-HOE140, but not by the B_2 antagonists D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK and HOE140. The inducible des-Arg^9-BK contractile response was suppressed by continuous *in vitro* exposure of the ileum to cycloheximide or actinomycin D, but neither inhibitor affected the contraction induced by BK, suggesting that the B_1 receptor could be induced *de novo*. *In vitro* and *ex vivo* treatment of the ileum with dexamethasone suppressed the induction of the contractile response to des-Arg^9-BK, but had no significant effect on the expression of B_1 receptor mRNA. Some protein kinase C inhibitors, i.e. H7 and calphostin C, suppressed the expression of B_1 receptor mRNA and diminished the contractile response to des-Arg^9-BK. These results suggest that the *de novo* synthesis of the B_1 receptor in the ileum preparation can be up-regulated at the transcriptional level (a process in which a specific isoform of protein kinase C may be involved). Additionally, these data suggest that the contractile response to des-Arg^9-BK involves a process sensitive to some post-transcriptional action of dexamethasone.

Keywords: Bradykinin B_1 receptor; Calphostin C; des-Arg^9-Bradykinin; Dexamethasone; H7; Rat ileum

1. Introduction

BK is well known for its potent biological activities, such as contraction or relaxation of vascular smooth muscles or isolated intestinal muscles from various mammalian species, induction of peripheral vascular permeability, and production of pain associated with inflammation [1–3]. Over two decades ago, Regoli *et al.* [4] reported that des-Arg^9-BK, a breakdown product of BK, caused the contraction of rabbit aortic strips under certain conditions. Subsequently, they classified the receptors for BK as B_1 and B_2 subtypes, based mainly upon pharmacological studies using antagonists [5].

cDNAs for B_2 and B_1 receptors of humans, rabbits, mice, and rats have been cloned [6–11]. Moreover, in addition to isolated blood vessels, several isolated organs, such as colon, intestine, urinary bladder, and murine trachea were reported to express the B_1 receptor after the animals had received bacterial LPS or were exposed to certain stimuli [12–20].

The significance of BK B_1 receptor induction in some cardiovascular disorders has been reported recently [21–24]. Expression of the B_1 receptor has been observed in patients after myocardial infarction [21]; B_1 receptor induction has been reported to cause hypotension in pigs after treatment with noxious stimuli [22]; expression of the

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Abbreviations: BK, bradykinin; des-Arg^9-BK, des-Arg^9-bradykinin; LPS, lipopolysaccharide; ACh, acetylcholine; IL, interleukin; MAP kinase, mitogen-activated protein kinase; RT–PCR, reverse transcriptase–polymerase chain reaction.

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B_1 receptor in the pulmonary vascular bed of cats has been demonstrated to be involved in the maintenance of vascular tone [23]; and the B_1 receptor has been shown to participate in the hypertensive response in spontaneously hypertensive rats [24]. In addition, several reports describe the induction of the B_1 receptor by inflammatory and infectious stimuli in rats [25,26]. In a study with B_1 receptor knockout mice a low response to inflammatory stimuli was observed [27]. All these facts suggest the importance of inducible molecules including the inducible BK B_1 receptor in body defense mechanisms. However, there have not been reports on the significance of B_1 receptor induction in relation to gastrointestinal disorders or carcinoma, even though induction of the B_1 receptor was reported to occur in the human ileum 24 hr following its isolation [18].

In the present paper, we reported that the contraction of the rat ileum is B_1 receptor-mediated, is time- and temperature-dependent, and requires the prior induction of B_1 receptor mRNA transcription. Additionally, using chemical agents we examine the mechanism of this BK B_1 receptor-mediated contractile response.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were purchased from Japan SLC and used at 7–10 weeks of age.

2.2. Agents

BK, des-Arg^9-BK, kallidin, des-Arg^{10}-kallidin, des-Arg^9-[Leu^8]-BK, D-Arg-[Hyp^3,Thi^{5,8}, D-Phe^7]-BK, and HOE140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK) were purchased from the Peptide Institute. Des-Arg^{10}-HOE140 (Research Biochemicals International), actinomycin D (Sigma), cycloheximide (Sigma), calphostin C (Calbiochem), H7 [1-(5-isoquinolynyl-sulfonyl)-2-methylpiperizine dihydrochloride; Sigma], PD98059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one; Calbiochem], Ro32-0432 {2-(8-[(dimethyl-amino)methyl]-6,7,8,9-tetrahydropyrido[1,2-*a*]indol-3-yl)-3-(1-methylindol-3-yl)maleinimide hydrochloride; Calbiochem}, HA1004 [*N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; Seikagaku-kogyo], dexamethasone phosphate (Banyu Pharmaceutical Co.), polymyxin B (Sigma), and LPS (*Escherichia coli*:B4; Sigma) were purchased from the indicated sources. Recombinant human IL-1α was a gift from the Dainippon Pharmaceutical Co. Actinomycin D, cycloheximide, calphostin C, H7, PD98059, Ro32-0432, and HA1004 were dissolved in DMSO solution at 10 mM, stored at −35°, and used at the desired concentrations by dilution with Tyrode's solution.

A kininase I inhibitor, DL-2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid (MERGETPA; Calbiochem [28]) was prepared at a final concentration of 10^{-4} M in Tyrode's solution.

2.3. Preparation of isolated ileum and the contractile response to BK and des-Arg^9-BK

Ileum was isolated, and several longitudinal sections, each 2.5 to 3 cm in length, were taken and washed extensively with Tyrode's solution. Several sections of ileum were preincubated in a vessel (500 mL) containing aerated Tyrode's solution at 37°, and then after the desired number of hours of incubation, an ileum strip was fixed with a 1-g weight load in a Magnus organ bath (15 mL) that had been filled with aerated Tyrode's solution at 37°. After equilibration for 15–30 min, agonists were applied. In all experiments for the contractile response, the contraction induced by a single dose of BK or des-Arg^9-BK given at 37° (except for experiments at different temperatures) was recorded by an isometric transducer (Star Medical Co.), and the magnitude of the contraction in response to each concentration of agonist was expressed as a percentage of that induced by ACh (10^{-5} M) [16]. The ileum was washed several times with fresh Tyrode's solution after the contractions reached a peak (90 s for BK and several minutes for des-Arg^9-BK), and then after the baseline recovery, the procedure was repeated approximately every 15–30 min.

