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Evidence for *in vitro* expression of B_1 receptor in the mouse trachea and urinary bladder

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1 Motor responses to des-Arg[9]-bradykinin and bradykinin were studied in the isolated mouse trachea (precontracted with carbachol, 10 μM) and the urinary bladder of either Swiss, C57Bl/6J or bradykinin B_2 receptor knockout (*Bk2r*$^{-/-}$) mice after 1–6 h *in vitro*. The expression of mRNA for the mouse B_1 receptor in tracheal and urinary bladder tissues was also studied by using Northern blot analysis.

2 In isolated tracheae, des-Arg[9]-bradykinin produced a relaxant response that increased over time: no response was observed after 1 h of incubation, whereas after 6 h the maximum response (1 μM) was 68–84% of the relaxation produced by isoproterenol (1 μM) in the three mouse strains. The relaxant response to bradykinin (1 μM) observed at 1 h (38–51% of isoproterenol) was increased (62–65% of isoproterenol) after 6 h in Swiss and C57Bl/6J mice, but was absent in *Bk2r*$^{-/-}$ mice. In the presence of cycloheximide, des-Arg[9]-bradykinin did not cause any response at 6 h.

3 Similar findings were obtained in the urinary bladder: at 1 h des-Arg[9]-bradykinin (1 μM) did not cause any motor effect, whereas at 6 h it caused a contraction that was 28–59% of that produced by carbachol (1 μM) in the three mouse strains. Cycloheximide blocked the response to des-Arg[9]-bradykinin. Bradykinin (1 μM) contracted urinary bladders at 1 h (34–35% of carbachol), as well as at 6 h (66–77% of carbachol) in Swiss and C57Bl/6J strains, but was without effect in *Bk2r*$^{-/-}$ mice.

4 Northern blot hybridization with a specific cDNA probe against mouse B_1 receptor mRNA using total RNA extracted from tracheae and urinary bladders freshly removed from Swiss and *Bk2r*$^{-/-}$ mice revealed minimal expression. However, marked hybridization was detected 150 min after *in vitro* exposure in both tissues.

5 Evidence is provided that *in vitro* exposure of mouse trachea and urinary bladder causes a time-dependent induction of B_1 receptors that cause relaxation and contraction, respectively.

Keywords: B_1 receptor; des-Arg[9]-bradykinin; B_2 receptor knockout mice; receptor induction

Abbreviations: BK, bradykinin; B_2 receptor knockout mice, *Bk2r*$^{-/-}$; GAPDH, glyceraldehyde-3-phosphate deshydrogenase

Introduction

Two types of receptors mediate the biological effects of kinins, the B_1 and the B_2 receptors (Regoli, 1987; Regoli & Barabe', 1980). B_2 receptors are constitutively present in a large variety of cells, including endothelial, smooth muscle and epithelial cells, fibroblasts, cells of exocrine glands and sensory neurones. Stimulation of B_2 receptors causes inflammatory responses either by direct stimulation of effector cells or by release of inflammatory mediators (Geppetti, 1993; Regoli & Barabe', 1980). In contrast, B_1 receptors are rarely expressed constitutively in vascular or extravascular tissues but, rather, their expression is induced by different conditions and interventions. B_1 receptors are present in rabbit (Regoli *et al*., 1977), rat (Marceau *et al*., 1980), pig (Beny *et al*., 1987), cow (De Kimpe *et al*., 1994), dog (Toda *et al*., 1987) and human tissues (Couture *et al*., 1981), and often they are upregulated after exposure of the tissue *in vitro* or *in vivo* to bacterial lipopolysaccarides (LPS), IL-1β or to ultraviolet irradiation (Marceau, 1995). Endogenous agonists of B_2 and B_1 receptors are bradykinin and kallidin, and the des-Arg[9] derivatives of these peptides, respectively (Marceau, 1995; Regoli, 1987). The possibility that kinins and B_1 receptors are involved in certain diseases including arthritis, hyperalgesia, and others has been postulated (Marceau, 1995).

B_2 receptor stimulation causes a variety of effects in the airways. Thus, the hypothesis that kinins and B_2 receptors may play a role in airway diseases such as asthma has been proposed (Barnes, 1992; Trifilieff *et al*., 1993). In contrast, a few studies have reported effects mediated by B_1 receptor in the lung, and these effects are mainly confined to the vasculature (Siebeck *et al*., 1989; Vianna & Calixto, 1998). Recently, a moderate relaxant response to des-Arg[9]-bradykinin was reported in mouse tracheal rings *in vitro* (Van Heuven-Nolsen *et al*., 1997). Another recent study showed that in the trachea of Balb/c mice B_1 receptor stimulation mediated a relaxant response (Li *et al*., 1998). However, in that study concentration-response curves were constructed in a non cumulative manner and, with this procedure, maximal responses were obtained after several hours of incubation in the organ bath (Li *et al*., 1998). There is evidence in the mouse that B_1 receptors may be upregulated by *in vitro* exposure of the tissue. Thus, the contractile response to des-Arg[9]-bradykinin increased after a few hours of incubation in isolated mouse stomach preparations (Nsa Allogho *et al*., 1995; 1998).

