The role of calcium signaling in early axonal and dendritic morphogenesis of rat cerebral cortex neurons under non-stimulated growth conditions

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Abstract

The effects of depolarizing stimuli on neurite outgrowth have been shown to depend on an influx of extracellular calcium. However, the role of calcium under non-stimulated growth conditions is less well established. Here we investigated the contribution of calcium signaling to early neuronal morphogenesis of rat cerebral cortex neurons at three levels by blocking L-type voltage sensitive calcium channels, by depleting intracellular calcium or by blocking myosin light chain kinase. Detailed quantitative morphological analysis of neurons treated for 1 day revealed that depletion of intracellular calcium strongly decreased the density of filopodia, arrested axonal outgrowth and strongly decreased dendritic branching. Preventing calcium influx through L-type voltage sensitive calcium channels and blocking of myosin light chain kinase activity selectively decreased dendritic branching. Our observations support an essential role for basal intracellular calcium levels in axonal elongation. Furthermore, under non-stimulated conditions calcium entry through L-type voltage sensitive calcium channels and myosin light chain kinase play an important role in dendritic branching. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Development and remodeling of connectivity within the CNS is strongly influenced by sensory information, which is relayed onto individual neurons as electric activity [26]. One way by which electric activity affects neuronal connectivity is by modulating neurite outgrowth. In neurons cultured from Helisoma or from rat dorsal root ganglia, electrical stimulation induced growth cone collapse and a cessation of neurite elongation [3,6]. In Xenopus spinal cord neurons and rat neocortical neurons chemical depolarization or electrical stimulation increased neurite outgrowth or branching [7,24,29]. The effects of depolarizing stimuli on neurite outgrowth and growth cone shape depend on influx of extracellular calcium [15,16,22]. The response of growth cones to intracellular calcium shows an optimum at which growth cone size and outgrowth rates are maximal (calcium set-point hypothesis; [15,16]). Below or above this optimum growth cones become small and neurites may stop elongating or retract. The different responses of neurons derived from various species or brain regions to depolarizing conditions may be explained by different optima for intracellular calcium levels. Since we found that in cerebral cortex neurons depolarization stimulates axonal outgrowth, we concluded that under normal growth conditions basal intracellular calcium levels must be suboptimal in these neurons [29]. Although the role of calcium-influx in depolarization-
induced neurite outgrowth is well established, it is less clear whether ‘spontaneous’ calcium-influx contributes to neurite outgrowth of cortical neurons under non-stimulated growth conditions. Investigations on spinal cord neurons of Xenopus in vitro and in vivo showed that also under ‘non-stimulated’ conditions neurite elongation is modulated by ‘spontaneous’ waves of calcium influx [8–10].

To investigate the role of calcium in the outgrowth of cultured cerebral cortex neurons under normal (non-stimulated) growth conditions, we blocked calcium-mediated signaling at several levels using pharmacological agents. The contribution of basal intracellular calcium levels to neurite outgrowth was measured following chelation of intracellular calcium with BAPTA-AM. The role of calcium entry through L-type voltage sensitive calcium channels (L-VSCCs) was measured in the presence of the selective blocker nifedipine. Previous observations showed that nifedipine can completely prevent depolarization-induced lamellipodia formation in cerebral cortex neurons [29]. Although opening of L-VSCCs requires depolarization, membrane potential is considerably higher in early neocortical neurons in culture than in mature neurons [2]. Moreover, blocking L-VSCCCs in cultured retinal ganglion neurons increased the length of the longest neurite while decreasing neurite branching [11]. Downstream of intracellular calcium we investigated the effects of blocking myosin light chain kinase (MLCK), a calcium/calmodulin-dependent kinase which acts as an important regulator of the actomyosin cytoskeleton. Since lamellipodia formation is dependent on rearrangement of the actin cytoskeleton, MLCK is a probable link between calcium signaling and rearrangement of the actin cytoskeleton, leading to long-term changes in neuronal morphology. We found that intracellular calcium levels were important for axonal outgrowth, dendritic branching and filopodia formation/maintenance during growth in the absence of extracellular stimulation. Under the same conditions, calcium entry through L-VSCCCs and the activity of MLCK contributed significantly to dendritic branching. Our observations show that calcium signaling also contributes to early neuronal morphogenesis of cultured cerebral cortex neurons under non-stimulated growth conditions.

