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Citrate modulates the regulation by Zn^{2+} of *N*-methyl-D-aspartate receptor-mediated channel current and neurotransmitter release

(electrophysiology/zinc/granule neurons)

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ABSTRACT **The effect of the two metal-ion chelators EDTA and citrate on the action of *N*-methyl-D-aspartate (NMDA) receptors was investigated by use of cultured mouse cerebellar granule neurons and *Xenopus* oocytes, respectively, to monitor either NMDA-evoked transmitter release or membrane currents. Transmitter release from the glutamatergic neurons was determined by superfusion of the cells after preloading with the glutamate analogue D-[^{3}H]aspartate. The oocytes were injected with mRNA isolated from mouse cerebellum and, after incubation to allow translation to occur, currents mediated by NMDA were recorded electrophysiologically by voltage clamp at a holding potential of −80 mV. It was found that citrate as well as EDTA could attenuate the inhibitory action of Zn^{2+} on NMDA receptor-mediated transmitter release from the neurons and membrane currents in the oocytes. These effects were specifically related to the NMDA receptor, since the NMDA receptor antagonist MK-801 abolished the action and no effects of Zn^{2+} and its chelators were observed when kainate was used to selectively activate non-NMDA receptors. Since it was additionally demonstrated that citrate (and EDTA) preferentially chelated Zn^{2+} rather than Ca^{2+}, the present findings strongly suggest that endogenous citrate released specifically from astrocytes into the extracellular space in the brain may function as a modulator of NMDA receptor activity. This is yet another example of astrocytic influence on neuronal activity.**

Glutamate receptors activated by *N*-methyl-D-aspartate (NMDA) have been implicated in a variety of functions in the central nervous system, such as learning and memory (1), as well as in neurodegenerative processes (2). NMDA receptors are functionally regulated by a number of factors, such as divalent cations. Mg^{2+} blocks in a voltage-dependent manner the NMDA-gated ion channel (3, 4) which is permeable to Ca^{2+} and Na^+ ions (5). Moreover, Zn^{2+} has been shown to inhibit the activity of the receptor, probably via two specific binding sites (6, 7). It is therefore likely that the free extracellular concentrations of these ions play a pivotal role in modulating the activity of the NMDA receptor, implicating both short and long-lasting effects (1). Zn^{2+} attenuates NMDA-induced neurotoxicity (7, 8). In addition to its inhibitory action on NMDA receptors, Zn^{2+} potentiates non-NMDA-induced electrophysiological responses (9, 10) and neuronal cell death (11).

In cell cultures it has been demonstrated that citrate, a constituent of the tricarboxylic acid cycle, is exclusively released from astrocytes in large amounts (12, 13). In addition, it has been found that neurons do not accumulate citrate (13), which may explain the relatively high concentration (0.4 mM) of citrate in the cerebrospinal fluid (14, 15). Since citrate is a chelator of divalent cations, it is conceivable that citrate might influence the excitable state of neurons via regulation of the concentration of free divalent cations and thus play a central role as an endogenous modulator of the NMDA receptor in the central nervous system. This would suggest a hitherto unknown regulatory function of a tricarboxylic acid cycle constituent. To address this important question, experiments were performed on cultured cerebellar granule cells (glutamatergic neurons) and mRNA-injected *Xenopus* oocytes. This allowed the actions of NMDA, as well as those of varying concentrations of Zn^{2+} and citrate, on neurotransmitter release and channel currents to be investigated. For comparison glutamate, kainate, and EDTA, a potent chelator of divalent cation, were used. The present study provides evidence that citrate acts as an endogenous modulator of NMDA receptor-mediated functions.

MATERIALS AND METHODS

Materials. Seven-day-old mice were obtained from the animal quarters of the Panum Institute, University of Copenhagen. Plastic tissue culture Petri dishes were purchased from Nunc, fetal bovine serum from Sera-Lab (Crawley Down, Sussex, U.K.), insulin from NOVO-Nordisk (Copenhagen), and penicillin from Leo (Copenhagen). D-[2,3-^{3}H]Aspartate (specific radioactivity, 35 Ci/mmol; 1 Ci = 37 GBq) was purchased from DuPont/NEN. Excitatory amino acids were obtained from Tocris Neuramin (Bristol, U.K.). All other reagents were from Sigma.

Cell Cultures. Cerebellar granule cells were cultured from 7-day-old mice, after dissociation of the tissue as described (16). The cultures were maintained in medium containing 50 μM kainate to destroy γ-aminobutyratergic neurons (17), and after 2 days in culture 20 μM 1-β-D-arabinofuranosylcytosine was added to the medium to prevent astrocytic contamination (16). Cells were routinely used after 6–7 days *in vitro*, at which time they have properties similar to those of mature granule cells (18).

