Selective blockade of NMDA-activated channel currents may be implicated in learning deficits caused by lead

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The effect of Pb^{2+} on glutamate receptor activity in rat hippocampal neurons was investigated with a view of explaining the cognitive and learning deficits produced by this heavy metal. Pb^{2+} (2.5–50 μM) selectively inhibited *N*-methyl-D-aspartate (NMDA)-induced whole-cell and single-channel currents in a concentration-dependent but voltage-independent manner, without significantly altering currents induced by either quisqualate or kainate. The frequency of NMDA-induced channel activation was decreased by Pb^{2+}. Neither glycine (10–100 μM), nor Ca^{2+} (10 mM) reversed the effect of Pb^{2+}. Pb^{2+} also inhibited the [^{3}H]MK-801 binding to rat hippocampal membranes in vitro. The elucidation of the actions of Pb^{2+} on the NMDA receptor ion channel complex provides important insights into the clinical and toxic effects of this cation.

Glutamate receptor; Rat hippocampal neuron; Lead poisoning; Single channel current; Heavy metal; Learning deficit

1. INTRODUCTION

The heavy metal Pb^{2+} is an environmental toxicant that poses a great threat to infant and child development, chiefly by causing a marked deficit in cognitive development [1–3]. Animal studies have indicated an impairment of the learning process after Pb^{2+} exposure [4–6]. Hippocampal damage has been linked to deficits in reversal learning in rats and in monkeys [7,8], and exposure of young monkeys to Pb^{2+} produced similar learning disorders [9]. Our current understanding of synaptic plasticity suggests that long-term potentiation (LTP) may underlie the processes of learning and memory [10–13] and several reports indicate that the NMDA subtype of glutamate receptors are involved in the process of LTP [14–16]. In view of these observations, we decided to examine the effect of Pb^{2+} on the glutamate receptor ion channel activity in cultured rat hippocampal neurons. The present study demonstrates that Pb^{2+} blocks the NMDA receptors located at glutamate synapses of rat hippocampal pyramidal neurons at concentrations which are capable of inducing neuropsychological disorders. A preliminary account of a part of this work has appeared in abstract form [17].

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Abbreviations: NMDA, *N*-methyl-D-aspartate; LTP, long-term potentiation; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Tissue culture*

Hippocampi of fetuses obtained from 16–18-day pregnant rats (Sprague-Dawley) were dissociated and plated according to the methods described by Lima-Landman and Albuquerque [18]. For recording channel currents, 7–21-day old cultures were used.

2.2. *Patch-clamp technique*

The recordings of both whole-cell and single-channel currents were made according to standard patch-clamp techniques [19] using an LM-EPC 7 patch-clamp system (List Electronic, FRG). The external solution had the following composition (mM): NaCl 165, KCl 5, $CaCl_2$ 2 (unless otherwise stated), Hepes 5, D-glucose 10, pH 7.3, 340 mOsm plus TTX (0.3 μM). The internal solution was composed of (mM): CsCl 80, CsF 80, CsEGTA 10, Hepes 10, pH 7.3, 330 mOsm. The patch microelectrodes were pulled from borosilicate capillary glass (World Precision Instruments, New Haven, CT). The microelectrodes when filled with the internal solution had resistances of 1–2 MΩ and 4–7 MΩ for whole-cell and single-channel experiments, respectively. No series-resistance compensation was used in the present experiments. The data were stored on FM tape (Racal 4DS) and filtered at 3 kHz. Whole-cell currents were analyzed using the PCLAMP program whereas analysis of single-channel currents were done using the IPROC-2 program.

Whole-cell currents were induced by fast applications of the agonists either alone or in combination with the indicated concentrations of Pb^{2+} (Cl^- salt). For rapid solution changes (about 100 ms), the outflow port of a U-shaped tube [20,21] was positioned near the cells (approximately 50 μm). We modified this system in order to obtain different outputs from the same port without moving the U-tube. The dead space for solution exchange was about 0.1 ml and the per-

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fusion rate was about 0.1 ml/min. Short pulses (1 s) of agonist applied to the cell surface gave reproducible and consistent responses under these conditions. These neurons were superfused at a rate of 1–2 ml/min with external solution.

