Bradykinin Sequesters B2 Bradykinin Receptors and the Receptor-coupled Go Subunits Goq and Goi in Caveolae in DDT1 MF-2 Smooth Muscle Cells*

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In this report, we show that the vasoactive peptide agonist bradykinin (BK) when bound to B2 BK receptors on DDT1 MF-2 smooth muscle cells promotes the recruitment and sequestration of the occupied receptors and the receptor-coupled G-protein α subunits Goq and Goi in caveolae. Association of ligand receptor complexes and Go subunits with caveolae was indicated by their co-enrichment on density gradients with caveolin, a marker protein for caveolae. Caveolin and Go subunits were monitored by immunoblotting, whereas receptors were monitored as ligand receptor complexes formed by labeling receptors with the agonist BK or the antagonist NPC17731 prior to cell disruption and caveolae enrichment. These complexes were detected with radioligand and by immunoblotting with BK antibodies. A direct interaction of Go subunits with caveolin was also indicated by their co-immunoprecipitation. Immunoelectron microscopy revealed that the enriched caveolin, Go subunits, and BK receptor complexes were present in structures of 0.1–0.2 μm. At 4 °C, BK and NPC17731 receptor complexes were detected in caveolae, and both complexes were sensitive to acid washing prior to cell disruption and caveolae enrichment. Elevation of the temperature to 37 °C increased the amount of BK receptor complexes in caveolae with a maximal response at 10 min (continuous labeling) or 20 min (single-round labeling), and the complexes became acid-resistant. These conditions also increased the amount of Goq and Goi in caveolae with a maximal response at 5–10 min. In contrast, the NPC17731 receptor complexes remained acid-sensitive and dissociated at this temperature, and antagonists did not increase the amount of Go subunits in caveolae. These results show that some agonists that act through G-protein-coupled receptors promote the association of their receptors and receptor-coupled Go subunits with caveolae.

Caveolae, or plasmalemma vesicles, are attracting a considerable amount of attention as specialized structures involved in signal transduction (1), internalization of small molecules by a process called phagocytosis (2, 3), and transcytosis (4). Caveolae are small flask-shaped invaginations of the plasma membrane (5, 6) that have been detected in most cells but are particularly abundant in adipocytes, endothelial cells, and muscle cells.

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††The abbreviations used are: G-protein, guanine nucleotide regulatory protein; BK, bradykinin; MEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MES, 2-N-morpholinoethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TBS, Tris-buffered saline; PI, phosphatidylinositol.

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Bradykinin Receptors, Ga Subunits, and Caveolae

study, we investigated the effect of B2 receptor antagonists and agonists on the association of B2 receptors, Goαq, and Gaq with caveolae in DDT1 MF-2 smooth muscle cells. This cell line was chosen since muscle is a rich source of caveolae (19), and BK binding to B2 receptors on these cells and their signaling and regulation have previously been characterized in some detail (43–45).

EXPERIMENTAL PROCEDURES

Materials—[2,3-prolyl-3,4-H]Bradykinin (110 Ci/mmol) and [prolyl-3,4-H]NPC17731 (55.5 Ci/mmol) were purchased from NEN Life Science Products, and [5,3H]N-[3-carboxamido-2-methylpropionyl]-bradykinin (17 Ci/mmol) was purchased from Amersham Corp. Monoclonal antibodies against caveolin were purchased from ICN (Casta Mesa, CA) and Transduction Laboratories (Lexington, KY). The former antibodies recognize both the 22- and 24-kDa isoforms of caveolin in DDT, MF-2 smooth muscle cells, whereas the latter antibodies recognize only the 24-kDa isoform. Polyclonal antibodies against Goq were generous gifts of Dr. Paul Sternweis (University of Texas Southwestern Medical School, Dallas). Polyclonal antibodies against Goq were obtained from Upstate Biotechnology (Lake Placid, NY), and polyclonal antibodies against BK were from Peninsula (Belmont, CA). Horseradish peroxidase-linked sheep anti-mouse IgG and donkey anti-antibody IgG were purchased from Amersham Corp. Cysteinylated antibodies against Gαi and Gαq were from Vector Laboratories, Inc. (Burlingame, CA). Streptavidin-Gold (10 nm) and protein A-Sepharose were from Sigma. Percoll was obtained from Pharmacia Biotech Inc. Optiprep was from Life Technologies, Inc. Protegol was purchased from British BioCell International (Phillipsburg, NJ). Immunodetection systems were obtained from Amersham Corp. and NEN Life Science Products. All other biochemicals were of the highest grade available.