Release Experiments. Release experiments were carried out as described (19, 20). In brief, cells were loaded with D-[^{3}H]aspartate (2 μCi per culture) for 30 min and then rapidly washed with Hepes-buffered saline without Mg^{2+} (HBS: 135 mM NaCl/5 mM KCl/1.5 mM $CaCl_2$/10 mM Hepes/6 mM glucose, pH 7.4). The cultures were superfused continuously with HBS at a flow rate of 2 ml/min at 37°C. Compounds were delivered in the superfusate. Cells were stimulated (300 μM NMDA) for 30 sec. Samples were collected every 30 sec and counted for radioactivity in a liquid scintillation spectrometer. Glycine in the release experiments as well as in the electrophysiological experiments was always used together with NMDA at a concentration of 10 μM.

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Abbreviations: NMDA, *N*-methyl-D-aspartate; HBS, Hepes-buffered saline.

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Electrophysiology. *Xenopus* oocytes were prepared, mRNA was injected, and electrophysiological responses were recorded as described (21). Recordings were typically made 5 or 6 days after injection of 75 ng of mRNA isolated from mouse cerebellum (22). For voltage-clamp measurements, oocytes were superfused with Ringer solution (115 mM NaCl/2 mM KCl/1.5 mM $CaCl_2$/5 mM Hepes, pH 7.4). Compounds were delivered in the superfusate. Recordings were made at room temperature and at a holding potential of −80 mV.

Determination of Formation Constants. The formation constants for the [Ca–citrate]$^-$ and [Zn–citrate]$^-$ complexes were determined in HBS (for composition see *Release Experiments*) by use of myrexide, a dye suitable for measuring free Ca^{2+} and Zn^{2+} concentrations spectrophotometrically (23).

RESULTS

The reported formation constants (log K) for the [Ca–EDTA]$^{2-}$ and the [Zn–EDTA]$^{2-}$ complexes and the corresponding constants for [Ca–citrate]$^-$ and [Zn–citrate]$^-$ are shown in Table 1. Since the constants for the citrate complexes reported previously may be somewhat ambiguous with regard to the preference of citrate to bind Zn^{2+} rather than Ca^{2+}, these constants were determined in the solutions used in the present study. It was found that citrate chelated Ca^{2+} with a log K of 4.6 and Zn^{2+} with a log K of 6.0 in HBS (Table 1). We conclude that EDTA and citrate have a preference to chelate Zn^{2+} rather than Ca^{2+}, with EDTA being a more efficient chelator than citrate.

Release of preloaded D-[^{3}H]aspartate in cerebellar granule cells was increased about 3-fold by exposure to 300 μM NMDA and 10 μM glycine (Fig. 1). When the granule cells were stimulated with NMDA in the presence of 1 mM citrate the increase in evoked transmitter release was 167% of that seen with NMDA in the absence of citrate (Fig. 1). Stimulation with NMDA and 100 μM EDTA produced a similar profile but with an even larger increase (245%) in the evoked release. No effect of these compounds was seen when NMDA was omitted from the superfusion medium (Fig. 1). Zn^{2+} dose-dependently induced an inhibition of transmitter release evoked by 300 μM NMDA with an IC_{50} value of around 5 μM (Fig. 2). Addition of 1 mM citrate or 100 μM EDTA changed the IC_{50} value for the inhibition by Zn^{2+} to 75 and 100 μM, respectively (Fig. 2). Moreover, EDTA (100 μM) caused, even in the presence of up to 25 μM Zn^{2+}, an increase in transmitter release (130%) compared with the situation where Zn^{2+} and EDTA were omitted from the superfusion medium (100%). The effect of varying the concentrations of EDTA and citrate on the transmitter release induced by 300 μM NMDA in the presence of 50 μM Zn^{2+} is shown in Fig. 3. EDTA totally abolished the attenuation of transmitter release elicited by Zn^{2+} when the EDTA/Zn^{2+} molar ratio was >1. The corresponding effect of citrate was less pronounced, although a 50% recovery of the inhibition of Zn^{2+} was observed when 0.5–1 mM citrate was added. Moreover, 50 μM Zn^{2+} reduced the transmitter release induced by 10 μM glutamate to 49 ± 7% (n = 8) of the control value, and simultaneous addition of 1 mM citrate abolished this effect of Zn^{2+} (results not shown). When the NMDA receptor was blocked by 0.5 μM MK-801, the stimulatory action of glutamate on the transmitter release was no longer affected by Zn^{2+} (results not shown).