2.4. Preincubation of the ileum preparations at different temperatures

Several strips of ileum were incubated in aerated Tyrode's solution kept at 25, 28, or 37°, for up to 6 hr, and some preparations were taken at 1, 3, 4.5, or 6 hr, and fixed on a Magnus bath containing aerated Tyrode's solution at the corresponding temperature. Application of agonists was done at 25, 28, or 37°, and the contraction responses were expressed as a percentage of the contraction induced by ACh (10^{-5} M) in the same preparation. The magnitudes of contraction in response to ACh at 25, 28, and 37° were not significantly different.

2.5. Application of antagonists and inhibitors

The antagonists used for the bradykinin B_1 receptor were des-Arg^9-[Leu^8]-BK [5] and des-Arg^{10}-HOE140 [29]; and those for the B_2 receptor, D-Arg [Hyp^3,Thi^{5,8},D-Phe^7]-BK [30] and HOE140 [31,32]. The desired concentration of a given antagonist was added to the bath 2 min before the addition of the agonist, BK or des-Arg^9-BK.

Several strips of ileum were incubated in the vessels containing aerated Tyrode's solution for several hours in the presence of a specific inhibitor. During this period the inhibitor solution was changed several times. The inhibitors and concentrations used were calphostin C (10^{-6} M), H7 (10^{-4} M), PD98059 (3×10^{-5} M), Ro32-0432 (10^{-5} M), HA1004 (10^{-5} M), dexamethasone (10^{-5} M),

and IL-1α (10–100 units/mL). After incubating the ileum strips for 1–6 hr, the contractile response of each was tested. For this an ileum strip was transferred and fixed in a Magnus bath, which had been filled with aerated Tyrode's solution at 37° without the above test agents.

In the case of actinomycin D (2×10^{-6} M), cycloheximide (7×10^{-5} M), polymyxin B (30–100 mM), or LPS (10^{-5} M), the ileum was exposed to these inhibitors continuously during the isolation and the examination of the contractile response. To examine the *in vitro* degradation of BK and des-Arg^9-BK during the observation of the contractile response, we also used Tyrode's solution containing the kininase inhibitor, MERGETPA (10^{-4} M).

2.6. Ex vivo study for dexamethasone or LPS

In the case of *ex vivo* experiments with dexamethasone, rats received a sterile saline solution of dexamethasone (given intraperitoneally at a dose of 0.5 mg/kg) 2, 6, or 12 hr before the excision of the ileal segment. In some experiments, a sterile saline solution of LPS (3×10^{-5} g/kg, 24 hr before the excision) was also injected intraperitoneally. Excised ileum preparations were processed, and contraction was examined in the same way as described in Section 2.3.

2.7. Detection of mRNA for B_1 and B_2 receptors

After incubation of the ileum for 4.5 hr with an inhibitor or other agent (actinomycin D, cycloheximide, calphostin C, H7, PD98059, Ro32-0432, HA1004, or dexamethasone), the contractile response by BK or des-Arg^9-BK was tested, and then total RNA was extracted with chloroform and isopropyl alcohol from a TRIzol (GIBCO BRL) suspension of sonicated ileal samples. The RT–PCR was performed with the use of primers previously designed based on Sprague–Dawley rat genomic DNA [33].

For the B_1 receptor, the primers were 5′-AATGTCGACTGGCCCTTCGGAACTGAC-3′ (sense) and 5′-AATAAGCTTCAGGCCCAGGTCTGTGAT-3′ (antisense); and those for the B_2 receptor were 5′-AATGTCGACGACTGGCTGTTCGGAGAG-3′ (sense) and 5′-AATAAGCTTGGTGACAATATCCACGGC-3′ (antisense). The quality of RNA samples was evaluated by simultaneous RT–PCR of GAPDH as previously reported [34].

2.8. Determination of endotoxin

The amount of endotoxin (LPS) in the Tyrode's solution or incubation mixture was estimated by the use of a commercial kit, a Toxicolor System LS 200 set (Seikagaku-kogyo).

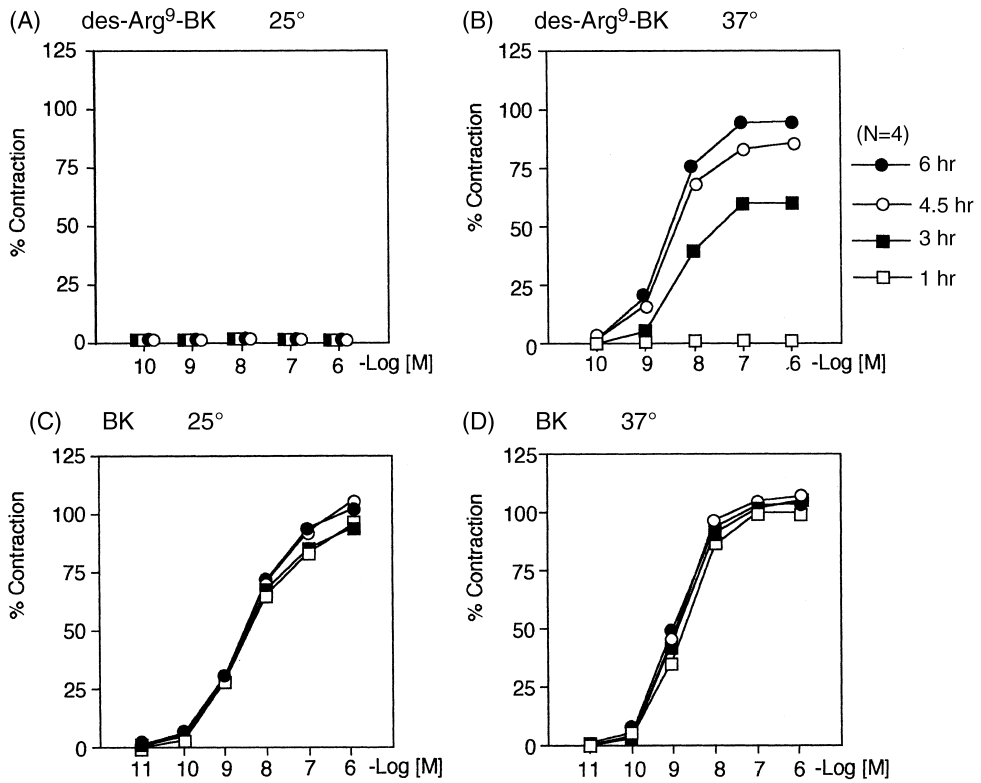

Fig. 1. Time- and temperature-dependent induction of contractile responses to des-Arg^9-BK in the rat ileum. Contractile responses of rat ileal longitudinal fragments to des-Arg^9-BK (A and B) and to BK (C and D) are expressed as a mean percentage of the contraction produced by 10^{-5} M acetylcholine. The ileal preparations were suspended in Tyrode's solution at 25 and 37° for up to 6 hr, and contractile responses to des-Arg^9-BK and BK were examined at 1, 3, 4.5, and 6 hr. Data are the means from preparations of 4 rats. Standard errors were approximately 2–5% at each point, but are not illustrated.

