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KCl activates mitogen-activated protein kinase in rabbit bailar artery

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Abstract

The objective of the present study was to investigate if MAPK can be activated by a non-receptor agonist KCl, which depolarizes membrane to increase intracellular Ca^{2+} and contracts cerebral arteries. Rabbit basilar arteries were used in isometric tension and western blot analysis studies. KCl produced a concentration-dependent contraction and an elevation of phospho-MAPK, which can be abolished by nicardipine, a voltage-dependent Ca^{2+} channel blocker, and by PD98059 or U0126, MAPK kinase inhibitors. Thus, MAPK can be activated by the elevation of intracellular Ca^{2+}, independent of the activation of either G-protein coupled receptors or receptor tyrosine kinase. KCl which not only depolarizes membrane potentials, opens voltage-dependent Ca^{2+}, and increases intracellular Ca^{2+}, but also, probably by elevation of intracellular Ca^{2+}, triggers the activation of MAPK which seems responsible for a predominant part of the contraction of KCl in the rabbit basilar arteries.

Mitogen-activated protein kinase (MAPK) is involved in the regulation of smooth muscle contraction. Two major pathways responsible for MAPK activation include signaling though G-protein coupled receptors and tyrosine kinase receptors. Phosphorylation of both threonine and tyrosine residues leads to activation of MAPK and subsequently to the phosphorylation of transcription factors c-myc, c-jun, and c-fos [4]. The mechanisms for MAPK-regulated smooth muscle contraction involve caldesmon. Caldesmon is a substrate of MAPK, and the phosphorylation of caldesmon reduces the affinity for actin-myosin and leads to dis-inhibition of actomyosin ATPase and contraction [5,18].

The role of MAPK pathway in cerebral arteries was studied using different receptor agonists as well as the agonists that were reported to produce prolonged contractions including endothelin-1, oxyhemoglobin, and hemolysate [17,23–25]. However, it is not clear if the activation of MAPK in cerebral arteries is solely by receptor agonists or MAPK can be activated by other means such as membrane depolarization and Ca^{2+} elevation. The objectives of the present study were to evaluate if MAPK can be activated by a non-receptor agonist KCl, which depolarizes membrane, opens voltage-dependent Ca^{2+}, and induces contraction in cerebral arteries.

Materials and methods

Isometric tension. All procedures were approved by the Animal Care and Use Committee at the University of Mississippi Medical Center.

New Zealand white rabbits ($n = 50$) of either sex, 4–6 pound weight, were anesthetized with an intravenous injection of thiopental (20 mg/kg) and euthanized by exsanguination. The basilar arteries were removed and cut into 3 mm rings in a dissecting chamber filled with modified Krebs–Henseleit bicarbonate solution, bubbled with 95% O_2 and 5% CO_2. No attempt was made to remove endothelial cells. The modified Krebs–Henseleit solution contained (mM): NaCl 120, KCl 4.5, $MgSO_4$ 1, $NaHCO_3$ 27, KH_2PO_4 1, $CaCl_2$ 2.5, and dextrose 10.

The rings were suspended at 500 mg resting tension (Radnoti transducer, Radnoti Glass, Monrovia, CA) between stainless steel hooks in 10 ml water-jacketed tissue baths (Radnoti Glass, Monrovia, CA) filled with modified Krebs–Henseleit buffer bubbled with 95% O_2/5% CO_2 at pH 7.4 and 37 °C. Rings were equilibrated for 90 min and bath solution was changed every 20 min. Tension was recorded continuously with a force–displacement transducer as described previously [22].

The tissues were divided into following groups: basilar arteries were pretreated with either nicardipine (1 μM), or PD98059 (30 μM), or

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U0126 (30 μM) for 30 min, and then contracted with KCl in a dose-dependent manner (10–120 mM). Each arterial ring was used only once with one antagonist to prevent any cross-reactions.

