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Non-stimulated Ca^{2+} leak pathway in cerebellar granule neurones

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Abstract

The aim of this study was to investigate the pathways of calcium influx routes in non-stimulated cerebellar granule neurones by use of standard microspectrofluorimetric techniques. Repetitive application of Ca^{2+}-free solutions for various time intervals induced decreases of resting cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) which were followed, on Ca^{2+} readmission, by a full recovery, always to the initial resting $[Ca^{2+}]_i$ levels. Use of drugs to deplete calcium stores (thapsigargin, alone or combined with low levels of ionomycin) did not cause release of Ca^{2+} from the intracellular stores nor enhanced the activity of the Ca^{2+} entry pathway. This influx was mainly independent of voltage operated calcium channels, since both L-type channel blockers (nitrendipine) and the hyperpolarizing agent pinacidil (a K^+-channel opener) were without effect. Contribution from glutamate receptors to this influx was eliminated since a combination of blockers of NMDA and AMPA glutamate receptors (NBQX and D-AP5) did not affect the properties of the Ca^{2+} response. The Ca^{2+} leak pathway was sensitive to micromolar levels of lanthanum and gadolinium, and to the compound 2-APB, features shared by several channels of the TRP superfamily. In summary, our results show the presence of a Ca^{2+} permeable pathway, active and patent in resting conditions in cerebellar granule neurones, and which is different from the voltage-operated calcium channels and not operated by depletion of the stores.

Keywords: Cerebellar granule neurones; Calcium signal; Calcium entry; Calcium leak

1. Introduction

Neuronal excitability depends on the transport of different ions across plasma membrane, including Ca^{2+} due to the multiple regulatory effects of this cation on ion channel physiology. Ca^{2+} signals associated to neurotransmitters and action potentials are largely based on calcium influx from extracellular medium. The two main pathways for this Ca^{2+} entry are voltage operated Ca^{2+} channels (VOCC) and receptor channels. Gating of these channels provide a fast and efficient method to transduce the spatial and temporal profile of neural excitation into regulatory actions conveyed by Ca^{2+} ions. Several aspects of VOCC, such as voltage gating, pharmacology and mechanisms of permeation and blockade, have been extensively studied in neuronal tissue.

Another route for calcium entry is through the TRP channels, a large family of widespread and mainly non-selective cationic channels [1]. Highly expressed in brain neurones, TRP channels have been proposed as the molecular counterpart of the mechanism termed capacitative calcium entry (CCE) or store operated calcium entry (SOC), an ubiquitous route for calcium entry in non excitable cells which is triggered by depletion of intracellular calcium pools [2]. This route is well established in other excitable systems (e.g., smooth muscle cells) [3], but reports about its presence in neurones are scarce, limited to cortical [4] and sensory neurones [5,6]. Although early reports and indirect data suggested an involvement of TRP channels in CCE [7], the issue is not fully resolved (cf. [1]).

Similar to any other cell type, neuronal resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is in part determined by the turnover of Ca^{2+} ions entering from extracellular medium through an ill-defined ''leak'' pathway. The aim of this work was to study the main Ca^{2+} influx mechanisms in cultured cerebellar granule neurones in non-stimulated conditions. Our data show that this cell type shows a resting Ca^{2+} leak that is clearly independent of VOCC and glutamate receptors, does not act as a classical

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capacitative entry system and shows a pharmacology profile resembling TRP channels.

2. Materials and methods

2.1. Cell culture

Cultures were prepared from post-natal (P5) rat pups using an enzymatic procedure, previously detailed [8]. The pups were sacrificed by cervical dislocation, in accordance with the local and national guidelines on animal care and use. The cerebellum was dissected in ice-cold, Ca^{2+}- and Mg^{2+}-free bicarbonate-based PBS, and then incubated in papain (7 U/ml, Wortinghton) for 15 min at 37 °C. The tissue was then triturated through fire-polished Pasteur pipettes, in the presence of DNase (700 units/ml, Sigma D 5025) and 4 mM $MgCl_2$. The final neuronal cell suspension was plated on 12 mm round coverslips coated with poly-D-lysine (25 μg/ml, Sigma), at an initial density of 0.25–0.3 millions/coverslip. After 2–3 h, when the neurones started to adhere to the coverslips, BME culture medium (Sigma B1522) supplemented with glucose (final concentration 32 mM), glutamine 2 mM, 10% heat inactivated horse serum (Sigma H1138) and a mixture of antibiotics (Sigma P4458) was added. After 24 h from plating, 10 μM cytosine arabinoside (Sigma) was added to restrict glial proliferation. The cultures were maintained in a humidified atmosphere of 5% CO_2–95% air at 37 °C in a tissue culture incubator, and were routinely used after 7–9 days of culture. The typical percentage of glial cells was 5%.

