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Dibucaine and Tetracaine Inhibit the Activation of Mitogen-activated Protein Kinase Mediated by L-type Calcium Channels in PC12 Cells

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Background: **An elevation of the intracellular calcium level, which is mediated by *N*-methyl-D-aspartate receptors and L-type Ca^{2+} channels both, activates the mitogen-activated protein (MAP) kinase signaling pathway involved in synaptic modification. It has recently been suggested that MAP kinase plays a role in coupling the synaptic excitation to gene expression in the nucleus of postsynaptic neurons. Because the effects of local anesthetics on cellular signal transduction in neuronal cells are not well-known, the authors investigated whether they affect the MAP kinase signaling pathway using PC12 cells.**

Methods: **The cells were stimulated with either 50 mM KCl or 1 μM ionomycin, and activated MAP kinase was thus immunoprecipitated. The immunocomplexes were then subjected to an Elk1 phosphorylation assay. Both the phosphorylation of MAP kinase and the induction of c-Fos were detected by immunoblotting.**

Results: **Pretreatment of the cells with 1 mM (ethylenedioxy)-diethyl-enedinitrilotetraacetic acid or 5 μ nifedipine blocked the MAP kinase activation induced by 50 mM KCl, whereas pretreatment with 2 μM ω-conotoxin GIVA did not. The expression of c-Fos induced by potassium chloride was also suppressed by dibucaine, tetracaine (concentrations that inhibited 50% of the activity of positive control [IC_{50}s] were 16.2 ± 0.2 and 73.2 ± 0.7 μM, respectively), and PD 98059, a mitogen-activated/extracellular receptor–regulated kinase inhibitor. Higher concentrations of dibucaine and tetracaine were needed to suppress the activation of MAP kinase induced by ionomycin (the IC_{50} values of dibucaine and tetracaine were 62.5 ± 2.2 and 330.5 ± 32.8 μM, respectively) compared with potassium chloride (the IC_{50} values of dibucaine and tetracaine were 17.7 ± 1.0 and 70.2 ± 1.2 μM, respectively). Although probable targets of these local anesthetics might be L-type Ca^{2+} channels or components between Ca^{2+} and Ras in MAP kinase pathway, the possibility that they directly affect MAP kinase still remains.**

Conclusions: **Dibucaine and tetracaine at clinical concentrations were found to inhibit the activation of MAP kinase and the expression of c-Fos mediated by L-type Ca^{2+} channels in PC12 cells. The suppression of MAP kinase pathway may thus be a potential target site for the actions of dibucaine and tetracaine, including the modification of the synaptic functions. (Key words: Local anesthetics; neuronal cells; signal transduction.)**

SIGNALS initiated by neurotransmitter release are transferred to the nuclei of the postsynaptic cells to activate specific programs of gene expression, thus influencing synaptic functions.[1,2]

It has recently been reported that mitogen-activated protein (MAP) kinase (also known as extracellular signal reactive kinase [Erk]) might play an important role in linking membrane depolarization to gene expression in the postsynaptic neurons.[3,4] MAP kinase is highly expressed in the central nervous system, primarily in such postsynaptic structures as neuronal cell bodies and the bases of the proximal dendrites.[5] MAP kinase has been shown to be activated by calcium influx through both *N*-methyl-D-aspartate (NMDA) receptors and L-type Ca^{2+} channels.[6,7] Membrane depolarization and the subsequent Ca^{2+} influx stimulate MAP kinase;[4,5,8,9] The Ca^{2+} influx stimulates Ras activation; Ras transmits its signal to mitogen-activated/extracellular receptor–regulated kinase (MEK) through Raf; MEK phosphorylates and activates MAP kinase. The activated MAP kinase is translocated to the nucleus and induces the expression of genes by phosphorylating and activating such transcriptional factors as c-Myc or Elk1 (fig. 1).[3,10,11]

The purpose of this study is to investigate whether local anesthetics affect the MAP kinase pathway activated by depolarization in neuronal cells. The rat pheochromocytoma cell line PC12 is considered to be a useful model for studying the mechanisms for a variety of calcium-dependent signaling events in the nervous system.[8,12–15] Here, we show the effects of dibucaine

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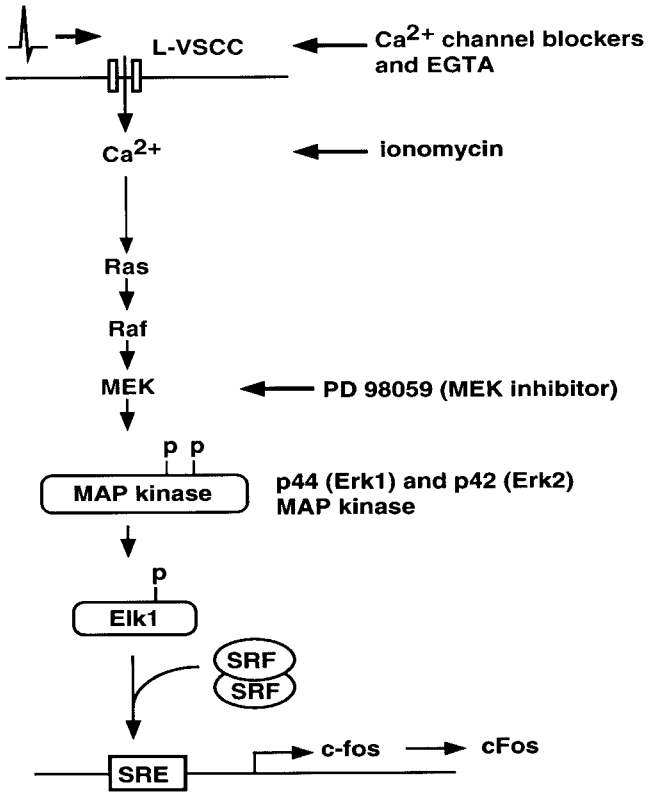

