Calcium-calmodulin mediates bradykinin-induced MAPK phosphorylation and c-fos induction in vascular cells

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Naidu, Padma S., Victoria Velarde, Christiana S. Kappler, Roger C. Young, Ronald K. Mayfield, and Ayad A. Jaffa. Calcium-calmodulin mediates bradykinin-induced MAPK phosphorylation and c-fos induction in vascular cells. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1061–H1068, 1999.—The vasoactive peptide bradykinin (BK) has been implicated in the pathophysiology of a number of vascular wall abnormalities, but the cellular mechanisms by which BK generates second messengers that alter vascular function are as yet undefined. Exposure of vascular smooth muscle cells (VSMC) to BK (10^{-7} M) produced a rapid and transient rise in intracellular calcium, which preceded an increase in tyrosine phosphorylation of mitogen-activated protein kinase (MAPK). MAPK activation by BK was observed as early as 1 min, peaked at 5 min, and returned to baseline by 20 min. Treatment of cells with the intracellular calcium chelator EGTA-acetoxymethyl ester inhibited BK-stimulated MAPK activation, suggesting that intracellular calcium mobilization contributes to the activation of MAPK. The calmodulin inhibitor W-7 also markedly inhibited BK-induced MAPK phosphorylation in the cytoplasm as well as in the nucleus. Moreover, the BK-induced increase in c-fos mRNA levels was significantly inhibited by the calmodulin inhibitor, indicating that calmodulin is required for BK signaling leading to c-fos induction. These results implicate the calcium-calmodulin pathway in the mechanisms for regulating MAPK activity and the resultant c-fos expression induced by BK in VSMC.

B2-kinin receptor; signal transduction; extracellular regulated kinases

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calmodulin complex on the cellular responses initiated by BK in VSMC.

METHODS

Cell culture. Rat aortic VSMC from male Sprague-Dawley rats (Charles-River Laboratories, Wilmington, MA) were prepared by a modification of the method of Majeck and Clowes (24). A 2-cm segment of artery cleaned of fat and adventitia was incubated in 1 mg/ml collagenase for 3 h at room temperature. The artery was then cut into small sections and fixed to a culture flask for explantation in a humidified atmosphere of 95% air-5% CO₂. Medium was changed every 3–4 days, and cells were passaged every 6–8 days by harvesting with trypsin-EDTA. Cell viability was assessed by standard dye exclusion techniques using 1% trypan blue. VSMC were identified by the following criteria. They stained positive for intracellular cytoskeletal fibrils of actin and smooth muscle cell specific myosin (indicative of contractile cell) and negative for factor VIII antigens. VSMC isolated by this procedure were homogeneous and were used in all studies between passage 2 and 4.

Measurement of intracellular calcium concentration by fura 2. VSMC grown to confluency on round glass coverslips were rendered quiescent by growing them in serum-free media for 2 days. For fura 2 loading, cells were incubated at 37°C for 30 min in DMEM containing 5 µM of fura 2-acetoxymethyl ester (AM; Molecular Probes, Eugene, OR). The cells were then washed in bathing solution 1 containing the following composition (in mM): 140 NaCl, 5 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES-Na, pH 7.4. In some experiments, EGTA and fura 2 were simultaneously loaded into the cells by coincubation of 50 µM EGTA-AM and 5 µM fura 2-AM at 37°C for 30 min. The coverslips were then mounted in the flow chamber, and the intracellular calcium concentration ([Ca²⁺]ᵢ) was determined fluorometrically by using an Attofluor Ratio Vision fluorescence microscope with a ×40 objective (Atto Instruments, Rockville, MD). Emission intensities at wavelength greater than 520 nm were obtained by alternatively exciting the fura 2 at 360 and 380 nm at 1 Hz. For each experiment, a control run, the ratio of intensities was obtained in 10–20 regions of interest. Relative concentrations of intracellular calcium were calculated from the ratio of emission intensity using the 360 and 380 nm excitation wavelength. Reported responses were normalized to baseline fluorescence for each region of interest.

