Human B1 and B2 Bradykinin Receptors and Their Agonists Target Caveolae-Related Lipid Rafts to Different Degrees in HEK293 Cells†

Maria E. Lamb,‡ Chongwu Zhang,§ Thomas Shea,‡ Donald J. Kyle,§ and L. M. Fredrik Leeb-Lundberg*,‡

Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78229-3900, and Department of Computational, Combinatorial, and Medicinal Chemistry, Purdue Pharma L.P., Cranbury, New Jersey 08512

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ABSTRACT: To address the targeting of G protein-coupled receptors to caveolae-related lipid rafts (CLR), we studied the human B2 (B2R) and B1 (B1R) bradykinin receptor subtypes in HEK293 cells. CLR were enriched on the basis of their unique buoyant density and composition of cholesterol, caveolin-1, and flotillin-1 but not clathrin. CLR contained B2R and B1R as determined by both receptor immunoblotting and the increase in specific activity of receptor agonist binding to cells at both 4 and 37 °C when binding was followed by CLR enrichment. B2R was highly enriched in this fraction, whereas B1R was not enriched. Furthermore, acid washing of cells prior to cell disruption minimally affected the CLR-associated B2R agonist binding, whereas it dissociated a major portion of the CLR-associated B1R agonist binding. In addition, when agonist binding at 4 °C was followed by an increase in the temperature to 37 °C, B2R agonist binding in CLR transiently increased, and this increase was dependent on the C-terminal domain. On the other hand, B1R agonist binding remained unchanged and was independent of the C-terminal domain. Our results show that B2R is constitutively targeted to CLR in HEK293 cells and appears to shuttle the agonist through these domains, whereas B1R may be there by default.

The cell membrane is composed of microdomains that are characterized by their unique protein and lipid composition and that are thought to serve specialized roles in protein trafficking and signal transduction. Clathrin-coated pits, the most extensively studied membrane microdomains, are shaped by the recruitment of clathrin to the membrane and formation of clathrin baskets, and these domains are involved in receptor-mediated endocytosis (*1*). Rafts are clathrin-independent liquid-ordered membrane assemblies of sphingolipids and cholesterol that associate with particular proteins while excluding others (*2*, *3*). Multiple types of rafts are thought to exist, but the best described are the caveolae. These rafts are characterized by their content of caveolins, which bind cholesterol, homooligomerize, and are thought to assist in shaping these rafts (*4*). Flotillin-1 heterooligomerizes with caveolin-1 and may also be detected in caveolae and possibly other rafts and participates in raft shaping (*5*, *6*). A number of signaling molecules physically interact with caveolins (*4*), but it is not clear if this interaction is always necessary for caveolae association. Nevertheless, caveolae and possibly related rafts have been proposed to serve as platforms for the formation of signaling complexes and facilitate optimal signal transduction (*7*, *8*).

Many heptahelical, G protein-coupled receptors (GPCR)[1] redistribute in the plasma membrane and sequester or internalize in the cell in response to agonist binding. Several such receptors and their cognate G proteins as well as their downstream effector proteins have been proposed to exist in caveolae (*9*). Still, little is known about the nature of this association. This sharply contrasts clathrin-coated domains, which have been characterized in considerable detail in terms of endocytosis and GPCR trafficking (*10*, *11*).

B1 and B2 receptors are the targets for kinins, which are proinflammatory peptides and among the most potent and efficacious vasodilator agonists known (*12*−*14*). The B2 receptor mediates the actions of BK and Lys-BK or KD, whereas the B1 receptor mediates the actions of the carboxypeptidase products desArg^{9}BK and desArg^{10}KD (*12*). These receptor subtypes are prototypical members of the rhodopsin family of GPCR but exhibit only 36% amino acid homology (*15*, *16*). Furthermore, they differ in that the B1 receptor is inducible (*17*), constitutively active (*18*), and desensitizes slowly (*19*), whereas the B2 receptor is ubiquitously expressed, exhibits no apparent constitutive activity (*18*, *20*), and desensitizes rapidly (*19*).

The original impetus for analyzing the association of BK receptors with lipid rafts was the observation that the majority of BK sequestered by the cell via the B2 receptor within 15 min remains associated with the plasma membrane rather than with endocytic vesicles (*21*). It was subsequently found that the B2 receptor-mediated sequestration of BK is almost entirely independent of both dynamin-1 and β-arrestin (*22*).

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* To whom correspondence should be addressed at the Division of Molecular Neurobiology, Wallenberg Neurosciences Center, Lund University, BMC, A12, SE-22184 Lund, Sweden. E-mail: fredrik.leeb-lundberg@mphy.lu.se.

‡ The University of Texas Health Science Center.

§ Purdue Pharma L.P.

[1] Abbreviations: BK, bradykinin; KD, kallidin; CLR, caveolae-related lipid rafts; PM, plasma membrane; PNS, postnuclear supernatant; GPCR, G protein-coupled receptor; WT, wild type; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid.

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The direct identification of BK and B2 receptors in caveolae-related lipid rafts led to the proposal that these microdomains are involved in receptor sequestration (*23*, *24*). The identification of various B2 and B1 receptor effectors such as $G\alpha_q$ (*23*, *25*) and endothelial nitric oxide synthase (*26*, *27*) in rafts also argues for the participation of these domains in receptor signaling.

To further address the role of caveolae-like lipid rafts in GPCR function and trafficking, we took advantage of yet another difference between the B2 and B1 receptor subtypes: the B1 receptor sequesters to a significantly lesser extent than the B2 receptor (*22*). Considering this difference, we hypothesized that the combined analysis of these receptors and their agonists in terms of raft association should tell us if receptors are located in these domains due to their random distribution in the plasma membrane or if a constitutive or active targeting mechanism exists. Since very few cell lines exist that constitutively express B1 receptors at a sufficiently high level, and to allow for receptor mutagenesis, we studied B1 and B2 receptors in transfected HEK293 cells.

EXPERIMENTAL PROCEDURES

Materials. [2,3-*prolyl*-3,4-^{3}H]bradykinin (90−114 Ci/mmol) and des(Arg^{10})[3,4-*prolyl*-3,4-^{3}H]kallidin (64−107 Ci/mmol) were obtained from NEN Life Sciences (Boston, MA). Monoclonal antibodies against caveolin-1 were purchased from ICN (Costa Mesa, CA) and Transduction Laboratories (Lexington, KY). The latter vendor was also the source of monoclonal antibodies against flottilin-1. Monoclonal antibodies against clathrin heavy chain were the kind gift of E. Lafer, University of Texas Health Science Center, San Antonio, TX. The original human B1 and B2 receptor clones in vector pcDNA3 (Invitrogen) were kindly provided by J. F. Hess, Merck Research Laboratories, West Point, PA. Bradykinin and desArg^{10}kallidin were from Bachem (Torrance, CA). Reagents for calcium phosphate transfections were purchased from Clontech (Palo Alto, CA). The Cholesterol CII cholesterol detection kit was purchased from Wako (Richmond, VA). All other chemicals were obtained as previously described (*22*, *23*).

