Anesthesiology 2002; 96:1191–201

Involvement of the Mitogen-activated Protein Kinase Family in Tetracaine-induced PC12 Cell Death

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Background: **To explore whether cytotoxicity of local anesthetics is related to apoptosis, the authors examined how local anesthetics affect mitogen-activated protein kinase (MAPK) family members, extracellular signal-regulated kinases (ERKs), c-Jun *N*-terminal kinases (JNKs)–stress-activated protein kinases, and p38 kinase, which are known to play important roles in apoptosis.**

Methods: **Cell death was evaluated using PC12 cells. Morphologic changes of cells, cellular membrane, and nuclei were observed. DNA fragmentation was electrophoretically assayed. Western blot analysis was performed to analyze phosphorylation of the MAPK family, cleavage of caspase-3 and poly(adenosine diphosphate–ribose) polymerase. Intracellular Ca^{2+} concentration was measured using a calcium indicator dye.**

Results: **Tetracaine-induced cell death was shown in a time- and concentration-dependent manner and characterized by nuclear condensation or fragmentation, membrane blebbing, and internucleosomal DNA fragmentation. Casepase-3 activation and phosphorylation of ERK, JNK, and p38 occurred in the cell death. PD98059, an inhibitor of ERK, enhanced tetracaine-induced cell death and JNK phosphorylation, whereas ERK phosphorylation was inhibited. Curcumin, an inhibitor of JNK pathway, attenuated the cell death. Increase of intracellular Ca^{2+} concentration was detected. In addition to the increase of ERK phosphorylation and the decrease of JNK phosphorylation, two Ca^{2+} chelators protected cells from death. Neither cell death nor phosphorylation of the MAPK family was caused by tetrodotoxin. Nifedipine did not affect tetracaine-induced apoptosis.**

Conclusions: **Tetracaine induces apoptosis of PC12 cells *via* the MAPK family. ERK activation protects cells from death, but JNK plays the opposite role. Toxic Ca^{2+} influx caused by tetracaine seems to be responsible for the cell death, but blocking of Na^{+} channels or L-type Ca^{2+} channels is unlikely involved in the tetracaine's action for apoptosis.**

ALTHOUGH local anesthetics are one of the old drugs being used clinically at present, they have been suggested to be neurotoxic or cytotoxic. Intrathecal administration of tetracaine or etidocaine, for example, caused histopathologic changes in rabbit spinal cord,[1] and lidocaine seems to directly induce rat dorsal root ganglion neuronal death[2] and attenuate the human eosinophil survival.[3] Chloroprocaine could also induce Schwann cell necrosis in rat peripheral nerve bundle.[4] Several clinical observations have also suggested that local anesthetics, such as lidocaine, given in the proximity of the neuronal structure, would cause transient dysfunction.[5,6] Since an *in vitro* study has suggested that dibucaine, tetracaine, lidocaine, and procaine would cause neuroblastoma apoptosis,[7] one may speculate that local anesthetics given into neuronal structures may induce apoptotic cell death in clinical practice.

Potential toxicity of local anesthetics on different cell types could be caused by various factors.[1–4,7] Cell death induced by chemical or physical stress stimuli seems to be categorized into two basic forms of cell death, apoptosis and necrosis, as defined based on morphologic and biochemical criteria.[8] Apoptosis is characterized by cellular shrinkage, membrane blebbing, nuclear condensation, and internucleosomal DNA fragmentation.[9] In contrast, necrosis is characterized by a rapid cell swelling and cell lysis, with random degradation of DNA.[9] Consistent with the notion that apoptosis is a gene-directed self-destruction program, alterations in gene expression are associated with apoptosis.[10] However, mechanisms involved in local anesthetic–induced cell death has not been clearly understood.

The mitogen-activated protein kinases (MAPKs) are a family of serine–threonine protein kinases.[11] They are important mediators for signal transduction from the cell surface to the nucleus[11] and are also thought to play important roles in the initiation and progression of cell death.[12–14] The first members identified in the family were p44 and p42 extracellular signal-regulated kinases (ERKs), known as ERK1 and ERK2. Phosphorylation of serine kinase Raf activates the downstream protein kinase, MAPK kinases (MEK1 and MEK2), which lead to activation and translocation of ERKs to the nucleus.[11,14] In the nucleus, the ERKs phosphorylate transcription factors Elk-1 and ATF-2. This signaling cascade is activated by growth factors[15] and stress stimuli[16] and plays an essential role in both growth factor and stress signaling.[14] Another class of MAPK family members, the stress-activated c-Jun *N*-terminal kinases (JNKs)–stress-activated protein kinases (p46-JNK1–p54-JNK2), are primarily responsive to stress stimuli.[17,18] JNKs activated by MAPKs (MKK3, MKK4,[19] and MKK7[20]) induce the phosphorylation of transcription factors, including c-Jun, Elk-1 and ATF-2, which regulate immediate early gene expression.[21] p38 kinase activated by MKK3 and MKK6 is also a member of the MAPK family[22] and can regulate tran-

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Received from the Departments of Anesthesiology and Critical Care Medicine and Biochemistry, Gifu University School of Medicine, Gifu City, Gifu, Japan; and Gifu International Institute of Biotechnology, Gifu City, Gifu, Japan. Submitted for publication December 18, 2000. Accepted for publication November 28, 2001. Supported in part by grant No. 11307027 from the Ministry of Education, Science, and Culture of Japan, Tokyo, Japan, and the Japan Society for the Promotion of Science, Tokyo, Japan. Presented in part at the 47th Annual Meeting of the Japan Society of Anesthesiologists, Tokyo, Japan, April 6–8, 2000.

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scription factors, including ATF-2, Elk-1, and CHOP.[14,23] Because local anesthetics have been reported to affect the phosphorylation of ERK in PC12 cells,[24,25] it is assumed that MAPK phosphorylation affected by local anesthetics may be associated with cell death.

Although cell death and transient neuronal dysfunctions caused by local anesthetics have been reported, mechanism(s) of their effects on intracellular signal molecules are still unclear. Thus, it is important to elucidate components of these signal transduction pathways and the mechanisms that govern the transmission of information. In the recent two decades, rat pheochromocytoma PC12 cells containing ion channels and receptors have been widely used as a neuronal cell line to study intracellular signal pathways and neuronal responses.[26,27] In addition, they have also been established as a model for neurotoxicologic studies.[27,28] To understand the processes of potential cytotoxicity of local anesthetics and to explore some clues for appropriate management, we examined whether local anesthetics such as tetracaine, bupivacaine, lidocaine, and procaine exert cytotoxic effects on PC12 cells. Because tetracaine at a clinically relevant concentration induced cell death of PC12 cells, we further examined whether ERK and JNK play crucial roles in the tetracaine-induced cell death and whether influx of Ca^{2+} would contribute to the process.

