

Bovine adrenal glomerulosa cells express such a low level of functional B₂ receptors that bradykinin does not significantly increase their aldosterone production

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Abstract

It was recently demonstrated that bradykinin (BK) stimulates aldosterone secretion in bovine adrenal glomerulosa (BAG) cells. The aim of the present study was to characterize the mechanism of action of BK on these cells. Binding experiments with the radioligand ¹²⁵I-[Tyr⁸]BK revealed the presence of a relatively small amount ($B_{\max}=180 \pm 55$ fmol/mg of protein) of high affinity ($K_d=0.65 \pm 0.17$ nM) binding sites. BK induced a time- and concentration-dependent increase of [³H]inositol trisphosphate ([³H]IP₃) in myo-[³H]inositol-labeled BAG cells. A maximal response was obtained with 10 nM BK and the EC₅₀ value was 1.0 ± 0.5 nM. ¹²⁵I-[Tyr⁸]BK binding and BK-induced IP₃ production were inhibited by the selective B₂ receptor antagonist Icatibant (1 μM) and unaffected by the selective B₁ receptor antagonist [DesArg⁹,Leu⁸]BK (1 μM). In fura-2 loaded BAG cells, BK (100 nM) induced a typical biphasic Ca²⁺ response composed of a rapid and transient increase of intracellular Ca²⁺ concentration [Ca²⁺]_i which slowly declined to a level that remained above basal level for about 5 min. In the presence of EGTA (2 mM), the rapid and transient calcium increase was unaffected whereas the plateau phase was abolished. Angiotensin II (Ang II, 100 nM) also

elicited a typical biphasic response in BAG cells. However the rapid and transient elevation of [Ca²⁺]_i was followed by a sustained plateau phase which remained above the basal level for more than 10 min. Although BAG cells express functional B₂ receptors, no secretion of aldosterone was observed after stimulation with 100 nM BK for 120 min. Under the same conditions Ang II increased by about 10-fold the basal level of aldosterone. The lack of effect of BK is probably attributable to its very transient effect on IP₃ production. Pretreatment of BAG cells with 100 nM BK for 20 min reduced by $70 \pm 10\%$ their total binding capacity. These results suggest a rapid and very efficient desensitization process. We conclude that BAG cells express functional B₂ receptors. The weak production of second messengers and the rapid desensitization process could explain why BK fails to increase aldosterone production in these cells. Since functional B₂ receptors are expressed in BAG cells it is likely that under some specific physiological or pathological conditions these receptors may play a significant role in aldosterone secretion. However these conditions remain to be determined.

Journal of Endocrinology (1998) **156**, 449–460

Introduction

The nonapeptide bradykinin (BK) has a wide range of biological effects on a number of different tissues and organs. BK is mostly known for its potent vasodilatory effect. BK is also involved in smooth muscle contraction, regulation of vascular permeability, pain transmission, cell proliferation and development of pathological states such as asthma and inflammation (Bhoola *et al.* 1992, Hall 1992).

BK is generated from kininogen by the proteolytic action of kallikreins which are present in many tissues. Two pharmacologically distinct types of kinin receptors (B₁ and B₂) have been identified (Regoli & Barabé 1980).

The B₁ receptor is selectively activated by BK analogs lacking the C-terminal Arg ([DesArg⁹]BK) whereas the B₂ receptor is primarily recognized by intact BK. The recent cloning of cDNAs for B₁ (Menke *et al.* 1994) and B₂ receptors (McEachern *et al.* 1991, Eggerickx *et al.* 1992, Hess *et al.* 1992) provided data agreeing with the pharmacological classification of these receptors. Cloning experiments have also revealed that these two receptors belong to the superfamily of seven transmembrane-domain, G protein-coupled receptors (Dohman *et al.* 1991, Strader *et al.* 1994). The activation of B₁ and B₂ receptors leads to the hydrolysis of polyphosphoinositides by phospholipase C (PLC) (Hong & Deykin 1980, Yano *et al.* 1984, Tilly

et al. 1987, Issandoue & Darbon 1991, Tropea *et al.* 1993, Schneck *et al.* 1994), generating the second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). DAG directly activates protein kinase C, whereas IP₃ indirectly activates calmodulin kinase through the release of Ca²⁺ from intracellular stores (Berridge 1993). It was demonstrated that B₂ receptors can also activate phospholipase A₂ (Hong & Levine 1978, Hong & Deykin 1980, Roscher *et al.* 1983), tyrosine phosphorylation (Leeb-Lunberg & Song 1991, 1993, Leeb-Lunberg *et al.* 1994) and membrane ionic fluxes (Reiser & Hamprecht 1982, Higashida *et al.* 1986).

It was shown by Staszewska-Barczak & Vane (1967) that the adrenal medulla could generate BK which could stimulate the release of catecholamines from chromaffin cells. More recently Scicli *et al.* (1991) identified, in canine adrenal cortex, a kallikrein-like kininogenase that was present at a concentration similar to that found in the adrenal medulla. Wang *et al.* (1996) demonstrated the presence of B₂ receptor mRNA in zona glomerulosa by *in situ* hybridization histochemistry. These studies suggested that BK could play a role in the adrenal cortex. Rosolowsky & Campbell (1992) reported that BK stimulates the release of aldosterone from cultured bovine adrenal glomerulosa (BAG) cells. They proposed that BK could participate in the regulation of aldosterone secretion. In more recent studies, neither Rudichenko *et al.* (1993) nor Malendowicz *et al.* (1995) could find any physiological correlation to suggest that in the rat, BK regulates aldosterone secretion *in vivo* or *in vitro*. However Malendowicz *et al.* (1995) clearly observed a rise of intracellular Ca²⁺ in BK-stimulated rat adrenal glomerulosa cells.

The purpose of the present study was to characterize BK receptors of BAG cells and to examine their mechanism of action. We demonstrate that BAG cells express a B₂ receptor. Stimulation of this B₂ receptor leads to the activation of PLC which is responsible for the generation of IP₃ and the mobilization of Ca²⁺. Under our experimental conditions, BK did not induce any aldosterone secretion by BAG cells. This could be attributed to the weak production of second messengers and the rapid desensitization process that inactivates the low-abundant B₂ receptor population expressed by BAG cells.