2.9. Statistical analysis

Data are expressed as means ± SEM of the indicated number of animals or experiments. Statistical analysis was conducted with Student's *t*-test or one-way analysis of variance followed by Dunnett's *t*-test.

3. Results

3.1. Time- and temperature-dependent induction of the functional activity and expression of mRNA for bradykinin B_1 receptor

We observed time-dependent contractile responses of isolated longitudinal rat ileum to des-Arg^9-BK, when the strips were incubated for several hours at 37° (Fig. 1B); the full response was reached after 6 hr of incubation. When incubated at 25°, the contraction by des-Arg^9-BK did not occur even after 6 hr (Fig. 1A); at 28° (data not shown), a contraction was not seen even after 3 hr but a small one emerged after 6 hr, which was approximately one-third of the magnitude of that at 37°. The contractile response to des-Arg^{10}-kallidin, another B_1 agonist, occurred in the same way as that to des-Arg^9-BK (data not shown). Contractile responses of the same ileal preparation to BK constantly occurred independently of time and temperature, as shown in panels C and D of Fig. 1.

These contractile responses to BK and des-Arg^9-BK did not change even in the presence of a kininase inhibitor, MERGETPA (10^{-4} M), in the Tyrode's solution of a Magnus bath, suggesting that these agonists had not been degraded in the Tyrode's solution during observation of contraction at 37°.

The pD_2 (a negative logarithm of the concentration of an agonist that induces 50% of the maximum response) ± SEM, obtained from the concentration–response curves of the ileum for BK, des-Arg^9-BK, and des-Arg^{10}-kallidin at 6 hr, were 9.19 ± 0.07 (N = 5), 8.80 ± 0.06 (N = 8), and 8.90 ± 0.05 (N = 3), respectively. The responses to the B_1 agonists, i.e. des-Arg^9-BK and des-Arg^{10}-kallidin, seemed to be less potent than the response to BK, in view of these pD_2 values. However, the magnitude of the maximal response at 10^{-6} M des-Arg^9-BK was almost the same as that of BK.

Next, we examined the expression of mRNAs for kinin B_1 and B_2 receptors in these preparations by the RT–PCR method, as shown in Fig. 2. B_1 receptor mRNA was not found in the ileum just after dissection, but became

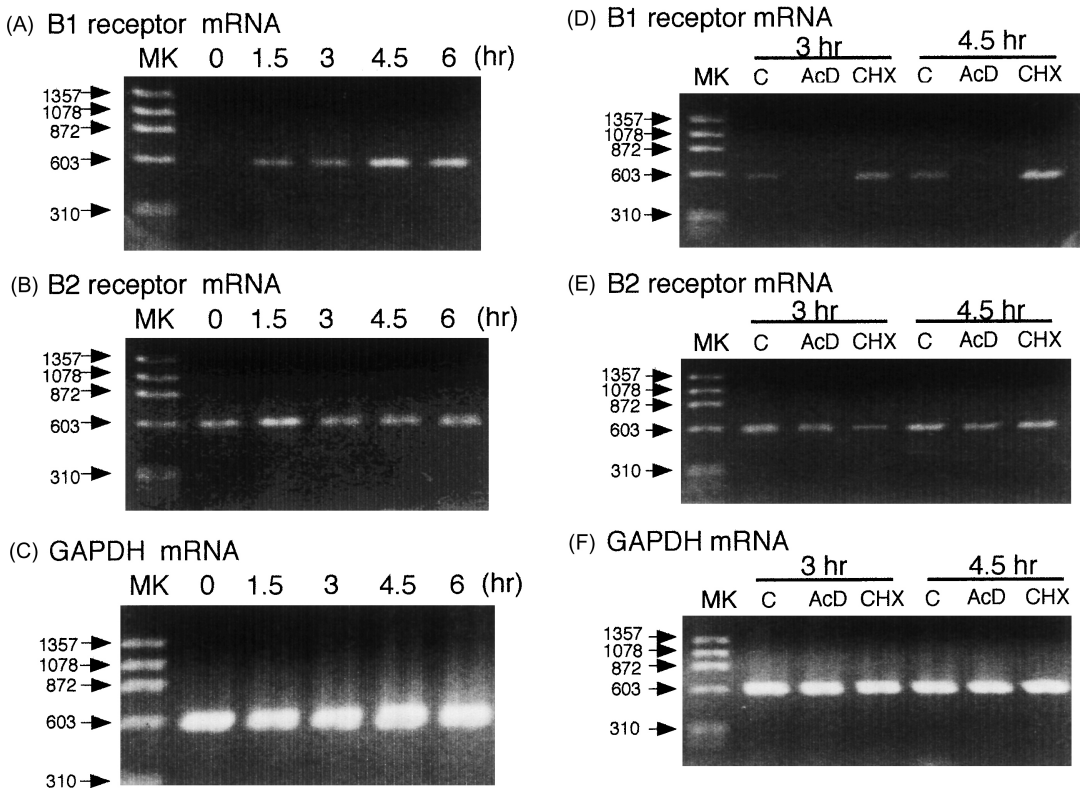

Fig. 2. Time-dependent expression of mRNA for the bradykinin B_1 receptor in comparison with that for the B_2 receptor and GAPDH (A–C) and effects of actinomycin D and cycloheximide on the expression of bradykinin B_1 and B_2 receptor and GAPDH mRNAs (D–F). Total RNA was extracted from each ileal preparation after suspension for 0, 1.5, 3, 4.5, or 6 hr at 37°, and subjected to RT–PCR for detection of mRNAs of B_1 (A) and B_2 (B) kinin receptors and GAPDH (C). For panels D–F, total RNA was extracted from each ileal preparation after suspension without (C) and with actinomycin D (AcD, 2×10^{-6} M) or cycloheximide (CHX, 7×10^{-5} M) for 3 and 4.5 hr, and subjected to RT–PCR for the detection of B_1 (D) and B_2 (E) kinin receptor and GAPDH (F) mRNAs. Typical results from 4 independent experiments (panels A–C) and from 3 independent experiments (panels D–F) are illustrated. Details are described in Section 2.

apparent at 1.5 hr and gradually increased with time up to 6 hr (Fig. 2A). Expression of the mRNA for this receptor preceded the emergence of the contractile activity. However, the B_2 receptor mRNA was seen immediately after the isolation of the ileum, and its intensity did not change up to 6 hr (Fig. 2B). The above results from the contractile response and the expression of mRNA indicate that both B_1 and B_2 receptors exist independently in the ileum incubated for several hours at 37°.

