

Involvement of the Ca^{2+} /calmodulin-dependent protein kinase II pathway in the Ca^{2+} -mediated regulation of the capacitative Ca^{2+} entry in *Xenopus* oocytes*

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Activation of the phosphoinositide transduction pathway induces capacitative Ca^{2+} entry in *Xenopus* oocytes. This can also be evoked by intracellular injection of $\text{Ins}(1,4,5)\text{P}_3$, external application of thapsigargin and/or incubation in a Ca^{2+} -free medium. Readmission of Ca^{2+} to voltage-clamped, thapsigargin-treated *Xenopus* oocytes triggers Ca^{2+} -dependent Cl^- current variations that reflect capacitative Ca^{2+} entry. Inhibition of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) by specific peptides markedly increased the amplitude of the transients, suggesting an involvement of the CaMKII pathway in the regulation of capacitative Ca^{2+} entry. Biochemical studies

provide evidence for the activation of CaMKII in response to the development of capacitative Ca^{2+} entry. In effect, a CaMKII assay *in vivo* allows us to postulate that readmission of Ca^{2+} to thapsigargin-treated oocytes can induce a burst of CaMKII activity. Finally, analysis of the Cl^- transient kinetics at high resolution of time suggests that CaMKII inhibition blocks the onset of the inactivation process without affecting the activation rate. We therefore postulate that CaMKII might participate in a negative feedback regulation of store-depletion-evoked Ca^{2+} entry in *Xenopus* oocytes.

INTRODUCTION

Variations in the concentration of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) are involved in many cellular responses of both excitable and non-excitable cells [1–5]. Activation of the phosphoinositide signalling pathway elevates the cytoplasmic concentration of $\text{Ins}(1,4,5)\text{P}_3$, which releases Ca^{2+} from intracellular stores. Emptying of intracellular stores by $\text{Ins}(1,4,5)\text{P}_3$ or inhibition of the endoplasmic Ca^{2+} -ATPase by pharmacological antagonists, such as thapsigargin or cyclopiazonic acid, opens a particular Ca^{2+} influx pathway in many cell types including *Xenopus* oocytes [1–3]. This Ca^{2+} influx pathway has been termed capacitative because it depends on the degree of depletion of the Ca^{2+} stores irrespective of the way in which it has been achieved. In the cell types in which it has been directly recorded, the capacitative Ca^{2+} entry current (I_{CRAC}) is very tiny [4,5] and is practically undetectable directly in *Xenopus* oocytes. However, *Xenopus* oocyte membrane presents many Ca^{2+} -activated Cl^- channels, which have been widely used as an amplification system to monitor rapid changes in $[\text{Ca}^{2+}]_i$ [6]. The resulting current [$I_{\text{Cl}(\text{Ca})}$] has been reported to be highly sensitive to Ca^{2+} concentration in the vicinity of the plasma membrane and seems to react as a linear function of the rate of rise of $[\text{Ca}^{2+}]_i$ [7]. When the oocyte is voltage-clamped at a suitable potential, even a tiny amount of Ca^{2+} entering the cell can give rise to currents of several microamperes. Therefore, as in many previous studies concerning capacitative Ca^{2+} entry in *Xenopus* oocytes, we measured the Ca^{2+} -evoked Cl^- currents as an index for I_{CRAC} .

There are a number of reports describing the regulation of capacitative Ca^{2+} entry by various ions, including Ca^{2+} itself, and/or protein phosphorylation [4]. Petersen and Berridge [8]

reported the regulation of capacitative Ca^{2+} entry by protein kinase C (PKC) in a Ca^{2+} -dependent manner.

In contrast, the phosphorylation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) has been investigated only with regard to $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} liberation. Zhang et al. [9] observed that low concentrations of Ca^{2+} augment the stimulation by $\text{Ins}(1,4,5)\text{P}_3$ of Ca^{2+} flux in permeabilized fibroblasts, an effect inhibited by CaMKII inhibitors. Here we investigated the effects of the inhibition of the CaMKII pathway on *Xenopus* oocytes in which capacitative Ca^{2+} entry was activated by irreversible store depletion. Basically, the amplitude of the Ca^{2+} -evoked Cl^- transients was clearly augmented after intracellular injection of CaMKII-inhibitory peptides. This result tends to demonstrate a possible involvement of CaMKII in the regulation of I_{CRAC} . Moreover, we biochemically investigated CaMKII activity in immature *Xenopus* oocytes by using the syntide-2 kinase assay [10], which has been adapted for experiments *in vivo*. We conclude that immature *Xenopus* oocytes present a pool of CaMKII that is mobilizable by the capacitative Ca^{2+} entry process.