Cell Culture and Treatments—DDT, MF-2 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin at 37 °C in 5% CO2. At 15 min before addition of agonist/antagonist, confluent cells were incubated in Leibovitz L-15 media containing 20 mm HEPES, pH 7.4, at 37 °C.

Enrichment of Caveolae—Two procedures were used for enrichment of caveolae. One procedure followed that of Sargiacomo et al. (18) with a few modifications (Method I). After treatment with agonist/antagonist, the cells were immediately transferred onto ice and washed with 10 ml of ice-cold PBS. All manipulations were then done at 4 °C. Cells were collected in PBS by scraping with a rubber policeman and washed twice in PBS by centrifugation at 250 × g for 5 min. The cell pellet was then Dounce homogenized (70 strokes) in 2 ml of 1% Triton X-100 in buffer A (25 mM MES, pH 6.7, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The homogenate was transferred to a 12-ml polycarbonate tube. On top of the sample was added 2 ml of 5% Optiprep in buffer B, and the gradient was centrifuged in a 60Ti rotor at 81,000 × g for 30 min. Each supernatant was collected and the pellet homogenized as described in Method I under “Enrichment of Caveolae.” The lysate was then centrifuged at 250 × g for 5 min. The supernatant was collected and used for immunoprecipitation at 4 °C as described previously (47). In short, lysates were incubated with anti-caveolin, anti-Gα (1:2,000), or anti-Gα (1:1,000) antibodies (1:2,000) overnight at 4 °C. In the case of caveolin immunoprecipitation, antibodies from ICN (1:2,000) and Transduction Laboratories (1:1,000) were combined. The lysates were then incubated with protein A-Sepharose beads for 1 h at 4 °C. In the case of anti-caveolin, the protein A-Sepharose beads had been precoupled to rabbit anti-mouse IgG. After incubation, the beads were washed twice with buffer A containing 3% BSA and then with 10 mM Tris-Cl, pH 7.4.

Immunoblotting—Samples were loaded in SDS-polyacrylamide gel electrophoresis buffer containing 6% β-mercaptoethanol for 5 min at 100 °C. Following electrophoresis on 12% polyacrylamide gels, the fractionated proteins were electroblotted onto 0.45-μm nitrocellulose membranes (Schleicher and Schuell) at ~500 mA for 45 min at 4 °C using a Genie electrophoretic blots (Idea Scientific) as described by Towbin et al. (48). The nitrocellulose membranes were blocked by incubation in 10% de-fatted milk in Tris-buffered saline (TBS) for 1 h. The membranes were then incubated as described in Method II. The membranes were then washed six times with TBS. The membranes were then incubated with anti-caveolin (1:1,000), anti-Gα (1:2,500), or anti-Gα (1:2,500) antibodies at 24 °C overnight. This was followed by a rinse in TBS as described above. Immunoreactive bands were visualized with immunodetection kits using peroxidase-labeled donkey anti-rabbit or sheep anti-mouse antibodies according to procedures described by the suppliers (Amersham Corp. and Transduction Laboratories). All blots were probed with antibodies against all the proteins. Stripping of blots between antibody staining was done by washing in 62.5 mM Tris-Cl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol for 45 min at 50 °C. The blots were then washed 3 × for 30 min in TBS. Residual immunoreactivity was checked with immunodetection kits in the absence of primary antibody.