3.2. Effect of B_1 and B_2 antagonists on the contractile response of rat ileum to BK and des-Arg^9-BK

We characterized pharmacologically the type of receptors involved in the contractile activities of des-Arg^9-BK (10^{-6} M) and BK (10^{-6} M) (Fig. 3) by using specific B_1- and B_2-receptor antagonists. As shown in panels A and B of Fig. 3, the B_2 antagonists, i.e. D-Arg-[Hyp^3,Thi^{5,8}, D-Phe^7]-BK and HOE140, inhibited the contractions induced by BK, but not those induced by des-Arg^9-BK. On the contrary, the B_1 antagonists, i.e. des-Arg^9-[Leu^8]-BK and des-Arg^{10}-HOE140, diminished only the contractile response to des-Arg^9-BK. The suppression of the contractile responses by B_1 and B_2 antagonists was concentration-dependent (data not shown). The experimental data thus indicate that the response to BK occurred via the B_2 receptor and that to des-Arg^9-BK via the B_1 receptor, even when the receptors co-existed in the ileum.

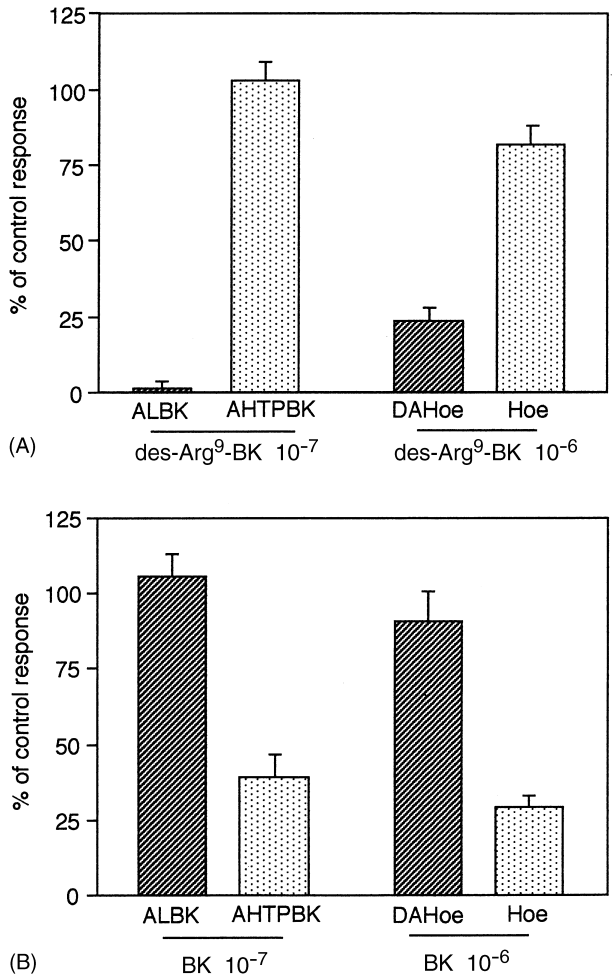

Fig. 3. Effect of bradykinin B_1- and B_2-receptor antagonists on the contractile responses induced by des-Arg^9-BK (A) or BK (B). Isolated ileal fragments were induced to contract after incubation for 4.5 hr at 37°. The ileum preparations fixed in the aerated Magnus bath were incubated for 2 min with an antagonist, i.e. des-Arg^9[Leu^8]-BK (ALBK, 10^{-6} M) or des-Arg^{10}-HOE140 (DAHoe, 10^{-6} M) as a B_1-receptor antagonist, and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK (AHTPBK, 10^{-6} M) or HOE140 (Hoe, 10^{-6} M) as a B_2-receptor antagonist; then various concentrations of des-Arg^9-BK or BK were added (10^{-7} or 10^{-6} M as representatives are shown). The percentage of acetylcholine-induced contractions was calculated as in Fig. 1. Values are the means ± SEM from 4–5 samples.

3.3. In vitro effects of actinomycin D and cycloheximide on the induction of the B_1 receptor

When actinomycin D (2×10^{-6} M) or cycloheximide (7×10^{-5} M) was added into the Magnus bath 3 min before the application of BK (1×10^{-6} M) or des-Arg^9-

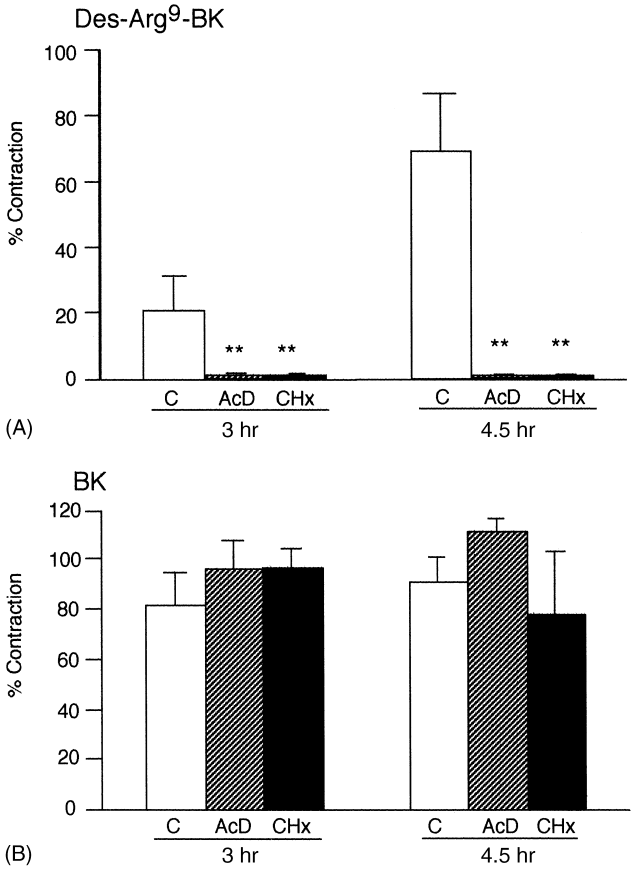

Fig. 4. Effects of actinomycin D and cycloheximide on the induction of contractile responses by des-Arg^9-BK in rat ileal fragments. The ileal preparations were preincubated in Tyrode's solution at 37° in the absence (C) or presence of actinomycin D (AcD, 2×10^{-6} M) or cycloheximide (CHx, 7×10^{-5} M) up to 3 or 4.5 hr, and then des-Arg^9-BK- (A) or BK-induced contractions (B) were examined. The percentage of acetylcholine-induced contractions was calculated as in Fig. 1. Data are the means ± SEM of the preparations from 3 rats. Key: (**) significantly different from control (C) at $P < 0.01$.

