Involvement of calmodulin in the activation of store-operated Ca^{2+} entry in rat hepatocytes

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Abstract The possible participation of calmodulin in the activation of store-operated Ca^{2+} entry (SOC) in single rat hepatocytes was investigated microspectrofluorimetrically. SOC was triggered after discharging intracellular Ca^{2+} stores using the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin in the absence of external Ca^{2+}. Re-admission of bath Ca^{2+} caused a rapid and pronounced Ca^{2+} entry. The calmodulin antagonists calmidazolium or CGS 9343B applied before the thapsigargin treatment inhibited SOC, whereas they were ineffective when added after the thapsigargin-induced Ca^{2+} transient. This study suggests that activation of calmodulin after the elevation of cytosolic Ca^{2+} associated with the emptying of Ca^{2+} stores is involved in the triggering of SOC in hepatocytes.

Key words: Store-operated Ca^{2+} entry; Ca^{2+} signaling; Calmodulin

1. Introduction

Calcium influx from the extracellular milieu is essential to ensure the cell's responsiveness to Ca^{2+}-mobilizing agonists in non-excitable cells. The dominant Ca^{2+} entry mechanism in non-excitable cells such as hepatocytes is mediated by the so-called store-operated Ca^{2+} entry (SOC), also dubbed capacitative Ca^{2+} entry (for recent reviews see [1,2]). This Ca^{2+} entry process is governed by the Ca^{2+} content of the agonist-sensitive intracellular Ca^{2+} stores, which, when depleted either by an agonist or by other means, activate a Ca^{2+} conductance at the plasma membrane. The membrane channel responsible for this Ca^{2+} influx has been identified first in Drosophila (*trp* gene [3]) and later in mammalian cells [4–6]. Despite intense work by numerous researchers, the nature of the mechanism that senses the filling state of the intracellular Ca^{2+} stores and communicates it to the cell membrane is still a matter of debate. A number of putative mechanisms have been proposed and partly demonstrated, but so far none is accepted as the unifying theory [1,2]. This could indicate that the linkage between the intracellular stores and the cell membrane is a complex mechanism, not necessarily common to all types of non-excitable cells.

In a physiological situation, the depletion of intracellular Ca^{2+} stores that is brought about by agonist stimulation in hepatocytes is accompanied by a sustained, transient or pulsatile elevation of the cytosolic Ca^{2+} concentration [7] depending on the type and concentration of the agonist. Many cellular responses that are controlled by Ca^{2+}-dependent pathways utilize calmodulin (CAM) as mediator of signaling [8,9]. CAM has been shown to activate multiple cellular processes either through direct interaction with target proteins or via activation of regulatory proteins such as kinases or phosphatases [10]. In the present study, we present evidence for the involvement of CAM in the activation of SOC in rat hepatocytes. The pathway used by CAM to activate SOC might include the CAM-dependent protein kinase II.

2. Materials and methods

Rat hepatocytes were isolated from male Wistar rats and attached to collagen-coated glass coverslips as described previously [11]. Intracellular Ca^{2+} was measured after loading of the cells with 4–6 μM fura-2 acetoxymethyl ester for 20 min at 37°C in Ham's F12 medium with 1% bovine serum albumin (BSA) and an additional incubation in a dye-free medium for another 10 min. Cells were then mounted on the stage of an inverted fluorescence microscope (Nikon, Tokyo, Japan) equipped with a 100×1.3 NA objective lens (Nikon) and a filter wheel (Lambda 10, Sutter Instrument, Novato, CA, USA) for rapid switching of excitation light between 340 nm and 380 nm. Emission fluorescence was reflected by a 400 nm dichroic mirror and filtered through a 520 nm long-pass filter. Fluorescent images were captured by a video camera with image intensifier (Videoscope Int., Washington, DC, USA) and digitized by an 8 bit image processor (Leutron, Glattburg, Switzerland) for quantitative analysis of intensity in selected regions of interest in single cells. Cells were superfused at 33°C in a thermostatted perfusion chamber. Calibration of cytosolic Ca^{2+} signal was accomplished in situ at the end of each experiment as previously described [12,13].