Fig. 1. The Ca^{2+} influx through L-type voltage sensitive Ca^{2+} channels activates the mitogen-activated protein (MAP) kinase signaling pathway. Synaptic transmission induces depolarization in the postsynaptic membrane and activates the L-type voltage sensitive Ca^{2+} channels (L-VSCC). The elevation of intracellular Ca^{2+} activates Ras by some as yet unknown mechanism. Ras transmits its signal to mitogen-activated/extracellular receptor–regulated kinase (MEK) through Raf. MEK phosphorylates and activates p44/p42 MAP kinase. The activated MAP kinase phosphorylates transcriptional factors, such as Elk1. The complex of phosphorylated Elk1 and the homodimer of serum response element (SRE) serum response factors binds to SRE and induces the transcription of c-Fos. Ionomycin elevates intracellular Ca^{2+} independent of L-type voltage sensitive Ca^{2+} channels and also activates MAP kinase pathway dependent on Ras activation.

and tetracaine on the activation of MAP kinase in PC12 cells.

Materials and Methods

The MEK inhibitor (PD 98059), phosphospecific MAP kinase antibody, phospho-p44/p42 MAP kinase monoclonal antibody, phosphospecific Elk1 antibody, anti-Elk1 antibody, active Erk2, and a p44/p42 MAP kinase assay kit were purchased from New England BioLabs (Beverly, MA). Protein A-agarose and anti-Erk1, anti-Erk2, and anti--c-Fos antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). w-Conotoxin GIVA was from the Peptide Institute (Osaka, Japan). Dibucaine, tetracaine, nifedipine, ionomycin, and adenosine triphosphate were purchased from Sigma (St. Louis, MO).

Cell Culture

PC12 cells were maintained in RPMI 1640 medium (Roswell Park Memorial Institute, developed by Dr. G. Moore) supplemented with 10% horse serum, 5% fetal bovine serum, 40 U/ml penicillin G, and 100 μg/ml streptomycin.

Detection of MAP Kinase Activity

PC12 cells were plated on 60-mm collagen-coated dishes (6×10^6 cells/dish) and incubated for 6–12 h in a low-serum medium (0.25% fetal bovine serum and 0.5% horse serum). After pretreatment with dibucaine or tetracaine for 10 min, the cells were stimulated with either 50 mM KCl or 1 μM ionomycin for 2 min, washed once with cold phosphate-buffered saline and lysed in 200 μl cell lysis buffer containing 20 mM tris(hydroxymethyl)-aminomethane (TRIS; pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM (ethylenedioxy)diethyl-enedinitrilotetraacetic acid (EGTA), 1% Triton X-100 (Sigma), 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 50 μM 4-amidinophenyl-methanesulphenyl fluoride (APMSF), and 1 μg/ml leupeptin. The cells were then scraped off and transferred to microcentrifuge tubes and sonicated with 3- $\times$ 5-s bursts in a Branson sonifier at low power in an ice bath. After centrifugation at 6,000*g* for 15 min at 4°C, the supernatant was transferred to a new tube and incubated with phospho-p44/p42 MAP kinase monoclonal antibody (1:200 dilution), which only detects MAP kinase phosphorylated at both threonine 202 and tyrosine 204, overnight at 4°C with gentle rocking. These phosphorylation sites are critical for the MAP kinase activity. Protein A-agarose was added for an additional 3 h. The immunoprecipitate was obtained by centrifugation at 6,000*g* for 1 min at 4°C and was washed twice with 0.5 ml cell lysis buffer and twice with 0.5 ml kinase buffer containing 25 mM TRIS (pH 7.5), 5 mM β-glycerophosphate, 2 mM 1,4-dithiothreitol, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$. The immunocomplexes were incubated with 1.2 μg Elk1 fusion protein, which is glutathione S-transferase fused to Elk1 codons 307–428, and 200 μM adenosine triphosphate in kinase buffer for 30 min at 30°C. The reaction was terminated by adding Laemmli buffer

(3 × concentration). After boiling for 5 min, the mixture was centrifuged at 6,000*g* for 2 min at 4°C. The supernatant was then subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted with phosphospecific Elk1 antibody, which detects only Elk1 phosphorylated at serine 383.

Detection of MAP Kinase Pphosphorylation

After stimulation with 50 mM KCl for 2 min, the cells were washed once with cold phosphate-buffered saline and lysed in radioimmunoprecipitation buffer containing 50 mM TRIS (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (Sigma), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 2 mM sodium vanadate, 50 mM NaF, 30 mM p-nitrophenyl phosphate, 40 μM 4-APMSF, 40 μM leupeptin, 1 μM pepstatin, and 30 μg/ml aprotinin. The cells then were incubated on ice for 20 min. The cell lysates were centrifuged at 6,000*g* for 20 min at 4°C and the supernatant was resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotted with phosphospecific MAP kinase polyclonal antibody that detects MAP kinase phosphorylated at tyrosine 204.