Single-channel recordings were done in the outside-out patch configuration. NMDA and $PbCl_2$ were added into the static bath and mixed thoroughly to achieve equilibrium conditions before making the recordings. The addition of the drugs was restricted to μl volumes so that any dilution that might occur was kept to a minimum (<5% in the present experiments). All experiments were performed at room temperature (20–22°C).

For the whole-cell experiments, the current amplitudes were measured and compared under different conditions. During the single-channel experiments, the channel open time, burst time and closed time and channel open probability were measured. A channel opening was detected when the current reached 50% of the estimated single-channel amplitude, and the closing when the current returned to the 50% level. To help eliminate noise points from analysis, it was further stipulated that the mean amplitude during a burst must be at least 80% of the estimated single-channel amplitude to be considered a valid event. For the purpose of counting the number of total openings and the total opening probability, the data were analyzed with a brief burst terminator (0.08 ms). Correction for the multiple level of openings was done arbitrarily by doubling the number of such multiples and adding it to the singles. The open probability was calculated by fitting the sampled points of the open state in the total amplitude histogram either to single or double Gaussian fits of the distribution. The area obtained from the above fits was converted to the total open duration in seconds by multiplying them by the sampling interval (0.08 ms). Given the total recording time of that patch, the total open probability of all the channels under the patch could thus be calculated. The values obtained by this method were comparable to those estimated by the frequency of individual openings even though it did not take into consideration the settings defined for detection of the open events.

2.3. *Binding studies*

Adult male Wistar rats were killed by decapitation, and the hippocampus was isolated and homogenized in 50 vols of cold Tris-HCl buffer (5 mM, pH 7.4). The homogenate was centrifuged at 17000 × *g* for 15 min. The pellet was suspended at a concentration of 1 mg protein/ml and used without further treatment. (+)-[^{3}H]MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]-cyclo-hepten-5,10-imine maleate; 30 Ci/mmol; Dupont-NEN) was used as a probe for the ion channel of the NMDA receptor complex [22,23]. Binding was measured by a filtration procedure. Membranes (100 μg protein) were incubated in a medium containing 5 mM Tris-HCl, pH 7.4, [^{3}H]MK-801 (1 nM, unless otherwise indicated), and any competing ligands as required by the experiment. Nonspecific binding was determined by including 10 μM unlabelled MK-801 in a parallel series of tubes. After a 1-h incubation at room temperature, the suspension was filtered through glass fiber filters (no.32; Schleicher & Schuell, Keene, NH) using a Brandel (Gaithersburg, MD) filtration manifold. The filters were washed twice with cold buffer and their radioactivity content determined by liquid scintillation counting. Assays were routinely carried out in the absence and presence of 100 μM glutamate, which stimulated specific binding by 40–70%; nonspecific binding was not affected by glutamate.

3. RESULTS AND DISCUSSION

To determine the nature of interaction of Pb^{2+} with the glutamate receptor, whole-cell currents evoked by NMDA, quisqualate or kainate were recorded from hippocampal neurons. When administered as an admixture with NMDA, Pb^{2+} depressed the peak amplitude of the NMDA-activated whole-cell currents in a concentration-dependent manner (fig.1, table 1). The IC_{50} of Pb^{2+} for peak depression of NMDA currents was about 10 μM (n = 15). The effect of Pb^{2+} on the NMDA receptor was detected at concentrations as low as 2.5 μM, and almost complete abolition of the responses was observed at 50 μM. The inhibitory effect of Pb^{2+} was seen at both hyperpolarized and depolarized membrane potentials, and a partial recovery of these responses occurred in about 5 min with nearly complete recovery in 20 min (fig.1, table 1). The amplitude of the currents elicited by either quisqualate or kainate was slightly reduced at 50 μM of Pb^{2+}. Higher concentrations of Pb^{2+} (250 μM) produced about 30% depression of the currents (n = 6) induced by both quisqualate and kainate (fig.1). Thus, Pb^{2+} appears to have a selective blocking action at the NMDA-type of glutamate receptor. Earlier studies using the endplates from frog and mammalian muscles, also indicated a very weak inhibitory action of Pb^{2+} occurring only at high concentrations of this ion (100–200 μM) at the postsynaptic nicotinic acetylcholine receptors [24,25].

