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Local Anesthetics Inhibit Muscarinic Receptor-mediated Activation of Extracellular Signal-regulated Kinases in Rat Pheochromocytoma PC12 Cells

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Background: **Because protein phosphorylation is a key mechanism for controlling cellular functions and extracellular signal-regulated kinase (ERK) plays a role in cellular signal transduction, the authors wanted to determine whether local anesthetics interfere with biochemical signaling molecules.**

Methods: **Protein tyrosine phosphorylation and ERK activation induced by carbachol, an agonist for muscarinic acetylcholine receptors, were examined in rat pheochromocytoma PC12 cells, a model for investigating signal transduction. Carbachol-induced tyrosine-phosphorylated proteins of 44 and 42 kd were determined by Western blot analysis and identified as activated ERK1 and ERK2 using anti-ERK antibody. The ERK activation was blocked by preincubation with atropine or an M_3 muscarinic acetylcholine receptor antagonist 4-diphenyacetooxy-1, 1-dimethylpiperidinium, indicating that is was mediated by M_3 muscarinic acetylcholine receptor activation. Then, in the presence of local anesthetic, the carbachol-induced tyrosine phosphorylation and ERK activation were evaluated. The effects of three Na^+ current-modifying reagents on carbachol-induced ERK activation were also evaluated.**

Results: **Procaine (10^{-4} to 10^{-3} M) inhibited carbachol-induced tyrosine phosphorylation and ERK activation in a concentration-dependent manner. Although tetracaine, lidocaine, and bupivacaine similarly suppressed carbachol-induced tyrosine phosphorylation and ERK activation, neither tetrodotoxin, veratridine, nor ouabain affected the carbachol-induced ERKs activation. Both ERKs were also activated by 4β-phorbol 12-myristate 13-acetate, an activator of protein kinase C, and fluoroaluminate (AlF_4^-), respectively, but procaine did not affect ERK activation induced by these two substances. The inhibition of carbachol-induced ERK activation by procaine was not modified by a phosphatase inhibitor, calyculin A.**

Conclusions: **The current results indicate that local anesthetics inhibit the activity of the signal-transducing molecule(s) leading to M_3 muscarinic acetylcholine receptor–mediated ERK activation in PC12 cells. Such action is unlikely to be a result of the drug's action on Na^+ channels or on the electrochemical gradients of the neuronal cell membrane. (Key words: Mitogen-activated protein kinase; Na^+ current; pirenzepine.)**

THE action of local anesthetics is thought to be principally through electrophysiologic currents that travel through ion channels that are important for the clinical efficacy of the drugs.[1] It is generally accepted that local anesthetics exert their anesthetic and toxic effects by inhibiting voltage-gated Na^+ channels, yet unknown mechanisms may be involved. Ion channels are regulated by protein phosphorylation,[2] which is an important mechanism in controlling cellular functions.[3] Recent reports indicate that local anesthetics could interact with membrane phospholipids and proteins and then affect various cellular activities.[1] Furthermore, local anesthetics are reported to interfere with or modulate some important biochemical signaling molecules, such as acetylcholine receptors (AChR),[4] guanosine 5′-triphosphate–binding proteins (G proteins),[5] protein kinase C (PKC),[6,7] and adenosine 3′,5′-cyclic monophosphate.[8] Despite such recent findings, the molecular mechanism underlying local anesthetic actions is not completely understood.

The mitogen-activated protein kinase (MAPK) cascades were recently identified in mammalian cells and play important roles in cellular signal transduction.[9] Exposure of cells to proliferative or stressful stimuli elicits a complex response involving one or more distinct phosphorylation cascades culminating in the activation of

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MAPK.[9] In mammalian cells, p44MAPK and p42MAPK, now known as ERK1 and ERK2, respectively, are the typical and best studied members of the MAPK family and are activated by phosphorylation on serine and threonine residues by MAPK kinase.[10] The stimulation of ion channels or receptors on the surface of membranes initiates a sequence of activation of PKC, Ras, Raf, and MAPK kinase, which in turn activates ERK.[10] Recently, several studies were performed to determine whether ERK plays a role in anesthesia-related phenomena, such as the effects of opioids in human neuroblastoma cells[11] and alcohol action in PC12 cells.[12] Rat pheochromocytoma PC12 cells contain Na^+, K^+, and Ca^{2+} channels and several membrane-bound receptors, including muscarinic and nicotinic ACh receptors,[13] and have been used widely to investigate signal transduction.

Inhibition of muscarinic signaling has been suggested to explain some states of general anesthesia.[14] We also know that muscarinic AChRs (mAChRs) are widely distributed in the peripheral and central neuronal systems and play a role in motor function and the processing of sensory information in the human spinal cord.[15] Although the mechanisms for local anesthetic blockade of nervous conduction have been established, the mechanism of epidural and spinal anesthesia is unknown and may be more complex than simply the inhibition of Na^+ channels.[1] To identify the molecular mechanisms responsible for the actions of local anesthetics, we evaluated the effects of local anesthetics on muscarinic receptor-induced protein tyrosine phosphorylation and ERK activation in PC12 cells. Because we found that local anesthetics inhibit carbamoylcholine chloride (carbachol)–induced ERK activation, but not 4β-phorbol 12-myristate 13-acetate (PMA)–induced activation, we also evaluated the effects of tetrodotoxin, an Na^+ channel blocker; veratridine, an activator of Na^+ channels; and ouabain, an Na^+–K^+ pump inhibitor, on CCh-induced ERK activation.

Materials and Methods

Materials

Procaine hydrochloride, tetracaine hydrochloride, lidocaine hydrochloride, bupivacaine hydrochloride, atropine sulfate salt, tetradotoxin, veratridine, ouabain, calyculin A, carbachol, and PMA were purchased from Sigma Chemical Company (St. Louis, MO). Pirenzepine dihydrochloride and 4-diphenyacetooxy-1,1-dimethylpiperidinium, methiodide, and pertussis toxin were obtained from Research Biochemicals International (Natick, MA). Dulbecco modified Eagle medium and horse serum were from Life Technologies (Grand Island, NY). Fetal bovine serum was from Nippon Bio-supply Center (Tokyo, Japan). Anti-phosphotyrosine mouse monoclonal antibody was from Upstate Biotechnology Incorporated (Lake Placid, NY). Anti-ERK mouse monoclonal antibody was from Affiniti Research Products Limited (Nottingham, UK). The goat anti-mouse immunoglobulin G horse-radish peroxidase-coupled secondary antibody and the enhanced chemiluminescence system used for Western blot analysis were obtained from Amersham Life Science (Buckinghamshire, UK). Other reagents were of the highest quality available.