2.2. Imaging experiments

For imaging experiments, the cultured neurones were transferred to a HEPES-based medium (also used as the perifusion buffer), containing (mM): NaCl 140, KCl 4.7, $CaCl_2$ 2, $MgCl_2$ 1.1, glucose 10 and HEPES 10, pH 7.4. The neurones were loaded with fura-2AM (5 μM) at 30 °C for 30 min, and another 30 min were allowed for de-esterification. All experiments were performed at room temperature. Cells were excited at 340/380 nm with a computer controlled filterwheel (Lambda-10, Sutter Instruments), and emitted fluorescence was collected with a 500 nm long-pass filter. Images were taken using a Peltier cooled CCD from Hamamatsu (HisCa C-4880, Hamamatsu Photonics), at 10-bit digital output, allowing separation of 1024 grey levels. Excitation and emission conditions, as well as image capture and posterior analysis, were controlled by dedicated software (Argus HisCa, Hamamatsu Photonics, Japan). When cells were perfused with Ca^{2+}-free solution which composition was similar to the HEPES-buffered medium described above but Ca^{2+} was replaced by 0.5 mM EGTA. Due to uncertainties regarding real K_d for fura-2 inside cells, we present all the results as raw fluorescence ratio (F_{340}/F_{380}). Some increases are given as increase of ratio fluorescence ($\Delta F_{340}/F_{380}$).

3. Results

The main protocol to assess the non stimulated calcium influx in cerebellar granule neurones is shown in Fig. 1. Fura-2 loaded neurones were perfused with pulses of Ca^{2+}-free solution (with added 0.5 mM EGTA) of variable duration between 2 and 15 min, after which external Ca^{2+} (2 mM) was readmitted. As seen in Fig. 1A, removal of extracellular Ca^{2+} induced a $[Ca^{2+}]_i$ decrease, while on Ca^{2+} readmission intracellular Ca^{2+} returned to values close to, or slightly higher than the original resting values. Successive repetitions of the manoeuvre, of shorter or longer duration, showed, as shown in Fig. 1B, a tight homeostatic control, with $[Ca^{2+}]_i$ decreasing to a well maintained new steady-state level when extracellular Ca^{2+} was removed. Similarly, readdition of extracellular Ca^{2+} determined an increase in $[Ca^{2+}]_i$ to values comparable to the initial resting $[Ca^{2+}]_i$ values, irrespective of the duration of Ca^{2+} removal from the extracellular medium (compared to the initial resting $[Ca^{2+}]_i$, the values of $[Ca^{2+}]_i$ following Ca^{2+} readmission were 100.1 ± 0.8% at 2.5 min, 101.9 ± 1.1% at 5 min and 104.9 ± 0.9% at

