Bradykinin Signalizing Counteracts cAMP-Elicited Aquaporin 2 Translocation in Renal Cells

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Bradykinin (BK) is one of the most important peptides regulating vascular tone, water, and ionic balance in the body, playing a key role in controlling BP. It is interesting that patients with essential hypertension excrete less BK than normotensive individuals. For elucidating the mechanism by which BK regulates renal water transport that contributes to its antihypertensive effect, aquaporin 2 (AQP2)-transfected collecting duct CD8 cells, expressing the BK type II receptor (BK2R), were used as an experimental model. In CD8 cells, BK pretreatment impaired forskolin-induced AQP2 translocation to the apical plasma membrane. For clarifying the signal transduction cascade associated with this effect, whether BK induced an increase in cytosolic calcium, via the G protein Gq, known to be coupled to BK2R, first was investigated. Spectrofluorometry using fura-2-AM revealed that 100 nM BK elicited a significant increase in Ca2+ which was abolished by the receptor antagonist HOE-140. BK acts through BK2R coupled to both Gq and Gi13, a known upstream effector of Rho protein. In CD8 cells, BK causes an increase in Rho activity, likely as a result of G13 activation. This results in stabilization of the cortical F-actin network, thus impairing AQP2 trafficking. These effects counteract physiologic vasopressin stimulation, which instead has an opposite effect on actin network organization through Rho inactivation.


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fiber formation, impairing AQP2 translocation to the plasma membrane (21).

In the rabbit collecting duct cell line RC.SVtsA58 expressing endogenous BK2R receptors, BK inhibited dDAVP-dependent cAMP production. This event was mimicked by PGE2 and suppressed with indomethacin, suggesting that the signal transduction initiated by BK included PGE2 synthesis (13). Moreover, in those cells, BK induced an increase in intracellular calcium and a decrease in cAMP production upon vasopressin stimulation, independent of PGE2 synthesis (22). In this work, CD8 cells, obtained after stable transfection of rabbit collecting duct cell line RC.SV3 with rat AQP2 water channels (23), were used to elucidate the signal transduction associated with BK activation and its effect on AQP2 trafficking.

Materials and Methods

Antibodies
AQP2 affinity-purified antibodies were obtained as described previously (23). Rabbit antisera were raised against the synthetic peptides corresponding to the 15 COOH-terminal amino acids of rat AQP2 (CELHSPQSLPRGSKA), including the ser 256. Ga13 and RhoA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Actin cytoskeleton was visualized using TRITC-Phalloidin (Sigma Aldrich, Milan, Italy).

Intracellular Calcium Measurements
Alterations of intracellular calcium concentration induced by BK pretreatment were determined by a spectrophotometric technique as described (24). CD8 cells were grown on glass coverslips. The coverslips were inserted into a specially designed cuvette, and the exposed area of the cell monolayer was 6.3 mm². Cells were perfused with BK in the bath solution, and intracellular calcium concentration was calculated from the emission fluorescence ratio of the two excitation wavelengths using the formula (Ca²⁺)i = Kd (R – Rmin)/(Rmax – R), where Kd (dissociation constant) of Fura-2 for Cai was 224 nM. Each sample was calibrated by the addition of 50 µM digitonin (Rmax) followed by 10 mM EGTA/Tris (Rmin).

Immunofluorescence
CD8 cells were grown on glass coverslips and fixed with 4% paraformaldehyde in PBS for 20 min and processed for immunofluorescence as described (17). For AQP2 visualization, AQP2 affinity-purified antibodies were used and revealed with fluorescein-conjugated goat anti-rabbit IgG (10 µg/ml in PBS). Alternatively, after blocking, actin cytoskeleton was visualized by incubation with phalloidin-TRITC (100 µg/ml, 45 min). AQP2 was detected with an epifluorescence microscope (TE 2000S, Nikon Instruments, Florence, Italy) equipped with a CCD camera (Princeton Instruments MicroMax 512BFT, Princeton, NJ) using a Delta RAM Highspeed Multil wavelength Illuminator for excitation (Photo Technology International PTI, South Brunswick, NJ). The xz planes were obtained by deconvolution using Autodeblur software (Universal Imaging Corp., West Chester, PA). The fluorescence intensity was analyzed by using Metamorph software, and the statistical analysis was performed by using a one-way ANOVA and Tukey multiple comparison test.