Detection of cFos

For c-Fos detection, PC12 cells (1×10^7 cells/dish) were plated on collagen-coated dishes, stimulated with 50 mM KCl at 37°C for 60 min, washed with cold phosphate-buffered saline three times, and lysed in lysis buffer containing 10 mM TRIS (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 20 μM 4-amidinophenylmethanesulphenyl fluoride, 30 μM leupeptin, and 30 μg/ml aprotinin. After centrifugation at 6,000*g* for 5 min at 4°C, the pellet was resuspended in 2 × Laemmli buffer, boiled for 5 min, and sonicated briefly. The presence of c-Fos was determined by Western blot analysis.

Western Blot Analysis

The samples were subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and were electrotransferred to the nitrocellulose membrane. The membranes were incubated with primary antibodies either for 45 min at room temperature or overnight at 4°C. The blots were probed with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG) antibody for 1 h. Antibody binding was detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotechnology, Uppsala, Sweden).

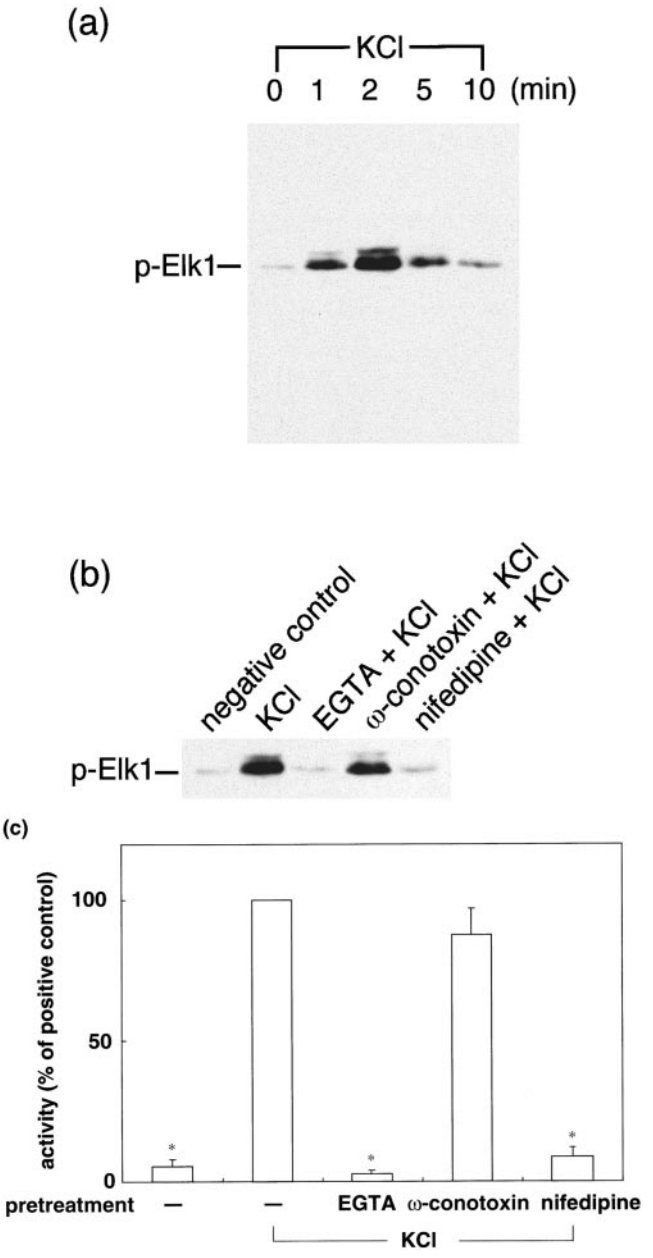

Fig. 2. (*A*) The time course of the KCl-induced activation of MAP kinase. (*B*) The effects of EGTA and calcium channel blockers on the KCl-induced activation of mitogen-activated protein (MAP) kinase. PC12 cells were stimulated with 50 μM KCl for the indicated periods of time or with KCl for 2 min at 37°C after pretreatment with 1 mM EGTA, 2 mM ω-conotoxin GIVA, or 5 mM nifedipine for 5 min. The MAP kinase activity was detected as described in Materials and Methods. Protein blots were probed with phosphospecific Elk1 antibody. Typical results of three independent experiments are shown. (*C*) A densitmetric analysis of the MAP kinase activity. Activation is expressed as a percentage of the activation caused by 50 mM KCl without pretreatment (positive control). Values are mean ± SEM of three independent experiments. *Different form positive control at $P < 0.05$.

Statistical Analysis

Western blots were scanned into the program Adobe Photoshop 4.0 (San Jose, CA) using an OPAL Ultra scanner (Heidelberg Jpapan, Tokyo, Japan) and then analyzed with NIH Image version 1.6 software (National Institutes of Health, Bethesda, MD). The results are expressed as a percentage of the density of the band obtained after stimulation with potassium chloride (KCl) or ionomycin without pretreatment (positive control) and presented as the mean ± SEM. Differences were analyzed using an unpaired *t* test. $P < 0.05$ was considered to be significant. The data points in concentration-dependent inhibition of dibucaine and tetracaine on the MAP kinase activity were fitted according to a four-paramater logistic model described by De Lean *et al.,*[16] and the concentrations that inhibit 50% of the activity of positive control (IC_{50}s) were derived from these fits.