Materials and Methods

Materials

A PC12 cell line was donated by Dr. Yoshinori Sugimoto (Shirakawa Institute of Animal Genetics, Fukushima, Japan). Tetracaine hydrochloride, procaine hydrochloride, lidocaine hydrochloride, bupivacaine hydrochloride, EGTA, and BAPTA-AM were purchased from Sigma Chemical Company (St. Louis, MO). BAPTA-AM was first diluted in dimethyl sulfoxide as a stock solution (10 mM) and added to culture media for 60 min before tetracaine treatment. Dulbecco modified Eagle medium and horse serum were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum was obtained from Nippon Bio-supply Center (Tokyo, Japan). PD98059 were obtained from Calbiochem (San Diego, CA). Phospho-ERK (Thr202–Tyr204) antibody, phospho–stress-activated protein kinases–JNK (Thr183–Tyr185) antibody, and phospho-p38 (Thr180–Tyr182) antibody were obtained from New England Biolabs (Beverly, MA). Caspase-3 antibody and poly(adenosine diphosphate–ribose) polymerase (PARP) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat antirabbit immunoglobulin, horseradish peroxidase-linked whole antibody, and enhanced chemiluminescence system were obtained from Amersham Life Science (Buckinghamshire, United Kingdom). Hoechst 33258 (bisbenzimide) staining dye was obtained from Wako, Inc. (Osaka, Japan). Other reagents were of the highest quality available.

Cell Culture of PC12 Cells

Monolayer cultures of PC12 cells were maintained in 100-mm-diameter tissue culture dishes in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal bovine serum and 5% (vol/vol) horse serum in humidified atmosphere containing of 5% CO_2, at 37°C. Stock cultures were subcultured routinely at a cell density 2–3 $\times$ 10^6 per dish at least once a week, and culture media were renewed every 2 days.

PC12 Cell Death Assay

PC12 cells were plated in 35-mm-diameter tissue culture dishes at 2 $\times$ 10^5 cells per dish and grown for 4 days and then exposed to the culture media containing indicated agents in humidified atmosphere containing of 5% CO_2, at 37°C for indicated times. After treatment, the cells were collected, washed twice with cold phosphate-buffered saline (PBS), and then suspended with 500 μl PBS. Cell death of PC12 cells was assessed by bright-field microscopy using a 0.5% trypan blue dye exclusion method. The death was determined by counting three randomly selected fields per sample. Three independent experiments were performed in triplicate on separate occasions with different cultures.

Analysis of Tetracaine-induced Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) activity released from injured cells was assessed using a commercially available kit (Kyokuto, Tokyo, Japan) as described by the manufacturer. PC12 cells were plated in 35-mm-diameter tissue culture dishes at 2 $\times$ 10^5 cells per dish and grown for 4 days and then exposed to the culture media containing indicated agents in humidified atmosphere containing of 5% CO_2, at 37°C for indicated times. The cells in media were removed by centrifugation (900*g*, 5 min), and LDH activity in the media was determined.

Scanning Electron Microscopy

PC12 cells were washed twice with PBS at 37°C and fixed with 1% glutaraldehyde (in PBS) for 30 min at 4°C. After the cell suspension had been centrifuged, the pellet was postfixed in a solution of 2% OsO_4 in PBS buffer for 60 min at 4°C, then dehydrated through graded concentrations of acetone and critical-point dried from liquid carbon dioxide. The material was mounted, coated with gold, and viewed in a JSM-U3 scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Assay of Nuclear Morphology

After treatment described above, the cells were collected, fixed with 1% glutaraldehyde for 30 min at 4°C, washed with PBS, and stained with 1 μM Hoechst 33258

for 5 min. The nuclei were analyzed under a nonconfocal fluorescence microscope (Olympus BX60; Olympus Ltd., Tokyo, Japan), with excitation at 360-nm ultraviolet illumination.

Analysis of DNA Fragmentation

Cells were cultured on 100-mm-diameter dishes, and treated cells were collected in cold PBS. After centrifugation (1,500*g*, 5 min), the pellet was resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1% Triton X-100) for 10 min at 4°C. The supernatant was saved after centrifugation (16,000*g*, 20 min) and treated with DNase-free RNase (10 μg/ml) and proteinase K (0.1 mg/ml) for 2 h at 37°C. Genomic DNA was precipitated by the addition of 1:6 (vol/vol) of 5 M NaCl and isopropyl alcohol overnight at −20°C, pelleted, and redissolved in loading buffer. DNA samples were then size-fractionated on a 2% agarose gel.

Freeze-fracture Electron Microscopy

PC12 cells were fixed with 1% glutaraldehyde in PBS buffer at 37°C for 30 min, washed twice with PBS, and then resuspended with 30% fresh glycerol in PBS overnight. Cells were frozen in Freon-12 and transferred to liquid nitrogen (−195°C). Frozen samples were fractured and shadowed with platinum-carbon in a freeze-fracture apparatus (Hitachi HF-1; Hitachi Ltd., Tokyo, Japan). Replicas were cleaned with hypochlorite solution and then examined with a JEM 1010 electron microscope (JEOL Ltd.).

Measurement of Intracellular Free Ca^{2+}

PC12 cells were plated in 35-mm-diameter tissue culture dishes at 2×10^5 cells per dish and grown for 4 days. The cells were incubated with the culture media containing the cytoplasmic calcium indicator, Fluo-3 AM (3 μM; Nacalai Tesque, Inc., Kyoto, Japan) for 120 min. Treated cells were collected by centrifugation (900*g*, 5 min) and suspended in 2 ml PBS. The fluorescence was measured using Versa Fluorometer (Bio-Rad, Tokyo, Japan) with excitation at 490 nm and emission at 510 nm.

Western Blot Analysis

PC12 cells were subcultured in 60-mm-diameter tissue culture dishes at 10^6 cells per dish and grown for 4 days. After treatment, the cells were washed twice in cold PBS and lysed with buffer A (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecylsufate [SDS], 150 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulgronyl fluoride, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 10 mM NaF, and 1 mM Na_2MoO_4). After incubation for 30 min at 4°C, the suspension was centrifuged at 13,000*g* for 20 min to obtain the cell extract. Proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose membrane. After blocking of the membranes with TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 2% bovine serum albumin, membranes were incubated with the indicated antibodies at room temperature for 90 min and then with the goat antirabbit immunoglobulin horseradish peroxidase-linked whole antibody at room temperature for 60 min. Detection was performed with enhanced chemiluminescence system.

Statistical Analysis

The data are expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance, followed by the Fisher least significant difference test. Differences were considered to be statistically significant at $P < 0.05$.