Finally, an examination of the shape of the Ca^{2+} -evoked Cl^- current transients reveals that the most prominent effect of CaMKII blockade is mainly focused on the inactivating phase of I_{CRAC} . Therefore our results led us to propose a negative feedback mechanism by Ca^{2+} on capacitative Ca^{2+} entry through CaMKII activation.

MATERIALS AND METHODS

Preparation of oocytes

Adult *Xenopus laevis* were anaesthetized in tricaine methane

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; CaM, calmodulin; CaMKII, Ca^{2+} /CaM-dependent protein kinase II; CaMKII(290–309), CaMKII fragment 290–309; CRAC, Ca^{2+} -release-activated Ca^{2+} ; $I_{\text{Cl}(\text{Ca})}$, Ca^{2+} -dependent Cl^- current; I_{CRAC} , CRAC current; MLCK(488–511), myosin light chain kinase fragment 488–511; PKC, protein kinase C; RRL, rabbit reticulocyte lysate; SC(290–309), peptide with the same amino-acid sequence as CaMKII(290–309), used as nonsense peptide.

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* This article is dedicated to the memory of Franck Fournier who passed away on 19 August 1995.

sulphonate (0.2% MS 222). Pieces of ovary were surgically removed and placed in ND96 medium of the following composition (in mM): NaCl 96, CaCl₂ 1.8, KCl 2, MgCl₂ 2, Hepes 5, titrated to pH 7.4 with NaOH. Oocytes were then treated for 2–3 h with collagenase (2 mg/ml; type IA) in Ca²⁺-free medium to remove follicular cells. Stage V and VI oocytes [11] were selected and maintained in ND96 supplemented with gentamycin (50 µg/ml) at 20 °C for up to 5 days.

Electrophysiological measurements

Changes in [Ca²⁺] were monitored by electrophysiological recording of $I_{Cl(Ca)}$ as described elsewhere [12]. Briefly, oocytes were impaled by two microelectrodes filled with 3 M KCl (0.5–1 mΩ) and voltage-clamped at –80 mV with a Geneclamp 500 voltage-clamp amplifier (Axon Instruments, Burlingame, CA, U.S.A.). Data acquisition and analysis were conducted with the pClamp software package (version 5.7.1; Axon Instruments). Experiments were performed at room temperature (18–22 °C).

Depletion of intracellular Ca²⁺ was performed by oocyte incubation in Ca²⁺-free ND96 medium supplemented with thapsigargin (1 µM; 0.01% DMSO) for at least 2 h as previously reported [13]. To achieve a Ca²⁺-free perfusate, Ca²⁺ was replaced by Mg²⁺ and EGTA (1 mM) was added. The peptide SC(290–309), with the sequence TFKLNRLAKGRIKLLKAMALT has the same amino acid composition as CaMKII fragment 290–309 [CaMKII(290–309)] (LKKFNARRKLLKGAILTTMLA) but does not correspond to anything, to our knowledge. It was therefore used as nonsense peptide throughout the study.

Syntide-2 phosphorylation assay *in vivo*

This assay is adapted from the syntide-2 kinase assay *in vitro* described in [14,15]. This test consists of the phosphorylation *in vivo* of syntide-2, a peptide with a sequence (PLARTLSVAG-LPGKK) similar to that of phosphorylation site 2 of glycogen synthetase, which contains the consensus motif (RXXS/T) for CaMKII [16,17]. To perform the assay *in vivo*, both radioactivity and substrate peptide were injected directly into oocytes. Briefly, dried batches of [³²P]P_i (5 mCi; Amersham, Little Chalfont, Bucks., U.K.) were resuspended in various assay solutions including water, syntide-2, syntide-2 plus myosin light-chain kinase fragment 488–511 [MLCK(488–511)], syntide-2 plus CaMKII(290–309), and syntide-2 plus SC(290–309). All the peptide solutions listed were made up in water to a final concentration of 20 mg/ml, corresponding to 8.6 mM for both CaMKII(290–309) and SC(290–309), and 9 mM for MLCK(488–511). *Xenopus* oocytes that had been kept in zero-Ca²⁺/thapsigargin medium for 2 h were injected with 50 nl of one of the previously described solutions and incubated for at least 15 min to reach phosphate equilibrium. Capacitative Ca²⁺ entry was then activated by transferring the oocytes into a Ca²⁺-containing medium for 2 min. The reaction was stopped by crushing the oocytes in 50 µl of ice-cold 10% (v/v) trichloroacetic acid, followed by incubation for 1 h at 0 °C to achieve protein precipitation. Owing to its structure, the syntide-2 peptide does not precipitate in trichloroacetic acid solution. Samples were centrifuged at 10000 g and the supernatant was immediately spotted on Whatman P81 phosphocellulose paper. Paper sheets were washed three times with water, dried and subjected to Čerenkov counting.