Mutation and Transfection. Mutations were done using a polymerase chain reaction−ligation−polymerase chain reaction protocol as described previously (*20*). HEK293 cells were grown in DMEM supplemented with 10% heat-inactivated horse serum in 10% CO_2 at 37 °C. Transient transfection was done with the calcium phosphate precipitate method in the presence of 15−25 μg of cDNA per 150 mm dish (*22*). This protocol yielded a transfection efficiency of 70−80% as determined in parallel transfection with cDNA encoding green fluorescent protein (unpublished observations). Cells were used 64 h after transfection.

Production of Antisera. Peptides encompassing B1 receptor residues 317−353 [B1R(317−353)] and B2 receptor residues 310−364 [B2R(310−364)] were synthesized at Purdue Pharma L.P. (Cranberry, NJ) following previously reported procedures (*28*). Female New Zealand White rabbits were immunized with unconjugated peptides according to established procedures. Initial antigen injection with Freund's complete adjuvant was followed by two booster injections with Freund's incomplete adjuvant. IgG was purified using a protein A resin as described by the manufacturer (Pierce).

Enrichment of Caveolae-Related Lipid Rafts. Enrichment of CLR followed the procedure of Smart et al. (*29*) as refined by Uittenbogaard et al. (*30*) with a few modifications as previously described (*23*). After agonist exposure, the cells were immediately transferred onto ice and washed with 10 mL of ice-cold buffer A (20 mM Tricine, pH 6.8, 250 mM sucrose, 1 mM EDTA). From thereon, all manipulations were done at 4 °C. Cells were collected in buffer A by scraping with a rubber policeman and washed once in the same buffer by centrifugation at 250*g* for 5 min. The cells were homogenized in a glass/Teflon homogenizer (30 strokes) in 2 mL of buffer A. This is referred to in the text as the cell lysate (LYS). The homogenate was then centrifuged at 1300*g* for 10 min in a microfuge. The supernatant was collected and the pellet homogenized and centrifuged again as described above. The two supernatants were combined, and they are referred to in the text as the postnuclear supernatant fraction (PNS). It was placed on top of 20 mL of 30% Percoll in buffer A and centrifuged in a 50.2Ti rotor at 81000*g* for 30 min. The opaque band at 4.5 cm from the bottom of the tube was collected in a 12 mL polyallomer tube, and the volume was adjusted with buffer A to a total of 2 mL. The sample was sonicated by three sets of two 15 s bursts with 10 s between bursts and 4 min between each set. This is referred to in the text as the plasma membrane fraction (PM). To this sample was then added 0.16 mL of buffer A and 1.84 mL of 50% Optiprep in buffer B (120 mM Tricine, pH 6.8, 250 mM sucrose, 6 mM EDTA), and on top was layered a 7 mL linear gradient of 10−20% Optiprep in buffer A. The gradient was centrifuged in an SW41Ti rotor at 58000*g* for 90 min. The top 5 mL of the gradient was collected and mixed with 4 mL of 50% Optiprep in buffer B in a 12 mL polyallomer tube. On top of the sample was added sequentially 1 mL of 15% and 0.6 mL of 5% Optiprep in buffer A, and the gradient was centrifuged in the SW41Ti rotor at 58000*g* for 90 min. The opaque band just above the 5/15% interface was then collected and is referred to in the text as the caveolae-related lipid rafts (CLR). Protein content of the fractions was determined using the methods of Lowry and Bradford.

Monitoring of Receptor Agonist Complexes. Two protocols were used to monitor the association of receptor agonist complexes with various fractions of the cell. One protocol called "continuous agonist exposure" allows for the monitoring of multiple rounds of agonist receptor complexes. In this protocol, cells were washed with DMEM, pH 7.2, 1 mM 1,10-phenanthroline, 0.1% BSA, and 140 μg/mL bacitracin and then incubated in the same buffer with a saturating concentration of [^{3}H]agonist (∼3 nM) for various times at 37 °C in the absence and presence of 1 μM nonradioactive agonist (*22*). Following enrichment of various cell fractions, the amount of [^{3}H]agonist associated with the fractions was determined by counting for radioactivity in a Beckman LS5000TD scintillation counter.

Another protocol called "one round agonist exposure" allows for the monitoring of one round of complexes. In this protocol, cells were first incubated for 90 min at 4 °C with saturating concentrations of [^{3}H]agonist (∼3 nM) in Leibovitz's L-15 medium, pH 7.4, 20 mM Hepes, 1 mM 1,10-phenanthroline, 0.1% BSA, and 140 μg/mL bacitracin in the absence and presence of 1 μM nonradioactive agonist. The cells were then rinsed with ice-cold DMEM to remove free

radioligand. The amount of [^{3}H]agonist specifically associated with the fraction after this incubation was referred to as "basal" receptor binding. The labeled cells were then incubated in DMEM for various times at 37 °C.

In a few experiments, the accessibility of the [^{3}H]agonist to the extracellular environment was determined using acid washing as previously described (*21*, *22*). In short, after washing the cells with ice-cold PBS and 0.3% BSA, they were incubated for 6 min with 0.05 M glycine, pH 3.0 at 4 °C, washed twice with buffer A, and then disrupted and fractionated as described above under Enrichment of Caveolae-Related Lipid Rafts. The [^{3}H]agonist associated with cellular fractions following the acid wash of the cells was considered sequestered by the fraction and inaccessible to the extracellular environment.

Immunoblotting. For immunoblotting of subcellular fractions, aliquots were taken and mixed with SDS−polyacrylamide gel sample buffer containing 5% β-mercaptoethanol and heated for 5 min at 100 °C. Samples were then electrophoresed on 8%, 10%, or 12% polyacrylamide gels. The gels were then electroblotted onto 0.45 μm nitrocellulose membranes and stained with anti-B2R(310−364) (1:500), anti-B1R(317−353) (1:500), anti-clathrin (1:100), anti-flotillin-1 (1:500), and anti-caveolin-1 (1:1000) (ICN). Immunoreactive bands were visualized with an immunodetection kit using peroxidase-labeled sheep anti-mouse or donkey anti-rabbit antibodies as described by the supplier (Perkin-Elmer Life Sciences).

Determination of Cholesterol Content. Cholesterol was extracted from the samples as described (*31*). Briefly, to each 0.25 mL sample was added 1 mL of hexane−2-propanol (3:2), and the resultant sample was incubated at room temperature for 30 min. The organic phase was saved, and the aqueous phase was re-extracted. The organic phases were combined and dried to completeness with nitrogen. The sample was then solubilized in 600 μL of 1% Triton X-100 in chloroform and dried with nitrogen. This was solubilized in 300 μL of water, and total cholesterol was determined using the Cholesterol CII kit as described by the vendor (Wako).

Data Analysis. Where indicated, data are presented as the mean ± SE and were compared using Student's *t* test.