Table 1. Formation constants for Zn^{2+} and Ca^{2+} complexes formed by chelation with EDTA or citrate

Metal ion	log K		Ref.
	EDTA	Citrate	
Ca^{2+}	10.5–11.0	3.2–4.9	24
		4.6	This work
Zn^{2+}	15.3–16.7	3.6–4.9	24
		6.0	This work

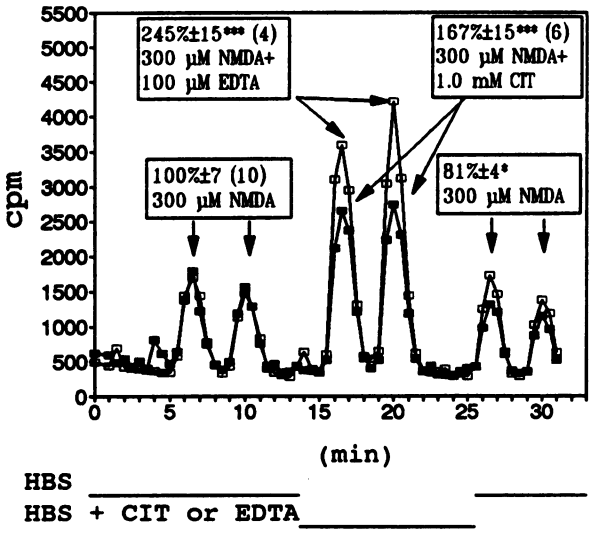

FIG. 1. Experiments showing the release of D-[^{3}H]aspartate from cultured cerebellar granule cells exposed to 300 μM NMDA and 10 μM glycine in the presence or absence of 1 mM citrate [■] or 100 μM EDTA [□] at the time indicated by arrows. Prior to the stimulations, citrate (CIT) and EDTA were added to the superfusate as indicated by horizontal bars.

To confirm the above results in another model system, *Xenopus* oocytes were injected with mRNA from cerebellum, and subsequent electrophysiological responses to application of excitatory amino acids were recorded. The effect of Zn^{2+} on responses induced by 300 μM NMDA plus 10 μM glycine or 100 μM kainate is shown in Table 2 and Fig. 4. Also in this model system Zn^{2+} produced a pronounced inhibition of the NMDA response. This inhibitory action of Zn^{2+} could be abolished by addition of citrate, and the presence of citrate even led to a larger response than that observed with NMDA and glycine added alone (Table 1 and Fig. 4). No such effect of Zn^{2+} or citrate was observed when 100 μM kainate was used to induce channel currents in the oocytes (Table 1). Likewise,

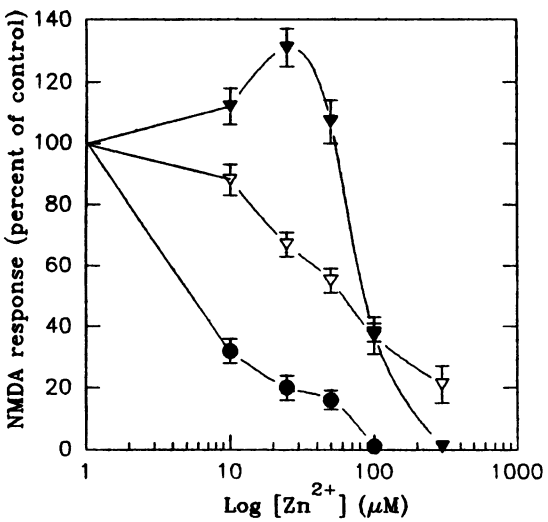

FIG. 2. Dose–response curves for the inhibitory action of Zn^{2+} (●), Zn^{2+} + 1 mM citrate (▽), and Zn^{2+} + 100 μM EDTA (▼) on release of D-[^{3}H]aspartate from cultured cerebellar granule cells induced by 300 μM NMDA and 10 μM glycine. Results are expressed as percent of control (no added Zn^{2+}, citrate, and EDTA) and represent the mean ± SEM of eight experiments.

