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N-methyl-D-aspartate-induced $^{45}Ca^{2+}$ release from pre-labelled adult rat hippocampus *in vivo*

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Abstract. We report the results of microdialysis experiments investigating the NMDA-induced release of intracellular Ca^{2+} in different brain regions. Microdialysis probes were implanted stereotaxically into the striatum, thalamus and hippocampus dentate gyrus (DG) of adult rats. Dialysates were analysed for alterations in the concentration of ionized Ca^{2+} in an initially calcium-free medium and for changes in ^{45}Ca efflux from the pre-labelled endogenous Ca^{2+} pools. The application of 5 mM of NMDA to the dialysis medium for 20 min in the striatum, resulted in increases in Ca^{2+} and ^{45}Ca concentrations by 25% and 35% respectively. After NMDA perfusion in the hippocampus DG and in the thalamus, decreases in the Ca^{2+} concentration to 65.6% and 38.6% of the basal level respectively, were accompanied by increases in ^{45}Ca efflux, exceeding 1,500% of the basal level in the hippocampus. Cell swelling, and the corresponding reduction of the extracellular space volume was insufficient to explain the huge increase in ^{45}Ca efflux. Thus, our experiments demonstrated that *in vivo* in the rat hippocampus DG, NMDA induces the release of ^{45}Ca to the extracellular space from unidentified intracellular calcium stores.

Key words: adult rats, Ca mobilization, hippocampus, thalamus, striatum, microdialysis, NMDA

INTRODUCTION

Activation of excitatory amino acid (EAA) receptors sensitive to N-methyl-D-aspartate (NMDA) induces opening of the large-conductance Ca^{2+} channels and a massive Ca^{2+} influx into neurones (Mayer and Westbrook 1987). This causes an increase in intracellular calcium concentration ($[Ca^{2+}]i$) (Bouchelouche et al. 1989, Courtney et al. 1990, Holopainen et al. 1990, Ciardo and Maldelesi 1991). Although influx of extracellular Ca^{2+} greatly participates in elevation of $[Ca^{2+}]i$ in neurones, the role of intracellular calcium pools may also be substantial. Recent *in vitro* data indicate that NMDA receptors may mediate Ca^{2+} mobilization from the intracellular pools (Lei et al. 1992, Dayanithi et al. 1993, Frandsen and Schousboe 1993, Simpson et al. 1993). The role of increased $[Ca^{2+}]i$ in EAA neurotoxicity has been demonstrated (Choi 1985, 1988, 1995). Dantrolene, an inhibitor of intracellular Ca^{2+} mobilization has been shown to prevent excitotoxic and ischemic neuronal damage *in vitro* and *in vivo* (Frandsen and Schousboe 1991, 1992, Lei et al. 1992, Zhang et al. 1993).

A rise in $[Ca^{2+}]i$ activates compensatory mechanisms with extrusion of Ca^{2+} from the cell through the action of an ATP-driven Ca^{2+} pump and an Na^+/Ca^{2+} antiporter (Gill 1981, Blaustein 1988). However recent data, Arens et al. (1992), indicates that in rat hippocampal slices *in vitro* stimulation of NMDA receptors prevents the presumed activation of intracellular Ca^{2+} release *via* Na^+/Ca^{2+} exchange.

A method for brain microdialysis, combined with the detection of calcium ions in dialysates and, the measurement of ^{45}Ca efflux from pre-labelled tissues (Łazarewicz et al. 1986), allows us to apply agonists directly to selected brain regions and to detect extracellular calcium transients, including ^{45}Ca release from pre-labelled cells. Thus, in this study *in vivo* microdialysis combined with ^{45}Ca and ionized Ca^{2+} measurements was used in attempts to demonstrate the NMDA-evoked intracellular ^{45}Ca release to the extracellular space in the adult rat striatum, thalamus and hippocampus. These brain structures differentially express the NMDA receptors and such indicators of different intracellular Ca^{2+} pools as ryanodine receptors and calcium binding proteins. The detection of $[^{14}C]$sucrose efflux allowed us to correct for changes in the extracellular space volume. The results of this study have been partially reported previously in abstract form (Łazarewicz et al. 1994).