RESULTS

Enrichment of Caveolae-Related Lipid Rafts. Fractionation of HEK293 cells to enrich for caveolae-related lipid rafts was done according to the procedure of Smart et al. (*29*) as refined by Uittenbogaard et al. (*30*) and as previously described by us (*23*). This procedure, which has been used extensively by multiple investigators to enrich for caveolae-related rafts (e.g., refs *23*, *25*, and *32*−*35*), yields in sequence at least four fractions including cell lysate (LYS), postnuclear supernatant (PNS), plasma membranes (PM), and caveolae membranes. Microheterogeneity is thought to exist among lipid rafts, presumably in part due to their varying content of caveolins. Considering that none of the available fractionation procedures are specific for any one type of raft, due to their very similar buoyant densities, the material in the caveolae membrane fraction is hereafter referred to as caveolae-related lipid rafts (CLR). Immunoblotting of PM and CLR from HEK293 cells revealed that CLR is highly enriched in both flotillin-1 and caveolin-1 immunoreactivity (Figure 1A,B). Furthermore, the concentration of cholesterol in CLR is approximately 10-fold higher than that in PM (Figure 2). On the other hand, CLR is completely devoid of any immunoreactivity toward clathrin heavy chain (Figure 1C). Thus, CLR isolated from HEK293 cells constitute unique plasma membrane microdomains that are clearly distinct from clathrin-containing domains and resemble cholesterol-enriched lipid rafts such as caveolae.

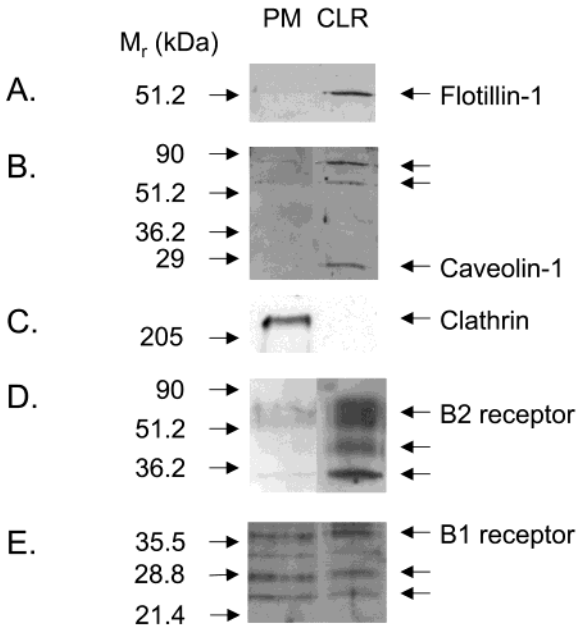

FIGURE 1: Analysis of subcellular fractions from HEK293 cells for clathrin, flotillin, caveolin-1, B1 receptors, and B2 receptors. Cells were fractionated into plasma membrane (PM) and caveolae-related lipid rafts (CLR) as described under Experimental Procedures. Samples of PM and CLR were then evaluated by immunoblotting for flotillin (3.5 and 3.5 μg of protein) (A), caveolin-1 (3.5 and 3.5 μg of protein) (B), clathrin heavy chain (1.8 and 45 μg of protein) (C), B2 receptor (3.5 and 3.5 μg of protein) (D), and B1 receptor (3.4 and 3.4 μg of protein) (E). Molecular mass standards are indicated on the left, and the various proteins analyzed are indicated on the right. The results are representative of at least three different experiments.

Identification of Receptor Agonist Complexes in Caveolae-Related Lipid Rafts. Two methods were used to detect human B1 and B2 receptors in HEK293 cell fractions including immunoblotting of receptors and binding of receptor agonists. Immunoblotting with a polyclonal antibody against the B2 receptor C-terminal residues 310−364 [anti-B2R(310−364)] resulted in the identification of receptor species with molecular masses of ~70, 44, and 32 kDa, which were highly enriched in CLR (Figure 1D). The broad band at ~70 kDa is identical in size to the BK-cross-linked bovine myometrial B2 receptor identified by anti-BK immunoblotting (*36*) and the human foreskin fibroblast B2 receptor identified by receptor immunoblotting (*37*). The smaller species are assumed to be proteolytic products of the ~70 kDa receptor species. Immunoblotting with an antibody against the B1 receptor C-terminal residues 317−353 [anti-B1R(317−353)] resulted in the identification of receptor-specific species with molecular masses of 38, 28, and 24 kDa (Figure 1E). In contrast to the B2 receptor, the B1 receptor species were not enriched in CLR. The size of the 38 kDa species is identical to that of the immunoblotted FLAG-tagged human

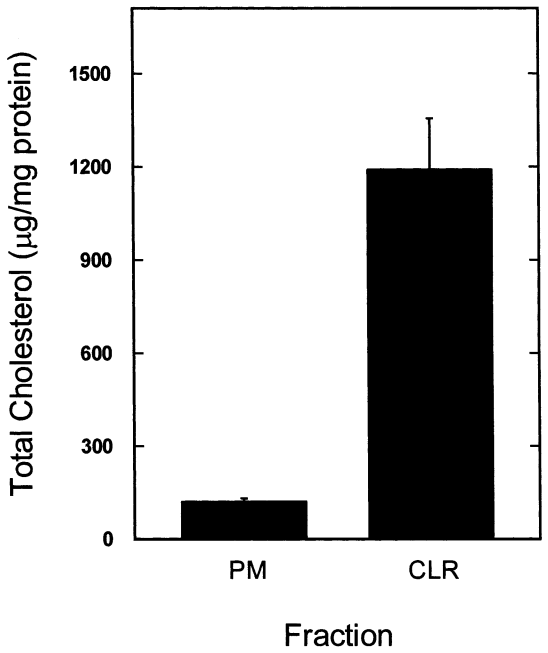

FIGURE 2: Analysis of subcellular fractions from HEK293 cells for total cholesterol. Cells were fractionated into plasma membrane (PM) and caveolae-related lipid rafts (CLR) as described under Experimental Procedures. Samples of PM and CLR were then evaluated for total cholesterol. The results are averages ± SE of at least three experiments.