Bath solution contained (mM): 145 NaCl, 5.6 KCl, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 10 HEPES, 10 glucose. A corresponding Ca^{2+}-free solution contained 0.1 mM EGTA without added Ca^{2+}. Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR, USA). Ham's F12 medium, BSA, thapsigargin, ionomycin, W-7, calmidazolium (i.e. Compound R24571) and KN-62 were from Sigma (St. Louis, MO, USA). J-8 was from Alexis Corp. (Läufelfingen, Switzerland). CGS 9343B was kindly provided by Ciba Geigy (Basel, Switzerland). The other chemicals were from Fluka (Buchs, Switzerland).

Data are presented as means ± S.E.M. from separate preparations. When appropriate, unpaired Student's *t*-test was performed to assess the statistical significance of differences, and a value of P lower than 0.05 was considered significant.

3. Results and discussion

In a first step, the presence of SOC was tested in our isolated rat hepatocyte preparation. Fig. 1A shows that when Ca^{2+} was removed from the bath and subsequently re-added without any other manipulation, the cytosolic Ca^{2+} concentration was practically not affected, indicating that the hepatocyte cell membrane possesses a low permeability for Ca^{2+} in resting conditions. Addition of thapsigargin (1 μM), a classical inhibitor of the endoplasmic reticulum (ER) Ca^{2+}-ATPase, discharged the intracellular Ca^{2+} stores and produced an as-

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Abbreviations: BSA, bovine serum albumin; ER, endoplasmic reticulum; SOC, store-operated Ca^{2+} entry; CAM, calmodulin

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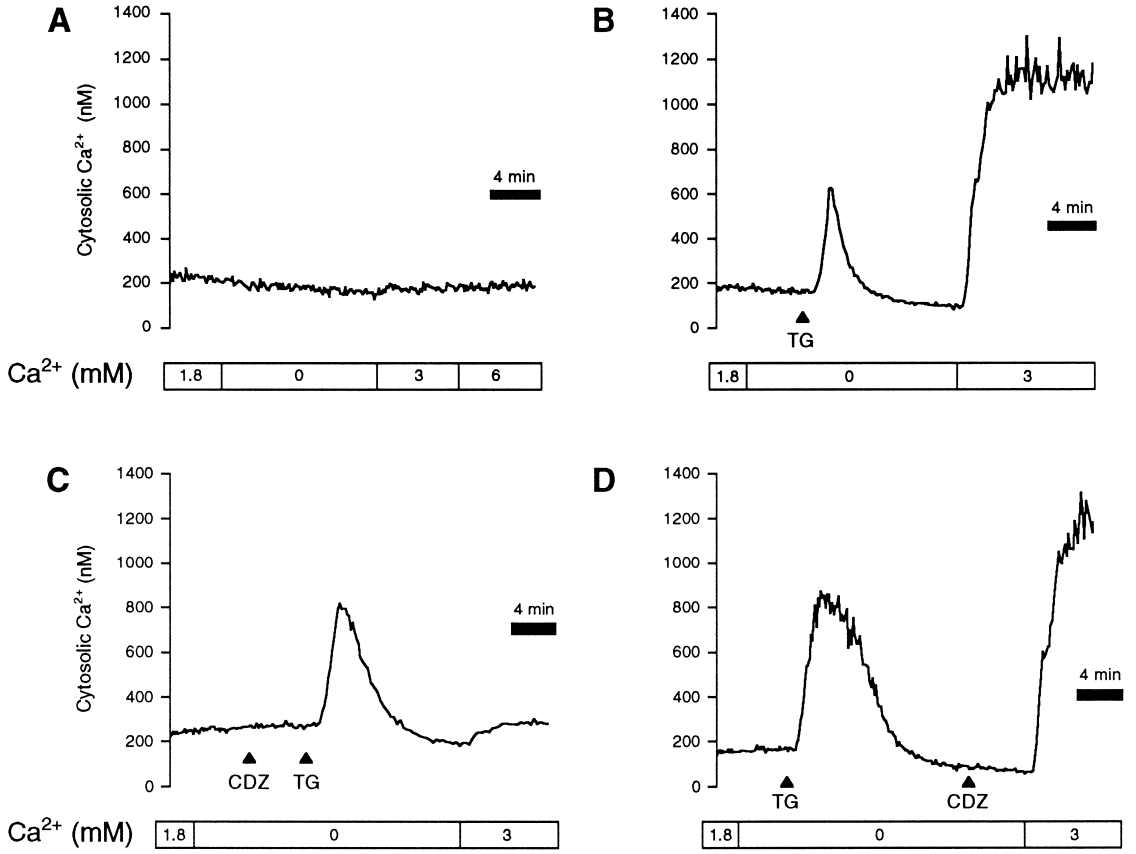