Western blot. The basilar arteries were removed under surgical microscope. After dose-dependent treatment with KCl (10–90 mM) for 5 min, arteries were immediately frozen in liquid nitrogen. The arteries were homogenized in (mM) 50 Tris–HCl, pH 7.5, 100 NaCl, 5 EDTA, 1 phenylmethylsulfonyl fluoride (PMSF), and 100 μl IGEPAL CA-630 for 20 min at 4 °C. The insoluble materials were removed by centrifugation at 13,000*g* for 10 min at 4 °C. The samples (30 μg protein) were applied to 12.5% sodium dodecyl sulfate–polyacrylamide-gel electrophoresis (SDS–PAGE). After electrophoretic transfer of the separated polypeptides to nitrocellulose membrane, the membranes were blocked using 5% non-fat milk in Tween-PBS (physiological buffer solution (PBS) containing 0.1% Tween 20) for 1 h. The membranes were washed with Tween-PBS and incubated at room temperature for 2 h in a 1:1000 dilution of mouse anti-phosphoMAPK antibodies ($p44^{ERK} + p42^{ERK}$ (ERK1/2)), which recognize the activated MAPK. Nitrocellulose membranes were later washed with Tween-PBS and incubated with 1:5000 dilution of sheep anti-mouse IgG antibody, linked with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, England) was used for visualization of protein bands. The results were quantified by Quantity One software (BioRad, Hercules, CA).

MAPK activity assay. For a normal MAPK activity in the rabbit basilar arteries, the arteries were treated with saline for 5 min. For KCl-induced MAPK activity, the arteries were treated with KCl (90 mM) for 5 min. For other studies, the arteries were preincubated with PD98059 (30 μM), or U0126 (30 μM), or nicardipine (1 μM), or in Ca^{2+}-free solution (Ca^{2+} free plus 2 mM EGTA) for 30 min and then exposed to KCl (90 mM) for 5 min. After the treatment, all the arteries were immediately frozen in liquid nitrogen. The MAPK activity was studied according to the method described in the MAPK assay manual (New England Biolabs, Beverly, MA). In brief, arteries were sonicated in lysis buffer provided in the kit, and the lysate was incubated with Immobilized Phospho—p44/p42 MAPK Monoclonal Antibodies overnight at 4 °C. After the incubation, the samples were centrifuged, thus removing all proteins except phosphoMAPK, and the pellets were incubated with Elk-1 fusion protein for 30 min at 30 °C. The samples (30 μl) were applied to 12.5% SDS–PAGE and then transferred to the nitrocellulose membrane. The membrane was incubated with phospho-Elk-1 antibodies overnight and then for 1 h with HRP-conjugated anti-rabbit secondary antibodies. The phospho-Elk-1 protein bands were visualized with LumiGLO. The density of the bands was quantified with Quantity One software (BioRad, Hercules, CA).

Data analysis. Data are expressed as the means ± the standard error of the mean. Statistical differences between the control and other groups were compared using a one-way analysis of variance (ANOVA) and then the Tukey–Kramer multiple comparison procedure, if significant variance was found. A probability value (P) of less than 0.05 was considered statistically significant.

Results

KCl-induced contractions in the basilar arteries

KCl (10–120 mM) produced concentration-dependent contractions in the presence of normal extracellular Ca^{2+} solution in the rabbit basilar arteries. KCl failed to produce any contraction in the Ca^{2+}-free solution (data not shown). Nicardipine (1 μM, 30 min preincubation), a voltage-dependent Ca^{2+} channels inhibitor, abolished the contraction to KCl ($P < 0.01$, ANOVA) (Fig. 1).