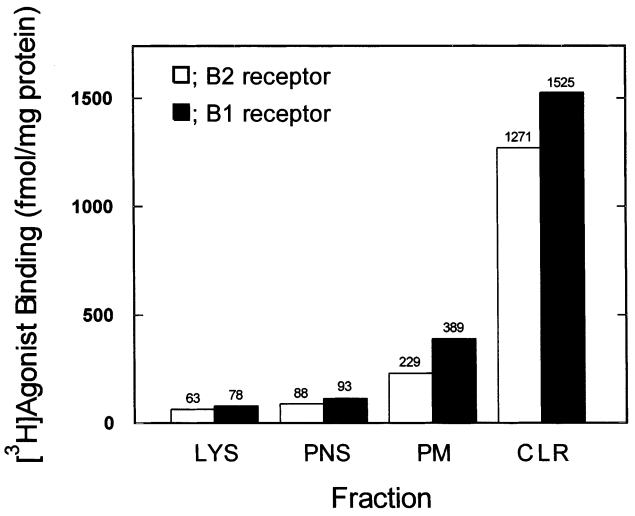

FIGURE 3: Specific binding activity of [^{3}H]BK and [^{3}H]des-Arg^{10}KD in subcellular fractions from HEK293 cells. Cells expressing WT B2 receptors (open bars) or WT B1 receptors (filled bars) were subjected to continuous exposure to [^{3}H]BK and [^{3}H]des-Arg^{10}KD, respectively, for 15 min at 37 °C as described under Experimental Procedures. The cells were then fractionated into lysate (LYS), postnuclear supernatant (PNS), plasma membrane (PM), and caveolae-related lipid rafts (CLR). Samples of LYS, PM, PNS, and CLR were then analyzed for the specific activity (fmol/mg of protein) of agonist binding. The results are representative of at least three different experiments.

B1 receptor expressed in these cells (*18*), indicating that it represents the intact B1 receptor. Again, the smaller species are assumed to be proteolytic products of the 38 kDa receptor species (Figure 1E). In each case, the detection of the immunoreactive species was blocked by preincubation of the antibody with the antigen (data not shown). In all, these results show that both B2 and B1 receptors are present in CLR in naive HEK293 cells, but only B2 receptors are specifically targeted to these domains.

One way to detect binding of receptor agonist in CLR was to continuously expose B2 and B1 receptor-expressing cells to a saturating concentration (~3 nM) of [^{3}H]BK and [^{3}H]desArg^{10}KD, respectively, for 15 min at 37 °C prior to cell disruption and fractionation, a protocol referred to as continuous agonist exposure. This protocol revealed that the specific activity of B2 receptor binding was considerably higher in CLR compared to PM (Figure 3). The specific activity of B1 receptor binding was also higher in CLR, and these results were observed over relatively wide ranges of expressed receptor binding levels (Table 1). Coincubation with nonradiolabeled agonists prior to cell disruption and fractionation revealed that 97−99% of the enriched agonist depended on receptor binding for enrichment in CLR. The appearance of agonist in CLR was rapid (minutes) in both B2 and B1 receptor-expressing cells. The B2 receptor binding level peaked at 15 min and then declined slowly (Figure 4A), whereas the B1 receptor binding level was maximal at 5 min and remained at that level for ≥30 min (Figure 4B).

To determine the degree of extracellular accessibility of the CLR-associated agonists, agonist-labeled cells were acid-washed prior to cell disruption and fractionation as previously described (*22*, *23*). Figure 5 shows that CLR-associated B2 receptor agonist was almost completely resistant to acid washing (80 ± 3%), whereas CLR-associated B1 receptor agonist exhibited only a limited level of acid resistance (31 ± 9%). Thus, agonists targeted to CLR by the B1 receptor remain within CLR in a state relatively accessible from the exterior of the cell, whereas agonists targeted to CLR by the B2 receptor become sequestered within these domains. Indeed, using acid resistance as the parameter for agonist association with CLR, the B2 receptor agonist is clearly more enriched in these compartments (19 ± 3-fold) compared to the B1 receptor agonist (10 ± 3-fold) (Table 1).

Kinetics of Association of Receptor Agonist Complexes with Caveolae-Related Lipid Rafts. Significant B2 and B1 receptor binding was also detected following exposure of cells to agonist for 90 min at 4 °C, a protocol termed one round agonist exposure, indicating that resident receptors are present in CLR in naive cells (Table 1). Elevating the temperature of cells subjected to one round agonist exposure to 37 °C allows for monitoring the fate of the CLR-associated receptor agonist. Doing so led to a transient increase in the CLR-associated B2 receptor agonist by approximately 2-fold (Figure 6A). On the other hand, the amount of CLR-associated B1 receptor agonist did not change (Figure 6B). The B2 receptor results match those seen in DDT$_1$ MF-2 cells, which natively express this receptor (*23*). While these results may be interpreted in several ways, one possible explanation is that the B2 receptor actively shuttles bound agonist within or through CLR, whereas the B1 receptor does not.

Figure 7 shows that the immunoreactivity of the antibodies was completely dependent on expression of the B2 and B1 receptors as there was no immunoreactivity in mock-transfected cells. Treatment of the B2 receptor-expressing cells with 1 μM BK for up to 30 min had no significant effect on the amount of the ~70 kDa B2 receptor-specific species in the CLR fraction (Figure 7A). The same result was obtained with the 38 kDa B1 receptor-specific species

Table 1: Specific Binding Activity of [^{3}H]DesArg^{10}KD and [^{3}H]BK to Human WT, Truncated, and Chimeric B1 and B2 Receptor Constructs in Subcellular Fractions of HEK293 Cells[a]

	fmol/mg of protein at 37 °C					fmol/mg of protein at 4 °C		
construct	LYS	CLR	CLR_A	CLR/LYS	CLR_A/LYS	LYS	CLR	CLR/LYS
WT B1								
A	45	1313	690	29	15	12	242	20
B	58	1547	137	27	2	27	936	35
C	28	698	357	25	13			
D	5	92	76	18	15			
E	11	191	43	17	4			
				23 ± 2	10 ± 3[b]			
WT B2								
A	41	529	752	13	18	36	544	15
B	40	483	438	12	11	37	489	13
C	56	615	488	11	9	146	4116	28
D	88	1978	1567	22	18			19 ± 5
E	40	648	993	16	25			
F	74	2009	1520	27	21			
G	28	299	957	11	34			
				16 ± 2	19 ± 3			
B1Stop320								
A	97	4610	701	48	7	26	1646	63
B	76	1993	482	26	6	40	1149	29
C	39	707	525	18	14			
				31 ± 9	9 ± 2			
B1(B2ICIV)								
A	357	12731		36		177	8670	49
B	56	1081		19		149	6339	43
C	116	3581		31		203	8898	44
				29 ± 5				45 ± 2
B2Stop314								
A	36	411	251	11	7	27	76	3
B	16	133	110	8	7	36	148	4
C	15	105	76	7	5			
				9 ± 1[c]	6 ± 1[c]			
B2(B1ICIV)								
A	67	656		10		70	560	8
B	42	113		3		131	818	6
C	22	204		9				
				7 ± 2[c]				

[a] Cells expressing each of the receptor constructs were incubated with radioligand for 15 min at 37 °C or 90 min at 4 °C. Activity of the radioligand (fmol/mg of protein) was then measured in the LYS fraction and CLR fraction with (CLR_A) and without acid washing. Results from two to seven independent transfection experiments (A, B, ...) are shown for each construct. The ratios of activities in each experiment and ratio mean ± SE are also shown. [b] $p < 0.005$ vs CLR/LYS for WT B1. [c] $p < 0.05$ vs WT B2R.

