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Aspirin inhibits human bradykinin B_2 receptor ligand binding function

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ABSTRACT

The bradykinin B_2 receptor, a member of the G protein-coupled receptors superfamily, is involved in a variety of physiological functions, including vasodilation, electrolyte transfer in epithelia, mediation of pain, and inflammation. The effect of aspirin on bradykinin binding to cell-surface receptor and on signal transduction were studied in CHO-K1 cells, stably expressing the human B_2 receptor. Cell-surface organization of the receptor was assessed by immunoprecipitation and Western blot analysis in CHO-K1 cells expressing N-terminally V5-tagged B_2 receptor. We found that the widely used analgesic, anti-thrombotic, and anti-inflammatory drug aspirin alters the B_2 receptor ligand binding properties. Aspirin reduces the apparent affinity of the receptor for [^{3}H]-bradykinin by accelerating the dissociation rate of [^{3}H]-bradykinin–receptor complexes. In addition, aspirin reduces the capacity of unlabeled bradykinin or the B_2 receptor antagonist icatibant to destabilize pre-formed [^{3}H]-bradykinin–receptor complexes. Kinetic and reversibility studies are consistent with an allosteric type of mechanism. Aspirin effect on B_2 receptor binding properties is not accompanied by alteration of the cell-surface organization of the receptor in dimers and monomers. Aspirin does not influence the receptor ability to transduce bradykinin binding into activation of G-proteins and phospholipase C. These results suggest that aspirin is an allosteric inhibitor of the B_2 receptor, a property that may be involved in its therapeutic actions.

1. Introduction

Bradykinin has a variety of physiological effects including vasodilation and endothelial activation, modulation of water and electrolyte transports in epithelia, and mediation of pain [1,2]. The physiological role of the kallikrein–kinin system has been well documented in the cardiovascular system, where kinins produced in arteries through the action of kallikrein participate in the control of arterial blood flow, and are involved in vascular remodeling, and in angiogenesis [3–6]. Through its vascular, chemotactic and pain producing effects, BK is also involved in inflammation [7].

BK exerts its effects by interacting with two different subtypes of G protein-coupled receptors, B_1 and B_2 [8]. The B_2 receptor is constitutively synthesized in tissues, contrary to the B_1 receptor, and mediates most of the BK effects described

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Abbreviations: GPCR, G protein-coupled receptor; BK, bradykinin; B_2 receptor, bradykinin receptor B_2 subtype; B_1 receptor, bradykinin receptor B_1 subtype; CHO-K1 cells, K1 type of Chinese hamster ovary cells; IPs, inositol phosphates.

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so far. We have previously shown that BK binds to the B_2 receptor in a negative cooperative manner, as a consequence of interaction between receptor molecules triggered by occupancy of the ligand binding site [9–11]. This phenomenon constitutes an early desensitization mechanism. The specific B_2 receptor antagonist icatibant also triggers allosteric interaction resulting in BK–receptor complex dissociation [9]. However, whether other classes of pharmacological agents can modulate B_2 receptor function is not known. Recently, several GPCRs, have been reported to be positively or negatively modulated by compounds acting on sites topographically distinct from the orthosteric site used by the endogenous ligands [12–14]. These compounds are termed allosteric modulators. We tested several candidate compounds for interaction with the B_2 receptor and found that aspirin (acetylsalicylic acid), influenced the binding of BK to the receptor.

Aspirin is a widely used drug with analgesic, antiplatelet, and anti-inflammatory properties. It is often used in pathological situations where kinin receptors are activated, such as ischemic heart disease or inflammation. It has been reported previously that aspirin is an allosteric inhibitor of the endothelin ETA receptor, a member of the GPCR family [15]. In the present study, we show that aspirin, at therapeutic concentration, is a negative modulator of the B_2 receptor ligand binding function, bringing a second example of a GPCR influenced by this compound.

2. Materials and methods

2.1. Materials

The CHO-K1 cells were from American Type Culture Collection, Rockville, MD, USA. Lipofectamine 2000 and monoclonal anti-V5 antibody were from Invitrogen, Leek, Netherlands. Fetal calf serum (lot no. S01190S0180) was from Biowest, ABCYS-Paris, France. Antibiotic cocktail for cell culture, igepal, protease inhibitor cocktail, bovine serum albumin (A-4378), aspirin (acetylsalicylic acid) were from Sigma Aldrich Chimie, Saint Quentin Fallavier, France. BK was from Alexis, San Diego, California. Icatibant was a generous gift from Hoechst, Germany. [^{3}H]-bradykinin (64 Ci/mmol), [^{3}H]-myoinositol (14 Ci/mmol), [^{35}S]-GTPγS (1065 Ci/mmol) and ECL kit were from Amersham Biosciences, Buckinghamshire, UK. Protein G magnetic beads were from Dynal, Oslo, Norway. Protein markers were from New England BioLabs-Ozyme, Saint-Quentin en Yvelines, France. Polyacrylamide was from Interchim, Montluçon, France. Peroxydase-conjugated secondary antibody was a goat anti-mouse IgG from Jackson ImmunoResearch Laboratories, Pennsylvania, USA.

2.2. Cell culture and receptor expression

The experiments described below were performed on CHO-K1 cells transfected with previously cloned human B_2 receptor cDNA placed under the control of cytomegalovirus promotor into the eucaryotic expression vector pcDNA3 [9,16]. A human B_2 receptor cDNA construct, having an exogenous V5 epitope coding sequence at the aminoterminal extremity of the receptor, just after the methionine start codon, was used in experiments involving receptor immunoprecipitation [17]. Lipofectamine 2000 reagent was used for transfection according to manufacturer instructions. G418-resistant cell clones expressing the recombinant receptors were selected on the basis of their ability to bind [^{3}H]-BK. These clones as well as parental CHO-K1 cells were grown at 37 °C in Ham's F12 medium, supplemented with 10% (v/v) fetal calf serum, antibiotics (penicillin 0.2 unit/ml, streptomycin 20 pg/ml and amphotericin B 0.5 mg/ml) and 0.5 mM glutamine, in a humid atmosphere of 95% air and 5% CO_2. Cells were used at confluence, *i.e.* 48–72 h after cell passage.