Results

Tetracaine-induced PC12 Cell Death

PC12 cells were exposed to tetracaine at concentrations from 0.2 to 1 mM. Tetracaine induced PC12 cell death in a time- and concentration-dependent fashion (fig. 1A). More than 50% of cells were dead after treatment with 1 mM tetracaine for 4 h; the cell death was reached at 90% after 10 h of treatment. Similar to cell death, LDH released from cells treated with tetracaine for 10 h was also in concentration-dependent manner (figs. 1A and B). Using a scanning microscope, cellular morphology changes were observed in response to tetracaine (fig. 2). After treatment of 1 mM tetracaine for 4 h, some blebs arised from cell surfaces and microvilli vanished, and after 8 h, pseudopodia (budding) emitted off cells. Nuclei were examined by staining with Hoechest 33258 (fig. 3). In the presence of 1 mM tetracaine, the nuclei of dead cells showed condensation, and some appeared fragmented. DNA fragmentation was apparent by gel electrophoresis by 2 h after treatment with 1 mM tetracaine (fig. 4). Freeze-fracture electron micrographs showed that intramembranous particles, which are considered to represent integral membrane proteins,[29] are markedly aggregated, and smooth intramembranous particle–free areas appeared on the protoplasmic fracture face in tetracaine-treated cell membrane, in contrast to untreated cell membrane, in which numerous intramembranous particles were randomly distributed (figs. 5A and B).

Activation of Caspase-3 in the Tetracaine-induced Cell Death

Caspase-3 has been identified as a key protease activated by cleavage *via* upstream proteases, and it disrupts homeostatic processes and initiates an orderly disassembly of cells, including degradation of genomic DNA.[30] The cleavage of PARP by caspase-3 is a well-characterized event in the apoptosis of animal cells.[31,32] In the

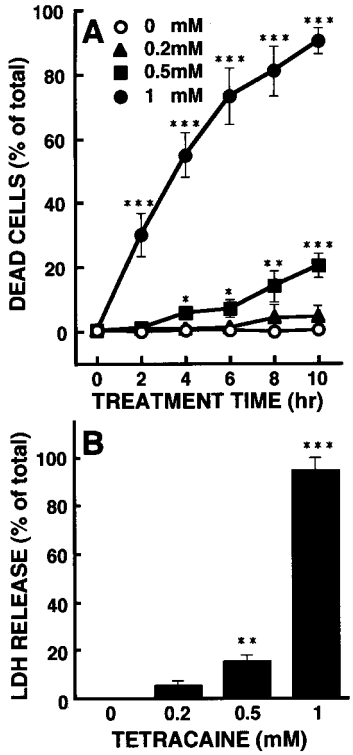

Fig. 1. (*A*) Time course of tetracaine-induced cell death. PC12 cells were treated with indicated concentrations of tetracaine for indicated periods and then stained with 0.5% trypan blue. Cell death was evaluated. Data represent mean ± SD of three different experiments, each conducted in duplicate. ***P < 0.0001 *versus* 0 mM, **P < 0.0005 *versus* 0 mM, *P < 0.05 *versus* 0 mM at the same time point. (*B*) Tetracaine-induced lactate dehydrogenase (LDH) release from PC12 cells. Cultured cells were challenged with indicated concentrations of tetracaine for 10 h, and LDH activity released in the media was assayed. Data shown are mean ± SD from two different experiments, each conducted in triplicate. ***P < 0.0001 *versus* 0 mM, **P < 0.0005 *versus* 0 mM.

tetracaine-induced cell death, caspase-3 proform breakdown and the cleavage of PARP were detected after tetracaine treatment for 4 h. An 89-kd signature fragment of PARP was observed at 8 h (fig. 6).

Effect of Local Anesthetics on the PC12 Cell Death

PC12 cells were exposed to procaine, lidocaine, bupivacaine, or tetracaine for 10 h (table 1). Tetracaine at 1 mM induced cell death; 1 mM of other local anesthetics tested here did not influence cell survival. By increasing the concentration to 2 mM, three drugs except procaine induced different degrees of cell damage, and their relative potencies in the cell death assay followed the order tetracaine > bupivacaine > lidocaine.

Tetracaine-induced Mitogen-activated Protein Kinase Family Phosphorylation

Mitogen-activated protein kinase family phosphorylation in PC12 cells treated with 1 mM tetracaine was examined by Western blot analysis. ERK1 and ERK2

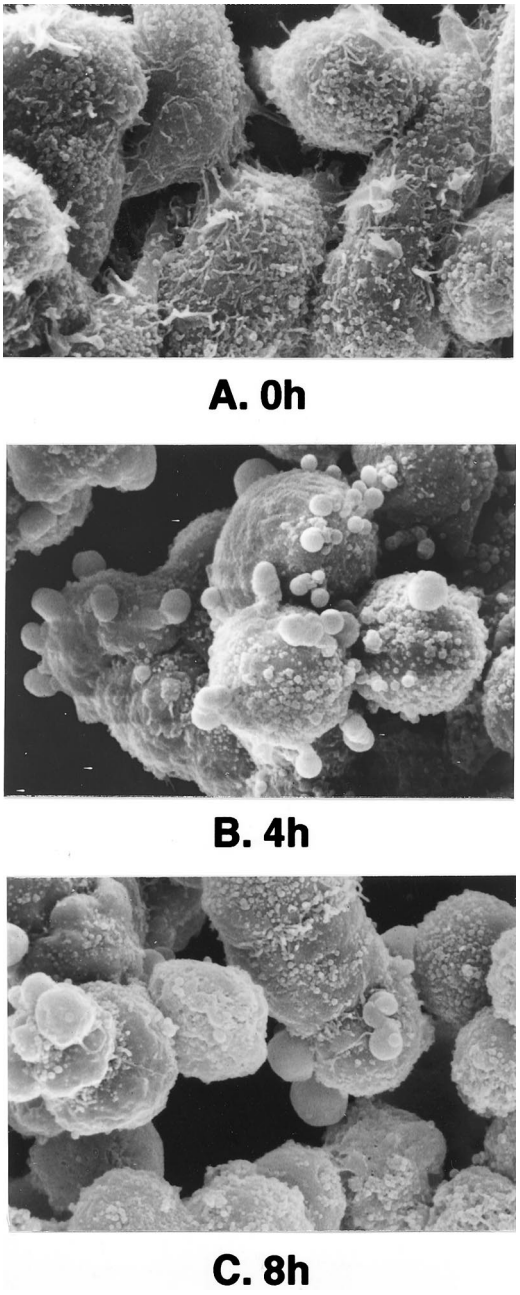

Fig. 2. Tetracaine-induced changes in surface morphology of PC12 cells. Cell morphology was viewed in a JSM-U3 scanning electron microscope. (*A*) Normally cultured PC12 cells have a variable number of microvilli. (*B*) After treatment with 1 mM tetracaine for 4 h, some blebs appear on cells and microvilli vanish (*B*). (*C*) After treatment for 8 h, some cells are developing buds. (Magnification ×3,500.)

were phosphorylated 30 min after exposure to the drug. The ERK phosphorylation decreased after reaching a peak at 60 min (fig. 7A). Tetracaine also resulted in JNK1 and JNK2 phosphorylation within 30 min. After a greater decreasing at 2 h, both phosphorylated JNKs gradually increased again (fig. 7B). p38 phosphorylation was also induced within 30 min, and the maximal phosphorylation was observed at 30 min (fig. 7C).