In the present paper we have investigated whether B_1 receptors of the mouse trachea could be upregulated as a

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function of the *in vitro* incubation time. Thus, we studied the relaxant response to des-Arg^9-bradykinin and bradykinin in tracheae precontracted with carbachol after 1–6 h of incubation in an *in vitro* apparatus for organ bath studies. Expression of the messenger RNA (mRNA) for the mouse B_1 receptor was studied in tracheae freshly taken from the mouse or in tracheae maintained for 150 min in the organ bath apparatus. For comparison, a similar investigation was performed in the mouse isolated urinary bladder, in which contraction to bradykinin and des-Arg^9-bradykinin and expression of the mRNA for the B_1 receptor were studied. Upregulation of B_1 receptors was studied in the Swiss albino mouse strain. We also investigated whether the absence of B_2 receptors could affect the process of upregulation of B_1 receptors. For this purpose, experiments were performed in mice in which the B_2 receptor gene was disrupted by gene targeting and homologous recombination (*Bk2r*$^{-/-}$) (Borkowski *et al*., 1995) and in one of the mouse strains used to produce the *Bk2r*$^{-/-}$, C57Bl/6J mice.

Methods

Animals

Mice of both sexes (18–25 g), kept in a temperature-controlled room with a 12 h light-dark cycle, received food and water *ad libitum*. Mice of the albino Swiss strain (Morini, Reggio Emilia, Italy) and of the black C57Bl/6J strain (Charles River, Lecco, Italy) were used. *Bk2r*$^{-/-}$ mice have been generated by homologous recombination and gene targeting from J129Sv mice and stem cells were implanted in C57Bl/6J mouse blastocystis (Borkowski *et al*., 1995). The *Bk2r*$^{-/-}$ mice were a generous gift of Dr F. Hess (Merck Research Laboratories, Rahway, NJ, U.S.A.).

Organ bath studies

Mice were sacrificed by injection of xylazine (100 mg ml^{-1}, 50 μl, i.m.). Trachea and urinary bladder were removed and carefully cleared of fat and connective tissue. Trachea rings and strips of urinary bladder were mounted in 5 ml organ baths containing a modified Krebs solution in mM (NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 0.5, $NaHCO_3$ 25, $NaHPO_4$ 1 and glucose 11.1) maintained at 37°C, and oxygenated with a mixture of 95% O_2 and 5% CO_2. To prevent peptide degradation captopril (1 μM) and phosphoramidon (1 μM) were present in the bath. Tissues were fixed to the base of the organ bath and connected to an isometric force transducer. An optimal tension of 1 g was applied to the trachea and of 0.7 g to the urinary bladder. During the initial stabilization period (30 min) tissues were washed three times. Relaxation to bradykinin and des-Arg^9-bradykinin was measured in tracheae precontracted with carbachol (10 μM). Cumulative concentration response curves to bradykinin (1 nM–1 μM) and its analogue des-Arg^9-bradykinin (1 nM–1 μM) were constructed by applying increasing concentration of the agonist as soon as a plateau was reached with the previous concentration. In the trachea the relaxant response to kinins was expressed as the percentage of the relaxant response to isoproterenol (1 μM). In the urinary bladder contractile responses to kinins were expressed as the percentage of the contraction to carbachol (1 μM). In some experiments indomethacin (5 μM) was added to the organ bath 45 min prior to the addition of the stimulus. Other experiments were performed in the continuous presence of cycloheximide (100 μM).

Bradykinin B_1 receptor cDNA probe synthesis

cDNA probe was synthesized as previously described for B_2 receptor cDNA probe (Schmidlin *et al*., 1998). Briefly, reverse transcription with MMLV-RT Rnase H$^-$ (Stratagene, La Jolla, CA, U.S.A.) was performed using total RNA extracted from mice kidney (see below). PCR was run for 35 cycles of amplification with three steps of 1 min each: denaturation at 96°C, annealing at 63°C and elongation at 72°C with Taq DNA polymerase (Promega, Madison, WI, U.S.A.) and with the specific primers 5′ AAATCTACCTGGCTAACTTGG 3′ and 5′ GTCCTGGATCACTCTCACCTG 3′, whose sequences correspond to nucleotides 326–346 and 937–957 of the mice bradykinin B_1 receptor gene (Pesquero *et al*., 1996). PCR products were analysed on a 1% agarose gel. After extraction, the 632 nucleotide PCR product was subcloned in pGEM-T plasmid (Promega, Madison, WI, U.S.A.). PCR product sequence analysis revealed a 100% homology with the mouse B_1 gene without any significant homology with other known genes. Sequence comparisons with gene bank were done using the NCBI Blast program.

Northern blot

Total RNA was isolated by the guanidinium isothiocyanate method as described by Chomczynski & Sacchi (1987). Total RNA was separated in a 1% agarose / 1 M formaldehyde gel, and transferred to a nylon membrane (Stratagene, La Jolla, CA, U.S.A.) in 20 × SSC. Prehybridization was performed for 4 h at 42°C in a prehybridization solution containing 50% formamide, 0.01% SDS (Sodium Dodecyl Sulphate), Denhardt's solution 2 × and SSC 5 ×. Hybridization was carried out with ^{32}P-random primer-labelled B_1-receptor and glyceraldehyde-3-phosphate deshydrogenase (GAPDH) cDNAs at 10^6 c.p.m. ml^{-1} over-night at 42°C in a solution containing 2.5% dextran sulphate, 10% salmon sperm (10 mg ml^{-1}) (Stratagene, La Jolla, CA, U.S.A.), 0.01% SDS, Denhardt's solution 4 ×, 50% formamide and SSC 5 ×. Filters were washed twice with SSC 2 × and 0.01% SDS at 42°C for 15 min, and SSC 1 × and 1% SDS at 50°C for 30 min before autoradiography exposure at −70°C with Kodak X-AR 5 film.