BK (1×10^{-6} M), the contractile responses of the ileal fragments to these agonists were completely unaffected, showing that neither actinomycin D nor cycloheximide has a direct inhibitory effect on BK- or des-Arg^9-BK-inducible contractions. However, the des-Arg^9-BK (1×10^{-6} M)-inducible contraction was suppressed almost completely when the ileal preparations were preincubated in the presence of actinomycin D (2×10^{-6} M) for 3 or 4.5 hr (Fig. 4A). Furthermore, the expression of mRNA for the B_1 receptor was suppressed almost completely by the presence of actinomycin D (Fig. 2D). In contrast, cycloheximide (7×10^{-5} M) completely inhibited the induction of the contractile response to des-Arg^9-BK (1×10^{-6} M) without significantly affecting the expression of B_1 receptor mRNA (Figs. 2D and 4A). However, neither agent affected either the expression of B_2 receptor mRNA or the B_2 receptor-driven contractile response significantly (Figs. 2E and 4B).

3.4. Effects of LPS, polymyxin B, or IL-1α in Tyrode's solution and the effect of ex vivo treatment with LPS

The amounts of endotoxin (LPS) in the Tyrode's solution with or without ileum strips incubated for 0, 3, or 6 hr at 37° were measured. The results (the mean of 2 samples) were as follows: without ileum strips, 11.8, 12.0, and 12.5 ng/mL, respectively, and with the strips, 23.3, 24.3, and 28.3 ng/mL, respectively. These results indicated that endotoxin did not increase during a 6-hr incubation.

As shown in Table 1, the presence of polymyxin B (30–100 mM), an endotoxin antagonist, in the organ bath solution throughout the incubation did not affect the induction of the B_1-receptor-mediated contraction significantly. Neither exogenous LPS added up to 2×10^{-6} g/mL to the Tyrode's solution nor the *ex vivo* treatment of rats with LPS (3×10^{-5} g/kg) 24 hr before the excision had any effect on the time course of induction of the contractile response to des-Arg^9-BK. Also, the presence of IL-1α (10–100 units/mL) during the incubation did not have a significant effect on the induction of the contraction caused by des-Arg^9-BK.

3.5. Ex vivo and in vitro effects of dexamethasone on the functional activity of the B_1 receptor and the expression of mRNA

As shown in Fig. 5A, the isolated ileum of rats that had received dexamethasone (0.5 mg/kg, i.p.) 2 hr before being

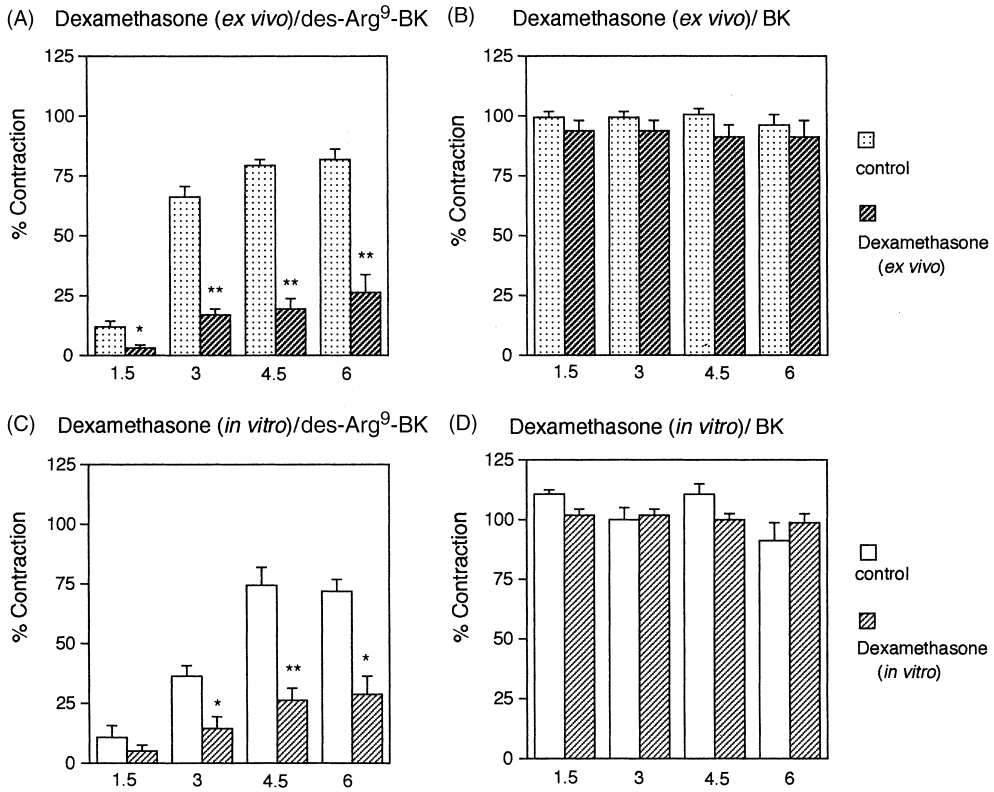

Fig. 5. Effects of dexamethasone on the induction of contractile responses of rat ileal fragments as elicited by des-Arg^9-BK and BK. In panels A and B, the ileal segments were isolated from rats that had received 0.5 mg/kg of dexamethasone (hatched columns) or vehicle (dotted columns) 2 hr before being killed, and fixed in an organ bath after the indicated times of preincubation in Tyrode's solution at 37° (*ex vivo*). In panels C and D, ileal segments isolated from normal rats were incubated in the presence (hatched columns) or absence (open columns) of dexamethasone (10^{-5} M), for the indicated times (*in vitro*), and then fixed in the bath to examine contractions in fresh Tyrode's solution at 37°. Contractions were induced by 10^{-6} M des-Arg^9-BK (A and C) or 10^{-6} M BK (B and D), and expressed as a percentage of the contractions induced by 10^{-5} M acetylcholine. Values are means of samples from 5 rats (*ex vivo*) or 4 rats (*in vitro*) ± SEM. Key: (∗) and (∗∗) significantly different from the corresponding vehicle treatment at $P < 0.05$ and $P < 0.01$, respectively.