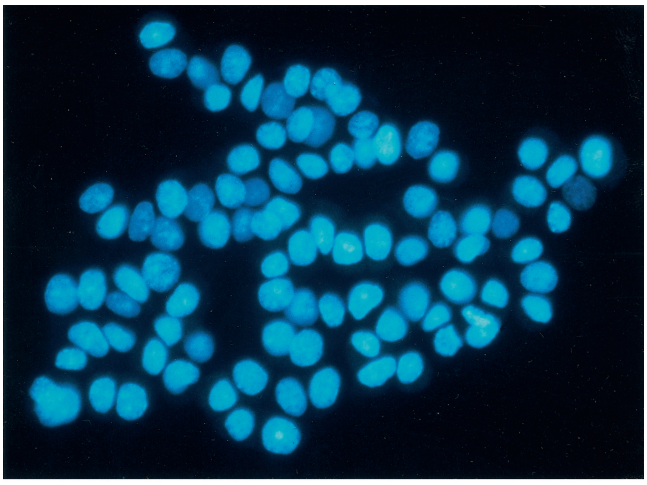

Fig. 3. Tetracaine-induced nucleus changes in PC12 cells. Cells were treated with or without 1 mM tetracaine for 4 h at 37°C and then stained with Hoechst 33258 (1 μM in phospate-buffered saline) and photographed under a fluorescent microscope. Normal nuclei were slightly stained blue, and nuclei of dead cells appear white because of shrinkage. Condensed and fragmented nuclei appeared in tetracaine-treated cells. (Magnification ×660.)

Effects of PD98059 on Tetracaine-induced Cell Death and Phosphorylation of the Mitogen-activated Protein Kinase Family

PD98059, a MEK1 inhibitor, was used to explore which role ERKs play in the cell death and enhanced the tetracaine-induced cell death and LDH activity in the media (figs. 8A and B). Significantly larger numbers of dead cells were observed in the presence of 50 μM PD98059 than in its absence ($P < 0.001$). PD98059 with the absence of tetracaine was hardly detected to affect cell survival (data not shown).

PD98059 at 50 μM completely blocked the tetracaine-induced ERK phosphorylation (fig. 8C). However, in the presence of PD98059, phosphorylation of JNK1 and JNK2 was enhanced at every time point (fig. 8D).

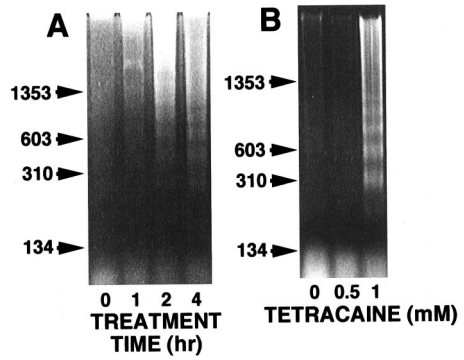

Fig. 4. Tetracaine-induced intranucleosomal DNA fragmentation. Cells were exposed to 1 mM tetracaine for indicated times (*A*) and to indicated concentration of tetracaine for 4 h (*B*). After tetracaine treatment, genomic DNA was separated on a 2% agarose gel. DNA fragments were visualized by ultraviolet fluorescence. Molecular size (base pairs) is indicated on the left.

Effects of Lower Concentrations of Tetracaine on Mitogen-activated Protein Kinase Phosphorylation in PC12 Cells

Tetracaine at lower concentrations (0.2 and 0.5 mM) also activated ERK phosphorylation, which took the same time course as the drug at higher concentration (1 mM; fig. 9A). Furthermore, the tetracaine-induced ERK activity occurred in a concentration-dependent manner (fig. 9A). PD98059 potently enhanced the effects of tetracaine at lower concentration on the PC12 cell survival (figs. 9B and C). The JNK phosphorylation could not be detected in the lower concentration treatment (data not shown). In the presence of 50 μM PD98059, 0.5 mM tetracaine induced a detectable JNK phosphorylation (fig. 9D), but 0.2 mM tetracaine did not (data not shown).

Effects of Curcumin on Tetracaine-induced Cell Death and Phosphorylation of Mitogen-activated Protein Kinase Family

Curcumin, a dietary pigment in curry, has been indicated to be a potent inhibitor of JNK activation by interfering with the molecule(s) upstream of JNK in the signal transduction pathway, and it also inhibits ERK activation at a higher concentration.[33,34] Curcumin at 10 μM partially inhibited the tetracaine-induced LDH activity released from damaged PC12 cells (fig. 10A) and the JNK phosphorylation (fig. 10B), but also slightly reduced the ERK phosphorylation (fig. 10C).

Tetracaine-induced Increase of Intracellular Ca^{2+} Concentration and Effects of Calcium Chelators on Tetracaine-induced Mitogen-activated Protein Kinase Phosphorylation and Lactate Dehydrogenase Release

Tetracaine-treatment caused an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PC12 cells, which nearly peaked at 15 min (fig. 11A). Both EGTA, a calcium-specific chelator, and BAPTA-AM, a chelator of intra-

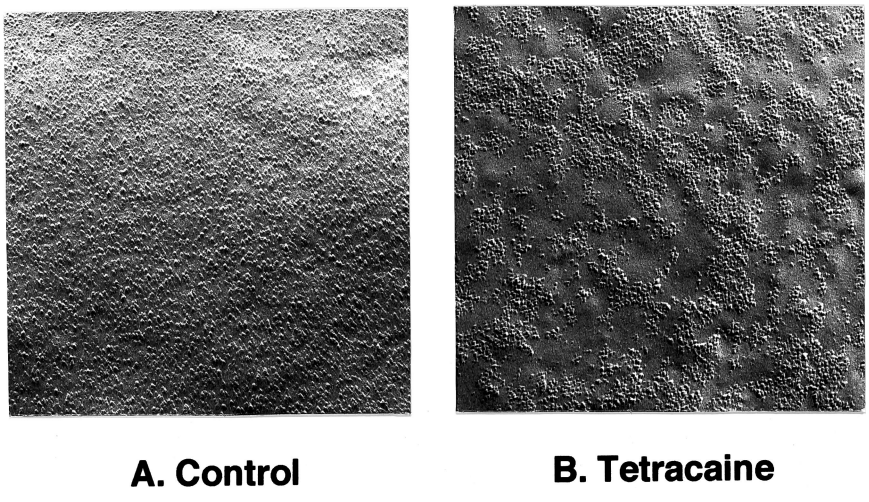

Fig. 5. PC12 cells were treated with or without 1 mM tetracaine for 8 h and then examined with electron microscope (JEM 1010). (*A*) Freeze-fracture image of untreated PC12 cell (Control). (*B*) Freeze-fracture electron micrograph of tetracaine-treated cell membrane (Tetracaine). (Magnification ×40,000.)

cellular calcium, significantly attenuated the action of tetracaine (fig. 11B). To understand the roles of increasing of $[Ca^{2+}]_i$ in tetracaine-induced apoptosis, the two calcium chelators were used. Tetracaine-induced LDH release from PC12 cells was diminished by minimizing extracellular Ca^{2+} concentration with EGTA (fig. 12A). EGTA potentiated tetracaine-induced ERK phosphorylation and decreased JNK phosphorylation (figs. 12B and C). BAPTA-AM at 5 μM also reduced the tetracaine-induced cell damage (fig. 13A) and the JNK phosphorylation (fig. 13B). The tetracaine-induced ERK phosphorylation did not show dramatic increase (fig. 13C).