Materials

All peptides were purchased from Bachem (Budendorf, Switzerland). Carbachol, cycloheximide, captopril, phosphoramidon, isoproterenol and indomethacin were from Sigma (St. Louis, MO, U.S.A.).

Statistical analysis

All data are means ± s.e.mean. Statistical analysis was performed by the Student's *t*-test for unpaired data. Statistical significance was accepted at a level of $P<0.05$.

Results

General

In baseline conditions both bradykinin and des-Arg^9-bradykinin did not cause any detectable motor effect in tracheae of Swiss mice after either 1, 2, 3, 4, 5 or 6 h of incubation (not shown). After 1 h of incubation, addition of carbachol (10 μM) resulted in a prompt contraction that

reached a plateau after 5–10 min. The contraction to carbachol was 1.15±0.13 g ($n=9$) (0.93±0.08, $n=12$ in C57Bl/6J mice and 1.32±0.09, $n=12$ in *Bk2r*$^{-/-}$ mice). Six reproducible contractions to carbachol (10 μM), each separated by a 1 h interval, were obtained in all the three mouse strains (not shown). Addition of isoproterenol (1 μM) caused a relaxation that varied between 55 and 75% of the contraction produced by carbachol in the three mouse strains, and relaxations obtained after 1 and 6 h were comparable (not shown).

After 1 h in the organ bath, isolated strips of urinary bladder of the Swiss mouse strain responded to carbachol (1 μM) with a prompt contraction (1.65±0.18 g, $n=12$). Carbachol-induced contractions at 2, 3, 4, 5 and 6 h were not statistically different from the contraction observed at 1 h (not shown). Similar results were obtained in C57Bl/6J mice and in *Bk2r*$^{-/-}$ mice (not shown).

Effect of des-Arg^9-bradykinin and bradykinin in mouse isolated tracheal rings

After 1 h of incubation des-Arg^9-bradykinin (1 nM–1 μM) did not cause any detectable relaxation in tracheae from mice of the Swiss strain precontracted with carbachol (10 μM). A relaxation to des-Arg^9-bradykinin was observed usually after 2–3 h of incubation, and the effect of des-Arg^9-bradykinin increased over time (Figure 1A). At 6 h a detectable relaxation was produced by 10 nM des-Arg^9-bradykinin. The maximum relaxation produced by des-Arg^9-bradykinin (1 μM) was 68±5% of the relaxation produced by isoproterenol (1 μM) (Figure 1A). The relaxant response to des-Arg^9-bradykinin observed at 6 h was completely abolished in preparations exposed to cycloheximide (Table 1), and markedly inhibited in the presence of the B_1 receptor antagonist, [Leu^8]des-Arg^9-bradykinin (1 μM) (Table 1). Similar results were obtained in C57Bl/6J mice (Figure 1B and Table 1) and in *Bk2r*$^{-/-}$ mice (Figure 1C and Table 1). In the latter strain of mice the maximal response to des-Arg^9-bradykinin after 6 h of incubation (84±4% of isoproterenol, $n=6$) was not statistically different from that obtained in C57Bl/6J mice (73±3% of isoproterenol, $n=6$). The relaxant response to des-Arg^9-bradykinin (1 μM) at 6 h was abolished by indomethacin (5 μM, for 45 min) (Table 1).

After 1 h of incubation, bradykinin (1 nM–1 μM) caused a concentration-dependent relaxation of tracheae from mice of the Swiss strain precontracted with carbachol (10 μM). Maximum relaxation produced by 1 μM bradykinin was 39±7% ($n=5$) of the relaxation induced by isoproterenol (1 μM) (Figure 2A). After 6 h of incubation the relaxation produced by bradykinin was slightly increased: maximum relaxation was 65±9% of the isoproterenol-induced relaxation ($n=5$, $P<0.05$) (Figure 2A). The relaxant response to bradykinin observed at 6 h was not significantly affected by cycloheximide (100 μM) (Table 1), and by the presence of the B_1 receptor antagonist, [Leu^8]des-Arg^9-bradykinin (1 μM) (Table 1). Similar results were obtained in C57Bl/6J mice (Figure 2B and Table 1). In contrast, in *Bk2r*$^{-/-}$ mice, administration of bradykinin (1 nM–1 μM) did not cause any relaxation when tested either after 1 or 6 h of incubation. In the presence of indomethacin (5 μM) for 45 min the relaxant response to bradykinin (1 μM) was abolished at both 1 and 6 h (Table 1).

In additional experiments, we tested the hypothesis that B_1 receptor upregulation was induced by exposure to the agonist. In parallel experiments in which the tracheae of Swiss mice were challenged with des-Arg^9-bradykinin (1 nM–1 μM at 1, 2, 3, 4 and 5 h), the response to des-Arg^9- bradykinin (1 μM at 6 h) was not significantly different (61±7% of isoproterenol, $n=5$) from the response observed in tracheae challenged with the vehicle of des-Arg^9-bradykinin, following the same protocol (57±7% of isoproterenol, $n=5$).