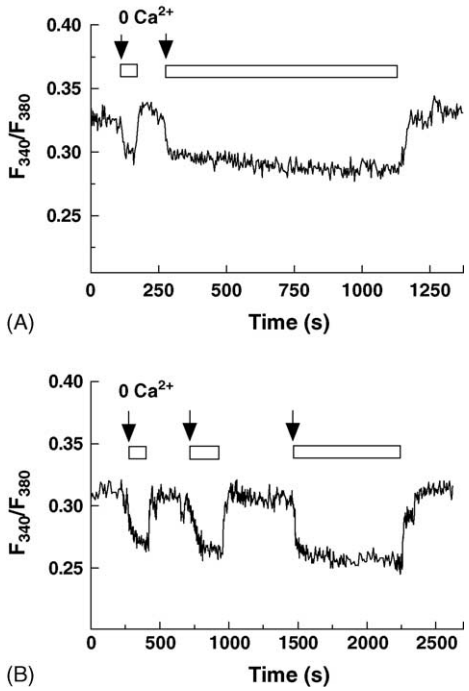

Fig. 1. Calcium influx restores resting $[Ca^{2+}]_i$ in non stimulated cerebellar granule neurones. Fura-2 loaded granule neurones were perfused with pulses of Ca^{2+}-free solution containing 0.5 mM EGTA at different time points. Note that Ca^{2+} restoration induced return of $[Ca^{2+}]_i$ to original, resting level, irrespective of pulse duration and time of application. Traces are representative of four independent experiments.

12 min). Furthermore, the rate of Ca^{2+} entry, measured as the slope of the linear phase of the $[Ca^{2+}]_i$ increase following Ca^{2+} readdition, was similar on Ca^{2+} readmission after 2 min or 15 min ($0.00153 \pm 6 \times 10^{-5}$ units of ratio $F_{340}/F_{380} \times s^{-1}$ at 2 min versus $0.00163 \pm 6 \times 10^{-5}$ units of ratio $F_{340}/F_{380} \times s^{-1}$ at 215 min ($n = 76$, $p > 0.08$, paired t-test).

It is well established that exposure of cells to a Ca^{2+}-free extracellular medium can activate CCE [3,9,10]. To control for this possibility in our conditions we performed a series of experiments using well-established protocols to trigger controlled activation of CCE, such as incubation of cells with thapsigargin (TPS, 1 μM), a specific inhibitor of the Ca^{2+} pump of the stores [11], in a Ca^{2+}-free medium. Fig. 2 shows that this treatment did not change the response of the neurones to the removal of extracellular Ca^{2+} and, upon Ca^{2+} readmission, $[Ca^{2+}]_i$ returned to levels close to resting values, without an additional $[Ca^{2+}]_i$ increase. Also, TPS did not accelerate the rate of $[Ca^{2+}]_i$ rise: the slope for $[Ca^{2+}]_i$ rise was similar in the absence (0.00103 ± 0.00019 ratio units $\times s^{-1}$) and in the presence of TPS (0.00106 ± 0.00015 ratio units $\times s^{-1}$, $p > 0.7$, paired t test, $n = 13$). Given that in some models a part of the intracellular Ca^{2+} stores are insensitive to thapsigargin, we performed a series of experiments in the presence of 50 nM ionomycin, a Ca^{2+} ionophore which can release Ca^{2+} from internal stores [12,13]. As shown in Fig. 2B, the effect is similar to thapsigargin alone.

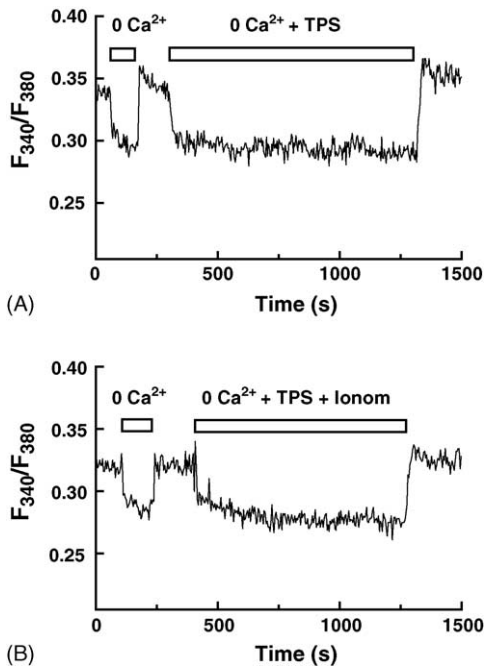

Fig. 2. Capacitative calcium entry is not active in non-stimulated cerebellar granule neurones. After an early Ca^{2+} removal pulse, cells were perfused with Ca^{2+}-free solution containing 1 μM thapsigargin (TPS) alone (A) or combined with 50 nM ionomycin (B). Subsequent Ca^{2+} re-addition returned $[Ca^{2+}]_i$ back to resting levels. Traces are representative of 433 cells (13 experiments, A) and 452 cells (17 experiments, B).