Results

MAP Kinase Activity Stimulated with Potassium Chloride Depolarization

When the PC12 cells were depolarized by KCl, MAP kinase was activated transiently as shown in figure 2A. The activity reached a maximum at 2 min and then decreased within 5 min. The MAP kinase activation depended on the Ca^{2+} influx through voltage-sensitive calcium channels. The pretreatment of the cells with either 1 mM EGTA or 5 μM nifedipine blocked the MAP kinase activation, whereas the pretreatment of the cells with 2 μM ω-conotoxin GIVA did not (figs. 2B and C). These findings, therefore, indicate that the activation of MAP kinase is mediated by L-type Ca^{2+} channels but not by N-type Ca^{2+} channels in PC12 cells.

Effects of Dibucaine and Tetracaine on MAP Kinase Activation Induced by Potassium Chloride

Dibucaine and tetracaine suppressed the activation of MAP kinase stimulated with KCl in a dose-dependent manner (figs. 3A and B). The pretreatment of cells with dibucaine or tetracaine inhibited the activation of the MAP kinase. The IC_{50} values for dibucaine and tetracaine were 17.7 ± 1.0 μM and 70.2 ± 1.2 μM, respectively (fig. 3C). The local anesthetics had no effect on the time dependence of MAP kinase activation (data not shown). The changes in the pH of the medium caused by adding dibucaine or tetracaine were not significant (approximately 0.05).

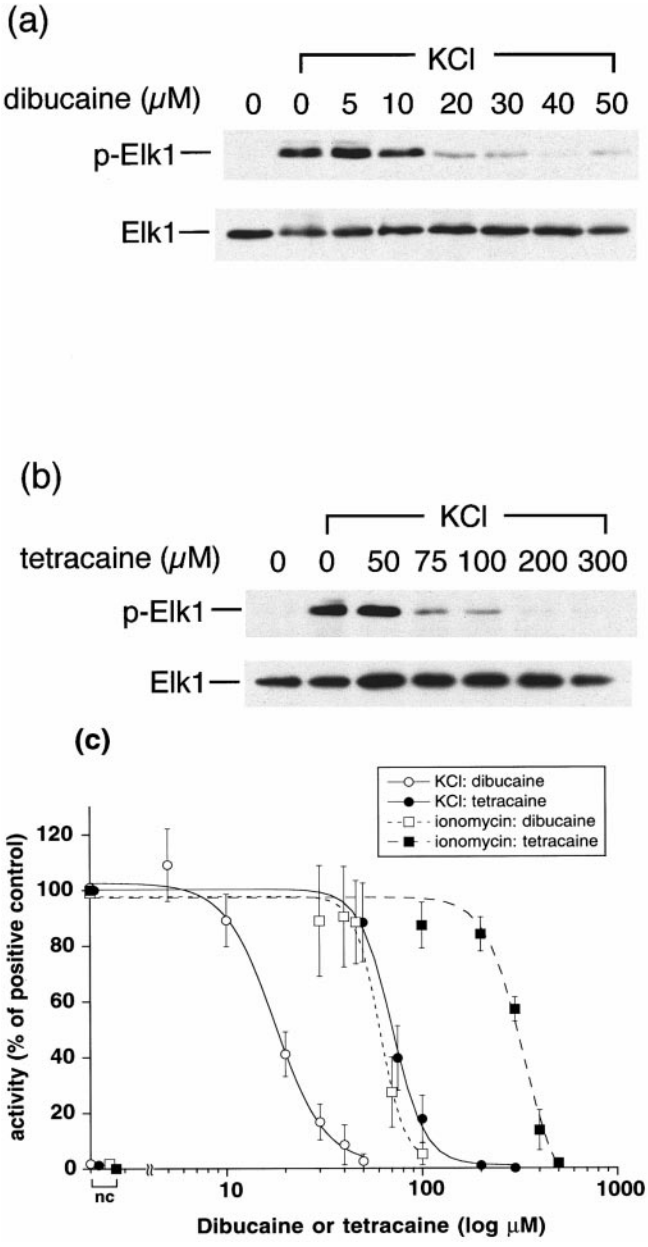

Fig. 3. Inhibition of KCl-induced mitogen-activated protein (MAP) kinase activation by dibucaine and tetracaine. The cells were pretreated with dibucaine or tetracaine for 10 min and then stimulated with 50 mM KCl for 2 min at 37°C. The MAP kinase activity was detected as described in Materials and Methods. (*A, B*) Protein blots were probed with phosphospecific Elk1 antibody (*upper*) and anti-Elk1 antibody (*lower*). Typical results of six independent experiments are shown. (*C*) A densitmetric analysis of the MAP kinase activity. Activation is expressed as a percentage of the activation caused by 50 mM KCl or 1 μM ionomycin without pretreatment (positive control). Values are mean ± SEM of six independent experiments. The lower values at negative control (nc) represent the percentage of activity of nonstimulated cells.