2. Materials and methods

2.1. Tissue culturing and fluorescent dye labeling

Culturing procedures were modified from Ramakers et al. [28]. Cerebral cortices were dissected from embryonic day 18 (E18) rats, cut into about 1 mm³ cubes and incubated with 1 ml 0.25% trypsin for 30 min in a CO₂ incubator at 36°C. After removal of most of the trypsin solution, 1 ml DMEM was added together with 200 μl soy bean trypsin inhibitor and 50 μl DNAse I (500 units). After 1 min 2 ml DMEM was added and the tissue blocks were dissociated by trituration with a Pasteur pipette. The cells were pelleted by centrifugation for 5 min at 1000 rpm. The pellet was resuspended in glia conditioned medium (GCM, see below) and a small aliquot (0.5 ml) was incubated with Dil (Molecular Probes, Eugene, USA; 50 μg/ml) for 30 min at 36°C. Meanwhile, the larger part of the cell suspension was counted and diluted to 3 million cells/ml in GCM. The stained cells were quickly pelleted for 10 s in an eppendorf centrifuge and washed three times by resuspension in 1 ml GCM containing 0.2% bovine serum albumin (BSA) and centrifugation. After the last centrifugation step, the cells were resuspended in 1 ml GCM and counted. The stained cells were diluted with the suspension of non-stained cells to obtain a ratio of one stained cell per 500 non-stained cells. A total of 50 μl of cell suspension was pipetted into glass rings (inner diameter 7 mm; height 1.3 mm) placed on 12 mm round coverslips coated overnight with polyethylene imine (Fluka, 10 mg/ml), 1 ml DMEM was added together with 200 μl GCM. After 1 min 2 ml DMEM was added and the tissue blocks were incubated with 1 ml 0.25% trypsin for 30 min in a CO₂ incubator at 100% humidity.

GCM was produced by incubating primary glial cultures for 4 days with a chemically defined medium consisting of 75% DMEM and 25% Ham’s F12, from which glutamate and aspartate were deleted and to which were added: insulin (10 μg/ml); human apo-transferrin (50 μg/ml), thioctic acid (0.25 μg/ml), tocopherol (10 μg/ml), retinol (1 μg/ml), biotin (0.1 μg/ml), sodium pyruvate (100 μg/ml), glutamine (30 μg/ml; all from Sigma) and penicillin/streptomycin (Gibco BRL, Bethesda, USA). The glial cultures (consisting mainly of astrocytes) were established from postnatal day one to three rat cerebra, dissociated by trituration and seeded at a density of one brain (two cerebra) per 175 cm² tissue culture flask in 50 ml D10 medium, consisting of DMEM with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (all media from Gibco).

2.2. Treatments

The cerebral cortex cultures (n=6) were treated for 24 h with BAPTA-AM (10 μM final concentration; Sigma), nifedipine (10 μM; Sigma), ML-7 (3 μM; Sigma), or vehicle (dimethyl sulfoxide; final concentration 0.1%; Sigma). BAPTA-AM enters the cells by means of its lipophilic acetoxymethyl (AM)-tail, and is retained as BAPTA after hydrolysis of the AM-tail [35]. BAPTA is highly selective for calcium (affinity 10³ higher for Ca²⁺ than for Mg²⁺). Although the active concentration of BAPTA within the cells is not known, similar concentrations of BAPTA-AM significantly lower basal calcium concentrations in thymocytes [14]. Nifedipine is a highly selective antagonist of L-type voltage-sensitive calcium channels which is commonly used at 10 μM. At this concentration it decreased calcium uptake by 50% in cultured forebrain neurons [21]. ML-7 is a selective antagonist of MLCK.
inhibitor of myosin light chain kinase with a $K_i$ of 0.3 $\mu$M [32]. The concentration we used (3 $\mu$M) is still 7- and 14-fold lower than the $K_i$ for inhibiting protein kinase A and protein kinase C, respectively [32].

