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 \(\alpha 4\beta 2\) and \(\alpha 7\) nAChRs. It also triggered glutamate release via activation of \(\alpha 7\) nAChRs. Pb\(^{2+}\) (0.1 and 1 \(\mu\)M) 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 \(\alpha 7\) and \(\alpha 4\beta 2\) nAChRs were mimicked by the protein kinase C (PKC) activator phorbol-12-myristate-13-acetate (1 \(\mu\)M) 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 \(\alpha 4\beta 2\) nAChR activation in interneurons that synapse onto other interneurons synapsing onto pyramidal neurons. Long-lasting inhibition of \(\alpha 7\) 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 \(\mu\)g/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 \(\geq 45\ \mu\)g/dl (Nightingale, 1991). However, chelation therapy does not protect these children or those with Pb\(^{2+}\) blood levels between 10 and 45 \(\mu\)g/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, at

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, \(\alpha\)-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$^{2+}$-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$^{2+}$ 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$^{2+}$ (Swanson et al., 1997).

Pb$^{2+}$ alters synaptic transmission in numerous preparations by disrupting the activity of Ca$^{2+}$-regulated proteins, including protein kinases, voltage-gated Ca$^{2+}$ channels (VGCC), and Ca$^{2+}$-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$^{2+}$ 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$^{2+}$ 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 α7*-nAChRs (Vizi and Kiss, 1998; Pereira et al., 2002), both of which are sensitive to inhibition by micromolar concentrations of Pb$^{2+}$ (Guilarte and Miceli, 1992; Uijjara 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$^{2+}$. Thus, the present study was designed to 1) determine whether Pb$^{2+}$ affects modulation by nAChRs of GABAergic or glutamatergic transmission, and, if so, by what mechanism(s); 2) compare the sensitivity to Pb$^{2+}$ of whole-cell currents and transmitter release triggered by about 100 to 150 and 60 to 80 agonist-containing external solution was calculated as the area under the time courseHEPS, 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-ethyleneglycol-bis[b-aminobutyl ether]-N,N'-tetraacetic acid, and 10 mM HEPS (pH 7.3 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 MΩ.

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$_2$ and 10 mM HEPS (pH 7.3 adjusted with CsOH). Nystatin was dissolved in dimethyl sulfoxide (50 mg/ml) and diluted in this pipette solution to a final concentration of 200 μg/ml.

All experiments were performed at room temperature (22–25°C). Agonist-containing solutions were applied to the neurons via a glass U-tube (Uijjara and Albuquerque, 1992). The U-tube was positioned at about 100 to 150 and 60 to 80 μm away from the neurons from which PSCs and whole-cell currents were recorded, respectively. Specific antagonists and Pb$^{2+}$ (as PbCl$_2$) were applied to the neurons via the bath perfusion and in admixture with the agonists via the U-tube.

Data Analysis. PSCs 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 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 PSCs 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 PSCs 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.

Macroscopic 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 Merek 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), Gó 7874, and Ro-31-8425 were purchased from Calbiochem (La Jolla, CA). Acetylicholine 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 CNQX (the 10 mM stock solution of CNQX had 12.2 mM NaOH). Picrotoxin, PMA, Gó 7874, and Ro-31-8425 were dissolved in dimethyl sulfoxide at concentrations 10,000-fold higher than the desired final concentration. Solutions containing PbCl₂ were prepared just before application to the neurons.

**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 GABA₆ 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 GABA₆ receptor antagonist, spontaneous postsynaptic currents recorded from cultured hippocampal neurons are sensitive to blockade by CNQX (10 μM) (Braga et al., 1999a). These currents, herein referred to as EPSCs, are mediated by glutamate released from spontaneously firing neurons that synapse onto the neurons from which recordings are obtained. Under our experimental conditions, contribution of NMDA receptors to glutamatergic synaptic events is negligible (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 GABA₆ 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 presynaptic α7* nAChRs facilitates the spontaneous release of glutamate from hippocampal neurons in culture and in slices (Radcliffe et al., 1999).