F-Actin Co-Sedimentation Assay
F-actin co-sedimentation was performed as described previously (25). Briefly, total membrane and cytosol fractions were prepared from CD8 cells. Cells were scraped and resuspended in homogenization buffer that contained 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors. Cells were homogenized using a 22-gauge needle, and nuclei were removed by centrifugation at 800 × g for 10 min. Membrane and cytosol fractions were obtained by centrifugation for 1 h at 4°C at 150,000 × g. The membrane fraction was resuspended in homogenization buffer at a protein concentration of 2 mg/ml. Protein concentrations of cytosol fractions were equilibrated for protein content, and formation of F-actin was initiated by a 50-fold polymerization buffer that contained 200 mM MgCl₂, 4 M KCl, and 100 mM ATP. The samples were incubated for 1 h at 37°C, and F-actin was pelleted by ultracentrifugation for 1 h at 4°C at 150,000 × g. The F-actin-containing pellets were rinsed with homogenization buffer. Membrane, cytosol, and F-actin fractions were separated by 13% SDS-PAGE and immunoblotted with G13-specific antibodies.

Affinity Precipitation of Cellular GTP-Rho. The purification of glutathione-s-transferases–Rho binding domain (GST-RBD) was performed as described previously (19,21). For evaluating Rho activity, CD8 cells were left either untreated or stimulated with 10⁻⁴ M forskolin for 15 min at 37°C. In addition, cells were preincubated with 0.1 µM BK for 15 min at 37°C in the absence or in the presence of forskolin. Alternatively, CD8 cells were preincubated with 100 µM indomethacin for 5 h and then incubated with 100 nM BK for 15 min. Cells were washed with ice-cold buffer that contained 150 mM NaCl and 10 mM Tris-buffered saline (pH 7.4) and processed for the affinity precipitation of cellular GTP-Rho as described previously (19,21). Bound Rho proteins were detected by Western blotting using a mAb against RhoA. The densitometric analysis was performed using Scion Image Software for Windows (Frederick, MD). Statistical analysis was performed by one-way ANOVA and Tukey multiple comparison test.

For affinity precipitation of GTP-RhoA from rat or rabbit kidney tubule, tubule suspensions were obtained as described previously (26). Briefly, kidney papillae were rapidly minced and digested in a buffer that contained 118 mM NaCl, 16 mM HEPES; 17 mM Na-HEPES, 14 mM glucose, 3.2 mM KCl, 2.5 mM CaCl₂, 1.8 mM MgSO₄, and 1.8 mM KH₂PO₄ (pH 7.4) in the presence of 0.2% collagenase and 0.2% hyaluronidase at 37°C for 90 min. After 45 min of incubation, 0.001% DNase I was added. The suspension then was centrifuged at 200 × g for 8 min to obtain tubular element of papilla. Half of the obtained tubules then were incubated for 15 min with 100 nM BK and subjected to the affinity precipitation as described above.

Actin Polymerization Assay
Actin polymerization was analyzed as described previously (27–29). Briefly, CD8 cells were left untreated and stimulated with forskolin (10⁻⁴ M) for 15 min. In addition, cells were pretreated either with HOE-140 or with U73122 and then with BK in the presence or in the absence of forskolin for 15 min at 37°C. The treatments were stopped by adding 450 µl of 3.7% paraformaldehyde, 0.1% Triton X-100, 0.25 µM TRITC-phalloidin in 20 mM potassium phosphate, 10 mM PIPES, 5 mM EGTA, and 2 mM MgCl₂ (pH 6.8). After staining for 1 h, the cells were washed three times with PBS, and 800 µl of methanol was added overnight. The fluorescence (540/565 nm) was read in an RF-5301PC fluorimeter. The values then were analyzed with a one-way ANOVA and Tukey multiple comparison test.

Results

BK Causes Dose-Dependent Increases in Intracellular Calcium via PLC Activation
BK has been shown to interact with BK2R, leading to Ca²⁺ release from intracellular stores by activating the PLC system (14,15,30,31). Therefore, changes in intracellular calcium (Ca³⁺)
were measured in CD8 cells (23) that were grown on coverslips to confluence, loaded with 10 μM Flura-2-AM. Figure 1A illustrates representative responses evoked by BK. Perfusion with BK induced a rapid increase in Ca, in a dose-dependent manner from 100 nM to 1 μM BK. In CD8 cells that were perfused with 100 nM BK, the Ca, concentration increased more than threefold over basal levels (from 78.8 ± 7.7 to 310.7 ± 42 nM; n = 3). When cells were treated with BK in the absence of extracellular calcium, the peak amplitude of the BK-induced Ca, was not affected, indicating that BK induces calcium release from intracellular stores (Figure 1A, inset). BK failed to elevate Ca, in cells that were pretreated with thapsigargin, indicating that the endoplasmic reticulum is the intracellular calcium store involved (data not shown).

