Activation of a capacitative Ca^{2+} entry pathway by store depletion in cultured hippocampal neurones

Alexandre Bouron*

Department of Pharmacology, University of Bern, Friedbuehlstrasse 49, Bern, Switzerland

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Abstract **Intracellular Ca^{2+} ($[Ca^{2+}]_i$) changes were measured in cell bodies of cultured rat hippocampal neurones with the fluorescent indicator Fluo-3. In the absence of external Ca^{2+}, the cholinergic agonist carbachol (200 μM) and the sarco-endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin (0.4 μM) both transiently elevated $[Ca^{2+}]_i$. A subsequent addition of Ca^{2+} into the bathing medium caused a second $[Ca^{2+}]_i$ change which was blocked by lanthanum (50 μM). Taken together, these experiments indicate that stores depletion can activate a capacitative Ca^{2+} entry pathway in cultured hippocampal neurones and further demonstrate the existence of such a Ca^{2+} entry in excitable cells.**

Key words: Hippocampus; Capacitative calcium entry; Store-operated channel

1. Introduction

The activation of metabotropic receptors, via the downstream phospholipase C/inositol 1,4,5-trisphosphate (IP_3)-dependent signalling cascade, releases Ca^{2+} from intracellular stores. In non-excitable cells, depleted Ca^{2+} compartments can be refilled by a store-operated Ca^{2+} influx also designated capacitative Ca^{2+} entry [1,2].

Neurones possess various intracellular Ca^{2+} stores such as the endoplasmic reticulum, mitochondria or the nuclear envelope [3,4]. The release of Ca^{2+} from internal compartments is thought to play a central role in Ca^{2+} signalling and neuronal physiology [4]. It is therefore of importance to characterise the mechanisms by which neurones can replenish their stores. The store-operated Ca^{2+} influx has long been regarded as a specific property of non-excitable cells. However, several important pieces of evidence for the existence of such a Ca^{2+} entry in neurones have been accumulated. For example, metabotropic receptors give rise to biphasic intracellular Ca^{2+} signals consisting of a fast Ca^{2+} transient spike, most likely due to the release of Ca^{2+} from IP_3-sensitive stores, followed by a plateau phase of slower kinetics during which intracellular Ca^{2+} ($[Ca^{2+}]_i$) remains elevated. This latter point indicates the existence of different Ca^{2+} routes [2]. In agreement with this hypothesis, the presence of a store-operated Ca^{2+} entry has been demonstrated in neuronal cell lines [5–7]. For instance, in neuroblastoma cells thapsigargin and the muscarinic agonist carbachol activate a Ca^{2+} current [6] similar to I_{CRAC}, a Ca^{2+} release-activated current which is commonly described as a Ca^{2+} influx through store-operated channels. Furthermore, two recent studies showing a store-operated Ca^{2+} entry in adrenal chromaffin cells and dorsal root ganglion (DRG) neurones [8,9] clearly confirmed that store depletion can activate a capacitative Ca^{2+} entry in excitable cells.

The present study was undertaken to verify the existence of a store-operated Ca^{2+} influx in CNS neurones. Ca^{2+} signals were detected in cell bodies of cultured rat hippocampal neurones by means of the Ca^{2+} indicator Fluo-3. In Ca^{2+}-free medium, the muscarinic agonist carbachol or thapsigargin transiently elevated $[Ca^{2+}]_i$. A subsequent readdition of Ca^{2+} into the external medium increased $[Ca^{2+}]_i$ which indicates a Ca^{2+} entry pathway through the plasma membrane. This influx could be blocked by lanthanum (La^{3+}), a cation known to inhibit store depletion-operated Ca^{2+} channels [10]. Taken together, these results suggest the existence of a capacitative Ca^{2+} entry pathway through the plasma membrane of cultured hippocampal neurones. This influx can be activated by the metabotropic receptor agonist carbachol and by $[Ca^{2+}]_i$ store depletion.

2. Materials and methods

2.1. Cell preparation

Mixed cell cultures of glial cells/hippocampal neurones from 3–5-day-old rats were prepared and maintained as described previously [11,12].

2.2. Imaging system

Cultured hippocampal cells were transferred from the culture medium to a standard solution containing (in mM) 135 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 30 glucose, pH 7.4 (NaOH). The depolarising medium contained 50 mM KCl. In this case, the NaCl concentration was reduced to maintain the osmolarity. Cells were incubated with 5–10 μM Fluo-3/AM at 37°C for 30–50 min. They were then rinsed with the standard solution and the coverslip was mounted on an experimental chamber and placed on the stage of an inverted microscope (Axiovert 100) connected to a laser scanning imaging system (LSM 410, Zeiss AG, Germany). Cells were superfused continuously by a gravity-driven system with the standard solution. A 488 nm excitation wavelength focused through a ×40 Neofluar objective lens (numerical aperture 1.4, Zeiss AG, Germany) was provided by an argon laser. Emitted fluorescence was collected at 2-s intervals at 530 nm through a 515–560-nm filter [12,13]. Analysis of somatic $[Ca^{2+}]_i$ signals was performed in visually identified hippocampal neurones.