Fig. 1. Inhibition of store-operated Ca^{2+} entry by the CAM antagonist calmidazolium. The effects of switching from a Ca^{2+}-containing bath medium to a Ca^{2+}-fee solution and back on cytosolic Ca^{2+} was tested in a control condition (A), and in a situation where the intracellular stores had been depleted (B) by 1 μM thapsigargin (TG). Addition of the CAM antagonist calmidazolium (CDZ, 10 μM) before the thapsigargin-induced Ca^{2+} transient effectively inhibited Ca^{2+} entry after re-addition of Ca^{2+} to the bath (C), whereas its application *after* the thapsigargin-induced Ca^{2+} transient was ineffective (D). The application of the drugs (arrowheads) and the manipulation of bath Ca^{2+} concentration (mM) in the experiments are given inside the graphs. The traces represent typical results obtained from the experiments summarized in Fig. 2.

sociated transient Ca^{2+} rise in the cytosolic compartment (Fig. 1B). After this maneuver, re-admission of Ca^{2+} in the bath caused a rapid and substantial entry of Ca^{2+}. As observed in other studies [1], this procedure revealed in rat hepatocytes the existence of a Ca^{2+} influx component that becomes activated by depletion of intracellular Ca^{2+} stores. Another inhibitor of ER Ca^{2+}-ATPase, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone, had the same effects ($n=4$, data not shown) indicating that the observed activation of Ca^{2+} entry was not specific to thapsigargin, but was due to the depletion of the intracellular Ca^{2+} stores.

In order to test whether CAM was involved in the signaling between the intracellular stores and the cell membrane entry channels, the effects of classical CAM antagonists were tested. Fig. 1C shows an experiment where the CAM inhibitor calmidazolium (10 μM) [14] applied before the discharge of stores prevented Ca^{2+} entry after subsequent re-admission of Ca^{2+} in the bath.

Activation of CAM normally occurs after an elevation of Ca^{2+} which binds to and activates CAM. In order to investigate whether the thapsigargin-induced cytosolic Ca^{2+} transient was necessary for the CAM inhibitor to show its effects, the experimental protocol was modified such that the calmidazolium was added after the Ca^{2+} transient, when cytosolic Ca^{2+} was back to baseline (Fig. 1D). In this situation, calmidazolium did not produce inhibition and re-admission of Ca^{2+} to the bath resulted in the same rapid and large Ca^{2+} influx as in the control condition (Fig. 1B). The significance of this result is discussed below.

A series of CAM antagonists were tested for their ability to inhibit SOC using the protocol described in Fig. 1C,D. Besides calmidazolium, the inhibitors tested were CGS 9343B [15], W-7, and J-8 [16]. In addition, the substance KN-62, a specific inhibitor of the CAM-dependent protein kinase II [17], was tested using the same experimental protocol. The antagonists were applied at a dose usually used in other studies (e.g. [18–20]). J-8 was used at a concentration close to its IC_{50} of 3 μM [16].