Effects of Dibucaine and Tetracaine on MAP Kinase Phosphorylation Induced by Potassium Chloride

To confirm that dibucaine and tetracaine affect the MAP kinase signaling pathway, we evaluated the effects of local anesthetics on MAP kinase phosphorylation induced by KCl. As shown in figure 4, pretreatment with dibucaine or tetracaine inhibited the phosphorylation of both Erk1 (44 kd) and Erk2 (42 kd). The IC_{50} values for dibucaine to inhibit the phosphorylation of Erk1 and Erk2 were 7.6 ± 0.8 μM and 13.1 ± 2.1 μM, respectively, and the IC_{50} values for tetracaine to inhibit the phosphorylation of Erk1 and Erk2 were 82.6 ± 13.1 μM and 101.3 ± 3.2 μM, respectively (fig. 4C). These values were comparable to those obtained from the experiment concerning MAP kinase activation.

Effects of Dibucaine and Tetracaine on MAP Kinase Activation Induced by Ionomycin

To evaluate the probable sites of action of dibucaine and tetracaine on MAP kinase pathway, we evaluated the effects of these local anesthetics on the activation of MAP kinase after the elevation of intracellular Ca^{2+} using a Ca^{2+} ionophore. If intracellular Ca^{2+} was increased with 1 μM ionomycin, a transient activation of MAP kinase was also observed (fig. 5A). Pretreatment of cells with dibucaine or tetracaine suppressed the activation of MAP kinase induced by ionomycin (figs. 5B and C). However, the IC_{50} values for dibucaine and tetracaine (62.5 ± 2.2 m and 330.5 ± 32.8 μM, respectively) were approximately four times higher than those obtained in inhibition of KCl-induced MAP kinase activation (fig. 3C)

Effects of Dibucaine and Tetracaine on c-Fos Induction

KCl-induced c-Fos expression was inhibited by 1 mM EGTA or 5 mM nifedipine (fig. 6). Pretreatment of the cells with 50 mM MEK inhibitor (PD 98059) also suppressed the c-Fos induction, suggesting that MAP kinase may be involved in the signaling pathway that links Ca^{2+} influx to gene expression. As shown in figure 7, dibucaine and tetracaine also inhibited the depolarization-induced c-Fos expression. The IC_{50} values for dibucaine and tetracaine (concentrations that inhibit 50% of the c-Fos expression induced by KCl without pretreatment) were 16.2 ± 0.2 mM and 73.2 ± 0.7 mM, respectively (fig. 7C). According to the trypan blue exclusion test, more than 95% of the cells were viable after incubation with 20 mM dibucaine, 200 mM tetracaine, or 50 mM MEK inhibitor for 60 min.