2.3. Materials

Cell culture reagents were obtained from Sigma Chemie AG (Buchs, Switzerland) or Gibco BRL (Life Technologies, Basel, Switzerland). Tetrodotoxin (TTX) was purchased in Alomone Labs (Jer-

*Present address: CNRS UMR 5091, Université de Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France.
Fax: (33)-5-56 98 61 82.
E-mail: alexandre.bouron@pcs.u-bordeaux2.fr

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usalem, Israel), Fluo-3/AM was from Molecular Probes (Leiden, The Netherlands), and thapsigargin from Calbiochem (Juro Supply AG, Lucerne, Switzerland). All experiments were performed at room temperature 7–21 days after the dissociation of the neonatal hippocampi.

3. Results

The experiments were carried out at room temperature with cultured hippocampal neurones dissociated from 3–5-day-old rats. Unless otherwise indicated, the external medium contained TTX (0.4 μM) to prevent the action potential-dependent entry of Ca^{2+}. It also contained the AMPA/kainate receptor antagonist CNQX (10 μM) to inhibit spontaneously occurring excitatory synaptic inputs.

Stimulation of hippocampal cholinergic muscarinic receptors leads to phosphatidylinositol hydrolysis and $[Ca^{2+}]_i$ mobilisation [14,15]. In the presence of 2 mM external $CaCl_2$, 200 μM of the muscarinic agonist carbachol, a concentration that maximally induces the accumulation of *myo*-inositol-1-phosphate [14], failed to evoke any $[Ca^{2+}]_i$ transients in 90% of the pyramidal cells tested. This latter point is in agreement with a previous report showing that intracellular Ca^{2+} stores of cultured hippocampal neurones are empty at rest [16]. To increase the number of responsive neurones, the cells were then superfused for 1 min with a depolarising medium containing 50 mM KCl but without TTX and CNQX. The high K^+ medium strongly elevated $[Ca^{2+}]_i$ which slowly decayed to the resting level (not shown). Five to 10 minutes after the washout of the high K^+ solution, a second application of carbachol transiently increased $[Ca^{2+}]_i$ in 70% of the pyramidal neurones tested (12 out 17 cells). Fig. 1 illustrates such experiments where the muscarinic agonist was added before and following depolarisation with high external K^+. The carbachol-induced mobilisation of Ca^{2+} consisted of a biphasic $[Ca^{2+}]_i$ signal characterised by a rapid elevation of $[Ca^{2+}]_i$ followed by a smaller plateau phase of slower kinetics. Similar experiments were also performed in the absence of external Ca^{2+}. Under these conditions, none of the cells tested re-

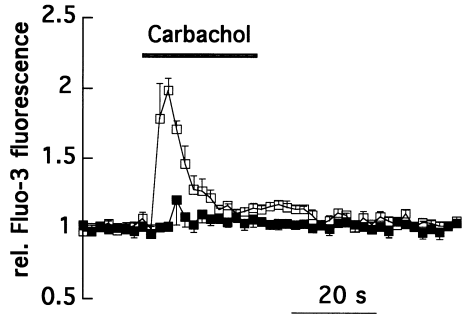

Fig. 1. Intracellular Ca^{2+} stores of cultured hippocampal neurones are empty at rest. Cytosolic Ca^{2+} changes $[Ca^{2+}]_i$ were measured by means of the Ca^{2+} indicator Fluo-3 in cell bodies of pyramidal hippocampal neurones dissociated from neonatal rats. The graph shows the normalised fluorescence as a function of time. The external medium contained 2 mM Ca^{2+}, 0.4 mM TTX and 10 μM CNQX. The muscarinic cholinergic agonist carbachol (200 μM) was added when indicated by the horizontal bar before (■) or after the superfusion of a depolarising medium containing 50 mM KCl, and no added TTX and CNQX (□). For each cell, $[Ca^{2+}]_i$ transients were detected in the same region of interest before and after the KCl challenge. Mean ± S.E.M. from five pyramidal cell bodies. A similar observation was made in two other experiments.