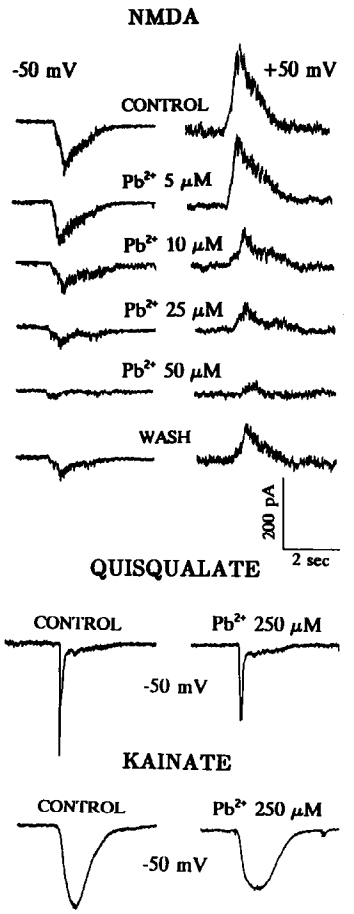

Fig.1. Effect of $PbCl_2$ on whole-cell currents evoked by 50 μM each of NMDA, quisqualate and kainate from rat hippocampal neurons. Both inward and outward currents evoked by NMDA under control condition, in the presence of graded concentrations of $PbCl_2$, and 5 min after wash, were obtained from the same neuron. Quisqualate and kainate responses were each obtained from separate neurons in the presence of 1 mM Mg^{2+} and 50 μM APV.

Table 1

Effect of different concentrations of $PbCl_2$ on the whole-cell currents evoked by NMDA from rat hippocampal neurons

Drug	% of control response	
	−50 mV	+50 mV
50 μM NMDA	100 (15)	100 (9)
50 μM NMDA + 1 μM Pb^{2+}	101.5 ± 1.5 (3)	–
50 μM NMDA + 5 μM Pb^{2+}	84.8 ± 2.7 (6)*	81.4 ± 5.3 (6)
50 μM NMDA + 10 μM Pb^{2+}	45.5 ± 2.4 (15)	46.4 ± 2.6 (9)
50 μM NMDA + 25 μM Pb^{2+}	30.1 ± 2.0 (6)	22.7 ± 2.1 (6)
50 μM NMDA + 50 μM Pb^{2+}	9.6 ± 1.0 (15)	10.1 ± 0.6 (9)
Wash (after 5 min)	47.7 ± 3.7 (12)	42.9 ± 2.5 (9)
Wash (after 20 min)	105.0 ± 2.8 (3)	–

Values presented are mean ± SE percentage of the control responses. The number of observations (n) obtained from a total of 15 neurons are indicated in parentheses after each value. * $P < 0.01$