These results indicate that our protocols containing TPS and ionomycin can be used to explore the properties of a Ca^{2+} entry pathway that mediates the increases in $[Ca^{2+}]_i$ on readmission of extracellular Ca^{2+}, in conditions in which no additional capacitative calcium entry is activated. In addition, the presence of TPS and ionomycin, by preventing the possible sequestration of Ca^{2+} ions into the intracellular stores [14], dissects out the possible interference of these stores when assessing the Ca^{2+} entry pathway. Therefore, the rest of this study assessing the resting, leak Ca^{2+} influx pathway was performed by using a Ca^{2+}-free medium that contained TPS and ionomycin.

One possible route for the resting Ca^{2+} influx are the VOCC channels, and almost all VOCC subtypes have been identified in the cerebellar granule neurones [8,15]. In addition, we have recently shown that in excitable smooth muscle cells removal of extracellular Ca^{2+} depolarizes plasma membrane, thus activating L-type Ca^{2+} channel in addition to "classical" SOC channels [3]. As seen in Fig. 3, nitrendipine, a selective inhibitor for L-type Ca^{2+} channels, did not affect the rate or amplitude of the $[Ca^{2+}]_i$ increase that followed the readmission of extracellular Ca^{2+}. At the concentration used in these experiments (1 μM), nitrendipine was effective in blocking the Ca^{2+} entry evoked by a strong KCl depolarization (not shown). A scatterplot graph comparing the values of $[Ca^{2+}]_i$ after Ca^{2+} readmission in the absence ("control") or presence of nitrendipine (Fig. 3C), shows no difference. A further confirmation of the lack of effect of VOCC inhibition is illustrated in Fig. 3D, when nitrendipine was added well after the readmission of extracellular Ca^{2+} (in effect, during normal "resting" conditions) without any effect on the $[Ca^{2+}]_i$.

As the cerebellar granule neurones express all the known types of voltage-activated calcium channels [15], it was still possible that even in the presence of nitrendipine, other, non-L VOCC could be activated by the exposure to the Ca^{2+}-free solution. To rule out this possibility we repeated the same protocol as above, but using the K^+ channel opener pinacidil, which induces hyperpolarization in excitable cells, such as gallbladder smooth muscle [3] or neurons [16]. Fig. 3B–C shows that, similar to nitrendipine, pinacidil (10 μM) did not affect the Ca^{2+} leak in granule neurones. This observation, together with the fact that pinacidil did not affect the $[Ca^{2+}]_i$ when administered in the resting, non-stimulated conditions (not shown), supports the view that the VOCC are not significantly involved in mediating the leak Ca^{2+} influx active in resting conditions. Another possible pathway for the Ca^{2+} influx observed in our experimental conditions is activation of glutamate receptors, which are present in this tissue and evoke strong $[Ca^{2+}]_i$ increases [17]. To investigate the possible activation of these receptors during Ca^{2+} removal we added to the perfusing medium a cocktail of well-established ionotropic glutamate receptors blockers: NBQX (10 μM) and D-AP5 (50 μM) [18]. Fig. 4 shows