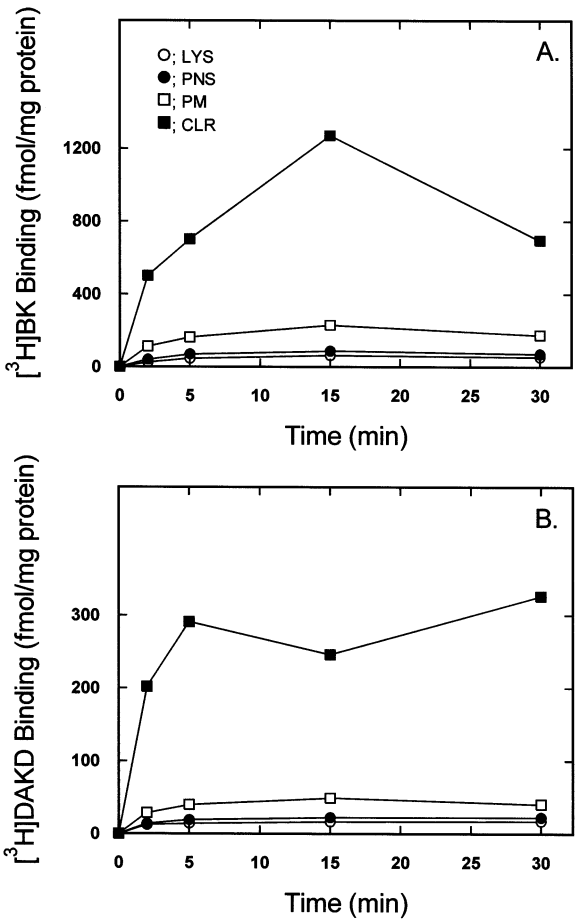

FIGURE 4: Time courses of association of specific binding of [^{3}H]BK and [^{3}H]desArg^{10}KD in subcellular fractions from HEK293 cells using continuous agonist exposure. Cells expressing WT B2 receptors (A) or WT B1 receptors (B) were subjected to continuous exposure to [^{3}H]desArg^{10}KD and [^{3}H]BK, respectively, for various times at 37 °C as described under Experimental Procedures. The cells were then fractionated into lysate (LYS), postnuclear supernatant (PNS), plasma membrane (PM), and caveolae-related lipid rafts (CLR). Samples of LYS, PM, PNS, and CLR were then analyzed for the specific activity (fmol/mg of protein) of agonist binding. The results are representative of at least three different experiments.

when B1 receptor-expressing cells were treated with 1 μM desArg^{10}KD (Figure 7B). Thus, agonist binding apparently does not perturb the net amount of either B2 or B1 receptors in CLR.

Role of the Receptor C-Terminal Domain in the Association of Receptor Agonist Complexes with Caveolae-Related Lipid Rafts. The GPCR C-terminal tail plays important roles in both receptor signaling and regulation. Indeed, the B1 and B2 receptor tails contain motifs for both palmitoylation and caveolin binding, two events that may participate in caveolae association (*4*). To begin to evaluate the molecular determinants for GPCR targeting to CLR, we made a series of constructs in which the C-terminal tails in the B1 and B2 receptors were truncated at various points. As shown in Figure 8, the B2 receptor was truncated after Arg^{313} (B2Stop314), and the B1 receptor was truncated after Phe^{319} (B1Stop320). We also created chimeric receptor constructs in which the C-terminal tails were exchanged between the two receptors at the conserved residues B2Gly^{309} and B1Gly^{316} (Figure 8) to make B2(B1ICIV) and B1(B2ICIV). All of the chimeric and truncation constructs bound their respective agonists with high affinity (*18*, *38*). Furthermore, all of the constructs were functionally active except B2Stop314, which was inactive. Table 1 shows that, when monitored by continuous agonist exposure at 37 °C for 15 min, agonist binding to B2(B1ICIV) and B2Stop314 was enriched in CLR at specific activities that were 56% and 44% lower, respectively, than that of binding to the WT B2 receptor. The B2Stop314 binding that was associated with CLR remained sequestered in these domains (Figure 5 and Table 1). In contrast, the specific activities of binding to B1(B2ICIV) and B1Stop320 were not significantly different from that to the WT B1 receptor (Table 1). Furthermore, CLR-associated agonist binding to B1Stop320 remained almost completely acid labile (Figure 5 and Table 1).

We also evaluated the effect of the receptor C-terminal domain on the kinetics of CLR association of one round of receptor-bound agonist. The relatively lower specific activities of B2Stop314 and B2(B1ICIV) binding in CLR observed

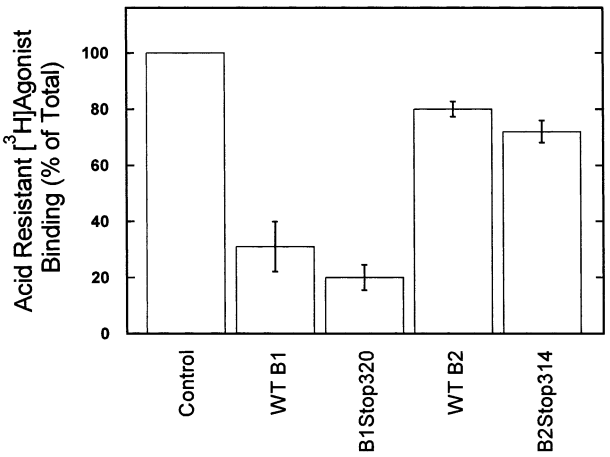

FIGURE 5: Effect of acid washing on the specific binding of [^{3}H]BK and [^{3}H]desArg^{10}KD in caveolae-related lipid rafts from HEK293 cells. Cells expressing WT B2 receptors (B2), WT B1 receptors (B1), B2Stop314, or B1Stop320 were subjected to continuous exposure to [^{3}H]BK or [^{3}H]desArg^{10}KD for 15 min at 37 °C as described under Experimental Procedures. After being washed with buffer or glycine hydrochloride, pH 3.0, the cells were enriched for caveolae-related lipid rafts (CLR). Samples were then analyzed for the specific activity of agonist binding. The results are expressed as percent of control, where 100% control is the total amount of specific binding of [^{3}H]desArg^{10}KD to WT B1 receptors and B1Stop320 and of [^{3}H]BK to WT B2 receptors and B2Stop314 in CLR in the absence of low pH washing. The results are averages ± SEM of at least three experiments.