2.3. Cell membrane preparation

After washing three times with PBS, cells grown in 10 cm Petri dishes were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 0.3 ml EDTA, protease inhibitor cocktail (1:500 dilution), 10 mM captopril, 0.08 unit/ml aprotinin), homogenized by 10 strokes of a "B" glass Dounce homogenizer, and centrifuged (100 × *g* for 5 min) at 4 °C. Supernatants were recovered and centrifuged (20,000 × *g* for 20 min) at 4 °C. Membranes were recovered in appropriate volume of membrane buffer (50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, protease inhibitor cocktail (1:500 dilution), 10 mM captopril, 0.08 unit/ml aprotinin), and stored at −80 °C until use. Protein concentration was determined using the method of Bradford.

2.4. Radioligand binding assay in intact cells

All experiments were started by washing cells grown in 48-well plates with 0.25 ml of modified Hank's balanced salt solution (HBSS: 127 mM NaCl, 5 mM KCl, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 20 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 10 mM sodium acetate, 0.8 mM $MgSO_4$, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.1% BSA, pH 7.4) and by pre-incubation for 30 min at 25 °C in 0.1 ml of solution A (HBSS containing 0.4 M sucrose to block receptor internalization [9,18], and protease inhibitors (10 mM captopril, 0.08 unit/ml aprotinin). When tested, aspirin was added during the pre-incubation period and during additional incubation step(s).

In a first set of experiments, [^{3}H]-BK binding was determined after incubating cells in 0.1 ml of solution A in the absence or presence of increasing concentrations of aspirin (0.3–20 mM) with a fixed concentration of [^{3}H]-BK (4nM). Incubation pH remained between 6.8 and 7.4 upon aspirin addition. Reactions were allowed to proceed for 20 min at 25 °C. Cells were then washed twice with 0.5 ml of ice-cold HBSS to remove unbound [^{3}H]-BK [9].

In association kinetic experiments, cells were incubated for various times up to 30 min with 4 nM [^{3}H]-BK in the absence or presence of aspirin (10 mM). In saturation analysis of [^{3}H]-BK binding, cells were incubated for 20 min in the absence or presence of aspirin (10 mM) with varying concentrations (0.1–20 nM) of [^{3}H]-BK.

In reversibility experiments, cells exposed 30 min to 10 mM aspirin were washed twice with 0.5 ml HBSS. They were then incubated for up to 60 min in 0.1 ml of aspirin-free solution A. Binding of [^{3}H]-BK (4 nM) was then performed for 20 min in the absence of aspirin. Control cells were exposed or not to

aspirin, then immediately incubated with [^{3}H]-BK in the presence or absence of 10 mM aspirin.

In dissociation kinetic experiments, cells were first exposed to [^{3}H]-BK (4 nM) for 20 min in the absence or presence of aspirin (10 mM). Non-bound [^{3}H]-BK was removed by two washes with 0.5 ml of ice-cold HBSS. Cells were then incubated in 0.5 ml of solution A with or without aspirin (10 mM) for various times up to 90 min (time-course), or in 0.5 ml of the same solutions containing increasing concentrations (0.05–100 nM) of unlabeled BK or the specific B_2 antagonist icatibant for a fixed time of 10 min. Experiments were terminated by measuring the radioactivity released in the medium and the radioactivity that remained associated to the cells, as previously described [9]. Results were expressed as the ratio between the cell-associated radioactivity and the total specific binding (cell-associated radioactivity plus medium radioactivity).

In all experiments, nonspecific binding was determined using 1000-fold excess of unlabeled BK and subtracted from total binding. It never exceeded 10% of total binding. Cell protein content was determined using the method of Bradford to express data in fmol [^{3}H]-BK bound/mg protein where necessary.

2.5. Phospholipase C assay in intact cells

Phospholipase C activity was measured in cells grown in 24-well plates and incubated overnight with 1 μCi/ml [^{3}H]-myoinositol [10]. The extra-cellular unincorporated radioactivity was removed by three washes with 0.5 ml of HBSS. Cells were incubated for 30 min in 0.3 ml of solution A in the absence or presence of aspirin (10 mM), then for 10 min with 10 mM LiCl, and for 15 min with 10 mM LiCl plus increasing concentrations of BK (0.1–20 nM) added in 0.3 ml of the same solutions. All incubations were at 37 °C, as previously described. The reactions were terminated by adding 0.2 ml of 7.5% (w/v) perchloric acid and the cells were scraped and transferred into glass tubes. The different hydrophilic compounds (inositol, glycerophosphoinositol and inositol phosphates) were separated from phospholipids by centrifugation after adding 1 ml of chloroform/methanol (2v/1v). Inositol phosphates were separated from inositol and glycerophosphoinositol by AG 1-X8 anion exchange chromatography, after neutralizing the hydrophilic phase with a mixture of KOH–HEPES. Results were expressed as the ratio between radioactivity measured in inositol phosphates and the total radioactivity incorporated into the cells [10].

Corresponding receptor occupancy was estimated in parallel in cells grown on 48-well plates by [^{3}H]-BK binding at 37 °C with the same peptide concentrations (0.1–20 nM). In these experiments, the cells were incubated for 30 min in 0.1 ml of solution A in the absence or presence of aspirin (10 mM), then for 15 min with [^{3}H]-BK contained in 0.1 ml of the same solutions.