Materials and Methods

Materials

Culture media and collagenase were from GIBCO BRL (Great Island, NY, USA). DNase I was from Sigma (St Louis, MO, USA). The Ca²⁺ fluorescent indicator fura-2 acetoxymethyl ester (fura-2 AM) was purchased from Calbiochem (San Diego, CA, USA). Myo-[³H]inositol (83 Ci/mmol) was obtained from Amersham

Radiochemical (Arlington Heights, IL, USA). Anion exchange resin AG 1-X8 (formate form) was from Bio-Rad (Richmond, CA, USA). BK, [Tyr⁸]BK, [DesArg⁹]BK, [DesArg⁹,Leu⁸]BK and Icatibant ([D-Arg⁰,Hyp³,β-(2-thienyl)-Ala⁵, D-Tic⁷,Oic⁸]BK; HOE-140) were purchased from Bachem California (Torrance, CA, USA). Antibovine adrenal P450_{11β} antibodies were generously provided by Dr J G Lehoux (Université de Sherbrooke, Québec, Canada). Antialdosterone antibodies were generously provided by Dr Alain Bélanger (Centre Hospitalier de l'Université Laval, Québec, Canada). Sheep antirabbit IgG-fluorescein antibody F(ab')₂ fragment was from Boehringer Mannheim GmbH (Mannheim, Germany). All other reagents were from Sigma. [¹²⁵I]-[Tyr⁸]BK (1000 Ci/mmol) was prepared with Iodogen and purified by high performance liquid chromatography on a C18 column, as previously described (Boulay *et al.* 1994).

Cell culture

Bovine adrenal glands were obtained at a nearby slaughterhouse. BAG cells were prepared as described by Boulay *et al.* (1992). Briefly, outer 0.5 mm slices of bovine adrenal cortex were minced into 1 mm² fragments and digested with 2 mg/ml collagenase and 0.2 mg/ml DNase I, followed by a mechanical dispersion. This procedure was repeated five times. After two washes, BAG cells were purified on a Percoll gradient (20%). The gradient was prepared by centrifugation at 35 000 g for 30 min at 4 °C. BAG cells were poured on top of the Percoll gradient and centrifuged at 500 g for 15 min at 4 °C. BAG cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) and washed by centrifugation at 300 g for 10 min at 4 °C.

BAG cells were resuspended in DMEM supplemented with 10% fetal bovine serum, 1% GIBCO Insulin-Transferrin-Selenium-X (serum supplement containing insulin 1 g/l, transferrin 0.55 g/l and selenium 0.7 mg/l), 50 U/ml penicillin, 60 mg/ml streptomycin and 2 mM L-glutamine. Cells were plated at a density of 1.5 × 10⁵ cells per well (15 mm diameter wells) for binding assays and for phosphatidylinositol hydrolysis assays. They were plated at a density of 1 × 10⁶ cells per cover slip (13 × 22 mm) for Ca²⁺ measurements. Cells were cultured at 37 °C, in a CO₂ incubator (5% CO₂-95% air) and the medium was changed after 24 h. Cell viability before plating (trypan blue exclusion) was always superior to 80%. Most of the experiments described in this study were performed with cells grown to confluence in 24-well culture plates (about 6 × 10⁵ cells/well). At the end of each experiment, representative wells were trypsinized and cells were counted with a hemocytometer. Each experiment contained its own controls, so that no corrections were needed for variations in cell density from one experiment to another.

Immunofluorescence studies

BAG cells cultured on glass coverslips were washed twice with ice-cold PBS. Cells were then fixed and permeabilized with methanol for 10 min at -20°C . After 10 min of rehydration with PBS at room temperature, non-specific binding sites were blocked with PBS containing 1% BSA for 30 min. Cytochrome P450_{11 β} was revealed with antiovine adrenal P450_{11 β} antibody (50 $\mu\text{g}/\text{ml}$). Incubation lasted for 1 h at room temperature and was followed by three successive washes with PBS. BAG cells were then incubated for 1 h with a sheep antirabbit IgG-fluorescein antibody (1:30). After three washes with PBS, coverslips were mounted on microscope slides and immunofluorescence was evaluated with a Leitz DM RBE microscope (Leica GmbH, Wetzlar, Germany) equipped for epifluorescence. Pictures were obtained with a Kodak TMX 100 film.

Flow cytometry

BAG cells were gently digested with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) for 10 min at 37°C . Cells were then washed and permeabilized with ice-cold PBS containing 0.05% saponin for 10 min at 4°C . Afterwards, cells were incubated for 30 min at room temperature, in PBS, with antiovine adrenal P450_{11 β} antibodies (10 $\mu\text{g}/\text{ml}$), washed by centrifugation at 200 g and labeled for 30 min at room temperature, in PBS, with a 1:30 dilution of a sheep antirabbit IgG-fluorescein antibody. Cells were then analyzed with a Becton-Dickinson flow cytometer.

Binding assays and receptor autoradiography

BAG cells were incubated in binding buffer (25 mM Tris-HCl pH 7.0, 250 mM dextrose, 1 mM phenanthroline-1,10, 1 mM captopril, 140 $\mu\text{g}/\text{ml}$ bacitracin, 1 mM dithiothreitol and 1 mg/ml BSA). Incubations were performed for 1 h (or as indicated) at 22°C in a final volume of 500 μl , in the presence of ^{125}I -[Tyr⁸]BK (0.2 nM or as indicated) and selected concentrations of unlabeled ligands. Non-specific binding was determined in the presence of 1 μM [Tyr⁸]BK. Incubations were terminated by two successive immersions of the plates in ice-cold washing buffer. Cell-associated radioactivity was then evaluated by γ counting following solubilization of the cells with 0.1 M NaOH. For receptor autoradiography studies, cell-associated radioactivity was evaluated with a Kodak BioMax MS film that was exposed for 8 h at -80°C .

IP₃ production

Cells were labeled for 20 h in inositol-free DMEM containing 20 $\mu\text{Ci}/\text{ml}$ myo- ^3H inositol. Cells were then

stimulated with BK or angiotensin II (Ang II) and incubations were terminated at selected times by addition of ice-cold perchloric acid (5% v/v). After centrifugation at 15 000 g for 15 min, the supernatants, containing water-soluble inositolphosphates (IPs) were extracted with a mixture of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine (1:1). The samples were vigorously mixed and centrifuged at 15 000 g for 1 min. The upper phase containing the IPs was applied to an AG 1-X8 resin column and IPs were sequentially eluted by addition of ammonium formate/formic acid mixtures of increasing ionic strength as described by Berridge *et al.* (1983).

Fluorometric monitoring of intracellular Ca²⁺

Measurement of intracellular Ca²⁺ ($[\text{Ca}^{2+}]_i$) variations in BAG cells was done according to the method of Morgan-Boyd *et al.* (1987) with some modifications. Briefly, cells grown on cover slips were washed with PBS containing 137 mM NaCl, 3.5 mM KCl, 0.9 mM CaCl₂, 1 mM MgCl₂, 3.5 mM NaH₂PO₄, 16.5 mM Na₂HPO₄ and 5.5 mM dextrose, pH 7.4 at room temperature. Cells were incubated with fura-2-AM (2 μM) for 45 min at 37°C in Medium 199 (M199) containing 25 mM Hepes (pH 7.4) and 3 mg/ml BSA. After loading, excess fura-2-AM was removed by washing. The cover slips were immobilized and the cells were superfused with PBS at a rate of 2 ml/min. Fura-2 fluorescence (excitation at 340 nm and 380 nm and emission at 510 nm) was recorded in a Hitachi F-2000 spectrofluorimeter.