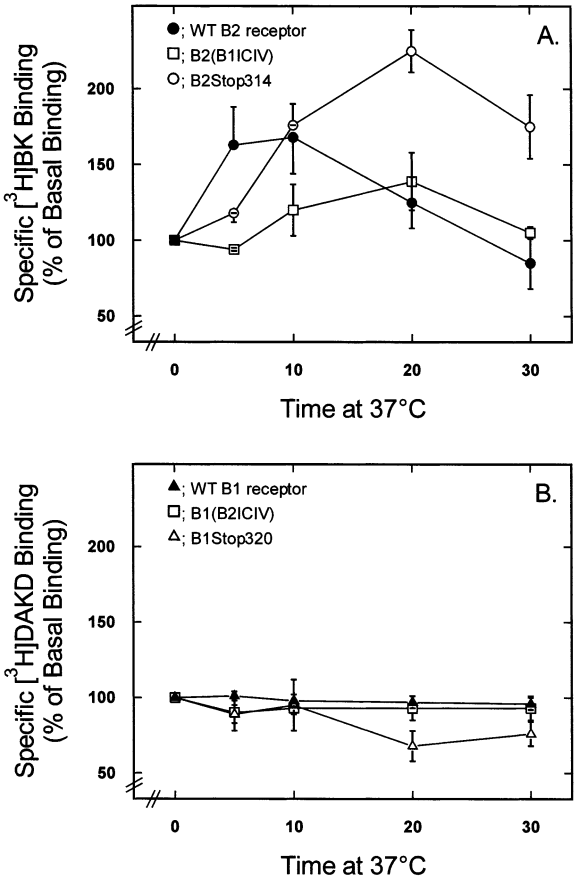

FIGURE 6: Time courses of association of specific binding of [^{3}H]BK and [^{3}H]desArg^{10}KD in caveolae-related lipid rafts from HEK293 cells using one round agonist exposure. (A) Cells expressing WT B2 receptors (36−37 fmol/mg of protein), B2Stop314 (27−36 fmol/mg of protein), and B2(B1ICIV) (70−131 fmol/mg of protein) were subjected to one round agonist exposure to [^{3}H]BK and then incubated for various times at 37 °C. (B) Cells expressing WT B1 receptors (12−27 fmol/mg of protein), B1Stop320 (26−40 fmol/mg of protein), and B1(B2ICIV) (149−203 fmol/mg of protein) were subjected to one round agonist exposure to [^{3}H]desArg^{10}KD and then incubated for various times at 37 °C. In both (A) and (B), the cells were then fractionated into caveolae-related lipid rafts (CLR). Samples were then analyzed for the specific activity of agonist binding. The results are expressed as percent of basal binding, where 100% basal binding is the amount of specific radiolabeled agonist binding in CLR following incubation at 4 °C for 90 min in cells expressing WT B2 receptors (489−544 fmol/mg of protein), WT B1 receptors (242−936 fmol/mg of protein), B2Stop314 (76−148 fmol/mg of protein), B1Stop320 (1149−1646 fmol/mg of protein), B2(B1ICIV) (560−818 fmol/mg of protein), and B1(B2ICIV) (6339−8898 fmol/mg of protein). The results are averages ± SE of at least two experiments.

during continuous agonist exposure at 37 °C were also observed when binding was established at 4 °C (Table 1). B2Stop314 binding behaved similarly to the WT B2 receptor binding in that it transiently increased by approximately 2-fold in CLR (Figure 6A). However, the truncation caused a significant delay in the increase. Indeed, the peak increase in WT B2 receptor binding occurred at about 5 min, whereas the peak increase in the B2Stop314 binding occurred at about 20 min. A delay in the response was also observed with B2-(B1ICIV). In addition, this response was also significantly reduced. On the other hand, B1Stop320 and B1(B2ICIV) binding behaved almost identically to the WT B1 receptor binding (Figure 6B). In all, these results show that the rate and amount of agonist B2 receptor association and sequestration in CLR are dependent on the receptor C-terminal tail. However, other receptor domains seem to participate as well since the substitution of the B2 receptor tail in the B1 receptor did not produce a response in this receptor.

DISCUSSION

In this study, we probed the role of lipid rafts in GPCR trafficking in HEK293 cells by taking advantage of the different degrees of sequestration of the human B2 and B1 receptor subtypes (*22*). Both receptors and their agonists are found in CLR as determined by both receptor immunoblotting of CLR and the increase in specific activity of receptor-dependent radiolabeled agonist binding at 4 and 37 °C upon CLR enrichment. However, the B2 receptor is highly enriched in CLR, whereas the B1 receptor is not enriched in this fraction. Therefore, some receptors, like the B2 receptor, are constitutively and specifically targeted to CLR, whereas others, like the B1 receptor, exist in CLR apparently as a result of their random distribution in the plasma membrane.

Several GPCR have been reported to associate with lipid rafts including the B2 BK (*23*, *24*), B1 BK (*39*), ET_A endothelin (*40*), cholecystokinin type A (*41*), β_2-adrenergic (*35*, *42*), m2 muscarinic (*43*), calcium-sensing (*34*), A_1 adenosine (*33*), and EDG-1 (*44*) receptors. Due to the microheterogeneity that is thought to exist among lipid rafts and the variation in techniques used to analyze the association of these receptors with rafts, which include their coenrichment with caveolin-containing light buoyancy fractions, co-immunoprecipitation with caveolins, colocalization with caveolin based on confocal fluorescence microscopy, and/or identification in uncoated vesicles by immunoelectron-microscopy, it is still unclear to what degrees different

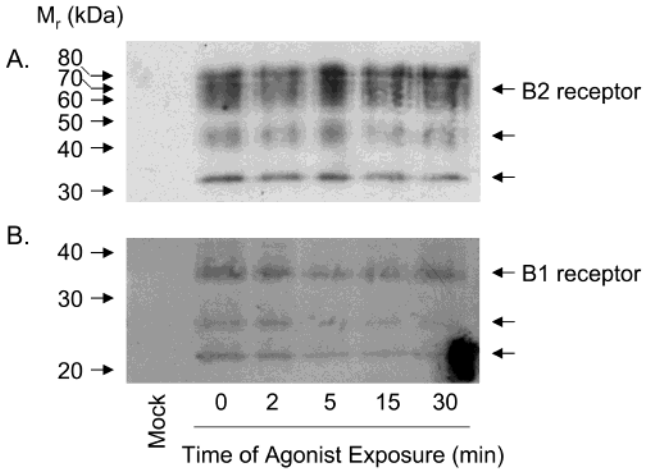

FIGURE 7: Effect of agonist exposure on B1 and B2 receptors in caveolae-related lipid rafts from HEK293 cells. Cells expressing WT B2 receptors (A) or WT B1 receptors (B) were subjected to continuous exposure to 1 μM BK and desArg^{10}KD, respectively, for various times at 37 °C as described under Experimental Procedures. The cells were then fractionated into caveolae-related lipid rafts (CLR). Samples of CLR, at equal amounts of protein, from the various time points were then evaluated by immunoblotting for B2 receptor (0.3 μg of protein) and B1 receptor (1.4 μg of protein). Molecular mass standards are indicated on the left, and the B1 and B2 receptors are indicated on the right.

receptors associate with these rafts and if the same or different types of rafts are involved.