2.6. [^{3}H]-BK binding and [^{35}S]-GTPγS assays in isolated cell membranes

Membranes (10 μg/assay) were incubated at 25 °C for 30 min without or with aspirin (10 mM) in 50 μl of a solution containing 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 10 mM captopril, 0.08 unit/ml aprotinin, 0.01 mg/ml leupeptin, 1 mM EDTA, 100 mM NaCl.

For [^{3}H]-BK binding assay, the incubation was prolonged for 35 min following the addition of [^{3}H]-BK (4 nM, final concentration) in 50 μl of the same solutions.

For [^{35}S]-GTPγS binding assay, the incubation was prolonged for 20 min following the addition of 40 μl of the same solutions containing (stimulated condition) or not (basal condition) BK (4 nM, final concentration), and then for further 15 min after addition of [^{35}S]-GTPγS (10 nM, final concentration) in 10 μl of the same solutions.

For both assays, the reaction was stopped by adding 2 ml of ice-cold washing solution (50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$) and the incubation medium was immediately filtered onto Whatman glass fiber (0.8–1.2 μm) presoaked overnight in 50 mM Tris–HCl, pH 7.4 buffer that contained ([^{3}H]-BK binding assay) or not ([^{35}S]-GTPγS binding assay) polyallylamine (0.1 mg/ml). The filters were then rinsed five times with 2 ml of washing solution, and the remaining radioactivity was measured by liquid scintillation.

2.7. Cell-surface receptor immunoprecipitation and Western blot

The technique used for immunoprecipitation of cell-surface receptors was that of Hilairet et al. [19] with slight modifications [11]. Experiments were performed on cells expressing N-terminally V5-tagged B_2 receptor grown in 6-well plates [17]. The cells were washed three times with PBS. They were incubated in 1 ml of solution A in the absence or presence of 10 mM aspirin for 30 min at 25 °C, then with or without 100 nM BK or icatibant in 0.25 ml of the same solutions in the presence of monoclonal anti-V5 antibody (1:500 dilution) for 20 min at 25 °C. After two washes in solution A and two washes in PBS at 4 °C, the cells were lysed in 0.25 ml of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% (v/v) igepal, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate, protease inhibitor cocktail (1:500 dilution) and 10 mM iodoacetamide), rocked for 45 min, and centrifuged (15,000 × *g* for 20 min). Cell-surface receptor-anti-V5 antibody complexes were then precipitated at 4 °C by rocking the supernatant for 2 h after adding 30 μl of a protein G magnetic beads suspension. The immunoprecipitate was washed three times with PBS and resuspended in 30 μl of a solution containing 10 mM sodium phosphate buffer pH 7, 10 mM iodoacetamide and 1% (w/v) SDS. After adding 30 μl of a 2× non-reducing sample buffer (125 mM Tris–HCl pH 6.8, 25% (v/v) glycerol, 5% (w/v) SDS, 0.002% bromophenol blue) and heating (100 °C for 5 min), the proteins contained in 10 μl as well as protein markers were resolved by 10% (w/v) SDS-PAGE for 50 min, transferred to nitrocellulose membrane, and subjected to immunoblotting using anti-V5 antibody (1:5000 dilution) and a goat anti-mouse peroxydase-conjugated secondary antibody (1:5000 dilution) as previously described [11]. Finally, proteins were visualized *via* chemiluminescence reactions using the ECL kit.

2.8. Data analysis

In saturation experiments, because binding saturation was not achieved with the highest [^{3}H]-BK concentration and

because the Scatchard plots of the binding data were non-linear as observed before [9], the binding values at the three highest [^{3}H]-BK concentrations were used in Scatchard coordinates [20] to estimate the maximal binding capacity (Bmax). Then, by using the estimated Bmax and the overall binding values in the Hill transformation [21], it is possible to estimate the [^{3}H]-BK concentrations corresponding to half-saturation (Kd app) and the Hill coefficients (nHill) for the binding reaction.

In dissociation experiments data were fitted by non-linear regression analysis with sigmoidal dose–response variable slope equation (Graph Pad^{TM} Prism software) to estimate the concentrations of unlabeled BK and icatibant (EC_{50}) necessary for half-reduction of the complexes, and the corresponding Hill coefficients of the dissociation curves.

Experiments were repeated at least three times, each time with duplicate or triplicate determinations. All values are means ± S.E.M. The effect of aspirin on kinetic parameters was assessed by ANOVA or unpaired Student's *t* test.

3. Results

3.1. *Aspirin decreases BK binding to B_2 receptor*

Aspirin induced a concentration-dependent reduction of [^{3}H]-BK binding (Fig. 1). The inhibition curve was steep and a concentration of 20 mM aspirin inhibited [^{3}H]-BK binding by ≈95%. Half maximum inhibition was observed with 8.7 ± 1.8 mM aspirin in these experimental conditions. Incubation solution containing acetic acid at pH 6.8 instead of aspirin had no effect on BK binding (not shown).

Association kinetic analysis performed with 4 nM [^{3}H]-BK and 10 mM aspirin showed that aspirin inhibition developed over time, despite the fact that the cells were pre-treated for 30 min with aspirin before adding [^{3}H]-BK (Fig. 2). The inhibition was 18% after 1 min, and progressively increased until 20 min up to about 40%. BK binding reached equilibrium at 20 min in the presence or in the absence of aspirin. The reversibility of aspirin effect (Fig. 3) was studied as described in Section 2. No effect of aspirin could be observed once aspirin was removed from the incubation medium, suggesting that the effect of aspirin is rapidly reversible or that this effect only occurs when the receptor binds BK.