Obtaining phosphorylated syntide-2 *in vitro*

The syntide-2 peptide was phosphorylated *in vitro* and radio-labelled by a Ca²⁺-independent mutant of CaMKII. A plasmid

(pT7-7) encoding the mutant ending at Leu-290 of rat brain CaMKII α holoenzyme was constructed as described [15]. The construct was linearized by *Pst*I and transcribed *in vitro* with T7 RNA polymerase in accordance with the manufacturer's instructions (Amersham). Translation *in vitro* was conducted for 50 min at 30 °C with 1 µg of the transcript in 15 µl of rabbit reticulocyte lysate (RRL; Amersham). The phosphorylation reaction was performed as follows: 3 µl of the programmed RRL described above was added to 40 µl of the following mixture: Hepes (50 mM), MgCl₂ (10 mM), ATP (100 µM), syntide-2 (1.5 mg/ml final concentration) and 150 µCi of [γ -³²P]ATP (specific radioactivity 3000 Ci/mmol; Amersham). The mixture was incubated for 1 h at room temperature, after which the reaction was stopped by adding 1 vol. of trichloroacetic acid (10%, v/v). The supernatant was recovered after a 15 min centrifugation at 10000 g and spotted on P81 paper. The sheets were then washed, dried and counted by Čerenkov radiation.

Phosphopeptide analysis

Syntide-2 was eluted from the phosphocellulose paper with 1 M NaCl. The eluate was loaded on a C₁₈ reverse-phase HPLC column (0.2 cm × 10 cm; P. E. Brownlee) equilibrated in 0.1% (v/v) trifluoroacetic acid (solvent A). Elution was performed by a 60 min gradient from solvent A to solvent B [60% CH₃CN/0.08% (v/v) trifluoroacetic acid]. Elution was monitored at 220 nm and fractions were collected manually in polypropylene tubes. Radioactivity was determined by Čerenkov radiation.

Electrospray ionization mass spectra were obtained on a Fisons Trio 2000 mass spectrometer by scanning from 400 to 1000 m/s. Data were processed with the Mass Lynx software and the instrument was calibrated with horse heart myoglobin.

Phosphorylated syntide-2 was hydrolysed at 110 °C in 5.7 M HCl for 100 min. Phosphoaminoacid analysis was performed by two-dimensional thin-layer electrophoresis at pH 1.9 and 3.5 as described [14].

Chemicals

All chemicals were purchased from Sigma unless otherwise stated. CaMKII(290–309) was from Bachem (Bubendorf, Switzerland) and MLCK(488–511) and SC(290–309) were kindly given by J. Méry (CRBM, CNRS, Montpellier, France). Both were dissolved in water.

RESULTS

Modulation of capacitative Ca²⁺ entry through a CaMKII pathway

Capacitative Ca²⁺ entry has been reported to be activated by the depletion of intracellular Ca²⁺ stores. The means by which we depleted the intracellular stores was to inhibit the cells' Ca²⁺ uptake mechanism by application of the endoplasmic Ca²⁺-ATPase inhibitor thapsigargin, so that stores were emptied of Ca²⁺ by the passive leak pathway [13].