Protein Staining—Some of the blots were stained with Protogold prior to immunoblotting. After transfer of the proteins, the nitrocellulose was incubated at 37 °C for 30 min in TBS containing 2% Nonidet P-40 and then washed 3 × for 5 min with TBS and 2 × for 1 min with H2O. Protogold (1.5 ml) was then added to the nitrocellulose and agitation continued for 30 min. For subsequent immunoblotting, the blot was washed 2 × for 20 min with TBS.

Radioligand Receptor Complexes—Cells were incubated with various concentrations of [3H]BRK and [3H]NPC17731 in Leibovitz L-15 media containing 20 mM HEPES, pH 7.4, 0.1% BSA, and the protease inhibitors trypotide (1 μM), bacitracin (140 μg/ml), and 1,10-phenanthroline (1 mM). Nonspecific binding was determined in the presence of 1 μM BK or 1 μM HOE140. Two protocols were used to form and follow radioligand receptor complexes. In one protocol, termed “continuous labeling,” cells were exposed continuously to radioligands for various times at 37 °C. Following rinsing of the cells with 2 × 3 ml of ice-cold PBS, 0.3% BSA to remove free radioligand, caveolae were enriched by Method II (see above under “Enrichment of Caveolae”). In a second protocol, termed “single-round labeling,” cells were first exposed to radioligands for 90 min at 4 °C. Following rinsing of the cells, caveolae were then enriched by Method II either immediately or following further incuba-
tion of the cells for various times at 37 °C. In both protocols, the caveola fraction was counted for radioactivity in a Beckman LS5000TD scintillation counter.

In some cases, cell disruption was preceded by a wash in low pH buffer (50 mM glycine, pH 2.0). As originally described by Hendershot et al. (49) and Ascoli (50), and subsequently by us for specific [3H]BK binding (43, 44), radioactivity associated with the cells following such a wash is considered intracellular and inaccessible to the extracellular environment.

PI Hydrolysis—Cells were assayed essentially as described (51). Briefly, confluent cells were incubated with 2 μCi/ml [3H]inositol in DMEM containing 10% fetal bovine serum at 37 °C for 48 h in 5% CO₂. The cells were then incubated for an additional 24 h in DMEM, 0.5% fetal bovine serum. The cells were then washed four times with 1 ml of DMEM and incubated in DMEM, 50 mM LiCl for 30 min. Following replacement with 2 ml of the same medium, the cells were incubated with 1 μM BK for various times at 37 °C. Inositol phosphates were then extracted and isolated using anion exchange chromatography.

Data Analysis—Autoradiograms were subjected to densitometry using a computer-coupled video system, and the data were analyzed by the NIH Image program (version 1.55).

RESULTS

Identification of Gaq and Gai in Caveolae—Multiple methods were used to evaluate the association of Ga subunits with caveolae in naive DDT1 MF-2 smooth muscle cells. In all methods, caveolae were monitored by immunoblotting with antibodies for the 22- and 24-kDa isoforms of caveolin, an integral protein component of caveolae (7–11). One method involved co-enrichment with caveolae on density gradients. On one type of gradient (Method I), enrichment of caveolae depends on both their buoyant density and resistance to 1% Triton X-100 as described by Sargiacomo et al. (18), and on another type of gradient (Method II), enrichment depends exclusively on their unique buoyant density as described by Smart et al. (20). As described previously by other investigators in different cells (18–20), Gaq and Gai were co-enriched with caveolae on both types of gradients as determined by immunoblotting with caveolin and Ga subunit-specific antibodies (data not shown). Furthermore, immunoelectron microscopy of fractions enriched for caveolae by Method I revealed that anti-caveolin and anti-Gaq antibodies immunostained structures of similar morphology and size (0.1–0.2 μm) (data not shown).

Another method involved co-immunoprecipitation of Gaq and Gai and caveolin. Fig. 1 shows that immunoprecipitation of 1% Triton X-100 lysates of cells with anti-caveolin antibodies resulted in co-immunoprecipitation of both Gaq and Gai. Furthermore, immunoprecipitation with anti-Gaq antibodies resulted in co-immunoprecipitation of Gaq and caveolin. Note that these immunoprecipitates were stained with anti-caveolin antibodies (Transduction Laboratories) that recognize only the 24-kDa caveolin isoform. Finally, immunoprecipitation with anti-Gaq antibodies resulted in co-immunoprecipitation of Gaq and caveolin. In contrast, protein A-Sepharose did not precipitate either Gaq or caveolin. In all, these results show that Gaq and Gai subunits are present in caveolae in naive DDT1 MF-2 smooth muscle cells.