Cell Culture of PC12 Cells

A PC12 cell line was supplied by Dr. Y. Sugimoto (Shirakawa Institute of Animal Genetics, Fukushima, Japan). Monolayer cultures of the 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 a humidified atmosphere containing of 5% carbon dioxide at 37°C. Stock cultures were subcultured routinely at a cell density of 2–3 $\times$ 10^6/dish at least once a week, and culture media were renewed every 2 days.

Western Blot Analysis of Protein Tyrosine Phosphorylation and Extracellular Signal-regulated Kinase Activation in PC12 Cells

PC12 cells were subcultured in 60-mm-diameter tissue culture dishes at 1 $\times$ 10^6 cells/dish and grown for 4 days. The cells were washed twice with 2 ml buffer A (25 mM Hepes, *p*H 7.4, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 1 mg/ml bovine serum albumin) and preincubated in 3 ml buffer A with or without local anesthetics or Na^+ current–modifying reagents (tetrodotoxin, veratridine, and ouabain) at 37°C for 10 min. The cells were stimulated with 1 mM carbachol or 200 nM PMA at 37°C for durations indicated in each experiment. The reaction was terminated by aspiration of the reaction buffer and washing twice with 2 ml ice-cold phosphate-buffered saline (8% NaCl, 0.2% KCl, 2.88% $Na_2HPO_4 \cdot 12H_2O$, 0.2% KH_2PO_4, *p*H 7.4). The washed cells were scraped quickly into 120 μl RIPA buffer (10 mM Tris-HCl, *p*H 7.4, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfronyl fluoride, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 10 mM NaF, and 0.1 mM Na_2MoO_4) and transferred to a microcentrifuge tube. After incubation on ice for 30 min, the suspension was centrifuged at 13,000*g* for 20 min to obtain the cell extract. Seventy micrograms of protein was

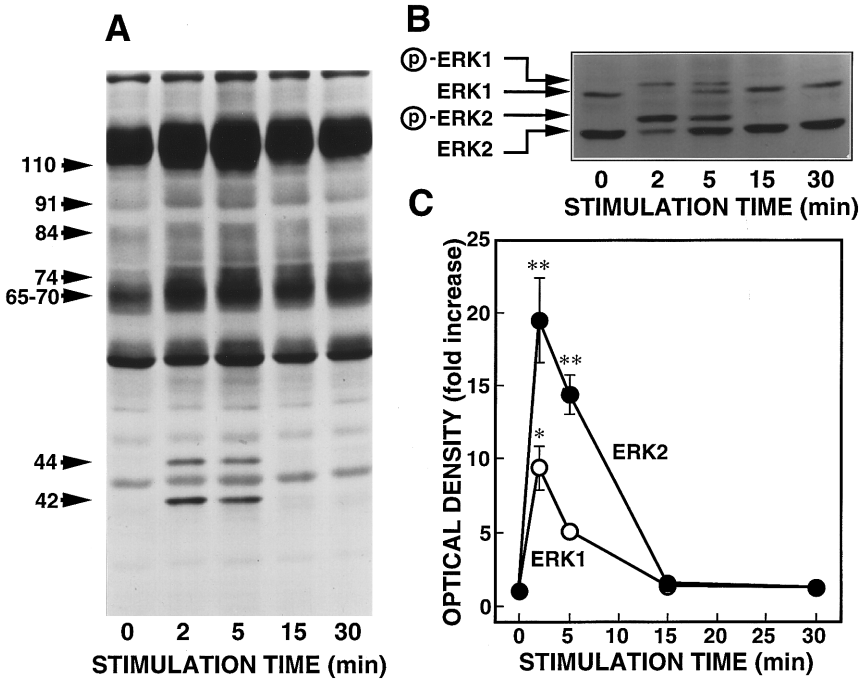

Fig. 1. Carbachol-induced protein tyrosine phosphorylation and extracellular signal-regulated kinase (ERK) activation in PC12 cells. (*A*) The cells were stimulated with 1 mM carbachol for the indicated times. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis with anti-phosphotyrosine antibody were performed. Carbachol induced rapid and transient increases in tyrosine phosphorylation of several proteins. The molecular mass markers, in kilodaltons, are indicated on the left. (*B*) By using anti-ERK antibody, 44- and 42-kd phosphoproteins were identified as active forms of ERK1 and ERK2. The upper two arrows on the left indicate ERK1 (Ⓟ, active forms of ERK1) and the lower two arrows indicate ERK2 (Ⓟ, active forms of ERK2). (*C*) The density of bands of activated ERKs was measured by the densitometer (○ ERK1; ● ERK2). Data represent the mean ±SD from three experiments. *$P < 0.05$ *versus* 0 min; **$P < 0.0001$ *versus* 0 min.

subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) and transferred electrophoretically to a nitrocellulose membrane. After the membranes were blocked with TBS-T (10 mM Tris-HCl, *p*H 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 2% bovine serum albumin, membranes were incubated with anti-phosphotyrosine antibody or anti-ERK antibody at room temperature for 90 min and then with the goat anti-mouse immunoglobulin G horseradish peroxidase–coupled secondary antibody at room temperature for 60 min. Detection was performed using an enhanced chemiluminescence system. The density of protein bands was analyzed using a densitometer (Densitograph; Atto Corporation, Tokyo, Japan).

Statistical Analyses

Data are presented as the mean ± SD from three experiments. Differences between values were evaluated using analysis of variance; when $P < 0.05$, differences were considered significant.

Results

Tyrosine Phosphorylation of Cellular Proteins and Extracellular Signal-regulated Kinase Activation in Response to Carbachol in PC12 Cells

Treatment of cells with 1 mM carbachol induced rapid and transient increases in tyrosine phosphorylation of several proteins with approximate molecular weights of 111, 91, 84, 74, 65–70, 44, and 42 kd. Among these, two phosphoproteins with molecular masses of 44 and 42 kd were most distinct, and phosphorylation of both pro-