Next, the effect of the specific BK2R antagonist, HOE-140, on the BK-dependent calcium increase was analyzed. We first verified whether sequential BK stimulations caused comparable Ca, increases. As shown in Figure 1B, after a 15-min washout from the first challenge, BK elicited a reproducible effect on Ca, (Figure 1B). HOE-140 (100 nM) added to the perfusate for 15 min reduced the BK-evoked Ca, increase by approximately 45% (from 310.7 ± 4.0 to 145 ± 15.2 nM; Figure 1, C and E). HOE-140 (1 μM) completely abolished the BK response (Figure 1, C, bottom, and E). These data suggest that BK elevates Ca, by activating BK2R. The PLC inhibitor U73122 was applied to test whether BK increases Ca, by activating PLC. BK failed to increase Ca, in cells that were pretreated for 30 min with 10 μM U73122, indicating that BK-induced elevation in Ca, is mediated by the PLC pathway (Figure 1, D and E).

**BK Antagonizes Forskolin-Induced AQP2 Translocation**

To investigate whether BK treatment might affect AQP2 trafficking, we analyzed cellular localization of AQP2 by immunofluorescence in CD8 cells under different experimental conditions. In basal conditions, AQP2 was localized mainly intracellularly, whereas incubation with forskolin caused translocation of AQP2 to the apical plasma membrane, as described previously and confirmed using an antibody that recognizes the extracellular C-loop of the AQP2 protein (23,32,33) (Figure 2A). Preincubation with BK inhibited forskolin-induced AQP2 translocation (Figure 2, A and xz reconstruction in the inset). HOE-140 pretreatment completely abolished the effect of BK upon forskolin stimulation, indicating that the action of BK is specifically mediated by BK2R activation (Figure 2, A and xz

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**Figure 1.** Effect of bradykinin (BK) on intracellular calcium (Ca,) concentration. (A) Dose-dependent increase in the Ca, concentration elicited by 100 nM to 1 μM BK. (Inset) Effect of BK on Ca, in the absence of extracellular calcium. (B) Effect of repetitive BK type II receptor (BK2R) stimulations on Ca, increases. After a 15-min washout from the first challenge, BK elicited a reproducible effect on Ca,. (C) Dose-response effect of HOE-140, a selective BK2R inhibitor, on Ca, increases. (D) Inhibitory effect of U73122, a specific inhibitor of phospholipase-C (PLC), on Ca, increases. (E) Means ± SE, n = 3, of three independent experiments. Data were analyzed by one-way ANOVA.
BK Modulates Ga13 Cellular Distribution

The data suggest that the Gq/PLC pathway activated by BK is not responsible for the impairment of AQP2 trafficking observed after forskolin stimulation. Because it has been shown that BK-induced stress fiber formation in fibroblasts is mediated by Ga13 (16), we next investigated whether BK treatment affects Ga13 cellular localization. Cell fractionation followed by Western blotting analysis of cellular fractions demonstrated that BK caused a significant enrichment of Ga13 in the particulate fraction compared with cytosolic fractions from control cells (Figure 3). A concomitant decrease in the soluble fraction was observed. Forskolin stimulation resulted in a redistribution of Ga13 from the particulate to the cytosolic fraction, whereas BK abolished the effect of forskolin on Ga13 subcellular localization (Figure 3 and densitometric analysis on the right). These data indicate that BK modulates the membrane association of Ga13 in renal CD8 cells. Although translocation of cytoplasmic proteins to plasma membrane-bound signaling complexes has emerged as a hallmark in some signaling pathways (34), the results shown here and those from other studies (35) suggest that some Gα may function similarly and be activated in this manner. It is interesting that plasma membrane localization of Ga13 required palmitoylation, a posttranslational dynamic and reversible modification (35). Moreover, G proteins, traditionally thought to be transducer molecules confined to the plasma membrane, are also present on intracellular vesicles as αβγ heterotrimers, and we and others have suggested that they participate in various intracellular transport pathways (32,36).

In NIH3T3 cells, transfection with the constitutively active Ga13 greatly increased the affinity between Ga13 and F-actin, indicating that affinity between F-actin and Ga13 might be considered an important indicator of Ga13 activity (25).