Aldosterone secretion

BAG cells were washed twice and incubated in M199 with 3.5 mM K⁺, 25 mM Hepes (pH 7.4), 1 mg/ml BSA, 0.1 mg/ml bacitracin, 1 mM phenanthroline-1,10, 1 mM captopril, 1 mM dithiothreitol and 100 nM BK or Ang II for different periods of time at 37°C . The aldosterone content of the medium was measured by RIA (Connolly *et al.* 1980).

Desensitization experiments

BAG cells were washed twice with 500 μl PBS and incubated in 500 μl M199 containing 25 mM Hepes (pH 7.4), 1 mM phenanthroline-1,10, 1 mM captopril, 140 $\mu\text{g}/\text{ml}$ bacitracin, 1 mM dithiothreitol and 1 mg/ml BSA. Incubations were performed at 37°C with selected concentrations of BK. After different periods of time, BAG cells were washed twice with 500 μl PBS and incubated in 500 μl of an acid solution (150 mM NaCl, 50 mM glycine, 1 mM CaCl₂ and 5.5 mM dextrose, pH 3.0) for 30 min at 4°C . We controlled that this acid wash completely dissociated receptor-bound BK. Cells were then rinsed with

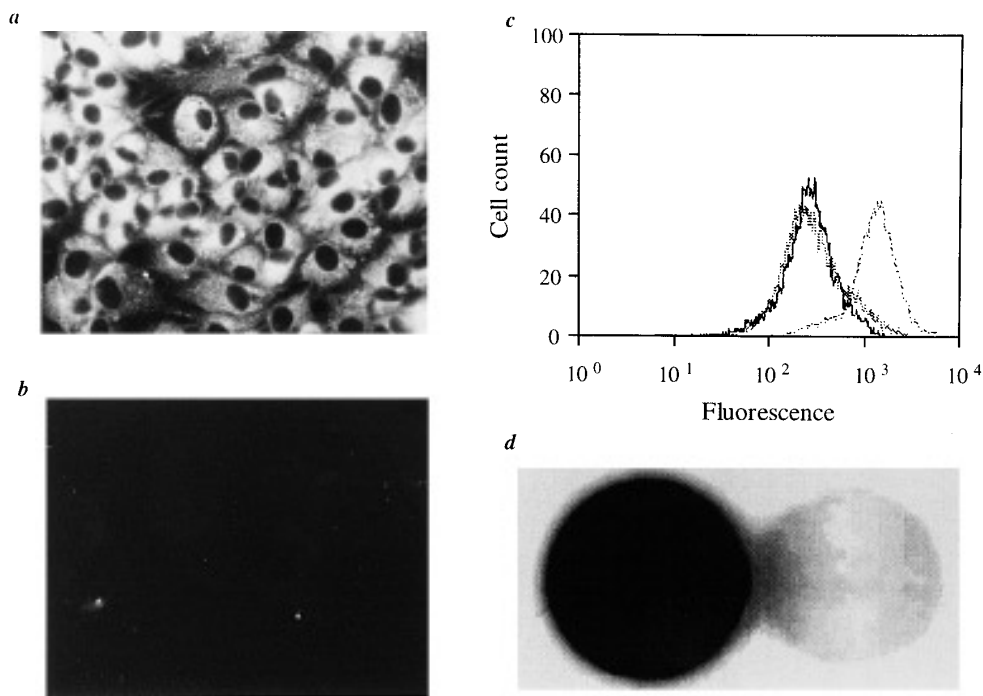


Figure 1 Characterization of BAG cells. Cells were incubated with an antiovine adrenal P450_{11 β} antibody and immunofluorescence and flow cytometry studies performed. In immunofluorescence, cells incubated with the specific antibody are shown in (a). (b) Cells were only stained with secondary antirabbit IgG-fluorescein antibody to evaluate non-specific staining. In flow cytometry analysis (c), cells were incubated with specific antibody before (solid line) or after permeabilization (dotted line) or incubated only with secondary antirabbit IgG-fluorescein antibody (dashed line). Autoradiograms of ¹²⁵I-[Tyr⁸]BK binding on BAG cells grown on six-well plates are shown in (d). Left well shows total binding; right well shows non-specific binding (in the presence of 1 μ M [Tyr⁸]BK).

the washing buffer (25 mM Tris-HCl pH 7.0 and 250 mM dextrose) and binding studies were performed as described under 'Binding assays'.

Data analysis

Experimental data resulting from representative experiments with different cells preparations are expressed as the mean \pm standard deviation (s.d.) of triplicate values. When the error bar is not seen, the symbol is larger than the error. Binding data were analyzed by both the Scatchard plot method and by a curve-fitting program using weighted non-linear least squares to find the values for each parameter that minimized the weighted sum of the squares (SCAFIT). When needed, data were analyzed by Student's *t*-test. *P* values of <0.05 were considered to be statistically significant.

Results

Characterization of BAG cells

In order to characterize our cell preparation we performed immunofluorescence studies with an antibody against

adrenal P450_{11 β} , the enzyme responsible for the last step of aldosterone synthesis in BAG cells (Fig. 1a). The antibody labeled the whole cell population grown on the plate. The majority of cytochromes P450_{11 β} detected were mostly confined within the cells, as deduced from the intensely stained structures. To evaluate non-specific fluorescence, cells were incubated exclusively with the sheep antirabbit IgG-fluorescein antibody (Fig. 1b). Under these conditions, no specific labeling was observed. To further characterize our cell preparation, flow cytometry was used (Fig. 1c). Permeabilized cells showed an important shift of fluorescence intensity after labeling with the antiovine adrenal P450_{11 β} antibody, indicating their high level of expression of the steroidogenic enzyme. Non-permeabilized cells showed a lower level of fluorescence similar to that of permeabilized cells incubated exclusively with the secondary antibody. Measurement of the steroidogenic products revealed that these cells produce low levels of aldosterone under basal conditions and no detectable cortisol (data not shown). These results indicated that our cell preparation was highly enriched in BAG cells. *In situ* binding of ¹²⁵I-[Tyr⁸]BK on BAG cells is illustrated in Fig. 1d. Specific binding was abundant and evenly distributed throughout the whole surface of the

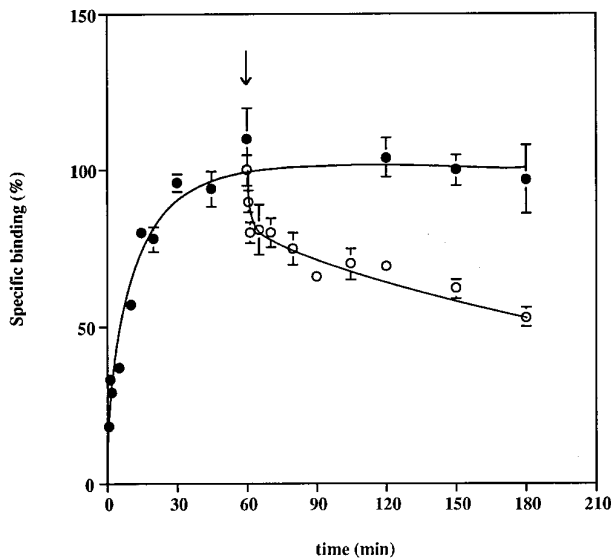


Figure 2 Kinetic analysis of ^{125}I -[Tyr⁸]BK binding to BAG cells. Association was initiated by incubating the cells for different periods of time with ^{125}I -[Tyr⁸]BK (0.2 nM, 150 000 c.p.m.) in a final volume of 500 μl , at 22 °C (●). At the time indicated by the arrow, 1 μM [Tyr⁸]BK was added to some aliquots to initiate dissociation (○). Values are percent of maximal specific binding (100%=5000 c.p.m.). Non-specific binding was determined in the presence of 1 μM [Tyr⁸]BK. Mean \pm s.d. of triplicate determinations; representative of three similar experiments.

plate indicating that the binding sites are expressed at the surface of most of the cells. ^{125}I -[Tyr⁸]BK binding was specific, as indicated by its strong inhibition in the presence of 1 μM [Tyr⁸]BK.