The amplitude of the Ca^{2+} rise (in nM) after re-admission of Ca^{2+} to the bath was used as a measurement of SOC. In the control group, Ca^{2+} entry increased cytosolic Ca^{2+} concentration by 1070 ± 76 nM. Fig. 2A shows that application of all CAM antagonists of this series before the thapsigargin-induced Ca^{2+} discharge reduced the subsequent Ca^{2+} entry by more than 75%, confirming the results presented in Fig. 1C with calmidazolium.

It has been shown by several groups that some CAM antagonists as well as KN-62 could directly block voltage-gated calcium channels (e.g. [21,22]). To our knowledge such an interaction has not been demonstrated with SOC channels of hepatocytes. However, because the results of Fig. 2A do not allow us to discriminate between a CAM-mediated effect

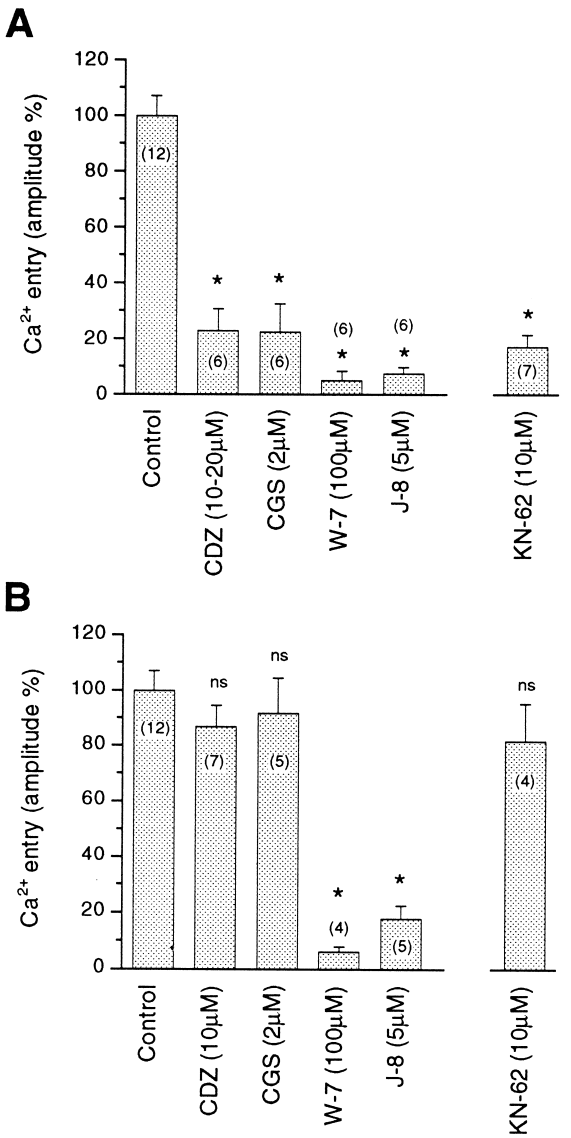

Fig. 2. Effect of various CAM antagonists on store-operated Ca^{2+} entry. The effect of selected CAM antagonists on store-operated Ca^{2+} entry is presented for a situation where the inhibitor was applied before (A) and after (B) the thapsigargin-induced Ca^{2+} transient. Calmidazolium (CDZ), CGS 9343B (CGS), W-7 and J-8 are compared at concentrations indicated in the graph. The amplitude of cytosolic Ca^{2+} concentration change in percent after re-addition of Ca^{2+} was compared to control condition, 100% corresponding to 1070 ± 76 nM Ca^{2+}. The number of experiments for each group is given in the graph. $*P < 0.0001$; ns, statistically not significant.