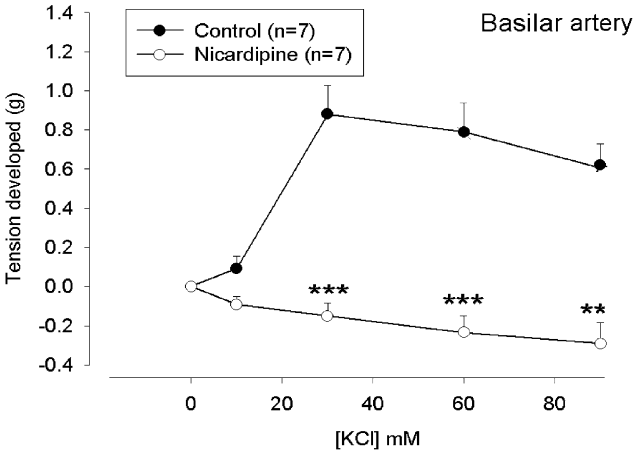

Fig. 1. Inhibitory effects of nicardipine on the KCl-induced contraction in the basilar arteries. The arterial rings were preincubated with 1 μM nicardipine for 30 min and then contracted with KCl in a dose-dependent manner. Nicardipine completely abolished KCl-induced contraction ($P < 0.01$, ANOVA). N = number of rings. *** indicates $P < 0.01$.

Effects of PD98059 on the contractions of the basilar arteries

MAPK kinase inhibitor PD98059 (30 μM, 30 min preincubation) completely abolished (90–105% inhibition) KCl-induced contraction of the rabbit basilar arteries (Fig. 2).

Effects of U0126 on the contractions of the basilar arteries

U0126 (30 μM, 30 min) partially but significantly inhibited KCl-induced contraction in the basilar arteries (51–62% inhibition) (Fig. 3).

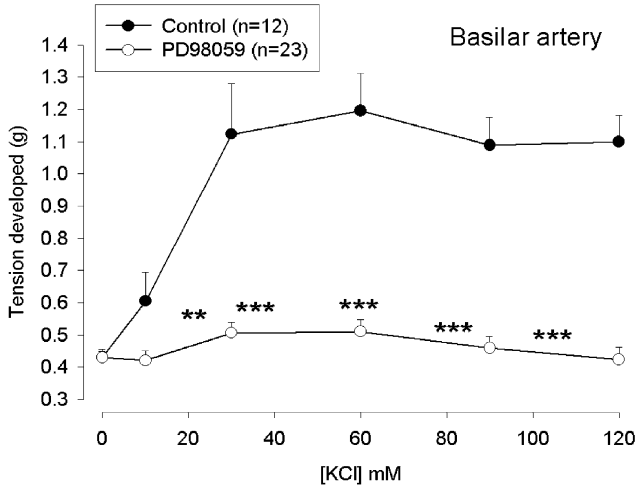

Fig. 2. Effects of PD98059 on KCl-induced contraction in the basilar arteries. After preincubation of the rings with 30 μM PD98059 for 30 min, contractions were induced with KCl (10–120 mM). PD98059 completely abolished KCl-induced contractions in the basilar arteries ($P < 0.02$–0.01, ANOVA). **, *** indicate $P < 0.05$ and 0.01, respectively.

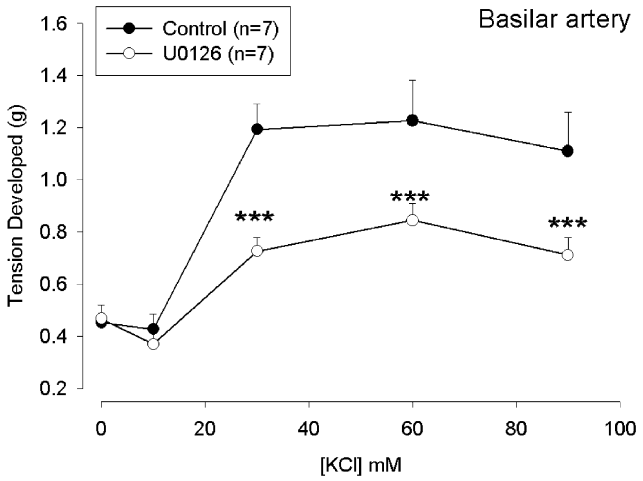

Fig. 3. U0126 significantly inhibited KCl-induced contraction in the basilar arteries. The arterial preparations were preincubated with 30 μM U0126 and then contacted with 10–120 mM KCl. U0126 significantly inhibited contractions to KCl in the basilar arteries ($P < 0.01$, ANOVA). *** indicates $P < 0.01$.