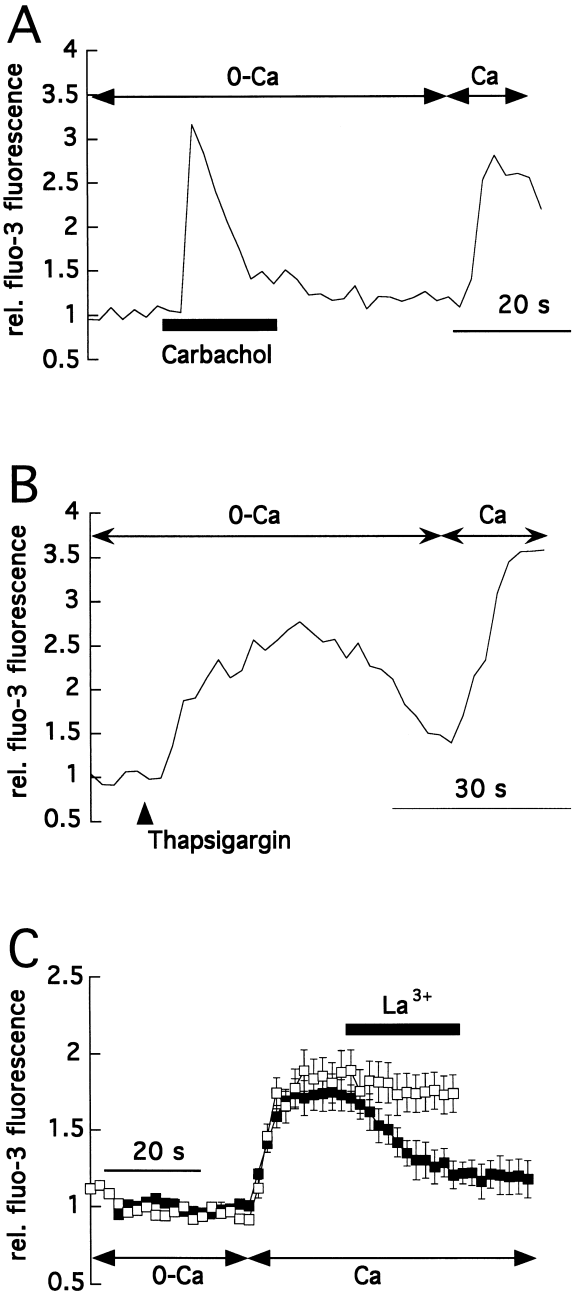

Fig. 2. Carbachol and thapsigargin induce a capacitative entry of Ca^{2+}. A–C: Normalised Fluo-3 fluorescence measured in individual hippocampal cell bodies as a function of time. The external medium contained TTX (0.4 μM) and CNQX (10 μM) without or with 2 mM Ca^{2+}. A: Carbachol (200 μM) was added when indicated by the horizontal bar. It transiently elevated $[Ca^{2+}]_i$. B: Thapsigargin (0.4 μM), added when indicated by the arrowhead, also increased $[Ca^{2+}]_i$. A capacitative Ca^{2+} entry was observed in only three out of the 12 carbachol-treated cells tested whereas it could be observed in all thapsigargin-treated cells tested ($n = 46$). C: Summary graph showing the effect of La^{3+} (50 μM) on the capacitative Ca^{2+} entry. Thapsigargin (0.4 μM) was added in the absence of external Ca^{2+}. $[Ca^{2+}]_i$ first slowly increased (not shown) and then returned to the basal level. Upon readdition of Ca^{2+}, the capacitative Ca^{2+} entry was blocked by 50 μM La^{3+} (■, $n = 9$ cells bodies). La^{3+} was added when indicated by the horizontal bar. Similar experiments were performed but without introducing 50 μM La^{3+} (□, $n = 12$ cell bodies). Mean ± S.E.M.

sponded to the first application of carbachol. However, the percentage of responsive neurones increased to 25% (eight out of 32 cells) when the second application of carbachol was preceded by a KCl challenge. In this case, the high external K^+ solution contained 2 mM $CaCl_2$ (without TTX and CNQX). These observations further illustrate that under control conditions intracellular Ca^{2+} stores of cultured hippocampal neurones are empty [16]. In the following experiments, a high K^+-containing depolarising solution was always added for 1 min 5–10 min prior to the addition of $[Ca^{2+}]_i$-mobilising drugs.