To understand the molecular mechanism of action of Pb^{2+} at the NMDA-activated channels, single channel recordings under outside-out patch-clamp conditions were made from hippocampal neurons. These studies indicated a marked reduction in the frequency of NMDA-activated channel openings (fig.2). The inhibitory effect could be detected at Pb^{2+} concentrations as low as 500 nM. Quantitative analysis of the effect of Pb^{2+} performed at a concentration range between 5 and 20 μM indicated a statistically significant reduction in the frequency of activation of NMDA channels (table 2). Frequency of activation was estimated either by counting the rate of individual openings or by obtaining the total open probability, and both methods gave very similar results (table 2). The inhibitory effect of Pb^{2+} appeared within seconds after its addition, and the NMDA responses remained inhibited as long as Pb^{2+} was present. Unlike the quick onset of action, the reversal of Pb^{2+}-induced inhibition measured under single-channel recording conditions, was much slower. On repeated washing of the outside-out patches for more than 30 min, only a partial recovery (30–40%, n = 5 patches) of the responses was achieved. These results indicate that the action of Pb^{2+} at the NMDA-type glutamate channels is slowly reversible. The delay in the recovery time of Pb^{2+}'s action could have significance in that chronic exposure to the heavy metal, as occurs in vivo, can have a long-lasting effect at the NMDA receptors. Neuropsychological deficits have been reported in children [26] whose blood Pb^{2+} concentration was in the range of 1.5–2.5 μM (30–50 μg/dl). Selective accumulation of Pb^{2+} in the rat hippocampus, compared to blood or other brain regions [27–29], implies that concentrations of Pb^{2+} in the analogous regions in children, could reach levels high enough to inactivate the NMDA receptors. Indeed, increased Pb^{2+} concentrations have been reported in the hippocampi of Pb^{2+}-poisoned children [30].

Table 2

Effect of different concentrations of $PbCl_2$ on the frequency of activation of NMDA-evoked currents obtained from single channel recordings

Group	Percent of initial 10 min recording		
	11–20 min 5 μM	21–30 min 10 μM	31–40 min 20 μM
Open frequency			
Control	110 ± 5	111 ± 19.6	75 ± 14.6
Pb^{2+}	64 ± 10.7	27 ± 3.7	14 ± 3.6
Open probability			
Control	105 ± 11	100 ± 15.5	79 ± 13.5
Pb^{2+}	57 ± 10.5	20 ± 3.9	11 ± 1.7

Responses are presented as percent of the initial value obtained during the first 10 min recording in each patch. Values are the mean ± SE of either the frequency of individual openings or the total open probability obtained from 5 patches in each group. In the control group, patches were exposed to 10 μM NMDA throughout the recording session, whereas in the Pb^{2+} group, the patches were exposed to NMDA first then to increasing concentrations of $PbCl_2$ at the end of 10 min of recording, while maintaining the same concentration (10 μM) of NMDA. In both methods of analysis significant reduction ($P < 0.05$) (Student's t-test) is seen in the Pb^{2+} group

To further examine the kinetic interaction of Pb^{2+} with the NMDA-type channels, the effects of this cation were studied under a wide range of membrane potentials on the predominant, high conductance (40–45 pS in our study) channels using low concentrations of NMDA (5–10 μM). The time constants of open time, burst time and closed times analyzed in the absence and presence of Pb^{2+} are shown in fig.3. The closed time distribution disclosed a very fast (< 100 μs), an intermediate (0.15–1.5 ms) and a long (10–1000 ms) exponential component. Both open and burst time histograms showed short and long components. In this study the long component of the open and burst times was analyzed because this component remained consistent in several patches studied. Under control conditions, the plot of the mean burst time vs membrane potential was linear. The value for burst time ranged from 9.5 to 13 ms over the range of potentials evaluated (−80 to +60 mV) (fig.3B), indicating very little voltage dependency. This is consistent with results of a previous report [31] for NMDA-activated channels from mouse central neurons. However, in contrast to their results [31], the channel open times decreased sharply with membrane hyperpolarization in a nonlinear manner (fig.3A). In addition, the intermediate closed time increased exponentially with hyperpolarization (fig.3C) and the number of events per burst also increased at negative potentials under our control conditions (fig.3G). This effect resembles the blocking action of Mg^{2+} at NMDA-type channels [32] or bispyridinium compound-induced block at the nicotinic receptor channels [33,34] in contrast to that reported for the large-conductance glutamate receptor

ion channels in rat cerebellar granule neurons [35]. Even though Mg^{2+} was not added to our extracellular medium in experiments where NMDA was used as the agonist, a small contamination of about 1–3 μM of Mg^{2+} could have originated from other salts used. Alternatively, some other unidentified blocking molecule may be responsible for the behavior of the channel kinetics observed under our control conditions or the observed channel kinetics may represent the intrinsic gating mechanism of the NMDA channels. In