2.3. Sampling and analysis of neuronal morphology

Following treatment between 24 and 48 h the cultures were fixed overnight at 4°C in 4% paraformaldehyde and 0.3% glutaraldehyde in phosphate buffered saline (PBS: 10 mM sodium phosphate buffer, pH 7.3 in 150 mM sodium chloride). After several rinses in PBS the cultures were mounted in Mowiol embedding medium consisting of 10% (w/v) Mowiol 4-88 (Calbiochem, San Diego, CA) and 25% (v/v) glycerol in 0.1 M Tris HCI pH 8.5, and stored at 4°C in the dark. The cultures were coded and embedded in a random manner, so that the experimenter who sampled the neurons was unaware of their treatment. Sampling was performed in a systematic, random manner by meandering through the cultures from left to right in non-overlapping lanes (i.e., with at least 300 $\mu$m distance between the centers of the lanes), and recording every DiI labeled cell that qualified as a neuron. Cells were identified as neurons if they had a fairly round soma with a diameter of less than 10 $\mu$m and at least one thin (equal or less than 2 $\mu$m in diameter) extension, with a minimal length equaling the soma diameter (Figs. 1 and 3). Previous immunocytochemical studies have shown that the non-neuronal axons can easily be excluded on the basis of these criteria and contribute less than 2% to the total cell population measured along the axon path; it corresponds to the net distance traveled by the 'fastest' growth cone. For the dendrites the same parameters were measured as for the axons. Since none of the parameters showed a normal distribution, groups were statistically compared using the non-parametric Mann–Whitney test. Statistical testing was performed using the SPSS program [25]. Effects were considered statistically significant (in the text or legends) if $P<5\%$.

3. Results

3.1. Control neurons at 48 h in culture

DiI staining resulted in continuous staining of neurons, including neurites and filopodia (Fig. 1). Neurons were clearly polarized, showing one long neurite which was several times longer than the other neurites. The long neurite was defined as the axon, according to Dotti et al. [5]. At 48 h in culture, the average neuronal cell body measured 110 $\mu$m$^2$ (Fig. 2a) and contained five dendrites (Figs. 1 and 2d). The total axon was on average 200 $\mu$m long (Fig. 2b), and contained six branch points (Fig. 2e). Intermediate segments were 15 $\mu$m long (Fig. 2h), while terminal segments measured about 23 $\mu$m (Fig. 2k). The axon contained one lateral filopodium every 16 $\mu$m (Fig. 2g). Dendrites were about 35 $\mu$m in length (Fig. 2c) and contained 0.8 branch points on average (Fig. 2f). Intermediate segment length (measured only in branched dendrites) was 7 $\mu$m (Fig. 2i), while terminal segment length (representing total dendrite length when dendrites were not...
branched) measured 16 \mu m (Fig. 2I). Dendrites contained one filopodium per 15 \mu m (Fig. 2J). These values are well comparable to values obtained in similar cultures of the same age in a previous study [29], except that in the present study the number of dendritic branches was almost doubled.