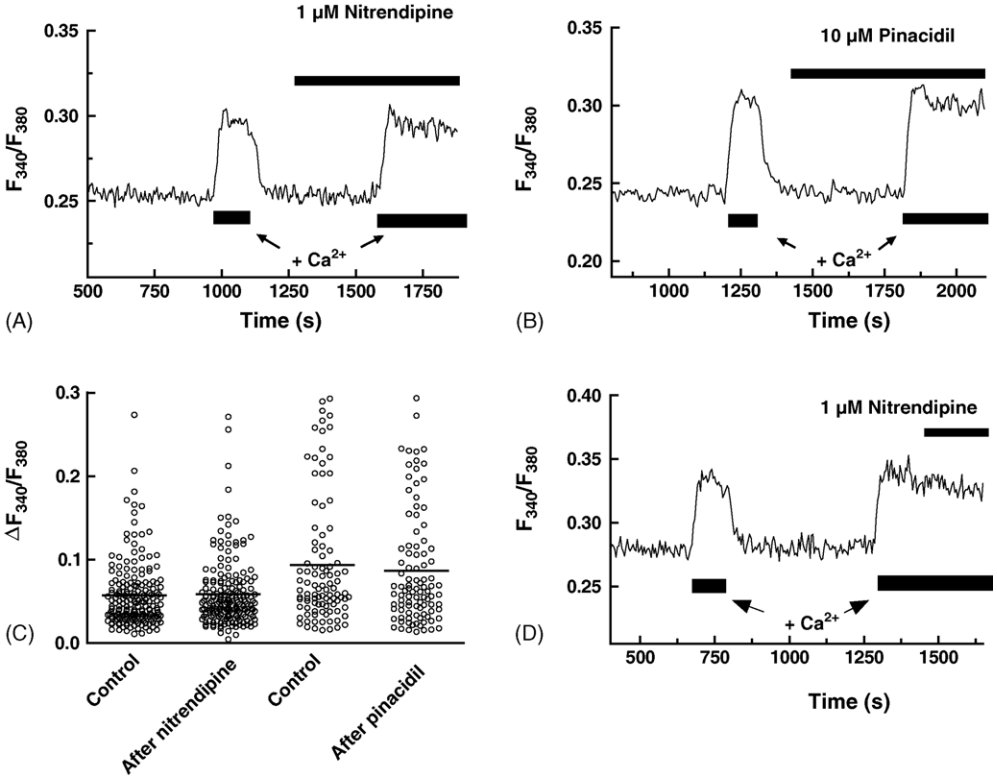

Fig. 3. Voltage operated Ca^{2+} channels do not participate in non stimulated calcium leak. Granule neurones were pretreated with 1 μM thapsigargin and 50 nM ionomycin in Ca^{2+}-free medium for 700–800 s before application of two pulses of external calcium separated by a Ca^{2+}-free interval. Treatment with 1 μM nitrendipine (A) or 10 μM pinacidil (B) before the second application was without effect on calcium entry. Traces are from individual cells representative of 184 cells (nitrendipine) or 98 cells (pinacidil) from four experiments. Panel C shows scatterplot and mean value (horizontal line) corresponding to the first calcium pulse (labelled as control) and the second pulse depicted in panels A and B. Panel D shows a typical trace showing lack of effect of nitrendipine when applied on top of the entry-induced plateau (representative of three experiments).

that this treatment had no effect on Ca^{2+} leak, which showed the same profile and amplitude as in control experiments.

Channels belonging to the TRP superfamily have been reported to be sensitive to a series of different blockers, including trivalent cations and 2-APB [19]. 2-APB is a $InsP_3$ receptor blocker frequently used as a relatively specific inhibitor for capacitative Ca^{2+} entry [13,20], with

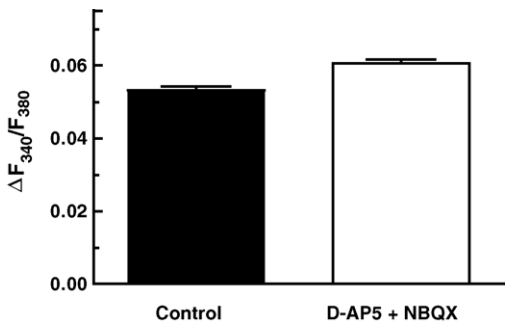

Fig. 4. Glutamate receptors do not mediate resting influx. After Ca^{2+} depletion, cells were perfused with Ca^{2+} containing solution. For NBQX (10 μM) and D-AP5 (50 μM)-treated cells the inhibitors were present from the beginning of the experiment, even before application of Ca^{2+}-free solution. Histogram bars represent increase in fura-2 fluorescence upon Ca^{2+} readmission. $n = 121$ cells, seven experiments.

