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Research Report

Nitric oxide modulates calcium entry through P/Q-type calcium channels and *N*-methyl-D-aspartate receptors in rat cortical neurons

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Abstract

Voltage-gated calcium channels (VGCC) and *N*-methyl-D-aspartate receptors (NMDAR) account for most of the depolarization-induced neuronal calcium entry. The susceptibility of individual routes of calcium entry for nitric oxide (NO) is largely unknown. We loaded cultured rat cortical neurons with fluo-4 acetoxymethylester to study the effect of the NO synthase inhibitor *N*ω-nitro-L-arginine and the NO donor *S*-nitroso-*N*-acetylpenicillamine on the intracellular calcium concentration ($[Ca^{2+}]_i$). The potassium-induced $[Ca^{2+}]_i$ increase was amplified by *N*ω-nitro-L-arginine and attenuated by *S*-nitroso-*N*-acetylpenicillamine. This modulation was abolished by either the P/Q-type VGCC antagonist ω-agatoxin IVA or by the NMDAR antagonist MK-801, but not by N-type (ω-conotoxin GVIA) or L-type (nimodipine) VGCC blockers. These results suggest that NO can modulate neuronal calcium entry during depolarization by interacting with P/Q-type VGCC and NMDAR.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Other neurotransmitters

Keywords: Nitric oxide; Voltage-gated calcium channel; Neocortex; *N*-methyl-D-aspartate receptor; Excitotoxicity; Cell culture

1. Introduction

Intracellular calcium is critically involved in almost all aspects of the neuronal life cycle, including development, differentiation, migration, communication, plasticity, and death. Moreover, changes of intracellular calcium pathways have been implicated in a plethora of neurological diseases, such as migraine, epilepsy, ischemia, cerebral hemorrhage, and Alzheimer's disease [21]. Nitric oxide (NO) shows a similarly ubiquitous role in health and disease of the central nervous system, and, perhaps not surprisingly, it has been shown that NO is fundamentally involved in many aspects of calcium signaling in the neuroglial network [12,20,30]. However, the effect of the tissue NO level on the intracellular calcium level following neuronal activation and the individual calcium channels that govern this effect are largely unknown. In this study, we focused on the susceptibility for NO of different routes of calcium entry into cultured rat cortical neurons following depolarization. Using the intracellular calcium indicator fluo-4 acetoxymethylester (fluo-4 AM), we applied inhibitors of voltage-gated calcium channels (VGCC) and *N*-methyl-D-aspartate (NMDA) receptors since most of the intracellular calcium surge following neuronal depolarization is mediated by these channels [25,28]. Our data indicate that depolariza-

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tion-induced neuronal calcium entry is modulated by NO interacting with P/Q-type VGCC and NMDA receptors, but not with N-type or L-type VGCC.

2. Materials and methods

All experiments were performed according to institutional guidelines and were approved by the local ethics committee.

2.1. Cell culture and calcium measurements

All cell culture media and supplements were purchased from Biochrom (Berlin, Germany). Primary rat cortical neurons were isolated from fetal rats at embryonic day 16–18 as described previously [1]. Neuronal cultures were kept for 11 days in vitro at 36.5 °C and 5% CO_2. To evaluate changes in the relative intracellular calcium concentration ($[Ca^{2+}]_i$), cells were loaded with fluo-4 AM (10 μM; Molecular Probes, Leiden, Netherlands) for 60 min and then rinsed three times with PBS. Prior to recording, the culture medium was exchanged for a modified HBSS consisting of (in mM): NaCl 137, KCl 5, $NaHCO_3$ 3, Na_2HPO_4 0.6, KH_2PO_4 0.4, $CaCl_2$ 1.4, $MgSO_4$ 0.8, HEPES 20, glucose 5.6, glycine 0.005 (pH 7.4). Fluorimetric recordings were performed using the CytoFluor multi-well plate reader (Applied Biosystems, Darmstadt, Germany). Baseline fluorescence was recorded at the beginning of the experiments, and subsequent $[Ca^{2+}]_i$ changes were determined in relation to baseline (F/F_0). Each group consisted of $n = 36$ wells from $n = 6$ plates. Following determination of basal $[Ca^{2+}]_i$ levels, the cells were depolarized for 3 min by switching to HBSS containing increased extracellular potassium concentration ($[K^+]_o$) (35 mM; $[Na^+]_o$ was lowered reciprocally to maintain isoosmolarity). $[Ca^{2+}]_i$ changes were determined for at least 10 min at intervals of 30 s following depolarization. Statistical comparisons were performed by comparing the peak and basal fluo-4 fluorescence values (F/F_0) using ANOVA followed by Bonferroni t test. $P < 0.05$ was accepted as statistically significant.