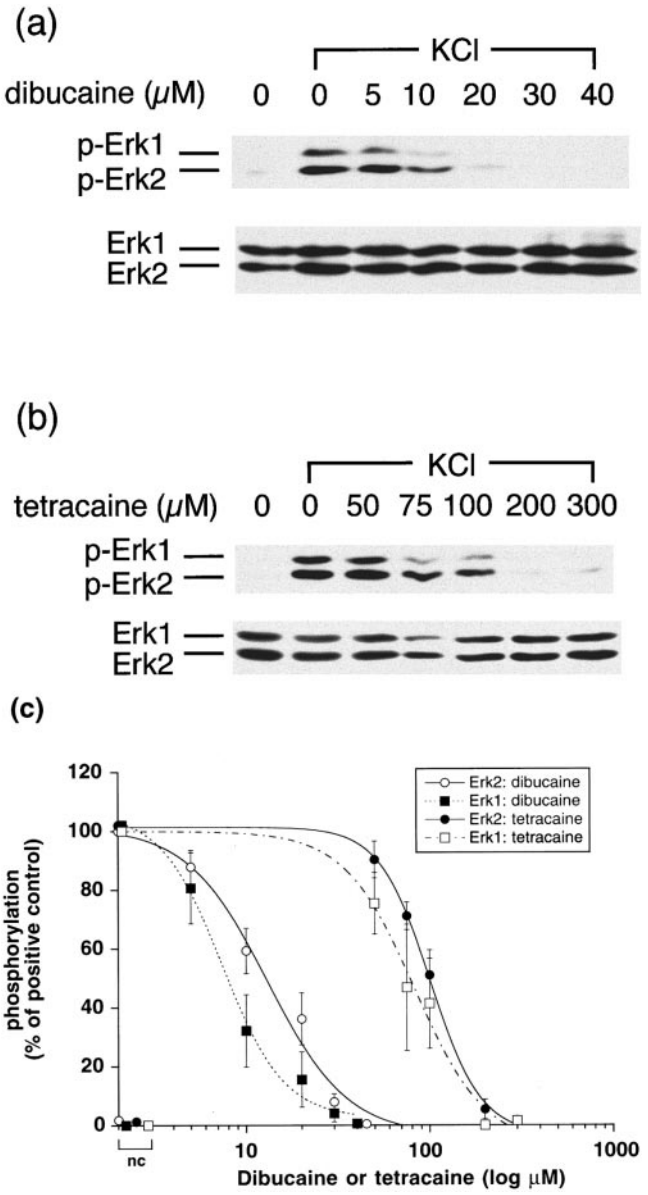

Fig. 4. Dibucaine and tetracaine inhibited the phosphorylation of mitogen-activated protein (MAP) kinase induced by KCl. The cells were stimulated with 50 mM KCl for 2 min at 37°C after pretreatment with dibucaine or tetracaine for 10 min. The phosphorylation of MAP kinase was detected as described in Materials and Methods. (*A, B*) The blots were probed with phosphospecific MAP kinase antibody (*upper*) and a mixture of anti-Erk1 and anti-Erk2 antibodies (*lower*). Typical results of six independent experiments are shown. (*C*) A densitmetric analysis of the phosphorylation of MAP kinase. Phosphorylation of Erk1 and Erk2 is expressed as a percentage of that caused by 50 mM KCl without pretreatment (positive control). Values are mean ± SEM of six independent experiments. The lower values at negative control (nc) represent the percentage of activity of nonstimulated cells.

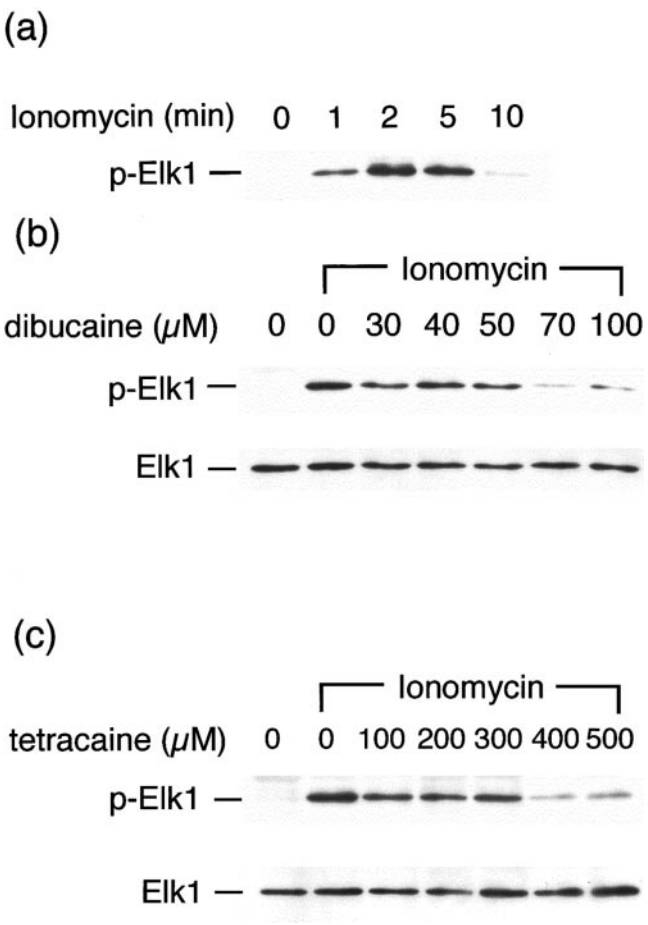

Fig. 5. Time course of the activation of mitogen-activated protein (MAP) kinase induced by ionomycin and inhibition of ionomycin-induced MAP kinase activation by dibucaine or tetracaine. (*A*) The cells were stimulated with 1 μM ionomycin for indicated periods of time at 37°C. The MAP kinase activity was detected as described in Materials and Methods. Typical results of three independent experiments are shown. (*B, C*) The cells were stimulated with 1 μM ionomycin for 2 min at 37°C after pretreatment with dibucaine or tetracaine for 10 min. The MAP kinase activity was detected as described in Materials and Methods. Protein blots were probed with phosphospecific Elk1 antibody (*upper*) and anti-Elk1 antibody (*lower*). Typical results of six independent experiments are shown.

Discussion

In this study, we showed that dibucaine and tetracaine inhibited the activation of MAP kinase and the expression of c-Fos induced by KCl in PC12 cells. Both the activation of MAP kinase and the expression of c-Fos induced by KCl depended on L-type Ca^{2+} channels. Because MEK inhibitor blocked KCl-induced c-Fos expression, it appears that the calcium signal mediated by L-type Ca^{2+} channels is transmitted, at least in part, to the nucleus through the MAP kinase pathway.

Converse *et al.*[17] reported that during spinal anesthesia, the mean concentration of tetracaine in human cerebrospinal fluid is 0.8–12 mg/dl; namely, 26.7–400 μM.[17] Therefore, the concentrations of the local anesthetics that inhibited MAP kinase activation induced by KCl seemed to be within the normal clinical ranges.