Figure 1(A) depicts the iterative application of 1.8 mM CaCl₂ in 2 min pulses, each separated by an interval of 3 min, on a voltage-clamped oocyte (holding potential, –80 mV) that has been pretreated with thapsigargin. Readmission of Ca²⁺ to the bathing solution evokes inward-directed Cl[–] current transients that have been reported to reflect the capacitative Ca²⁺ entry process [8]. On-line injection of MLCK(488–511), a well-described calmodulin (CaM) antagonist [14], markedly increased the amplitude of the Ca²⁺-evoked Cl[–] current transients, suggesting the possible involvement of a CaM-dependent pathway in

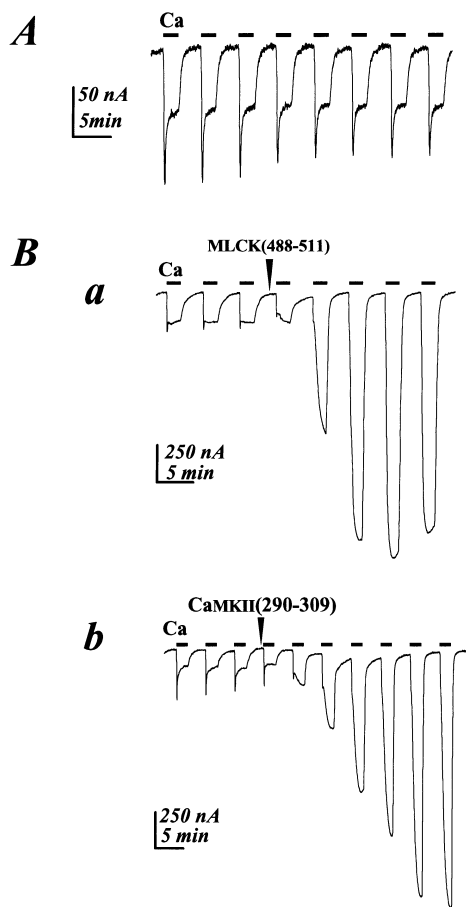


Figure 1 Capacitative Ca²⁺ entry and its regulation by a CaM/CaMKII pathway

Pulsed extracellular application of 1.8 mM Ca²⁺ evoked Ca²⁺ influx-triggered Cl⁻ currents in thapsigargin-treated *Xenopus* oocytes under voltage-clamped conditions at -80 mV. Ca²⁺ pulses of 2 min are indicated by dark bars above the current traces. (A) Under control conditions, extracellular Ca²⁺ pulses elicited Cl⁻ current transients whose amplitude quickly reached a steady state. (B) The magnitude of the Ca²⁺-evoked Cl⁻ current transients was markedly enhanced after intracellular injection of MLCK(488-511) (a) or CaMKII(290-309) (b). Both peptides were injected at a concentration of 10 mg/ml in the microinjection pipette; their final intracellular concentration can be estimated as 40 μ M.

the regulation of the capacitative Ca²⁺ entry (Figure 1B, a). After injection of MLCK(488-511), the transients remained sensitive to La³⁺ (1 mM), a trivalent cation that has been shown to inhibit capacitative Ca²⁺ entry in many cell types, including *Xenopus* oocyte [8]. Moreover, the use of CaMKII(290-309), a specific CaMKII inhibitory peptide [16], clearly elicited the same effects on the Cl⁻ current transients as those described with MLCK(488-511) (Figure 1B, b). Taken together, these results suggest that CaMKII takes part in a negative regulation of capacitative Ca²⁺ entry. In that sense the CaMKII pathway would represent a possible link by which Ca²⁺ negatively regulates its own entry.

Specificity and dose-effect relationships of the CaMKII inhibitory peptides

The peptide concentrations used in this study might seem to be rather high; however, they are expressed as intra-pipette concentrations. Oocytes were injected with roughly 10 nl of peptide

