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Evidence for BK_1 bradykinin-receptor-mediated prostaglandin formation in osteoblasts and subsequent enhancement of bone resorption

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1 The effects of the BK_1 bradykinin (BK)-receptor agonist des-Arg^9-BK on bone resorption and prostaglandin formation in osteoblasts have been studied.

2 Des-Arg^9-BK (1 μM) stimulated the release of ^{45}Ca from prelabelled neonatal mouse calvarial bones and the formation of prostaglandin E_2 (PGE_2) in calvarial bones. The stimulatory effect on bone resorption and PGE_2 formation could be totally inhibited by indomethacin, flurbiprofen and hydrocortisone.

3 The BK_1 receptor antagonist des-Arg^9-Leu^8-BK (10 μM) inhibited des-Arg^9-BK (0.01–0.1 μM)-induced release of ^{45}Ca from prelabelled neonatal mouse calvarial bones, while leaving BK (0.1–1 μM)-induced ^{45}Ca release unaffected.

4 In isolated osteoblast-like cells from neonatal mouse calvarial bones, des-Arg^9-BK (1 μM) induced a slowly developing increase in PGE_2 formation that was significantly different from untreated controls after 24 h. Treatment with BK caused a rapid burst (within minutes) of PGE_2 formation.

5 Des-Arg^9-Leu^8-BK (10 μM) selectively inhibited des-Arg^9-BK (1 μM)-induced PGE_2 and prostacyclin formation in isolated osteoblast-like cells incubated for 72 h. Des-Arg^9-Leu^8-BK did not affect BK and Lys-BK (1 μM)-induced PGE_2 and prostacyclin formation in isolated osteoblast-like cells incubated for 72 h.

6 These data indicate that osteoblasts are equipped with BK_1-receptors mediating enhanced prostaglandin formation and subsequent bone resorption.

Introduction

We have previously reported that the inflammatory mediator bradykinin (BK) is capable of stimulating bone resorption *in vitro* (Gustafson & Lerner, 1983). The stimulatory effect of BK on bone resorption is abolished by several inhibitors of prostaglandin formation, suggesting that the effect on bone resorption is mediated via enhanced endogenous prostaglandin formation in bone tissue (Gustafson & Lerner, 1983; Lerner *et al.*, 1987). This view is compatible with the observations that prostaglandin E_2 (PGE_2) and prostacyclin are potent stimulators of bone resorption *in vitro* (reviewed in Raisz & Martin, 1984). Furthermore, BK stimulates prostaglandin formation in isolated osteoblast-like cells from neonatal mouse calvarial bones and in the cloned murine osteoblastic cell lineage MC3T3-E1 (Lerner *et al.*, 1989), as well as in isolated human osteoblast-like cells (Ljunggren *et al.*, 1990). Based upon these findings, we have proposed that BK may serve as a mediator of the inflammatory induced bone resorption seen in areas of chronic inflammatory processes, e.g. rheumatoid arthritis, periodontitis and osteomyelitis.

There are at least two subtypes of BK receptors, the BK_1- and BK_2 receptors (Regoli & Barabé, 1980). These receptors have been pharmacologically characterized by using different kinin analogues with agonistic and antagonistic properties (Regoli & Barabé, 1980; Vavrek & Stewart, 1985). By comparing the rank order potency of such analogues, we have recently found that osteoblasts are equipped with BK_2-receptors mediating prostaglandin formation (Ljunggren & Lerner, 1987). However, we have also found that the BK_1-receptor agonist des-Arg^9-BK enhances bone resorption *in vitro* (Lerner *et al.*, 1987). The aim of this study was therefore to determine whether des-Arg^9-BK induced bone resorption was due to a partial BK_2 agonistic effect, or if there may exist a BK_1-receptor-mediated mechanism involved in inflammatory induced bone resorption.