Although dibucaine and tetracaine inhibited the activation of MAP kinase induced by KCl and ionomycin, IC_{50} values for dibucaine and tetracaine obtained from the experiment using ionomycin were higher than those obtained from the experiment using KCl. Because it has been reported that Ras is necessary to induce the acti-

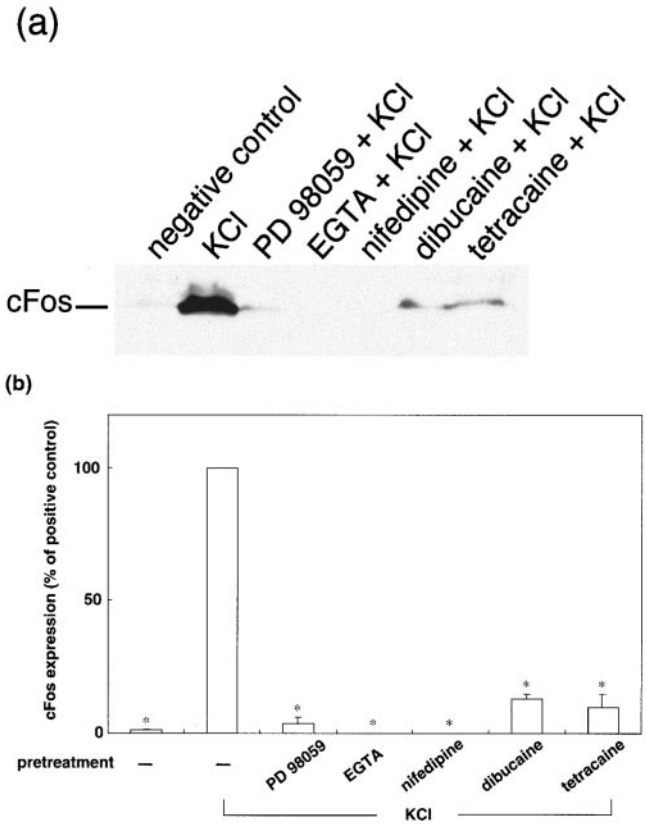

Fig. 6. The effects of dibucaine, tetracaine, EGTA, nifedipine, and mitogen-activated/extracellular receptor–regulated kinase (MEK) inhibitor on the KCl-induced expression of c-Fos. The cells were incubated with 50 mM KCl for 60 min at 37°C after pretreatment with 20 μM dibucaine, 200 μM tetracaine, 1 mM EGTA, or 5 μM nifedipine for 10 min, and with 50 μM MEK inhibitor (PD 98059) for 30 min. The expression of c-Fos was detected as described in Materials and Methods. (*A*) Protein blots were probed with anti-c-Fos antibody. The typical results of three independent experiments are shown. (*B*) A densitmetric analysis of the expression of c-Fos. c-Fos expression is expressed as a percentage of that caused by 50 mM KCl without pretreatment (positive control). Values are mean ± SEM of three independent experiments. *Different from positive control at $P < 0.05$.

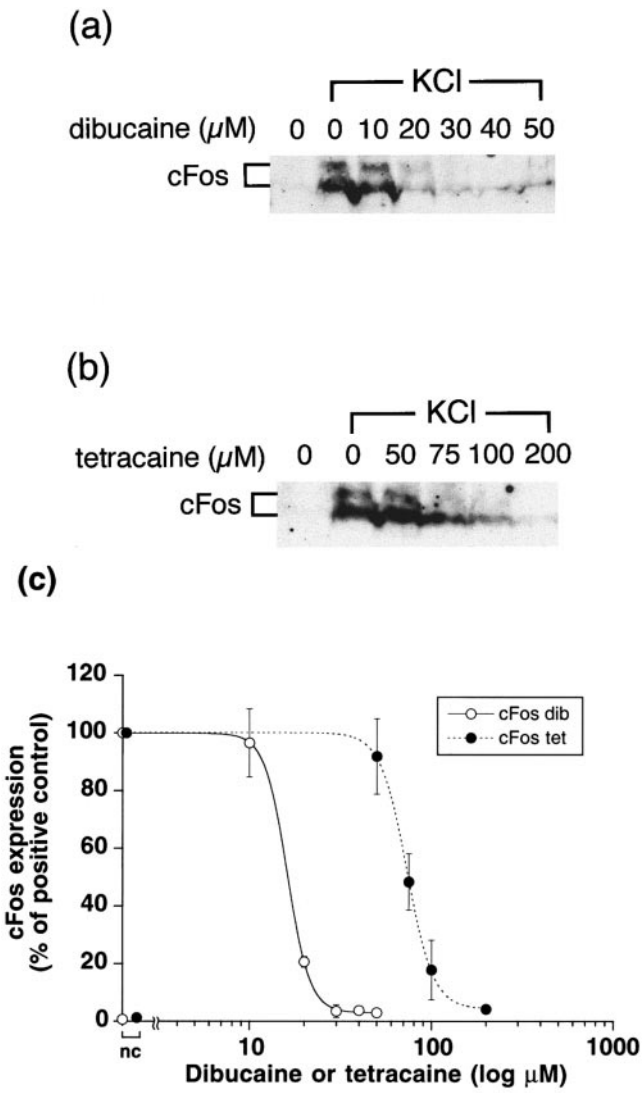

Fig. 7. Dose-dependent effects of dibucaine of tetracaine on the expression of c-Fos induced with KCl. The cells were stimulated with 50 mM KCl for 60 min at 37°C after pretreatment with dibucaine or tetracaine for 10 min. c-Fos was detected as described in Materials and Methods. (*A, B*) Protein blots were probed with anti–c-Fos antibody. Typical results of three independent experiments are shown. (*C*) A densitmetric analysis of the phosphorylation of mitogen-activated protein kinase. Phosphorylation is expressed as a percentage of that caused by 50 mM KCl without pretreatment (positive control). Values are mean ± SEM of three independent experiments. The lower values at negative control (nc) represent the percentage of activity of nonstimulated cells.

vation of MAP kinase by ionomycin,[9] there is a possibility that dibucaine and tetracaine at lower concentrations primarily affect upstream of Ras in MAP kinase pathway: L-type Ca^{2+} channels or yet unknown mechanisms between Ca^{2+} and Ras.