KCl causes phosphorylation of MAPK in the basilar arteries

KCl (30–90 mM) induced a marked MAPK phosphorylation demonstrated by immunostaining with antibody detecting dually phosphorylated form of ERK1/2 in the basilar arteries ($P < 0.05$–0.02, ANOVA). A maximal MAPK phosphorylation was caused by 90 mM of KCl and exceeded control levels about four times (Fig. 4).

U0126, PD98059, nicardipine, or Ca^{2+}-free solution inhibited MAPK activity

KCl (90 mM) caused a significant increase of MAPK activity in the basilar arteries (Fig. 5). Preincubation of the basilar arteries with PD98059 (30 μM), or U0126 (30 μM), or nicardipine (1 μM), or in Ca^{2+}-free solution for 30 min, followed by treatment of the samples with KCl (90 mM) did not cause any statistically significant changes in MAPK activity, as compared to control levels.

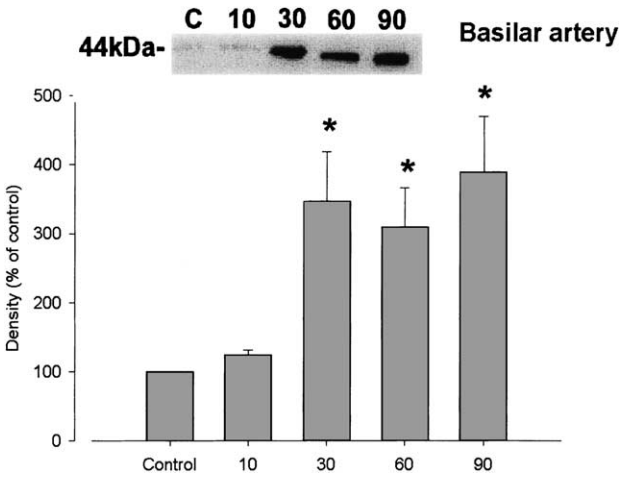

Fig. 4. Phosphorylation of MAPK by KCl (10–90 mM). Lower dose of KCl (10 mM) did not induce significant elevation of phospho-MAPK levels. Higher doses of KCl (30–90 mM) induced significant elevation of phospho-MAPK levels in the rabbit basilar arteries. * indicates $P < 0.05$.

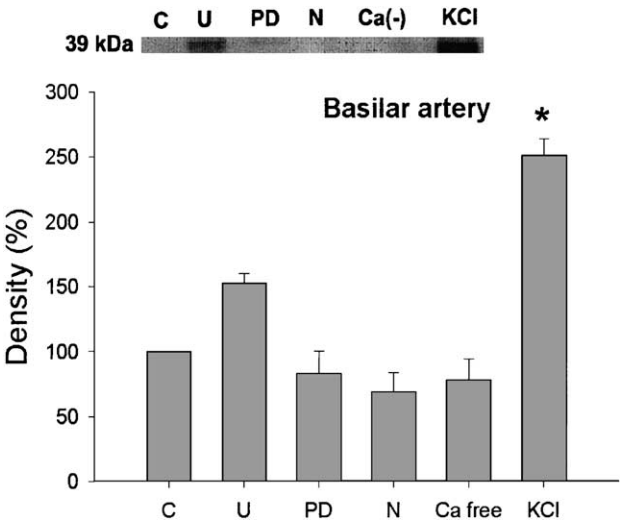

Fig. 5. Effects of inhibitors on MAPK activity. The basilar arteries were preincubated with PD98059 (30 μM), U0126 (30 μM), nicardipine (1 μM), or in Ca^{2+}-free solution for 30 min, and then exposed to KCl (90 mM) for 5 min. Treatment of the basilar arteries with KCl (90 mM) without other inhibitors caused statistically significant increase in MAPK activity ($P < 0.05$, ANOVA). The MAPK activity levels in the inhibitor treated groups are not statistically different from that of controls. * indicates $P < 0.05$.