**Pb²⁺ Blocks ACh-Trigged 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 αβ2- 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. **, p < 0.01 according to the paired Student's t test.

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.
upon exposure of the neurons to ACh (1 mM) was approximately 4-fold higher than that recorded before the agonist pulse. However, in the presence of Pb2+ (0.1 and 1 μM), the frequency of ACh (1 mM)-triggered IPSCs was not significantly higher than the baseline frequency of IPSCs recorded before the application of ACh (Fig. 3). Furthermore, in contrast to the rapidly reversible reduction by Pb2+ (100 nM) of the frequency of spontaneous IPSCs, Pb2+-induced inhibition of ACh-triggered IPSCs was slowly reversible (Fig. 5). The reversibility was only partial (Figs. 3, A–C, and 5), and the ACh-triggered IPSCs recorded after washing of the neurons with nominally Pb2+-free external solution were completely blocked by DHβE (100 nM; Fig. 3C). Pb2+ (100 nM) also reduced the frequency of choline (10 mM)-induced IPSCs by 82.8 ± 2.5% (n = 3 neurons), and this effect was irreversible upon 20-min washing of the neurons with nominally Pb2+-free external solution. The following findings support the concept that the portion of the ACh-triggered IPSCs that remained blocked after washing of the neurons is due to the irreversible blockade by Pb2+ of α7* nAChR activity in GABAergic neurons. First, ACh-triggered IPSCs recorded after washing of neurons with nominally Pb2+-free external solution were completely blocked by the αβ2 nAChR-selective antagonist DHβE (Fig. 3C). Second, α7* nAChRs seem to contribute to approximately 30 to 35% of the ACh-induced GABA release, and this corresponds to the percentage of the ACh response that remains blocked after washing of the neurons with nominally Pb2+-free external solution. Third, GABA release triggered by the α7 nAChR-selective agonist choline was blocked by Pb2+, but could not be reversed even after a 20-min washing of the neurons with nominally Pb2+-free external solution.

Under control conditions, the frequency of EPSCs recorded upon exposure of the neurons to ACh (1 mM) was approximately 3-fold higher than that recorded before the agonist pulse (Fig. 4C). After perfusion of the neurons with external solution containing Pb2+ (0.1 or 1 μM), the frequency of ACh (1 mM)-triggered EPSCs was approximately the same as the frequency of EPSCs recorded before the agonist pulse (Fig. 4, A–C). The effect of Pb2+ (100 nM and 1 μM) on ACh-triggered EPSCs could not be reversed, even after 30- to 60-min washing of the neurons with nominally Pb2+-free external solution (Fig. 4, A–C).

**Differential Sensitivity to Pb2+ of Whole-Cell Currents and IPSCs Triggered by Activation of α7* nAChRs.** To determine the sensitivity to Pb2+ of postsynaptic and presynaptic responses resulting from activation of α7* nAChRs, we compared the magnitude of the effect of Pb2+ on the amplitude of ACh (1 mM)-evoked type IA currents (i.e., whole-cell currents subserved by somatodendritic α7* nAChRs) to the magnitude of the effect of the heavy metal on glutamate released by ACh (1 mM)-induced activation of α7* nAChRs.

![Fig. 3. Effects of Pb2+ on ACh-triggered IPSCs.](image-url)
Membrane potential, 3C were used to quantify the effects of Pb2\(^{+}\)-induced blockade of ACh-triggered EPSCs. A and B, sample recordings of EPSCs obtained from neurons perfused with picrotoxin (100 nM) -containing external solution. The frequency of spontaneous EPSCs and of ACh-triggered EPSCs. 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.

Perfusion of cultured hippocampal neurons for ≥ 5 min with external solution containing either 0.1 or 1 \(\mu\)M 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 \(\mu\)M (Mike et al., 2000b). In contrast, under the same experimental condition, Pb2\(^{+}\) (1 \(\mu\)M) blocked by 100% EPSCs resulting from \(\alpha7^{+}\) nAChR activation in glutamatergic neurons synapsing onto the neurons under study (Fig. 6).