BK Promotes Sequestration of Gaq and Gai in Caveolae—Intracellular signals elicited by BK through B2 receptors have been shown to be mediated by both Gaq and Gai types of α subunits (36–42). To evaluate agonist regulation of Gaq and Gai association with caveolae and a direct role for these structures in BK signaling, we assayed for changes in the level of Gaq and Gai in caveola-enriched fractions of DDT1 MF-2 cells before and after continuous exposure of the cells to BK. Fig. 2, A–C, shows plots of caveola fractions obtained in parallel by Method I from cells exposed to 1 μM BK for various times at 37 °C and which were then sequentially stained with anti-caveolin, anti-Gaq, and anti-Gai antibodies. Exposure of an equal number of cells with BK for up to 20 min did not change significantly the amount of enriched caveolin (Fig. 2A).

Restraining the same blot with anti-Gaq antibodies revealed that BK transiently increased the amount of enriched Gaq (Fig. 2B). The amount of Gaq was slightly increased at 2 min, peaked at 5 min, and then had partially returned to base line at 20 min. A duplicate blot showed that BK also transiently increased the amount of enriched Gaq (Fig. 2C), and the time course of this event was identical to that of the increase in Gaq. The peak fold increases in enrichment of Gaq and Gai were 3.14 ± 0.07 (n = 6) and 20.9 ± 7.8 (n = 3).

Fig. 3, B–D, shows plots of caveola fractions obtained in parallel by Method II from cells exposed to 1 μM BK continuously for various times and which were then sequentially stained with anti-caveolin, anti-Gaq, and anti-Gai antibodies. As shown in Fig. 3B, this method resulted in slight variations in the recovery of caveolin. Probing a duplicate blot for total protein with Protogold shows that this variation correlated with the amount of total blotted protein (Fig. 3A). Two major protein bands at 22- and 24-kDa on the protein blot co-migrated with the 22- and 24-kDa caveolin bands on the immunoblot. Drying of the protein blot caused the blot to shrink and the bands on this blot to appear at a slightly higher molecular mass than the bands on the immunoblot. Assuming that the protein bands are caveolin, these results indicate that Method II yields a relatively pure preparation of caveolin. Reprobing the Protogold blot with antibodies against Ga subunits revealed the presence also of Gaq, and Gai (Fig. 3, C and D). Normalizing for the amount of caveolin on the blot, Fig. 3E...
shows that 1 μM BK also transiently increased the relative amounts of Goq and Gai enriched by this method. The time courses of the BK responses were similar to those obtained using Method I (Fig. 2). The peak fold increases in enrichment of Goq and Gai were 1.76 ± 0.16 (n = 2) and 2.27 ± 0.20 (n = 2). Together, these results indicate that BK stimulates a transient increase in the association of Goq and Gai with caveolae in DDT1 MF-2 smooth muscle cells. Fig. 4 shows a typical representation of the temporal relationship between BK-stimulated translocation of Goq to caveolae and BK-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis, an event presumed to be mediated by Goq activity. Clearly, the latter response occurs significantly earlier than the former.

**Bradykinin Regulation of Goq and Gai Association with Caveolae Is Agonist-specific and Mediated by the B2 BK Receptor**—To determine the specificity of the BK-stimulated increase in the association of Goq with caveolae, DDT1 MF-2 cells were also incubated with HOE140, a high affinity B2 BK receptor-specific antagonist. By sequential staining of the blots with anti-caveolin and anti-Goq antibodies followed by normalizing for the amount of caveolin on the blots, exposure of cells to 1 μM HOE140 was shown not to change subsequent enrichment of Goq by Method I (Fig. 5). On the other hand, this antagonist completely inhibited the increase in enrichment of Goq, in response to 0.1 μM BK. HOE140 also inhibited the BK-stimulated increase in enrichment of Gai (data not shown). The conclusions drawn from these results are 2-fold. First, the BK-stimulated increase in the association of Goq and Gai with caveolae is mediated specifically through the B2 receptor. Second, the B2 receptor-mediated increase is specific for agonists, i.e. the response requires receptor binding as well as receptor-mediated Ga activation to occur.