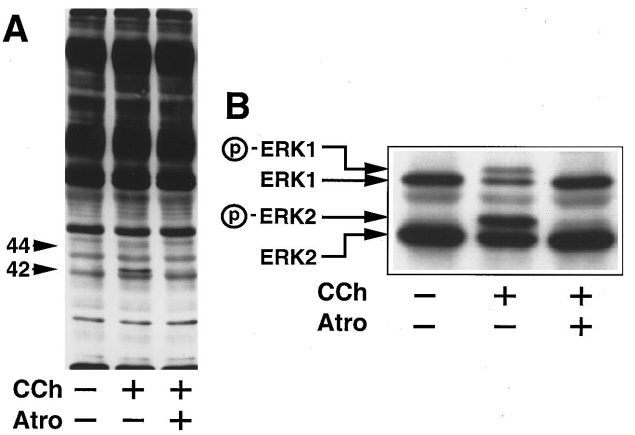

Fig. 2. Muscarinic receptor–mediated tyrosine phosphorylation and activation of extracellular signal-regulated kinase (ERKs). PC12 cells were treated with the absence (−) or the presence (+) 50 μM atropine (Atro) for 10 min and then stimulated with 1 mM carbachol for 2 min. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis with anti-phosphotyrosine antibody or anti-ERK antibody were performed. (*A*) The presence of atropine abolished tyrosine phosphorylation of 44- and 42-kd proteins. The molecular mass markers of p44 and p42 expressed in kilodaltons are indicated on the left. (*B*) Atropine completely blocked the activation of ERK1 and ERK2. The upper two arrows on the left indicate ERK1 (Ⓟ, active forms of ERK1) and the lower two indicate ERK2 (Ⓟ, active forms of ERK2).

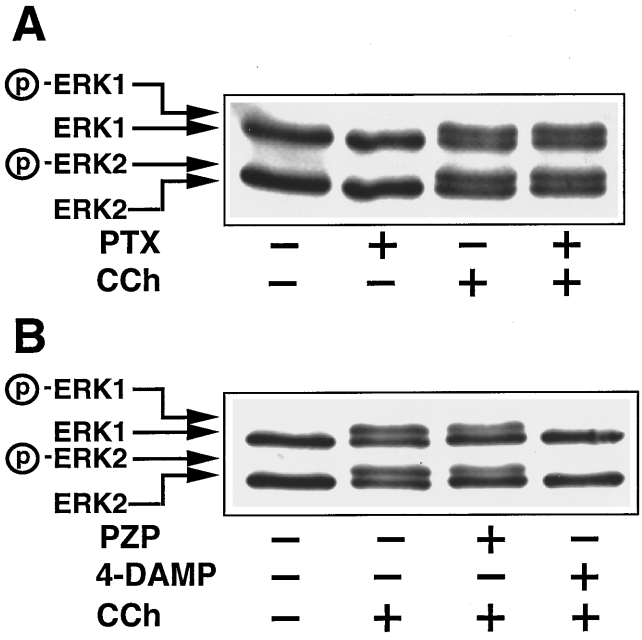

Fig. 3. The muscarinic acetylcholine receptor (mAChR) subtype involved in carbachol-induced extracellular signal-regulated kinase (ERK) activation in PC12 cells. (*A*) Carbachol-induced ERK activation insensitive to pertussis toxin. The cells were treated with or without 10 ng/ml pertussis toxin (PTX) for 21 h and then stimulated with the presence (+) or the absence (−) of 1 mM carbachol for 2 min. (*B*) The effects of mAChR antagonists on carbachol-induced ERK activation. The cells were stimulated with or without 1 mM carbachol in the presence (+) or the absence (−) of 10 μM pirenzepine (PZP) or 4-diphenyacetooxy-1,1-dimethylpiperidinium (4-DAMP). The antagonists were added 10 min before the addition of carbachol. Ⓟ-ERK1 and Ⓟ-ERK2 indicate activated ERK1 and ERK2.

teins reached a peak 2 min after stimulation of carbachol (fig. 1A). These two proteins were identified as active forms of ERK1 and ERK2 by Western blot analysis using anti-ERK antibody. As shown in figure 1B, two bands of ERKs were presented as doublets 2–15 min after carbachol stimulation. Tyrosine-phosphorylated bands of 44- and 42-kd proteins were overlapped with upper bands of ERKs doublets, respectively (figs. 1A and 1B). As determined by densitometric analysis, active forms of ERKs reached the maximum (about 20 times more than nonstimulated levels for ERK2 and 10 times more than nonstimulated levels for ERK1) at 2 min and returned to the basal level 15 min after stimulation (fig. 1C).

Such carbachol-induced tyrosine phosphorylation of 44- and 42-kd proteins was blocked completely by preincubation with 50 μM atropine. This indicates that carbachol-induced activation of ERK1 and ERK2 was through the mAChRs in PC12 cells (figs. 2A and 2B). The mAChR-mediated ERK activation was insensitive to pertussis toxin (fig. 3A). Pretreatment of PC12 cells with pertussis toxin (10 ng/ml) for 21 h failed to suppress the carbachol-induced activation of ERK1 and ERK2. To determine which subtype of mAChRs was involved, the cells were pretreated with 10 μM pirenzepine, the M_1 receptor antagonist, and with 10 μM 4-diphenyacetooxy-1,1-dimethylpiperidinium, the M_3 receptor antagonist. The carbachol-induced activation of ERKs was blocked completely by 4-diphenyacetooxy-1,1-dimethylpiperidinium but not by pirenzepine (fig. 3B).

Effects of Local Anesthetics on Carbachol-induced Protein Tyrosine Phosphorylation in PC12 Cells

In PC12 cells preincubated with 1–10 × 10^{-4} M procaine for 10 min, carbachol-induced protein tyrosine

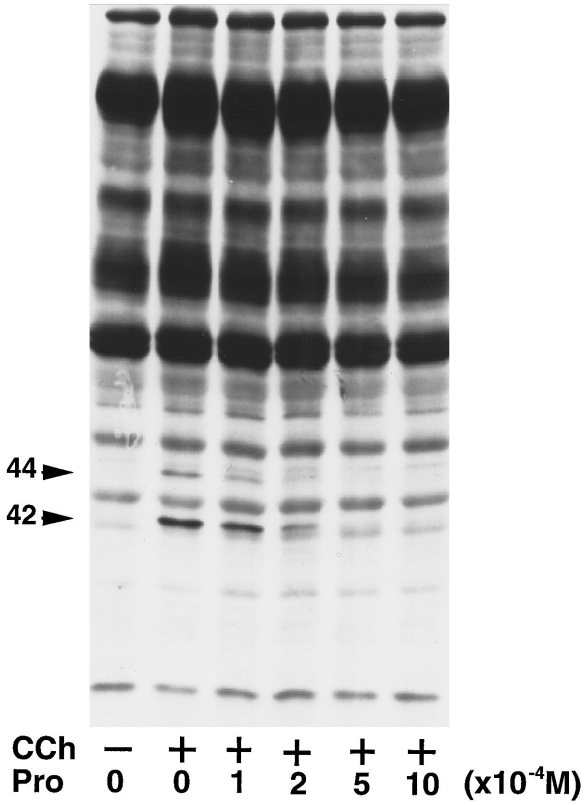

Fig. 4. The effects of procaine on carbachol-induced protein tyrosine phosphorylation in PC12 cells. The cells were treated with the indicated concentrations of procaine (Pro) for 10 min and then stimulated in the presence (+) or the absence (−) of 1 mM carbachol for 2 min. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis with anti-phosphotyrosine antibody were performed. The carbachol-induced protein tyrosine phosphorylation of 44- and 42-kd proteins was inhibited by procaine in a dose-dependent manner. The molecular mass markers of p44 and p42, expressed in kilodaltons, are indicated on the left.

