MARCEAU ET AL. SUPPLEMENTARY MATERIAL

Supplementary Methods

Cells. The institutional research ethics board approved the anonymous use of human umbilical cord segments obtained after normal deliveries. All culture surfaces were gelatin-coated. Primary cultures of smooth muscle cells (SMCs) were obtained from explants of deendothelialized human umbilical arteries maintained in medium 199 supplemented with antibiotics and 20% fetal bovine serum (cell culture media and reagents from Gibco-Invitrogen, Burlington, ON, Canada; FBS from Wisent, St. Bruno. QC, Canada). Cells were used between passages 3 to 6. They expressed the marker α-actin (monoclonal antibody 1A4 from Sigma, St. Louis, MO, USA).

Binding assays. The binding of the agonist radioligang [^{3}H]Lys-des-Arg^9-bradykinin (-BK) ([^{3}H]des-Arg^{10}-kallidin, PerkinElmer Biosciences, Boston, MA, USA; 64-88 Ci/mmol) has been applied to intact human umbilical artery SMCs in 12-well plates. Cells were optionally submitted to various treatments (β-cyclodextrin, cholesterol, as mentioned in Supplementary Results), washed twice with the binding medium [consisting of Medium 199 supplemented with 0.1% BSA, 1 μM amastatin, 1 μM captopril, 1 μM phosphoramidon and sodium azide 0.02%, w/v] and filled with 1.0 ml of prewarmed (37°C) binding medium. The B_1 receptor ligand (0.125–4 nM) and cold competing peptide (1 μM concentrations of the agonist Lys-des-Arg^9-BK) for the determination of nonspecific binding) were added to the wells. After 60 min of incubation at 37°C, each well was washed three times with 2 ml of ice-cold phosphate-buffered saline (PBS), pH 7.4. One milliliter of 0.1 N NaOH was finally added to dissolve the cells. Radioactivity in the

resulting suspension was determined by scintillation counting (5 min per vial). The parameters of the Scatchard plots were calculated using a computer program (Tallarida and Murray, 1987).

Cell fractionation. To analyze radioligand and receptor redistribution to buoyant cell fractions, human umbilical artery SMCs (one 75-cm^2 flask in each gradient) were variously treated and prelabeled with a radioligand for 60 min at 37°C in serum-free medium 199, the agonist [^{3}H]Lys-des-Arg^9-BK or the antagonist [^{3}H]Lys-[Leu^8]des-Arg^9-BK (90 Ci/mmol; PerkinElmer; 1 nM of each ligand), supplemented or not by an excess of cold peptide (1 µM). Cell fractionation was performed entirely at 4°C. The medium was removed, the cells were washed twice with cold PBS pH 7.5, lyzed and scraped with Na_2CO_3 500 mM, pH 11, containing the protease inhibitor cocktail Complete Mini (Roche Molecular Biochemicals, Indianapolis, IN) used as directed (total lysis buffer volume of 2 ml). The cellular material recovered from scraping was homogenized (150 strokes in a glass-glass pestle) and sonicated (6 × 15 s). The rest of the separation was adapted from Smart et al. (1995) (first gradient centrifugation only) with some modifications. Briefly, the 2 ml cell lysate was mixed with 2 ml of 50% OptiPrep (Invitrogen) diluted in solution B (0.25 M sucrose, 6 mM EDTA, 120 mM Tris, pH 7.8). The mixture is placed at the bottom of a tube later filled with a 6 ml discontinuous linear gradient of OptiPrep (20 to 10% in buffer A [0.25 M sucrose, 1 mM EDTA, 20 mM Tris, pH 7.8]). This tube was centrifuged for 90 min at 52,000g. At the end, 10 1-ml fractions were collected (numbered from top to bottom) and used for radioactivity counting (400 µl), total protein determination (10 µl; BCA Protein Assay, Pierce) or protein immunoblotting (2-10 µl per track).

Immunoblotting. Ten µl of the 1-ml density gradient fraction were loaded in each track of 9 % polyacrylamide gels; the samples were separated and transferred overnight on PVDF membranes.

Immunoblot for COX-2, $G_{\alpha q}$ and caveolin-1 were applied (monoclonal antibodies from Calbiochem, dilution 1:1000; polyclonals from Upstate, 1:1000; polyclonals from Santa Cruz Biotechnology, 1:750, respectively).

