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Store-depletion and hyperforin activate distinct types of Ca^{2+}-conducting channels in cortical neurons

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ABSTRACT

Cortical neurons embryos (E13) from murine brain have a wide diversity of plasma membrane Ca^{2+}-conducting channels. For instance, they express several types of transient receptor potential channels of C-type (TRPC) and hyperforin, a potent TRPC6-channel activator, controls the activity of TRPC6-like channels. In addition, E13 cortical neurons possess plasma membrane channels activated in response to the depletion of internal Ca^{2+} pools. Since some TRPC channels seem to be involved in the activity of store-depletion-activated channels, we investigated whether hyperforin and the depletion of the Ca^{2+} stores control similar or distinct Ca^{2+} routes. Calcium imaging experiments performed with the fluorescent Ca^{2+} indicator Fluo-4 showed that the TRPC3 channel blocker Pyr3 potently inhibits with an IC_{50} of 0.5 μM the entry of Ca^{2+} triggered in response to the thapsigargin-dependent depletion of the Ca^{2+} stores. On the other hand, Pyr3 does not block the hyperforin-sensitive Ca^{2+} entry. In contrast to the hyperforin responses, the Ca^{2+} entry through the store-depletion-activated channels is down-regulated by the competitive tyrosine kinase inhibitors genistein and PP2. In addition, the immunosuppressant FK506, known to modulate several classes of Ca^{2+}-conducting channels, strongly attenuates the entry of Ca^{2+} through the store-depletion-activated channels, leaving the hyperforin-sensitive responses unaffected. Hence, the Zn^{2+} chelator TPEN markedly attenuated the hyperforin-sensitive responses without modifying the thapsigargin-dependent Ca^{2+} signals. Pyr3-insensitive channels are key components of the hyperforin-sensitive channels, whereas the thapsigargin-dependent depletion of the Ca^{2+} stores of the endoplasmic reticulum activates Pyr3-sensitive channels. Altogether, these data support the notion that hyperforin and the depletion of the Ca^{2+} pools control distinct plasma membrane Ca^{2+}-conducting channels. This report further illustrates that, at the beginning of the corticogenesis, immature cortical neurons express diverse functional Ca^{2+} channels.

1. Introduction

In the cortex of mouse embryo, the first post-mitotic neurons appear at E11–12 [1]. Even at this embryonic age, cortical neurons of the immature cortex possess a large repertoire of Ca^{2+}-conducting channels. For instance, E13 cortical cells express intracellular channels: IP3-sensitive receptors (IP3R) (IP3R1–IP3R3) and ryanodine-sensitive receptors (RyR) (RyR1–RyR3), with IP3R1 and RyR2 being the predominant proteins [2]. Hence, several types of plasma membrane Ca^{2+}-conducting channels are present in E13 cortical cells, like voltage-gated Ca^{2+} channels [3], NMDA receptors [4], and Ca^{2+}-conducting channels activated in response to the depletion of internal Ca^{2+} stores [5]. In addition, E13 cortical neurons have several types of TRPC channels [6] and possess functional diacylglycerol-sensitive channels that can be recruited by hyperforin [7], a plant extract known to activate TRPC6 channels without activating TRPC1, TRPC3, TRPC4, or TRPC5 channels [8].

Store-depletion-activated channels (also called store-operated channels, SOC) form a class of voltage-independent Ca^{2+} channels. They open in response to the emptying of the endoplasmic reticulum (ER) Ca^{2+} pools [9]. The molecular characterisation of the SOC has long been awaited but recent data revealed that Orai and Stim are two key molecular players involved in SOC activity [10,11]. However, the role of C-class transient receptor potential (TRPC) cations channels in this Ca^{2+} signalling is still discussed [12–14]. Indeed, some experimental data support the notion that TRPC channels, at least some of them, could participate in SOC activity.

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In the present report, the properties of the hyperforin-sensitive and the store-depletion-activated channels of cortical neurons were compared. The data gained provide new insights into these Ca^{2+}-conducting channels present in the immature cortex at the beginning of the neurogenesis. This study highlights the large repertoire of families of Ca^{2+}-conducting channels even at early stages of the corticogenesis.

2. Materials and methods

2.1. Primary cell cultures

Cultures of cortical neurons were prepared according to [5]. The procedures used were approved by the Ethical Committee of Rhône-Alpes Region and by the Ethical Committee of Grenoble (ComEth) (France). Briefly, embryos (E13) from C57BL6/J mice (vaginal plug was designated E0) were sacrificed and the brains were kept in an ice-cold Ca^{2+}- and Mg^{2+}-free Hank's solution supplemented with 33 mM glucose, 4.2 mM $NaHCO_3$, 10 mM HEPES, 1% penicillin/streptomycin. The isolated cortices were triturated by means of repetitive aspirations though a sterile and fire-polished Pasteur pipette. Four to five E13 mice (e.g. 8–10 cortices) were used for each culture. The cell suspension was then filtered through a 70 μm cell strainer (BD Falcon). Sterile glass cover-slips (Ø 16 mm, Marienfield, Germany) were coated with poly-L-ornithine (2 h at 37 °C) and washed twice with sterile water before plating the cells. Cells grown in a Neurobasal medium containing 2% B27, 1% penicillin/streptomycin and 500 μM glutamine.

