Pb$^{2+}$ via Protein Kinase C Inhibits Nicotinic Cholinergic Modulation of Synaptic Transmission in the Hippocampus

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ABSTRACT

The present study was designed to investigate the effects of Pb$^{2+}$ on modulation of synaptic transmission by nicotinic receptors (nAChRs) in the rat hippocampus. To this end, inhibitory and excitatory postsynaptic currents (IPSCs and EPSCs, respectively) were recorded by means of the whole-cell mode of the patch-clamp technique from rat hippocampal neurons in culture. Acetylcholine (ACh, 1 mM; 1-s pulses) triggered GABA release via activation of α4β2 and α7 nAChRs. It also triggered glutamate release via activation of α7 nAChRs. Pb$^{2+}$ (0.1 and 1 μM) blocked ACh-triggered transmitter release. Blockade by Pb$^{2+}$ of ACh-triggered IPSCs was partially reversible upon washing of the neurons. In contrast, even after 30- to 60-min washing, there was no reversibility of Pb$^{2+}$-induced blockade of ACh-triggered EPSCs. The effects of Pb$^{2+}$ on GABA release triggered by activation of α7 and α4β2 nAChRs were mimicked by the protein kinase C (PKC) activator phorbol-12-myristate-13-acetate (1 μM) and blocked by the indolocarbazole Gö 7874 (50 nM) and the bisindolylmaleimide Ro-31-8425 (150 nM), which are selective PKC inhibitors. After washing of fully functional neuronal networks that had been exposed for 5 min to Pb$^{2+}$, the irreversible inhibition by Pb$^{2+}$ of ACh-triggered glutamate release was partially overridden by a disinhibitory mechanism that is likely to involve α4β2 nAChR activation in interneurons that synapse onto other interneurons synapsing onto pyramidal neurons. Long-lasting inhibition of α7 nAChR modulation of synaptic transmission may contribute to the persistent cognitive impairment that results from childhood Pb$^{2+}$ intoxication.

Childhood Pb$^{2+}$ poisoning represents a major concern for public health, particularly because of the cognitive deficits that persist throughout the lives of people who are exposed at early ages to low levels of this pervasive environmental pollutant (Bellinger et al., 1987; Stokes et al., 1998). Although exposure of children to low Pb$^{2+}$ levels is not associated with overt physical signs of toxicity, it causes mental retardation with selective impairments of language, cognition, behavior,

and school performance (Lidsky and Schneider, 2003; Needleman, 2004). Intellectual abnormalities can be seen even in children who have blood Pb$^{2+}$ concentrations well below 10 μg/dl (Lanphear et al., 2000), the level of concern for exposure of children to Pb$^{2+}$ established in 1991 by the Centers for Disease Control and Prevention. The negative impact of low-level Pb$^{2+}$ intoxication on brain functioning increases with the duration of the exposure and is inversely proportional to the age at which children are exposed to the heavy metal (Lidsky and Schneider, 2003).

Currently, treatment of Pb$^{2+}$ intoxication relies on the use of chelators, which are recommended by the Food and Drug Administration for children with Pb$^{2+}$ blood levels ≥45 μg/dl (Nightingale, 1991). However, chelation therapy does not protect these children or those with Pb$^{2+}$ blood levels between 10 and 45 μg/dl from the developmental neurological impairments that result from exposure to the heavy metal (Rogan et al., 2001). Poor understanding of the means by which neuronal functions are affected by Pb$^{2+}$ accounts for the persistent cognitive impairment that results from childhood Pb$^{2+}$ intoxication.

ABBREVIATIONS: VGCC, voltage-gated Ca$^{2+}$ channels; NMDA, N-methyl-D-aspartate; nAChR, nicotinic acetylcholine receptor; PKC, protein kinase C; IPSC, inhibitory postsynaptic current; EPSC, excitatory postsynaptic current; ACh, acetylcholine; PMA, phorbol 12-myristate 13-acetate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; MLA, methyllycaconitine; DHβE, dihydro-β-erythroidine.
least in part, for the lack of effective therapeutic approaches to reverse the Pb\textsuperscript{2+}-induced cognitive deficits.

Establishment of neuronal circuitries in the developing central nervous system depends on the pattern of electrical activity going through the synapses. At early stages of brain development, most neurons fire spontaneously, and this spontaneous electrical activity, which ultimately controls synaptic strength, seems to be crucial for axon outgrowth, pruning of synaptic connections, and maturation of neuronal signaling properties (Moody, 1998). Therefore, it is conceivable that learning disabilities in children exposed to low levels of Pb\textsuperscript{2+} result from direct changes that the heavy metal imparts on synaptic activity particularly in the hippocampus, a major brain area that is involved in cognitive processing and accumulates significant amounts of Pb\textsuperscript{2+} (Swanson et al., 1997).

Pb\textsuperscript{2+} alters synaptic transmission in numerous preparations by disrupting the activity of Ca\textsuperscript{2+}-regulated proteins, including protein kinases, voltage-gated Ca\textsuperscript{2+} channels (VGCC), and Ca\textsuperscript{2+}-binding proteins that regulate mobilization and docking of synaptic vesicles (Atchison, 2003; Suszkiew, 2004). In primary hippocampal cultures, action potential-independent synaptic transmission is facilitated by nanomolar concentrations of Pb\textsuperscript{2+} due to an intracellular action of the heavy metal (Braga et al., 1999b). Also in these cultures, action potential-dependent synaptic transmission is inhibited by similar concentrations of Pb\textsuperscript{2+} due to blockade of VGCC (Braga et al., 1999a). In the hippocampus, however, synaptic transmission is modulated by ionotropic receptors such as N-methyl-D-aspartate (NMDA) receptors and \(\alpha7\) nAChRs (Vizi and Kiss, 1998; Pereira et al., 2002), both of which are sensitive to inhibition by micromolar concentrations of Pb\textsuperscript{2+} (Guilarte and Miceli, 1992; Ujihara and Albuquerque, 1992; Ishihara et al., 1995; Zwart et al., 1995; Marchioro et al., 1996; Mike et al., 2000b; Si and Lee, 2003).