and a direct blockade of Ca^{2+} entry channels, the CAM antagonists were tested using the protocol described in Fig. 1D, in which the addition of the antagonist was done after the thapsigargin-induced Ca^{2+} transient. A time delay of 5 min ± 8 s was respected between the addition of drugs and re-admission of Ca^{2+} in the bath. Fig. 2B shows that besides calmidazolium, CGS 9343B and KN-62 did not produce an inhibition of Ca^{2+} entry when applied after the transient. This result allows us to rule out a direct interaction of these three substances with the channel, which would occur regardless of when the drug is added. To exclude the possibility that the lack of inhibition exhibited by these substances under these conditions was due to a shorter exposure time than in the case of an application prior to the thapsigargin treatment, a 15-min delay between the addition of calmidazolium and the Ca^{2+} re-addition was tested and did not inhibit Ca^{2+} entry either ($n = 2$).

In contrast, W-7 and J-8, two closely related substances [16], inhibited Ca^{2+} entry both when applied before and after the thapsigargin-induced Ca^{2+} transient, suggesting the possibility of a direct blocking action of these substances on the Ca^{2+} entry channel in addition to their described effects on CAM. The differential effects of this series of compounds is consistent with the respective potency and selectivity of the drugs, which was reported to be significantly higher for calmidazolium and CGS 9343B than for W-7 [23].

KN-62, a specific inhibitor of CAM-dependent protein kinase II [17], is often used by others to identify the mechanism activated by CAM (e.g. [18]). The inhibition observed when KN-62 was applied *before* the thapsigargin-induced cytosolic Ca^{2+} transient, but not when applied *after* the transient, tends to support the conclusion that the CAM kinase II takes part in the signaling pathway, possibly by phosphorylating the SOC channel or an associated regulatory protein. This hypothesis is supported by the finding that depletion of cellular ATP prevents activation of SOC in hepatocytes and in other cell types [24,25].

The results presented in this study are consistent with a mechanism in which activation of CAM is a key step in the activation of SOC in rat hepatocytes and occurs during the thapsigargin-induced discharge of the intracellular Ca^{2+} stores. CAM in turn activates its target protein – possibly CAM kinase II – which places the SOC channel in an active state. Once in this active state, the Ca^{2+} entry channel is no longer sensitive to CAM antagonists or to CAM kinase II inhibitors. Sensitivity of SOC to CAM antagonism has been found other cell types. A correlation between the amplitude of the cytosolic Ca^{2+} transient evoked by thapsigargin and the extent of Ca^{2+} entry, and a sensitivity to W-7 have been established in fibroblasts [26]. In thyroid cells [19] KN-62, calmidazolium, and W-7 attenuated Ca^{2+} entry, although the CAM-independent mechanism of inhibition (i.e. direct blockade of Ca^{2+} entry channels) was considered dominant by the authors.

All CAM antagonists used in the present work, as well as KN-62, at the doses used in this study had an inhibitory effect on phenylephrine-induced Ca^{2+} oscillations either by reducing their frequency or by suppressing them completely (data not shown). In another study [20], the CAM antagonists CGS 9343B and calmidazolium were both found to reversibly inhibit Ca^{2+} oscillations in rat hepatocytes. The authors hypothesized that CAM interacted directly with the *myo*-inositol 1,4,5-trisphosphate receptor of the intracellular stores to provide the release process with cytosolic Ca^{2+} sensitivity and that CAM antagonism resulted in an inhibition of Ca^{2+} mobilization. In view of our present results, the inhibition of oscillations could be entirely explained by an inhibition of Ca^{2+} entry, a process that is known to be required for sustained oscillatory response [20].

Taken together, the results of this study with rat hepatocytes suggest that CAM becomes activated during the elevation of cytosolic Ca^{2+} that follows the discharge of intracellular stores and participates in the activation of SOC. The fact that inhibition of CAM after the Ca^{2+} transient has occurred did not prevent SOC speaks in favor of a mechanism in

which, once activated by a CAM-dependent mechanism, SOC remains so for a certain time.

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