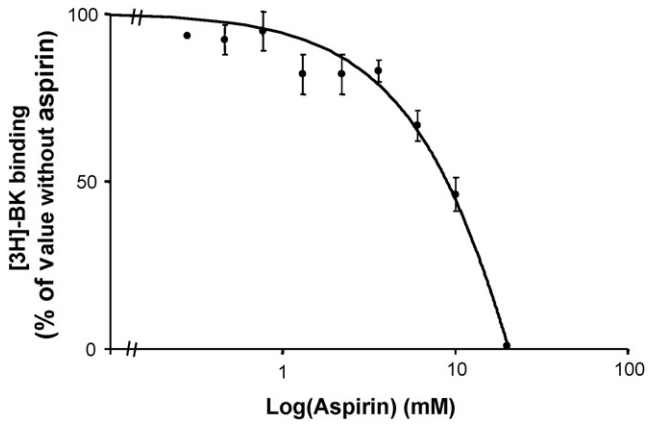

Fig. 1 – Inhibition by aspirin of [^{3}H]-BK binding to recombinant B_2 receptors expressed in intact CHO-K1 cells. Binding of [^{3}H]-BK (4 nM) was measured after 20 min incubation at 25 °C in the presence of increasing concentrations of aspirin. The specific binding obtained in aspirin-treated cells was expressed as percent of the specific binding obtained without aspirin (425 ± 5 fmol of [^{3}H]-BK/mg of protein).

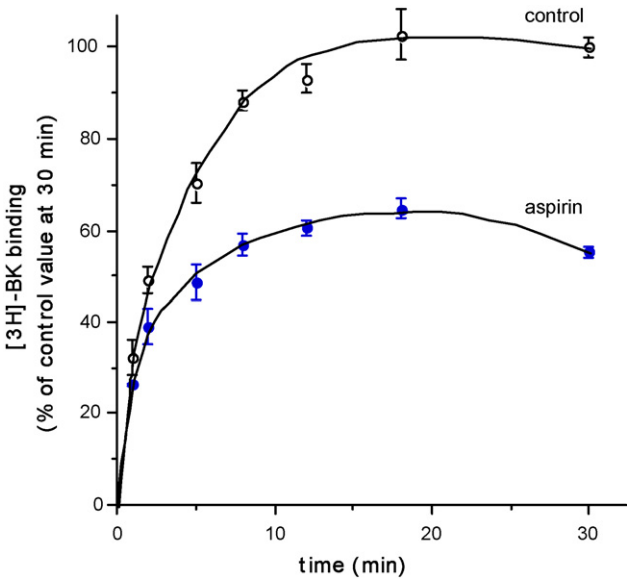

Fig. 2 – Association kinetics of [^{3}H]-BK to B_2 receptors in intact cells in the absence or presence of aspirin. Binding of [^{3}H]-BK (4 nM) was determined at 25 °C at different incubation times in the absence or presence of 10 mM aspirin. Results (specific bindings) are expressed as percent of the specific binding obtained at 30 min without aspirin (693 ± 80 fmol of [^{3}H]-BK/mg of protein).

Aspirin (10 mM) inhibited the binding of [^{3}H]-BK over a wide range of BK concentration (0.1–20 nM), (Fig. 4). Binding saturation was not achieved with the highest [^{3}H]-BK concentration. Bmax, estimated as described above, were 157 ± 12 and 187 ± 22% of the binding determined with 20 nM [^{3}H]-BK in the absence of aspirin and in aspirin-treated cells, respectively. Kd app and Hill coefficient were 10 ± 1 nM (control) and 29 ± 3 nM (aspirin) ($p < 0.01$), and 0.98 ± 0.10 (control) and 0.98 ± 0.10 (aspirin), respectively. Comparison of the above values indicates that aspirin neither changed the maximal binding capacity nor the Hill coefficient for [^{3}H]-BK binding, but decreased the apparent affinity of the B_2 receptor for [^{3}H]-BK.

3.2. *Aspirin destabilizes BK–B_2 receptor complex*

An interesting observation is that the dissociation rate of [^{3}H]-BK–B_2 receptor complexes (formed during 20 min incubation with 4 nM [^{3}H]-BK) was greater in the presence than in the absence of 10 mM aspirin ($p < 10^{-4}$, Fig. 5). This suggests that decreased stability of [^{3}H]-BK–receptor complex very likely contributes to the reduction in the receptor apparent affinity observed with aspirin.

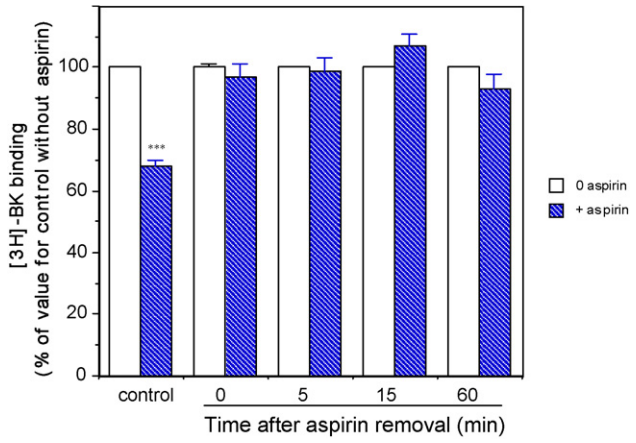

Fig. 3 – Reversibility of aspirin effect on [^{3}H]-BK binding in intact cells. Cells exposed (filled bars) or not (open bars) 30 min at 25 °C to 10 mM aspirin were either immediately incubated (control) for 20 min with 4 nM [^{3}H]-BK in the absence or presence of aspirin, respectively, or washed with aspirin-free solution then incubated for 0–60 min with aspirin-free solution before assaying [^{3}H]-BK binding in the absence of aspirin. The specific binding obtained in aspirin-treated cells was expressed as percent of the specific binding obtained in the corresponding non-treated cells (510 ± 21 fmol of [^{3}H]-BK/mg of protein). $^{***}p < 0.001$ compared to control value without aspirin (unpaired Student's *t* test).