Cytosolic and nuclear extraction of proteins. Cytosolic and nuclear proteins were extracted from VSMC by the technique of Andrews and Faller (2). Quiescent VSMC grown in 10-cm dishes were stimulated with BK (10⁻⁷ M) for various times. The cells were suspended in 250 µl of lysis buffer (20 mM Tris, 130 mM NaCl, 10% glycerol, 10 mM 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM Na vanadate, 100 µM aprotinin, 0.15 mg/ml benzamidine, pH 8.0, sonicated for 10 s and centrifuged at 13,000 g for 10 min. The supernatant was harvested as the cytosolic fraction. The pellet fraction was suspended in 500 µl of cold nuclear lysis buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF), incubated on ice for 20 min, and centrifuged at 15 min at 4°C. The protein concentration in the nuclear and cytosolic fractions were determined by the Lowry method (22).

Western blotting of MAP kinases. To measure MAPK activity in cytosol and nuclear fractions, 20–25 µg of soluble protein obtained as previously described was subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins in the gel were transferred to polyvinylidene difluoride membrane and immunoblotted with rabbit polyclonal phospho-specific MAPK antibodies (1:1,000 dilution, New England Biolabs, Beverly, CA) that detect p42MAPK and p44MAPK. Total MAPK was measured in the same membranes by stripping the membrane and immunoblotting with anti-MAPK antibodies (1:6,000 dilution). The immunoreactive tyrosine phosphorylated MAPK and Total MAPK were detected by the CDP-Star chemiluminescent system (New England Biolabs).

RESULTS

Activation of MAPK by BK. Treatment of VSMC with 10⁻⁷ M BK resulted in a time-dependent increase in tyrosine phosphorylation of p42MAPK and p44MAPK, compared with unstimulated cells (Fig. 1). MAPK phosphorylation was detectable as early as 1 min, peaked at 5 min, and declined to basal values by 20 min. BK treatment produced a seven- to eightfold increase in MAPK phosphorylation relative to basal activity. In subsequent experiments, MAPK measurements were carried out with BK stimulation for 5 min.

Calcium and calmodulin role in BK-induced MAPK activation. VSMC preloaded with fura 2 were monitored for changes in intracellular calcium after exposure to BK (10⁻⁷ M) in normal and calcium-free medium. BK evoked a rapid and transient rise in intracellular calcium that peaked at 1–30 s and gradually returned to baseline within 1 min (Fig. 2). This rise in intracellular calcium by BK occurred despite the removal of extracellular calcium from the media, suggesting that transmembrane calcium influx is not necessary for the rise in intracellular calcium. In support of this finding, preincubation of VSMC with the intracellular calcium chelator EGTA-AM (50 µM) for 30
EGTA-AM inhibited the BK-induced MAPK phosphorylation by about 50% (Fig. 3B). We also carried out experiments to study the effects of BK on MAPK activation in calcium-free medium to determine whether extracellular calcium is required for MAPK activation. The results show similar activation of MAPK by BK in calcium-containing as well as calcium-free media (Fig. 3C). Furthermore, addition of EGTA (3 mM) to the extracellular media did not alter MAPK activation in response to BK (Fig. 3C). Taken together, these data indicate that the rise in intracellular calcium, rather than calcium influx, contributes significantly to BK-induced MAPK activation in VSMC. The failure to completely block BK-induced MAPK phosphorylation by EGTA-AM, in the face of complete elimination of the rise in intracellular calcium, indicates that BK promotes MAPK activation via calcium-dependent as well as calcium-independent mechanisms.

In a separate experiment we investigated the effects of EGTA-AM on MAPK phosphorylation by agents that do not elicit release of intracellular calcium. VSMC were treated with phorbol 12-myristate 13-acetate (PMA 5 µM) for 5 min, in the presence and absence of EGTA-AM. PMA resulted in a 3.54 ± 0.35-fold increase in MAPK activation compared with unstimulated controls. A similar increase (3.71 ± 0.30) in MAPK activation was observed in response to PMA in the presence of EGTA-AM. This finding suggests that the inhibitory effect of EGTA-AM on MAPK activation is specific for agonists that stimulate rise in intracellular calcium such as BK.