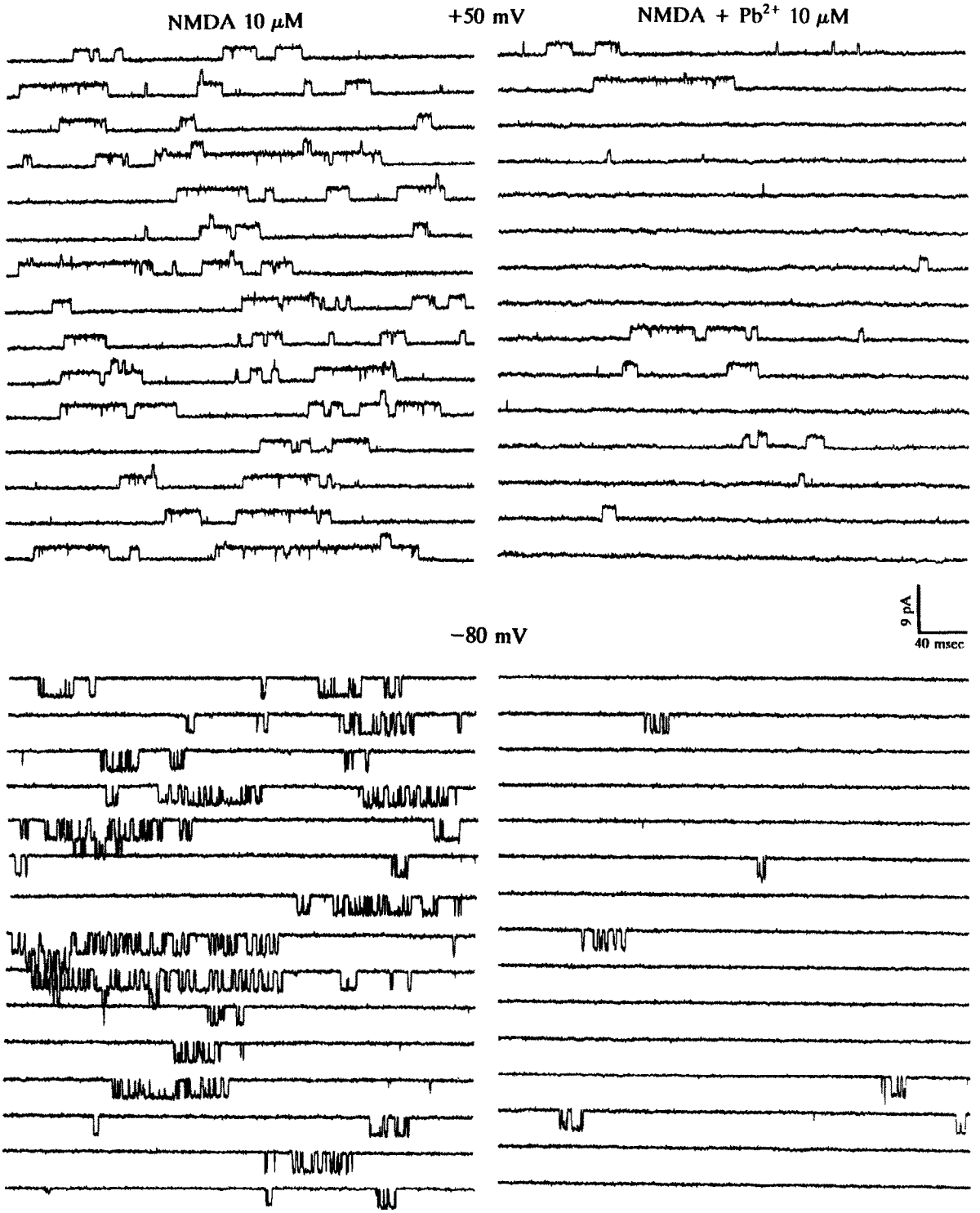

Fig.2. Samples of single channel recordings obtained from an outside-out patch from a rat hippocampal neuron. Channel currents activated by NMDA alone (left panels) and NMDA in the presence of 10 μM $PbCl_2$ (right panels) are shown on a continuous time scale in each panel. Note the reduction in the frequency of openings as the predominant effect of Pb^{2+} seen at both positive and negative membrane potentials. Data shown were filtered at 2.5 kHz.