To date, very little is known regarding signal transduction mechanisms that link nAChR activation to modulation of action potential-dependent transmitter release in the brain, and no studies have addressed how this modulatory process could be affected by Pb\textsuperscript{2+}. Thus, the present study was designed to (1) determine whether Pb\textsuperscript{2+} affects modulation by nAChRs of GABAergic or glutamatergic transmission, and, if so, by what mechanism(s); (2) compare the sensitivity to Pb\textsuperscript{2+} of whole-cell currents and transmitter release triggered by nAChR activation; and (3) examine the net effect of Pb\textsuperscript{2+} on nicotinic modulation of synaptic activity in the hippocampus. Glutamatergic and GABAergic postsynaptic currents were recorded by means of the patch-clamp technique from cultured hippocampal neurons, and the effects of acute exposure to Pb\textsuperscript{2+} on GABA and glutamate release evoked by nAChR activation were analyzed.

Results presented herein demonstrate that at nanomolar concentrations Pb\textsuperscript{2+} acts via protein kinase C (PKC) to inhibit action potential-dependent transmitter release triggered by \(\alpha7\) and/or \(\delta\) nAChR activation in hippocampal neurons. This effect, which cannot be easily reversed upon removal of Pb\textsuperscript{2+} from the extracellular compartment, causes a long-lasting disruption of the activity of large neuronal networks and can, therefore, contribute to the cognitive impairment induced by Pb\textsuperscript{2+}. These findings and the lack of therapeutic approaches to reverse the complex Pb\textsuperscript{2+}-PKC interactions emphasize the concept that preventive measures are still by far the best means to protect children against Pb\textsuperscript{2+}-induced neurological deficits.

Materials and Methods

Cultures of Hippocampal Neurons. Hippocampal neurons were harvested from 16- to 18-day-old fetal Sprague-Dawley rats and cultured according to the procedure described by Ujihara and Albuquerque (1992). Neurons cultured for 10 to 30 days were used in this study.

Electrophysiological Recordings. Whole-cell currents, IPSCs, and EPSCs were recorded through an LM-EPC-7 amplifier (List Electronics, Heidelberg, Germany) from cultured hippocampal neurons using the whole-cell mode of the patch-clamp technique (Hamill et al., 1981). After being filtered at 3 kHz (8-pole Bessel filter), the signals were stored on videocassette recording tapes, digitized at 50 \(\mu\)s, and analyzed off-line. The physiological solution used to perfuse the neurons at a rate of 2 to 4 ml/min had the following composition: 165 mM NaCl, 5 mM KCl, 2 mM CaCl\textsubscript{2}, 10 mM glucose, 5 mM HEPES, and 0.001 mM atropine (pH 7.3 adjusted with NaOH; 340 mOsM). Tetrodotoxin (200 nM) was added to this solution for recordings of agonist-evoked whole-cell currents. The physiological solution used to fill the patch pipettes had the following composition: 160 mM CsCl, 10 mM Cs-ethylglucosyl-bis(\beta-aminoethyl ether)-N,N'-tetraacetic acid, and 10 mM HEPES (pH adjusted with CsOH; 340 mOsM). The patch pipettes were pulled from borosilicate capillary glass (World Precision Instruments, Inc., Sarasota, FL), and when filled with physiological solution, had resistances in the range of 2 to 5 M\(\Omega\).

In some experiments, whole-cell currents were recorded from cultured hippocampal neurons under the perforated-patch configuration. In these experiments, the external solution had the same composition as that described above for recording of whole-cell currents, and the pipette solution was composed of: 60 mM CsCl, 75 mM Cs-methanesulfate 5 mM MgCl\textsubscript{2}, and 10 mM HEPES (pH adjusted with CsOH; 7.3 with CsOH). Nystatin was dissolved in dimethyl sulfoxide (50 mg/ml) and diluted in this pipette solution to a final concentration of 200 \(\mu\)g/ml.

All experiments were performed at room temperature (22–25°C). Agonist-containing solutions were applied to the neurons via a glass U-tube (Ujihara and Albuquerque, 1992). The U-tube was positioned at about 100 to 150 and 60 to 80 \(\mu\)m away from the neurons from which PSCs and whole-cell currents were recorded, respectively. Specific antagonists and Pb\textsuperscript{2+} (as PbCl\textsubscript{2}) were applied to the neurons via the bath perfusion and in admixture with the agonists via the U-tube.