**Involvement of PKC in the Effect of Pb2\(^{+}\) on ACh-Triggered GABA and Glutamate Release.**

To verify whether dilution of diffusible intracellular molecules accounted for the low sensitivity to Pb2\(^{+}\) of somatodendritic responses mediated by \(\alpha7^{+}\) 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 \(\mu\)M)-containing external solution reduced the amplitudes of type IA currents elicited by ACh (1 mM) by 41.1 ± 0.05% (mean ± S.E.M., \(n = 11\) neurons). Similarly, 5-min exposure to Pb2\(^{+}\) (10 \(\mu\)M) 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 \(\alpha7^{+}\) nAChRs could not be accounted for by dilution of diffusible intracellular components.
from control with according to the paired Student's t test. Release process that is modulated by the activation of somatodendritic region of neurons, and/or 2) affects the transmission controls the nAChR activity and is not present in the somatodendritic region of neurons, 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 EPSCs and IPSCs, 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 7877. 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 Pb²⁺ and Ro-31-8425 was not significantly different from that recorded in the presence of the PKC inhibitor alone. Therefore, PKC inhibition prevents Pb²⁺-induced blockade of transmitter released by nAChR activation.

The PKC activator PMA mimicked the effects of Pb²⁺ 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 μM). In the presence of PMA (1 μM), 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 Pb²⁺ on Nicotinic Cholinergic Modulation of the Activity of Neuronal Circuitries in Hippocampal Cultures.** It has been reported that α7* and α4β2* 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 Pb²⁺ 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 Pb²⁺ (100 nM)-containing external solution, the frequencies of spontaneous IPSCs and EPSCs were reduced by about 50 to 60%. Pb²⁺ (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 Pb²⁺-free external solution, the frequencies of spontaneous IPSCs and EPSCs returned to control levels, and approximately 30% of the Pb²⁺-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 Pb²⁺-free external solution (Fig. 9).

**Discussion**

**Pb²⁺ at Nanomolar Concentrations Inhibits Modulation by α7* and α4β2* nAChRs of Synaptic Transmission in Hippocampal Neurons.** The present study demonstrates that acute exposure of primary hippocampal cultures to nanomolar concentrations of Pb²⁺ causes inhibition of action potential-dependent transmitter release triggered by activation of α7* nAChR and/or α4β2* nAChRs. These effects cannot be explained by the VGCC block that underlies the Pb²⁺-induced inhibition of action potential-dependent synaptic transmission (Braga et al., 1999a). Maximal inhibition by Pb²⁺ of action potential-dependent transmitter release occurs in less than 1 min and is fully reversible within 1 min of removal of Pb²⁺. In contrast, maximal inhibition of nicotinic modulation of action potential-dependent transmitter release occurs after 10- to 20-min exposure of the neurons to Pb²⁺.

Furthermore, Pb²⁺-induced blockade of transmitter release triggered by α7* nAChR activation is irreversible and Pb²⁺-induced blockade of transmitter release triggered by α4β2* nAChR activation is only reversed after approximately 10 min of removal of Pb²⁺ from the extracellular compartment.

A long-lasting inactivation by Pb²⁺ 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 (Bielarczyk et al., 1996). In addition, changes in expression of NMDA receptor subunits observed in the hippocampus of young rats exposed to low Pb²⁺ levels (Nihei et al., 2000) might reflect compensatory mechanisms resulting from Pb²⁺-induced blockade of nicotinic modulation of glutamatergic transmission.

**Nicotinic Modulation of Synaptic Transmission Is More Sensitive than Somatodendritic Nicotinic Responses to Blockade by Pb²⁺: Involvement of PKC.** In hippocampal neurons, 30 μM Pb²⁺ reduces by no more than 20% the activity of somatodendritic α4β2* nAChRs, and the IC₅₀ for Pb²⁺-induced inhibition of somatodendritic α7* nAChRs is approximately 18 μM (Ishihara et al., 1995; Mike et al., 2000b). In contrast, Pb²⁺ at 100 nM caused approximately 90% inhibition of transmitter release triggered by activation of α7* and α4β2* nAChRs in neurons synapsing onto the cells under study.