Table 1
Effects of endotoxin, an endotoxin antagonist, and IL-1α on the induction of the contractile response of isolate rat ileum

Treatments[a]	Magnitude of contraction (% of Ach contraction)[b]		
	Time after start of suspension		
	1 hr	3 hr	6 hr
Polymyxin B (N = 4)			
0 mM	2.5 ± 2.0	57.5 ± 12.5	100.2 ± 2.5
30 mM	5.0 ± 5.1	43.8 ± 20.0	101.2 ± 5.0
100 mM	5.1 ± 2.5	30.0 ± 10.0	85.0 ± 5.4
LPS (N = 4)			
0 mg/mL	23.8 ± 16.7[c]	61.9 ± 11.9	92.9 ± 4.8
2 mg/mL	21.4 ± 14.3[c]	64.3 ± 14.3	100.0 ± 3.6
IL-1α (N = 3)			
0 units/mL	4.2 ± 2.0	64.6 ± 2.1	112.5 ± 5.2
10 units/mL	4.2 ± 2.2	68.8 ± 2.3	100.0 ± 5.0
50 units/mL	4.4 ± 2.0	70.8 ± 3.8	83.3 ± 4.5
100 units/mL	4.2 ± 1.9	81.3 ± 4.2	91.7 ± 5.5
LPS[d] (*ex vivo*, N = 4)			
Vehicle	9.5 ± 2.4	45.2 ± 11.9	79.8 ± 11.9
30 mg/kg	11.9 ± 7.1	35.7 ± 7.1	73.8 ± 14.3

[a] Ileal strips were suspended in Tyrode's solution in the presence or absence of polymyxin B, LPS, or human recombinant IL-1α for 1, 3, and 6 hr, and contraction in response to des-Arg^9-BK or BK (not shown) was examined.

[b] Contractions of the ileal strips to des-Arg^9-BK (10^{-6} M) are expressed as a percentage of those induced by acetylcholine (10^{-5} M). Values are means ± SEM from the indicated number of rats (N).

[c] The contractions were examined after a 2-hr incubation.

[d] LPS or vehicle was injected intravenously 24 hr before dissection of the ileum, and the isolated ileal strips were suspended in the organ bath containing Tyrode's solution.

killed showed suppressed induction of the contractile response to des-Arg^9-BK. The suppression was not complete, as some activity (approximately 1/4–1/5 of that of the control) remained. Similar inhibition was also seen when the ileal preparations isolated from normal rats were suspended in Tyrode's solution containing dexamethasone (1×10^{-5} M, Fig. 5C) *in vitro*. However, neither *ex vivo* nor *in vitro* treatment affected the BK-inducible contraction at any time examined (Fig. 5B and D). Neither *ex vivo* nor *in vitro* treatment with dexamethasone changed the expression level of B_1 receptor mRNA, as shown in Fig. 6A. Similar data on the contraction response and expression of mRNA were also obtained from preparations from rats pretreated with dexamethasone 6 or 12 hr before surgical excision of the ileum (data not shown).

3.6. Effects of protein kinase inhibitors on the induction of contraction and B_1 mRNA expression

An ileal fragment was incubated in Tyrode's solution containing protein kinase inhibitors for 4.5 hr, and then the contractile responses to BK and des-Arg^9-BK were examined with the fragment in Tyrode's solution without

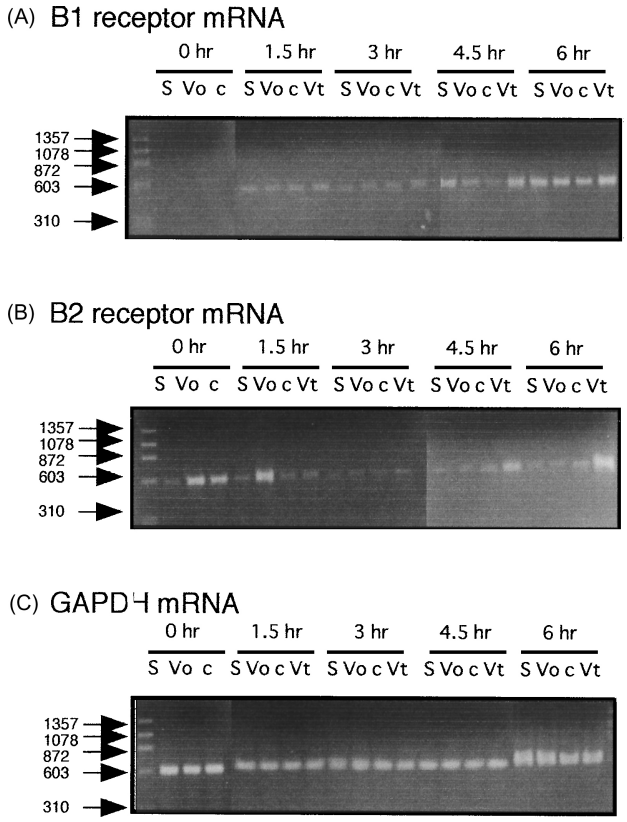

Fig. 6. Effects of dexamethasone on the expression of bradykinin B_1 and B_2 receptor mRNAs. Total RNA was extracted from each ileal preparation after incubation in Tyrode's solution in the presence or absence of dexamethasone for 0, 1.5, 3, 4.5, or 6 hr, and subjected to RT–PCR for the detection of B_1 and B_2 kinin receptor and GAPDH mRNAs. Ileal preparations were isolated from rats that had received saline (S) or dexamethasone (Vo) 2 hr before being killed and suspended in fresh Tyrode's solution; or preparations were isolated from non-treated rats, suspended in Tyrode's solution in the absence (c) or presence (Vt) of dexamethasone (10^{-5} M) for up to 6 hr. A typical result from 4 experiments is illustrated. Details are described in Section 2.