Data Analysis. IPSCs and EPSCs were analyzed using the pCLAMP6 software (Axon Instruments, Foster City, CA) and a suite of the Continuous Data Recording program (Dempster, 1989). Under each experimental condition, the baseline frequency of IPSCs and EPSCs was estimated using the Continuous Data Recording software by averaging the number of events recorded for 3 min before exposure of the neurons to ACh. At the beginning of the agonist pulse, there was a summation of events. The average frequency of events triggered by ACh was determined as the number of events recorded for 60 s after the end of the ACh pulse. The average number of events per minute recorded after this time was the same as the baseline frequency of events recorded before the agonist pulse. Normalizing the frequency of events triggered every 2 min by 1-s pulses of ACh to the baseline frequency of events recorded before the agonist pulse revealed that the effect of ACh on IPSCs or EPSCs did not run down with recording time (which varied from 60 to 90 min; \(n = 5\) neurons). Alternatively, using the pCLAMP6 software the net charge of IPSCs or EPSCs triggered by 1-s pulse of agonist-free or agonist-containing external solution was calculated as the area under the curve delimited by the pulse. Then, the net charge of events recorded during the agonist-free pulse was subtracted from the net charge of events triggered by the agonist. Subsequently, the base-
line-corrected net charge of agonist-triggered synaptic events recorded under control conditions was taken as 100% and compared with that recorded under different test conditions.

Macrosopic nicotinic currents recorded from neurons under the whole-cell or the perforated-patch configuration were characterized as type IA if they decayed to the baseline during the time the neurons were exposed to ACh. To correct for rundown, the amplitudes of type IA currents evoked by 1-s pulses of ACh (1 mM) applied to the neurons every 30 s for 10 min were plotted against recording time. Fitting the data points to a single- or a double-exponential function allowed for estimation of the amplitude of currents at any recording time. All results are presented as mean ± S.E.M., and differences between results obtained from a group of cells during a treatment and those obtained from the same cells before that treatment were statistically evaluated using the paired Student’s t test.

**Drugs and Toxins.** 6-Cyano-7-nitroquinoxaline-2,3-dione (CHQX) was obtained from Sigma/RBI (Natick, MA). Dihydro-β-erythroidine (DHBE) was a gift from Merck Sharp and Dohme (Rahway, NJ). Methyllycaconitine citrate (MLA) was kindly provided by Dr. M. H. Benn (Department of Chemistry, University of Calgary, Calgary, AB, Canada). Phorbol 12-myristate 13-acetate (PMA), Go 7874, and Ro-31-8425 were purchased from Calbiochem (La Jolla, CA). Acetylcholine chloride, atropine sulfate, PbCl₂, tetrodotoxin, and picrotoxin were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals except picrotoxin, PMA, and Ro-31-8425 were dissolved in double-distilled water, and the stock solutions (0.01–1 M) were kept frozen until ready to use. NaOH was used to dissolve choline (10 mM, 1-s pulse) in the external solution and could be attributed to blockade by Pb²⁺ of VGCC

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**Results**

Characterization of the Effect of nAChR Activation on Spontaneous IPSCs and EPSCs Recorded from Hippocampal Neurons in Culture. As reported previously (Braga et al., 1999a), in the presence of CNQX (10 μM), an antagonist of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors, spontaneous postsynaptic currents recorded from cultured hippocampal neurons are sensitive to blockade by the GABAₐ receptor antagonist picrotoxin (100 μM). These currents, herein referred to as IPSCs, are mediated by GABA released from spontaneously firing neurons that synapse onto the neurons from which recordings are obtained. Likewise, in the presence of picrotoxin (100 μM), a GABAₐ receptor antagonist, spontaneous postsynaptic currents recorded from cultured hippocampal neurons are sensitive to blockade by CNQX (10 μM) (Braga et al., 1999a).

Application of 1-s pulses of ACh (1 mM) to cultured hippocampal neurons continuously perfused with CNQX (10 μM)-containing external solution triggered a burst of IPSCs that showed signs of summation at the beginning of the agonist pulse and lasted much longer than the agonist pulse (Fig. 1A). The effect of ACh was completely abolished by 10- to 15-min perfusion of the neurons with external solution containing both the α7 nAChR-selective antagonist MLA (1 nM) and the α4β2 nAChR-selective antagonist DHβE (100 nM) (Fig. 1, A and B). The effect of MLA and DHβE on ACh-triggered IPSCs was reversible upon washing of the neurons for 15 to 20 min with external solution (Fig. 1A), and each antagonist individually was only capable of blocking part of the ACh-triggered IPSCs (Fig. 1B). MLA (1 nM) blocked about 35% of the effect of ACh, whereas DHβE blocked approximately 65% of the ACh effect. Considering that 1-s exposure of hippocampal neurons to nicotinic agonists does not alter the activity of postsynaptic GABAₐ receptors (Alkondon et al., 1999), the present results indicate that ACh-triggered IPSCs were the result of the interaction of the agonist with both α7 and α4β2 nAChRs present on neurons synapsing onto the neurons under study. These findings are in agreement with previous reports that presynaptic/preterminal nAChRs control GABA release from hippocampal neurons (Alkondon et al., 1999; Radcliffe et al., 1999). The results described above also suggested that the contribution of α4β2 nAChRs is larger than that of α7 nAChRs to modulation of GABA release. To strengthen this concept, the effects of the α7 nAChR-selective agonist choline on IPSCs were analyzed. Choline (10 mM, 1-s pulse) increased the frequency of IPSCs. However, enhancement of IPSC frequency by choline (10 mM) was 40.4 ± 10.9% (n = 3) of that induced by ACh (1 mM), although equipotent, nearly saturating concentrations of the agonists were used to activate α7 nAChRs (Mike et al., 2000a).