Effect of Nifedipine and Tetrodotoxin on Tetracaine-induced Cell Death

L-type Ca^{2+} channels have been demonstrated to exist in PC12 cells.[27] Thus, we examined role of the Ca^{2+} channels in tetracaine-induced apoptosis. Nifedipine, a selective blocker of L-type Ca^{2+} channels, neither induced the cell survival nor affected the action of tetracaine (table 2). Tetrodetoxin (0.5 mM) was introduced to examine whether blockade of Na^+ channel was involved in tetracaine-induced cell death, and we did not detect any effects of the Na^+ channel blocker on cell survival (table 2).

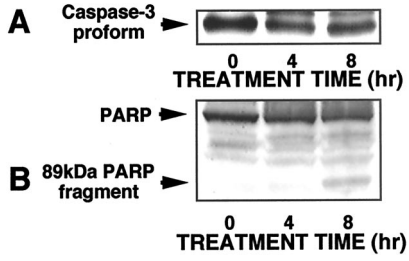

Fig. 6. Tetracaine-induced caspase-3 activation. PC12 cells were treated with 1 mM tetracaine for indicated times. Proteins (50 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Cleavage of proform of caspase-3 and poly(adenosine diphosphate–ribose) polymerase (PARP) was determined. Special bands are indicated by arrows on the left. Caspase-3 proform breakdown (*A*) and cleavage of PARP (*B*) were detected after 4 h of tetracaine treatment. An 89-kd signature fragment of PARP was clearly observed at 8 h (*B*).

Discussion

The major finding of the current study is that tetracaine, bupivacaine, and lidocaine, at their clinically relevant concentrations, induced PC12 cell death. The morphologic and biochemical changes associated with the tetracaine-induced cell death are characterized by plasma membrane bleb formation, chromatin condensation, nuclear fragmentation (or DNA ladder), and budding off of cellular fragments.[10] Caspase-3 activation occurred in the process of the tetracaine-induced cell death. Phosphorylations of the MAPK family, ERK, JNK, and p38 were also observed in the process. Inhibition of ERK phosphorylation with a MEK1 inhibitor potentiated the tetracaine-induced JNK posphorylation and cell death. Curcumin, a JNK inhibitor, attenuated the cell damage. Although blocking of Na^+ channels was not likely involved in the action of tetracaine, EGTA, a Ca^{2+} chelator, which *per se* did not affect ERK and JNK phosphorylation, enhanced tetracaine-induced ERK phosphorylation and decreased JNK phosphorylation and prevented cell death. BAPTA-AM partially reduced tetracaine-induced cell death.

Table 1. PC12 Cell Death Induced by Local Anesthetics

	Dead Cells (% of Total)	
	1 mM (10 h)	2 mM (10 h)
Control	0.5 ± 0.3	0.2 ± 0.2
Procaine	0.3 ± 0.3	0.2 ± 0.2
Lidocaine	0.5 ± 0.3	3.1 ± 1.5†
Bupivacaine	0.8 ± 0.2	36.5 ± 5.7*
Tetracaine	86.1 ± 7.4*	99.9 ± 0.1*

Data represent mean ± SD of three different experiments, each performed in duplicate.

* $P < 0.0001$ *versus* control. † $P < 0.0005$ *versus* control.

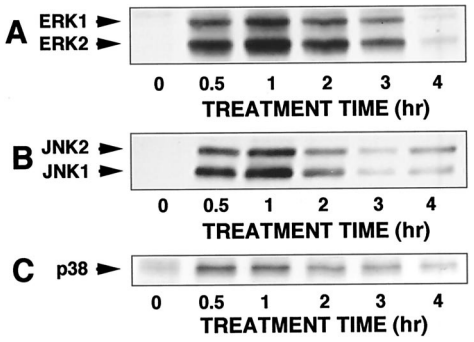

Fig. 7. Tetracaine-induced phosphorylation of the mitogen-activated protein kinase family. PC12 cells were challenged with 1 mM tetracaine for indicated times, and whole cell lysates were prepared. Proteins (70 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis were performed with phospho-ERK (*A*), phospho-JNK (*B*), or phospho-p38 antibody (*C*). Special bands are indicated by arrows on the left.

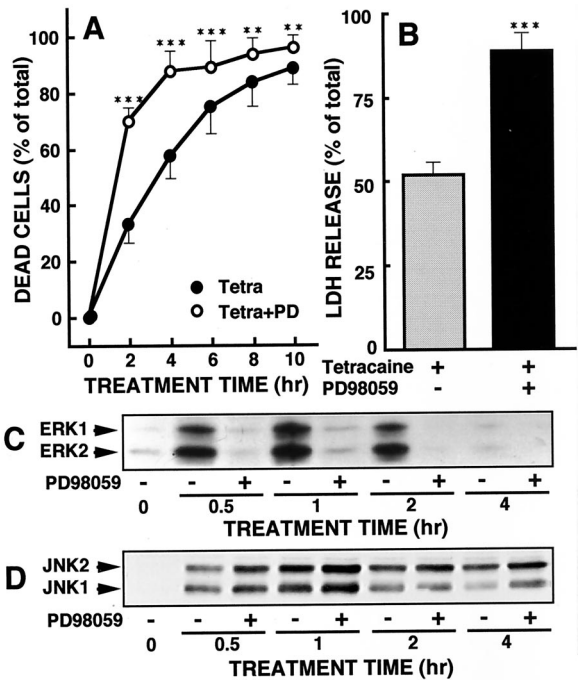

Fig. 8. (*A*) Effects of PD98059 on tetracaine-induced PC12 cell death. Cells were exposed to 1 mM tetracaine (Tetra) in the presence or absence of 50 μM PD98059 (PD) for indicated time points, and collected cells were washed and dyed by 0.5% trypan blue. Data are mean ± SD of three different experiments, each conducted in duplicate. ***$P < 0.0001$ *versus* tetracaine, **$P < 0.0005$ *versus* tetracaine at the same time point. (*B*) Effects of PD98059 on tetracaine-induced lactate dehydrogenase (LDH) release. PC12 cells were treated with or without 50 μM PD98059 in the presence of 1 mM tetracaine for 4 h. LDH activity released in the media was assayed. Data are mean ± SD from two different experiments, each conducted in triplicate. ***$P < 0.0001$ *versus* tetracaine. (*C* and *D*) Effects of PD98059 on the tetracaine-induced phosphorylation of ERKs and JNKs. Cells were challenged with 1 mM tetracaine in the absence (−) or presence (+) of 50 μM PD98059 for indicated time points, and the phosphorylation of ERKs and JNKs was analyzed with phospho-ERK antibody and phospho-JNK antibody, respectively. Special bands are indicated by arrows on the left. Tetracaine-induced phosphorylation of ERK1 and 2 was prevented (*C*), and phosphorylation of JNK1 and JNK2 was increased (*D*) using PD98059.