Binding studies

Kinetic binding experiments were performed on BAG cells. Cells were incubated with ^{125}I -[Tyr⁸]BK (0.2 nM) for different periods of time at 22 °C. A time-dependent increase of specific binding was observed (Fig. 2). An apparent steady state was reached within 1 h. A slow dissociation of the bound ligand was observed following the addition of 1 μM unlabeled [Tyr⁸]BK (Fig. 2). This slow dissociation rate indicates a high affinity interaction between BK and its receptor.

In saturation studies, incubation of cells with increasing concentrations of ^{125}I -[Tyr⁸]BK (Fig. 3, upper panel) resulted in the progressive saturation of the specific binding sites. Scatchard analysis of these binding data (Fig. 3, lower panel) was consistent with a single class of high affinity binding sites with a K_d of 0.65 ± 0.17 nM and a maximal binding capacity of 180 ± 55 fmol/mg protein.

The specificity of ^{125}I -[Tyr⁸]BK binding was analyzed in concentration-displacement experiments, shown in Fig. 4. Addition of increasing concentrations of unlabeled [Tyr⁸]BK inhibited tracer binding in a concentration-

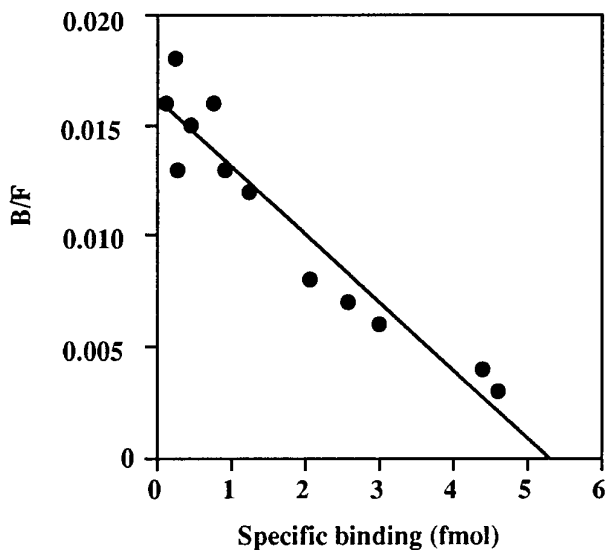
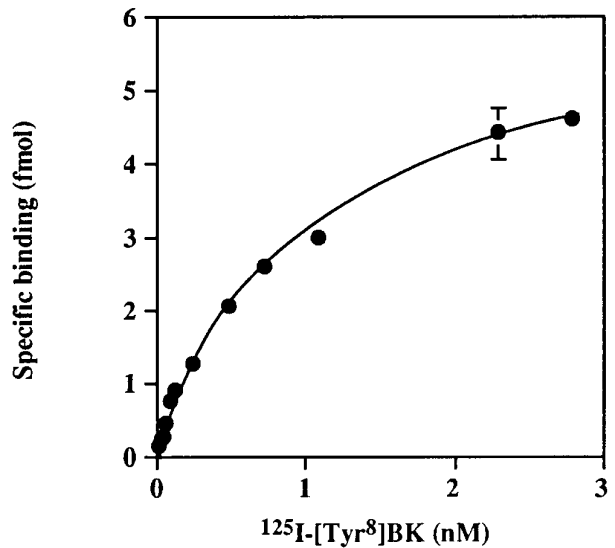


Figure 3 Saturation of ^{125}I -[Tyr⁸]BK binding sites on BAG cells. Cells were incubated at 22 °C for 1 h in the presence of increasing concentrations of ^{125}I -[Tyr⁸]BK (0.02–3 nM) (upper panel). Non-specific binding was determined in the presence of 1 μM [Tyr⁸]BK. Each point represents the mean \pm s.d. of triplicate values. Similar results were obtained in three separate experiments. Scatchard analysis of binding data is shown in the lower panel.

dependent manner. The half-maximal effective concentration needed to inhibit the tracer binding (IC_{50}) was 2.00 ± 0.75 nM. BK was 10 times more potent than [Tyr⁸]BK, with an IC_{50} of 0.18 ± 0.07 nM. Icatibant (B_2 receptor antagonist) was slightly less potent than BK with an IC_{50} of 0.3 ± 0.2 nM. Finally, [DesArg⁹]BK and

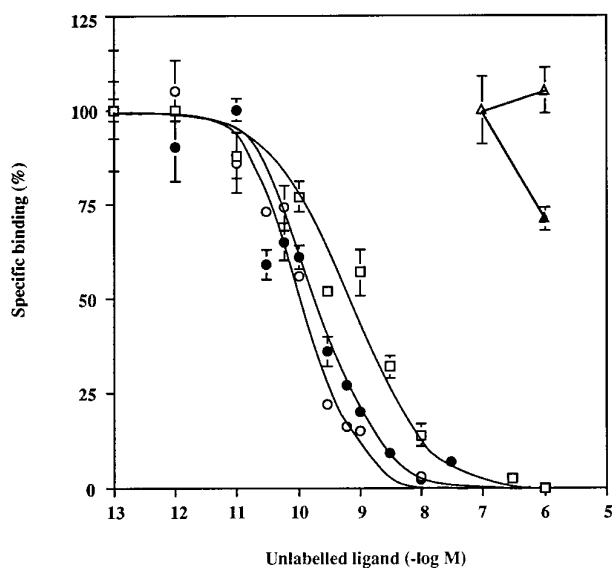


Figure 4 Competitive binding of ^{125}I -[Tyr⁸]BK to BAG cells. Cells were incubated for 1 h at 22 °C with ^{125}I -[Tyr⁸]BK (0.2 nM, 150 000 c.p.m.) and increasing concentrations of different ligands: [Tyr⁸]BK (□), BK (○), Icatibant (●), [DesArg⁹]BK (△) and [DesArg⁹,Leu⁸]BK (▲). Total binding was 8000 c.p.m. and non-specific binding was 3000 c.p.m. Non-specific binding was determined in the presence of 1 μM [Tyr⁸]BK. Values are percent of maximal specific binding (100%=5000 c.p.m.). Mean \pm s.d. of triplicate values; representative of three similar experiments.