inhibitors. Among the protein kinase inhibitors examined, PKC inhibitors H7 and calphostin C suppressed the induction of the contractile response of ileal fragments to des-Arg^9-BK, as shown in Fig. 7. H7 significantly suppressed, but calphostin C only slightly inhibited B_1 receptor mRNA expression (Fig. 8). However, HA1004 (1×10^{-5} M, data not shown, another C-kinase inhibitor), Ro32-0432 (an inhibitor of G-protein-coupled protein kinase 5 and a less potent PKC inhibitor), and PD98059 (an inhibitor of MAP kinase kinase [MAPKK]) did not suppress either the induction of contraction (Fig. 7A and B) or B_1 receptor mRNA expression (Fig. 8A). These results would suggest that a specific isoform of protein kinase C, but not PD98059-sensitive MAPKK, might be involved in the process leading to the induction of the B_1 receptor-mediated contractile response in this system. In contrast, the contractile response to BK and expression of B_2 receptor mRNA were not affected by these protein kinase inhibitors.

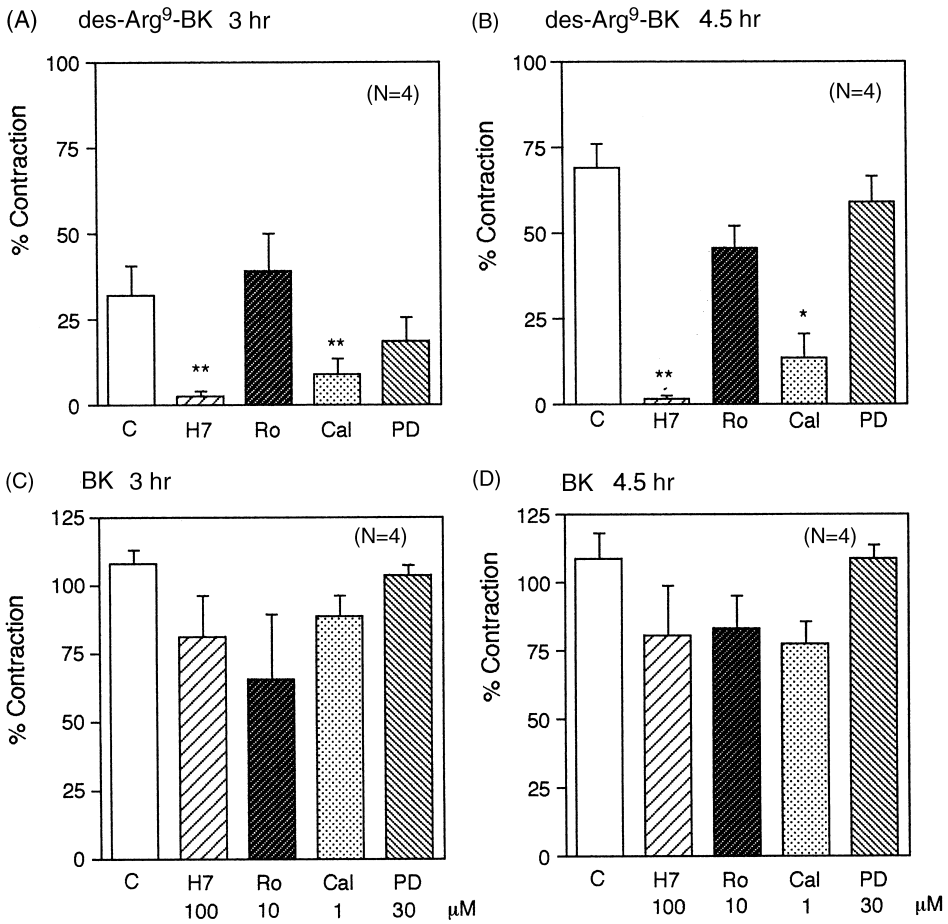

Fig. 7. Effects of protein kinase inhibitors on the induction of contractile responses to des-Arg^9-BK in rat ileal fragments. Longitudinal ileal fragments were incubated in Tyrode's solution at 37° up to 4.5 hr in the absence (C) or presence of the indicated concentration of H7, Ro32-0432 (Ro), calphostin C (Cal), or PD98059 (PD). The fragments were taken out after a 3- or 4.5-hr incubation, and contractile responses to des-Arg^9-BK (A and B) and BK (C and D) were examined in the Magnus bath containing fresh Tyrode's solution without these agents. Data are expressed as a percentage of the contractions induced by 10^{-5} M acetylcholine, and are shown as the means ± SEM of preparations from 4 rats. Key: (∗) and (∗∗) significantly different from control (C) at $P < 0.05$ and $P < 0.01$, respectively.

4. Discussion

The results of the present study indicate that the des-Arg^9-BK-inducible contractile response of the isolated rat ileum was time- and temperature-dependent, and that the activity was mediated through B_1 receptors, because it was inhibited by B_1-selective antagonists, but not by B_2-specific ones. In contrast, both the contractile response to BK and the expression of B_2 receptor mRNA were constantly present, showing neither an increase nor a decrease throughout the experiment.

The above results demonstrated the co-existence of the B_1 and B_2 receptors in rat ileum incubated in Tyrode's solution for several hours at 37°. Nevertheless, the contractile response elicited by BK did not change significantly even after the robust expression of the B_1 receptor mRNA after a 6-hr incubation. The presence of a kininase inhibitor, MERGETPA, did not increase the magnitude of the contraction induced by BK or des-Arg^9-BK. These facts indicate that BK and des-Arg^9-BK had not been degraded during the suspension in the Magnus bath, in contrast to the degradation found in an *in vivo* experiment [35]. Furthermore, the contractile activity of BK was inhibited by only B_2-specific antagonists, whereas that elicited by des-Arg^9-BK was diminished by only B_1-selective antagonists. Therefore, both peptides, BK and des-Arg^9-BK, seemed to express their own action on their respective B_2 and B_1 receptors.