**Identification of Ligand B2 Receptor Complexes in Caveolae**—Co-enrichment of B2 BK receptors with caveolae was monitored by radioligand receptor binding. To minimize the risk of disrupting the ligand receptor complex, these studies were done under detergent-free conditions (Method II). As shown in Fig. 6, lane 2, when B2 receptors were subjected to single-round labeling at 4°C (see "Experimental Procedures") with 3 nM [3H]BK, a high affinity agonist (K_D = 1.4 ± 0.2 nM), this procedure yielded a significant amount of radioligand (0.49 ± 0.04 fmol/10^6 cells, n = 3) in the caveolae-enriched 1-ml fraction (B), the bottom fraction above the interface in the final Optiprep gradient in Method II. This activity was specific for caveolae as no radioligand was detected in the adjacent caveolea-less 1-ml fraction (T) at the top of the gradient (Fig. 6, lane 1) or any other fraction of the gradient (data not shown). In the presence of an excess of BK (1 μM) or HOE140 (1 μM) during incubation of the cells with [3H]BK, no radioligand was detected in either fraction B (Fig. 6, lane 4) or fraction T (lane 3). Thus, the appearance of BK in the caveolae-enriched fraction required the interaction of BK with B2 receptors. A significant amount of radioligand (1.47 ± 0.03 fmol/10^6 cells, n = 3) in the caveolae-enriched fraction was also observed when receptors were labeled under identical conditions with 2 nM [3H]NPC17731, a high affinity B2 receptor antagonist (K_D = 0.44 ± 0.06 nM). Considering that B2 receptor-specific radioligand binding activity was detected in the caveolae-enriched fraction following incubation of cells at 4°C with either an agonist or an antagonist, these results indicate that a fraction of the B2 receptors in naive DDT1 MF-2 cells are present in caveolae. Further incubation of single-round [3H]BK-labeled
cells for 5 and 30 min at 37 °C increased the amount of ligand receptor complexes in fraction B (Fig. 6, lanes 6, 8, 10, and 12) but not in fraction T (lanes 5, 7, 9, and 11).

Further evidence for association of B2 receptors with caveolae was obtained by immunoelectron microscopy of caveolae-enriched fractions (Method II) using a polyclonal BK antibody (Peninsula) (Fig. 7). To optimize immunodetection of BK, cells were exposed to 1 μM BK continuously for 20 min at 37 °C. As shown in Fig. 7A, BK immunoreactivity appeared in distinctly shaped clusters of 0.1–0.2 μm. These clusters were not present in samples stained with normal rabbit serum (Fig. 7B). Structures of a similar size were specifically immunostained with antibodies against caveolin (Fig. 7, C and D).

**Sequestration of BK B2 Receptor Complexes in Caveolae**—To address the relationship between the caveolae-associated ligand receptor complexes and Gα subunits, the association of the receptor complexes with caveolae was followed at 37 °C. The amount of complexes in caveolae at each time point was normalized to the relative amount of caveolin in each sample as determined by immunoblotting. When receptors were subjected to continuous labeling with 3 nM [3H]BK at 37 °C, the appearance of BK receptor complexes in caveolae was rapid and reached a steady state level of 0.42 fmol/10⁶ cells at 10 min (Fig. 8A). Single-round labeling with 3 nM [3H]BK revealed that this steady state represented a continuous flux of complexes through caveolae. As shown in Fig. 8B, under these conditions elevation of the temperature to 37 °C resulted in a transient increase in the number of complexes in caveolae. The number was slightly increased at 10 min, peaked at 20 min (303 ± 47% of basal; n = 3), and then had almost completely returned to basal at 60 min. When receptors were subjected to single-round labeling with 2 nM [3H]NPC17731, elevation of the temperature to 37 °C resulted in the dissociation of the NPC17731 receptor.
complexes with a $t_{1/2} = 30 \pm 2$ min ($n = 2$) (Fig. 8B). No nonspecific binding of the two ligands was detected at any time point in the caveolae-enriched fraction (Fig. 8, A and B). These results show that at $37 \, ^\circ C$ BK receptor complexes migrate from non-caveolae locations in the plasma membrane through caveolae. In contrast, NPC17731 receptor complexes are unable to migrate to this compartment.