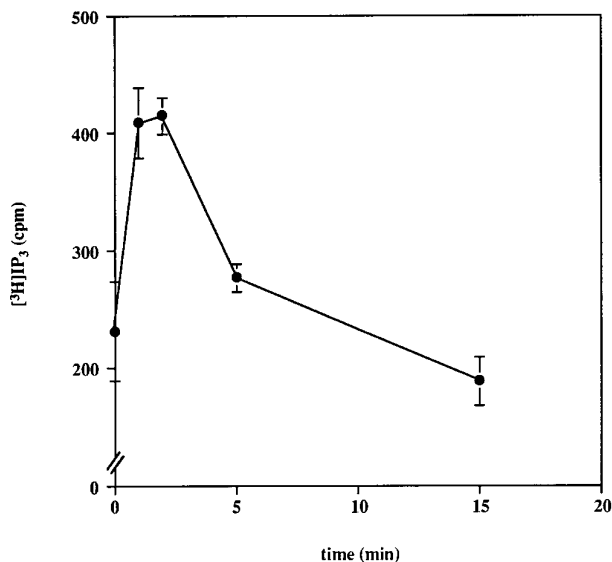


Figure 5 Time course of BK-induced ^3H IP₃ production in BAG cells. Cells preloaded with myo- ^3H inositol were incubated for different periods of time in the presence of 100 nM BK at 37 °C, in a final volume of 250 μl . At indicated times, incubations were stopped with perchloric acid and ^3H inositol phosphates analyzed as indicated in Materials and Methods. A typical experiment, performed in triplicate (mean \pm s.d.), representative of three similar experiments.

[DesArg⁹,Leu⁸]BK (B₁ receptor agonist and antagonist respectively) had no important inhibitory effect on ^{125}I -[Tyr⁸]BK binding, at a concentration as high as 1 μM .

Functional properties of BK receptors

The functional properties of BK receptors were evaluated by measuring IP₃ production, intracellular Ca²⁺ increase and aldosterone secretion. BAG cells incubated in the presence of BK (100 nM) for different periods of time, demonstrated a time-dependent increase of IP₃ production. A maximal IP₃ production was observed after 1–2 min but rapidly declined to a low level within 15 min of BK stimulation (Fig. 5). When cells were incubated for 2 min in the presence of increasing concentrations of BK, their IP₃ content increased progressively in a concentration-dependent manner (Fig. 6). The threshold concentration was around 0.1 nM and the maximal response was obtained with 10 nM BK. The half-maximal effective concentration (EC₅₀) for IP₃ production was 1.0 ± 0.5 nM. The specificity of BK effect on IP₃ production was also evaluated with different BK antagonists. As shown in Fig. 7, preincubation of BAG cells for 5 min in the presence of Icatibant (1 μM) abolished BK-induced IP₃ production whereas pretreatment for 5 min with [DesArg⁹,Leu⁸]BK (1 μM) caused no significant inhibitory

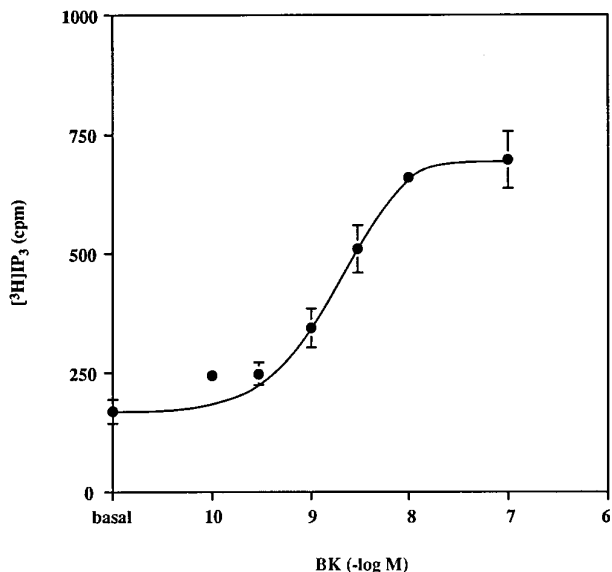


Figure 6 Concentration-response effect of BK on ^3H IP₃ production by BAG cells. Cells preloaded with myo- ^3H inositol were incubated for 2 min at 37 °C with increasing concentrations of BK (0.1–100 nM) in a final volume of 250 μl . The incubations were stopped with perchloric acid and ^3H inositol phosphates analyzed as indicated in Materials and Methods. A typical dose-response curve, performed in triplicate (mean \pm s.d.), representative of three similar experiments.

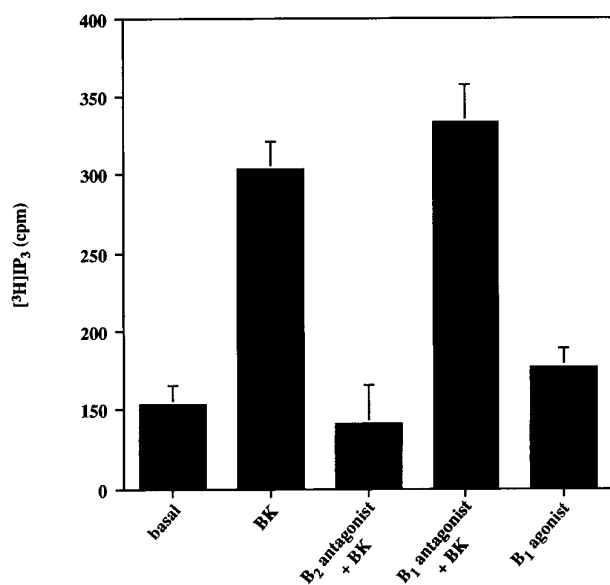


Figure 7 Specificity of BK-induced IP₃ formation in BAG cells. Cells preloaded with myo-[³H]inositol were preincubated for 5 min at 37 °C with vehicle, 1 μM [DesArg⁹,Leu⁸]BK (B₁ receptor antagonist) or Icatibant (B₂ receptor antagonist) before stimulation for 2 min at 37 °C with 100 nM BK or [DesArg⁹]BK, as indicated. Incubations were stopped with perchloric acid and [³H]inositol phosphate analyzed as indicated in Materials and Methods. A typical experiment, performed in triplicate (mean ± s.d.), representative of two similar experiments.

effect. Consistent with these results, [DesArg⁹]BK (100 nM) barely increased IP₃ level in BAG cells.