Sugiyama and Muteki[18] reported that local anesthetics inhibit the L-type Ca^{2+} channels in rat dorsal root ganglion cells, although the physiologic implications of this remain to be elucidated. In their report, the IC_{50} values for dibucaine and tetracaine were 34 and 79 μM, respectively, which are similar to the IC_{50} values for dibucaine and tetracaine in our study (17.7 and 70.2 μM, respectively).

In animal studies, a blockade of the L-type Ca^{2+} channels was reported to induce antinociceptive effects.[19–24] However, the mechanisms causing these effects have yet to be clarified. Although L-type Ca^{2+} channels are expressed in neurons, they do not seem to play a key role in neurotransmitter release.[25–27] L-type Ca^{2+} channels are localized to the base of the dendrites and neuronal cell bodies but not to the synaptic terminals,[28,29] thus suggesting that they mediate calcium-dependent signaling events in the postsynaptic neurons. In studies using cultured cortical neurons, the basal expression of specific immediate early genes were blocked by L-type Ca^{2+} channel antagonists and increased by L-type Ca^{2+} channel agonists.[30,31] In cultured hippocampal neurons, cultured spinal-cord neurons, and adrenal cromaffin cells, L-type Ca^{2+} channels activated by KCl induced the expression of brain-derived neurotrophic factor and proenkephalin genes.[32–34] These observations suggest that L-type Ca^{2+} channels appear to play a critical role in the coupling of synaptic excitation and gene expression. Although a relatively minor component of the calcium current evoked by synaptic activation is attributed to the L-type Ca^{2+} channels,[19] the calcium influxes caused by synaptic excitation may be highly localized and activate the specific calcium-dependent signaling pathways.

Recently, not only the elevation of intracellular calcium, but also the route of calcium entry has been reported to influence the expression of genes by activating the distinct regulatory elements in the promoter region.[35–37] For example, in cultured cortical neurons, the activation of L-type Ca^{2+} channels, but not NMDA receptors, increases cell survival by the enhanced expression of brain-derived neurotrophic factor,[38] thus suggesting the importance of L-type Ca^{2+} channels in the nervous system. It has been reported that the calcium influx *via* receptors activates gene transcription mediated by serum response element (SRE), and calcium entry *via* L-type Ca^{2+} channels activates gene transcription mediated by both cyclic adenosine monophosphate response element and SRE.[34–36] The signaling pathway that leads to SRE-dependent gene transcription is considered to include the activation of MAP kinase.[35–37,39]

Another possibility to explain the difference in the concentrations of local anesthetics between KCl and ionomycin to inhibit the activation of MAP kinase is that the level of intracellular Ca^{2+} elevation might be higher on stimulation with 1 mM ionomycin than with 50 mM KCl. Higher concentrations of Ca^{2+} may induce a strong activation of MAP kinase: This strong activation might necessitate higher concentrations of local anesthetics for inhibition. But this seems unlikely. According to Delorme *et al.*,[40] the peak intracellular Ca^{2+} concentrations measured using quin 2 after the addition of 50 mM KCl, 10 μM ionomycin (in the presence of 5% fetal bovine serum and 10% horse serum), and 50 nM ionomycin (in the absence of serum) are similar, probably because of protein binding of ionomycin. In our experiments, the cells were stimulated in the medium containing 0.25% fetal bovine serum and 0.5% horse serum. So, the peak concentration of intracellular Ca^{2+} after the addition of 1 μM ionomycin is not supposed to be much higher than that after the addition of 50 mM KCl. However, there might be differences in the distribution of Ca^{2+} within the cells between the two stimuli. The upstream pathways to the MAP kinase are supposed to be multiple,[4] and the difference in the distribution of Ca^{2+} might activate these different pathways, resulting in the difference in IC_{50} values for local anesthetics on stimulation with KCl from ionomycin. Therefore, although dibucaine and tetracaine at clinical concentrations possibly affect L-type Ca^{2+} channels and components between calcium and Ras in MAP kinase pathway, we cannot rule out the possibility that these local anesthetics might inhibit the MAP kinase directly. It has been reported that two homologous proteins of MAP kinase family, Erk1 and Erk2, with molecular masses of 44 and 42 kd, are expressed in the central nervous system.[41] Whereas Erk2 is widely expressed, Erk1 is observed in restricted regions.[42] Both of these MAP kinases are activated by phosphorylation by MEK, although the difference in the role of these MAP kinases is not well-known. In our findings, dibucaine and tetracaine inhibited the phosphorylation of both Erk1 and Erk2 during stimulation with KCl.

In conclusion, we showed that dibucaine and tetracaine at clinical concentrations inhibited the depolarization-stimulated MAP kinase activation and c-Fos induction mediated by L-type Ca^{2+} channels. MAP kinase may play an important role in gene expression and in control of synaptic functions. Our findings suggest that the inhibition of MAP kinase pathway might be one of the potential sites for the actions of local anesthetics in the nervous system.

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