The morphologic changes of the cell surface are unique as the standpoint of apoptosis. Intramembranous particle distribution was markedly aggregated, and smooth intramembraneous particle-free areas appeared on the protoplasmic fracture in the presence of tetracaine. It is generally thought that the primary sites of local anesthetic action are at the cellular plasma membrane of the neuronal structures[35]; tetracaine-induced morphologic changes are thus a very provocative finding. Because the time course for apoptotic cell death needed a longer time, it seems likely that tetracaine first triggers intracellular signaling pathways rather than its direct membrane effects, and the apoptosis-like morphologic changes are mediated by the intracellular signaling pathways including the MAPK family. On the process of such morphologic changes, movement of Ca^{2+} from the exterior through the plasma membrane[36–38] or from intracellular organella[39,40] into the cytosol could play crucial roles, and increased $[Ca^{2+}]_i$ regulates several key steps in the apoptotic signaling pathway in various cell types, including neurons.[39,40] Agents that suppress influx of Ca^{2+} can prevent apoptosis in PC12 cells,[41] such as unpermeable EGTA, which chelates extracellular Ca^{2+} and abolishes influx of Ca^{2+}.[38] Local anesthetics such as lidocaine have been indicated to cause $[Ca^{2+}]_i$ increase *via* Ca^{2+} influx through plasma membrane[2,7] and to kill cells.[7] In the current study, because EGTA abolished influx of Ca^{2+} and kept the cells alive, an increase in $[Ca^{2+}]_i$ from an extracellular source is thought to play an important role in the tetracaine-induced apoptosis. BAPTA-AM, an esterified EGTA analog, goes into cytosol, where it is deesterified and binds Ca^{2+}. Although local anesthetics were thought to block L-type Ca^{2+} channels,[42] and lidocaine at 30 mM would increase $[Ca^{2+}]_i$ *via* voltage-gated Ca^{2+} channels,[2] the effects of tetracaine seem not to be a result of its action on L-type Ca^{2+} channels, because nifedipine neither induced apoptosis nor inhibited the tetracaine-induced cell death. We found that the role of the increased $[Ca^{2+}]_i$ *via* plasma membrane from the exterior is crucial in tetracaine-induced cell death, but one may not exclude other mechanisms such as membrane fluidity changes and alteration of mitochondrial functions, which have been reported to be involved in apoptosis.[43–45] Indeed, it has been suggested that local anesthetics affect membrane fluidity[7] and mitochondrial functions.[46,47]

To examine consequences of phosphorylation of ERK and JNK pathways in tetracaine-induced PC12 cell death, we used PD98059, an inhibitor of MEK1, which lies immediately upstream of ERK and is responsible for ERK phosphorylation or activation.[11] PD98059 has been used to characterize the role of the ERK pathway in a lot of cell lines.[11,48,49] For example, it has been reported that

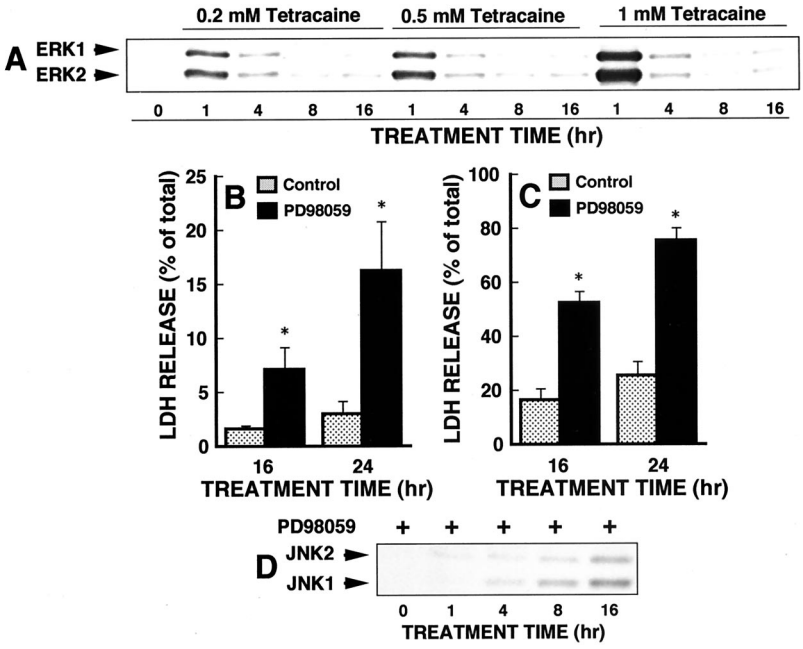

Fig. 9. (*A*) ERK phosphorylation induced by several concentrations of tetracaine. PC12 cells were exposed to 0.2, 0.5, or 1 mM tetracaine for indicated times, and then ERK phosphorylation was analyzed using phospho-ERK antibody. (*B* and *C*) The effect of PD98059 on lower concentration of tetracaine-induced lactate dehydrogenase (LDH) release. PC12 cells were pretreated with (PD98059) or without (control) 50 μM PD98059 for 60 min and then exposed to 0.2 or 0.5 mM tetracaine for indicated times, respectively. LDH activity released in the media was analyzed. Data are mean ± SD from three different experiments, each performed in triplicate. *$P < 0.05$ *versus* control at the same time points. (*D*) The effect of PD98059 on 0.5 mM tetracaine-induced JNK phosphorylation. PC12 cells pretreated with PD98059 were exposed to 0.5 mM tetracaine, and Western blot analysis was performed with phospho–stress-activated protein kinases–JNK antibody.

ERK activation protects neurons from glutamate toxicity, and PD98059 blocked the neuroprotection by inhibiting ERK activation.[50] ERK activation is necessary for prevention of PC12 cell death by *N*-acetylcysteine, a promoter of neuronal survival, and PD98059 could completely inhibit the cell survival promotion.[51] Thus, the current result indicating that inhibition of ERK phosphorylation by PD98059 increased tetracaine-induced cell death would suggest that ERK activation plays a protective role in the cellular survival pathway. Because EGTA pretreatment seems to protect PC12 cells from apoptosis by minimizing the toxic influx of Ca^{2+}, enhancement of ERK phosphorylation by EGTA might suggest the recovery of ERK phosphorylation inhibited by the toxic influx of Ca^{2+} (fig. 12). It is thus assumed that PC12 cells would activate ERK cascade to resist a potential action of tetracaine, but Ca^{2+} influx induced by tetracaine *per se* seems to overwhelm the protective cascade and then facilitate cell death.