Supplementary Results and Discussion

Human umbilical artery SMCs are an established model for the regulated expression of the B_1R (Moreau et al., 2007; Koumbadinga et al., 2010). A conventional carbonate-buffer extraction of lipid rafts followed by gradient centrifugation was applied in the present experiments. The performance of the technique was examined in experiments reported in the Supplementary Fig., panel A. The ten top fractions of the Optiprep gradient spanned a large range of density values and total proteins were abundant in two areas of the gradient: a light one (fractions 2-4) and a heavier one (fraction number ≥ 6). Endogenous caveolin-1 was mostly present in fractions 3 and 4, whereas protein $G_{\alpha q}$, a primary signaling partner of the B_1 receptor (Leeb-Lundberg et al., 2005), was slightly lighter on the average (maximal in fraction 2), as previously reported in other systems (Rashid-Doubell et al., 2007). Thus, the buoyant lipid raft markers are present in fraction numbers ≤ 5. COX-2, a endoplasmic reticulum protein, is expressed as a solubilized protein in dense fractions (≥ 6).

Cells pre-labeled with 1 nM of the agonist or antagonist radioligands were fractionated in the density gradient system and the specific binding of each was differentially distributed (Supplementary Fig. panel B). [^{3}H]Lys-des-Arg^9-BK was more abundant in buoyant lipid rafts,

and less abundant in soluble fractions than [^{3}H]Lys-[Leu^8]des-Arg^9-BK. Considering the lower amount of total proteins in fractions 1-5 (panel A), the agonist radioligand is highly concentrated in lipid rafts.

β-Cyclodextrin treatment (10 mM in serum-free medium for 60 min, followed by 30 min washout in serum-free medium) was applied to intact smooth muscle cells adherent to 12-well plates (Supplementary Fig. panel C). This system allows cholesterol extraction before the binding assay is applied to the intact cells (saturation curves). The major effect of β-cyclodextrin treatment was to decrease the affinity of the agonist radioligand [^{3}H]Lys-des-Arg^9-BK (K_D 4.4 ± 1.9 nM, vs. 0.17 ± 0.02 nM in controls; Scatchard plots, panel D). There was also an apparent increase of B_{max}, but the bound ligand quantity was uncertain for the higher radioligand concentration. A 30-min pretreatment of cells with cholesterol (1 mM) complexed with methyl-β-cyclodextrin apparently slightly increased the affinity of the B_1 receptor population solution (K_D 0.11 ± 0.01 nM), while somewhat decreasing the B_{max} (29.5 ± 0.6 fmol/mg protein, 39.9 ± 1.2 in controls). Successive treatments with β-cyclodextrin and complexed cholesterol allowed the recovery of an important fraction of the high affinity binding sites (K_D 0.24 ± 0.06 nM, B_{max} 22.6 ± 12.9 fmol/mg; Supplementary Fig. panels C and D), supporting the role of cholesterol in receptor affinity modulation by β-cyclodextrin.

In summary, the B_1R agonist radioligand is concentrated in cholesterol-rich domains of the SMC membrane. The high affinity of this radioligand for the B_1R is dependent on the integrity of these domains. It has been proposed that the major signaling partner of the B_1R, protein G_q, has a caveolar location (Oh and Schnitzer, 2001). This, along with morphological data indicating an

agonist-induced translocation of the B_1R to caveolae (see Main text), suggests that the high affinity state of the B_1R may be dependent on assembly with G_q.