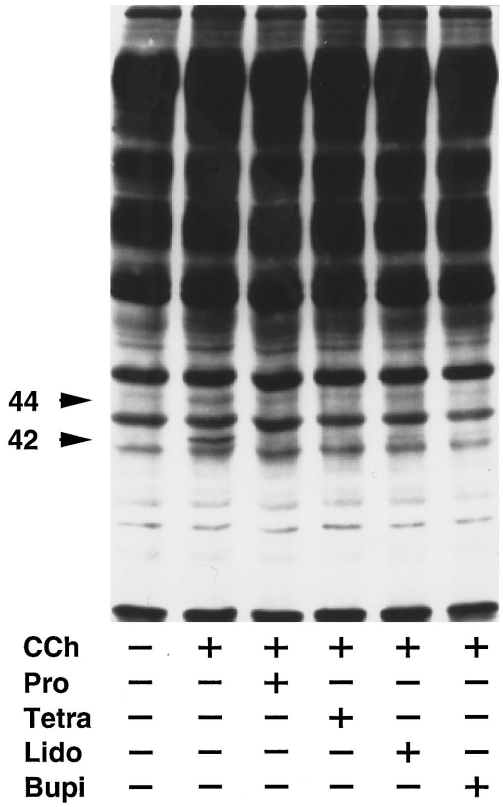

Fig. 5. The inhibitory effects of local anesthetics on carbachol-induced protein tyrosine phosphorylation in PC12 cells. The cells were treated in the presence (+) or the absence (−) of 5 × 10^{-4} M procaine (Pro), tetracaine (Tetra), lidocaine (Lido), or bupivacaine (Bupi) for 10 min and then stimulated with 1 mM carbachol for 2 min. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis with anti-phosphotyrosine antibody were performed. The carbachol-induced protein tyrosine phosphorylation of 44- and 42-kd proteins was inhibited by the presence of each local anesthetic. The molecular mass markers of p44 and p42, expressed in kilodaltons, are indicated on the left with arrows.

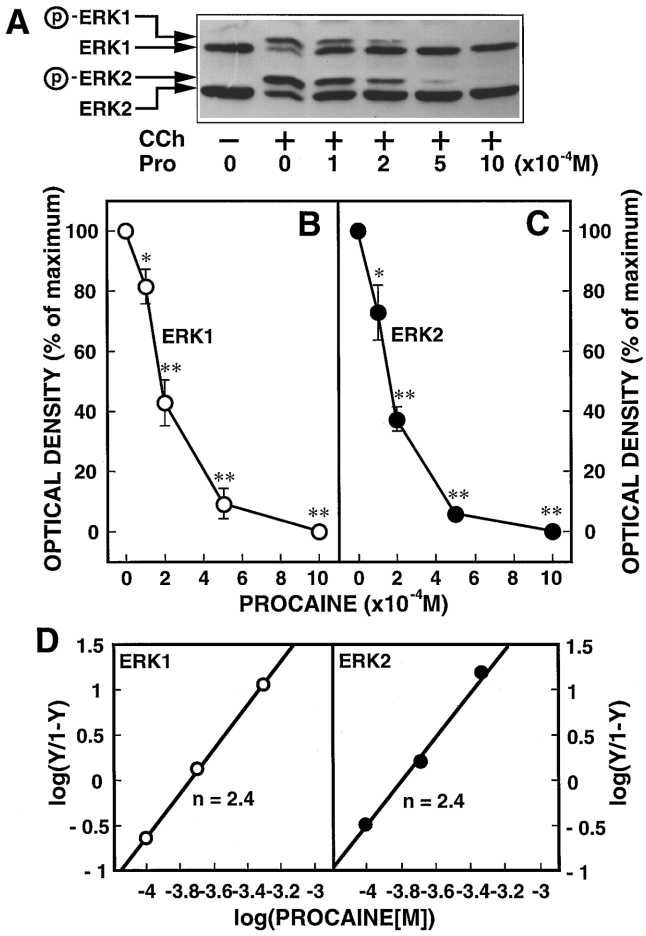

Fig. 6. The effects of procaine on carbachol-induced extracellular signal-regulated kinase (ERK) activation in PC12 cells. The cells were preincubated with the indicated concentration of procaine (Pro) for 10 min and then stimulated in the presence (+) or the absence (−) of 1 mM carbachol for 2 min. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis with anti-ERK antibody were performed. (*A*) Procaine dose dependently inhibited the activation of ERKs. The upper two arrows on the left indicate ERK1 (Ⓟ, active forms of ERK1) and the lower two arrows indicate ERK2 (Ⓟ, active forms of ERK2). The density of shifted bands (active forms) of (*B*) ERK1 and (*C*) ERK2 was measured by densitometric analysis and expressed as percentages of the result obtained in the absence of procaine. Data represent the mean ±SD from three different experiments. **P* < 0.05 *versus* 0 × 10^{-4} M; ***P* < 0.0001 *versus* 0 × 10^{-4} M. (*D*) The Hill plots of the procaine inhibition of the carbachol-induced ERK activation are shown. Y and 1−Y represent inhibited and noninhibited ERK activation, respectively. The Hill plots were made by computerized analysis of log [Y/1−Y] *versus* log procaine concentration, thereby yielding the Hill coefficients n = 2.4 for the effects of procaine on ERK1 and ERK2.

phosphorylation decreased significantly. Procaine suppressed tyrosine phosphorylation of ERKs in a concentration-dependent manner and almost completely abolished tyrosine phosphorylation of these proteins at 5 × 10^{-4} M (fig. 4). Pretreatment of PC12 cells with tetracaine, lidocaine, and bupivacaine (5 × 10^{-4} M) also inhibited carbachol-induced tyrosine phosphorylation of ERKs (fig. 5).