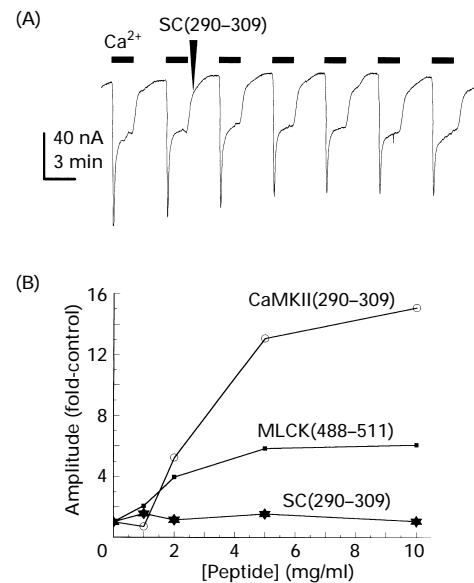


Figure 2 Specificity and dose-effect relations of the CaMKII inhibitory peptides

(A) The Ca²⁺-evoked Cl⁻ current transients were not affected by intracellular injection of the nonsense peptide SC(290-309) (10 mg/ml in the microinjection pipette). (B) The dose-effect relations for CaMKII(290-309), MLCK(488-511) and SC(290-309) were performed by measuring their ability to increase the amplitude of the Ca²⁺-evoked Cl⁻ current transients. The nonsense peptide SC(290-309) remained ineffective on the capacitative Ca²⁺ entry irrespective of its concentration. Note that the concentrations indicated on the x-axis correspond to the peptide concentration in the injection pipette, not the final concentration in the oocyte.

solutions at 10 mg/ml. The oocyte volume can be approximated to 1 μ l and therefore the final peptide concentration in the cell can be estimated as 40 μ M, which is consistent with other studies with such peptides [17]. With the aim of verifying the effects elicited by microinjection of CaMKII inhibitory peptides, we investigated the effects of SC(290-309). This peptide presents the same amino acid composition as CaMKII(290-309) but differs in its sequence (see the Materials and methods section). As shown in Figure 2(A), the injection of SC(290-309) into voltage-clamped thapsigargin-treated oocytes did not alter the pattern of Cl⁻ current transients induced by Ca²⁺ readmission. This experiment demonstrates that the effects caused by injection of CaMKII(290-309) depend not only on its amino acid composition but also on its sequence, which describes the inhibitory C-terminus of rat brain CaMKII α holoenzyme. Figure 2(B) shows the dose-effect relations of the different inhibitory peptides used in this study compared with those of the nonsense peptide SC(290-309). The capacitative Ca²⁺ entry process is clearly enhanced by both CaMKII inhibitors, whereas it remains insensitive to intracellular injection of SC(290-309). Moreover, the effects of CaMKII(290-309) and MLCK(488-511) seem to reach their maximum at a pipette concentration of approx. 10 mg/ml. Taken together, these results tend to establish the specificity of the well-known CaMKII inhibitors that were employed in this study with regard to the capacitative Ca²⁺ entry process.

CaMKII assay *in vivo*

The increase in CaMKII activity that was suggested by the electrophysiological data was investigated by a CaMKII assay *in vivo* (see the Materials and methods section). Our first aim was

Table 1 Phosphorylation of syntide-2 *in vivo*

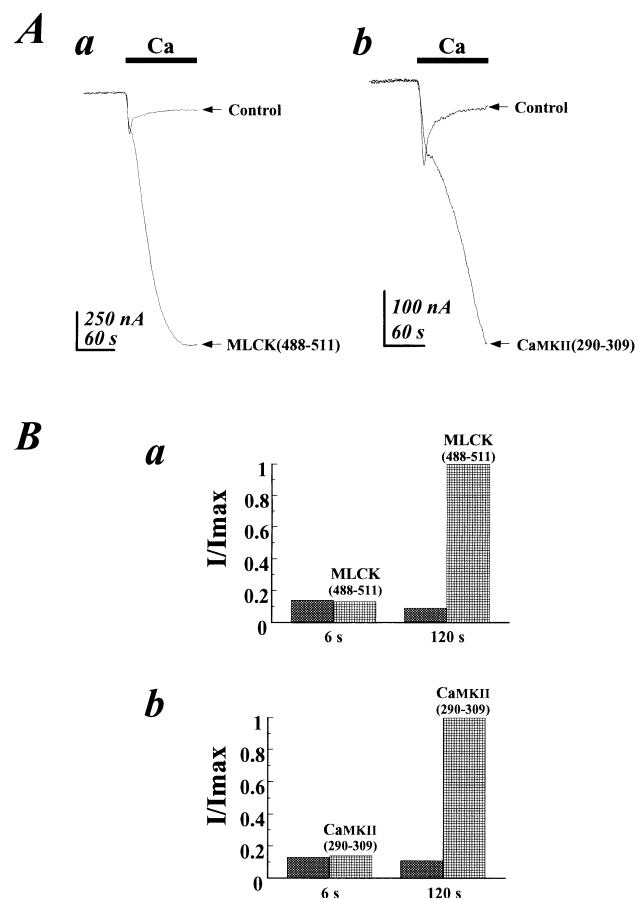
Table 1 summarizes the results of the phosphorylation of syntide-2 *in vivo* induced by Ca^{2+} readmission to zero- Ca^{2+} /thapsigargin-treated oocytes. Symbols: +, the compound is present as well as [^{32}P]P_i in the injection mixture; -, the compound is absent. Incorporation of radioactivity is expressed in c.p.m. counted on the P81 paper sheets as described in the Materials and methods section. Results are means \pm S.E.M.; *n* indicates the number of oocytes contributing to the mean.