contrast to Mg^{2+} [32], Pb^{2+} did not induce flickering of the channels during the open state nor change the mean channel open time over a potential range between −80 and +50 mV (fig.3A). Further, Pb^{2+} had no effect on the intermediate closed time even though the long closed interval was greatly prolonged. At the concentrations studied (up to 40 μM), Pb^{2+} failed to alter the single channel conductance. At negative membrane potentials (−60 mV and more negative potentials), Pb^{2+} reduced the number of openings per burst and also reduced the burst duration. However, the most striking effect of Pb^{2+} was seen in the form of a reduction in the channel activation which occurred at both hyperpolarized and depolarized membrane potentials. There was a reduction in the frequency of individual openings (fig.3E) and bursts (fig.3F), and a reduction in the overall probability of openings (fig.3H).

Evidence for a competitive interaction between Pb^{2+} and Ca^{2+} has been demonstrated in studies on synaptic transmission in bullfrog sympathetic ganglion [36] and rat muscles [25,37], and on synaptosomal uptake of choline [38]. To test such an interaction between Pb^{2+}

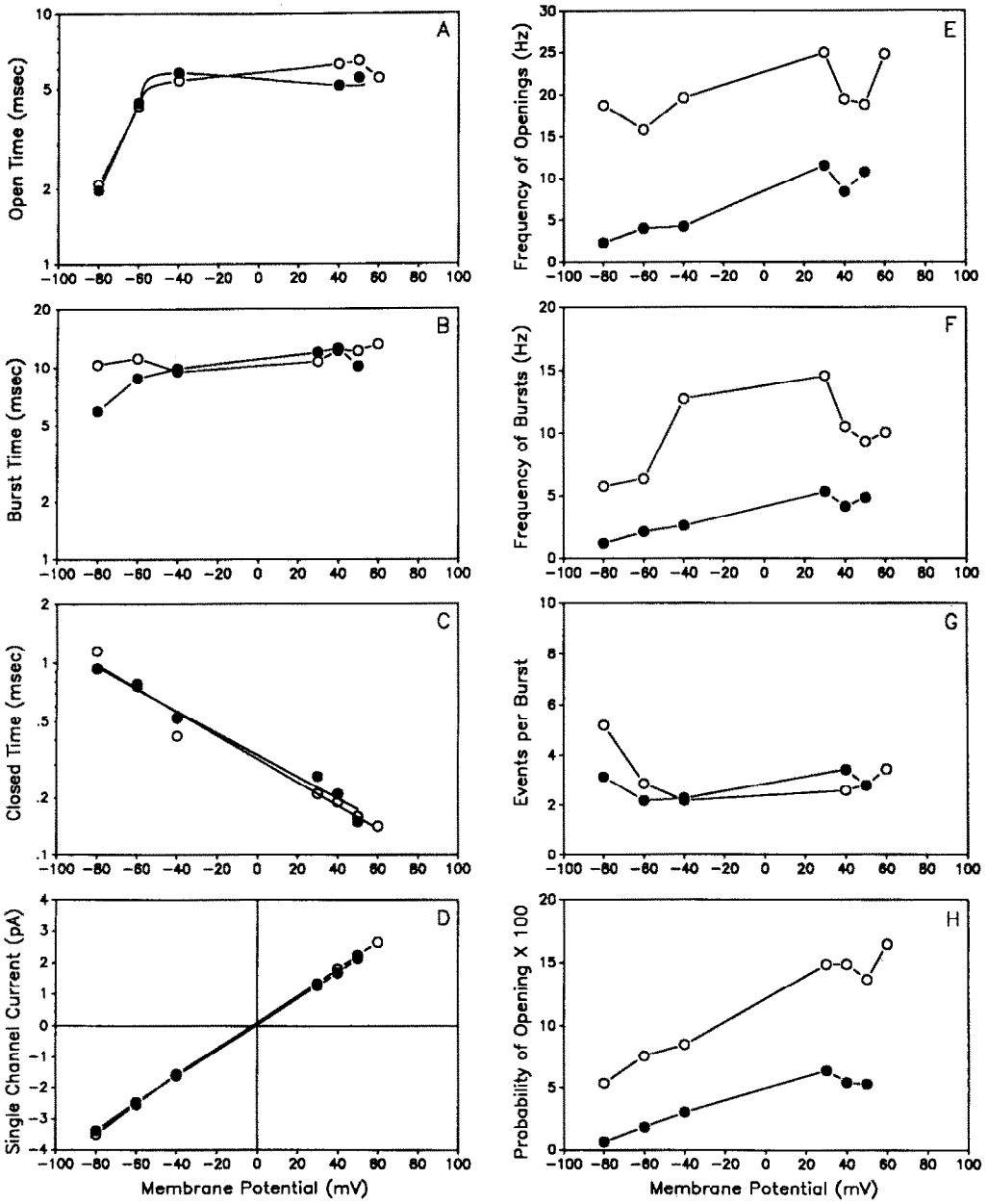

