The Mitogen-Activated Protein Kinase p38-2 Is Necessary for the Inhibition of N-Type Calcium Current by Bradykinin

Malgorzata A. Wilk-Blaszczak,¹ Bernd Stein,² Shuichan Xu,¹ Miguel S. Barbosa,² Melanie H. Cobb,¹ and Francesco Belardetti¹

¹Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235, and ²Signal Pharmaceuticals, Inc., San Diego, California 92121

Calcium influx via voltage-dependent calcium channels ($I_{Ca,V}$) links depolarization of excitable cells to critical cellular processes, such as secretion, contraction, and gene transcription. Fast regulation of $I_{Ca,V}$ (<1 sec) by G-protein-coupled receptors is a relatively well-defined mechanism, whereas slow (30–60 sec) actions of transmitters and hormones on the same current remain poorly understood. In NG108-15 cells, the kinetically slow inhibition of N-type $I_{Ca,V}$ by bradykinin (BK) requires the sequential activation of two G-proteins, heterotrimeric G_{13} and monomeric Rac1/Cdc42. We have now defined a role in this

pathway for the relatively fast-acting p38 mitogen-activated protein kinase (MAPK). The slow inhibition of $I_{Ca,V}$ by BK was suppressed specifically by SB203580, a compound that inhibits the p38 family of MAPKs. BK potently and selectively activated a newly discovered p38 family member, p38-2. These data provide the first evidence that a MAPK is involved in the regulation of $I_{Ca,V}$ by a receptor-mediated process.

Key words: G-protein; calcium channels; bradykinin; p38; MAPK; neuroblastoma x glioma; NG108-15; G_{13}

Depolarization of the neuronal membrane leads to the opening of voltage-activated calcium channels ($I_{Ca,V}$), followed by a rapid increase of the intracellular calcium concentration (Tsien et al., 1991). In turn, the cytoplasmic calcium signal controls a multitude of cellular functions (Kandel et al., 1991). This process is tightly regulated by a variety of hormones and neurotransmitters, in most cases acting on the calcium channels via receptors coupled to heterotrimeric G-proteins (Hepler and Gilman, 1992; Hille, 1992, 1994; Hescheler and Schultz, 1993; Wickman and Clapham, 1995; Jan and Jan, 1997). Despite such an important role, regulation of $I_{Ca,V}$ by G-proteins is understood only partially. For example, some of these G-protein actions are fast (<1 sec, time to peak) and appear to be mediated by membrane-delimited pathways (possibly involving a direct action of $\beta\gamma$ subunits on the channels) (Herlitze et al., 1996; Ikeda, 1996). Other G-protein effects on $I_{Ca,V}$ are quite slow (>30 sec), and are mediated by unknown signal transduction cascades (Hille, 1994).

We have used neuroblastoma x glioma hybrid cell line (NG108-15) (Hamprecht et al., 1985) to determine the signaling pathway that mediates the inhibition of $I_{Ca,V}$ by bradykinin (BK) (Brown and Higashida 1988a). In these cells, high voltage-activated $I_{Ca,V}$ comprises two components: ω -conotoxin (ω -CgTX)-sensitive (N-type) and dihydropyridine-sensitive (Tausig et al., 1992; Wilk-Blaszczak et al., 1994b). BK and Leu-Enkephalin (Leu-Enk) inhibit the ω -CgTX-sensitive component

via two heterotrimeric G-proteins, G_{13} and G_{oA} , respectively (Hescheler et al., 1987; Tausig et al., 1992; Wilk-Blaszczak et al., 1994b). Both of these effects are BAPTA- or EGTA-insensitive. G_{oA} produces fast inhibition, presumably by direct action of G_{oA} on the channels. In contrast, G_{13} inhibits $I_{Ca,V}$ with slow kinetics, via activation of monomeric G-protein Rac1/Cdc42 (Wilk-Blaszczak et al., 1997; see also Bourne et al., 1990, 1991; Ridley, 1995; Zhong, 1995; Lamaze et al., 1996; Machesky and Hall, 1996). In addition, BK, but not Leu-Enk, inhibits the dihydropyridine-sensitive component of $I_{Ca,V}$ via two additional BAPTA- or EGTA-sensitive pathways acting in parallel and mediated by $G_{q/11}$ and G_{i2} (Wilk-Blaszczak et al., 1996).