Inhibitory Effects of Local Anesthetics on Carbachol-induced Extracellular Signal-regulated Kinase Activation

Phosphorylation of ERK1 and ERK2 was prevented by procaine in a dose-dependent manner (fig. 6A). Some

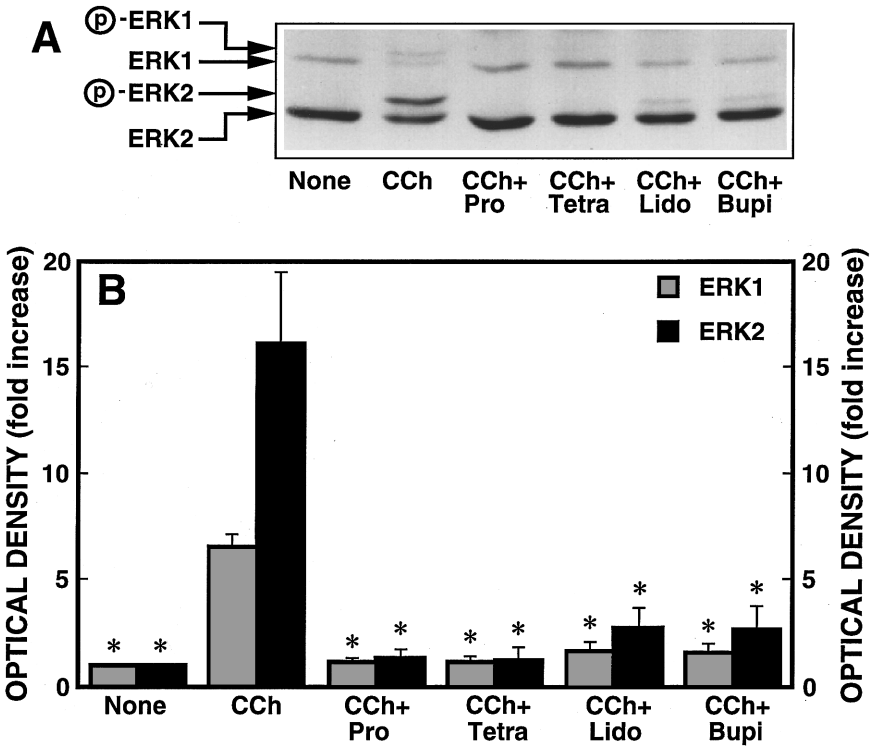

Fig. 7. The inhibitory effects of local anesthetics on carbachol-induced extracellular signal-regulated kinase (ERK) activation in PC12 cells. The cells were treated in the presence or the absence of 5×10^{-4} M procaine (Pro), tetracaine (Tetra), lidocaine (Lido), or bupivacaine (Bupi) for 10 min and then stimulated with or without 1 mM carbachol for 2 min. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis with anti-ERK antibody were performed. (*A*) The activated forms of ERKs were prevented by the presence of each local anesthetic. The upper two arrows on the left indicate ERK1 (Ⓟ, active forms of ERK1) and the lower two arrows indicate ERK2 (Ⓟ, active forms of ERK2). (*B*) The density of shifted bands (active forms) of ERK1 and ERK2 was measured using the densitometric analysis. Data represent the mean ± SD of three experiments. *$P < 0.0001$ *versus* carbachol.

significant effect ($P < 0.05$) was apparent with 10^{-4} M procaine. Activation of these ERKs was almost completely suppressed in the presence of 5×10^{-4} M procaine in PC12 cells (figs. 6B and 6C). The presence of tetracaine, lidocaine, and bupivacaine at 5×10^{-4} M similarly inhibited the activation of both ERKs (fig. 7). Although procaine and tetracaine appeared to be more potent than lidocaine and bupivacaine, the effects of each local anesthetic did not differ significantly ($P > 0.05$) at 5×10^{-4} M.

Effects of Procaine on 4β-Phorbol 12-Myristate 13-Acetate–induced Extracellular Signal-regulated Kinase Activation

A commonly used activator of PKC as a receptor-bypass stimuli, PMA caused activation of ERK1 and ERK2 and peaked 15 min after stimulation in PC12 cells (data not shown). We then considered whether procaine exerted inhibitory effects on PMA-induced ERK activation. Although 5×10^{-4} M procaine almost completely suppressed carbachol-induced ERK activation (fig. 8A), it did not have any effects on PMA-induced ERK activation (fig. 8B).

Effects of Na^+ Current–modifying Reagents on Carbachol-induced Extracellular Signal-regulated Kinase Activation

Carbachol-induced ERK activation was not affected by preincubating PC12 cells with tetrodotoxin or veratridine at 1×10^{-6} M (fig. 9A). In addition, at 5×10^{-3} M ouabain also had no effects on the carbachol-induced activation of ERKs (fig. 9B).

The Effects of Procaine on the AlF_4^--induced Extracellular Signal-regulated Kinase Activation and the Effect of Protein Phosphatase on Procaine-inhibited, Carbachol-induced Extracellular Signal-regulated Kinase Activation

Because we knew that the combination of NaF plus $AlCl_3$ (presumably acting as AlF_4^-) can directly activate G proteins,[16] PC12 cells were exposed to NaF plus $AlCl_3$ to determine whether the ERK cascade downstream of the G proteins in PC12 cells is influenced by procaine. AlF_4^- caused a time-dependent activation of ERK1 and ERK2 (data not shown). However, 5×10^{-4} M procaine did not affect the AlF_4^--induced ERK activation (fig. 10A). In addition, pretreatment of PC12 cells with 10 nM calyculin A, a potent phosphatase inhibitor, did not mod-