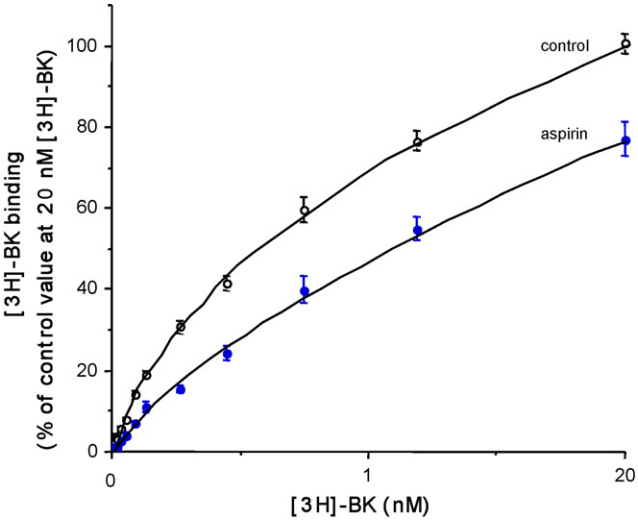

Fig. 4 – "Concentration-dependence" of [^{3}H]-BK binding in the absence or presence of aspirin in intact cells. [^{3}H]-BK binding was measured after 20 min incubation at 25 °C with eleven different concentrations of [^{3}H]-BK (0.1–20 nM) in the absence or presence of 10 mM aspirin. In each experiment, specific binding was expressed as percent of the specific binding (915 ± 150 fmol of [^{3}H]-BK/mg of protein) determined in the absence of aspirin with 20 nM [^{3}H]-BK.

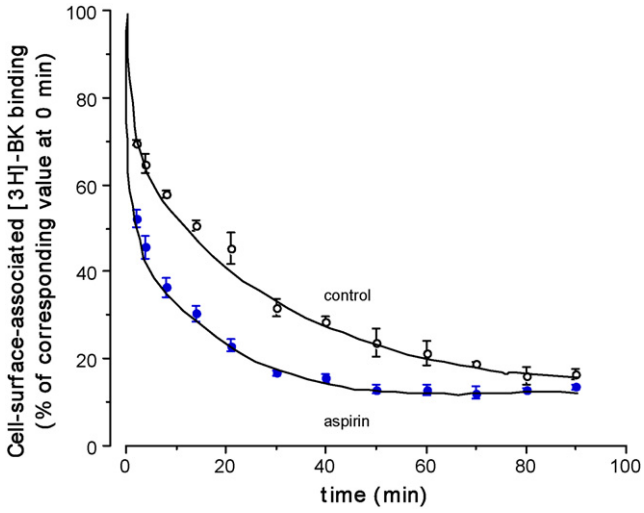

Fig. 5 – Aspirin promotes dissociation of [^{3}H]-BK–receptor complexes in intact cells. Specific binding fractions released in the medium and remaining associated to the cells were determined after a 20 min incubation at 25 °C with 4 nM [^{3}H]-BK in the absence or presence of 10 mM aspirin, followed by removal of non-bound [^{3}H]-BK, and incubation for the indicated times in the absence or presence of aspirin, respectively. The cell-associated specific binding was expressed as percent of the specific total binding (642 ± 32 fmol of [^{3}H]-BK/mg of protein (control) and 309 ± 12 fmol of [^{3}H]-BK/mg of protein (aspirin)). Effect of aspirin, $p < 10^{-4}$ (ANOVA).

3.3. *Aspirin influences the cooperativity in ligand binding*

We previously reported that unlabeled BK or the B_2 receptor antagonist icatibant increased the dissociation rate of [^{3}H]-BK–B_2 receptor complexes [9]. The experiments depicted in Fig. 6 were designed to examine whether aspirin interferes with the dissociating effect of these ligands. For this purpose, the dissociation of [^{3}H]-BK–B_2 receptor complexes (formed after 20 min incubation with 4 nM [^{3}H]-BK) was measured after 10 min incubation with increasing concentrations of unlabeled BK or icatibant (0.05–500 nM) in the absence or presence of aspirin (10 mM). In both absence and presence of aspirin, the addition of unlabeled BK or icatibant resulted in a concentration-dependent increase in the dissociation of [^{3}H]-BK–receptor complexes. However, a larger fraction of complexes remained in the presence of aspirin, indicating that unlabeled BK and icatibant are less potent in dissociating [^{3}H]-BK–B_2 receptor complexes when aspirin is present.

EC_{50} and Hill coefficients for BK and icatibant effects are presented in Table 1. EC_{50} values for both unlabeled BK and icatibant remained unchanged with aspirin. Hill coefficient of the dissociation curves did not change with aspirin in the case of BK, but was markedly reduced in the case of icatibant. This indicates that aspirin differentially influences the abilities of unlabeled BK and icatibant to dissociate [^{3}H]-BK–receptor complexes. Finally, it can be noted that, in absence of aspirin, the Hill coefficient for [^{3}H]-BK–B_2 complex dissociation was close to 2 for icatibant, and close to unity for BK.