Figure 8*a* and *b* show that BK (100 nM) increased [Ca²⁺]_i in BAG cells. A typical biphasic response was observed with a rapid and transient elevation of [Ca²⁺]_i followed by a slow decline of [Ca²⁺]_i which remained above the basal level for at least 5 min (Fig. 8*a*). In the absence of extracellular Ca²⁺, the rapid and transient elevation of [Ca²⁺]_i was still observed upon stimulation with BK, but the slow decline phase was abolished (Fig. 8*b*). Actually under these conditions, [Ca²⁺]_i rapidly declined to a level clearly below the basal level. As a control, Fig. 8*c* shows that Ang II (100 nM) could also increase [Ca²⁺]_i in BAG cells. Ang II elicited a typical biphasic response with a rapid and transient elevation of [Ca²⁺]_i followed by a sustained plateau phase which remained above the basal level for more than 10 min (Fig. 8*a*).

The steroidogenic effect of BK was then evaluated. BAG cells were stimulated with a high concentration of BK (100 nM) for different periods of time at 37 °C. As shown in Fig. 9, BK did not significantly increase aldosterone secretion whereas Ang II (100 nM) elicited a robust (10-fold over basal) steroidogenic response. It is important to note that no significant increase of aldosterone production was observed within the first few minutes of

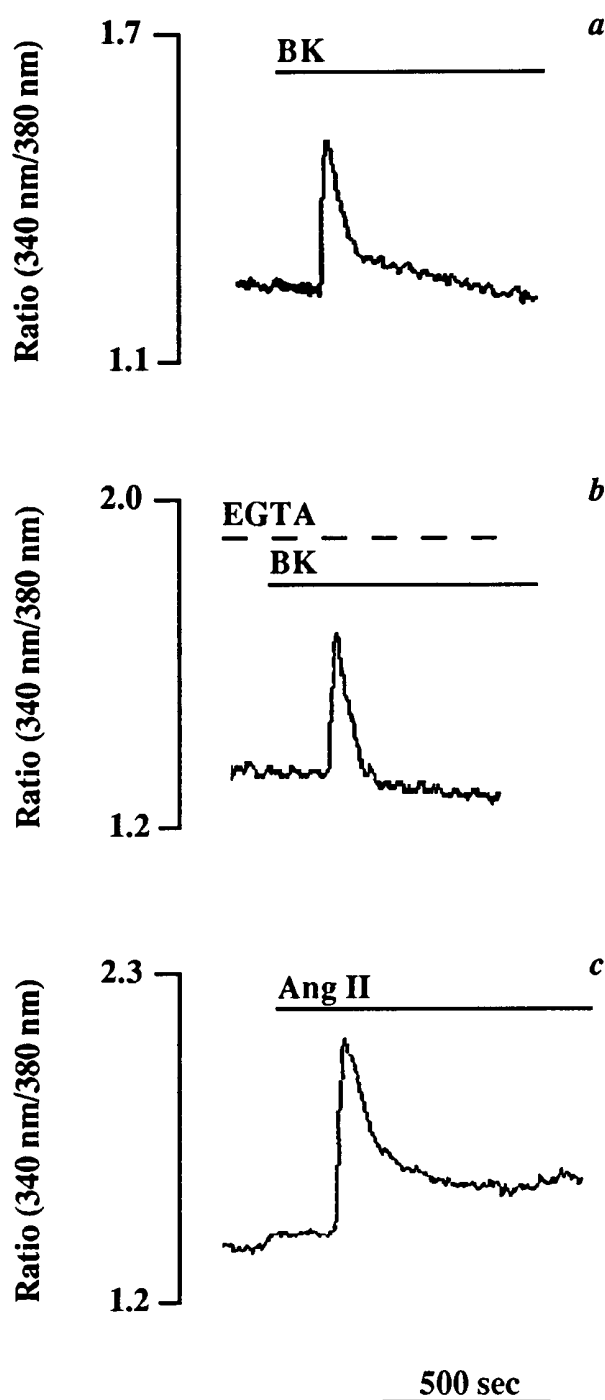


Figure 8 Ca²⁺ mobilization in BAG cells. Cells grown on coverslips were loaded with 2 μM fura-2-AM and stimulated at 22 °C with 100 nM BK in the absence (a) or presence (b) of 2 mM EGTA or with 100 nM Ang II (c). [Ca²⁺]_i measurements are expressed as the ratio of fluorescence values obtained on dual wavelength excitation of 340 nm and 380 nm. Fluorescence intensity was measured at an emission wavelength of 500 nm. Typical experiments, representative of four similar experiments.

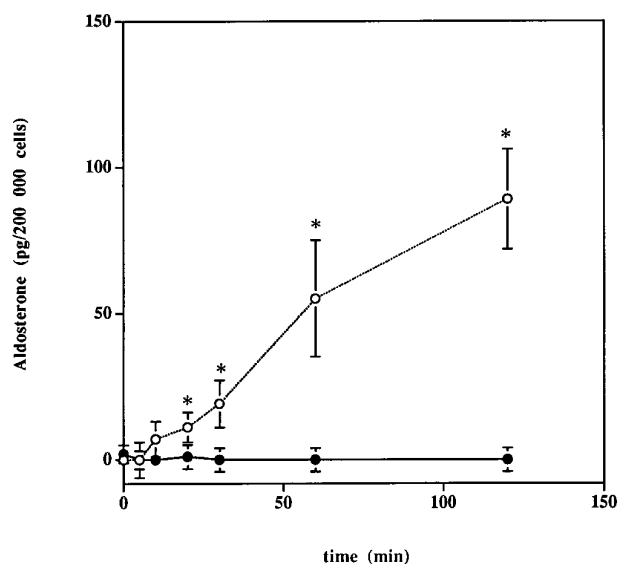


Figure 9 Time course of agonist-induced aldosterone secretion in BAG cells. Cells were stimulated for different periods of time at 37 °C with 100 nM BK (●) or Ang II (○). Incubation medium was removed and assayed for aldosterone by RIA. Values are aldosterone secretion over the basal level (basal level=15 pg). A typical experiment, performed in triplicate (mean ± s.d.), representative of four similar experiments. * $P < 0.05$, considered to be statistically significant.

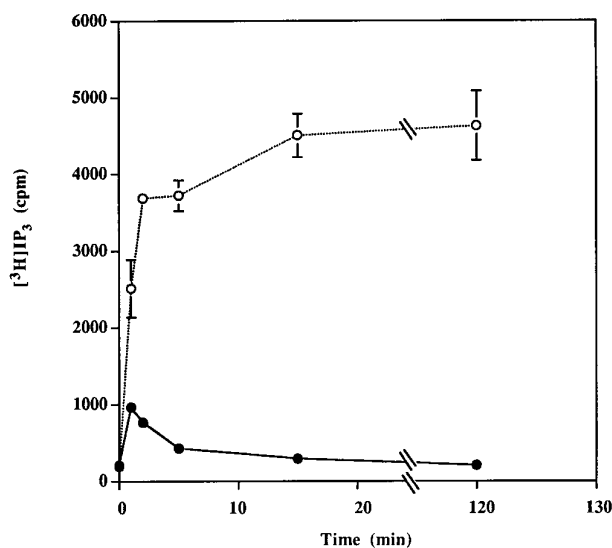


Figure 10 Time course of agonist-induced [^3H]IP₃ production in BAG cells. Cells preloaded with myo-[^3H]inositol were incubated for different periods of time in the presence of 100 nM BK (●) or Ang II (○) at 37 °C, in a final volume of 250 μl . At indicated times, incubations were stopped with perchloric acid and [^3H]inositol phosphates analyzed as indicated in Materials and Methods. A typical experiment, performed in triplicate (mean ± s.d.), representative of three similar experiments.

stimulation with Ang II. Co-stimulation of BAG cells with BK and Ang II did not potentiate the aldosterone secretion induced by Ang II alone (data not shown).