Discussion

The following results were obtained in the present study: (1) MAPK kinase inhibitors PD98059 and U0126 significantly inhibited KCl-induced contractions in the rabbit basilar arteries. (2) KCl produced *MAPK phosphorylation* in a concentration-dependent manner. (3) KCl enhanced significantly the *MAPK activity*. (4) MAPK kinase inhibitors, nicardipine, as well as Ca^{2+}-free conditions prevented the activation of MAPK. Thus, MAPK can be activated and contributed to the contraction in cerebral arteries by non-receptor agonist KCl. The possible mechanism for MAPK activation is the elevation of intracellular Ca^{2+} induced by KCl which depolarizes the membrane and opens voltage-dependent Ca^{2+} channels.

Activation of MAPK by receptor agonists

In most tissues, two major pathways are responsible for MAPK activation including signaling though G-protein coupled receptors and tyrosine kinase receptors. The adapter protein Grb2 links the tyrosine-phospho-

rylated receptor to SOS, which acts as a guanine nucleotide exchange factor for $p21^{ras}$, and the active GTP-bound $p21^{ras}$ stimulates Raf-1 kinase activity toward mitogen-activated protein kinase kinase (MEK). The MEK activation leads to MAPK phosphorylation on both threonine and tyrosine residues and subsequently to phosphorylation of transcription factors c-myc, c-jun, and c-fos. Tyrosine kinase receptors-associated substances such as EGF, PDGF, and growth hormone [3,10] are associated with the MAPK signaling. Another pathway for the activation of MAPK is by the activation of G-protein coupled receptors. For example, angiotensin II-induced activation of MAPK in vascular smooth muscle cells is mainly mediated by a Ca^{2+}/calmodulin-dependent tyrosine kinase through PI–PLC mediated Ca^{2+} release coupled with G_q [8]. Norepinephrine [20], endothelin 1 [20], 5-hydroxytryptamine [19], and angiotensin II [15,21] are all associated with G-protein coupled receptors and their signaling pathways include MAPK. However, receptor activation (either receptor tyrosine kinases or G protein coupled receptors) seems not the only way to activate MAPK. Membrane depolarization especially elevation of intracellular Ca^{2+} might enhance the activity of MAPK in smooth muscle cells.

Activation of MAPK by elevation of intracellular Ca^{2+}

Several previous studies indicated that either MAPK seems involved in contractions induced by non-receptor agonist or MAPK inhibitors seem not selective since PD98059 reduced the contraction by KCl *in peripheral arteries*. In swine carotid arteries, histamine and KCl increased MAPK precipitation (*neither phosphorylated MAPK nor MAPK activity*) of both 42 and 44 kDa isoforms (ERK1/2) in a time-dependent manner. Thus, membrane depolarization was suspected to play a role in the enhancement of MAPK precipitation [12]. Similar observation was reported in porcine carotid arteries that KCl induced a transient elevation of MAPK precipitation (again, not MAPK activity) accompanied by a sustained caldesmon phosphorylation and sustained contraction [1].

Controversial observation was reported that PD98059 (30 μM) did not reduce KCl-induced contraction of ferret aorta smooth muscle cells [6]. In the same study, Dessy et al. [6] concluded that that MAPK activation is not Ca^{2+}-dependent since, in the absence of extracellular Ca^{2+}, α-adrenoreceptor agonist phenylephrine increased the level of MAPK and phosphorylatedcaldesmon, and PD98059 (30 μM) abolished the caldesmon phosphorylation.