METHODS

Animals, microdialysis

Thirty six adult Wistar rats (200-250 g body weight) were used for these experiments. Local ethical committees approved all of the animal procedures used in this study. Microdialysis probes CMA/11 (CMA Microdialysis AB, Stockholm, Sweden), membrane length 1 mm, outer diameter 0.24 mm, were implanted stereotaxically into the selected brain areas of rats anaesthetized with urethane (1.25 g/kg i.p.). The coordinates for the tip of the cannula according to a stereotaxic atlas of Paxinos and Watson (1982) for implantations into the striatum were: 2.5 mm lateral, 0.7 mm anterior relative to bregma, and 5.5 mm ventral from dura. The probes were implanted into the dentate gyrus (DG)/CA4 subfields of the hippocampus according to coordinates: 2.3 mm lateral, 3.8 mm posterior relative to bregma, and 3.5 mm ventral from dura. The coordinates for implantations into the thalamus were: 2.0 mm lateral, 1.8 mm posterior to bregma and 6.5 mm ventral from dura.

The probes were perfused with Krebs Ringer bicarbonate (KRB) medium (NaCl 122 mM, KCl 3 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 0.4 mM, $NaHCO_3$ 25 mM, pH 7.4), containing either 1.3 mM $CaCl_2$ or nominal calcium free (no calcium was added), as indicated below. The rate of dialysis was 2.5 µl/min in $[^{45}Ca]$ experiments or 5 µl/min in $[^{14}C]$sucrose experiments.

Changes in ^{45}Ca efflux

After implantation, the probes were perfused for 1 h with KRB medium containing 1.3 mM (25 µCi) $^{45}CaCl_2$, followed by 150 min equilibration - per-

fusion with non-radioactive, calcium-containing KRB medium. Thereafter samples were collected every 5 min. After 20 - 30 min of measuring the control efflux, the medium containing 5 mM NMDA was introduced for 20 min. Then the control dialysis medium was reintroduced and samples were collected for an additional 70 - 80 min. The radioactivity of ^{45}Ca in each sample of dialysate was measured by liquid scintillation counting, and changes in ^{45}Ca efflux were calculated from the data as described previously (Łazarewicz et al. 1986). Briefly, after a 1 h loading with ^{45}Ca and a 2.5 h equilibration period, a steady-state decay in ^{45}Ca radioactivity in dialysates, represented by a straight line on the semilog plot vs. time was observed. These decay curves for individual experiments served as a basis for calculating changes in ^{45}Ca efflux evoked by NMDA. Thus, the radioactivity of samples collected during the first and last 20-30 min of the experiment were used to extrapolate the control values for each time period by a computer-aided semilog regression analysis, and all the results were expressed as a percentage of this extrapolated basal value. *In vitro* calibration (recovery tests) of the dialysis probe used in this study demonstrated 4.3% recovery of $^{45}Ca^{2+}$.

Total Ca^{2+} measurements

In these experiments ^{45}Ca was not used. After control dialysis for 3.5 h, a calcium-containing medium was substituted for a calcium-free medium. Then, after 30 min of wash-out, the experiments were scheduled as described for ^{45}Ca efflux measurements. The samples of dialysates were collected for a total calcium determination. Samples representing consecutive 20-30 min periods of each experiment, were pooled and the Ca^{2+} concentration was measured using ion selective microelectrodes, as described previously (Hagberg et al. 1984).

Changes in sucrose space volume

In separate experiments [^{14}C]sucrose was used to determine changes in the extracellular volumes during NMDA application (Katayama et al. 1992). The probes were perfused for 20 min with KRB containing 10 mM sucrose labelled with 25 μCi of [^{14}C]sucrose, followed by a 20 min wash-out perfusion with KRB medium. To increase the precision of [^{14}C]sucrose efflux measurements, samples were collected every 2 min, however the dialysis rate was twice that of calcium experiments (see above). The samples were collected for a total period of 50 min. Basal efflux for 10 min was followed by a 20 min perfusion with 5 mM NMDA, then by a perfusion with standard KRB medium for 15 min. The radioactivity of [^{14}C]sucrose in each sample of dialysate was measured by liquid scintillation counting. A one-compartmental straight line on the semilog plot was never observed in [^{14}C]sucrose samples. The efflux of radioactivity during the initial 10 min, was used as a basis, for calculating a basal [^{14}C]sucrose efflux. From the graphical analysis of a semilog plot of [^{14}C]sucrose radioactivity in consecutive samples vs. time, changes in the sucrose efflux were calculated and expressed as a percent of basal level. An *in vitro* calibration test, demonstrated a 2.2% [^{14}C]sucrose recovery.