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Methods

Measurement of bone resorption

Bone resorption was assessed by measuring the release of ^{45}Ca from neonatal mouse calvarial bones, prelabelled with 1.5 μCi ^{45}Ca 4 days prior to dissection (Reynolds, 1976; Lerner, 1987). The calvarial bones were dissected into four fragments and only the posterior 2/3 of the parietal bones were used. The sensitivity in the neonatal mouse calvarial bone resorption assay is the same whether we use calvarial halves or calvarial quarters (data not shown). The samples were preincubated for 24 h in CMRL 1066 culture medium containing indomethacin (1 μM). This was done in order to reduce basal ^{45}Ca release in untreated controls (Lerner, 1987). The bones were then thoroughly rinsed and subsequently incubated for 72 h in CMRL 1066 containing test substances or vehicle. After 72 h incubation, ^{45}Ca-release was determined by analyzing the amount of radioactivity in the media, using liquid scintillation. The bones were dissolved in HCl and the remaining radioactivity was measured. The results were expressed as the percentage release of initial ^{45}Ca (Lerner, 1987).

Isolation and culture of osteoblast-like cells from mouse calvarial bones

Calvarial bones from 2–3 days-old mice were dissected out and bone cells were isolated by sequential enzymatic digestion (Boonekamp *et al.*, 1984). The calvarial bones were transferred to Ehrlenmeyer flasks in a shaking water bath (37°C), treated with EDTA (0.4 mM) in phosphate buffered saline (PBS) for 3 × 10 min, washed with PBS and subsequently treated with bacterial collagenase (180 u ml^{-1}) in PBS without EDTA for 4 × 10 min. The cells from the two last digestion fluids were collected, centrifuged, resuspended in Alpha Modification of Eagle's Medium (α-MEM) with 10% Foetal Calf Serum (FCS), and seeded in 25 cm^2 tissue culture flasks. When confluent, the cells were detached with trypsin (1 mg ml^{-1}) and reseeded in

2 cm^2 multiwell culture dishes. Only cells from passages 1–2 were used in the experiments. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Determination of PGE_2 formation in calvarial bones

Calvarial bones were dissected, preincubated with indomethacin and rinsed as described above. The bones were thereafter incubated in CMRL 1066 with test substances or vehicle for 72 h. Samples from the incubation media were withdrawn, acidified to pH 3.5 and stored at −20°C. The amount of PGE_2 released from the calvarial bones into the medium, during the incubation, was then analyzed as described below.

Determination of PGE_2 and 6-keto-$PGF_{1\alpha}$ production in osteoblasts

Prior to the experiments, the cell layers were rinsed twice with prewarmed Tyrode solution and preincubated for 30 min in α-MEM without FCS. The preincubation media were then removed and fresh medium with test substances or vehicle was added (500 μl/well). At different time points, as stated in legends, the media were removed, acidified to pH 3.5 with HCl, frozen and stored at −20°C. The amounts of PGE_2 and prostacyclin (as assessed by analysis of the stable breakdown product 6-keto-$PGF_{1\alpha}$) in the media were analyzed with commercially available Radio Immuno Assay (RIA)-kits with [^{125}I]-PGE_2 and [^{125}I]-6-keto-$PGF_{1\alpha}$ as tracers. After the experiments, the cells were detached with trypsin and counted in a haemocytometer.

Statistics

Statistical evaluation of the data was performed by use of Student's *t* test for unpaired samples.

Materials

Hydrocortisone, BK, Lys-BK, des-Arg^9-BK, des-Arg^9-Leu^8-BK and serum albumin were from Sigma Chemical Co., St. Louis, MO, U.S.A.; CMRL 1066 medium, α-MEM and FCS from Flow Laboratories, Irvine, Scotland; bacterial collagenase (clostridium, type 1) from Wortington Biochemical, Freehold, NJ, U.S.A.; $^{45}CaCl_2$ (specific activity 20–30 Ci g^{-1}), PGE_2 and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), RIA-kits from New England Nuclear Chemicals, Dreieich, West Germany; multiwell plastic culture dishes from Costar, Cambridge, Mass, U.S.A. Flurbiprofen was kindly provided by Astra, Södertälje, Sweden. Indomethacin was a kind gift from Merck, Sharp and Dohme, Haarlem, The Netherlands.