2.2. Calcium imaging experiments with Fluo-4

The experiments were carried out according to experimental procedures described previously [7]. In these experiments, the baseline Fluo-4 fluorescence was recorded for ≥1 min and averaged (F0). The results are expressed as F/F0 as a function of time, with F being the Fluo-4 fluorescence. Data are presented as mean ± S.E.M., with *n* being the number of cell bodies analyzed. Recordings were made at room temperature 1–3 days after the plating of the cells. At that time, ≥75% of them displayed a depletion-activated Ca^{2+} entry [5]. Certain variability in the size of the thapsigargin-dependent Fluo-4 signals was observed from one batch of cultured cells to the other. Each treatment was compared to its proper control (non treated cells from the same batch of cells). In every case, and unless otherwise indicated, conditions to be tested were repeated on at least three batches of cultured cells.

2.3. Materials

Genistein, FK-506, calyculin A, methyl-β-cyclodextrin, and cholesterol were from Sigma–Aldrich (France). Fluo-4/AM was purchased from Molecular Probes (Invitrogen, France). The Neurobasal medium, B27, glutamine were from (Invitrogren, France). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) was from Calbiochem (Merck Chemicals, France). The ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trif luoromethyl)-1H-pyrazole-4-carboxylate (Pyr3) was kindly provided by Dr. Y. Mori (Kyoto University, Japan). Hyperforin, prepared as a mixture of hyperforin with its homologue adhyperforin (ratio 8:2), was prepared as a sodium salt. It was a kind gift from Dr. Willmar Schwabe GmbH & Co. (Karlsruhe, Germany). The hyperforin-dependent Fluo-4 responses reported in the present report were smaller than previously found [7]. Since the hyperforin extract is not pure, this may explain the variability between our two reports (present one and [7]) carried with two different batches of hyperforin.

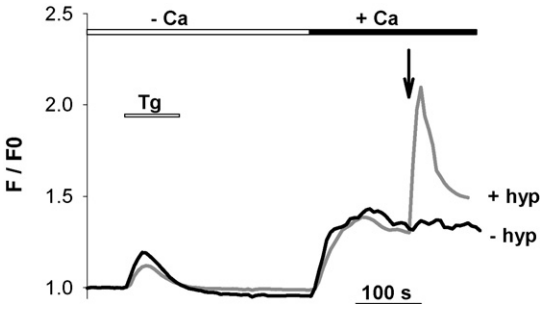

Fig. 1. Cortical neurons possess store-depletion-activated channels and hyperforin-sensitive channels. The figure shows somatic changes in fluo-4 fluorescence (F/F0) as a function of time in two cultured cortical neurons. Thapsigargin (Tg, 1 μM) was applied on a cell kept in a Ca^{2+}-free medium, washed away and 2 mM external Ca^{2+} was reintroduced (as illustrated by the horizontal black bar) which provoked a second Ca^{2+} rise through store-depletion-activated channels. Hyperforin (10 μM) or its vehicle (DMSO, 0.1%) was added (arrow) after the onset of the Tg-dependent Ca^{2+} entry. Similar data were obtained on 98 cells.

3. Results

We previously showed that E13 cortical neurons possess hyperforin-sensitive channels [7] and store-depletion-activated channels [5]. We first asked whether these two Ca^{2+} routes co-exist in the same cells. To this aim, cortical neurons were first treated with thapsigargin (Tg), a potent inhibitor of the endoplasmic reticulum Ca^{2+} pumps [15]. It caused a transient Fluo-4 signal reflecting the passive release of Ca^{2+} from internal pools followed by the extrusion of Ca^{2+} out of the cells. A subsequent superfusion with a Ca^{2+}-containing recording medium gave rise to a second Fluo-4 signal (Fig. 1). The addition of hyperforin (arrow, Fig. 1) during the Tg-dependent Ca^{2+} entry elicited a rapid and transient elevation of the Fluo-4 fluorescence as already reported [7,8]. The fact that these responses are additive suggests the coexistence of distinct Ca^{2+} routes. This latter hypothesis was tested below.

Hyperforin-sensitive channels of cortical neurons are insensitive to agents disturbing the actin cytoskeleton [7]. In the following experiments, cortical neurons were treated with cytochalasin D to inhibit actin filament polymerization. However, this agent did not affect the Tg-dependent Ca^{2+} release and entry (Fig. 2A). Another way to modify the actin filament network is to use phosphatase inhibitors such as calyculin A. Like cytochalasin D, pre-treating cells with calyculin A had no effect on the Tg-dependent Ca^{2+} release and entry (not shown, $n = 38$ cells). Caveolae have been shown to play important roles in Ca^{2+} signalling [16,17]. To determine whether the store-depletion-activated channels were located in caveolae-like structures, cortical neurons were kept 2 days in a culture medium supplemented with 20 μg/ml cholesterol. After this treatment, cells were transferred to (a cholesterol- and) nominally Ca^{2+}-free Tyrode solution and Tg was added to deplete the stores. Cholesterol had no effect on the Tg-dependent release and entry of Ca^{2+} (Fig. 2A). In another set of experiments, methyl-β-cyclodextrin was used as a modifier of caveolae structures. Here again, treating the cells with methyl-β-cyclodextrin did not affect the Tg-dependent release and entry of Ca^{2+} (not shown, $n = 35$ cells). In conclusion, like the hyperforin-sensitive channels, the store-depletion-activated channels of cortical neurons are not present in caveolae-like structures.

Regulation by tyrosine kinases seems to be a general feature of depletion-activated channels [18] but hyperforin-sensitive channels are not regulated by these kinases [7]. The involvement of tyrosine kinases in the Tg-dependent Ca^{2+} entry of embryonic cortical neurons was verified by using genistein and PP2, two tyrosine kinase inhibitors which do not affect the hyperforin-sensitive responses [7]. Both inhibitors depress the amplitude of the Tg-