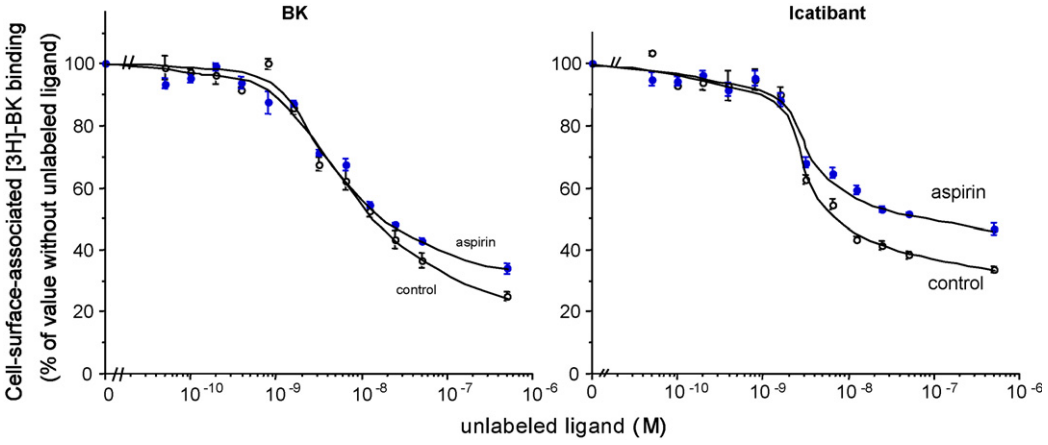

Fig. 6 – Aspirin affects unlabeled BK- and icatibant-promoted dissociations of [^{3}H]-BK–receptor complexes in intact cells. Specific binding fractions released in the medium and remaining associated to the cells were determined after a 20 min incubation at 25 °C with 4 nM [^{3}H]-BK in the absence or presence of 10 mM aspirin, followed by removal of non-bound [^{3}H]-BK and 10 min incubation with increasing concentrations of unlabeled BK (left panel) or icatibant (right panel) in the absence or presence of 10 mM aspirin, respectively. After calculation of the cell-associated specific binding as percent of the corresponding specific total binding, the results are given as percent of the value without unlabeled BK or icatibant (61.5 ± 1.4% (control) and 46.8 ± 1.0% (aspirin)).

3.4. *Aspirin has no effect on the cell-surface organization of B_2 receptor molecules*

To get insight into the molecular mechanism underlying the effect of aspirin on B_2 receptor binding properties, cells expressing N-terminally V5-tagged B_2 receptor were incubated without or with 10 mM aspirin, then without or with BK or icatibant (100 nM), as in binding experiments. Cell-surface receptor molecules were then immunoprecipitated and resolved on Western blot with anti-V5 antibody. As reported previously [11], the receptor was found as dimers (D) and two species of monomers, glycosylated on two (M2) or all of three (M3) extra-cellular potential N-glycosylation sites of the receptor (Fig. 7). Aspirin, BK or icatibant alone had no effect on the cell-surface organization of the B_2 receptor molecules.

Table 1 – Effects of aspirin on the dissociation of [^{3}H]-BK–B_2 complexes induced by BK or icatibant in intact CHO cells

		Inducing ligand	
		BK	Icatibant
EC50 (nM)	Control	5.8 ± 1.2	3.4 ± 0.4
	Aspirin	6.7 ± 1.2	4.3 ± 0.6
Hill coefficient	Control	0.97 ± 0.02	1.79 ± 0.09***
	Aspirin	0.97 ± 0.02	0.97 ± 0.01§§§

Values are estimates of BK or icatibant concentrations for half-reduction of [^{3}H]-BK–B_2 complexes (EC50), and of the Hill coefficient governing the dissociation elicited by BK or icatibant. These values were obtained from non-linear regression plots of Fig. 6 data with the sigmoidal dose–response variable slope equation (Graph Pad^{TM} Prism software). $^{***}p < 0.001$, icatibant *versus* BK. $^{§§§}p < 0.001$ aspirin *versus* corresponding control (unpaired Student's *t* test).

Aspirin had also no effect when combined with BK or icatibant, suggesting that it does not influence ligand binding by altering the oligomerization of the receptor molecules on the cell surface.

3.5. *Aspirin has no effect on B_2 receptor coupling efficiency*

To examine whether aspirin affects receptor coupling efficiency, BK binding and phospholipase C activity were determined in parallel in intact cells, in the absence or presence of 10 mM aspirin, by using the same concentrations range (0.1–20 nM) of [^{3}H]-BK for binding study and unlabeled BK for phospholipase C assay. Data are presented in Fig. 8. The IPs production increased with [^{3}H]-BK binding in both absence and presence of aspirin, but aspirin reduced the basal (no BK) phospholipase C activity. A similar relationship (inset) between receptor occupancy and phospholipase C activation was observed when the latter is expressed as the ratio between BK-stimulated and basal activities. This suggests that aspirin does not affect the ability of the receptor to transduce BK binding into phospholipase C activation.

Receptor coupling efficiency was also studied by parallel measurements of [^{3}H]-BK and [^{35}S]-GTPγS bindings. The experiments were performed without (basal condition) or with 4 nM unlabeled BK (stimulated condition) for [^{35}S]-GTPγS binding assay, and with 4 nM [^{3}H]-BK for [^{3}H]-BK binding assay. The data (Table 2) confirm that aspirin inhibits [^{3}H]-BK binding to B_2 receptor. They also show that interaction of BK with B_2 receptor resulted in an increase in [^{35}S]-GTPγS binding, which was of a lower magnitude when aspirin was present. It is worth noting that aspirin alone increased [^{35}S]-GTPγS binding, and the same result was also observed with membranes from non-transfected cells. This means that aspirin activates G protein(s) other than B_2 receptor-dependent G protein(s). The activation of such G