Morphological examination

The position of the probes in each experiment was determined by morphological examination. After experiments the rats were submitted to a deep barbiturate anesthesia and the brains were fixed in situ by intracardial perfusion with 4% neutralized formalin solution. After a minimum of 1 week the brains were transferred to ethanol (99.9%) and embedded in paraffin. Subsequently, 10 μm-thick cross sections were stained with cresyl violet.

Materials

N-methyl-D-aspartic acid (NMDA) was purchased from Sigma, St. Louis, MO, USA. All other chemicals were of analytical grade. $^{45}CaCl_2$ and [^{14}C]sucrose were obtained from Amersham, England.

Data analysis

A non-parametric Walsh test (Siegel 1956) was used for testing the statistical significance of the effects of NMDA application, when compared with basal levels. However, for graphical reason, data are presented in figures as mean values ± SEM.

RESULTS

Morphology

Morphological examination confirmed the successful implantation of the dialysis probes tips to locations in the central part of the striatum (caudoputamen), thalamus (ventral nuclear complex), and dorsal hippocampus (inner blade of the dentate gyrus). Implanting the probes did not produce extensive damage to the neurones located more than 30 μm from the trace of the microdialysis probe (results not shown).

Calcium ions

Basal concentrations of calcium ions in samples of dialysates which were collected from the adult rat striatum, thalamus, and hippocampus during dialysis perfusion prior to NMDA application, were 38.0 ± 9.0 μM, 22.5 ± 6.6 μM, and 25.9 ± 4.7 μM, respectively. Subsequent changes in Ca^{2+} concentrations were expressed as a percent of the basal values which were taken as 100% (Fig 1). In the samples of dialysate from the adult rat striatum, collected during the application of 5 mM NMDA to the perfusate, there was a transient 25% increase in Ca^{2+} concentration, which was followed by a return to the basal level in subsequent samples. In dialysates from the thalamus and hippocampus collected during NMDA perfusion, there was a decrease in Ca^{2+} concentration. A maximal decrease to 38.6% and 65.6% of the basal level, respectively, was found in samples collected 20 min after termination of the NMDA perfusion. In dialysates collected from the thalamus during the next 20 min, Ca^{2+} concentration remained considerably below the basal level, whereas in the hippocampus there was a complete recovery to the initial value (Fig. 1).

^{45}Ca efflux

During the whole period of dialysis perfusion to the adult rat striatum with 5 mM NMDA, a 30 - 40% increase in ^{45}Ca efflux was observed. This was followed by a rapid return to baseline with only non-significant decreases in ^{45}Ca efflux during the post-NMDA perfusion (Fig. 2, insert).

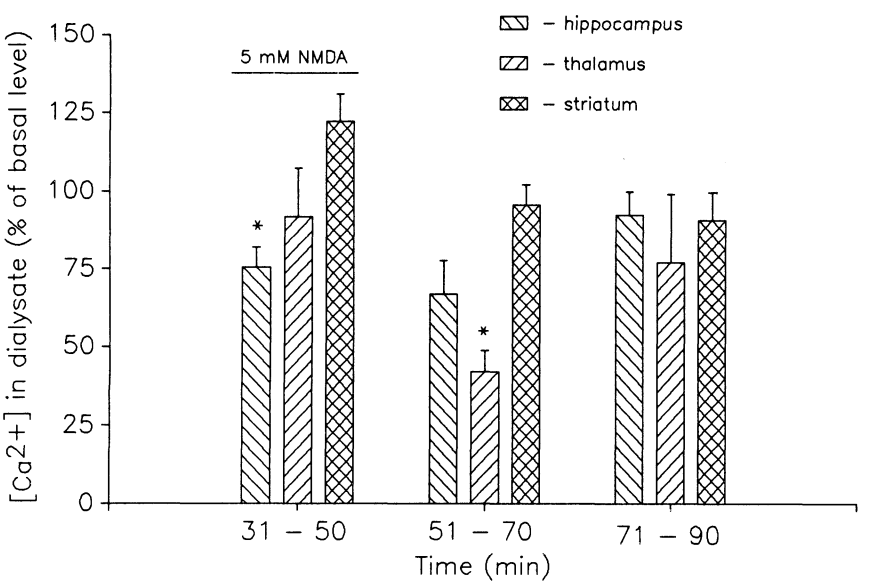

Fig. 1. Effect of 5 mM NMDA applied in the dialysis medium on Ca^{2+} concentration in dialysates of the adult rat striatum, thalamus, and hippocampus. Microdialysis probes were stereotaxically implanted into the selected areas of adult rats and perfused at 2.5 μl/min with nominal Ca^{2+}- free medium. NMDA was applied for 20 min after 30 min of the control perfusion. Pooled samples representing 20-min dialysates were analysed for Ca^{2+}. Results are means ±SEM (n=4). $^{*}P<0.05$.