### 3.2. Effects of intracellular calcium chelation with BAPTA-AM

Numerous studies have shown that intracellular calcium is a major determinant of neurite outgrowth [15,16,22]. To establish which aspects of early neuronal morphogenesis
Fig. 2. Effects of nifedipine and BAPTA-AM treatment on neuronal morphology at 48 h in culture. The neurons were treated with nifedipine or BAPTA-AM (both at 10 μM) or vehicle (controls; final concentration of DMSO: 0.1%) from 24 h onwards. Number of neurons (or axons) analyzed: controls: 96; nifedipine: 68; BAPTA-AM: 91. Number of dendrites analyzed: controls: 481; nifedipine: 335; BAPTA-AM: 264. Since many dendrites were not branched, the dendritic intermediate segment lengths were based on the following numbers of intermediate segments: Controls: 191; nifedipine: 97; BAPTA-AM: 77. Statistics (Mann–Whitney test): *P < 0.05; **P < 0.01; ***P < 0.001.
are affected by decreasing basal intracellular calcium levels, cerebral cortex cultures were treated with 10 μM BAPTA-AM at 24 h in culture and analyzed quantitatively at 48 h (Fig. 2). Intracellular calcium chelation had profound effects on most aspects of neuronal morphology (Figs. 1 and 2). Compared to control cultures cell body area was decreased by almost 25% (Fig. 2a), while the number of dendrites was decreased from five to three (−40%; Fig. 2d). Total axon length was decreased by 60% (Fig. 2b). Axonal branching was decreased by 75% (Fig. 2e). Surprisingly, intermediate segment length was not affected by the treatment (Fig. 2h), while terminal segment length showed a 25% increase (Fig. 2k). However, maximum axonal path length was strongly reduced (−50%; not shown), indicating that the growth cones were slowed down considerably. Total dendrite length tended to be somewhat decreased by calcium chelation (−13%; not statistically significant; Fig. 2c). Dendritic branching was decreased by 40% (Fig. 2f), but this was compensated by an increase in intermediate (25%; Fig. 2i) and terminal segment length (10%; Fig. 2l). The maximum dendritic path length was not affected by calcium chelation (not shown), indicating that in dendrites elongation rates were not affected by calcium chelation. The density of lateral filopodia was decreased to a similar extent along axons and dendrites (by −40 and −45%, respectively; Fig. 2g and h).

Comparison of axonal properties of neurons treated with BAPTA-AM from 24 to 48 h in culture to 24 h control neurons (Fig. 4) revealed that total axon length and axonal branching were very similar (compare Fig. 2b and e with Fig. 4b and e). Thus, calcium depletion seemed to have arrested axonal outgrowth at the 24 h level. The number of dendrites per neuron was also arrested at the 24 h control value (compare Figs. 2d and 4d). Although dendritic branching was strongly decreased upon calcium chelation, dendritic elongation and branching were still increased over the values at 24 h (compare Fig. 2c and f with Fig. 4c and f).

3.3. Effects of blocking L-type calcium channels

To test whether 'spontaneous' calcium influx through L-VSCCs plays any significant role during early neuronal morphogenesis under non-stimulated growth conditions, cultures were treated with 10 μM nifedipine between 24 and 48 h in culture and subsequently analyzed for changes in neuronal morphology (Fig. 2). Due to the strong variability in neuronal morphologies qualitative observations did not reveal alterations in the presence of nifedipine (Fig. 1). Nifedipine treatment did not affect the surface area of the neuronal cell body (Fig. 2a) or the number of dendrites per neuron (Fig. 2d). None of the axonal parameters were affected by nifedipine treatment: neither total axon length (Fig. 2b) nor the number of branch points per axon were changed (Fig. 2e). Also, the density of filopodia along axons was not altered by nifedipine treatment (Fig. 2g). In contrast, several dendritic parameters were affected: total dendrite length was decreased by 20% (Fig. 2c), while the number of branch points per dendrite was decreased by 25% (Fig. 2f). The length of intermediate and terminal segments of dendrites were not altered by nifedipine treatment (Fig. 2i and j). However, the maximum dendritic path length was decreased by 15% (not shown), indicating that growth cones showed some decrease in net extension rate. The density of filopodia along dendrites was not affected (Fig. 2j). These observations indicate that preventing the spontaneous influx of extracellular calcium through L-VSCCs affected dendritic branching as well as extension rates.