Results

Des-Arg^9-BK (1 μM) and BK (1 μM) stimulated the release of prelabelled ^{45}Ca from neonatal mouse calvarial bones during 72 h incubation (Table 1). In addition, BK and des-Arg^9-BK (1 μM) also enhanced the formation of PGE_2 in the calvarial bones (Table 1). Addition of indomethacin (1 μM) to the incubation medium totally inhibited BK and des-Arg^9-BK-induced formation of PGE_2 and release of prelabelled ^{45}Ca (Table 1). Two additional, structurally different, inhibitors of prostaglandin synthesis flurbiprofen (1 μM) and hydrocortisone (1 μM) also totally abolished des-Arg^9-BK-induced formation of PGE_2 and release of prelabelled ^{45}Ca from calvarial bones incubated for 72 h (Table 1).

Des-Arg^9-BK, dose-dependently, stimulated ^{45}Ca release from prelabelled neonatal mouse calvarial bones cultured for 72 h (Figure 1). The lowest concentration of des-Arg^9-BK causing a statistically significant effect was 10 nM and a maximal stimulatory effect (130% of controls) was seen at 1 μM (mean from 5 experiments; Figure 1). The BK_1-receptor antagonist des-Arg^9-Leu^8-BK (10 μM) totally inhibited the effect of des-Arg^9-BK at low concentrations (0.01–0.1 μM), and significantly reduced the effect of 1 μM des-Arg^9-BK (Figure 1). Des-Arg^9-Leu^8-BK had no effect by itself on ^{45}Ca release from prelabelled calvarial bones (Figure 1).

BK (0.1 and 1 μM)-induced release of ^{45}Ca from prelabelled calvarial bones was not sigificantly affected by des-Arg^9-Leu^8-BK (10 μM; Table 2).

In isolated osteoblast-like cells from neonatal mice, BK (1 μM), but not des-Arg^9-BK (1 μM), induced a rapid formation of PGE_2 (within minutes). An elevated amount of PGE_2 in the incubation media, withdrawn from cells treated with BK, was sustained for 48 h (Figure 2). In these long term incubations, a stimulatory effect of des-Arg^9-BK (1 μM) on the formation of PGE_2 developed slowly during culture, causing a significant stimulation after 24 h (Figure 2). The degree of stimulation at 24 and 48 h, however, was substantially less than that obtained with BK (Figure 2).

During 48 h incubation of osteoblast-like cells, the BK_1 receptor antagonist des-Arg^9-Leu^8-BK (10 μM) significantly inhibited des-Arg^9-BK (1 μM)-induced PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis (Table 3). BK (1 μM) and Lys-BK (1 μM)-induced formation of PGE_2 and 6-keto-$PGF_{1\alpha}$ in 48 h incu-

Table 1 Effect of bradykinin (BK) and des-Arg^9-BK on prostaglandin E_2 (PGE_2)-formation and ^{45}Ca-release in neonatal mouse calvarial bones

Agonist	*Conc.* (μM)	*Inhibitor*	*Conc.* (μM)	*PGE_2* (pg/bone)	*^{45}Ca* (%-release)
—	—	—	—	72.5 ± 9.8	27.1 ± 1.1
—	—	Indomethacin	1	14.8 ± 1.5[a]	24.6 ± 0.6
—	—	Flurbiprofen	1	12.5 ± 1.8[a]	25.0 ± 0.9
—	—	Hydrocortisone	1	38.8 ± 9.8	29.3 ± 1.6
BK	1	—	—	783.3 ± 46.3[a]	51.0 ± 2.3[a]
BK	1	Indomethacin	1	18.3 ± 5.5[a,b]	28.4 ± 0.6[b]
des-Arg^9-BK	1	—	—	301.0 ± 40.5[a]	41.3 ± 3.2[a]
des-Arg^9-BK	1	Indomethacin	1	12.8 ± 2.0[a,c]	28.2 ± 1.5[c]
des-Arg^9-BK	1	Flurbiprofen	1	22.3 ± 3.3[a,c]	30.0 ± 1.0[c]
des-Arg^9-BK	1	Hydrocortisone	1	37.5 ± 5.5[a,c]	33.8 ± 1.2[c]

Prelabelled neonatal mouse calvarial bones were dissected into four fragments using only the posterior two thirds of the parietal bones. After 24 h preincubation with indomethacin (1 μM) the samples were rinsed and incubated for 72 h in CMRL 1066 with test substances or vehicle. After the incubation, the amount of ^{45}Ca released into the medium was calculated by liquid scintillation and the amount of PGE_2 released to the medium was calculated by RIA.