Concerned about the fact that B₂ receptors on BAG cells fail to induce any significant increase of aldosterone secretion, we compared the effects of BK and Ang II on the activation of PLC. Figure 10 shows that Ang II elicited a much more important and sustained IP₃ production in BAG cells.

Desensitization of ^{125}I -[Tyr⁸]BK binding capacity

We verified whether the transient nature of BK-induced IP₃ production could be correlated with desensitization of B₂ receptors. BAG cells were pretreated with BK (100 nM) for different periods of time, and after an acid wash the subsequent ^{125}I -[Tyr⁸]BK binding activity was evaluated. Pretreatment with 100 nM BK caused a time-dependent decrease of ^{125}I -[Tyr⁸]BK binding (Fig. 11). Specific binding of ^{125}I -[Tyr⁸]BK decreased by about 70 ± 10% in 20 min. Longer pretreatment periods up to 1 h did not further attenuate ^{125}I -[Tyr⁸]BK binding capacity of BAG cells (Fig. 11). The concentration-dependent relationship of this desensitizing effect was studied. Pretreatment of BAG cells for 30 min with increasing concentrations of BK progressively decreased ^{125}I -[Tyr⁸]BK binding activity (Fig. 12). A maximal

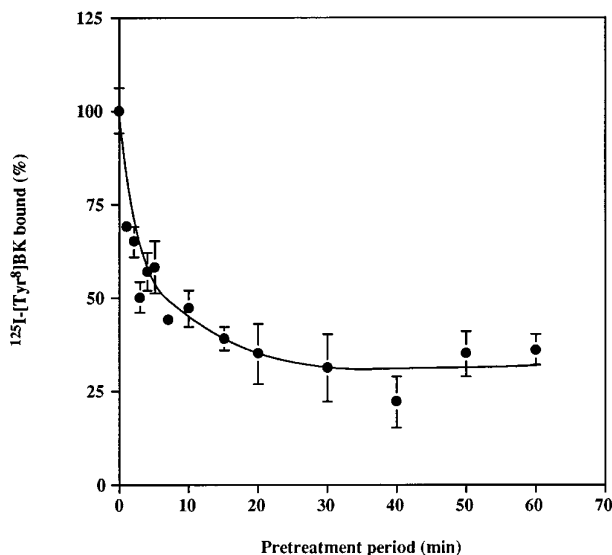


Figure 11 Rapid desensitization of ^{125}I -[Tyr⁸]BK binding activity of BAG cells. Cells were pretreated at 37 °C with 100 nM BK for various periods of time (0–60 min). After an acid wash (pH 3.0), cells were incubated for 1 h at 4 °C with ^{125}I -[Tyr⁸]BK (0.2 nM, 150 000 c.p.m.). The 100% represents specific ^{125}I -[Tyr⁸]BK binding (7000 c.p.m.) to cells pretreated in the absence of BK. Non-specific binding was determined in the presence of 1 μM [Tyr⁸]BK. Each point represents the mean ± s.d. of triplicate values. A typical experiment, representative of three similar experiments.

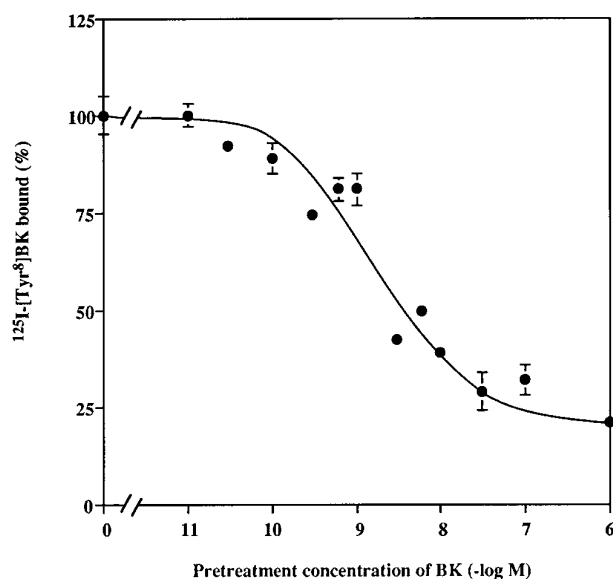


Figure 12 Concentration-dependent desensitization of ^{125}I -[Tyr⁸]BK binding activity of BAG cells. Cells were pretreated for 30 min at 37 °C with increasing concentrations of BK (0.01–1000 nM). After an acid wash (pH 3.0), cells were incubated for 1 h at 4 °C with ^{125}I -[Tyr⁸]BK (0.2 nM, 150 000 c.p.m.). The 100% represents specific ^{125}I -[Tyr⁸]BK binding (7000 c.p.m.) to cells pretreated in the absence of BK. Non-specific binding was determined in the presence of 1 μM [Tyr⁸]BK. Each point represents the mean \pm S.D. of triplicate values. A typical experiment, representative of three similar experiments.

decrease (70%) was observed with 30 nM BK and the half effective dose was 1.3 ± 0.6 nM.

Discussion

In this study, we characterized a specific binding site for BK on BAG cells. The binding of ^{125}I -[Tyr⁸]BK was time-dependent, reversible and saturable. Scatchard analysis revealed a relatively small population (180 ± 55 fmol/mg protein) of high affinity (0.65 ± 0.17 nM) binding sites. BK receptors, most especially B₂ receptors, have been characterized in a number of tissues. Binding studies have revealed a variable density (~ 50 – 400 fmol/mg protein) of high affinity (~ 0.1 – 5 nM) receptors in different tissues including guinea-pig ileum, guinea-pig lung, rat mesangial cells, rat and bovine uterine myometrium, bovine aortic endothelial cells, human fibroblasts and NG 108–15 cells (reviewed by Hall 1992). Three different groups (Rosolowsky & Campbell 1992, Malendowicz *et al.* 1995, Wang *et al.* 1996) using approaches such as *in situ* hybridization histochemistry and biological assays have previously suggested that the adrenal cortex expresses BK receptors. To our knowledge our binding studies are the first to provide direct evidence of BK receptors expression on BAG cells.

BK receptors have been classified on the basis of their affinity for agonists and antagonists as B₁ or B₂ types (Regoli & Barabé 1980). The pharmacological profile of ^{125}I -[Tyr⁸]BK binding sites on BAG cells is characteristic of a B₂ receptor. This conclusion is based on the ability of BK (a selective B₂ receptor agonist) and Icatibant (a selective B₂ receptor antagonist) to inhibit the specific binding of ^{125}I -[Tyr⁸]BK with high apparent affinities and on the inability of [DesArg⁹]BK (a selective B₁ receptor agonist) and [DesArg⁹,Leu⁸]BK (a selective B₁ receptor antagonist) to inhibit ^{125}I -[Tyr⁸]BK binding.