3.4. Effects of blocking myosin light chain kinase activity

Several protein kinases are activated in a calcium/calmodulin dependent manner, including myosin light chain kinase (MLCK) and calcium/calmodulin kinase II (Ca/CamK II). In a previous study it was found that blockers of MLCK, but not of Ca/CamK II can mimic the effects of calmodulin antagonists on growth cone motility [12]. Since MLCK is likely to influence neuronal morphogenesis through its effect on the actomyosin cytoskeleton, the role of MLCK in neurite outgrowth was investigated by treating the cultures with the selective MLCK antagonist ML-7 between 1 and 24 h. Qualitative observations did not reveal obvious effects of ML-7 (Fig. 3). ML-7 did not affect the area of the neuronal cell body (Fig. 4a) or the number of dendrites (Fig. 4d). Axonal parameters were not changed by ML-7 (Fig. 4b, e, h and k). Total dendrite length was not affected by ML-7 (Fig. 4c), even though the number of branch points and intermediate segment length were reduced (Fig. 4f and i). Since on average only one in four dendrites showed a branch point, dendritic length was not strongly affected by a decrease in intermediate segment length, as the number of intermediate segments was low. In this case, a small (statistically not significant) increase in terminal segment length, which determined about 75% of the total length of the dendrites, prevented a decrease in total dendritic length (Fig. 4l). Thus, blocking MLCK mainly affected dendritic branching.

4. Discussion

In a previous study we found that prolonged depolarization increased axon length and branching in cultured cerebral cortex neurons [29]. These effects were preceded by a rapid induction of lamellipodia within minutes after depolarization, indicating rearrangement of the actin cytoskeleton. Lamellipodia formation was dependent on an influx of extracellular calcium through L-VSCCs, and required intracellular calcium levels of 1 μM or higher.
Here we investigated whether calcium ions contribute to neurite outgrowth under non-stimulated conditions, by interfering with calcium signaling at three different levels. Several studies have shown effects of interference with calcium signaling on neurite elongation and growth cone shape [4,19,20,31]. Using a new method to quantitate neuronal morphology [29], we could establish the effects of manipulating calcium signaling in more detail. In addition, as we studied the effects of 24 h treatments, our observations extend previous studies which were limited to the effects of manipulating calcium signaling for up to 1 h. The method proved to be quite sensitive, revealing significant treatment effects on neuronal morphology that could not be observed qualitatively. Lowering of intracellular calcium arrested axonal outgrowth and branching, halted the net addition of dendrites and strongly decreased dendritic branching and the density of filopodia. Blocking calcium influx through L-VSCCs and blocking MLCK specifically decreased branching in dendrites.

Preventing calcium influx has been found to suppress the effects of depolarization and other stimuli on growth cone morphology and neurite extension [4,31]. In addition, outgrowth of axons under non-stimulated conditions is arrested by ‘spontaneous’ waves of calcium influx in spinal cord neurons of Xenopus and chicks [8–10]. Here we find that in cerebral cortex neurons net elongation of axons is prevented by lowering intracellular calcium. Decreasing basal intracellular calcium levels did not lead to net retraction, indicating that intracellular calcium is more important for the elongation than the maintenance of axons. Dendrites showed normal net elongation in the presence of BAPTA-AM, although branching was strongly reduced. The differential effect of calcium depletion on axons and dendrites led to a strong reduction in the polarity of the neurons: BAPTA-AM decreased the ratio between axon length and dendrite length from about 6 to 2.7. A similar decrease in polarity was observed in hippocampal neurons in which a strong calcium influx was induced with a calcium ionophore [23]. These findings are consistent with observations in chick dorsal root ganglion neurons which show that both calcium removal and calcium ionophores arrest previously elongating neurites [19]. Since during the first 3 days in culture axons grow much faster than dendrites [29], it is not surprising that treatments which reduce elongation primarily affect axons rather than dendrites.