Few studies examined the role of MAPK in cerebral arteries and the effect of non-receptor agonist such as *KCl on either the phosphorylated MAPK or the MAPK activity has not been reported*. Due to lack of tools in examining the phosphorylated MAPK or the MAPK activity previously, the relaxant effect of PD98059 on KCl-induced contraction in rat middle cerebral arteries was suspected as a non-selective action [13]. In the present study, we have demonstrated that KCl increased the level of phosphorylated MAPK and MAPK activity in the rabbit basilar artery, and MAPK kinase inhibitors PD98059 and U0126 abolished the effect of KCl on the MAPK activity and reduced KCl-induced contraction. The mechanism of KCl-induced MAPK activation might not be related to membrane depolarization [12] but rather depends upon an elevation of intracellular Ca^{2+}. Our results indicate that KCl did not produce contraction or MAPK activation in the absence of extracellular Ca^{2+}, and KCl-induced contraction and MAPK activation were abolished by nicardipine or by Ca^{2+}-free solution. Our study suggests that the inhibitory effect of MAPK kinase inhibitors such as PD98059 on KCl-induced contraction is not a non-selective action as suspected previously [13]. On the contrary, KCl activates MAPK by elevation of intracellular Ca^{2+}, and MAPK contributes to a large degree of KCl-induced contraction in the rabbit basilar artery. These observations in rabbit cerebral arteries were confirmed in peripheral arteries as well. KCl activated MAPK and MAPK inhibitors reduced the effect of KCl in the rabbit femoral artery (not shown).

The effect of PD98059 seems more potent than that of U0126 in isometric tension study even though both inhibitors showed similar inhibitory action on the MAPK activity. We do not have a good explanation for the difference in the extent of inhibition of contraction by PD98059 and U1026. Both of them cause their effects by inhibition of MAPK precursor MEK [7,9]. Comparative kinetic analysis of U0126 and PD98059 demonstrates that U0126 and PD098059 are noncompetitive inhibitors with respect to both MEK substrates and ERK. It was further suggested that they share a common or overlapping binding site. Quantitative evaluation of the steady-state kinetics of MEK inhibition by these compounds reveals that U0126 has approximately 100-fold more potent than PD98059 [9]. In rabbit basilar arteries, different potencies of PD98059 and U0126 on endothelin-1, or hemolysate induced contractions were reported in our previous publications [23–25].

Two issues remain to be resolved. First, how intracellular Ca^{2+} activates MAPK. One of the possibilities is that protein kinase C (PKC) might be involved in the signaling pathways for intracellular Ca^{2+} to activate MAPK. Membrane depolarization and elevation of intracellular Ca^{2+} induced by KCl (109 mM) activates PKC [14]. Since PKC and MAPK are closely interconnected, it can be speculated that activation of PKC probably leads to MAPK activation [12]. Apparently, other signaling pathways from intracellular Ca^{2+} to the activation of MAPK need to be explored.

Another question is the mechanism that is responsible for the effect of MAPK kinase inhibitors on KCl-induced contraction. PD98509 was demonstrated to affect intracellular Ca^{2+} directly in cerebral arteries by yet to be established mechanism [2]. In peripheral arteries, PD98059 attenuated the Ca^{2+} elevation induced by a G protein-coupled receptor agonist angiotensin II in rat mesenteric vascular smooth muscle cells [15,16]. These studies support a possible direct action of MAPK kinase inhibitors on intracellular Ca^{2+} or its regulatory mechanisms. Even though PD98059 but not U0126 directly blocked voltage-dependent Ca^{2+} channels in oocytes [11], it is unlikely, in cerebral arteries, that the effect of PD98059 is non-specific or blocks voltage-dependent Ca^{2+} channels as suspected previously [13]. The MAPK activity was not studied in these reports. Our data showed strong evidences that KCl increased MAPK phosphorylation and activity and MAPK kinase inhibitors PD98059 and U0126 abolished both MAPK activity and contraction induced by KCl.

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