Cytosolic fractions of CD8 cells were prepared, F-actin polymerization was induced, and F-actin interacting proteins were analyzed by Western blotting as described in Materials and Methods. Relative to the control, whereas forskolin stimulation caused a decrease in immunodetectable Ga13 in the F-actin-enriched fraction, BK induced a significant increase in Ga13 affinity to F-actin. It is interesting that the amount of Ga13 that co-sedimented with the F-actin fraction after forskolin stimulation in cells that were treated with BK was comparable to that observed in control cells (0.83 ± 0.04 mean ± SE; n = 12); in cells that were incubated with BK (0.57 ± 0.03), HOE-140 and BK (0.73 ± 0.08), and BK and forskolin (BK+FK; 0.77 ± 0.056); and in cells that were pretreated with U73122 and BK in the absence (U+FK; 0.52 ± 0.05) or in the presence of forskolin (U/BK+FK; 0.87 ± 0.11). Ratios >1, indicating a predominant localization of AQP2 at the plasma membrane, were found in forskolin-stimulated cells (FK; 2.80 ± 0.36) and in cells that were preincubated with HOE-140 before BK and forskolin stimulation (H/BK+FK; 2.91 ± 0.33).

Figure 2. Effect of BK on aquaporin 2 (AQP2) trafficking. (A) CD8 cells were left untreated (CTR) or incubated with forskolin (FK; 100 μM; 15 min). Alternatively, cells were preincubated with BK (BK100 nM; 15 min) or with BK and forskolin (15 min) in the presence or in the absence of HOE-140, a selective inhibitor of the BK2R. In addition, CD8 cells were preincubated with U73122 and BK in the presence or in the absence of forskolin. AQP2 was stained using anti-AQP2 antibodies and visualized by epifluorescence microscopy. The xz reconstructions were obtained by deconvolution using Autodeblur software (insets). (B) Ratios of cell membrane/intracellular membrane fluorescence signals. The intracellular and cell membrane immunofluorescence signal intensities were calculated using Metafphorm software and normalized to the background signal intensities (n = 12). Ratios >1 indicate a cell membrane localization of AQP2. (*P < 0.001 with respect to control). Values are expressed as means ± SE.
BK Activates Rho Signaling

The heterotrimeric G proteins of the G12/13 family mediated stress fiber formation through the small G protein Rho, and we recently demonstrated that Rho is an important protein for both diuretic and antidiuretic agents (19,21). The amount of active RhoA was determined by an affinity precipitation assay of active RhoA using the Rho-binding domain of Rhotekin fused to glutathione-s-transferases (37,38). Compared with control conditions (100%), the amount of precipitated active RhoA decreases upon forskolin stimulation (50.03 ± 9.985%; n = 3; Figure 5). In contrast, preincubation with BK resulted in a significant increase in the amount of active RhoA (150.6 ± 5.07%; n = 3). Moreover, in cells that were pretreated with BK in the presence of forskolin, the amount of active Rho was similar to that found in control conditions (91.45 ± 7.8%; n = 3; Figure 5A). These results are consistent with the view that BK signaling counteracts forskolin-induced Rho activation. However, it cannot be excluded that BK effect can be mediated by activation of PGE2 synthesis. In fact, we showed previously that PGE2 can activate Rho in rat renal cells (21), and BK enhances PGE2 production in mesangial cells (39). To clarify this point, we tested the BK effect in CD8 cells that were preincubated with indomethacin, a known inhibitor of COX1 and COX2 to inhibit PGE2 synthesis. Under these experimental conditions, BK still induced a significant increase in the amount of active Rho, suggesting a direct effect of BK on Rho in vitro (Figure 5B). To investigate whether these observations can be confirmed in a more physiologic system, we evaluated Rho activity in isolated rat and rabbit collecting ducts that were treated with BK. BK caused Rho activation in both samples, confirming the results obtained in the renal cell line (Figure 5C).

BK Treatment Causes Stress Fiber Formation

In CD8 cells, expression of constitutively active RhoA caused stress fiber formation, impairing forskolin-activated AQP2 trafficking (17,18). Therefore, we next evaluated the effect of BK on...
actin cytoskeleton organization. As shown in Figure 6A, forskolin stimulation caused a partial depolymerization of actin filaments, whereas incubation with BK stabilized actin cytoskeleton organization in the absence and in the presence of forskolin. Pretreatment with HOE-140 completely abolished the effect of BK on actin filaments, indicating that BK-induced stress fiber formation was specifically mediated by BK2R activation. The selective inhibitor of PLC, U73122, made no difference to the effect of BK. Quantification of the F-actin content confirmed that compared with the control condition (97 ± 3.07; n = 14), forskolin reduced F-actin content (64.6 ± 4.06; n = 14), whereas BK increased F-actin content in the absence (145.5 ± 9.7; n = 14) or in the presence of forskolin (119.7 ± 6.5; n = 14) (Figure 6B). Similar results were observed when the cells were pretreated with U73122 before BK treatment. The effect of BK was completely abolished in the presence of HOE-140. Pretreatment with HOE-140 alone or with U73122 did not alter F-actin organization (data not shown).