Whereas Rac1/Cdc42 act presumably downstream of G_{13} , it is not known how these G-proteins couple to calcium channels. In the present work, we have tested the hypothesis that Rac1/Cdc42 inhibit $I_{Ca,V}$ through activation of a mitogen-activated protein kinase (MAPK) (Robinson and Cobb, 1997). MAPK pathways are highly conserved, ubiquitous, and versatile signaling devices. They are activated by surface receptors via interplay of specific proteins that often include a monomeric G-protein. Until recently, MAPKs were thought to mediate the effects of growth factors and hormones on long-lasting cellular events, such as proliferation and differentiation. Evidence now has emerged that MAPKs can be activated by receptors coupled to heterotrimeric G-proteins (Crespo et al., 1994a,b; Shapiro et al., 1996) and play relatively fast regulatory roles, such as the response of yeast to osmotic stress (Ruis and Schuller, 1995; Waskiewicz and Cooper, 1995). In addition, p38 MAPK is activated by Rac and Cdc42 in cotransfection experiments (Zhang et al., 1995). These observations led us to test the hypothesis that a p38 MAPK mediates the inhibitory action of BK and G_{13} on $I_{Ca,V}$.

MATERIALS AND METHODS

Cultures. Cultures of NG108-15 cells were prepared as described in Hamprecht and co-workers (1985) and Wilk-Blaszczak and co-workers (1994b).

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Correspondence should be addressed to Dr. M. A. Wilk-Blaszczak, Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235.

Dr. Belardetti's present address: Glaxo Wellcome SpA, Via Fleming 4, 37135 Verona, Italy.

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Solutions. Extracellular solution for calcium currents included (in mM): 125 NaCl, 5.4 CsCl, 1.8 CaCl₂, 1.0 MgCl₂, 10 HEPES, 5.0 glucose, and 0.0005 TTX, pH 7.4 (with NaOH); for potassium currents (in mM): 125 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 20 HEPES, and 5.0 glucose, pH 7.4 (with NaOH); pipette solution for calcium responses (in mM): 112 CsCl, 1 MgCl₂, 10 EGTA, 30 HEPES, 3 ATP, and 0.1 GTP, pH 7.3 (with CsOH); for potassium responses (in mM): 115 KCl, 0.1 MgCl₂, 40 HEPES, 3 ATP, and 0.1 GTP, pH 7.3 (with KOH).

SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole]; SKF106978 [2-(4-methylsulfinyl)-3-[4-(2-methylpyridyl)]-6,7-dihydro[5H]pyrrolo[1,2-a]imidazole]; and PD98059 [2'-amino-3'-methoxyflavone] were dissolved in dimethylsulfoxide (DMSO), stored in 20 mM aliquots at -20°C , and reconstituted in pipette solution to the final concentration of 20 μM . They were dialyzed into the cell from the patch pipette for 20 min before transmitter application. Nordihydroguaiaric acid (NDGA) and indomethacin were dissolved in DMSO and then reconstituted in pipette solution at 5 μM . All chemicals were obtained from Sigma (St. Louis, MO), except nucleotides (Boehringer Mannheim, Indianapolis, IN), peptides (Peninsula, Belmont, CA), and PD98059 (Calbiochem, San Diego, CA). SB203580 and SKF106978 were the gift of Dr. John Lee (SmithKline Beecham).

Recording techniques. A plastic dish containing the cells was placed on the stage of an inverted microscope and superfused slowly with extracellular solution at room temperature (23°C). The standard whole-cell patch-clamp technique was used to isolate $I_{Ca,V}$ and to dialyze the cells with inhibitors or control solutions (Hamill et al., 1981). To measure the modulation of $I_{Ca,V}$, step pulses (0 mV, 100 msec) were delivered by an IBM-compatible PC every 10 sec from a holding potential of -90 mV (pCLAMP software; Axon Instruments, Foster City, CA). The $I_{Ca,V}$ evoked by each command step, after analog correction of the capacitive current, was digitized at 10 kHz by the computer, which also performed simultaneous linear subtraction of the residual capacitive current, as well as of the leakage current (P/4 protocol). The inhibition of $I_{Ca,V}$ by neurotransmitters was expressed as a percentage of the peak current inhibited at the maximum of the transmitter action. Only $I_{Ca,V}$ records that displayed net inward current at the end of the depolarizing pulse and without a decline of peak $I_{Ca,V}$ over the duration of the experiment were used for analysis.