complex effects in $[Ca^{2+}]_i$ signals. Although it was initially used on the basis of its ability to block agonist-evoked Ca^{2+} depletion, additional evidence showed that a primary target are Ca^{2+} entry pathways (for a review see [20]). Regarding Ca^{2+} channels, it has proven to either inhibit [21–23] or activate [20,24,25] different subsets of TRP superfamily channels. Therefore, we assayed its effects on the Ca^{2+} leak as shown in Fig. 5. Acute application of 2-APB after the re-establishment of the resting $[Ca^{2+}]_i$ that follows the readmission of extracellular Ca^2 induced a significant decrease of $[Ca^{2+}]_i$. This inhibitory effect was dose-dependent, both in terms of the size of the $[Ca^{2+}]_i$ depression and of the number of cells that responded to the administration of 2-APB. With 100 μM 2-APB, 90% of cells responded with a decrease of $[Ca^{2+}]_i$ that averaged 50% (see Fig. 5B). The effect was smaller with 30 μM 2-APB, when only around 50% of the recorded cells responded, with a significantly smaller depression of $[Ca^{2+}]_i$ (Fig. 5A and B).

Another type of generic Ca^{2+} channels inhibitors, effective at submillimolar concentrations in many cell types are the trivalent cations. Some members of TRP cationic channels are also sensitive to Gd^{3+} and La^{3+} at concentrations ranging from submicromolar to micromolar levels [26–28]. Therefore, we investigated the effect of these cations on the resting Ca^{2+} influx pathway in cerebellar

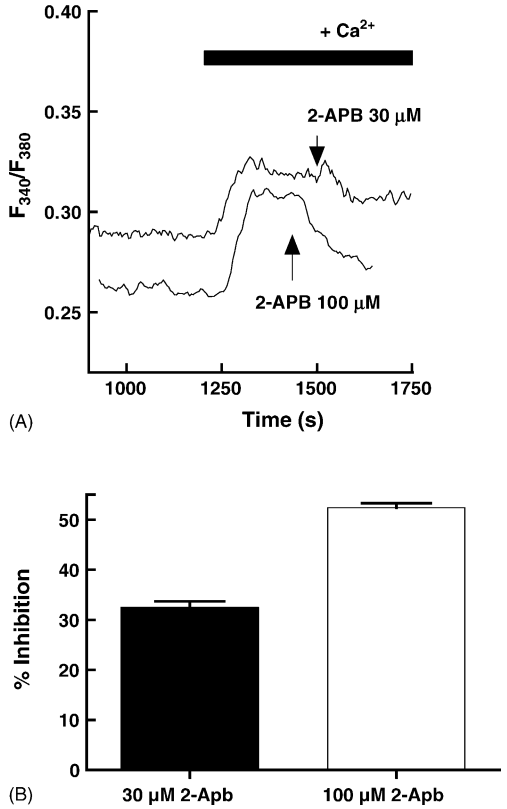

Fig. 5. 2-APB inhibits Ca^{2+}-depletion-evoked influx in cerebellar granule neurones. (A) Cells previously depleted with Ca^{2+}-free solution, thapsigargin and ionomycin were subsequently perfused with extracellular calcium. Arrowheads indicate moment of application of 30 and 100 μM 2-APB. (B) Shows mean ± S.E.M. of the inhibition of calcium entry in response to 2-APB. Percentage of inhibition was calculated as 2-APB-induced decay in F_{340}/F_{380} respect the increase evoked by Ca^{2+} entry. $n = 71$ cells/eight experiments (30 μM) and 69 cells/five experiments (100 μM).