Effect of des-Arg^9-bradykinin and bradykinin in mouse isolated urinary bladder strips

After 1 h of incubation des-Arg^9-bradykinin (1 nM–1 μM) did not cause any detectable motor response in isolated strips of urinary bladders from mice of the Swiss strain. A contraction to des-Arg^9-bradykinin was observed usually after 2–3 h of incubation, and this contraction increased over time. The maximum contraction produced by des-Arg^9-bradykinin

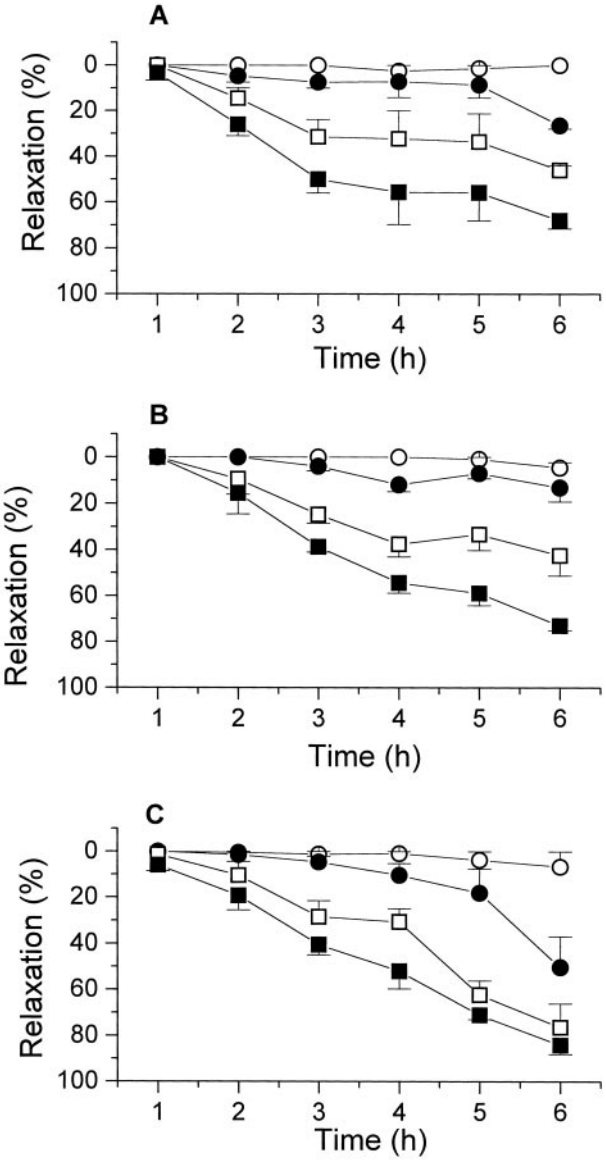

Figure 1 The relaxation of mouse isolated tracheal rings (precontracted with carbachol, 10 μM) from three different mouse strains (A), Swiss albino; (B), C57Bl/6J black, and; (C), bradykinin B_2 receptor knockout (*Bk2r*$^{-/-}$) induced by increasing concentrations of des-Arg^9-bradykinin (○, 1 nM; ●, 10 nM; □, 100 nM; ■, 1 μM) . The effect of the des-Arg^9-bradykinin was tested after 1–6 h of incubation in the organ bath *in vitro*. Relaxation was expressed as the percentage of the relaxation caused by isoproterenol (1 μM). Entries are mean±s.e.mean of at least five experiments.

Table 1 Effect of cycloheximide and [Leu^8]des-Arg^9-bradykinin and indomethacin on the relaxant and contractile effect produced by bradykinin (BK) and des-Arg^9-bradykinin (des-Arg^9-BK) in tracheae (precontracted with carbachol) and urinary bladders of three mouse strains

	Vehicle		*Cycloheximide, 100 μM*		*[Leu^8]des-Arg^9-BK, 1 μM*		*Indomethacin, 5 μM*	
	BK 1 μM	*des-Arg^9-BK 1 μM*	*BK 1 μM*	*des-Arg^9-BK 1 μM*	*BK 1 μM*	*des-Arg^9-BK 1 μM*	*BK 1 μM*	*des-Arg^9-BK 1 μM*
Mouse trachea (relaxation % of isoproterenol 1 μM)								
Swiss								
1 h	39±9	ND	42±8	ND	NM	NM	ND	ND
6 h	62±8	68±3	56±7	ND	61±7	11±4*	ND	ND
C57								
1 h	51±2	ND	45±9	ND	NM	NM	ND	ND
6 h	64±4	73±2	68±5	ND	56±3	5±2*	ND	ND
Bk2r$^{-/-}$								
1 h	ND	6±3	ND	ND	NM	NM	NM	ND
6 h	ND	84±4	ND	ND	NM	15±5*	NM	ND
Mouse urinary bladder (contraction % of carbachol 1 μH)								
Swiss								
1 h	20±5	ND	20±5	ND	NM	NM	28±6	ND
6 h	77±2	28±2	77±5	ND	64±5	4±1*	73±9	32±4
C57								
1 h	40±5	ND	40±5	ND	NM	NM	31±5*	ND
6 h	73±2	47±5	63±7	ND	68±9	9±2*	65±9*	51±6
Bk2r$^{-/-}$								
1 h	ND	ND	ND	ND	NM	NM	NM	ND
6 h	ND	51±4	ND	ND	NM	8±3*	NM	43±6

ND: not detectable. NM, not measured. Values are mean±s.e.mean of at least four experiments. *$P<0.05$ vs respective controls.