Discussion
Vasopressin increases urine concentration by regulating the expression of AQP2 in the apical membrane of the collecting duct principal cells, thus increasing water permeability (40). Activated GTP binding proteins of the Rho family play a key role in inhibiting this process by inducing actin polymerization close to the AQP2 vesicle fusion sites (17,18,21). Various diuretic compounds, such as PGE2, endothelin-1, and BK, are negative regulators of vasopressin-dependent water reabsorption. For some of them, it has become clear that there is an ultimate effect on AQP2 trafficking/expression. We recently demonstrated that in renal primary rat inner medullary collecting duct cells, the signal transduction underlying prostaglandin
EP3 receptor stimulation resulted in the activation of the Rho protein, which induced actin polymerization and inhibited vasopressin-dependent AQP2 translocation at the plasma membrane (21). Moreover, the physiologic involvement of PGE2 in inducing a defect in renal water concentrating ability through impairment of AQP2 trafficking has recently been confirmed in humans by our group (41). It is interesting that we have shown that physiologic or pharmacologic agonists of extracellular calcium-sensing receptors counteracted cAMP-induced AQP2 translocation in renal cells by inducing actin polymerization through Rho signaling (24).

BK induces prostaglandin release through phosphatidylinositol 3-kinase and mitogen-activated protein signaling by regulating COX-2 activity (42), and in rabbit collecting duct cells, BK also causes the release of arachidonic acid (43). In this contribution, we focused on the molecular basis for the diuretic effect induced by BK, with particular emphasis on the intracellular events elicited by BK in collecting duct cells and the possible involvement of AQP2 in this process.

We show here that, in renal cells, BK elicits a transient increase in intracellular calcium concentration by activating the Gq/PLC pathway. This might reduce the vasopressin-dependent increase in cAMP (22) through selectively inhibiting isoform 6 of adenyl cyclase (44), which was found to be enriched in the kidney medulla (45), in the thick ascending limb and in the collecting duct tubule (46). More recently, the presence of a single calcium-calmodulin sensitive adenyl cyclase isoform in inner medullary collecting duct, namely AC3, which is required for vasopressin induced increase in cAMP level, has been demonstrated (47). However, the obtained data demonstrate that BK impairs forskolin-induced AQP2 translocation even in the presence of PLC inhibitor (Figure 2), indicating that the increase in intracellular calcium is not responsible for BK inhibitory effect on AQP2 targeting.

Besides this observation, the novel finding emerging from this study is the functional involvement of the Rho/Gα13 pathway in BK signaling. Ga13 is an important upstream effector of Rho protein, and we have shown that Rho activation impairs AQP2 targeting to the plasma membrane in renal cells (19). Conversely, Rho inhibition causes F-actin depolymerization, thus facilitating AQP2 trafficking to the plasma membrane in renal cells (17–19, 21).

We show here that forskolin stimulation resulted in a decrease in the abundance of Ga13 in a membrane-enriched fraction, paralleled with an increase in the soluble fraction, suggesting that forskolin stimulation, by reducing Ga13 membrane association, results in a decrease in overall Ga13 activity. In contrast, BK treatment increased membrane-associated Ga13, indicating that BK signaling might activate Ga13. Previous studies also suggested that some Ga activation may occur through translocation of cytoplasmic proteins to plasma membrane-bound signaling complexes (35). BK-induced Ga13 activation was confirmed further with an additional experimental strategy. On the basis of the observation that the ability of Ga13 to mediate F-actin cosedimentation increased in NIH3T3 cells that were transfected with the constitutively dominant positive of Ga13 (25), we observed that, compared with the control cells, BK significantly increased the amount of Ga13 co-sedimented with F-actin, whereas forskolin stimulation had opposite effects. In renal cells, Ga13 activation might be responsible for stimulating Rho protein activity, as shown in other cell types (48), inducing stress fiber formation independent of the PLC/Ca2+ pathway (16). It is interesting that BK treatment resulted in activation of RhoA in native rabbit and rat collecting duct suspensions, indicating that the results obtained in vitro probably can be confirmed in vivo.

To conclude, we propose here a novel pathway for the effect of BK that can partially explain its diuretic effect through an impairment of AQP2 trafficking. We suggest that BK acts through BK2R coupled to both Gq and Ga13, a known upstream effector of Rho protein. Rho activation results in a stabilization of the F-actin network. These effects counteract the physiologic hormonal stimulation via cAMP formation, which leads to an increase in protein kinase A activity, which can phosphorylate and inactivate Ga13 (49), having an opposite effect on actin network organization.

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