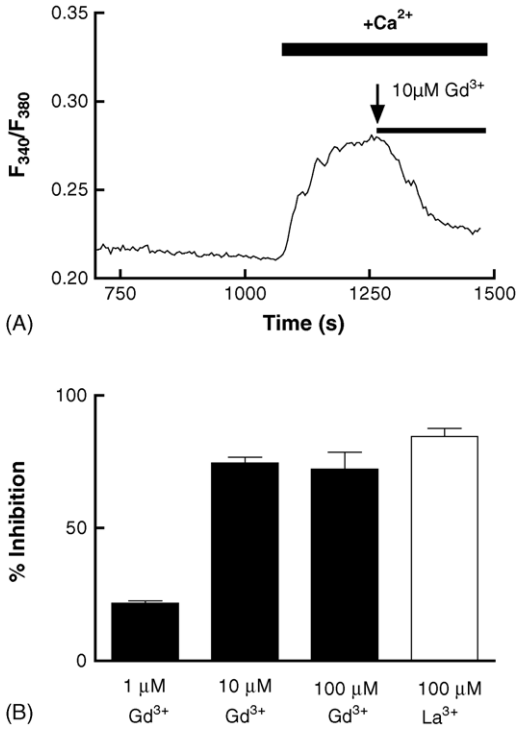

Fig. 6. Micromolar levels of lanthanum and gadolinium inhibit calcium entry in cerebellar granule neurones. (A) Effect of acute application of Gd^{3+} on top of the plateau response evoked by Ca^{2+} readmission in Ca^{2+}-depleted cells. The trace is representative of four experiments. (B) Mean ± S.E.M. of the percentage of inhibition induced by Gd^{3+} and La^{3+}; n = four to eight experiments.

cells. As shown in Fig. 6, both Gd^{3+} and La^{3+} exerted a dose-dependent, powerful inhibitory effect.

4. Discussion

Our data demonstrate the presence of a Ca^{2+} leak pathway in cultured cerebellar granule neurones maintained in resting, non-stimulated conditions. This calcium entry pathway appears independent of both voltage operated Ca^{2+} channels and from the glutamatergic ionotropic mediation calcium channels. This Ca^{2+} influx route is active in resting conditions, is not further activated by a capacitative calcium entry mechanism, and shares with TRP channels sensitivity to 2-APB and trivalent cations.

During neuronal stimulation and activity, the main pathways for calcium entry are the VOCC and the receptor channels, particularly the glutamate receptors. The aim of this work was to ascertain the nature of the Ca^{2+} entry during resting, non-stimulatory conditions, using the removal of extracellular calcium followed by its readdition as the experimental probe. A perineuronal reduction in the extracellular Ca^{2+} concentration has physiological meaning in the context of normal brain activity and can trigger a number of functional responses with important regulatory role [29]. In some cell types, removal of extracellular Ca^{2+} activates L-type VOCCs, as in smooth muscle cells [3] or cardiac cells [30]. In addition, removal of divalent cations increases the conductance of VOCC channels for monovalent cations [31], resulting in an increased excitability in some neuronal preparations [32]. However, in our experiments neither nitrendipine nor pinacidil, which should hyperpolarise the cells, affected the $[Ca^{2+}]_i$ recovery when Ca^{2+} was reapplied after the exposure to Ca^{2+}-free solutions, nor the levels of $[Ca^{2+}]_i$ when the drugs were administered acutely to cerebellar granule neurones in resting state, bathed in Ca^{2+}-normal solution. These data then are incompatible with the possibility that VOCC might play a significant role in mediating the Ca^{2+} leak pathway revealed by the removal/readmission of extracellular Ca^{2+}. The unblocking effect of a reduction of extracellular Ca^{2+} on monovalent cations permeability [33] has been reported not only for the VOCCs, but also for the capacitative Ca^{2+} entry [25] or for other cation channels, such as K^+ channels [34]. These effects are thought to be due to occupancy of negatively charged domains of the protein channel by Ca^{2+} ions while they traverse the pore, which are rendered accessible to monovalent cations in presence of low extracellular calcium [25].

Another mechanism triggered by the exposure of many cell types to Ca^{2+}-free media is the activation of CCE mechanism. Information regarding CCE in neurones is scarce, and has been described in cortical and some sensory neurones, as well as in spinal cord neurones (see [5,10]). This is compounded by the fact that in many central neurones the internal Ca^{2+} stores appear to fill only during neural stimulation [4,10,14,35]. Accordingly, in additional experiments (not shown) we were unable to release Ca^{2+} from internal stores when using the highly potent $InsP_3$-based stimulant quisqualic acid, for which specific receptors are present on cerebellar cells [36]. Note that application of thapsigargin and ionomycin also failed to release Ca^{2+} from the stores, as indicated by absence of Ca^{2+} transient upon application of the drugs (Fig. 2). Even previous stimulation of the cells with depolarizing pulses of KCl in order to fill the stores were without effect on the magnitude of the Ca^{2+} leak, contrary to hippocampal neurones, where this manoeuvre unmasked CCE [4]. We found that well-established protocols that deplete intracellular Ca^{2+} stores [35] did not affect the Ca^{2+} leak in our experimental conditions (cf. Fig. 1), supporting a view that CCE is negligible in this cell type. Of course, our data do not rule out the possible presence of capacitative entry in other compartments of the cerebellar granule neurones, given that we have used measurements in the cellular soma.