Analyses of the effects of Pb²⁺ on whole-cell currents evoked by activation of somatodendritic α7* nAChRs revealed that the heavy metal, by interacting directly with α7* nAChRs, causes a rapidly developing (τ,onset = 240 ms) and fully reversible blockade of the receptor activity (Mike et al., 2000b). They also indicated that, via an intracellular mechanism, Pb²⁺ causes a slowly developing and irreversible inhibition of somatodendritic α7* nAChRs (Mike et al., 2000b). Assuming that 1) α7* and α4β2* nAChRs regulating transmitter release are in preterminal/presynaptic neuronal compartments; and 2) a single intracellular mechanism underlies the slowly developing Pb²⁺-induced blockade 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 Pb²⁺ of transmitter
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 hippocampus

...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 (Séguéla et al., 1993; Moss et al., 1996). Second, direct phosphorylation by PKC of 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...
Pb\textsuperscript{2+} Blocks Nicotinic Modulation of Synaptic Transmission

Acute exposure of hippocampal neurons to Pb\textsuperscript{2+} (100 nM) resulted in simultaneous reduction of ACh-induced release of GABA and glutamate. After a 30-min washing of the neurons with nominally Pb\textsuperscript{2+}-free external solution, ACh-triggered GABA release was partially reversed. This finding is in agreement with results obtained from the analysis of GABAergic transmission when glutamatergic transmission was blocked by the AMPA receptor antagonist CNQX. However, also partially reversed was the ACh-triggered glutamate release. The latter finding contrasts with the irreversibility of Pb\textsuperscript{2+}-induced blockade of ACh-evoked glutamate release observed when GABAergic transmission was blocked by the GABA\textsubscript{A} receptor antagonist picrotoxin. The partial recovery of ACh-triggered glutamate release observed in the absence of the GABA\textsubscript{A} and AMPA receptor antagonists is likely to be a result of a previously described disinhibitory mechanism (Alkondon et al., 1999) that involves \( \alpha_4\beta_2 \) nAChR activation in an interneuron that, in turn, synapses onto glutamatergic neurons.

**Toxicological Relevance of Pb\textsuperscript{2+}-Induced Inhibition of Cholinergic Modulation of Synaptic Transmission.**

Numerous studies have investigated the complex interactions between Pb\textsuperscript{2+} 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 Pb\textsuperscript{2+}-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 Pb\textsuperscript{2+}, and concentrations of Pb\textsuperscript{2+} ranging from 25 to 100 nM have been found in the cerebrospinal fluid of humans not known to be occupationally exposed to Pb\textsuperscript{2+} (Swanson et al., 1997, and references therein).

The analysis of the effects of Pb\textsuperscript{2+} on synaptic transmission in the context of fully functional neuronal networks is essential for the understanding of how Pb\textsuperscript{2+} alters overall neuronal activity in the hippocampus. As reported herein, acute exposure of hippocampal neurons to 100 nM Pb\textsuperscript{2+} 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 Pb\textsuperscript{2+}, 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 \( \alpha_4\beta_2 \) nAChR-mediated disinhibition of pyramidal neurons. Thus, the most insidious residual effect of short-term exposure of hippocampal neurons to low levels of Pb\textsuperscript{2+} 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 Pb\textsuperscript{2+} particularly at early ages when the brain is highly plastic.

Considering that there is no available therapeutic ap-
proach to reverse the complex Pb2\textsuperscript{2+}-PKC interactions that account for some of the lifelong effects of Pb2\textsuperscript{2+} in the brain (Lasley and Gilbert, 2000), including the presently reported inhibition of nicotinic cholinergic modulation of synaptic transmission that outlasts removal of Pb2\textsuperscript{2+} from the extra-cellular 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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