Fig.3. Effect of $PbCl_2$ on the kinetics of the single channel currents evoked by NMDA (10 μM) from hippocampal neuron. Relationship between membrane potential and mean channel open time (A), mean channel burst time (B), mean channel intermediate closed time (C), single channel current (D), frequency of open events (E), frequency of burst events (F), events per burst (G), probability of opening (H), were obtained in the absence (open circles) and presence of 10 μM $PbCl_2$ (filled circles) from a single outside-out patch. Similar results were seen in 4 separate patches.

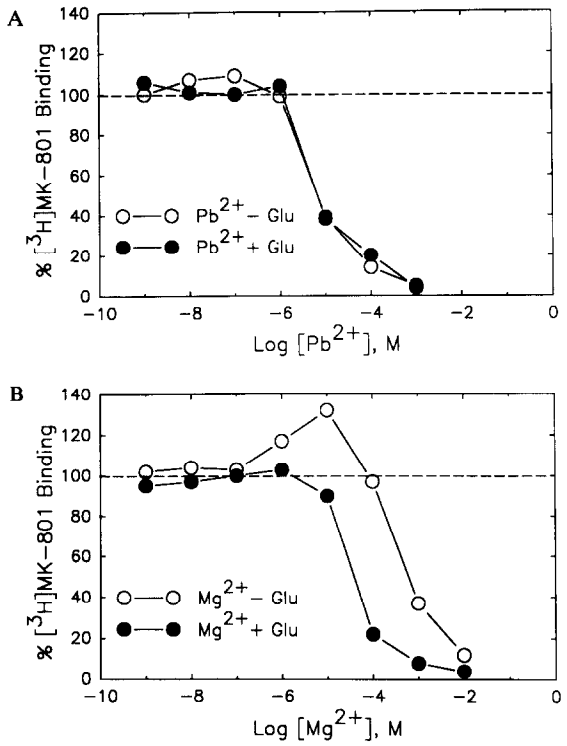

Fig.4. (A) Effect of $PbCl_2$ on [^{3}H]MK-801 binding to the ion channel of the NMDA receptor complex. Binding was measured in the absence (open circles) or presence (filled circles) of 100 μM glutamate. Binding is expressed as fraction of the total specific binding measured in the absence of Pb^{2+}; total specific binding was 45% greater in the presence of glutamate. Each point represents the mean of 3 determinations. (B) Effect of $MgCl_2$ on [^{3}H]MK-801 binding to the ion channel of the NMDA receptor complex. Binding was measured in the absence (open circles) or presence (closed circles) of 100 μM glutamate. Binding is expressed as fraction of total specific binding measured in the absence of Mg^{2+}. Each point represents the mean of 3 determinations.