Reduction of intracellular calcium also caused a considerable loss of filopodia from neurites. This is in line with studies which showed that calcium influx is necessary for the induction of filopodia [4]. Time-lapse video microscopy has shown that many sidebranches originate from lateral filopodia (Ramakers and Kerssens, unpublished observations), while depletion of lateral filopodia by disruption of the actin cytoskeleton reduced branching (Ramakers et al., submitted). Thus, it is possible that the decrease in dendritic branching is due to the loss of...
Fig. 4. Effects of ML-7 on neuronal morphology at 24 h in culture. The neurons were treated with ML-7 (3 μM) or vehicle (control; final concentration of DMSO: 0.1%) from 1 h onwards. Number of neurons (or axons) analyzed: controls: 39; ML-7: 33. Number of dendrites analyzed: controls: 102; ML-7: 106. Since many dendrites were not branched, the dendritic intermediate segment lengths were based on the following numbers of intermediate segments: controls: 19; ML-7: 12. Statistics (Mann–Whitney test): \# P<0.1; *P<0.05.
filopodia from dendrites. The same may apply to the loss of dendrites as their reduction was associated with a loss of filopodia from the cell body. Since the dendrites are constantly generated from the cell body and retracted ([5]; Ramakers, unpublished observations), it is likely that the loss of dendrites is due to a loss of filopodia, which serve as initiation points for the generation of dendrites [34]. In a previous study we also found that loss of dendrites was associated with a (prior) loss of filopodia from the cell body (Ramakers et al., submitted). Neurite elongation is mainly dependent on tubulin polymerization, while branching and the formation/maintenance of dendrites depends on actin polymerization (Ramakers et al., submitted). This indicates that lowering intracellular calcium affected both actin and tubulin polymerization, in line with previous effects of intracellular calcium on the actin and tubulin cytoskeleton [19].

Local clustering of L-VSCCs within growth cones has been shown to correlate with sites of neurite outgrowth [33,38]. We found that blocking L-VSCCs specifically decreased dendritic branching as well as elongation, resulting in lower total dendrite length, without affecting axons. A similar decrease in dendritic branching was found in retinal ganglion cells [11]. Thus, it appears that, at least under non-stimulated conditions, calcium influx through L-VSCCs plays a significant role in dendrites, but not in axons. This could be explained by an absence of VSCCs from axons or axonal growth cones, or a relatively minor contribution of VSCCs to calcium influx in axons. In adult rat cerebral cortex and hippocampal pyramidal neurons immunostaining showed L-VSCCs to be present on neuronal cell bodies and proximal dendrites, and little if any on axons [1,37]. Calcium imaging also demonstrated highest levels of calcium influx through L-VSCCs in the first 200 μm of the proximal dendrites of adult hippocampal pyramidal neurons [30]. Although no data are available about the distribution of L-VSCCs on immature neurons, our observations suggest that also in early neurons L-VSCCs may be located preferentially on dendrites.

Short-term and long-term effects of calcium on filopodia ([4,31]; present study) and lamellipodia [19,29], clearly indicate regulation of actin-dependent structures by calcium. The signaling pathways that couple calcium dynamics to rearrangement of the actin cytoskeleton are still obscure. MLCK constitutes a potential link between calcium signaling and the actomyosin cytoskeleton. MLCK is expressed in neurons and upregulated during axon degeneration [13]. Blocking MLCK in goldfish retinal explant cultures with ML-7 resulted in growth cone arrest [12]. In cerebral cortex neurons ML-7 decreased dendritic, but not axonal branching, similar to the effects of nifedipine. These differential effects suggest that dendrites are more susceptible to interference with the coupling between calcium influx and modulation of the actin cytoskeleton than axons. This is supported by findings that showed that the generation/maintenance of early dendrites, but not of axons, is strongly dependent on actin polymerization (Ramakers et al., submitted).

Taken together, our observations show that in cultured cerebral cortex neurons growing under non-stimulated conditions ‘basal’ intracellular calcium levels are necessary for axonal elongation to occur and contribute to dendritic branching and the generation/maintenance of filopodia. Dendritic branching under resting conditions is furthermore dependent on calcium influx through L-VSCCs and basal activity levels of MLCK. Apparently, these mechanisms are functional before the onset of synaptic communication in our cultures [27,36]. At an even earlier stage the migration of cerebellar granule cells is positively correlated with spontaneous fluctuations in calcium entry through VSCCs [17,18]. Thus, modulation of basal intracellular calcium levels plays an important role in various aspects of early neuronal differentiation. The involvement of VSCCs at this stage indicates a role for these channels in non-synaptic communication.

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References