The induction of B_1 receptor activity required prior expression of receptor mRNA. The *de novo* expression of B_1 receptor mRNA and receptor protein synthesis was time- and temperature-dependent since the contractile response to des-Arg^9-BK occurred 3 hr after the incubation at 37° and was not induced at 25°. This feature is in line with the results of actinomycin D and cycloheximide in the present study, which confirmed the steps of the B_1 receptor induction, i.e. the induction of B_1 receptor mRNA followed by *de novo* synthesis of the receptor protein. These features are in accord with previous reports, showing the time-dependent induction of the B_1 receptor in the rat ileum

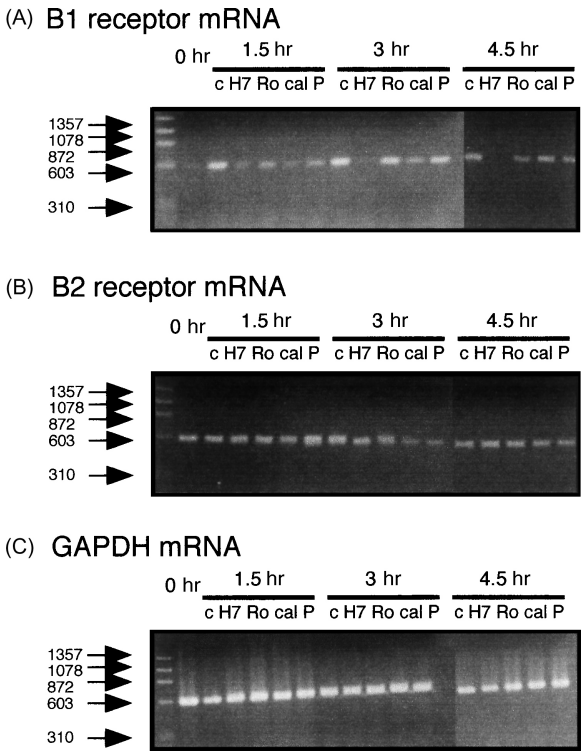

Fig. 8. Effects of protein kinase inhibitors on the expression of bradykinin B_1 and B_2 receptor mRNAs. Total RNA was extracted from each ileal preparation suspended for 0, 1.5, 3, or 4.5 hr in Tyrode's solution in the absence (c) or presence of H7, Ro32-0432 (Ro), calphostin C (cal), or PD98059 (P) at the concentrations described in Fig. 7, and subjected to RT–PCR for the detection of B_1 and B_2 kinin receptor and GAPDH mRNAs. A typical result of 4 experiments is illustrated. Details are described in Section 2.

[17], induction of the B_1 receptor after overnight incubation of human ileum at 37° [18], and suppression of B_1 receptor induction in rabbit aorta by protein synthesis inhibitors [19].

However, some details of the present results are different from the data in previous reports. For example, as for the involvement of endotoxin in the induction of the B_1 receptor, previous reports described the induction of a hypotensive response in rats that had received LPS 24 hr earlier [12,15]. In the present study, pretreatment of animals with LPS for 24 hr or the addition of LPS to the incubation mixture did not change the time course of the induction of the B_1 receptor. Also, we considered that ileal fragments might have made contact with LPS during surgical excision of the ileum. However, when we measured the endotoxin content in the Tyrode's solution, the amount of LPS was very small and did not increase during a 6 hr incubation of the rat ileum. Furthermore, the continuous contact between polymyxin B, an antagonist of LPS, and the ileal preparation had no effect on the induction of the B_1 receptor. Thus, it is possible that LPS is not involved in the induction of the B_1 receptor in the ileum, unlike the case for blood vessels [12,15,36].

It was also reported that *in vivo* treatment of mice with IL-1α could induce the B_1 receptor [36] and that lung fibroblasts expressed the receptor induced via the activation of NF-κB or AP-1 [20,37–40]. However, in the present study, the *in vitro* treatment with IL-1α did not affect the induction of the B_1 receptor. The present results indicate that there might be some difference in the regulation of the *BK* B_1 receptor gene between the vascular and gastrointestinal systems, for example, involvement of a cell type-specific factor [41]; further study is needed on this point.

Both *in vitro* and *ex vivo* treatment of the ileum with dexamethasone suppressed its des-Arg^9-BK-inducible contractile response but not its contractile response to BK. This suppression by dexamethasone was transcription-independent as the levels of both B_1 and B_2 receptor mRNA were unaffected by the treatment. This would suggest regulation at a non-genomic or post-transcriptional level [42]. This finding is in line with a previous report that dexamethasone modulated the post-transcriptional regulation of bradykinin receptor expression by TNFα in lung fibroblasts [38]. The present results suggest that the induction of the B_1 receptor may be regulated by factors dependent upon temperature and time, not only those affecting mRNA expression but also those participating in the post-transcriptional or non-genomic regulation of the receptor.

Some protein kinase C inhibitors, such as H7 and calphostin C, suppressed the expression of B_1 receptor mRNA thus inhibiting the induction of contraction, but other inhibitors did not affect the expression of this transcript. This selectivity suggests the involvement of a specific isoform of protein kinase C or one that is tissue-specific in the up-regulation of the B_1 receptor in the ileum. The presence of a phorbol ester in the organ bath did not affect B_1 receptor induction (data not shown), suggesting that the induction is complex in nature [20]. However, incubation of the ileal fragments with these inhibitors did not affect the contraction induced by the BK agonist directly, because addition of the inhibitors to the Magnus bath during the examination of the BK-induced contractions did not affect the response. Thus, a specific isoform of protein kinase C may be involved in the induction of the B_1 receptor in the rat ileum.

It was reported that PD98059 inhibited the contractile response to a B_1 agonist when a rabbit aorta preparation was exposed continuously to this inhibitor [40,41]. We also reported that a PD98059-sensitive MAP kinase pathway for intracellular Ca^{2+} elevation was involved in the signal transduction by stimulated B_1 and B_2 receptors in HEK293 cells overexpressing these receptors [33]. However, in the present study, PD98059 had no effect on either B_1 receptor induction or contraction, indicating that the PD98059-sensitive MAP kinase pathway may not be involved, although it could take part in the contraction of blood vessels [43]. The precise mechanism responsible for the induction of the B_1 receptor in the rat ileum appears to be

very complex and different from the mechanism in other systems, and hence requires further study.

In conclusion, this study showed that a time- and temperature-dependent *in vitro* induction of the contractile response to des-Arg^9-BK of the rat ileum occurred following the up-regulation of the expression of B_1 receptor mRNA. In contrast, the contractile response to BK was neither time- nor temperature-related and the level of the B_2 receptor mRNA was constant. Other data in this study suggest that a specific isoform of protein kinase C may be involved in the induction of the B_1 receptor in the rat ileum as well as possibly a dexamethasone-sensitive non-genomic, post-transcriptional, process.

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