In hippocampal neurons continuously perfused with external solution containing picrotoxin (100 μM), ACh (1 mM, 1-s pulse) triggered a burst of EPSCs that showed signs of summation at the beginning of the agonist pulse (Fig. 2A). The effect of ACh on EPSC frequency was not as robust as that on IPSC frequency (compare Figs. 2B and 1B) and was completely abolished by 10- to 15-min perfusion of the neurons with external solution containing MLA (1 nM) (Fig. 2, A and B). The effect of MLA on ACh-triggered EPSCs was reversible upon washing of the neurons for 15 to 20 min with external solution (Fig. 2A). Taking into account that 1-s exposure of hippocampal neurons to ACh (1 mM) does not alter the activity of postsynaptic glutamatergic receptors (Radcliffe et al., 1999, and references therein), it can be concluded that ACh-triggered EPSCs were the result of activation of α7 nAChRs present on neurons synapsing onto the neurons under study. These results concur with previous reports that activation of preterminal and/or postsynaptic α7 nAChRs facilitates the spontaneous release of glutamate from hippocampal neurons in culture and in slices (Radcliffe et al., 1999).

**Pb²⁺ Blocks ACh-Triggered IPSCs and EPSCs in Cultured Hippocampal Neurons.** Continuous perfusion for 10 to 20 min of cultured hippocampal neurons with external solution containing Pb²⁺ (100 nM or 1 μM) reduced substantially the frequencies of IPSCs (Fig. 3) and EPSCs (Fig. 4). The effect of Pb²⁺ on the frequencies of IPSCs (Fig. 5) and EPSCs (data not shown) was reversible within 1 min of washing of the neurons with nominally Pb²⁺-free external solution and could be attributed to blockade by Pb²⁺ of VGCC (Braga et al., 1999a). Evidence is now provided that after perfusion of the neurons with Pb²⁺-containing external solution, Pb²⁺ also inhibits ACh-triggered transmitter release and that this effect occurs independently of Pb²⁺-induced VGCC blockade.

In the absence of Pb²⁺, the frequency of IPSCs recorded
Fig. 1. Characterization of ACh-triggered IPSCs. A, sample recordings of IPSCs obtained from a cultured hippocampal neuron continuously perfused with CNQX (10 μM)-containing external solution. A 1-s pulse application of ACh (1 mM) to a large field of neurons synapsing onto the neuron under study resulted in a sizable, long-lasting increase in the IPSC frequency. After 10- to 15-min perfusion of the neurons with external solution containing the α4β2- and the α7-nAChR-selective antagonists DHβE (100 nM) and MLA (1 nM), respectively, ACh was unable to trigger IPSCs. The blocking effect of the antagonists was fully reversible after 15- to 20-min perfusion of the neurons with antagonist-free external solution. Contamination of these responses with postsynaptic nicotinic currents arising from activation of nAChRs present on the somatodendritic region of the neuron under study was very unlikely, because the neuron was voltage-clamped at -40 mV, and, at this holding potential, nicotinic whole-cell currents tend to be very small in amplitude due to rectification. B, quantification of the ACh-induced enhancement of the IPSC frequency and of the blockade of this effect by DHβE and/or MLA. Under each experimental condition, the baseline frequency of IPSCs recorded for 3 min before the agonist application was taken as 1 and used to normalize the frequency of IPSCs triggered by ACh (1 mM). Graph and error bars represent mean and S.E.M., respectively, of results obtained from three neurons. The dotted line represents the 100% control level for each experimental condition.

Fig. 2. Characterization of ACh-triggered EPSCs. A, sample recordings of EPSCs obtained from a cultured hippocampal neuron continuously perfused with picrotoxin (100 μM)-containing external solution. A 1-s pulse application of ACh (1 mM) to a large field of neurons synapsing onto the neuron under study increased substantially the frequency of EPSCs. A 10- to 15-min perfusion of the neurons with external solution containing the α7-nAChR-selective antagonist MLA (1 nM) resulted in the blockade of the ACh effect. The blocking effect of MLA was reversible after 15- to 20-min washing of the neurons with antagonist-free external solution. Membrane potential, -30 mV. B, quantification of the ACh-triggered increase in EPSC frequency and of the blockade of this effect by MLA. Under each experimental condition, the average baseline EPSC frequency recorded for 3 min before the agonist application was taken as 1 and used to normalize the frequency of EPSCs triggered by ACh (1 mM). Graph and error bars represent mean and S.E.M. of results obtained from three neurons. The dotted line represents the 100% control level for each experimental condition. ** indicates that results are different from control with p < 0.01 according to the paired Student’s t test. Contamination of the events with whole-cell currents arising from activation of somatodendritic nAChRs was minimized because, as stated under Materials and Methods, the average frequency of events triggered by ACh was determined as the number of events recorded for 60 s after the end of the ACh pulse.
Differential Sensitivity to \( \text{Pb}^{2+} \) of Whole-Cell Currents and IPSCs Triggered by Activation of \( \alpha 7^* \) nAChRs. To determine the sensitivity to \( \text{Pb}^{2+} \) of postsynaptic and presynaptic responses resulting from activation of \( \alpha 7^* \) nAChRs, we compared the magnitude of the effect of \( \text{Pb}^{2+} \) on the amplitude of \( \text{ACh} \) (1 mM)-evoked type IA currents (i.e., whole-cell currents subserved by somatodendritic \( \text{nAChR} \)s) to the magnitude of the effect of the heavy metal on glutamate released by \( \text{ACh} \) (1 mM)-induced activation of \( \alpha 7^* \) nAChRs.

In addition, the frequency of spontaneous IPSCs recorded for 3 min in the absence of \( \text{Pb}^{2+} \) was taken as 1 and used to normalize the frequency of spontaneous IPSCs recorded for 3 min after 5-min perfusion of the neurons with \( \text{Pb}^{2+} \)-containing external solution. Graph and error bars represent mean and S.E.M. of results obtained from three neurons. The dotted line represents the 100% control level for each experimental condition. * and ** indicate that, according to the paired Student’s \( t \) test, results were different from control with \( p < 0.05 \) and 0.01, respectively.