We previously used acid washing to show that total specific $[^3H]BK$ binding to DDT1 MF-2 cells at $4 \, ^\circ C$ is accessible to the extracellular environment, whereas at $37 \, ^\circ C$ the major portion of the binding is intracellular (43, 44). Thus, BK receptor complexes undergo internalization at $37 \, ^\circ C$. The sensitivity of the $[^3H]BK$ and $[^3H]NPC17731$ receptor complexes in caveolae to acid washing differed drastically at this temperature. As shown in Fig. 9, the $[^3H]BK$ receptor complexes were almost completely (84%) resistant to acid washing. On the other hand, the $[^3H]NPC17731$ receptor complexes were only minimally (27%) resistant to this treatment (Fig. 9). Thus, most of the BK receptor complexes in caveolae become inaccessible to the extracellular environment at $37 \, ^\circ C$, whereas most of the NPC17731 receptor complexes remain accessible at this temperature.

**DISCUSSION**

In this report, we show that B2 BK receptors and the receptor-coupled $G_\alpha$ subunits $G_\alpha_q$ and $G_\alpha_i$ are present in caveolae in DDT1 MF-2 smooth muscle cells. Addition of the agonist BK results in a receptor-mediated recruitment and sequestration of all three signaling components in this plasma membrane structure. BK antagonists are unable to promote this response but inhibit the BK-promoted response. Furthermore, the recruitment occurs after BK-stimulated PI hydrolysis suggesting that this response may not be required for the early signaling through the receptor.
cantly less than 100%, this value is probably an underestimate of the relative amount of receptors in caveolae in intact naive cells under basal conditions.

B2 BK receptors have been shown to activate and signal through both Gₐq and Gₐi (36–42). Gₐq-mediated BK stimulation of phospholipase Cβ activity leading to PI hydrolysis and subsequent intracellular Ca²⁺ mobilization is the best understood pathway (36, 37), and this pathway is most likely responsible for BK-stimulated PI hydrolysis in DDT₁ MF-2 cells (44). Even though a role for Gₐi in B2 BK receptor signaling in DDT₁ MF-2 cells has not yet been addressed, it is likely that the receptor is able to also couple to this Gₐ subunit in these cells.

In A431 cells, approximately half of the cellular phosphatidylinositol 4,5-bisphosphate appears to be confined in caveolae-like structures (56). Treatment of these cells with BK for 5 min resulted in an approximately 50% decrease in the phosphatidylinositol 4,5-bisphosphate level in these structures without a concomitant decrease in the plasma membrane phosphatidylinositol 4,5-bisphosphate level (56). These results together with ours suggest that the coupling of at least some G-protein-coupled receptors to phospholipase Cb may be restricted to caveolae. Furthermore, this interpretation is supportive of the original idea that caveolae localization represents a mechanism for amplifying cellular signals by increasing the proximity and coupling of receptors and effectors (1).