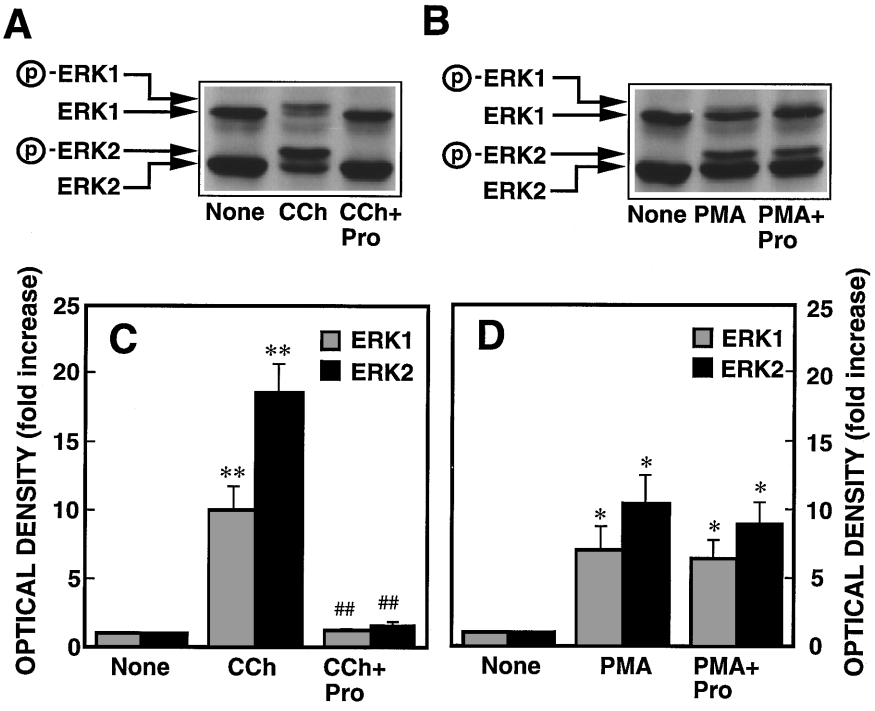

Fig. 8. Comparison of the effects of procaine on carbachol- and 4β-phorbol 12-myristate 13-acetate (PMA)–induced extracellular signal-regulated kinase (ERK) activation in PC12 cells. The cells were treated in the presence or the absence of 5×10^{-4} M procaine (Pro) for 10 min and then stimulated with 1 mM carbachol for 2 min (*A*) or 200 nM PMA for 15 min (*B*). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis with anti-ERK antibody were performed. The upper two arrows on the left indicate ERK1 (Ⓟ, active forms of ERK1) and the lower two arrows indicate ERK2 (Ⓟ, active forms of ERK2). The density of shifted bands (active forms) of ERKs by (*C*) carbachol or (*D*) PMA was analyzed by the densitometer, respectively. Data represent the mean ± SD of three experiments. **P* < 0.05 *versus* none; ***P* < 0.0001 *versus* none; ##*P* < 0.0001 *versus* carbachol.

ify the effects of procaine on carbachol-induced ERK activation (fig. 10B).

Discussion

The molecular mechanisms underlying local anesthetic action for neuronal activities are not well understood. We have presented data showing that carbachol induces ERK1 and ERK2 activation that is mediated by the activation of M_3 mAChR in PC12 cells and that procaine inhibits carbachol-mediated tyrosine phosphorylation and ERK activation in a concentration-dependent manner. Although tetracaine, lidocaine, and bupivacaine similarly inhibited carbachol-induced tyrosine phosphorylation and ERK activation, neither Na^+ channel modifiers (such as tetrodotoxin, an Na^+ channel blocker, or veratridine, an Na^+ channel activator) nor ouabain, an Na^+–K^+ pump inhibitor, affected ERK activation. The inhibitory effects of local anesthetic were not observed in both PMA-induced ERK activation and AlF_4^--induced ERK activation. In addition, a potent phosphatase inhibitor did not modify the effects of procaine on the carba-

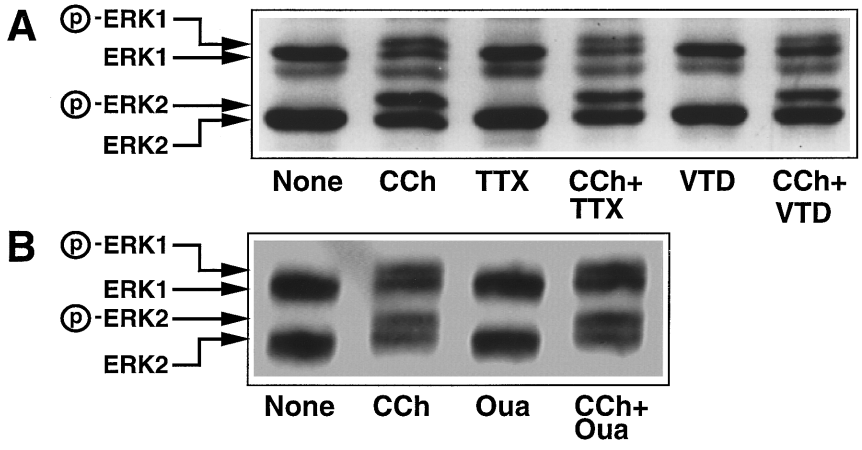

Fig. 9. The effects of (*A*) tetrodotoxin (TTX), veratridine (VTD), and (*B*) ouabain (Oua) on carbachol-induced activation of extracellular signal-regulated kinases (ERKs) in PC12 cells. The cells were treated in the presence or absence of 1×10^{-6} M tetrodotoxin, 1×10^{-6} M veratridine, or 5×10^{-3} M ouabain for 10 min and then stimulated with or without 1 mM carbachol for 2 min. Extracted proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-ERK antibody. The upper two arrows on the left indicate ERK1 (Ⓟ, active forms of ERK1) and the lower two arrows indicate ERK2 (Ⓟ, active forms of ERK2).

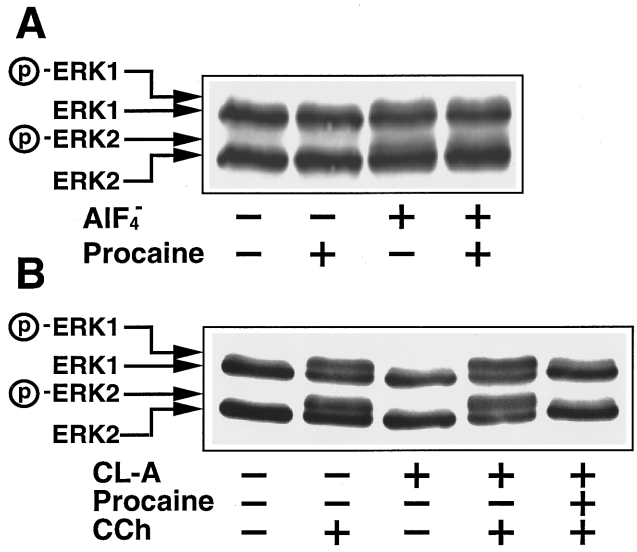

Fig. 10. The mechanisms of the effects of procaine on carbachol-induced extracellular signal-regulated kinase (ERK) activation in PC12 cells. (*A*) There were no effects of procaine on the AlF_4^--induced ERK activation. The cells were treated with or without 5×10^{-4} M procaine and then stimulated with 10 mM NaF plus 10 μM $AlCl_3$ for 30 min. (*B*) There were no effects of calyculin A on the procaine inhibiting the carbachol-induced ERK activation. The cells were exposed to the presence (+) or the absence (−) of 10 nM calyculin A for 10 min and then 5×10^{-4} M procaine was added. Ten minutes after procaine treatment, the cells were stimulated with 1 mM carbachol for 2 min. Proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-ERK antibody. The upper two arrows on the left indicate ERK1 (ⓟ, active forms of ERK1) and the lower two indicate ERK2 (ⓟ, active forms of ERK2).

chol-induced ERK activation. Therefore, the site(s) of inhibition of local anesthetics is probably located at mAChR or an interface between the receptors and G protein (fig. 11), rather than in the downstream of G protein. The ERK signaling cascade was recently shown to play an important role in the receptor-mediated signaling processes in neuronal cells.[10,11] The current results would provide an important clue for better understanding the molecular mechanism of the local action of an anesthetic.