Supplementary References

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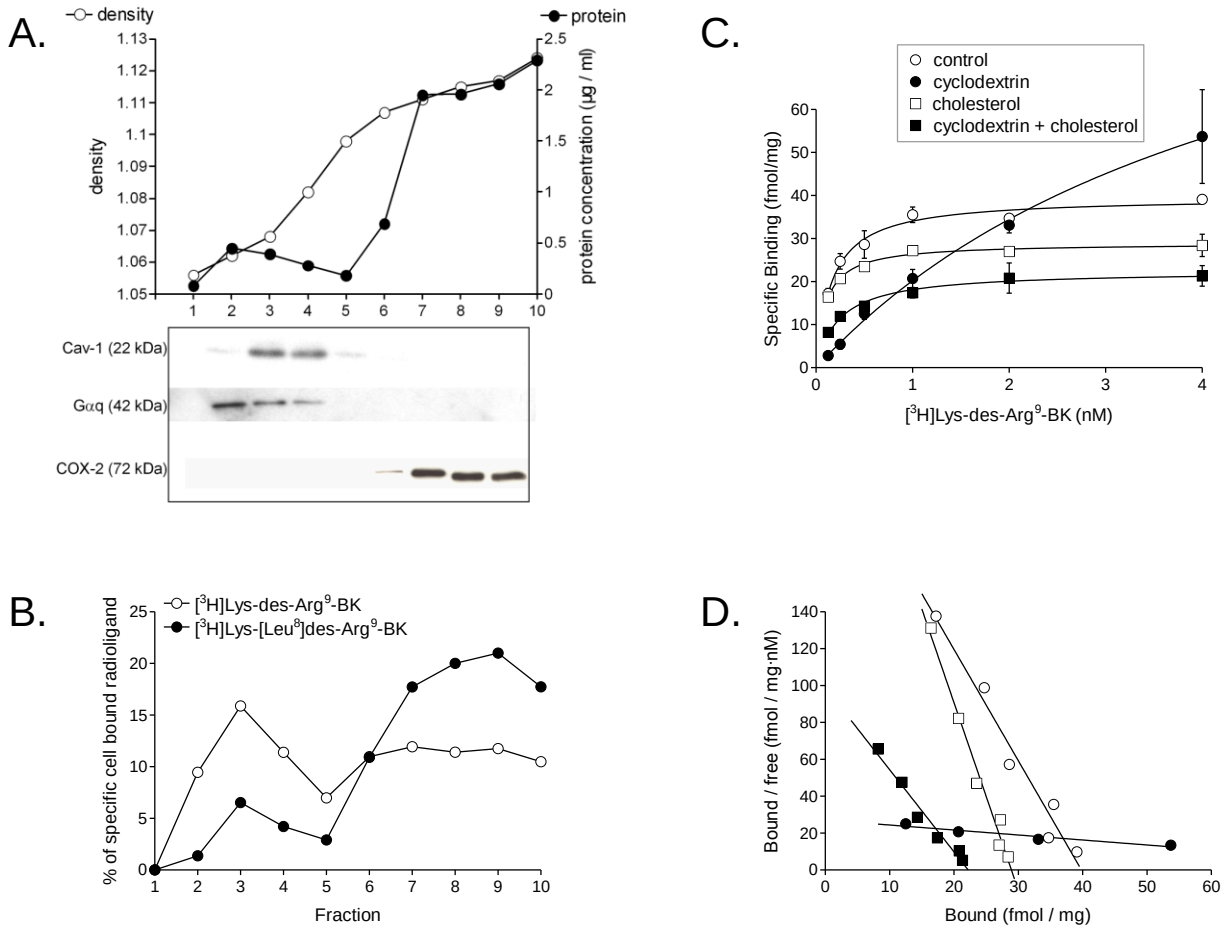

Supplementary figure. A. Comigration of total proteins (top) and specific proteins (immunoblots of 10 µl of each fraction, bottom, fractions 1 to 9 only, middle panel) in buoyant SMC fractions from the density gradient centrifugation in the procedure applied to recover cholesterol-rich rafts. The fraction number (from tube top, 1 ml) is indicated, as well at the density of each fraction (top). B. Migration of radioactivity in buoyant cell fractions from the density gradient centrifugation procedure applied to recover rafts (see text for details). SMC maintained in the complete culture medium were extracted after a treatment with the agonist [^{3}H]Lys-des-Arg^9-BK or the antagonist [^{3}H]Lys-[Leu^8]des-Arg^9-BK (1 nM each) at 37°C (60 min in serum-free medium) (100% = 14.8 fmol for the agonist ligand, 5.3 fmol for the antagonist). C. Effect of successive treatments with β-cyclodextrin (10 mM, 60 min) and cholesterol/β-cyclodextrin complex (1 mM, 30 min, indicated as cholesterol) on the binding sites of [^{3}H]Lys-des-Arg^9-BK in SMCs (full saturation curves). Results (average ± s.e.m. of two experiments composed of duplicatas) are expressed in fmol/mg total cell protein to compensate for some cell loss in wells treated with β-cyclodextrin alone. D. Scatchard plots derived from the averaged binding data in panel C. See text for analysis.