and Ca^{2+}, we raised the extracellular concentration of Ca^{2+} from 2 mM to either 4 or 10 mM after eliciting the blocking action of Pb^{2+}. Although a reduction in the single channel conductance and a transient increase in the frequency was observed, the inhibitory action of Pb^{2+} was maintained. Additionally, prior addition or removal of Ca^{2+}, did not prevent or enhance respectively, the blocking action of Pb^{2+} on the NMDA receptor, suggesting that the effect of this metal ion was not mediated by a competitive interaction with Ca^{2+} at the NMDA channels. Raising the concentration of NMDA 2–10-fold (5 patches) failed to antagonize the action of Pb^{2+} on the frequency of activation, indicating that Pb^{2+} did not compete for the agonist binding site as in the case with 2-amino-5-phosphono valerate (APV) [39]. These results also rule out the possibility that the reduction in the frequency is not a consequence of a lowered NMDA concentration due to complex formation between the cation and the amino acid [32]. A Mg^{2+}-like channel-blocking effect [32] is also unlikely because neither a voltage-dependent block nor the presence of flickering and open time reduction was observed in the presence of Pb^{2+}. Addition of glycine (10–100 μM) in the extracellular medium after eliciting Pb^{2+}-induced block, failed to antagonize the inhibitory effect of Pb^{2+} (observed in 6 patches), suggesting that the glycine site [40] is not involved in the action of Pb^{2+}.

MK-801 has been reported to be a potent and selective noncompetitive blocker of the NMDA channels [22,41]. Access of [^{3}H]MK-801 to its binding site inside the channel is controlled by drugs that act at the NMDA receptor and modify the opening of the channel [42]. The divalent cations Zn^{2+} and Mg^{2+} have been reported to affect the binding kinetics of [^{3}H]MK-801 to putative NMDA-type channels in brain membranes [42,43]. In the present study, we have examined the effect of Pb^{2+} on the binding of [^{3}H]MK-801 to the rat brain hippocampal membranes and compared its effect with that of Mg^{2+}. As depicted in fig.4, Pb^{2+} inhibited the binding of [^{3}H]MK-801 in a concentration-dependent manner with an IC_{50} value close to 7 μM, and this effect was unaltered by inclusion of the agonist, glutamate, to the medium. Mg^{2+} was found to poorly inhibit the binding of [^{3}H]MK-801 (IC_{50} = 700 μM), but its inhibition was significantly enhanced in the presence of glutamate (IC_{50} = 40 μM). These results are consistent with the notion that Mg^{2+} interacts with the open state of the NMDA channels [32,42]. The failure of glutamate to shift the inhibitory concentration-response curve of Pb^{2+} (fig.4) indicates that Pb^{2+} acts predominantly at a closed conformation of the NMDA receptor which is consistent with a lack of effect of Pb^{2+} on the mean channel open time of NMDA-induced single-channel currents. In addition, the effect of Pb^{2+} resembles that of Zn^{2+} in that both cations produce a voltage-independent block of the NMDA channels [44] and they exhibit similar blocking potencies (Pb^{2+} being more potent than Zn^{2+}). Among several divalent cations, Zn^{2+} was found to be the most potent in inhibiting the binding of [^{3}H]MK-801 [43]. Our study reveals that Pb^{2+} is even more potent than Zn^{2+} in this action. It appears from these findings that Pb^{2+} and Zn^{2+} may bind to a similar site in the NMDA-receptor channel complex.

In summary, the present results demonstrate for the first time a blocking effect of Pb^{2+} on the NMDA subtype of glutamate receptors. The concentrations at which the blockade occurs are comparable to that found in lead-poisoned children. Ample evidence in the literature is now available to indicate the key role of NMDA receptors in the processes of learning and memory and also the impairment of such processes by Pb^{2+}. Therefore the blocking action of Pb^{2+} on the NMDA receptor ion channel certainly is an important clue for the exploration of the clinical effects of this cation.

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