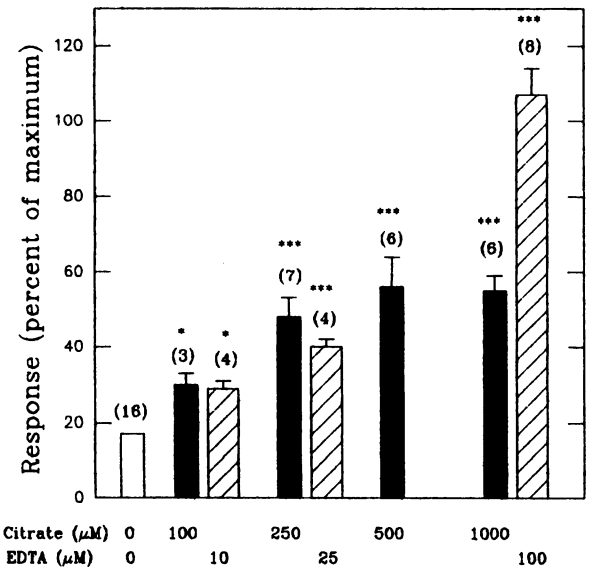

FIG. 3. Effect of various concentrations of citrate (black bars) and EDTA (hatched bars) on D-[^{3}H]aspartate release from cultured cerebellar granule cells induced by 300 μM NMDA and 10 μM glycine in the presence of 50 μM Zn^{2+} (open bars). Results are expressed as percent of the control value (100%, no added Zn^{2+}) and are averages of the number of experiments given in parentheses, with SEM values indicated by vertical bars. Asterisks indicate statistically significant differences from the control (*, $P < 0.05$; ***, $P < 0.001$; ANOVA F test).

kainate-induced transmitter release from cerebellar granule cells was not affected by Zn^{2+} (results not shown).

DISCUSSION

The observed potentiating action of citrate or EDTA on the evoked release of D-[^{3}H]aspartate from cerebellar granule cells (Fig. 1) is probably related to the ability of EDTA and citrate to chelate endogenously released Zn^{2+}. Release of endogenous Zn^{2+} has been shown to occur during neuronal activity in hippocampal slices (25) and in the hippocampal mossy-fiber zone of the rat brain *in situ* (26) at concentrations sufficiently high to block NMDA-mediated excitation (9, 10). In addition, the concentration of Zn^{2+} at the synapses of the CA3 region of the hippocampus has been estimated to be as high as 100–300 μM, whereas the concentration in the brain in general is in the range 1–20 μM (27). The observation that

Table 2. Effect of Zn^{2+} and citrate on excitatory amino acid-induced currents in *Xenopus* oocytes injected with mRNA from mouse cerebellum

Conditions (with 1.5 mM Ca^{2+})	Current, % of control	n
NMDA (control)	100 ± 11	8
NMDA + Zn^{2+}	38 ± 3***	8
NMDA + Zn^{2+} + citrate	160 ± 15**	8
Kainate (control)	100 ± 1	8
Kainate + Zn^{2+}	97 ± 1	8
Kainate + Zn^{2+} + citrate	103 ± 1	7

Experiments performed in *Xenopus* oocytes as outlined in Fig. 4 are summarized, showing the effect of 50 μM Zn^{2+} or 50 μM Zn^{2+} plus 1 mM citrate on currents induced by 300 μM NMDA and 10 μM glycine (NMDA) or 100 μM kainate in oocytes injected with mRNA from mouse cerebellum. Results are expressed as percent of control (100%, no added Zn^{2+} and citrate) and represent the mean ± SEM of n experiments on individual oocytes. Asterisks indicate statistically significant differences from the control (**, $P < 0.01$; ***, $P < 0.001$; ANOVA F test).