A store-operated Ca^{2+} entry pathway has been described in various excitable cells such as neuronal cell lines, adrenal chromaffin cells and DRG neurones [5–9]. Therefore, it was of interest to verify whether such capacitative Ca^{2+} entry could also exist in CNS neurones. In the presence of a Ca^{2+}-free solution, carbachol (200 μM) transiently elevated $[Ca^{2+}]_i$ (Fig. 2A). A subsequent addition of 2 mM Ca^{2+} caused a second $[Ca^{2+}]_i$ rise in 25% of the hippocampal neurones tested ($n = 12$) (Fig. 2A). In another set of experiments, the sarco-endoplasmic reticulum Ca^{2+} (SERCA) pump inhibitor thapsigargin was used to deplete intracellular Ca^{2+} stores [17]. Cells were kept in a Ca^{2+}-free medium. The addition of thapsigargin (0.4 μM) slowly increased $[Ca^{2+}]_i$. The addition of 2 mM Ca^{2+} activated an influx of Ca^{2+} (Fig. 2B) in all pyramidal cell bodies tested ($n = 46$). In a last set of experiments, the Ca^{2+} channel blocker lanthanum (La^{3+}) was used to inhibit the capacitative Ca^{2+} entry pathway. Micromolar concentrations of La^{3+} reversibly blocked this pathway in bovine aortic endothelium cells [10]. As before, the cells were first bathed in a Ca^{2+}-free medium. Thapsigargin (0.4 μM) slowly increased $[Ca^{2+}]_i$ (not shown) which returned to the basal level. When added, 2 mM external Ca^{2+} produced a robust Ca^{2+} signal that could be blocked by La^{3+} (50 μM, $n = 9$) (Fig. 2C).

4. Discussion

The release of Ca^{2+} from internal stores influences many neuronal processes [4]. Therefore, a better understanding of the Ca^{2+} entry pathways through the plasma membrane of nerve cells is of crucial interest. Neurones express a great diversity of voltage-gated Ca^{2+} channels. Dihydropyridine-sensitive Ca^{2+} channels are thought to mediate a sustained Ca^{2+} entry at resting membrane potentials in hippocampal neurones [18]. This Ca^{2+} influx could play a role in maintaining resting $[Ca^{2+}]_i$ and in refilling intracellular Ca^{2+} compartments. However, the existence of a steady-state Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels and operating at resting membrane potentials is still debated. Recent studies demonstrated that voltage-gated Ca^{2+} channel blockers do not affect this Ca^{2+} influx which is activated by depletion of caffeine-sensitive Ca^{2+} stores [9,19]. A similar caffeine-sensitive store-operated Ca^{2+} pathway has recently been identified in cultured DRG neurones [9]. In addition, a voltage-independent current carried by Ca^{2+} and Na^+ could be measured in bovine adrenal chromaffin cells in response to store depletion induced by thapsigargin or Ca^{2+} chelators [8]. Taken together, these observations provide functional evidence for the existence of a Ca^{2+} influx through voltage-independent channels of excitable cell membranes and activated by store depletion. The experiments described in the present study show that a similar Ca^{2+} entry pathway operates in cultured hippocampal neurones. It can be activated in response to store depletion induced by the IP_3-generating agonist carbachol as well as the SERCA pump inhibitor thapsigargin.

The *Drosophila* and mammalian TRP channels are thought to function as store-operated Ca^{2+} channels [2]. However, the exact molecular identity of the channels underlying the capacitative Ca^{2+} entry is unknown [1]. Another important unresolved issue concerns the mechanism of activation of these channels. Since the capacitative entry pathway can be activated by caffeine [9,19] or thapsigargin ([8]; present study), phospholipase C activation and IP_3 production are most likely not the signalling molecules linking store depletion and the Ca^{2+} influx [1]. In the light of recent experiments, two models (although not mutually exclusive) can be proposed to explain the gating of capacitative Ca^{2+} entry channels. Functional data indicate an interaction between the plasma membrane and the endoplasmic reticulum [20]. On the other hand, the second model suggests a secretory-like mechanism involving the insertion of the capacitative Ca^{2+} entry channel into the plasma membrane [21,22]. Neither a diffusible messenger nor a GTP-dependent process seems to be involved in the gating of the channels [22,23].

The experiments of the present study showing that intracellular Ca^{2+} stores of cultured hippocampal neurones are depleted at rest are in agreement with previous observations (see [16]) and suggest a constant Ca^{2+} leak from the stores. Therefore, depleted intracellular compartments should activate the capacitative Ca^{2+} entry pathway. However, the capacitative Ca^{2+} influx was only observed after carbachol or thapsigargin treatment. It could be hypothesised that the capacitative Ca^{2+} channels are recruited by a rapid and short-lasting elevation of $[Ca^{2+}]_i$. According to this scheme, the continuous leak of Ca^{2+} from the stores would not provide a signal capable of activating this pathway.

The physiological significance of a neuronal store depletion-activated Ca^{2+} entry is not clearly established. This influx of Ca^{2+} may affect membrane excitability or Ca^{2+}-dependent processes. For instance, it maintains Ca^{2+} oscillations in DRG neurones [9]. In the light of recent experiments showing that a capacitative Ca^{2+} entry modulates exocytosis in adrenal chromaffin cells [8] it would be interesting to determine whether this Ca^{2+} entry pathway could also modulate neurotransmitter release and synaptic plasticity in the hippocampus.

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