(1 μM) was 28±3% of the contraction produced by carbachol (1 μM) (Figure 3A). The contractile response to des-Arg^9-bradykinin observed at 6 h was completely abolished in preparations exposed to cycloheximide (Table 1), and markedly inhibited in the presence of the B_1 receptor antagonist, [Leu^8]des-Arg^9-bradykinin (1 μM) (Table 1). Results obtained in C57Bl/6J (Figure 3B) mice and in *Bk2r$^{-/-}$* (Figure 3C) mice were similar to those reported in mice of the Swiss strain (Figure 3A and Table 1). In *Bk2r$^{-/-}$* mice the maximum response to des-Arg^9-bradykinin obtained at 6 h (59±4% of carbachol, $n=6$) was not statistically different from that obtained in C57Bl/6J mice (56±5% carbachol, $n=6$). Indomethacin (5 μM, for 45 min) did not affect the contraction caused by des-Arg^9-bradykinin (1 μM) at 6 h (Table 1).

After 1 h of incubation of urinary bladders from mice of the Swiss strain, bradykinin (1 nM–1 μM) caused a concentration dependent contraction of isolated strips of urinary bladder (Figure 4A). The maximum contraction produced by 1 μM bradykinin at 1 h was significantly lower (34±2% of carbachol, $n=6$) than the contraction produced at 6 h (66±4% of carbachol, $n=6$, $P<0.05$). The contractile response to bradykinin observed at 6 h was not significantly affected by cycloheximide (100 μH) (Table 1), and by the presence of the B_1 receptor antagonist, [Leu^8]des-Arg^9-bradykinin (1 μM) (Table 1). Indomethacin (5 μM) did not affect the contraction caused by bradykinin at 6 h (Table 1). Similar results were obtained in C57Bl/6J mice (Figure 4B and Table 1). In contrast, in *Bk2r$^{-/-}$* mice administration of bradykinin (1 nM–1 μM) did not cause any motor effect when tested either at 1 or 6 h.

In parallel experiments in which the urinary bladders of Swiss mice were challenged with des-Arg^9- bradykinin (1 nM–1 μM, at 1, 2, 3, 4 and 5 h) the response to des-Arg^9-bradykinin (1 μM at 6 h) was not significantly different (38±5% of carbachol, $n=5$) from the response observed in tracheae challenged with the vehicle for des-Arg^9-bradykinin, following the same protocol (42±5% of isoproterenol, $n=5$).

Northern blot analysis

Total RNA was extracted, and subjected to Northern analysis using 40 μg of RNA per lane. RNA was transferred to nylon membranes, and hybridized with a ^{32}P-labelled B_1 receptor cDNA probe as described under Methods. For standardization, the same blot was probed again with a cDNA fragment of the GAPDH gene. Figure 5 shows that hybridization of a cDNA probe, derived from the mouse B_1 receptor mRNA, by Northern blot with total RNA extracted from tissues (tracheae and urinary bladders) freshly removed from mice was minimal. However, hybridization markedly increased (by 3–4 fold) 150 min after *in vitro* exposure in both tissues. Expression was similar in tissues taken from both Swiss and *Bk2r$^{-/-}$* mice.

Discussion

In the present study we demonstrated that des-Arg^9-bradykinin causes a relaxation of mouse isolated tracheal rings precontracted with carbachol and a contraction of mouse isolated urinary bladder strips. Several pieces of evidence favour the hypothesis that these effects of des-Arg^9-bradykinin are mediated by B_1 receptors, including experiments with *Bk2r$^{-/-}$* strain and experiments with the B_1 receptor antagonist, [Leu^8]des-Arg^9-bradykinin. To our knowledge this is the first evidence that B_1 receptor activation results in a contraction of the mouse urinary bladder. However, the most important finding of the present paper is the demonstration of the upregulation of B_1 receptor in both tracheal and urinary bladder preparations and that this upregulation is a function

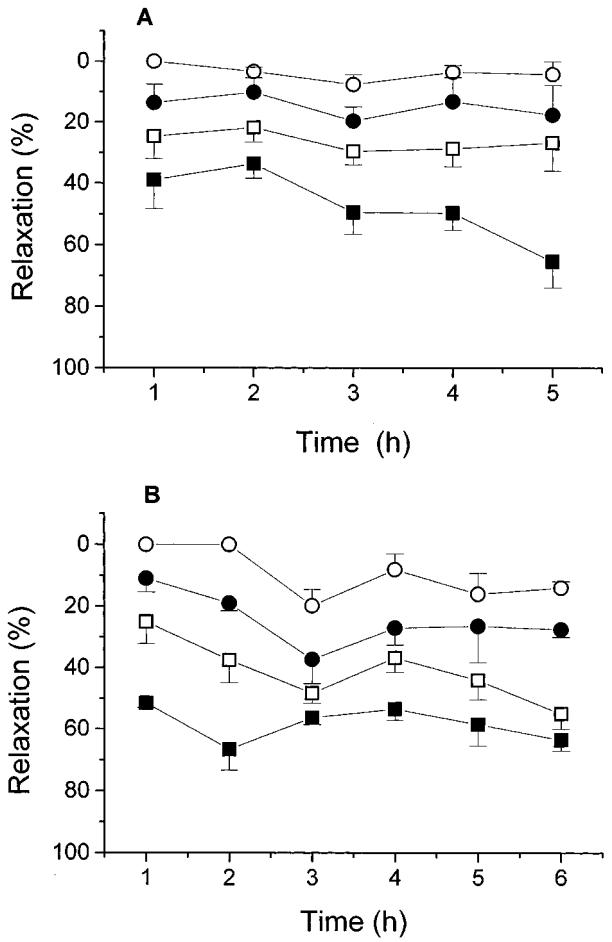

Figure 2 The relaxation of mouse isolated tracheal rings (precontracted with carbachol, 10 μM) from two different mouse strains (A), Swiss albino, and (B), C57Bl/6J black induced by increasing concentrations of bradykinin (○, 1 nM; ●, 10 nM; □, 100 nM; ■, 1 μM). The effect of the bradykinin was tested after 1–6 h of incubation in the organ bath *in vitro*. Relaxation was expressed as the percentage of the relaxation caused by isoproterenol (1 μM). Entries are mean ± s.e.mean of at least five experiments.