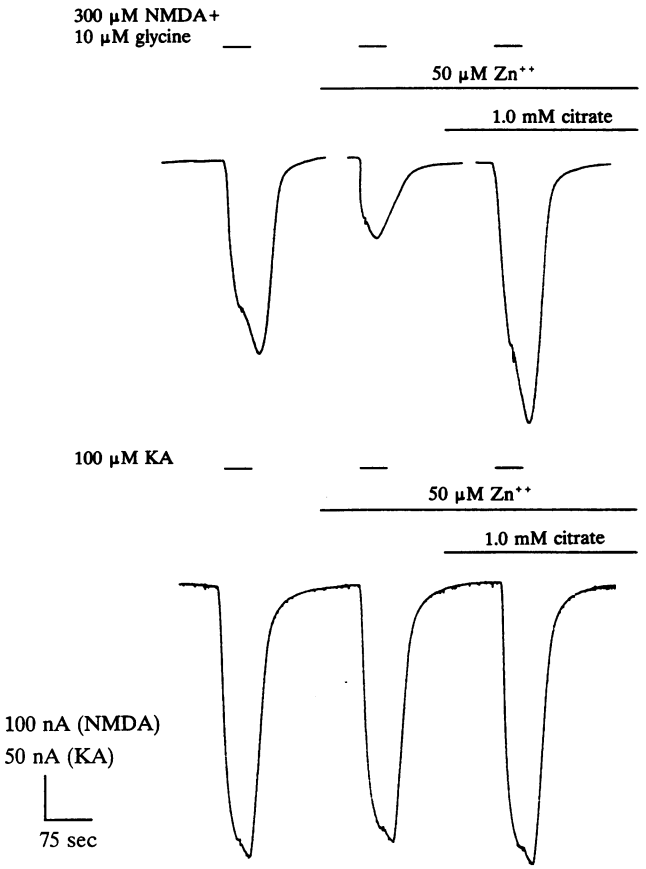

FIG. 4. NMDA (*Upper*) and kainate (*Lower*)-induced currents in a *Xenopus* oocyte injected with cerebellar mRNA recorded in the presence or absence of 50 μM Zn^{2+} or 50 μM Zn^{2+} plus 1 mM citrate. Data were obtained from an oocyte clamped at a holding potential of −80 mV. Downward deflections of the baseline indicate inward currents induced by 300 μM NMDA and 10 μM glycine or 100 μM kainate (KA) during the periods indicated by horizontal bars.

EDTA produced a larger increase in transmitter release than citrate, even at an EDTA concentration 10 times lower than that of citrate, corresponds well with the preference of citrate and EDTA to chelate Zn^{2+} rather than Ca^{2+}, with EDTA being the most efficient chelator (Table 1). The increase in transmitter release elicited by NMDA in the presence of 100 μM EDTA and up to 50 μM Zn^{2+} (Fig. 2) is also consistent with the high formation constant for the $[Zn–EDTA]^{2-}$ complex. By using the constants given in Table 1, the free Zn^{2+} and Ca^{2+} concentrations were calculated to be approximately 2.3 nM and 1450 μM, respectively, when the initial concentrations of Ca^{2+}, Zn^{2+}, and EDTA were 1500 μM, 50 μM, and 100 μM, respectively. By using the same initial concentrations and 1 mM citrate instead of EDTA, the concentrations of free Zn^{2+} and Ca^{2+} were calculated to be 2.0 μM and 581 μM, respectively. This may explain the finding that citrate was less efficient than EDTA in reversing the inhibitory action of Zn^{2+}, since 2 μM Zn^{2+} would still be able to partly inhibit the NMDA response (Figs. 2 and 3). Moreover, it can be concluded that the free Ca^{2+} concentration is high enough to allow a sufficient Ca^{2+} influx through the NMDA receptor to elicit transmitter release.

In *Xenopus* oocytes injected with mRNA from cerebellum, Zn^{2+} also produced a pronounced inhibition of the NMDA response, in accordance with the findings above and previous results with oocytes (9) and hippocampal neurons (28). In the oocyte system, citrate abolished the attenuation by Zn^{2+} and even led to a response larger than that observed with NMDA alone (Fig. 4 and Table 2). It has been reported that Zn^{2+} has

a stimulating action on responses mediated by non-NMDA receptors (9, 28), although the potentiation by Zn^{2+} of responses to kainate at low concentrations of Zn^{2+} was at most 10% (28). Under the experimental conditions used in the present study, no such effect of Zn^{2+} or citrate was observed when kainate was used to induce channel currents in oocytes (Table 2) or transmitter release from cerebellar granule cells (results not shown).

These results demonstrate that EDTA and citrate can, by a chelating action, influence in a reversible manner the attenuation of NMDA receptor activity produced by Zn^{2+}. However, since NMDA receptor activity is attenuated not only by Zn^{2+} but also by Mg^{2+} (3, 4), citrate might exert some of its action *in vivo* by chelating Mg^{2+}. The formation constant (log *K*) for $[Mg\text{–citrate}]^-$ is reported to be 3.2–3.3 (24)—i.e., much lower than that for the $[Zn\text{–citrate}]^-$ constant (Table 1). Therefore it is likely that chelation of Mg^{2+} by citrate *in vivo* may play only a minor role. On the basis of the chelation of Zn^{2+} and the high concentration of citrate in the cerebrospinal fluid, it is attractive to suggest a role of citrate as an endogenous modulator of glutamate-mediated excitation through the NMDA receptor. In this context, it is of particular interest that astrocytes may have a pronounced regulatory role, since citrate is synthesized and released exclusively from these cells (12, 13). This is another example of the many modulatory actions of astrocytes on neuronal activity (29). It may be of importance to investigate whether citrate can chelate Zn^{2+} bound both to the external surface and within the channel of the NMDA receptor complex. Further studies of these possible functions of citrate therefore appear warranted.

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