The MAPKs are a family of serine–threonine protein kinases that are activated in response to various stimuli and play important roles in cellular signal transduction.[9] Among three major MAPK cascades, ERK, Jun N-terminal kinase/stress-activated protein kinase, and p38, which was identified recently,[10] the ERK studied in the current investigation has been shown to be involved in development, proliferation, plastic functions such as long-term potentiation,[17] and apoptosis.[18] PC12 cells are stimulated with carbachol *via* heterotrimetric G protein–coupled mAChR,[19,20] followed by the direct binding of the adapter protein Grb2 to Sos.[10] Then the Grb2–Sos complex activates the nucleotide exchange activity on Ras, which leads to activation of the Raf–ERK pathway.[10] In addition to this pathway, the Ras-independent pathway may involve PKC.[10]

Phospholipase C activated by heterotrimeric G protein acts on phosphatidylinositol 4,5-bisphosphate to produce inositol(1,4,5)-triphosphate and diacylglycerol, both of which serve as second messengers to mobilize calcium and activate PKC, respectively.[21,22] Protein kinase C could be involved in the ERK activation by Raf phosphorylation on serine residues.[23] Our current findings that carbachol induced tyrosine phosphorylation of p42- and p44-kd proteins and ERK activation in PC12 cells correspond with results observed in oligodendrocytes, the myelin-producing cells of the central nervous system.[24] Atropine and 4-diphenyacetooxy-1,1-dimethylpiperidinium, an M_3 mAChR antagonist, blocked concentration-dependent MAPK activation induced by carbachol. Activation of ERKs is implicated in the transmission of the signal for mAChR.[24–26] Although it is not clear whether the inhibition of M_3 mAChR-mediated tyrosine phosphorylation and ERK activation with procaine, lidocaine, bupivacaine, and tetracaine at clinically relevant concentrations is related to their anesthetic action, to toxic side effects, or both, many pharmacologic properties of local anesthetics can be attributed to their actions on signal transduction molecules.

Local anesthetics are known to bind to mAChRs[27] and nicotinic AChRs.[1] With open channels, anesthetic binding to the AChR occurs immediately after agonist stimulation.[1,27] Cohen-Armon *et al.*[19,20] have found with membrane prepared from brain that the binding of agonists to mAChRs and to the Na^+ channels in the open state is a coupled event mediated by G proteins. When an agonist-like carbachol binds to its specific receptor, mAChR, the G protein mediates the activity of transmembrane channels through which ions flow, leading to changes in transmembrane voltage that could trigger signal transmission, and thus signal transduction molecules could be altered. Inhibitors such as procaine, as a cationic inhibitor of the AChR, can bind to the inhibitory site before the channel opens and regulates the ion permeability of the AChR.[1,27] Local anesthetics can allosterically interact with mAChR,[28,29] and it has been reported that lidocaine interacts with primary and allosteric recognition sites on mAChR.[29] After we plotted a curve fit for Hill equation, we found that the Hill coefficient for the procaine con-

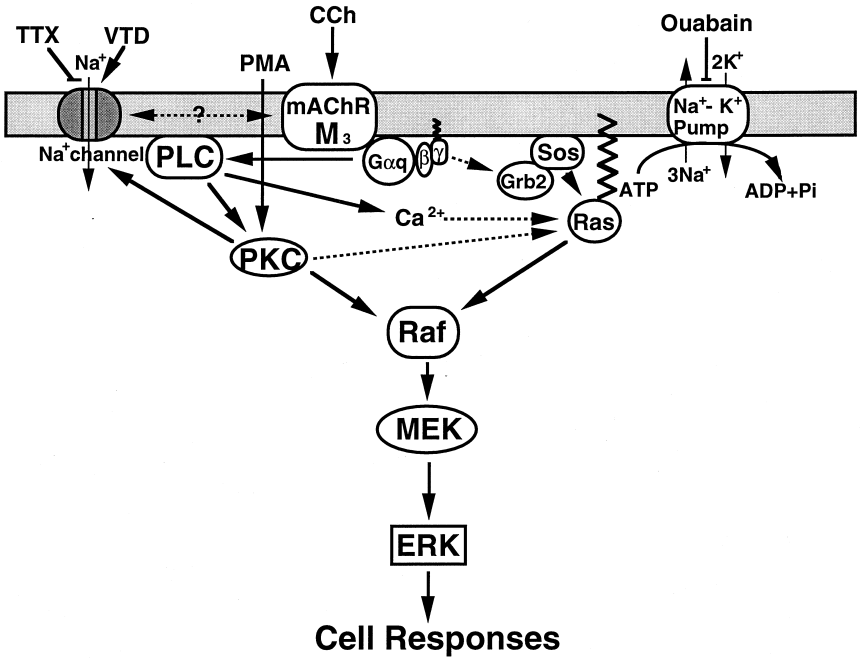

Fig. 11. A hypothetical scheme of the effects of local anesthetics on muscarinic receptor–mediated extracellular signal-regulated kinases (ERKs) in PC12 cells. The stimulation of muscarinic receptors in the cellular membranes initiates a sequence of activation of protein kinase C, Ras, Raf, and mitogen-activated protein kinase (MEK) that in turn activates ERKs. Local anesthetics perturb the cascade of the m_3 AChR-mediated ERK activation. In addition, the action of local anesthetics is independent of their effects on the voltage-dependent Na^+ channels. Inhibition of Na^+–K^+ pumps does not affect the muscarinic acetylcholine receptor–mediated ERK activation.

centration–inhibition relation was 2.4 (fig. 6D), it can be assumed that the inhibition of local anesthetic on ERK activation involves multiple sites of action. The current observations may suggest that suppression of mAChRs would be involved, at least in part, in the local anesthetic inhibition of carbachol-induced ERK activation.