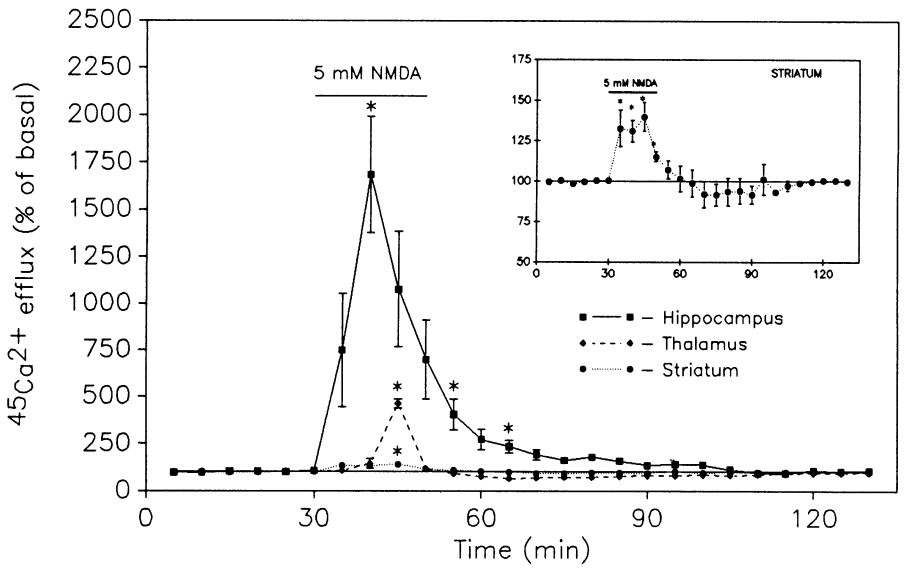

Fig. 2. Effect of 5 mM NMDA on ^{45}Ca efflux from the adult rat striatum (see also insert), thalamus, and hippocampus. After ^{45}Ca labelling of the brain areas through the dialysis medium and equilibration, samples were collected in 5 min intervals. NMDA was applied as indicated by the horizontal bar. For other explanations see Fig. 1. Data expressed as % of basal values. Results are means ±SEM (n=4). $^{*}P$<0.05.

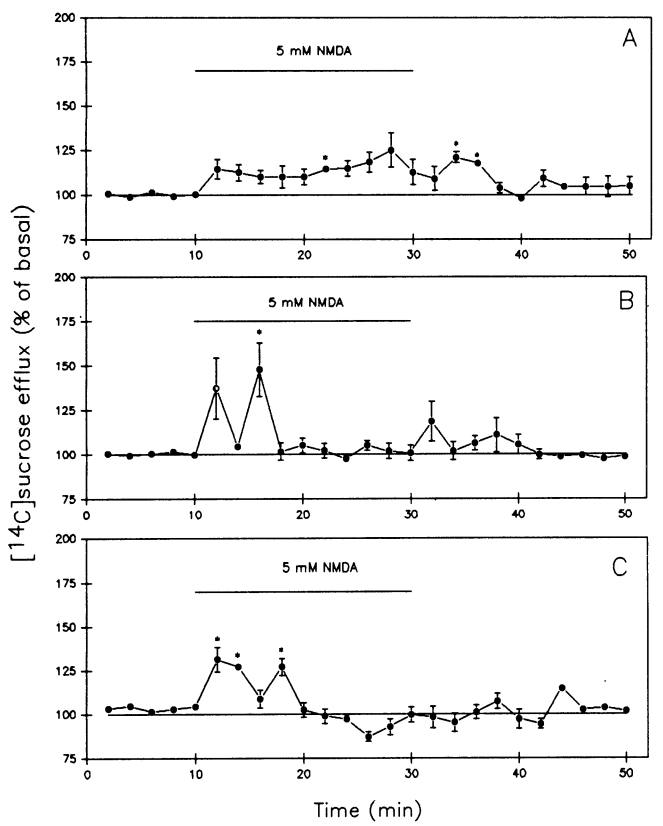

Fig. 3. Effects of 5 mM NMDA on [^{14}C]sucrose efflux from the adult rat striatum (A), thalamus (B), and hippocampus (C). After [^{14}C]sucrose labelling of the brain areas through the dialysis medium and equilibration, samples were collected in 2 min intervals. NMDA was applied as indicated by the horizontal bar. Perfusion rate: 5 µl/min. Other explanations as for Figs. 1 and 2. Data expressed as % of basal values. Results are means ± SEM (n=4). $^{*}P$<0.05.