Current-voltage relationships were obtained by applying 50 msec command pulses (every 5 sec) from -90 mV to the various test voltages in the presence or absence of BK. To measure $I_{K,BK}$ (the transient, voltage-independent K^{+} current activated by BK) (Brown and Higashida 1988a; Wilk-Blaszczak et al., 1994a), the cells were held at -40 mV. The $I_{K,BK}$ was measured as the maximal outward current after application of BK. Only cells that displayed a positive holding current at the end of the perfusion were used for analysis.

Application of transmitters. BK and Leu-Enk were pipetted directly into the bath in aliquots of 200 μl (0.1 μM) (Wilk-Blaszczak et al., 1994b). To eliminate the effect of variability among cells on the size of the responses, cells dialyzed with inhibitor and control solutions were alternated.

Kinase assay. For the activation of p38 and p38-2 MAPK by BK, cells were incubated at room temperature and then rapidly frozen in liquid nitrogen, subsequently thawed on ice, and lysed with a lysis buffer containing (in mM): 20 Tris Cl, 10 EGTA, 60 β -glycerophosphate, 10 MgCl₂, 2 dithiothreitol, 1 vanadate, 1 phenylmethylsulfonyl fluoride, 1% Triton X-100, and 10 $\mu\text{g}/\text{ml}$ leupeptin and aprotinin, pH 7.6. For the other treatments used, cells were incubated with PBS in the absence or presence of stimuli at 37°C for 30 min, and then washed with PBS and lysed with lysis buffer. To perform immune complex assays of p38 MAPKs, soluble extract (300 μg) was incubated with protein A Sepharose conjugated with either anti-p38-2 or anti-p38 antibodies. Polyclonal antibodies selective to p38 (p297) were generated against purified recombinant protein, as described (Khokhlatchev et al., 1997) and did not recognize p38-2 as judged by immunoblotting. Antibodies to p38-2 were raised using a 14-amino acid C-terminal peptide (Stein et al., 1997) and did not cross-react with p38. The Sepharose beads were washed twice with lysis buffer, twice with 0.25 M Tris Cl, pH 7.6, 0.1 M NaCl, and once with kinase assay buffer containing (in mM): 20 HEPES, 1 benzamidine, 1 dithiothreitol, and 10 MgCl₂, pH 7.6. The activities of immunoprecipitated p38 and p38-2 were detected by a standard kinase assay using GST-ATF2 (1–254) as a substrate (Frost et al., 1996). For the *in vitro* test of the inhibition of kinases by SB203580 recombinant, purified GST-p38 and GST-p38-2 (both 0.5 μg) were preincubated for 30 min with increasing concentrations of SB203580, as indicated, and then tested in a kinase reaction with 1 μg of purified recombinant GST-ATF2 and 50 nM

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Phosphorylated proteins were separated by SDS-PAGE on 10% gels and then subjected to autoradiography. Incorporation of $[\text{}^{32}\text{P}]\text{phosphate}$ was quantitated with a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). GST-ATF2 phosphorylation in the absence of SB203580 was set to 100%.

Statistical analysis. The values of inhibition $I_{Ca,V}$ by transmitters are distributed not normally and therefore are displayed as cumulative histograms. The curves were generated by plotting the transmitter response for the cell (percentage of current inhibited, x-axis) versus the percentage of cells in the treatment group that had larger responses than this value (McGehee et al., 1992; Wilk-Blaszczak et al., 1994a,b, 1997). This method provides direct information about the scattering of the data. For the purpose of immediate comparison, response indices (in arbitrary units equal to the areas under the respective cumulative response curves) are presented in the figures. Statistical comparisons of the responses were performed using the log-rank test (Koch et al., 1985). Responses to Leu-Enk not included in the figures are presented as mean $\pm$ SD.

RESULTS

SB203580, an inhibitor of p38 MAPK, selectively blocks the inhibition of $I_{Ca,V}$ by BK

To determine the second messengers used by BK in the slow inhibition of $\omega\text{-CGTX}$ -sensitive component of $I_{Ca,V}$, we isolated G_{13} mediated pathway using high concentration of EGTA in the recording pipette (10 mM). Under these conditions, two additional parallel pathways used by BK and mediated by $G_{q/11}$ and G_{12} are suppressed (Wilk-Blaszczak et al., 1994b, 1996). We used SB203580, a highly specific inhibitor of p38 and p38-2 MAPKs (Lee et al., 1994; Cuenda et al., 1995; Kumar et al., 1997) to test the role of this MAPK in the G_{13} mediated effect of BK.