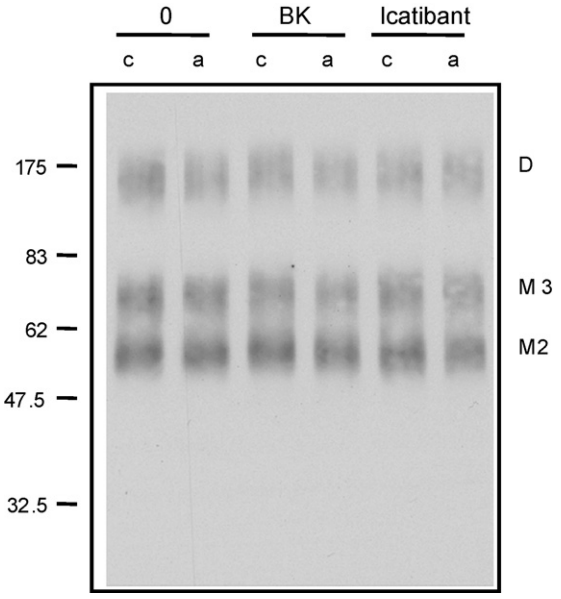

Fig. 7 – Aspirin does not influence cell-surface organization of B_2 receptor molecules. After incubating CHO-K1 cell lines expressing N-terminally V5-tagged B_2 receptor for 20 min at 25 °C with anti-V5 antibody in the absence (0) or presence of 100 nM unlabeled BK (BK) or icatibant (icatibant), and in the absence (c) or presence of 10 mM aspirin (a), cell-surface receptor molecules were immunoprecipitated with anti-V5 antibody, run on 10% (w/v) SDS-PAGE, and analyzed on Western blot with anti-V5 antibody. D corresponds to dimers, and M2 and M3 to di- and tri-glycosylated monomers, respectively. Molecular standard masses (in kDa) positions are indicated on the left side of the panel. The results are representative of experiments repeated three times.

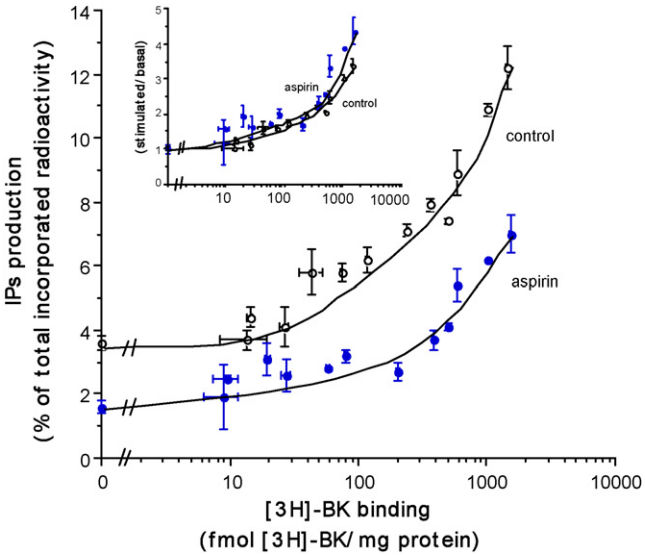

Fig. 8 – Aspirin does not alter B_2 receptor coupling efficiency in intact CHO-K1 cells expressing B_2 receptor. In each experiment, phospholipase C activity (ordinates) and [^{3}H]-BK binding (abscissa) were measured in parallel, after 15 min incubation of the cells with the same increasing unlabeled BK (phospholipase C) and [^{3}H]-BK (binding) concentrations (0.1–20 nM). *Inset*: inositol phosphates production was expressed as the ratio between the stimulated production and the corresponding basal production (3.6 ± 0.2% for control and 1.6 ± 0.2% for aspirin).

protein(s) might be responsible for the reduced basal phospholipase C activity observed in intact cells treated by aspirin. In any case, the ratio between the BK-dependent increase in [^{35}S]-GTPγS binding and [^{3}H]-BK binding did not vary in the presence of aspirin, supporting the conclusion that aspirin does not impair B_2 receptor coupling efficiency.

4. Discussion

The notion that GPCR function can be altered by compounds, termed allosteric modulators as opposed to orthosteric ligands that use the natural ligand binding site, has emerged during the last decade [12–14]. Allosteric modulators of GPCRs described so far are chemically very different in nature. However, there is high specificity regarding modulators acting on a given GPCR. Brucine and gallamine, are active on muscarinic acetylcholine receptors [22–24]. Amiloride is active on dopamine receptors, α-adrenergic receptors and adenosine receptors [25–29] whereas oleamide is active on 5-hydroxytryptamine receptor [30]. We found that none of these compounds influenced the human B_2 receptor (data not shown). By contrast, aspirin was able to influence the ligand binding properties of this receptor.

All experiments of the present work were performed in intact cells pre-treated for 30 min with sucrose to prevent receptor internalization [9,18], or on purified cell plasma membranes, to study receptor molecules located on cell surface. Aspirin decreased the stability of BK–receptor complexes resulting in a decrease in the apparent affinity of the receptor for BK. No effect of aspirin was observed in cells previously exposed to aspirin, if BK binding was monitored in the absence of aspirin. This can either mean that aspirin effect on BK binding is rapidly reversible, or that the receptor needs to bind BK to be in an aspirin-sensitive configuration. Association kinetic data are rather in favor of the second hypothesis, because the effect of aspirin developed progressively with time during BK binding, even after a long 30 min pre-exposure of cells to aspirin.