At least a fraction of Gₐq and Gₐi in DDT₁ MF-2 cells physically interacts with caveolin. These results agree with recent observations in vitro and in recombinant systems (57). In these studies, caveolin-1 was found to physically interact specifically with the GDP-bound form of Gₐs in vitro and act as a GDP dissociation inhibitor. In addition, wild-type Gₐ expressed in Madin-Darby canine kidney cells co-fractionated with caveolin, whereas a constitutively active Gₐ mutant did not. Furthermore, addition of GTPγS resulted in the quantitative exclusion of wild-type Gₐs from caveolin-enriched fractions. These observations led to the hypothesis that caveolin-1 holds Gₐ subunits in an inactive state, and Gₐ subunit activation terminates this holding and releases the Gₐ subunit from caveolin-1 (34). We did not investigate the association of Gₐq and Gₐi in caveolae in the interval of BK-stimulated PI hydrolysis and when, accord-

![Fig. 7. Anti-BK and anti-caveolin immunoelectron microscopy of fractions enriched for caveolae from BK-stimulated cells by Method II.](image)

A, cells were first exposed to 1 μM BK continuously for 20 min and then processed according to Method II as described under “Experimental Procedures.” The caveolae-enriched fraction on the final Optiprep gradient was then subjected to immunoelectron microscopy using anti-BK (A), irrelevant rabbit antibody (B), anti-caveolin antibodies (C), or irrelevant mouse antibody (D) and then detected with Streptavidin-Gold (10 nm). The bar at the bottom of A indicates 0.5 μm.

![Fig. 8. Time courses of association of BK and NPC17731 receptor complexes in caveolae under conditions of continuous labeling and single-round labeling.](image)

A, cells were subjected to continuous labeling with 3 nM [³H]BK in the absence (●) and presence (○) of 1 μM BK for various times at 37 °C. The cells were then processed according to Method II as described under “Experimental Procedures.” The caveolae-enriched fraction on the final Optiprep gradient was then counted for radioactivity. B, cells were subjected to single-round labeling with 3 nM [³H]BK (circles) or 2 nM [³H]NPC17731 (triangles) in the absence (●, ▲) and presence (○, △) of 1 μM BK and then incubated for various times at 37 °C. The cells were then processed according to Method II as described under “Experimental Procedures.” The caveolae-enriched fraction on the final Optiprep gradient was then counted for radioactivity. The results are expressed as % of Basal where 100% basal represents the total amount of [³H]BK (0.49 ± 0.04 fmol/10⁶) or [³H]NPC17731 (1.47 ± 0.03 fmol/10⁶) in the caveolae-enriched fraction after incubation of cells for 90 min at 4 °C. The result is the average ± S.E. of two ([³H]NPC17731) or three ([³H]BK) experiments.
approximately 0.5 fmol/10^6 cells at 10 min. To determine if this steady state was relatively rapid and reached a steady state level of approximately 15 s. Thus, BK-promoted translocation of the two Gα subunits to caveolae does not seem to be required for the early signaling of the receptor. Instead, the Gα subunit translocation parallels B2 receptor-a dissociation with caveolin-1 to temporarily hold the Gα subunit from caveolin-1. However, such an alternative may not be detectable in these cells as B2 receptors in a native system probably couple to and directly activate relatively few Gα subunits.

Our interpretation of the BK-promoted increase in the level of Gαq and Gα11 in caveolae is that BK causes a translocation of these subunits from the cytosol or non-caveolae regions of the plasma membrane to caveolae. The time courses of these responses, which both occur with τ1/2 of 2–5 min, do not parallel Gαq-mediated BK stimulation of PI hydrolysis in these cells which occurs with a τ1/2 of approximately 15 s. Thus, BK-promoted translocation of the two Gα subunits to caveolae does not seem to be required for the early signaling of the receptor. Instead, the Gα subunit translocation parallels B2 receptor-mediated BK internalization (43) and receptor sequestration (44) in these cells. Internalized BK is rapidly degraded suggesting that these events are involved in terminating BK action (43). The sequestered receptors rapidly (τ1/2 ≈ 15 min) return to the plasma membrane upon removal of BK (44) suggesting that they are only superficially sequestered in the cell. Indeed, at least a fraction of the sequestered receptors can be accessed by treatment of the plasma membrane fraction with the CHAPS detergent (44).