Rises in intracellular free calcium concentration result in the reversible formation of a calcium-calmodulin complex. A major mechanism for calcium signal transduction is via activation of calmodulin and calmodulin-dependent protein kinase II. To evaluate the requirement for calmodulin in the activation of MAPK in response to BK, VSMC were pretreated for 45 min with a calmodulin inhibitor W-7 (30 µM). The inhibitor was used at concentrations that achieved half-maximal inhibition of calmodulin (13). Once again, BK produced a marked increase in tyrosine phosphorylation of MAPK. This was inhibited by W-7 to a level not significantly different from unstimulated control cells (Fig. 4A). Another study was also carried out to assess the effects of a second calmodulin inhibitor, calmidazolium, on BK-induced MAPK phosphorylation. Treatment of VSMC with BK (10⁻⁷ M) resulted in a 2.45 ± 0.16-fold increase in MAPK phosphorylation compared with unstimulated controls (P < 0.006). However, in the presence of calmidazolium (10 µg), the increase in MAPK activation in response to BK was reduced to 1.81 ± 0.13 (P < 0.03 BK vs. BK-calmidazolium, respectively, n = 3).

We next explored the role of the calmodulin kinase II inhibitor KN-93 (30 µM) on MAPK activation in re-
response to BK (Fig. 4B). VSMC pretreated with the calmodulin kinase II inhibitor KN-93 for 30 min were followed by stimulation with either BK (10^{-7} M) or angiotensin II (ANG II, 10^{-7} M) for 5 min. MAPK activation in response to ANG II has been shown to involve activation of calcium-calmodulin protein kinase II pathway (1). The results shown in Fig. 4 demonstrate that both BK and ANG II produced a significant increase in MAPK phosphorylation compared with unstimulated cells (P < 0.001, n = 6). This increase in MAPK phosphorylation in response to either BK and/or ANG II was completely abolished in the presence of the calmodulin kinase II inhibitor KN-93 (Fig. 4). KN-93 had no significant effect on basal phosphorylation of MAPK. These findings suggest that MAPK activation by BK seems to occur via a calmodulin-calmodulin kinase II-dependent mechanism.

Nuclear phosphorylation of MAPK by BK is calmodulin dependent. The nuclear targets for MAPK include the phosphorylation of Elk-1/TCF, which in turn leads to transcriptional activation at the serum response element and induction of c-fos mRNA levels (12). For MAPK to influence c-fos expression, it requires translocation to the nucleus. To assess the role of calmodulin in BK-induced nuclear phosphorylation of MAPK, we measured the tyrosine phosphorylation of MAPK in

Fig. 3. Role of intracellular calcium in BK-induced MAPK activation. VSMC were pretreated with EGTA-AM (50 µM) for 30 min (A), nifedipine (1 µM) for 1 min (B), and/or in extracellular calcium-free medium ± EGTA, followed by BK (10^{-7} M) stimulation for 5 min (C). MAPK phosphorylation in the cell lysate was measured by immunoblots using anti-phosphotyrosine-MAPK antibodies. Total MAPK was measured in the same immunoblot by stripping membrane and reincubating with anti-total MAPK antibodies. Blots are representative of 6 separate experiments. Bar graphs represent intensities of both p42mapk and p44mapk bands measured in densitometer relative to total MAPK and expressed as percent phosphorylation relative to control. *P < 0.01 vs. control. #P < 0.01 vs. BK.

Fig. 4. Role of calmodulin in BK-induced MAPK activation. VSMC were pretreated for 45 min with calmodulin inhibitor W-7 (30 µM, A) and/or calmodulin kinase II inhibitor KN-93 (30 µM, B), followed by either BK or angiotensin II (ANG II) stimulation (10^{-7} M) for 5 min. MAPK phosphorylation in the cell lysate was measured by immunoblots using anti-phosphotyrosine-MAPK antibodies. Total MAPK was measured in the same immunoblot by stripping membrane and reincubating with anti-total MAPK antibodies. Blots shown are representative of 5 separate experiments. Bar graphs represent intensities of both p42mapk and p44mapk bands measured in densitometer relative to total MAPK and expressed as percent phosphorylation relative to control. *P < 0.005 vs. control. #P < 0.005 vs. BK. †P < 0.005 vs. ANG II.
the cytosol and nuclear extracts of VSMC treated with 10−7 M BK, in the presence and absence of calmodulin inhibitor W-7. Treatment of VSMC with BK for 5 min resulted in a marked increase in tyrosine phosphorylation of MAPK in both cytosolic and nuclear fractions (Fig. 5). Pretreatment of VSMC with W-7 significantly decreased both cytosolic and nuclear MAPK phosphorylation in response to BK. Phosphorylated p42mapk and p44mapk in the cytosol of W-7-treated cells were reduced by approximately 50% compared with BK-stimulated cells. Nuclear phosphorylation of p42mapk and p44mapk in response to BK was also significantly reduced by the calmodulin inhibitor.