Our finding that lanthanum, gadolinium and 2-APB inhibit the non-stimulated Ca^{2+} leak suggest the possibility that a set of TRP channels could be involved, consistent with previous data showing the presence of TRPC channels in cerebellum [37,38]. Although 2-APB has been extensively used as a non specific inhibitor of CCE and a $InsP_3$ antagonist, there is evidence that its primary (though not unique) point of action is the extracellular face of the plasma membrane ([20]) and electrophysiological data reveals a direct inhibition of TRP channels [21,23]. In addition to inhibition of intracellular $InsP_3$ receptors [39] and Ca^{2+}-ATPases of the endoplasmic reticulum [40], 2-APB activates some channels of the TRP superfamily [20,24,25], and some authors have postulated its target is a regulatory protein upstream of TRP channels [41]. Therefore, our results with 2-APB must be interpreted with caution. However, two strong arguments clearly open the possibility of TRP involvement in the Ca^{2+} leak described in this study. First, the available data clearly show that 2-APB does not act on VOCC [42] or on the other Ca^{2+} permeable channels, such as ionotropic receptors [41] or arachidonic acid-activated channels [43], and this facilitates the use of this drug to target the activity of the CCE and TRP channels. Second, the side-effects of 2-APB cannot explain the $[Ca^{2+}]_i$ decrease observed in our experiments. Actually, blockade of $InsP_3$ receptors or capacitative entry are not operative in our experimental conditions, and activation of TRP subsets is contradictory to our observation.

Regarding the effect of La^{3+} and Gd^{3+}, our results are also compatible with the participation of TRP channels in the Ca^{2+} leak pathway. Gadolinium ions are direct inhibitors for some TRP channels, ranging from submicromolar [19,26] to submillimolar (200 μM [27,28]) concentrations, even when the stores are not depleted [26]. Direct comparison of non capacitative TRP-mediated influx with capacitative entry shows that the former is poorly sensitive to low micromolar Gd^{3+} [28,44] but is blocked by ~100 μM, in keeping with the effects of 10–100 μM Gd^{3+} used in this study. Likewise, micromolar levels of lanthanum inhibit systems expressing TRPC channels [45], and levels of 100 μM La^{3+} have been reported to be necessary to block TRPC3-mediated influx [27,28]. In summary, our results with 2-APB and lanthanides suggest the possibility that the resting Ca^{2+} leak in cerebellar granule neurones is mediated by TRP channels, although they are not conclusive to identify the Ca^{2+} pathway.

The data reported here indicate that resting $[Ca^{2+}]_i$ is controlled by a tonic, background calcium influx route which is open and patent in non stimulated cells. Background Ca^{2+} channels have been previously reported in muscle [46] and in some neurones including cerebellar granule neurones from *mdx* mice [47], but at the moment the possible role is unknown. In muscle it has been reported that leak channels are involved in capacitative entry [46], which seems to be different from our observations. Our data raise the possibility that TRP channels function as background influx channels for control of $[Ca^{2+}]_i$ in the non stimulated state in neurones. Expression of TRP channels is related to the proliferative state of the cells [48], and we have recently shown in an excitable model that calcium signals can quickly modulate TRPC transcription (Pozo and Camello, manuscript in preparation). Therefore, is conceivable that cellular activity (e.g., calcium signals) continuously modulates background Ca^{2+} permeability through rapid changes in TRP expression, thus supplying a fast adaptative mechanism to match metabolic and signalling status of the cell. However, more studies are necessary to identify the molecular basis of the resting Ca^{2+} leak in cerebellar neurones.

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