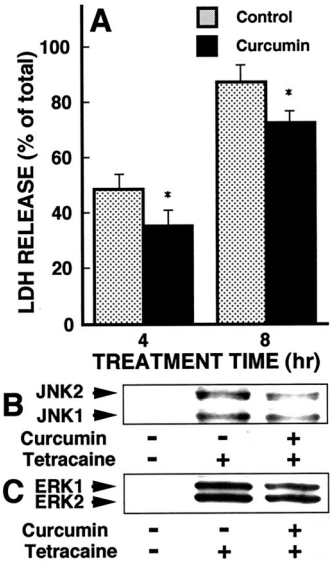

Fig. 10. (*A*) Effect of curcumin on tetracaine-induced lactate dehydrogenase (LDH) release. PC12 cells were incubated with (curcumin) or without (control) 10 μM curcumin for 1 h and then treated with 1 mM tetracaine for indicated times. LDH activity released in the media was analyzed. Data are mean ± SD from three different experiments, each performed in triplicate. *$P < 0.05$ *versus* control at the same time points. (*B* and *C*) Effects of curcumin on tetracaine-induced phosphorylation of the mitogen-activated protein kinase family. Curcumin-incubated PC12 cells were exposed to 1 mM tetracaine for 1 h, and phosphorylation of JNK (*B*) or ERK (*C*) was analyzed.

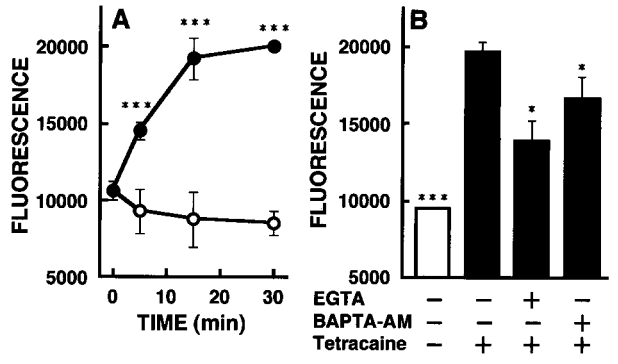

Fig. 11. (*A*) Tetracaine-induced increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). PC12 cells were loaded with 3 μM Fluo-3 AM for 120 min and treated with (filled circles) or without (open circles) 1 mM tetracaine for indicated times. (*B*) Effects of chelators on tetracaine-induced increase in $[Ca^{2+}]_i$. EGTA (1 mM) or BAPTA-AM (5 μM) was added in the loading media for the last 30 min (EGTA +) or 60 min (BAPTA-AM +), and then cells were exposed to 1 mM tetracaine (+) or culture media (−) for 15 min. $[Ca^{2+}]_i$ (as fluorescent intensity units) was measured. Data are mean ± SD from two different experiments, each performed in duplicate. ***$P < 0.0001$ *versus* control (*A*), *$P < 0.05$ *versus* only presence of tetracaine, ***$P < 0.0001$ *versus* only presence of tetracaine (*B*).

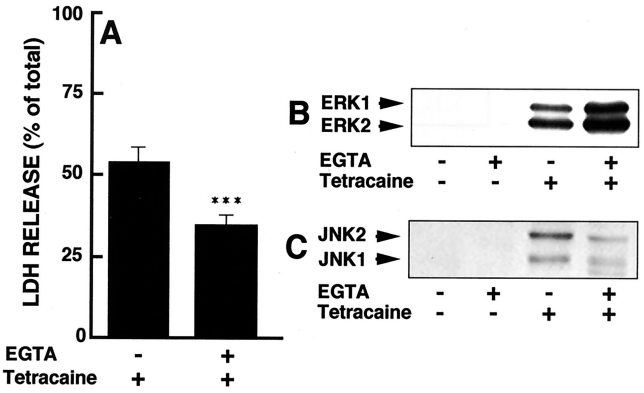

Fig. 12. Effects of EGTA on the tetracaine-induced lactate dehydrogenase (LDH) release and phosphorylation of the mitogen-activated protein kinase family. PC12 cells were pretreated with (+) or without (−) 1 mM EGTA for 30 min and then treated with (+) or without (−) 1 mM tetracaine for 4 h, and LDH activity released in the media was analyzed. Data are mean ± SD from two different experiments, each conducted in triplicate. ***$P <$ 0.0001 *versus* only presence of tetracaine (*A*). After pretreatment described as above, cells were subjected to media in the presence or absence of 1 mM tetracaine for 1 h. Proteins (70 μg) from whole cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and Western blot analysis was performed with phospho-ERK (*B*) or phospho–stress-activated protein kinases–JNK antibody (*C*).

It has been indicated that JNK cascade is involved in apoptosis in response to distinct stimuli in various cell types.[23] Inhibition of c-Jun, the transcription factor activated by the JNK pathway, could protect the neurons from apoptosis,[52] and increased activity of c-Jun seems to be sufficient to trigger apoptotic cell death in fibroblasts.[53] Activation of JNK cascade has been reported to occur in the apoptosis induced by environmental stresses, ionizing radiation, hydrogen peroxide, ultraviolet C radiation, heat shock, tumor necrosis factor α, as well as PD98059.[54] The current results indicating that curcumin inhibited tetracaine-induced JNK phosphorylation and cell death are in agreement with those of other reports.[33,34] In addition to inhibiting ERK phosphorylation, PD98059 enhanced JNK phosphorylation and magnified tetracaine-induced cell death. The current findings may suggest that tetracaine-induced apoptotic cell death would occur as a result of losing balance of signaling molecular activities between survival signal, ERK cascade, and death signal, JNK cascade. Attenuation of JNK phosphorylation by EGTA and BAPTA-AM would

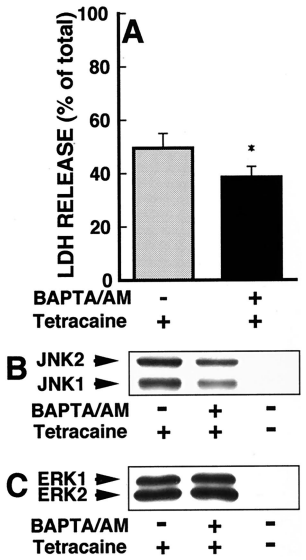

Fig. 13. PC12 cells were pretreated with (+) or without (−) 5 μM BAPTA-AM for 1 h and then treated with (+) or without (−) 1 mM tetracaine for 4 h, and LDH activity was analyzed. Data are mean ± SD from three different experiments, each conducted in triplicate. *$P <$ 0.05 *versus* only presence of tetracaine (*A*). After pretreatment described as above, cells were subjected to media in the presence or absence of 1 mM tetracaine for 1 h. Proteins (70 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and Western blot analysis was performed with phospho-ERK (*B*) or phospho–stress-activated protein kinases–JNK antibody (*C*).