BK-induced c-fos mRNA expression is regulated by calmodulin. To evaluate whether the induction in c-fos mRNA levels by BK are calmodulin dependent, we measured c-fos mRNA levels in VSMC pretreated with W-7 for 45 min, followed by BK (10−7 M) stimulation for 30 min. As shown in Fig. 6, c-fos mRNA levels were undetectable at 0 min but were markedly induced within 30 min of BK stimulation. However, in the presence of the calmodulin inhibitor W-7, the induction of c-fos mRNA by BK was significantly suppressed (6,996 ± 470 vs. 4,235 ± 452 densitometric units; BK vs. BK-W-7, respectively, P < 0.002). β-actin mRNA levels measured in the same cells were not altered by any of the treatments (7,515 ± 1,330, 10,013 ± 288, 6,869 ± 444, 7,490 ± 181, densitometric units, control vs. BK vs. W-7 vs. W-7-BK, respectively, Fig. 6).

DISCUSSION

The findings of the present study demonstrate that activation of MAPK and induction of c-fos in response to BK challenge in VSMC involve a rise in intracellular calcium and activation of the calcium-calmodulin complex. The peak activation of MAPK by BK was observed at 5 min, whereas the rise in intracellular calcium elicited by BK peaked within 30 s and declined to baseline by 60 s. EGTA-AM binding of calcium and the calmodulin inhibitor did not entirely block MAPK activation, indicating that intracellular calcium rise and calmodulin activation is only one of the upstream second messengers utilized by BK to activate MAPK in
VSMC. In the present studies, we have also shown the induction of c-fos by BK involves activation of MAPK by a calcium-calmodulin-dependent pathway.

One of the earliest postreceptor events in BK transmembrane signaling is elevation in intracellular calcium. Our data suggest that this \( [\text{Ca}^{2+}]_i \) rise produced by BK in VSMC probably occurs through release of calcium from intracellular stores. From other available data, it is likely that the BK B2 receptor initiates calcium release following activation of phospholipase C-\( \beta \) through pertussis toxin-insensitive \( G_\text{q} \), which converts phosphatidylinositol \( 4–5\)-disphosphate into Ins(1,4,5)P3 and diacylglycerol (20). Ins(1,4,5)P3 in turn can act as an intracellular second messenger by binding to specialized tetrameric Ins(1,4,5)P3 receptors that span the endoplasmic reticular membrane to trigger release of calcium from the endoplasmic reticulum (3). Support for this mechanism comes from our finding that removal of extracellular calcium did not abrogate the rise in \( [\text{Ca}^{2+}]_i \) elicited by BK rather than an influx of calcium through L-type calcium channels is required for MAPK activation. Furthermore, removal of calcium from the extracellular medium and or addition of EGTA to the extracellular medium did not alter the response of BK to activate MAPK. Taken together, our data seem to suggest that mobilization of calcium from intracellular stores by BK is sufficient to activate MAPK. Our findings support previous published data demonstrating that the rise in intracellular calcium elicited by thapsigargin, ANG II, and ionomycin contributes to the activation of MAPK (1, 6, 9). Although the cellular mechanism(s) through which calcium activates MAPK is not clearly defined, the involvement of Ras, Raf and MEK has been suggested (34). We have preliminary evidence to suggest that MAPK activation by BK is inhibited by a specific inhibitor of MEK (unpublished observations).