The present study also demonstrated that BK receptors on BAG cells are coupled to PLC. BK induced a rapid and concentration-dependent production of IP₃. A maximal production of IP₃ (3- to 5-fold above basal level) was obtained with 10 nM BK. As concluded from binding studies, the high efficiency of BK and the lack of activity of [DesArg⁹]BK indicate that PLC activation is mediated through a B₂ receptor. This conclusion is also supported by the potent inhibitory effect of Icatibant and the lack of effect of [DesArg⁹,Leu⁸]BK on BK-induced PLC activation, as previously shown in other cell types (Ransom *et al.* 1992, Tatakis *et al.* 1992).

The functional activity of B₂ receptors on BAG cells was also demonstrated by the typical biphasic $[\text{Ca}^{2+}]_i$ elevation induced by BK. As observed in many other systems (for review see Berridge 1993), an initial transient rise of Ca^{2+} (probably due to IP₃-mediated Ca^{2+} mobilization from intracellular stores) was followed by a more sustained phase (probably due to the entry of external Ca^{2+} since it was abolished in the presence of 2 mM EGTA). Similar results were obtained by Malendowicz *et al.* (1995) who showed that BK could increase $[\text{Ca}^{2+}]_i$ in rat zona glomerulosa cells. Altogether these results lead to the suggestion that BK could participate in the regulation of aldosterone secretion by BAG cells.

Although we clearly demonstrated the presence of functional B₂ receptors on BAG cells, no significant steroidogenic effect could be elicited by BK. Ang II, a well characterized steroidogenic agent (Fraser *et al.* 1979) elicited a robust 10-fold increase in aldosterone secretion by these cells. The reason why BK, contrary to Ang II, was unable to increase steroidogenesis is probably related to the mechanism of activation utilized by both types of receptors. Aldosterone, contrary to certain types of hormones, is not stored in cells. The major part of secreted aldosterone comes from *de novo* synthesis. As it has been demonstrated by Kojima *et al.* (1984), it takes at least 10 min (in our study almost 20 min) before a significant amount of aldosterone can be observed during cell stimulation with Ang II and this secretion involves different temporal activation of two distinct components of the pathway. The transient rise of $[\text{Ca}^{2+}]_i$ which activates calmodulin kinase is largely responsible for an initial transient aldosterone secretion. The subsequent activation of PKC by $[\text{Ca}^{2+}]_i$ and DAG is responsible for the sustained aldosterone

secretion (Kojima *et al.* 1984). Therefore to produce a significant amount of aldosterone, the signaling mechanism must be activated for a certain period of time in order to maintain a significant production of the synergistic second messengers IP₃ and DAG.

We showed that a maximal concentration of BK elicited a 3- to 5-fold increase of IP₃ while under similar conditions Ang II elicited a more robust ~20-fold increase of IP₃, as previously shown by Balla *et al.* (1988) and Boulay *et al.* (1990). We also demonstrated that contrary to Ang II which exerts a long lasting effect on IP₃ production, the effect of BK is waning after 2 min and is considerably decreased after 5 min of stimulation (Fig. 10). A similar transient effect of BK on IP₃ production was also observed in other cell types (Lambert *et al.* 1986, Portilla & Morrison 1986, Fu *et al.* 1988, Bascands *et al.* 1991). Some authors suggested that it could be due to proteolytic degradation of BK. This suggestion cannot explain the transient effect of BK since readdition of BK (15 min after a first stimulation) failed to restore IP₃ production (data not shown). Measurements of [Ca²⁺]_i showed that although both BK and Ang II could elicit a typical biphasic response, Ang II produced a more sustained plateau phase which was maintained for at least 10 min after stimulation whereas the effect of BK was waning within 5 min. Taken together, these results suggest that BK-induced IP₃ production and [Ca²⁺]_i increase are not important enough to support aldosterone secretion. The weaker efficacy of BK is probably due in part to the low level of B₂ receptors expression on BAG cells. We have shown in the present study that BAG cells express about 10 000 B₂ receptors/cell, which is about 15-fold lower than the level of expression of AT₁ receptors, by these same cells, determined in our previous study (Boulay *et al.* 1990).

We showed that pretreatment of BAG cells with different concentrations of BK for different periods of time caused a rapid and efficient loss of B₂ receptor binding activity. These results strongly suggest that the loss of PLC activation following BK stimulation is due to receptor desensitization. Receptor desensitization is a well characterized phenomenon (Benovic *et al.* 1988, Lohse *et al.* 1990, Liggett 1991) that has been observed for different types of G protein-coupled receptors. It has been previously demonstrated that B₂ receptors undergo rapid internalization (Roscher *et al.* 1983, Wolsing & Rosenbaum 1991, Munoz & Leeb-Lunberg 1992, Pradaude *et al.* 1995), a process which is considered to be one of the first steps in receptor desensitization (Lefkowitz *et al.* 1990). Further studies are necessary to better characterize the precise mechanisms involved in B₂ receptor desensitization in BAG cells. In that respect BAG cells should constitute a good model for such studies since we showed that they possess a very efficient mechanism of B₂ receptor desensitization.

In summary, our results demonstrate the presence of B₂ receptors on BAG cells. These receptors are coupled to

PLC and thus increase the formation of IP₃ and the mobilization of intracellular Ca²⁺. However BK could not induce a significant aldosterone production by BAG cells. This is probably due to the low level of B₂ receptor expression and to an efficient mechanism of B₂ receptor desensitization. These results could explain why we and other groups (Rudichenko *et al.* 1993, Malendowicz *et al.* 1995) did not observe any BK-induced aldosterone secretion. It has been previously suggested that BK could play a role in the regulation of aldosterone secretion (Rosolowsky & Campbell 1992). Since functional B₂ receptors are expressed in adrenal glomerulosa cells, it is likely that under some specific physiological or pathological conditions these receptors may play a significant role in aldosterone secretion. Further studies should attempt to identify such conditions.

Acknowledgements

The authors would like to thank respectively, Dr Alain Bélanger (Centre Hospitalier de l'Université Laval, Québec, Canada) and Dr Jean-Guy Lehoux (Université de Sherbrooke, Québec, Canada) for their generous gift of antialdosterone and antibovine adrenal P450_{11β} antibodies. This work is part of the PhD thesis of L C and was supported by grants from the Medical Research Council of Canada (MRCC) and the Heart and Stroke Foundation of Québec (HSFQ). L C and D E R are recipients of studentship awards from the Heart and Stroke Foundation of Canada (HSFC). M P is a recipient of studentship awards from the MRCC. G G is an MRCC scientist.

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Received 13 February 1997

Revised manuscript received 6 May 1997

Final version received 8 August 1997

Accepted 9 October 1997