Table 2. Effect of Nifedipine and Tetrodotoxin on Tetracaine-induced Cell Death

	Dead Cells (% of Total)
Control	0.3 ± 0.7
Tetrodotoxin	0.4 ± 0.5
Nifedipine	0.7 ± 1.0
Tetracaine	57.1 ± 4.3*
Nifedipine + tetracaine	63.3 ± 9.7*

PC12 cells were treated with or without 5 μM nifedipine or 0.5 mM tetrodotoxin for 30 min and then challenged with or without 1 mM tetracaine for 4 h. Lactic dehydrogenase activity released in the media was analyzed. Data shown are mean ± SD from two different experiments, each performed in triplicate.

* $P <$ 0.0001 *versus* control.

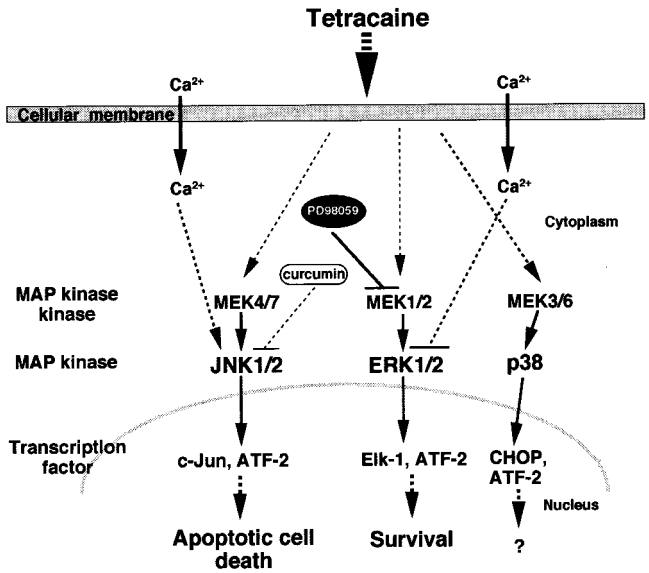

Fig. 14. A model for the tetracaine-induced mitogen-activated protein (MAP) kinase family phosphorylation. Tetracaine may target on cellular membrane and activate ERK, JNK, and p38 pathways. PD98059 prevented MEK1 phosphorylation and blocked ERK activation. JNK phosphorylation and cell death were partially inhibited by curcumin. Tetracaine-induced toxic Ca^{2+} influx may decrease ERK phosphorylation and be involved in JNK.

suggest that overinflux of Ca^{2+} might be upstream of JNK phosphorylation. p38 activation has been demonstrated not only to correlate with cell death,[12,55] but also to play important roles in cell survival[48,56] and the process of preconditioning.[57] Thus, before investigating further in detail, we could not postulate which role p38 played in tetracaine-induced cell death.

In our previous studies, local anesthetics have been found to suppress important signal molecules, such as phospholipase D[58] and muscarinic receptor-mediated ERK activation.[24] ERK activation, which was found to be blocked with tetracaine (0.5 mM), lasted for 15 min (peaked at 2 min) after carbachol stimulation.[24] The time course of KCl-stimulated ERK activation, which was completely blocked by tetracaine at 0.2 mM, just lasted for 10 min.[25] In the current study, tetracaine-induced ERK phosphorylation was observed to last for several hours. Because of differences among these time courses, one can speculate that these pathways of ERK activation should differ. Tetracaine suppresses carbachol- or KCl-stimulated ERK activation by affecting the muscarinic receptor or L-type calcium channels.[24,25] In addition, it remains to be elucidated whether tetracaine can direct act ERK *per se* so far. It seems likely that tetracaine has different effects on distinct ERK pathways by diverse mechanisms. Thus, the activation of ERK cascade as a protective pathway may be a response of the cell itself to an irritant such as tetracaine by some unknown mechanism. Although the mechanism(s) for cell death other than apoptosis might be involved in PC12 cell death in the presence of tetracaine, we could exclude the possibility that necrotic cell death occurs in prolonged exposure of local anesthetics in any important way.

Local anesthetics used clinically rarely produce localized nerve damage. Reports of neurologic injury after single-dose and continuous spinal anesthesia suggest that clinically relevant concentrations of local anesthetics would cause neuronal injury.[5,6,59] Although tetracaine at 0.2 mM scarcely induced cell death, ERK activation might indicate that cells responded to this concentration of the drug to active protective signal pathway to resistant extrairritant. It is well known that concentrations of three major clinical local anesthetics—lidocaine, bupivacaine, and tetracaine—for peripheral and central anesthesia are 1–5% (approximately 37–185 mM), 0.5–0.75% (approximately 15–23 mM), and 0.25–0.5% (approximately 8–17 mM), respectively. Because of vascular absorption by regional circulation and dilution by cerebrospinal fluid, their anesthetic concentrations are definitely lower than the used concentrations. During spinal anesthesia, the mean concentration of tetracaine in human cerebrospinal fluid has been reported to be approximately 0.02–0.4 mM.[60] Because neuronal tissues would be exposed to local anesthetics at much higher concentrations than their anesthetic concentrations, it may be possible that apoptotic cell death around their injected sites could occur. Local anesthetics have been considerably safely used; however, prolonged use of local anesthetics for spinal or epidural anesthesia seems to be no longer simply safe and anesthesiologists should be wary of injecting them at extremely high concentrations in the vicinity of the neuronal structure, especially near the spinal cord. Because of significant differences between *in vitro* observations and clinical observations, at clinically useful doses, effects of local anesthetics on neuronal structures might be different. Thus, more *in vitro* and *in vivo* studies are needed.

In summary, the current study indicates that tetracaine, bupivacaine, and lidocaine at millimole concentrations caused PC12 cell death, and tetracaine-induced cell death featured plasma membrane bleb formation, nuclear fragmentation, or condensation, chromatin fragmentation, and budding off of cellular fragments. Caspase-3 activation was observed in the tetracaine-induced cell death. Phosphorylation of the MAPK family, ERKs, JNKs, and p38 occurred in the process; ERK cascade and JNK cascade may play opposite roles to protect from or result in apoptosis (fig. 14). Toxic influx of Ca^{2+} may damage cells by decreasing tetracaine-induced ERK phosphorylation and facilitating JNK phosphorylation. The action of local anesthetics on cell survival does not likely result from their effects on voltage-gated sodium channels and L-type calcium channels. The implications will help in understanding the mechanisms of the neurotoxicity of local anesthetics.

The authors thank Takashi Naganawa (Technician, Department of Biochemistry, Gifu University School of Medicine, Gifu City, Gifu, Japan) for technical assistance.

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