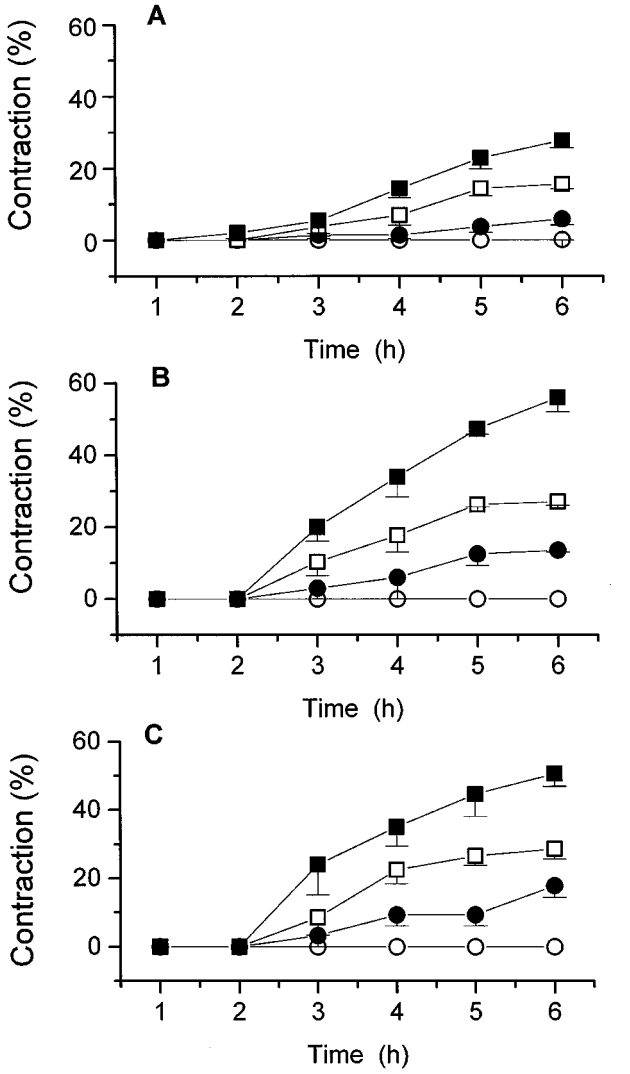

Figure 3 The contraction of mouse isolated strips of urinary bladders from three different mouse strains (A), Swiss albino; (B), C57Bl/6J black, and (C), bradykinin B_2 receptor knockout (*Bk2r*$^{-/-}$) induced by increasing concentrations of des-Arg^9-bradykinin (○, 1 nM; ●, 10 nM; □, 100 nM; ■, 1 μM). The effect of the des-Arg^9-bradykinin was tested after 1–6 h of incubation in the organ bath *in vitro*. The contraction was expressed as the percentage of the contraction caused by carbachol (1 μM). Entries are mean ± s.e.mean of at least five experiments.

of the *in vitro* incubation time of the tissue. Most isolated smooth muscle preparations do not respond to des-Arg^9-bradykinin during the first hour of exposure in an *in vitro* apparatus. Then, in a time-dependent manner these same preparations become more responsive to des-Arg^9-bradykinin, thus suggesting that the B_1 receptor has been upregulated. In this respect, a typical, and extensively studied, preparation is the isolated rabbit aorta. In this tissue, the contractile response to phenylephrine remains practically stable over time, whereas the contraction induced by des-Arg^9-bradykinin, a response that is not visible during the first hour of incubation, becomes evident after 3–6 h of incubation (Bouthillier *et al.*, 1987; Marceau *et al.*, 1980; Regoli *et al.*, 1978). Exposure to inflammatory cytokines, such as interleukin-1β, accelerates, whereas protein synthesis and mRNA inhibitors, including cycloheximide or actinomycin D, block the appearance of the des-Arg^9-bradykinin-mediated response (Bouthillier *et al.*, 1987; Marceau, 1995; Marceau *et al.*, 1980; Regoli *et al.*, 1978). The present experiments suggest that, similar to the rabbit aorta, upregulation of the B_1 receptor occurs in both the mouse trachea and urinary bladder after several hours of incubation.