Two transmitter responses were tested sequentially for each cell whenever possible. The inhibition of $I_{Ca,V}$ by BK was measured first (Fig. 1*A₁*, *B₁*) and by Leu-Enk thereafter (Fig. 1*A₂*, *B₂*). This protocol was adopted because these two inhibitory pathways use distinct signaling mechanisms to converge on the same component of $I_{Ca,V}$, making the response to Leu-Enk an appropriate control for any experimental manipulations of the pathway of BK (see also Wilk-Blaszczak et al., 1994b). We found that in cells dialyzed with SB203580 (20 μM), the inhibition of $I_{Ca,V}$ by BK was blocked, whereas that to Leu-Enk was unaffected (Fig. 1*A₁*, *A₂*). Application of SKF106978, an inactive analog of SB203580 (20 μM), did not block either response (Fig. 1*B₁*, *B₂*). These were consistent observations, as shown in Figure 1*C,D*, where a summary of all the experimental results is presented. Moreover, application of SB203580 had no significant effects on the peak $I_{Ca,V}$ (Fig. 2*A*) or the holding current (data not shown). The inhibitions of $I_{Ca,V}$ by two transmitters in control conditions (with SKF106978 for either BK or Leu-Enk, and with SB203580 for Leu-Enk) were comparable with inhibitions in the presence of 0.1% DMSO (the vehicle for the inhibitor) (Fig. 1*C,D*) or in its absence (Wilk-Blaszczak et al., 1997). These observations suggest that a p38 MAPK plays a necessary role in the signal transduction pathway used by BK and G_{13} to inhibit $I_{Ca,V}$.

The inhibitory effect of SB203580 is pathway-specific

The inhibitory action of SB203580 on the response to BK could result from the direct block of the BK-sensitive portion of $I_{Ca,V}$, rather than of p38 MAPK. However, under the present experimental conditions (10 mM EGTA in the pipette) (Wilk-Blaszczak et al., 1994b, 1996), BK and Leu-Enk converge on the same component of $I_{Ca,V}$, and any direct effect of the inhibitor on $I_{Ca,V}$ would suppress both transmitter responses. This was not the case (Fig. 1*A₁*, *A₂*). In addition, the time courses of peak $I_{Ca,V}$ during the intracellular dialysis with SB203580 ($n = 11$, Fig. 2*A*) were of similar to those observed during the dialysis with SKF106978

Figure 1. Inhibition of $I_{Ca,V}$ by BK is blocked by SB203580, the inhibitor of p38 MAPK. *A*, *B*, Sequential responses to BK (*A*₁, *B*₁) and Leu-Enk (*A*₂, *B*₂) in two cells. One was dialyzed with SB203580 (20 μ M; *A*), the other with SKF106978 (20 μ M; *B*). The $I_{Ca,V}$ was activated by a 100 msec test command to 0 mV, applied every 10 sec from a holding potential of -90 mV. Leakage- and capacitance-subtracted current traces are displayed, showing $I_{Ca,V}$ before (Con) and at the peak of BK action (0.1 μ M). The continuous line marks the zero current. *C*, *D*, Summary of the responses to BK and Leu-Enk. Cumulative histograms (*C*) and response indices (*D*) are displayed. The cumulative distributions were constructed as follows. For each cell, the inhibition of the $I_{Ca,V}$ by the transmitter (in percentage; see Materials and Methods) was plotted on the horizontal axis (X). For each X , the fraction of responses (% Responses X) larger than a given X was calculated and used to build the cumulative distribution. For example, the cumulative distribution in *C*₁ shows that ~80% of the cells dialyzed with DMSO had a response to BK greater than zero (open squares), whereas only ~20% of the SB203580-treated cells had a response greater than zero (filled circles). The response indices (*D*) are proportional to the areas under the corresponding cumulative distribution. The numbers on the left of each bar indicate the number of cells studied. The asterisk denotes a statistically significant difference ($P < 0.001$) from the controls (log-rank test).

($n = 10$, data not shown) and control solution (for example, see Wilk-Blaszczak et al., 1994b, 1997). In most experiments, the peak current increased during intracellular dialysis, irrespective of the treatment (Fig. 2*A*). Thus, the suppressive action of SB203580 on the response to BK was not attributable to an effect of the inhibitor on the $I_{Ca,V}$ itself.