*N*ω-nitro-L-arginine (L-NNA; Sigma, Deisenhofen, Germany), *S*-nitroso-*N*-acetylpenicillamine (SNAP; Sigma), dizocilpine (MK-801; Sigma), ω-conotoxin GVIA (Bachem, Heidelberg, Germany), NMDA (Sigma), and ω-agatoxin IVA (Peptide Institute, Osaka, Japan) were solubilized in HBSS. Nimodipine (Sigma) was solubilized in DMSO (final concentration <0.1%) and then added to HBSS. The concentrations of all reagents were adopted from those reported in the literature [3–6,9,13–19,23,24,27,28,32]. The cell cultures were preincubated with these reagents for 30 min before depolarization.

2.2. Immunocytochemistry

Cell cultures were fixed (4% PFA, 15 min at RT) and incubated overnight at 4 °C with primary antibodies against MAP-2 (1:500; Sigma), TUJ1 (1:500; Covance, Berkeley, CA), glial fibrillary acidic protein (GFAP, 1:500; Dako, Hamburg, Germany), CNPase (1:100; Sternberger, Lutherville, MD), and ED1 (1:1000; Serotec, Düsseldorf, Germany). Secondary antibodies conjugated with Alexa Fluor 488 or Texas Red (Molecular Probes) were added at a dilution of 1:250 for 1 h at RT; omission of primary antibodies served as negative control. The sections were coverslipped with Immuno-fluore (MP Biomedicals, Eschwege, Germany) and examined under a conventional fluorescence microscope (DMRA; Leica, Bensheim, Germany) equipped with standard fluorescein and Texas Red filter sets. Cells from four independent preparations were counted at 20× magnification (10 visual fields/preparation). Data are given as mean ± SD.

3. Results

3.1. Characterization of cortical cell cultures

We used antibodies that specifically recognize neuronal (MAP-2, TUJ1), oligodendroglial (CNPase), astroglial (GFAP), and microglial (ED1) antigens in order to determine the purity of our cultures. Immunocytochemical analysis revealed that neurons by far exceeded any other cell population in our preparations (neurons: 77.2 ± 5.8%; astrocytes: 18.5 ± 2.1%; oligodendrocytes 2.3 ± 8.1%; microglia: 0.2 ± 1.0%). Fig. 1 shows a representative example. Thus, only astrocytes could have added measurably to changes in fluo-4 AM fluorescence besides cortical neurons. However, the astrocytic expression of VGCC and NMDA receptors is, if at all, very low. Thus, it is assumed that the $[Ca^{2+}]_i$ changes were predominantly related to neurons.

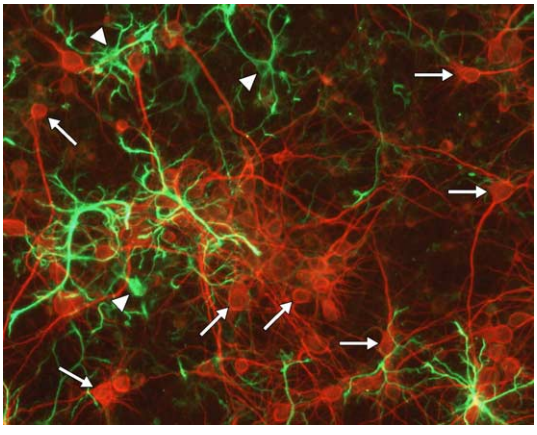

Fig. 1. Neurons are the predominant cell type in rat primary cortical cultures. Immunocytochemical analysis was performed using antibodies that specifically recognize neuronal (MAP-2, arrows) and astroglial (GFAP, arrowheads) antigens. Secondary antibodies conjugated with Alexa Fluor 488 (green) and Texas Red (red) were used for visualization. Note that the number of neurons exceeded the number of astrocytes.