The application of 5 mM NMDA to the thalamus and to the hippocampus induced a brief, massive increase in ^{45}Ca efflux, by 450% and 1,600%, respectively. After termination of NMDA perfusion there was a rapid recovery to the basal ^{45}Ca efflux rates (Fig. 2).

[^{14}C]sucrose efflux

A perfusion with 5 mM NMDA induced 20% - 30% increases in [^{14}C]sucrose efflux from all three brain structures, which was followed by a rapid return to the baseline after termination of NMDA perfusion (Fig. 3 A, B and C).

DISCUSSION

The results of this *in vivo* study demonstrate that in the hippocampus and to a lesser extent in the thalamus, but not in the striatum of adult rats, NMDA application induces enhanced release of intracellular ^{45}Ca. To detect this phenomenon, which may reflect mobilization of intracellular calcium pools, we utilised a complex microdialysis approach combined with measurements of total and radioactive calcium and the estimation of changes in the volume of extracellular space.

The measurements of ^{45}Ca efflux have been used previously in several *in vitro* studies to visualize the release of intracellular calcium (Borle 1972,

Łazarewicz et al. 1977, Łazarewicz and Kanje 1981, Authi et al. 1993). During the present *in vivo* experiments, after a prolonged labelling of the endogenous pool of Ca^{2+} with ^{45}Ca and subsequent washing with non-radioactive KRB medium, the bulk of ^{45}Ca remains inside the cell. Thus, an increase in ^{45}Ca efflux in response to NMDA, which exceeds similar changes in ionized Ca^{2+} concentrations, may reflect an enhanced release of more radioactive ^{45}Ca from the intracellular compartment. The nature of the changes in ^{45}Ca efflux to dialysates may not be discussed without information on changes in the total extracellular Ca^{2+} concentration. For the ionized Ca^{2+} measurements the rat brain structures were dialysed against a nominal calcium-free medium, thus calcium transients in the dialysates reflected changes in extracellular Ca^{2+} concentration in the vicinity of the dialysis probe. To take into account the influence of NMDA-evoked cell swelling on concentrations of extracellular components (Heinemann 1986), we measured [^{14}C]sucrose efflux. As it has been previously shown by Katayama et al. (1992), an increase in [^{14}C]sucrose efflux in the rat hippocampus during ischemia reflects stimulation of the NMDA receptors leading to cell swelling.

Our present results demonstrate that the application of NMDA induces approximately 20%-30% increase in the efflux of [^{14}C]sucrose from all the brain regions tested. The NMDA-induced shrinkage of the extracellular space may only slightly modify changes in calcium concentrations and in the ^{45}Ca efflux which we observed in the hippocampus and thalamus. However they may explain increases in extracellular calcium concentration and in ^{45}Ca release in the striatum.

The lack of a significant decrease in extracellular Ca^{2+} evoked by NMDA in the striatum indicates that activation of the striatal NMDA receptors does not induce a prolonged destabilization of calcium homeostasis in neurones that could be detected by our methods. NMDA induced a massive and long lasting decrease in the extracellular calcium concentration in the thalamus and hippocampus. Previously, using the same method, we have demonstrated NMDA-evoked decreases in extracellular calcium concentration in the rabbit hippocampus (Łazarewicz et al. 1995). Similar decreases in extracellular calcium concentrations evoked by NMDA application in slices of the rat hippocampus have been demonstrated using calcium-sensitive microelectrodes (Heinemann et al. 1988, Arens et al. 1992). Thus, a large NMDA-induced reduction of Ca^{2+} concentration in dialysates of the hippocampus and the thalamus found in this study reflects the decrease in extracellular calcium concentration caused by the influx of calcium to neurones.