The values are means ± s.e.mean for 5–6 samples.

[a] Significantly different from untreated controls ($P < 0.01$).

[b] Significantly different from BK-treated bones ($P < 0.01$).

[c] Significantly different from des-Arg^9-BK-treated bones ($P < 0.01$).

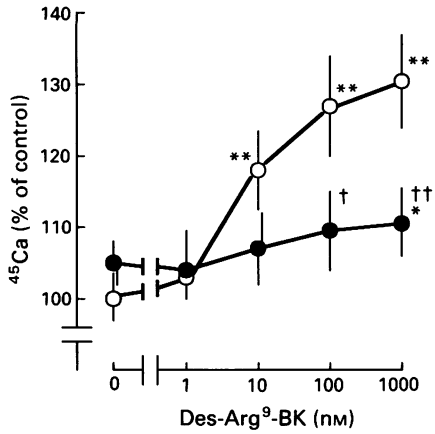

Figure 1 Effect of the BK_1 antagonist des-Arg^9-Leu^8-BK on des-Arg^9-BK-induced bone resorption. Prelabelled neonatal mouse calvarial bones were dissected into four fragments using only the posterior two thirds of the parietal bones. The fragments were preincubated in indomethacin (1 μM) for 24 h. Thereafter the bones were rinsed and incubated for 72 h submerged in 2.5 ml CMRL 1066 culture medium with test substances or vehicle. The amount of ^{45}Ca released into the culture media was analysed by liquid scintillation. (○) Des-Arg^9-BK; (●) des-Arg^9-BK + des-Arg^9-Leu^8-BK (10 μM). Values represent pooled data (means with ± s.e.mean shown by vertical lines) from 5 separate experiment with 5–7 bone fragments in each group. The data are presented as % of control with the control values set as 100%. Basal resorption in the 5 experiments (means ± s.e.mean) were 19.40 ± 1.97%; 25.92 ± 1.50%; 33.34 ± 1.90%; 34.71 ± 2.88% 41.18 ± 1.44%. Significantly different from untreated controls: * $P < 0.05$; ** $P < 0.01$. Significantly different from des-Arg^9-BK: † $P < 0.05$; †† $P < 0.01$.

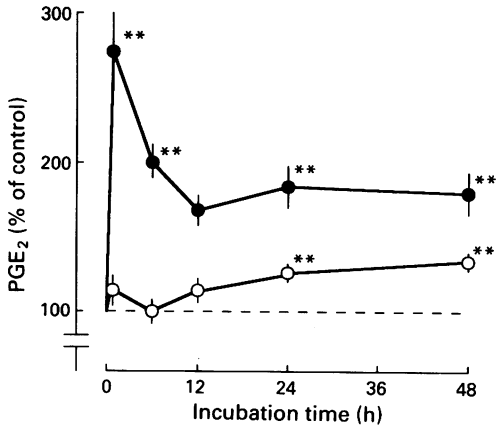

Figure 2 Long term time course of bradykinin (BK) and des-Arg^9-BK-induced prostaglandin E_2 (PGE_2) formation in isolated osteoblast-like cells. Osteoblast-like cells were isolated from neonatal mouse calvarial bones by sequential enzymatic digestion with bacterial collagenase. The cells were seeded in multiwell culture dishes. Prior to the experiments, the cell layer were rinsed and the cells were subsequently incubated in α-MEM containing 1% FCS with or without kinins. At different time points, the media were withdrawn and acidified to pH 3.5 and frozen. The cells were detached with trypsin and counted in a haemocytometer. PGE_2 in the incubation media was analyzed by RIA. Symbols represent pooled data from three different experiments and are expressed as % of control with the control values set at 100%. The mean amount of PGE_2 ± s.e.mean in the control wells (pg/1000 cells) after 48 h was 2.27 ± 0.18 (55,000 cells/cm^2), 0.90 ± 0.01 (62,000 cells/cm^2) and 15.52 ± 0.86 (28,800 cells/cm^2). (●) BK (1 μM); (○) des-Arg^9-BK (1 μM). Significantly different from untreated controls ** $P < 0.01$.

bation of osteoblast-like cells was not affected by des-Arg^9-Leu^8-BK (10 μM; Table 3); nor were the basal levels of PGE_2 and 6-keto-$PGF_{1\alpha}$ affected by des-Arg^9-Leu^8-BK.