**Fig. 3.** Effects of \( \text{Pb}^{2+} \) on \( \text{ACh} \)-triggered IPSCs. A and B, sample recordings of IPSCs obtained from neurons continuously perfused with \( \text{CNQX} \) (10 \( \mu \text{M} \))-containing external solution. After 10- to 15-min perfusion of the neurons with 100 \( \text{nM} \) (A) or 1 \( \mu \text{M} \) (B) \( \text{Pb}^{2+} \)-containing external solution, there was a reduction of the frequency of spontaneous IPSCs and of \( \text{ACh} \)-triggered IPSCs. After 15- to 20-min washing of the neurons with nominally \( \text{Pb}^{2+} \)-free external solution, \( \text{Pb}^{2+} \)-induced reduction of the frequency of spontaneous IPSCs was fully reversible, whereas \( \text{Pb}^{2+} \)-induced blockade of \( \text{ACh} \)-triggered IPSCs was only partially reversible. Membrane potential, −30 mV. C, quantification of the effects of \( \text{Pb}^{2+} \) on the frequency of spontaneous IPSCs and on \( \text{ACh} \)-triggered IPSCs. For each experimental condition, the frequency of \( \text{ACh} \)-triggered IPSCs was normalized to the frequency of IPSCs recorded for 3 min before the automatic application. In addition, the frequency of spontaneous IPSCs recorded for 3 min in the absence of \( \text{Pb}^{2+} \) was taken as 1 and used to normalize the frequency of spontaneous IPSCs recorded for 3 min after 5-min perfusion of the neurons with \( \text{Pb}^{2+} \)-containing external solution. Graph and error bars represent mean and S.E.M. of results obtained from three neurons. The dotted line represents the 100% control level for each experimental condition. * and ** indicate that, according to the paired Student’s \( t \) test, results were different from control with \( p < 0.05 \) and 0.01, respectively.
Perfusion of cultured hippocampal neurons for ≥5 min with external solution containing either 0.1 or 1 µM Pb²⁺ produced either no reduction or approximately 20% reduction of the peak amplitude of type IA currents, respectively (Fig. 6). The IC₅₀ for Pb²⁺ in reducing the amplitude of type IA currents has been reported to be approximately 18.9 µM (Mike et al., 2000b). In contrast, under the same experimental condition, Pb²⁺ (1 µM) blocked by 100% EPSCs resulting from α₇* nAChR activation in glutamatergic neurons synapsing onto the neurons under study (Fig. 6).

To verify whether dilution of diffusible intracellular molecules accounted for the low sensitivity to Pb²⁺ of somatodendritic responses mediated by α₇* nAChRs, some experiments were performed in hippocampal neurons under the perforated-patch configuration. (i.e., a patch-clamp configuration that maintains intact the intracellular contents). Perfusion for 5 min of whole-cell patched neurons with Pb²⁺ (10 µM)-containing external solution reduced the amplitudes of type IA currents elicited by ACh (1 mM) by 41.1 ± 3.49% (mean ± S.E.M., n = 11 neurons). Similarly, 5-min exposure to Pb²⁺ (10 µM) of neurons under the perforated-patch configuration reduced the amplitudes of ACh (1 mM)-evoked type IA currents by 46.3 ± 3.49% (mean ± S.E.M., n = 4 neurons). Therefore, the low sensitivity to Pb²⁺ of somatodendritic responses mediated by α₇* nAChRs could not be accounted for by dilution of diffusible intracellular components.

Involvement of PKC in the Effect of Pb²⁺ on ACh-Triggered GABA and Glutamate Release. Blockade of ACh-triggered transmitter release by Pb²⁺ (0.1 and 1 µM) is unlikely to be mediated by a direct interaction of the heavy metal with α₇* and α₄β²* nAChRs. First, only at micromolar concentrations does Pb²⁺ interact directly with these nAChRs (Ishihara et al., 1995; Mike et al., 2000b). Second, blockade resulting from the direct interaction of Pb²⁺ with α₇* nAChRs occurs within 1 s and is fully reversible (Mike et
al., 2000b), whereas Pb²⁺-induced blockade of glutamate release triggered by activation of α₇* nAChRs occurs within 20 min and is irreversible (Fig. 4). The slow onset and the slow reversibility of the effect of Pb²⁺ on ACh-triggered transmitter release are consistent with an intracellular mode of action. Thus, it can be hypothesized that the differential effect of Pb²⁺ on presynaptic and postsynaptic nicotinic responses recorded from hippocampal neurons results from the interactions of Pb²⁺ with an intracellular target that 1) directly controls the nAChR activity and is not present in the somatodendritic region of neurons, and/or 2) affects the transmitter release process that is modulated by the activation of nAChRs.

The finding that a low extracellular concentration (100 nM) of Pb²⁺ was sufficient to block more than 90% of the ACh-evoked transmitter release indicated that such an intracellular target should have high affinity for Pb²⁺. PKC, in addition to binding Pb²⁺ with very high affinity (Markovac and Goldstein, 1988), modulates the activity of neuronal nAChRs (Fenster et al., 1999, and references therein) and proteins that regulate the transmitter release process (Atchison, 2003; Suszkiw, 2004, and references therein). Thus, experiments were designed to determine the effects of the PKC inhibitor Gö 7874 (Kleinschroth et al., 1995) or Ro-31-8425 (Wilkinson et al., 1993) on Pb²⁺-induced blockade of ACh-triggered glutamate and GABA release.