In the experiments presented, $I_{Ca,V}$ was evoked by the depolarization to a fixed voltage (0 mV) (see Materials and Methods).

Figure 2. Effects of SB203580 on $I_{Ca,V}$. *A*, Action of SB203580 on peak $I_{Ca,V}$ for the entire duration of the experiment. Time course of the peak current during intracellular dialysis with SB203580 (20 μ M), at the end of which BK and Leu-Enk were applied sequentially (bars, both at 0.1 μ M). Between 0 and 20 min, $I_{Ca,V}$ was activated every 30 sec. In the remaining portion of the time course, every 10 sec. Other parameters are as in Figure 1. *B*, *C*, Action of the inhibitor over a broad range of membrane potentials. They show peak $I_{Ca,V}$ -voltage relations in two cells, before (open circles) and during (closed circles) application of BK (0.1 μ M), and after intracellular dialysis of SKF106978 (*B*, 20 μ M) or SB203580 (*C*, 20 μ M). The current-voltage curves were obtained using a series of 50 msec commands (V_t) of increasing amplitude, at 5 sec intervals. The other explanations are as in Figure 1.

To examine whether SB203580 suppresses the response to BK by shifting the current-voltage ($I-V_m$) relationship of $I_{Ca,V}$, we examined the inhibition produced by BK over a broad range of membrane potentials (V_m) in the presence and absence of the p38 MAPK inhibitor. In the cells dialyzed with SB203580 (20 μ M; $n = 2$), the $I-V_m$ curve was similar to that observed in the presence of the inactive analog (20 μ M SKF106978) (Fig. 2*B,C*) or to that obtained after dialysis with pipette solution (for example, see Wilk-Blaszczak et al., 1997). However, application of BK in the presence of the p38 inhibitor failed to inhibit $I_{Ca,V}$ over the entire range of V_m values examined, whereas in the presence of SKF106978 it produced an inhibition comparable with that observed in cells dialyzed with pipette solution (Fig. 2*B,C*) (Wilk-Blaszczak et al., 1997).

The specificity of the blocking action of SB203580 on the G_{13} pathway was tested additionally using a second response to BK, transient activation of a voltage-independent K^+ current ($I_{K,BK}$) (Brown and Higashida, 1988a). This second response is mediated by $G_{q/11}$ (Wilk-Blaszczak et al., 1994a), which activates phosphatidylinositol metabolism leading to an increase of the intracellular Ca^{2+} concentration (Brown and Higashida, 1988b; Smrcka et al., 1991). $I_{K,BK}$ was measured in cells dialyzed either with the inhibitor SB203580 or with the inactive analog SKF106978 (Fig. 3*A*₁, *A*₂). In all cells examined, the intracellular application of

Figure 3. SB203580 does not block activation of $I_{K,BK}$ by BK. In *A*₁ and *A*₂, examples of the activation of $I_{K,BK}$ by BK (0.1 μM) from two cells, after intrapipette dialysis with SB203580 (20 μM; *A*₁) or SKF106978 (20 μM; *A*₂). Data were acquired at 100 Hz. *B*, Cumulative histograms and response indices (inset) are displayed for all the $I_{K,BK}$ responses.

Figure 4. The inhibition of $I_{Ca,V}$ by BK is not attenuated by inhibitors of eicosanoid and ERK pathways. Cumulative histograms (*A*) and response indices (*B*) of the inhibitions of $I_{Ca,V}$ by BK in cells dialyzed with indomethacin (5 μM; filled circles), NDGA (5 μM; filled squares), PD98059 (20 μM; open circles), or DMSO (0.1%; open squares). DMSO controls are the same as those displayed in Figure 1*C*₁ and *D*₁. Other explanations are as in Figure 1.

SB203580 did not reduce the amplitude of $I_{K,BK}$, compared with the application of SKF106978 (Fig. 3*B*).