The calcium-binding protein calmodulin is implicated in the regulation of many cellular signaling pathways triggered by calcium, including progression of cell cycle and regulation of cell proliferation (17, 23, 39). Calmodulin binds to and activates several cellular proteins in response to a rise in intracellular calcium. The results of the present study indicate that activation of cytosolic and nuclear MAPK in response to BK are mediated through a calmodulin-dependent pathway. Support for this pathway comes from the findings that inhibition of calmodulin with the cell-permeable inhibitors W-7 and/or calmidazolium significantly reduced MAPK activation in response to BK. The calmodulin kinase II inhibitor KN-93 also inhibited the response of BK to stimulate MAPK activation. Our data are in agreement with a recent study by Abraham et al. (1) that implicated a role for calmodulin-dependent kinase II in MAPK activation in response to ionomycin stimulation.

Our results indicate that one of the nuclear targets for calmodulin is the induction of the protooncogene c-fos, which binds with c-jun to form the AP-1 complex transcription factor, thereby regulating the expression of genes containing this element (18). The present data show that treatment of VSMC with the calmodulin inhibitor W-7 significantly reduced the increase in c-fos mRNA level observed in response to BK stimulation. This is the first indication that BK stimulates c-fos induction via a calmodulin-dependent pathway. Although the induction of c-fos transcription can be mediated by several cis-activating elements that are recognized by proteins that are activated in response to an external signal, the sequence of events leading to c-fos induction by BK has not been completely defined. Because calmodulin has been shown to be present in the nucleus, it is possible that calmodulin through its binding to nuclear calcium could influence c-fos expression directly or indirectly by activating calmodulin kinase II, which has been shown to contribute to c-fos expression (45).

Another pathway through which BK could induce c-fos expression is via activation of MAPK through a calmodulin-dependent pathway. Support for such a pathway comes from the finding that the calmodulin inhibitor blocks not only BK-induced p42\text{mapk} and p44\text{mapk} phosphorylation in the cytoplasm but also phosphorylation of p42\text{mapk} and p44\text{mapk} in the nucleus. Once nuclear p42\text{mapk} and p44\text{mapk} are activated, they result in the phosphorylation of p62\text{TCF/Eik-1} proteins leading to enhanced c-fos transcription (12). In this regard, a recent study by El-Dahr et al. (10), showed that the tyrosyl phosphorylation of Eik-1 in response to BK is mediated via MAPK activation in mesangial cells. Furthermore, the calmodulin-kinase cascade has been shown to activate transcription through phosphorylation of Eik-1 (11). An alternative pathway through which BK can induce c-fos expression is through intracellular calcium mobilization and hence MAPK activation. In this regard, our findings indicate that the intracellular calcium chelator EGTA-AM decreased (35%) c-fos mRNA expression in response to BK. Other studies have reported that the increase in c-fos expression in response to endothelin or ionomycin can be attenuated in the presence of 1,2-bis(2-aminophenoxy)ethane N,N,N',N' -tetraacetic acid, an intracellular calcium chelator (32, 43).

In addition to calcium binding, phosphorylation of calmodulin may regulate its mode of action. Tyrosine phosphorylation of calmodulin by Src kinases and/or the insulin receptor kinase was shown to enhance biological activity, whereas serine and threonine phosphorylation decreased calmodulin activity (36). In this
regard, BK could activate calmodulin by two distinct regulatory pathways, one through a rise in intracellular calcium and the other via activation of cytoplasmic tyrosine kinases such as Src, PYK2, and or focal adhesion kinase 125 (21).

In summary, our results suggest that activation of the B2 receptor by BK initiates multiple signals and subsequent cross-talk between second messengers leading to the release of intracellular calcium, which binds to calmodulin and results in the activation and nuclear translocation of MAPK. Activated nuclear MAPK can phosphorylate TCF/ Elk-1, resulting in induction of the transcription factor c-fos, leading to further downstream signaling events that can regulate VSMC function. A better understanding of the signal transduction pathways by which BK modulates vascular function provides the basis for investigating the paracrine or autocrine effects of BK on vascular smooth muscle cell proliferation in states of vascular injury and disease.

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