Discussion

Prolonged incubation with the BK_1 receptor agonist des-Arg^9-BK stimulated prostaglandin formation in isolated osteoblast-like cells from neonatal mouse calvarial bones. This was not due to a weak agonistic effect on BK_2-receptors since the BK_1 antagonist des-Arg^9-Leu^8-BK, in long term incubations, selectively inhibited des-Arg^9-BK induced prostaglandin synthesis whereas BK and Lys-BK induced prostaglandin synthesis was unaffected. Kinetic analysis revealed that the stimulatory effect of BK on PGE_2 synthesis was seen very rapidly (within minutes) whereas that of des-Arg^9-BK was observed first after a time lag of 24 h. The decreasing treated-versus-control ratio for BK-treated cells between 2–6 h (Figure 2) was due to a slow increase in PGE_2 formation in the control cells and not to decreasing amounts of PGE_2 in the culture media. The selective inhibitory action of des-Arg^9-Leu^8-BK and the different time courses indicate the presence of both BK_1 and BK_2 receptors in osteoblasts linked to prostanoid formation. This is further supported by our finding that both BK and des-Arg^9-BK are stimulators of ^{45}Ca release from prelabelled neonatal mouse calvarial bones *in vitro*, by a mechanism that was abolished by agents that inhibit prostaglandin synthesis (e.g. indomethacin and hydrocortisone). In addition, des-Arg^9-Leu^8-BK inhibited des-Arg^9-BK- but not BK-induced ^{45}Ca release from the calvarial bones. Thus, the data obtained in neonatal mouse calvarial bones are compatible with the data obtained in the isolated osteoblast-like cells.

Although the effect of BK in most cells is due to an action on BK_2 receptors (Regoli & Barabé, 1980), osteoblasts are not the only cell type which express BK_1 receptors. Thus it has been reported that this BK receptor subtype is present in isolated arterial vessels (Churchill & Ward, 1986; Guimaraes *et al.*, 1986; Rhaleb *et al.*, 1989). In addition there are reports showing that the BK_1 receptor is induced and expressed by the cells due to tissue damage or exposure to various substances e.g. lipopolysaccharides and interleukin-1β (IL-1) (Regoli *et al.*, 1978; 1981; Barabé *et al.*, 1982; Deblois *et al.*, 1988). It is therefore possible that the isolated osteoblasts synthesize and express BK_1 receptors due to damage during the isolation and culture procedures or due to an autocrine regulation by IL-1 synthesized during culture. Such a mechanism is supported by the lag period in the kinetics of des-Arg^9-BK induced formation of prostanoids. The view, however, that the presence of BK_1 receptors is only due to induction, has been challenged by data obtained with different preparations of iso-

Table 2 Effect of des-Arg^9-Leu^8-BK on BK-induced ^{45}Ca release from prelabelled neonatal mouse calvarial bones

		^{45}Ca-release (% of control)	
Agonist	*Conc.* (μM)	−des-Arg^9-Leu^8-BK	+des-Arg^9-Leu^8-BK
—	—	100.0 ± 3.9	106.1 ± 4.3
BK	0.1	128.6 ± 8.9[a]	149.2 ± 12.2[a]
BK	1.0	160.1 ± 13.9[a]	145.7 ± 9.5[a]

Calvarial bones from neonatal mice were dissected into four fragments using only the posterior two thirds of the parietal bones. After 24 h preincubation with indomethacin (1 μM) the samples were rinsed with Tyrode solution and subsequently incubated for 72 h in CMRL 1066 with test substances or vehicle. Thereafter the % release of ^{45}Ca into the culture media was analysed by liquid scintillation. Values represent pooled data from 3 different experiments and are expressed as % of control with the ^{45}Ca release in untreated controls set as 100%. The basal ^{45}Ca % release in untreated controls (mean ± s.e.mean) for the 3 experiments were 41.18 ± 1.44 ($n = 5$); 25.92 ± 1.51 ($n = 6$); 19.40 ± 1.97 ($n = 6$).
Values represent means ± s.e.mean for 17 samples from three different experiments.
[a] Significantly different from untreated controls ($P < 0.01$).