Inhibitions of extracellular signal-regulated kinase (ERK) or arachidonic acid metabolism do not suppress the response to BK

BK is a pleiotropic agonist acting through a variety of signaling mechanisms, some of which might contribute to the inhibitory pathway of G_{13} in conjunction with p38 MAPK. For example, p38 MAPK might act on the channels via activation of phospholipase A_2 and production of eicosanoids (Kennedy et al., 1996; Kramer et al., 1996; Xing et al., 1997; Zhang et al., 1997). We have examined the possible role of two signaling pathways activated by BK in the inhibitory effect of G_{13} , activation of the ERK and stimulation of arachidonic acid metabolism (Ahn et al., 1992; Schror, 1992; Busse et al., 1994; Clark and Murray, 1995).

We used PD98059, a specific inhibitor of the ERK pathway, to test the hypothesis that ERK might play a role in the response to BK (Dudley et al., 1995). We observed that application of PD98059 (20 μM) did not suppress the inhibitory action on $I_{Ca,V}$ of either BK (Fig. 4*A,B*) or Leu-Enk (31.3 ± 24.1 , $n = 4$ for PD98059; 29.4 ± 12.9 , $n = 5$ for DMSO). These observations indicate that the ERK pathway plays no role in the inhibitory response to BK.

We have then examined the role of arachidonic acid metabo-

lites (eicosanoids) in the inhibition of $I_{Ca,V}$ by BK. We have applied indomethacin (5 μM) intracellularly to block the cyclooxygenase pathway and NDGA (5 μM) to block the lipoxygenase pathway of arachidonic acid. Application of either inhibitor did not suppress the inhibition of $I_{Ca,V}$ by BK (Fig. 4*A,B*) compared with control cells dialyzed with DMSO-containing pipette solution. The inhibition of $I_{Ca,V}$ by Leu-Enk also was not suppressed by the inhibitors (32.4 ± 13.6 , $n = 9$ for NDGA; 44 ± 8.7 , $n = 7$ for indomethacin; 32.5 ± 12.8 , $n = 10$ for DMSO). Taken together, these data reinforce the idea that a p38 MAPK plays an unique role in the inhibitory action of BK, without the involvement of ERK or arachidonic acid pathways.

BK activates a new member of p38 MAPK family

Taken together, our data indicate that p38 MAPK mediates the inhibition of $I_{Ca,V}$ produced by BK. To assay directly the activity of this MAPK and its regulation by BK in NG108-15 cells, we performed immune complex kinase assays with antibodies that recognize selected MAPK isoforms (Fig. 5). We found that two members of the p38 family of MAPKs, p38 and p38-β, were not activated by BK (Fig. 5*A*₂). Lack of significant stimulation by BK was not attributable to low-level expression of these proteins, because immunoblot analysis revealed that they were present (data not shown), nor to their lack of regulation, because they were normally activated by osmotic shock in NG108-15 cells (Fig. 5*B*₂). Rather, we found that BK potentially activated a newly discovered p38 family member, p38-2 MAPK (Fig. 5*A*₁), which also is SB203580-sensitive (Fig. 5*D*) (Stein et al., 1997). This regulatory effect is unique to BK, because in contrast to p38 and p38-β MAPKs, p38-2 is not activated by osmotic shock (Fig. 5*B*₁). These data are consistent with the idea that BK uses a specialized MAPK pathway that includes p38-2 to regulate $I_{Ca,V}$ (Fig. 5*E*). This model is reinforced by the observations that the activation of p38-2 by BK was transient, much like the effect on $I_{Ca,V}$ of continuous application of this transmitter (Fig. 5*C*), and that BK did not activate two other MAPKs, ERK and Jun N-terminal kinase (data not shown). More generally, these findings indicate that p38 and p38-2 are regulated differentially and target distinct effectors.

DISCUSSION

In summary, we have shown that a unique p38-2 MAPK plays a necessary role for the inhibitory effect of BK on $I_{Ca,V}$. This conclusion is supported by several sets of observations. First, both the modulation of $I_{Ca,V}$ by BK and the activity of p38-2 MAPK are blocked specifically by SB203580. Second, BK potentially and selectively activates p38-2 MAPK, and the time course of this effect is similar to that of the inhibition of $I_{Ca,V}$ by BK.

Previous work has shown that SB203580 is a highly selective inhibitor of the p38 family of MAPKs, acting *in vivo* via competition with ATP (Wilson et al., 1997; Young et al., 1997). No inhibition of Jun N-terminal kinase, ERK, or other kinases has been found (Cuenda et al., 1995, 1997; Kumar et al., 1997). A single amino acid difference between p38 and p38-2 and other MAPKs is responsible for this selectivity (Wilson et al., 1997). In addition, this series of compounds has been used extensively as a probe to evaluate physiologic events that depend on the p38 MAPK pathway (Cuenda et al., 1995; Shapiro and Dinarello, 1995; Beyaert et al., 1996; Hazzalin et al., 1996; Kumar et al., 1996; Saklatvala et al., 1996).