Exposure for 15 min of the hippocampal neurons to Gö 7874 at 50 nM, a concentration sufficient to block by 100% the activity of PKC while having negligible effect on other protein kinases (Kleinschroth et al., 1995), caused no significant change in the frequency of spontaneously occurring IPSCs and EPSCs (data not shown). Likewise, the effects of Pb²⁺ (100 nM) on the frequencies of spontaneous IPSCs and EPSCs were not influenced by 15-min exposure of the hippocampal neurons to 50 nM Gö 7874 (Figs. 3, 4, and 7). These findings suggested that 1) the basal activity of PKC in cultured hippocampal neurons had no significant effect on spontaneous GABAergic or glutamatergic transmission, 2) the sensitivity of VGCC to blockade by Pb²⁺ was not significantly altered by the PKC inhibitor, and 3) Gö 7874 itself did not bind Pb²⁺. The effects of ACh on the frequencies of IPSCs and EPSCs were also unaffected by the PKC inhibitor Gö 7874 (Figs. 3, 4, and 7, A–C). However, after a 15-min perfusion of the neurons with external solution containing both Gö 7874 (50 nM) and Pb²⁺ (100 nM), ACh (1 mM) could still increase by approximately 3- and 4-fold the frequencies of IPSCs and EPSCs, respectively (Fig. 7, B and C). Thus, it can be concluded that blockade by Pb²⁺ of transmitter released by nAChR activation involves a PKC-dependent mechanism. To lend further support to this conclusion, experiments were carried out using Ro-31-8425 at 150 nM, a concentration sufficient to cause selective full inhibition of PKC activity.
(Wilkinson et al., 1993). Results obtained from these experiments were similar to those reported above for Gö 7874. As shown in Fig. 8, A and C, the baseline-corrected net charge of ACh- or choline-triggered IPSCs recorded in the presence of both Pb\(^{2+}\) and Ro-31-8425 was not significantly different from that recorded in the presence of the PKC inhibitor alone. Therefore, PKC inhibition prevents Pb\(^{2+}\)-induced blockade of transmitter released by nAChR activation. The PKC activator PMA mimicked the effects of Pb\(^{2+}\) on GABA release triggered by ACh or choline (Fig. 8B). The agonist was applied to the hippocampal neurons before (control) and after 20- to 25-min perfusion with external solution containing PMA (1 \(\mu\)M). In the presence of PMA (1 \(\mu\)M), the baseline-corrected net charge of IPSCs triggered by ACh (1 mM) or choline (10 mM) was 41.4 ± 2.33 and 29.8 ± 4.00% (mean ± S.E.M., \(n = 3\) neurons), respectively, of that triggered by either agonist in the absence of the phorbol ester (Fig. 8, B and C).

**Eﬀects of Pb\(^{2+}\) on Nicotinic Cholinergic Modulation of the Activity of Neuronal Circuitries in Hippocampal Cultures.** It has been reported that \(\alpha_7^*\) and \(\alpha_4\beta_2^*\) nAChRs, by virtue of their involvement in modulation of GABA release, can be part of inhibitory and disinhibitory mechanisms in the hippocampus (Alkondon et al., 1999). Disinhibition, which is the consequence of activation of an interneuron that synapses onto another interneuron that, in turn, synapses onto a pyramidal neuron, can easily be seen in fully active neuronal circuitries, i.e., in the absence of GABA\(_A\) and AMPA receptor antagonists.

In the absence of CNQX and picrotoxin, IPSCs and EPSCs were recorded simultaneously from single neurons dialyzed with the methanesulfonate-containing internal solution (see Materials and Methods). Replacement of chloride with methanesulfonate in the internal solution shifts the reversal potential of GABAergic currents toward a more negative membrane potential (i.e., approximately −44 mV) and does not alter the reversal potential of glutamatergic currents, which remains close to 0 mV (Alkondon et al., 1999). Thus, in recordings obtained from neurons dialyzed with methanesulfonate-containing internal solution and voltage clamped between −44 and 0 mV, GABAergic and glutamatergic events occur as upward and downward deflections, respectively. Using this experimental paradigm, the effects of Pb\(^{2+}\) on ACh-triggered GABA and glutamate release could be analyzed simultaneously in a near-physiological condition.

Using the methanesulfonate-containing internal solution, the frequencies of IPSCs and EPSCs were recorded from single neurons by extracellular recording and microdialysis with a methanesulfonate-containing external solution (see Materials and Methods). Replacement of chloride with methanesulfonate in the external solution and voltage clamped between −44 and 0 mV, GABAergic and glutamatergic events occur as upward and downward deflections, respectively. Using this experimental paradigm, the effects of Pb\(^{2+}\) on ACh-triggered GABA and glutamate release could be analyzed simultaneously in a near-physiological condition.

Using the methanesulfonate-containing internal solution, the frequencies of IPSCs and EPSCs were substantially enhanced by ACh (1 mM) in neurons voltage clamped at −20 mV (Fig. 9). When neurons were perfused for 10 to 20 min with Pb\(^{2+}\) (100 nM)-containing external solution, the frequencies of spontaneous IPSCs and EPSCs were reduced by about 50 to 60%. Pb\(^{2+}\) (100 nM) also blocked the ACh-triggered IPSCs and EPSCs (Fig. 9).