A differential effect of NMDA on extracellular Ca^{2+} concentration and ^{45}Ca efflux was found in the rat hippocampus DG and in the thalamus. Since application of NMDA induces a concomittant decrease in Ca^{2+} concentration and a very large increase in ^{45}Ca efflux, the latter effect evidently reflects the release of more radioactive intracellular calcium to the extracellular space. This event is accompanied by the NMDA-evoked influx of extracellular calcium to neurones. Our unpublished data demonstrates that in the rat hippocampus DG the NMDA-induced increase in ^{45}Ca efflux is dependent on NMDA concentration, sensitive to NMDA receptor antagonists and to amiloride derivatives inhibiting Na^+/Ca^{2+} exchange (Rybkowski and Łazarewicz, in preparation). A neuronal origin for the bulk of NMDA-activated calcium fluxes is indisputable. However, in light of the recently described expression of NMDA receptors on glia (Luque and Richards 1995, Porter and McCarthy 1995), and of previous data on KCl and neurotransmitter-induced ^{45}Ca release form isolated and cultured glia (Łazarewicz et al. 1977, Łazarewicz and Kanje 1981), a possible participation of glia cannot be ruled out.

A simple explanation of the effect of NMDA-evoked ^{45}Ca release from the pre-labelled brain structures as an exchange between the extracellular and more radioactive intracellular calcium does not apply to all experimental data. It has been demonstrated in our previous studies in the rabbit hippocampus that NMDA application induces a decrease in ^{45}Ca efflux which is dependent on

NMDA concentrations and inhibited by NMDA receptor antagonists, and follows a corresponding decrease in ionized Ca^{2+} concentration in dialysate (Łazarewicz and Salińska 1993, Łazarewicz et al. 1995). Moreover, an NMDA-induced decrease in extracellular Ca^{2+} concentrations in rat CA1, without signs of activation of Ca^{2+} extrusion from cells, has been reported by Arens et al. (1992). These differences may result from distinct properties of cellular calcium homeostasis in the rat hippocampal CA1 and DG, and in the rabbit hippocampus. The differences may concern the various components of the complex calcium homeostasis machinery such as the expression of calcium binding proteins, calcium stores in the endoplasmic reticulum, and calcium release mechanisms. Intracellular ^{45}Ca, which is released from the rat hippocampus during NMDA stimulation, may originate from different pools. NMDA-induced mobilization of Ca^{2+}/ryanodine sensitive pool of calcium in the endoplasmic reticulum has been recently described in cultured neurones (Frandsen and Schousboe 1992, Dayanithi et al. 1993, Simpson et al. 1993). A displacement of Ca^{2+} bound to intracellular calcium binding proteins is also possible (Baimbridge 1992, Chard et al. 1993).

Differences between the various rat brain structures investigated with respect to the influence of NMDA on extracellular Ca^{2+} concentrations and ^{45}Ca efflux, should also be considered in light of the differential expression of NMDA receptors, calcium binding proteins and vesicular Ca^{2+} stores. The differential effects of NMDA in these rat brain regions cannot be explained by fundamental differences in the distribution of NMDA receptors, as they are abundant in all subregions of hippocampus, thalamus and striatum (Monaghan and Cotman 1985, Monaghan et al. 1988, Brose et al. 1993, Greenamyre et al. 1984, Beaton et al. 1992, Jacobson and Cottrell 1993). The high density of ryanodine, but not of trisphosphoinositol (IP3) receptors, has been found in the rat DG, they are also present in the striatum, whereas the thalamus expresses few ryanodine and IP3 receptors (Sharp et al. 1993, Smith and Nahorski 1993). On the other hand, a high density of calbindin-D_{28k} immunoreactivity has been found in the DG of the rat hippocampus (Baimbridge et al 1982, Sloviter 1989, Andressen at al. 1993), whereas this calcium binding protein is expressed heterogeneously in the rat striatum and in the thalamus (Frassoni et al. 1991, Liu and Graybiel 1992, Arai et al. 1994). Thus, based on these data, a role for the ryanodine-sensitive intracellular calcium pool in the endoplasmic reticulum and of calbindin-D_{28k} in the mechanism of NMDA-induced ^{45}Ca release may be proposed. Our subsequent studies will be focused on the identification of intracellular ^{45}Ca pools that participate in the NMDA-induced ^{45}Ca release in the adult rat hippocampus DG, where this effect is highly pronounced and coincides with the expression of ryanodine receptors and calbindin-D_{28k}.

In conclusion, the results of this study indicate that a large NMDA-induced influx of extracellular calcium to cells of the rat hippocampus and thalamus is accompanied by the release of intracellular calcium pre-labelled with ^{45}Ca to the extracellular space. Calcium that is released as a result of NMDA-stimulation may originate from various intracellular Ca^{2+} pools including calcium stores in the endoplasmic reticulum and Ca^{2+} displaced from calcium binding proteins.

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