We compared the CLR association of two physiologically related but structurally distinct receptors, the B2 and B1 BK receptors, and used a lipid raft fraction (CLR) enriched from HEK293 cells according to the procedure of Smart et al. (*29*) as refined by Uittenbogaard et al. (*30*). This fraction is caveolae-related since it is enriched in cholesterol, caveolin-1, and flotillin-1 but not in clathrin. It is also enriched in B2 receptors and in B2 receptor-bound BK. Incubation at 37 °C leads to an increase in the amount of CLR-associated BK.

The net amount of CLR-associated B2 receptors does not change upon agonist stimulation. The apparently conflicting kinetics of BK and B2 receptor association with CLR during BK stimulation may be reconciled by the fact that different techniques were used to monitor the two. The reversibly bound BK needs to be trapped, presumably in a properly oriented vesicle, to remain associated with the CLR fraction during the relatively extensive fractionation procedure, whereas the receptor does not. Indeed, specific clusters of BK immunoreactivity are observed in CLR as determined by immunoelectron microscopy (*23*). Therefore, a likely scenario is that B2 receptors exist both outside and inside the "cave" of the raft or in any of the subcompartments of which lipid rafts such as caveolae have been proposed and observed to be composed (*45*, *46*). On the other hand, BK binds to receptors independently of their localization within the raft. However, since different parts of rafts probably vesicularize differently, BK may be trapped and, consequently, enriched only when bound to receptors in parts where it can be trapped. Conversely, receptors may be enriched independently of their raft locale. Hence, an increase in the amount of BK in CLR without a change in the amount of receptor may be due to the redistribution of receptor−agonist complexes within the raft. Indeed, the sequestration of BK in CLR that occurs upon raising the temperature to 37 °C may very well involve such redistribution.

In contrast to the B2 receptor, the B1 receptor is not enriched in the CLR fraction. Furthermore, elevation of the temperature to 37 °C does not change the amount of desArg^{10}KD binding in CLR. In addition, a major portion of the CLR-associated desArg^{10}KD does not become sequestered in these domains at this temperature. Thus, B1 receptors may exist in rafts simply due to their random distribution in the plasma membrane.

To begin to address the mechanism of receptor targeting to CLR, we searched for motifs upon which CLR association has been reported to be dependent and which may differ in the B2 and B1 receptors. The role of the C-terminal domain in GPCR regulation is well described. Both the B2 and B1 receptor C-terminal domains contain motifs for acylation at Cys^{324,329} and Cys^{330}, respectively, a covalent modification necessary for some proteins to associate with caveolae (*26*, *27*, *47*). A putative caveolin binding motif (ΦXΦXXXXΦ and ΦXXXXΦXXΦ, where Φ is aromatic amino acid Trp, Phe, Tyr) is also present in both the B2 (Phe^{312}XXXXTrp^{317}-XXTyr^{320}) and the B1 receptors (Phe^{319}XXXXTrp^{324}-XXTyr^{327}) (*4*). Several effector proteins that associate with caveolin-1 and -3 contain this motif, including caveolin-1 and -3 themselves, but not caveolin-2, and the motif binds the caveolin scaffolding domain, a 41 amino acid region of the cytoplasmic N-terminal domain of the caveolins (*4*). The B2 receptor tail also contains a Ser/Thr cluster, which is phosphorylated in vivo in response to agonist (*48*) and is critical for receptor desensitization (*20*) and total receptor sequestration (*48*). A similar cluster is absent in the B1 receptor, which is not subject to any significant phosphorylation (*49*), desensitization (*19*), and sequestration (*22*).

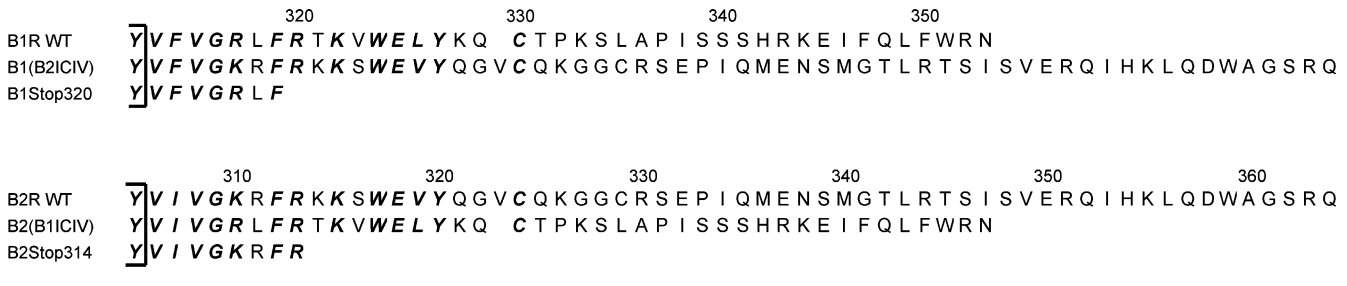

FIGURE 8: Amino acid sequence alignments of the C-terminal tails of the human WT and truncated B2 and B1 receptors. Indicated is the predicted junction of the seventh transmembrane domain and the C-terminal tail (half bracket) and conserved residues between the receptor subtypes (bold italics). The numbering of residues in the B2 and B1 receptors is according to Hess et al. (*15*) and Menke et al. (*16*), respectively.

Truncation of the B2 receptor C-terminal domain decreased the amount of B2 receptor agonist in CLR. This decrease may be due to slower CLR transit since truncation also delayed the transient increase in agonist binding following one round agonist exposure. This decrease occurred at both 4 and 37 °C, arguing that truncation leads to fewer receptors in raft locales capable of binding and trapping the ligand. Thus, the B2 receptor C-terminal domain seems to contain an epitope that participates in receptor targeting to CLR or the transit of agonist receptor complexes through CLR. Substitution of the B1 receptor C-terminal tail in the B2 receptor decreased the amount and the rate of B2 receptor agonist targeting to CLR. The C-terminal tail does not seem to be the only domain responsible for CLR targeting since substitution of the B2 receptor tail in the B1 receptor did not produce a response in the B1 receptor. Also, truncation of the C-terminal domain had no effect on B1 receptor CLR association.

In summary, B2 and B1 receptors both reside in CLR in HEK293 but do so to drastically different degrees. Considering that fractions enriched for cholesterol, caveolin-1, and flotillin-1 by density centrifugation are most probably heterogeneous, we cannot conclusively state with which type of raft(s) these receptors associate. The B2 receptor is constitutively and specifically targeted to these structures, where it sequesters the agonist through a mechanism that involves the receptor C-terminal domain. On the other hand, the B1 receptor is not specifically targeted to CLR, and the B1 receptor agonist associated with CLR remains to a large extent stationary and accessible in these structures.