The ability of des-Arg^9-bradykinin to relax the trachea was prostanoid-dependent (i.e., abolished by indomethacin). Indirect mechanisms (prostanoids or nitric oxide release) have been shown to mediate relaxant effects of kinins in the guinea-pig and rat airways (Figini *et al.*, 1995; Frossard & Barnes, 1990; Frossard *et al.*, 1989; Schmpler & Calixto, 1994). In particular, a cyclo-oxygenase-sensitive mechanism has been demonstrated to mediate des-Arg^9-bradykinin-induced relaxation in isolated mouse trachea (Li *et al.*, 1998; Van Heuven-Nolsen *et al.*, 1997). Thus, the question arises as to whether the increased relaxant response to des-Arg^9-bradykinin was not due to the upregulation of the B_1 receptor, but rather to the upregulation of cyclo-oxygenase. Various observations exclude this hypothesis. Firstly, the relaxant response to bradykinin, already present after 1 h of incubation was also abolished by indomethacin pretreatment. Thus, the absence of des-Arg^9-bradykinin-induced relaxation in the first few hours of incubation seems to be unrelated to cyclo-oxygenase down-

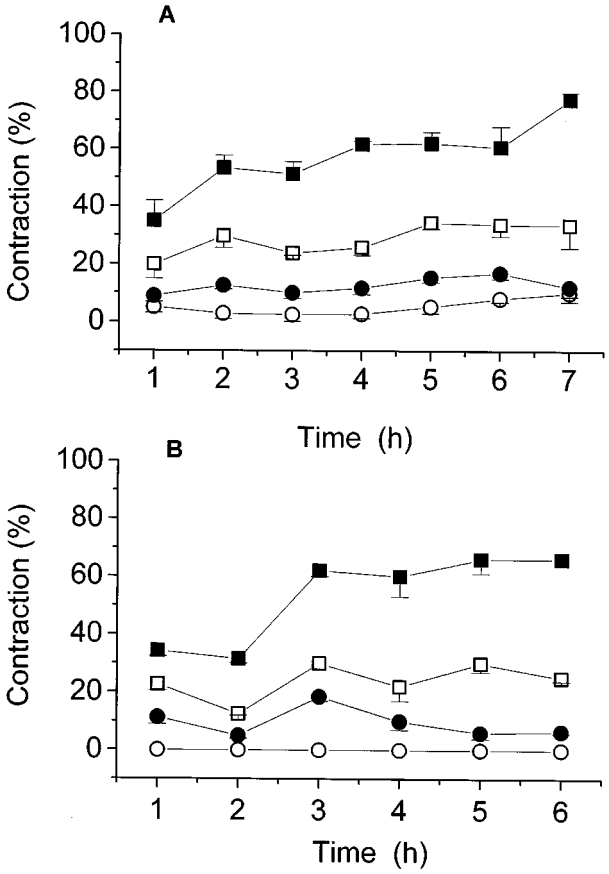

Figure 4 The contraction of mouse isolated strips of urinary bladder from two different mouse strains (A), Swiss albino, and (B), C57Bl/6J black induced by increasing concentrations of bradykinin (○, 1 nM; ●, 10 nM; □, 100 nM; ■, 1 μM). The effect of the bradykinin was tested after 1–6 h of incubation in the organ bath *in vitro*. The contraction was expressed as the percentage of the contraction caused by carbachol (1 μM). Entries are mean ± s.e.mean of at least five experiments.

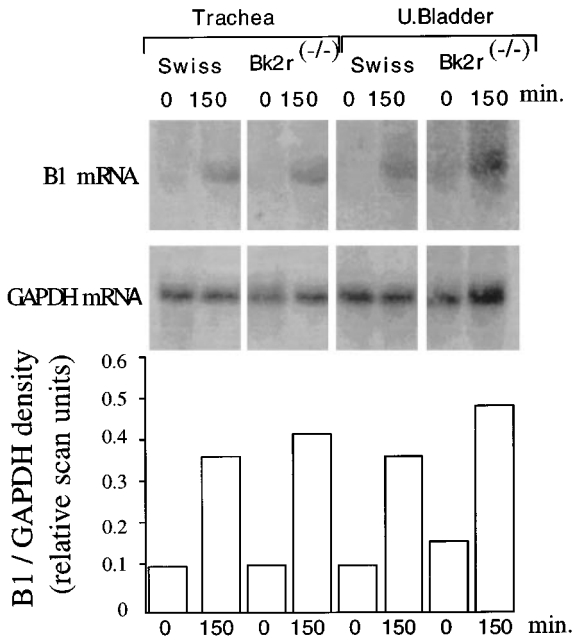

Figure 5 The steady state level of B_1 mRNA of mouse isolated tracheal rings and strips of urinary bladder of two different mouse strains (Swiss albino and *Bk2r*$^{-/-}$ mice) after 0 or 150 min of incubation in an organ bath. Top: Total RNA was extracted, and subjected to Northern analysis using 40 μg of RNA per lane. RNA was transferred to nylon membranes, and hybridized with a ^{32}P-labelled B_1 receptor cDNA probe as described under Methods. For standardization, the same blot was probed again with a cDNA fragment of the glyceraldehyde-3-phosphate deshydrogenase (GAPDH) gene. Blot shown is representative of three independent experiments. Bottom: The optical density of the B_1 receptor mRNA signals standardized by GAPDH optical density.

regulation. Secondly, although contraction to des-Arg^9-bradykinin observed in the urinary bladder was completely resistant to indomethacin, it was markedly upregulated.

The relaxant and contractile responses to bradykinin in the trachea and bladder, respectively, are putatively due to the activation of B_2 receptors. The increase in the response to bradykinin as a function of the incubation time was much less evident than that of des-Arg^9-bradykinin. Because B_2 receptors may be also upregulated and their expression increased by different conditions (Schmidlin *et al*., 1998) it is possible that incubation *in vitro* may cause a moderate upregulation of B_2 receptors in the mouse trachea and urinary bladder. However, additional factors, including changes in receptor-G protein coupling and in intracellular signalling pathways may be also involved.