These results are consistent with aspirin being an allosteric modifier of the B_2 receptor. However, it may be difficult to distinguish on the basis of kinetic data mechanisms based on allosteric interaction from those related to chemical modification. Thus, the compound SCH-202676 firstly described as an allosteric modulator of a variety of structurally distinct GPCRs, was then suggested to be rather a protein modifier influencing GPCRs function *via* thiol modification [31,32]. Aspirin has been found to be active, besides the bradykinin B_2 receptor, on the endothelin ETA receptor but not on the

Table 2 – Effects of aspirin on [^{35}S]-GTPγS binding to membranes of cells expressing the B_2 receptor

	Control	Aspirin
Cells expressing human B_2 receptors		
[^{3}H]-BK binding (A)	477 ± 53	228 ± 39*
[^{35}S]-GTPγS binding		
BK-dependent (B)	174 ± 13	98 ± 14*
B/A	0.36 ± 0.04	0.42 ± 0.05
Basal	363 ± 15	446 ± 30
Non-transfected cells		
[35S]-GTPγS binding, basal	331 ± 13	504 ± 16**

[^{35}S]-GTPγS and [^{3}H]-BK binding assays were performed at 25 °C in parallel using the same membrane preparations. [^{3}H]-BK binding (A) was measured after incubating the membranes for 35 min with 4 nM [^{3}H]-BK in the absence or presence of 10 mM aspirin. For [^{35}S]-GTPγS binding assay, membranes were incubated without or with aspirin for 20 min in the absence (basal condition) or presence (stimulated condition) of 4 nM BK, then for 15 min after adding [^{35}S]-GTPγS to determine the BK-dependent [^{35}S]-GTPγS binding (B), *i.e.* the difference between the stimulated and basal [^{35}S]-GTPγS bindings. Basal binding of [^{35}S]-GTPγS in the absence and presence of aspirin was also determined using membranes from non-transfected CHO-K1 cells. Values for the bindings (expressed in fmol/mg protein) and for the ratio B/A are the means ± S.E.M. of three independent experiments, each performed in triplicate. *$p < 0.05$, **$p < 0.01$, *versus* corresponding control value (unpaired Student's *t* test).

endothelin ETB receptor, the purinergic P2Y2 receptor or the neuromedin B-preferring bombesin receptor [15]. Moreover, the present study documents that the effect of aspirin is quickly reversible upon drug removal, and is not observed after a 30 min pre-incubation with the receptor followed by aspirin withdrawal before BK addition. These features are not consistent with aspirin acting as a chemical modifier of GPCRs, or as a membrane modifier, and are rather suggestive of an allosteric type of mechanism.

Besides the effect of aspirin described here, other allosteric modifications can influence BK binding to the B_2 receptor. One of these allosteric modifications results from the interaction of the receptor with specific G proteins [9], as for any GPCR [12]. In addition, BK itself or the specific B_2 receptor antagonist icatibant trigger interactions between receptor molecules that accelerate the dissociation of [^{3}H]-BK–B_2 complexes [9]. To try to gain further mechanistic insight into the allosteric regulation of B_2 receptor we examined whether the aspirin- and the ligand-triggered modulations of the complex dissociation interfere with each other. We found that aspirin reduced the potency of BK or icatibant for destabilizing pre-formed [^{3}H]-BK–B_2 receptor complexes. Interestingly, the kinetics of aspirin effect on either BK or icatibant actions differed. Indeed, the Hill coefficient describing the ligand-induced dissociation of [^{3}H]-BK–B_2 receptor complexes did not change with aspirin for BK, but was reduced in the case of icatibant. This observation is likely to be related to the fact that BK and icatibant act *via* distinct binding sites on the B_2 receptor [6,33,34], so that aspirin may differentially interfere with the dissociating effect of these two ligands. The concept that BK and icatibant act *via* distinct sites is further supported in the present study by the difference observed, in the absence of aspirin, in the Hill coefficients for BK and icatibant-induced [^{3}H]-BK–B_2 complex dissociations. Hill coefficient was indeed close to 2 for icatibant, and close to 1 for BK, consistent with the fact that icatibant may either occupy one or two sites, whereas BK can only occupy one site when inducing [^{3}H]-BK–B_2 receptor dissociation. The modulations of BK binding induced by orthosteric ligands or by aspirin occur without apparent modification in the arrangement of the B_2 receptor molecules into dimers and monomers. After immunoprecipitation and Western blot, the receptor is found on the cell surface as both dimers and monomers. Assuming that there is only one BK binding site on each receptor molecule, the observation of BK-induced BK–B_2 receptor complex dissociation implies that BK reduces the affinity of the occupied receptors *via* binding to free receptors. This could be achieved within the dimers by trans-conformational change [35,36] whereas separated monomers need to interact with each other for the establishment of the required conformational change. For icatibant-induced BK release, similar mechanisms may be involved. But, it is also possible that icatibant-induced BK release occurs in individual monomers, since icatibant acts on a site distinct from that of BK. The same mechanistic hypotheses as for icatibant can be made for aspirin. In addition, aspirin may interact directly with the receptor or *via* associated proteins such as G proteins, even though aspirin does not modify the transduction of BK binding into phospholipase C activation. Aspirin may also interact with BK. In any case the study suggests the presence on the BK–B_2 receptor complex of an allosteric site accommodating aspirin and having possibly alternate ligands of higher affinity.

Aspirin is widely used as an analgesic, anti-inflammatory and anti-thrombotic agent acting by means of cyclooxygenase-dependent as well as cyclooxygenase-independent mechanisms [37,38]. The proinflammatory and pain promoting effects of BK involves the B_2 and B_1 receptors [2,6,7]. Since we found that aspirin reduces the affinity of B_2 receptor for BK, it is conceivable that the anti-B_2 receptor effect of the drug participates in the analgesic and anti-inflammatory effect of aspirin although this is speculative. It can be pointed out that the anti-BK effect was observed for aspirin concentrations of 1 mM and above (Fig. 1). The anti-inflammatory effect of aspirin requires achieving plasma acetylsalicylate and salicylate concentrations of 1–3 mM, while the anti-thrombotic effect occurs at lower drug concentration [39,40,41]. The comparison of these concentrations with those inhibiting BK binding in our experimental conditions, makes the anti-BK effect of aspirin of putative therapeutic relevance in inflammation. In thrombosis prevention however, while inhibition of BK action can be expected to be detrimental, aspirin concentration may be too low for triggering anti-B_2 receptor effect.

Conflicts of interest

The authors state no conflict of interest.

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