The above observations prompted us to investigate the involvement of caveolae in the cellular processing of the BK B2 receptor complex. Under conditions of continuous labeling at 37°C, the appearance of BK receptor complexes in caveolae was relatively rapid and reached a steady state level of approximately 0.5 fmol/10^6 cells at 10 min. To determine if this steady state represented a flux of complexes through caveolae, cells subjected to single-round labeling with BK were incubated for various times at 37°C. Under these conditions, the amount of BK receptor complexes increased transiently in caveolae. These results provide direct evidence that BK promotes the flux of receptors through caveolae. This response is agonist-specific as receptor complexes with the antagonist NPC17731 were unable to migrate to caveolae.

Caveolae can exist in different shapes from relatively flattened structures to clear invaginations to pinched off vesicles (58). Little is known about any physiological regulation of the shape of the caveolae even though depletion of cholesterol, an important component of caveolae, results in the flattening of any invaginated caveolae (59). It has been hypothesized that ligands for receptors that localize to caveolae regulate the shape of these structures, but no evidence has so far been presented that this type of regulation occurs. To address this issue, ligand receptor complexes on intact cells were subjected to acid washing. Caveolae were then enriched from these cells and assayed for ligand receptor complexes. At 4°C, both BK and NPC17731 receptor complexes in caveolae were sensitive to acid washing indicating that the lumen of the caveolae in which the complexes presumably reside at this temperature is accessible to the extracellular environment. At 37°C, the NPC17731 receptor complexes in caveolae remained sensitive to acid washing. On the other hand, the BK receptor complexes were almost completely resistant to this treatment. Thus, at this temperature the BK receptor complexes must be sequestered in caveolae in a form not accessible to the extracellular environment. One possible explanation for these results is that the BK receptor complex promotes the closure of the caveolae. Furthermore, these results provide direct evidence that caveolae represents one compartment through which the BK receptor complex is internalized.

The above results show that caveolae appears to play an important role in the signaling and processing of BK receptor signaling components. As discussed above, the receptors and Gα subunits present in caveolae under basal conditions may be important in the initial receptor-G-protein coupling. The BK-promoted translocation of these components to caveolae in DDT freeze-fracture cells occurred after the initial BK-stimulated PI hydrolysis. Thus, if translocation leads to amplification of the receptor signal, such amplification must occur secondary to initial receptor-G-protein coupling. Alternatively, translocation may represent a mechanism for terminating the cellular signal. On a similar time scale, exposure of DDT freeze-fracture cells to BK results in heterologous desensitization of the BK response (44-45). This desensitization is observed as an attenuation of the responsiveness to both BK and norepinephrine, an α1-adrenergic receptor agonist which also stimulates PI hydrolysis, and phosphorylation of the α1-adrenergic receptor (45). A vast amount of information available in the literature indicates that heterologous desensitization seems to be a multistep process involving both translocation and covalent modification of multiple proteins in the signaling pathway. Our information presents one potential scenario in which BK-promoted association of Gαq and Gα11 with caveolae is part of the mechanism in which this agonist heterologously desensitizes the cell to further stimulation by various agonists that utilize these Gα subunits for signaling. In this scenario, the increased association of Gα subunits with caveolae may promote an inactive, desensitized state of the subunits by promoting their physical interaction with caveolins-1 to temporarily hold the Gα subunits in a GDP-bound state. In the same scenario, the recruitment and sequestration of the BK B2 receptor complex in caveolae represents the initial step in internalization of the complex. Whether or not caveolae is the only compartment through which the BK receptor complex traverses in the internalization pathway remains to be established.

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**Fig. 9. Effect of acid washing on [3H]BK and [3H]NPC17731 receptor complexes in caveolae at 37°C.** Cells were subjected to single-round labeling with 3 nM [3H]BK or 2 nM [3H]NPC17731 for 20 min at 37°C. Following acid washing, cells were processed according to Method II as described under "Experimental Procedures." The caveolae-enriched fraction on the final Optiprep gradient was then counted for radioactivity. The results are expressed as % of Control where 100% control is the total amount of [3H]BK (0.55 ± 0.13 fmol/10^6 cells) or [3H]NPC17731 (1.12 ± 0.44 fmol/10^6 cells) in the caveolae-enriched fraction. The result is the average ± S.E. of three experiments.