Similar to the findings reported in the previous sections, after a 30-min washing of the neurons with nominally Pb\(^{2+}\)-free external solution, the frequencies of spontaneous IPSCs and EPSCs returned to control levels, and approximately 30% of the Pb\(^{2+}\)-induced inhibition of ACh-triggered IPSCs could not be reversed (Fig. 9). However, in contrast to the findings reported in previous sections, only approximately 20% of the ACh-triggered glutamate release remained blocked after washing of the neurons with nominally Pb\(^{2+}\)-free external solution (Fig. 9).

### Discussion

**Pb\(^{2+}\) at Nanomolar Concentrations Inhibits Modulation by \(\alpha_7^*\) and \(\alpha_4\beta_2^*\) nAChRs of Synaptic Transmission in Hippocampal Neurons.** The present study demonstrates that acute exposure of primary hippocampal cultures to nanomolar concentrations of Pb\(^{2+}\) causes inhibition of action potential-dependent transmitter release triggered by activation of \(\alpha_7^*\) nAChR and/or \(\alpha_4\beta_2^*\) nAChRs. These effects cannot be explained by the VGCC block that underlies the Pb\(^{2+}\)-induced inhibition of action potential-dependent synaptic transmission (Braga et al., 1999a). Maximal inhibition by Pb\(^{2+}\) of action potential-dependent transmitter release occurs in less than 1 min and is fully reversible within 1 min of removal of Pb\(^{2+}\). In contrast, maximal inhibition of nicotinic modulation of action potential-dependent transmitter release occurs after 10- to 20-min exposure of the neurons to Pb\(^{2+}\). Furthermore, Pb\(^{2+}\)-induced blockage of transmitter release triggered by \(\alpha_7^*\) nAChR activation is irreversible and Pb\(^{2+}\)-induced blockage of transmitter release triggered by \(\alpha_4\beta_2^*\) nAChR activation is only reversed after approximately 10 min of removal of Pb\(^{2+}\) from the extracellular compartment.

A long-lasting inactivation by Pb\(^{2+}\) of nicotinic modulation of synaptic transmission could account for the apparent cholinergic hippocampal deafferentation that persists long after initial exposure of young rats to low levels of lead acetate (Biellarczyk et al., 1996). In addition, changes in expression of NMDA receptor subunits observed in the hippocampus of young rats exposed to low Pb\(^{2+}\) levels (Nihei et al., 2000) might reflect compensatory mechanisms resulting from Pb\(^{2+}\)-induced blockage of nicotinic modulation of glutamatergic transmission.

**Nicotinic Modulation of Synaptic Transmission Is More Sensitive than Somatodendritic Nicotinic Responses to Blockade by Pb\(^{2+}\): Involvement of PKC.** In hippocampal neurons, 30 \(\mu\)M Pb\(^{2+}\) reduces by no more than 20% the activity of somatodendritic \(\alpha_4\beta_2^*\) nAChRs, and the IC\(_{50}\) for Pb\(^{2+}\)-induced inhibition of somatodendritic \(\alpha_7^*\) nAChRs is approximately 18 \(\mu\)M (Ishihara et al., 1995; Mike et al., 2000b). In contrast, Pb\(^{2+}\) at 100 nM caused approximately 90% inhibition of transmitter release triggered by activation of \(\alpha_7^*\) and \(\alpha_4\beta_2^*\) nAChRs in neurons synapsing onto the cells under study.

Analyses of the effects of Pb\(^{2+}\) on whole-cell currents evoked by activation of somatodendritic \(\alpha_7^*\) nAChRs revealed that the heavy metal, by interacting directly with \(\alpha_7^*\) nAChRs, causes a rapidly developing (\(t_{\text{onset}} = 240\) ms) and fully reversible blockade of the receptor activity (Mike et al., 2000b). They also indicated that, via an intracellular mechanism, Pb\(^{2+}\) causes a slowly developing and irreversible inhibition of somatodendritic \(\alpha_7^*\) nAChRs (Mike et al., 2000b). Assuming that 1) \(\alpha_7^*\) and \(\alpha_4\beta_2^*\) nAChRs regulating transmitter release are in preterminal/presynaptic neuronal compartments; and 2) a single intracellular mechanism underlies the slowly developing Pb\(^{2+}\)-induced blockage of whole-cell currents and transmitter release triggered by nAChR activation, it can be hypothesized that such a mechanism prevails in preterminal/presynaptic regions of hippocampal neurons. Alternatively, the high sensitivity to Pb\(^{2+}\) of transmitter...
release triggered by α7* nAChR activation could be explained by Pb²⁺-induced changes in proteins that link nAChR activation to the transmitter release process.

The finding that the PKC inhibitors Gö 7874 and Ro-31-8425 prevented Pb²⁺-induced inhibition of nicotinic cholinergic modulation of action potential-dependent transmitter release indicated that this effect of Pb²⁺ is mediated by a PKC-dependent mechanism. To date, at least 12 PKC isoforms have been identified and classified as classical, new, or atypical PKCs (Tanaka and Nishizuka, 1994). High levels of classical PKC isoforms are found in mossy fiber terminals and in axons of hippocampal interneurons, and some PKC isoforms, particularly the ε isoform, are present predominantly in nerve terminals rather than in the somata of central nervous system neurons (Tanaka and Nishizuka, 1994; Majewski and Iannazzo, 1998). Therefore, the PKC prevalence in preterminal/presynaptic neuronal compartments could account for the high sensitivity to inhibition by Pb²⁺ of transmitter released by nAChR activation. The membrane association of PKCs in the brain (Tanaka and Nishizuka, 1994) can also explain the finding that the magnitude of Pb²⁺-induced blockade of somatodendritic α7* nAChR activity is unaltered by dialysis of the neuronal intracellular contents.