Constitutive B_1 receptors seem to mediate plasma protein exudation in mouse pleura (Vianna & Calixto, 1998). The B_1 receptor in the trachea was, however, clearly inducible. Thus, it is possible that inducible (airways) and constitutive (pleural vessels) B_1 receptors are present in different areas of the respiratory system. Different mechanisms may be responsible for the upregulation of receptor mediated responses. In the present case, evidence is provided that upregulation of B_1 receptors both in the trachea and urinary bladder is due to *de novo* synthesis of the receptor protein, because cycloheximide ablated selectively the response to des-Arg^9-bradykinin in the trachea and urinary bladder. More importantly, the Northern blot analysis of the mRNA for the B_1 receptor showed a minimum expression of the mRNA in tissues freshly taken from the mouse, whereas after 150 min of incubation in the organ bath a much higher expression of the mRNA was measured. The time required for translational and post-translational processes of the mRNA is conceivable with the maximum response found after 6 h of incubation in both the trachea and bladder. To our knowledge this is the first demonstration of increased mRNA for the B_1 receptor in mouse tissue that parallels the increased functional response to des-Arg^9-bradykinin and one of the few examples in any animal species. This finding corroborates the hypothesis that incubation *in vitro* is a process that leads to *de novo* synthesis of B_1 receptors (Marceau, 1995). The transcription factor, NF-κB, and mitogen-activated protein (MAP) kinases seem to be involved in the induction of B_1 receptors in vascular smooth muscle cells in culture (Ni *et al*., 1998), in isolated rabbit aortic preparations (Larrivee *et al*., 1998) and in non-vascular cells, including human embryo lung fibroblasts (Zhou *et al*., 1998). The possibility that NF-κB and MAP kinases are involved in the upregulation of B_1 in the mouse trachea and urinary bladder remains to be studied. A recent paper reported that B_1 receptor is upregulated by its own agonist in human fibroblasts (Schanstra *et al*., 1998). The present findings do not support this mechanism of induction. The reasons for the difference, including human vs mouse cells or fibroblasts in culture vs whole tissue specimens, remain to be determined.

In the present study three different mouse strains were used. The ability of trachea and urinary bladder to express B_1

receptors is not apparently restricted to one individual mouse strain since both Swiss albino and C57Bl/J6 strains were able to show comparable response to des-Arg^9-bradykinin in the trachea and urinary bladder. We also studied the response to des-Arg^9-bradykinin in *Bk2r*$^{-/-}$ in order to investigate two hypotheses. First, we wondered whether disruption of B_2 receptor could be a sufficient reason to upregulate B_1 receptor *in vivo*. Second, we asked whether the ability of the tissue to upregulate the response to des-Arg^9-bradykinin (and to express the mRNA for the B_1 receptor) could be different in *Bk2r*$^{-/-}$ and in wild type mice. Experiments did not support any of these hypotheses: as observed in the two wild type mouse strains, in *Bk2r*$^{-/-}$ responses to des-Arg^9-bradykinin after 1–2 h of incubation were absent and expression of mRNA in freshly taken tissues was minor. In addition, the time course of the upregulation of the response to des-Arg^9-bradykinin in the *Bk2r*$^{-/-}$ strain and in the mouse strain (the C57Bl/J6 mice) used to produce the knockout mice, was comparable. In conclusion, the study of *Bk2r*$^{-/-}$ mice did not offer any evidence for a vicarious role of B_1 receptors when the B_2 is absent, at least *in vitro* in the mouse trachea and bladder.

In a previous paper the urinary bladder of C57/black mice was proposed as a monoreceptor preparation, where only bradykinin B_2 receptors are present (Nsa Allogho *et al*., 1995). Present findings obtained in two different wild type mouse strains, including the C57/black, and more importantly, in *Bk2r*$^{-/-}$ mice showed clearly that B_1 receptors are upregulated in this tissue. The failure of previous investigators to detect a B_1 receptor mediated response in the mouse urinary bladder (Nsa Allogho *et al*., 1995) might be due to the relatively short incubation time used in that study. It has been proposed recently that the mouse trachea is a new preparation that allows the study of B_1 receptors (Li *et al*., 1998). In that paper the response to des-Arg^9-bradykinin, detected after 45 min of incubation, was minor and it was considered to undergo marked tachyphylaxis. Therefore, concentration-response curves were constructed in a non cumulative manner, leaving at least a 30 min interval between challenges of increasing concentrations of des-Arg^9-bradykinin (Li *et al*., 1998). The present data question this view and give a different interpretation of the results obtained by Li and coworkers (1998). The response to des-Arg^9-bradykinin in the first 1–2 h of incubation is minor and the graded response is not evident because a few receptors are probably expressed on the plasma membrane at that time point. However, after 6 h of incubation expression of B_1 receptors is high and the graded response to des-Arg^9-bradykinin may be clearly shown. Thus, concentration response curves obtained in a non cumulative manner (Li *et al*., 1998) may reflect the time dependent upregulation of B_1 receptor. Obviously, different results of previous (Li *et al*., 1998) and present study may reflect additional factors, including the difference in the mouse strain used. The present data would suggest that to wait for the appropriate incubation time *in vitro*, is the most convenient way to study B_1 receptor in the isolated mouse trachea and urinary bladder. Because of the putative role of B_1 receptors in asthma and in urinary tract diseases, important issues deriving from the present study are those related to the possibility to upregulate B_1 receptors *in vivo* in these two tissues and their study in pathophysiological models of diseases.

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