REFERENCES

1. Mellman, I. (1996) *Annu. Rev. Cell Dev. Biol. 12*, 575−625.
2. Kurzchalia, T. V., and Parton, R. G. (1999) *Curr. Opin. Cell Biol. 11*, 424−431.
3. Pike, L. J., Han, X., Chung, K.-N., and Gross, R. W. (2002) *Biochemistry 41*, 2075−2088.
4. Smart, E. J., Graf, G. A., McNiven, M. A., Sessa, W. C., Engelman, J. A., Scherer, P. E., Okamoto, T., and Lisanti, M. P. (1999) *Mol. Cell. Biol. 19*, 7289−7304.
5. Bickel, P. E., Scherer, P. E., Schnitzer, J. E., Oh, P., Lisanti, M. P., and Lodish, H. F. (1997) *J. Biol. Chem. 272*, 13793−13802.
6. Volonte, D., Galbiati, F., Li, S., Nishiyama, K., Okamoto, T., and Lisanti, M. P. (1999) *J. Biol. Chem. 274*, 12702−12709.
7. Shaul, P. W., and Anderson, R. G. (1998) *Am. J. Physiol. 275*, L843−L851.
8. Ostrom, R. S., Post, S. R., and Insel, P. A. (2000) *J. Pharmacol. Exp. Ther. 294*, 407−412.
9. Anderson, R. G. W. (1998) *Annu. Rev. Biochem. 67*, 199−225.
10. Krupnick, J. G., and Benovic, J. L. (1998) *Annu. Rev. Pharmacol. Toxicol. 38*, 289−319.
11. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) *Annu. Rev. Biochem. 67*, 653−692.
12. Regoli, D., and Barabe, J. (1980) *Pharmacol. Rev. 32*, 1−46.
13. Proud, D., and Kaplan, A. P. (1988) *Annu. Rev. Immunol. 6*, 49−83.
14. Bhoola, K. D., Figueroa, C. D., and Worthy, K. (1992) *Pharmacol. Rev. 44*, 1−80.
15. Hess, J. F., Borkowski, J. A., Young, G. S., Strader, C. D., and Ransom, R. W. (1992) *Biochem. Biophys. Res. Commun. 184*, 260−268.
16. Menke, J. G., Borkowski, J. A., Bierilo, K. K., MacNeil, T., Derrick, A. W., Schneck, K. A., Ransom, R. W., Strader, C. D., Linemeyer, D. L., and Hess, J. F. (1994) *J. Biol. Chem. 269*, 21583−21586.
17. Marceau, F., Hess, J. F., and Bachvarov, D. R. (1998) *Pharmacol. Rev. 50*, 357−386.
18. Leeb-Lundberg, L. M., Kang, D. S., Lamb, M. E., and Fathy, D. B. (2001) *J. Biol. Chem. 276*, 8785−8792.
19. Mathis, S. A., Criscimagna, N. L., and Leeb-Lundberg, L. M. (1996) *Mol. Pharmacol. 50*, 128−139.
20. Fathy, D. B., Leeb, T., Mathis, S. A., and Leeb-Lundberg, L. M. (1999) *J. Biol. Chem. 274*, 29603−29606.
21. Munoz, C. M., and Leeb-Lundberg, L. M. (1992) *J. Biol. Chem. 267*, 303−309.
22. Lamb, M. E., De Weerd, W. F., and Leeb-Lundberg, L. M. (2001) *Biochem. J. 355*, 741−750.
23. de Weerd, W. F., and Leeb-Lundberg, L. M. (1997) *J. Biol. Chem. 272*, 17858−17866.
24. Haasemann, M., Cartaud, J., Muller-Esterl, W., and Dunia, I. (1998) *J. Cell Sci. 111*, 917−928.
25. Oh, P., and Schnitzer, J. E. (2001) *Mol. Biol. Cell 12*, 685−698.
26. Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G., and Michel, T. (1996) *J. Biol. Chem. 271*, 6518−6522.
27. Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J. E., and Sessa, W. C. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 6448−6453.
28. Kyle, D. J., Martin, J. A., Farmer, S. G., and Burch, R. M. (1991) *J. Med. Chem. 34*, 1230−1233.
29. Smart, E. J., Ying, Y. S., Mineo, C., and Anderson, R. G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10104−10108.
30. Uittenbogaard, A., Ying, Y., and Smart, E. J. (1998) *J. Biol. Chem. 273*, 6525−6532.
31. Uittenbogaard, A., Shaul, P. W., Yuhanna, I. S., Blair, A., and Smart, E. J. (2000) *J. Biol. Chem. 275*, 11278−11283.
32. Lasley, R. D., Narayan, P., Uittenbogaard, A., and Smart, E. J. (2000) *J. Biol. Chem. 275*, 4417−4421.
33. Mineo, C., Gill, G. N., and Anderson, R. G. (1999) *J. Biol. Chem. 274*, 30636−30643.
34. Kifor, O., Diaz, R., Butters, R., Kifor, I., and Brown, E. M. (1998) *J. Biol. Chem. 273*, 21708−21713.
35. Rybin, V. O., Xu, X., Lisanti, M. P., and Steinberg, S. F. (2000) *J. Biol. Chem. 275*, 41447−41457.
36. Herzig, M. C., and Leeb-Lundberg, L. M. (1995) *J. Biol. Chem. 270*, 20591−8.
37. Abd Alla, S., Quitterer, U., Grigoriev, S., Maidhof, A., Haasemann, M., Jarnigan, K., and Muller-Esterl, W. (1996) *J. Biol. Chem. 271*, 1748−1755.
38. Kang, D. S., and Leeb-Lundberg, L. M. F. (2002) *Mol. Pharmacol.* (in press).
39. Sabourin, T., Bastien, L., Bachvarov, D. R., and Marceau, F. (2002) *Mol. Pharmacol. 61*, 546−553.
40. Okamoto, Y., Ninomiya, H., Miwa, S., and Masaki, T. (2000) *J. Biol. Chem. 275*, 6439−6446.
41. Roettger, B. F., Rentsch, R. U., Pinon, D., Holicky, E., Hadac, E., Larkin, J. M., and Miller, L. J. (1995) *J. Cell Biol. 128*, 1029−1041.
42. Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S., Strosberg, A. D., and Benedetti, E. L. (1989) *Eur. J. Cell Biol. 50*, 340−352.
43. Feron, O., Smith, T. W., Michel, T., and Kelly, R. A. (1997) *J. Biol. Chem. 272*, 17744−17748.
44. Igarashi, J., and Michel, T. (2000) *J. Biol. Chem. 275*, 32363−32370.
45. Rietveld, A., and Simons, K. (1998) *Biochim. Biophys. Acta 1376*, 467−479.
46. Kosawada, T., Skalak, R., and Schmid-Schonbein, G. W. (1999) *J. Biomech. Eng. 121*, 472−479.
47. Parat, M.-O., and Fox, P. L. (2001) *J. Biol. Chem. 276*, 15776−15782.
48. Pizard, A., Blaukat, A., Muller-Esterl, W., Alhenc-Gelas, F., and Rajerison, R. M. (1999) *J. Biol. Chem. 274*, 12738−12747.
49. Blaukat, A., Herzer, K., Schroeder, C., Bachmann, N., Nash, N., and Muller-Esterl, W. (1999) *Biochemistry 38*, 1300−1309.

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