Table 3 Effect of the BK_1 receptor antagonist des-Arg^9-Leu^8-BK on BK, Lys-BK and des-Arg^9-BK induced prostaglandin formation in isolated osteoblast-like cells

Agonist	*Conc.* (μM)	*Addition*	*Conc.* (μM)	*Prostaglandin formation* (pg/1000 cells) PGE_2	*6-keto-$PGF_{1\alpha}$*
—	—	—	—	57.2 ± 9.6	7.6 ± 0.4
—	—	des-Arg^9-Leu^8-BK	10	47.7 ± 6.6	7.1 ± 0.3
BK	1	—	—	158.4 ± 4.6[a]	22.3 ± 0.7[a]
BK	1	des-Arg^9-Leu^8-BK	10	153.3 ± 8.4[a]	20.8 ± 1.3[a]
Lys-BK	1	—	—	129.5 ± 21.6[a]	20.3 ± 1.3[a]
Lys-BK	1	des-Arg^9-Leu^8-BK	10	136.4 ± 2.9[a]	21.2 ± 1.2[a]
des-Arg^9-BK	1	—	—	108.8 ± 12.1[a]	12.9 ± 2.3[a]
des-Arg^9-BK	1	des-Arg^9-Leu^8-BK	10	64.3 ± 6.4[b]	10.1 ± 0.7[c,d]

Osteoblast-like cells were insolated from neonatal mouse calvarial bones by sequential enzymatic digestion. Prior to the experiment, the cell layers were rinsed with Tyrode solution and subsequently incubated in α-MEM containing 1% FCS with kinins or vehicle for 48 h. After the experiment, the incubation media were withdrawn, acidified to pH 3.5 and stored at −20°C. The amounts of prostaglandin E_2 (PGE_2) and 6-keto-$PGF_{1\alpha}$ in the culture media were analysed with RIA.
Values represent means ± s.e.mean for 6 wells.
[a] Significantly different from untreated controls ($P < 0.01$).
[b] Significantly different from des-Arg^9-BK ($P < 0.01$).
[c] Significantly different from untreated controls ($P < 0.05$).
[d] Significantly different from des-Arg^9-BK (0.05).

lated arterial vessels indicating that BK_1 receptors in these organs are not generated *de novo*, but present from the beginning of the isolation (Churchill & Ward, 1986; Guimaraes *et al.*, 1986; Rhaleb *et al.* 1989). The recent report that the osteoblastic osteosarcoma cell line ROS 17/2.8 is equipped with BK_1 receptors specifically binding des-Arg^9-BK (Santora, 1989), indicates that BK_1 receptors may be a constituent feature of osteoblasts.

BK can stimulate prostaglandin biosynthesis in various organs including kidney, heart, lung, blood vessels, spleen and uterus (for rev. see Nasjletti & Malik, 1979). In these organs the prostaglandin response has been observed only after stimulation with BK and analogues with affinity for the BK_2 receptor. Bone tissue is the only tissue, to our knowledge, where it has been directly shown that BK_1 receptors mediate enhanced prostaglandin synthesis. Indirect evidence, however, that the BK_1 receptor may stimulate prostaglandin synthesis has been reported from studies on the rabbit mesenteric and dog renal arteries, where the relaxant effects of des-Arg^9-BK can be blocked by indomethacin (Churchill & Ward, 1986; Rhaleb *et al.*, 1989).

In conclusion, our data indicate that both BK_1 and BK_2 receptors on osteoblasts are mediating an increase in prostaglandin synthesis. The large differences in time course of the response to specific agonists for the two receptors indicate that different signal transducing systems are linked between receptor occupancy and prostaglandin synthesis. It is very interesting to note that, like our observations on prostaglandin biosynthesis, the effect of des-Arg^9-BK on relaxation of dog renal arteries is considerably slower than that caused by BK (Rhaleb *et al.*, 1989). Our data, together with those of others (Regoli *et al.*, 1981; Deblois *et al.*, 1988) may suggest that in areas adjacent to inflammatory processes, bone tissue may become more sensitive to kinins due to upregulation of the number of BK_1 receptors in addition to the already present BK_2 receptors.

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