Modulation of Na^+ channels by PKC is likely to have important effects on signal transduction and synaptic transmission in the central nervous system.[2] The action of ion channels and Na^+–K^+ pumps, which are closely associated with PKC activity, has a central role in the regulation of neuronal excitability, so PKC has been considered as a major site of signal transduction molecules for anesthetic action.[6,30] The increased activity of PKC modulates neuronal signal transduction by phosphorylation of several membrane proteins, including voltage-dependent Na^+ and other ion channels.[31] The phosphorylation of Na^+ channels (α- subunits) could occur by altering PKC activity.[32] Although PKC-activated Raf is thought to be Ras independent, stimulation of PKC in COS cells leads to Ras activation followed by formation of the Ras–Raf-1 complex,[33] suggesting that PKC could activate ERKs by a mechanism distinct from that initiated by mAChR stimulation by carbachol. Activation of ERKs *via* the M_3 mAChR subtype, such as M_1 and M_5, is independent of intracellular or extracellular Ca^{2+} but depends in part on PKC.[26]

The effects of local anesthetics on PKC, however, are complicated. In a recent *in vivo* study, Nivarthi *et al.*[6] found that the PKC activity of the spinal cord was increased with clinically relevant concentrations of intrathecal procaine and tetracaine. In *in vitro* experiments, dibucaine and tetracaine competitively inhibit the binding of PMA to its receptor.[34] Unlike staurosporin, an inhibitor of PKC, neither 10^{-5} to 10^{-3} M ropivacaine, 10^{-3} M bupivacaine, nor $1–3 \times 10^{-3}$ M lidocaine exerts any effects on the PMA-induced inhibition of phosphoinositide breakdown in human SK-N-MC neuroblastoma cells.[35] Because Na^+ channels in PC12 cells could be blocked completely by 5×10^{-4} M procaine,[36] the fact that PMA-induced ERKs activation was not affected by procaine suggests that PMA-stimulated PKC activity remained intact in our experiments. Therefore, inactivation or activation of PKC, in the presence of local anesthetics, might not influence the effects on ERKs in any important way. Therefore, the effects of local anesthetics on carbachol-induced ERK activation is unlikely to be involved in the inhibition *via* Na^+ channels or the PKC-dependent pathways at the concentration used in the current experiments.

We can only speculate why the Na^+ current-modifying reagents did not affect mAChR-mediated ERK activation. Several Na^+ transport pathways, such as the Na^+ channel and the Na^+–K^+ pump, are implicated in changes in the intracellular Na^+ concentration. The membrane depolarization of the voltage-gated Na^+ channel affects G

protein–coupled mAChR only if the voltage-gated Na^+ channels can be activated,[20] and the blockade of Na^+ channels by tetrodotoxin decreases muscarinic agonist binding to the receptors, whereas an Na^+ channel activator such as veratridine increases the binding.[19]

Furthermore, it has been reported that the tyrosine phosphorylation process seems to directly control the Na^+–K^+ pump activity in the proximal convoluted tubule,[37] and veratridine stimulates phosphoinositide breakdown, which is inhibited by local anesthetic.[38] Therefore, we assumed that tetrodotoxin, veratridine, and ouabain could have some effect on muscarinic signal transduction of ERKs. However, we observed no effect in the presence of those Na^+-modifying reagents. It is possible that such Na^+-modifying reagents may not affect cellular events *via* pertussis toxin–insensitive G proteins, such as carbachol-induced ERK activation. In human neutrophils, tetrodotoxin and veratridine have been reported not to affect PMA-induced O_2^- generation, but eight local anesthetics, including procaine, suppress it in their concentration-dependent and lipid solubility–dependent manners.[39]

The application of carbachol reduces the peak Na^+ current without changing the voltage dependency of the channels, and the activation of mAChR strongly modulates Na^+ channel activity.[40] In addition, the channel-mediated Na^+ entry and Na^+–K^+ pump activity are functionally interdependent,[41] and binding to mAChR is voltage dependent.[20] Therefore, it is conceivable that the stimulation by carbachol might have already altered the channel functions and Na^+–K^+-pump functions of PC12 cells, so neither tetrodotoxin, veratridine, nor ouabain may cause any change in mAChR-mediated events. Although we cannot completely exclude the possibility that Na^+-modifying reagents could affect ERK activation, the inhibition of mAChR-induced ERK activation by local anesthetics seems likely to be independent of blockade of Na^+ currents and electrochemical gradients across the PC12 cell membrane.

It is unclear whether a potential interaction with M_3 mAChR plays any role in local anesthetic action or state. As shown in the current results and by findings of other investigations, M_3 mAChRs are coupled to a pertussis toxin–insensitive G protein and seem to regulate the activation of membrane-bound guanylyl cyclase.[14,42] The M_3 mAChRs have been reported to exist extensively in the spinal cord[43] and in brain regions, including the cerebral cortex, caudate nucleus, globus pallidus, the substantia nigra, and the hypothalamus,[44] and to play some role in neuronal cellular activities, such as N-methyl-D-aspartate receptor-mediated adenosine release in the cortex.[44] An activator of G protein, AlF_4^-, caused time-dependent ERK1 and ERK2 activation, but 5×10^{-4} M procaine did not affect the AlF_4^--induced activation (fig. 10A). Neither did a phosphatase inhibitor modify the effects of procaine on the carbachol-induced ERK activation. Therefore, these data may provide further evidence that the inhibition of muscarinic signaling by local anesthetics that we observed could exist in some specific sites of interaction with M_3 mAChR signaling molecules, probably at the mAChR, an interface between mAChR and G protein, or membrane lipid microdomains around the receptors and G-protein molecules, but this is unlikely because of the effect on G proteins *per se*. The inhibition of M_3 mAChR-mediated ERK activation should be considered a convincing candidate for one target of local anesthetic action. Because little work has been done on the local anesthetic effects on ERK signaling, further studies are needed to determine the precise sites of local anesthetic action on the ERK signaling molecules.

In conclusion, the local anesthetics procaine, lidocaine, tetracaine, and bupivacaine, at their clinically relevant concentrations, inhibit carbachol-induced ERK activation, which is implicated in the transmission of signals *via* M_3 mAChR-mediated cellular functions in PC12 cells. The target(s) that local anesthetics affect may be upstream of G proteins, including mAChR. Our findings may provide evidence that the inhibition of the ERK cascade is crucial for the action of local anesthetics. The lack of effect of Na^+-current–modifying reagents on the muscarinic receptor-mediated ERK activation may suggest a great diversity of electrophysiologic and pharmacologic properties of local anesthetic action in signaling.

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