We have shown here that the blocking action of SB203580 is pathway-specific, because two additional pathways, one used by

Figure 5. The p38-2 MAPK is activated by BK in NG108-15 cells. *A*, *B*, Immune complex assay of p38-2 MAPK (*A₁*, *B₁*) and p38 MAPK (*A₂*, *B₂*), after stimulation of intact cells with control extracellular solution (*C*), BK (10 μ M), interleukin-1 β (IL1 β , 10 ng/ml), or 0.7 M NaCl. For the treatments with BK or the controls in *A₁* and *A₂*, the cells were treated for the time given above the lane (5 min for *C*, in *A₂*). *C*, Time course of the peak $I_{Ca,V}$ in the continuous presence of BK (1 μ M). The horizontal bar denotes the presence of BK in the bath. The parameters of the voltage-clamp are as explained in Figure 1. *D*, SB203580 blocks the activity of p38 (dark bars) and p38-2 (light bars). Each bar represents means of at least two independent measurements of kinase activity. *E*, Proposed pathway for the inhibition of $I_{Ca,V}$ by BK.

Leu-Enk to inhibit the same component of $I_{Ca,V}$ that is inhibited by BK, and the other used by BK to activate $I_{K,BK}$, were not affected by SB203580. Furthermore, SB203580 did not act distally by blocking calcium channels or altering their voltage-dependency. We concluded that a p38 MAPK mediates the inhibitory response to BK and validated this model by identifying the kinase that is likely to mediate this response. We have shown in *in vitro* studies that BK regulates p38-2 selectively, without affecting two other members of the same family of MAPKs, p38 and p38- β . The latter MAPKs, recognized by the same antibody, are regulated via separate pathways, because unlike p38-2, they respond to osmotic shock. This specificity of regulation appears unique to neuronal cells, because in recombinant systems also, p38-2 is sensitive to osmotic shock (Stein et al., 1997). Finally, the link between p38-2 MAPK and $I_{Ca,V}$ is reinforced by similar time courses of their regulation by BK.

We have also examined the hypothesis that p38-2 might act on $I_{Ca,V}$ in concert with other pathways. For example, release of eicosanoids can be produced via activation of phospholipase A_2 by MAPK, and these highly active signaling molecules might be responsible for the inhibition of $I_{Ca,V}$. However, this is unlikely here, because the response to BK is not blocked by inhibitors of the main pathways for the production of eicosanoids. Similarly, we have shown that ERK is not involved in this response to BK.

We need to elucidate now two portions of the pathway used by BK to inhibit $I_{Ca,V}$. The first is proximal and concerns the mechanism of the coupling between G_{13} , Rac1/Cdc42, and p38-2 MAPK. Additional kinases might be involved, such as an src-homolog between G_{13} and Rac1/Cdc42 (Diverse-Pierluissi and Dunlap, 1996) and a PAK-like kinase further downstream (Zhang et al., 1995). The second portion of this signaling pathway to be clarified is distal and involves the mechanism of coupling between p38-2 and the calcium channels. It will be interesting to examine whether p38-2 phosphorylates directly the channel protein that also is the target of other kinases (Gray et al., 1997; Zamponi et al., 1997).

Our studies define a novel role for MAPK pathways in the regulation of ion channels and indicate that p38-2, a specific stress-activated MAPK, is involved in the regulation of $I_{Ca,V}$ by G-protein-coupled receptor. Whereas recent studies have shown that MAPKs can play relatively fast regulatory roles in response to G-protein activation, our studies extend these actions to the regulation of neuronal excitability and synaptic interactions. Thus, in nerve cells, MAPKs could mediate both the effects of neurotrophins (Figurov et al., 1996; Stoop and Poo 1996; Martin et al., 1997) and some of the relatively slow (by channel standard) actions of neurotransmitters. The mechanisms to be possibly mediated by a p38 MAPK are the central synaptic plastic events that accompany chronic pain and inflammation, an important form of stress in higher organisms (Dray and Perkins, 1993; Zieglergaensberger and Toelle, 1993).

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