PKC is activated and inhibited by Pb²⁺ at picomolar and nanomolar concentrations, respectively (Markovac and Goldstein, 1988; Suszkiew, 2004). If Pb²⁺-induced blockade of nicotinic cholinergic modulation of transmitter release resulted from inhibition of basal PKC activity in hippocampal neurons, the PKC inhibitors Gö 7874 and Ro-31-8425 should have mimicked the effect of Pb²⁺ on ACh-triggered release of GABA and glutamate. Instead, the effect of Pb²⁺ on nicotinic cholinergic modulation of action potential-dependent transmitter release was mimicked by the PKC activator PMA. Therefore, it is more likely that intracellular Pb²⁺ levels achieved after acute exposure of the neurons to 100 nM or 1 μM Pb²⁺ are sufficient to activate PKC, which in turn phosphorylates nAChRs, proteins associated with the receptors, and/or proteins linking the receptors to the action potential-dependent transmitter release process, rendering nicotinic modulation of synaptic transmission inactive.

Direct phosphorylation of nAChRs by PKC reduces receptor activity in sympathetic neurons (Downing and Role, 1987). However, some lines of evidence favor the concept that Pb²⁺-induced blockade of nicotinic cholinergic modulation of action potential-dependent transmitter release is not a result of PKC-mediated phosphorylation of the nAChRs. First, α7 nAChR subunits do not have PKC consensus sequences and cannot be phosphorylated by PKC (Séguela et al., 1993; Moss et al., 1996). Second, direct phosphorylation by PKC of α4β2 nAChRs ectopically expressed in oocytes and human embryonic kidney 293 cells increases receptor activity by reducing the rate of desensitization and deactivation (Fenster et al., 1999).

**A Disinhibitory Mechanism Surmounts Partially the Long-Lasting Inhibition by Pb²⁺ of Glutamate Release Triggered by α7* nAChR Activation in the Hippocampus.** In a near-physiological condition, exposure of hippocam-
Pb²⁺ Blocks Nicotinic Modulation of Synaptic Transmission

Numerous studies have investigated the complex interactions between Pb²⁺ and PKC and some of the effects of such actions on receptor expression, neuronal function, and synaptic plasticity (Lasley and Gilbert, 2000, and references therein). This is, however, the first report that Pb²⁺-PKC interactions cause disruption of nicotinic cholinergic modulation of action potential-dependent release of GABA and glutamate in the hippocampus. The toxicological relevance of this finding is emphasized by the fact that inhibition of nicotinic modulation of synaptic transmission can be detected upon acute exposure of hippocampal neurons to nanomolar concentrations of Pb²⁺, and concentrations of Pb²⁺ ranging from 25 to 100 nM have been found in the cerebrospinal fluid of humans not known to be occupationally exposed to Pb²⁺ (Swanson et al., 1997, and references therein).

The analysis of the effects of Pb²⁺ on synaptic transmission in the context of fully functional neuronal networks is essential for the understanding of how Pb²⁺ alters overall neuronal activity in the hippocampus. As reported herein, acute exposure of hippocampal neurons to 100 nM Pb²⁺ induces the following changes in action potential-dependent synaptic events: 1) reversible blockade of spontaneous transmitter release; and 2) long-lasting inhibition of nicotinic cholinergic modulation of GABA and glutamate release. After removal of Pb²⁺, action potential-dependent, spontaneous transmitter release is fully restored and nicotinic cholinergic facilitation of GABAergic transmission is partially reversed in all neuronal networks that are modulated by nAChR activity. In contrast, nicotinic cholinergic modulation of glutamatergic transmission is partially restored only in some circuitries via 4β2 nAChR-mediated disinhibition of pyramidal neurons. Thus, the most insidious residual effect of short-term exposure of hippocampal neurons to low levels of Pb²⁺ will be a long-lasting reduction of glutamatergic synaptic transmission. Given the involvement of the glutamatergic system in learning and memory, an enduring reduction of glutamatergic activity could underlie cognitive deficits that persist throughout the lives of patients who are exposed to Pb²⁺ particularly at early ages when the brain is highly plastic.

Fig. 9. Effects of Pb²⁺ on ACh-triggered IPSCs and EPSCs in fully functional neuronal circuitries. Top traces, sample recordings of IPSCs and EPSCs obtained from a neuron internally dialyzed with the methanesulfonate-containing internal solution (see Materials and Methods) and voltage clamped at −20 mV. At this membrane potential, EPSCs occur as downward deflections and IPSCs occur as upward deflections. The neuron was exposed for 1 s to ACh before (control), 15 min after the start of the perfusion with Pb²⁺, and at 20 min after washing with nominally Pb²⁺-free external solution. Bottom graph, frequencies of ACh-triggered EPSCs and IPSCs recorded before, during, and after exposure of the neurons to Pb²⁺ were estimated according to the protocol described in Fig. 4. Graph and error bars represent mean and S.E.M., respectively, of results obtained from three neurons. The dotted line represents the 100% control level for each experimental condition. ** indicate that, according to the paired Student’s t test, results were different from control with p < 0.01.
proach to reverse the complex Pb2⁺-PKC interactions that account for some of the lifelong effects of Pb2⁺ in the brain (Lasley and Gilbert, 2000), including the presently reported inhibition of nicotinic cholinergic modulation of synaptic transmission that outlasts removal of Pb2⁺ from the extracellular compartment, it is all the more important that primary prevention of exposure of children to this ubiquitous environmental pollutant remains at the forefront in public health (Needleman, 2004).

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