| [Ca ²⁺] (mM) | Syntide-2 | MLCK(488–511) | CaMKII(290–309) | SC(290–309) | Radioactivity (c.p.m.) | <i>n</i> |
|--------------------------|-----------|---------------|-----------------|-------------|------------------------|----------|
| 0 | – | – | – | – | 30.53 \pm 15.25 | 10 |
| 0 | + | – | – | – | 184.50 \pm 22.50 | 8 |
| 0 | + | + | – | – | 25.00 \pm 7.00 | 4 |
| 0 | + | – | + | – | 31.00 \pm 16.00 | 4 |
| 0 | + | – | – | + | 219.00 \pm 19.00 | 4 |
| 1.8 | – | – | – | – | 23.50 \pm 12.25 | 7 |
| 1.8 | + | – | – | – | 1293.02 \pm 264.62 | 12 |
| 1.8 | + | + | – | – | 32.00 \pm 12.00 | 4 |
| 1.8 | + | – | + | – | 26.00 \pm 14.00 | 4 |
| 1.8 | + | – | – | + | 1324.04 \pm 242.22 | 9 |

to recover the peptide and to ensure that it had been properly phosphorylated. To that end, samples phosphorylated *in vivo* (oocyte) and *in vitro* (RRL) were applied to a reverse-phase HPLC column. Approx. 80 % of the radioactivity co-eluted with the main UV peak and the retention times observed for the peptides phosphorylated *in vivo* and *in vitro* were each identical with that of the initial peptide (results not shown). The different fractions were subjected to electrospray ionization MS to determine the molecular masses of the main species. The expected values were obtained: 1505 Da for the oocyte sample and 1503 Da for the RRL sample (theoretical molecular mass 1507.8 Da). Furthermore, enough phosphorylated substrate was present in the RRL sample to be detected at 1583 Da, corresponding to the addition of an 80 Da phosphoryl group. As final confirmation, the nature of the phosphorylated residue was determined after acid hydrolysis and two-dimensional thin-layer electrophoresis. As expected, phosphorylated Ser was detected in both RRL and oocyte samples (results not shown). These experiments attest that syntide-2 can be recovered nearly intact after microinjection into *Xenopus* oocytes and that it is actually phosphorylated in response to the capacitative Ca^{2+} entry process. The syntide-2 test *in vivo* has been performed in different conditions, summarized in Table 1. Even in the absence of Ca^{2+} in the bathing solution, a basal CaMKII activity could be recorded that was inhibited by MLCK(488–511) and CaMKII(290–309) but not by SC(290–309). A 6-fold increase in syntide-2 phosphorylation was observed when Ca was readmitted to the oocytes' bathing solution. As above, this increase was antagonized by both MLCK(488–511) and CaMKII(290–309) but not by SC(290–309). In total, these results demonstrate that CaMKII can be activated by the development of the capacitative Ca^{2+} entry process.

Implication of the CaMKII pathway in the process of the inactivation of capacitative Ca^{2+} entry

The effects of both MLCK(488–511) and CaMKII(290–309) on the responses to extracellular Ca^{2+} were analysed at a higher resolution of time to investigate the kinetics of individual responses. In the control situation the Ca^{2+} -evoked Cl^- transients displayed a peaked waveform with a time to peak of 6 ± 1 s (*n* = 12). When MLCK(488–511) or CaMKII(290–309) was injected into the oocytes the peaked pattern vanished and changed to a smooth, slowly activating waveform that coincided with a large increase in the amplitude of the response (Figure 3A). In some

**Figure 3** Regulation of I_{CRAc} inactivation kinetics by CaMKII

(A) Effects of intracellular injection of MLCK(488–511) (a) or CaMKII(290–309) (b) are shown at a higher resolution of time in comparison with control conditions on thapsigargin-treated oocytes. Altering the CaMKII pathway clearly blocked the inactivation kinetics (peptides were injected at a concentration of 10 mg/ml in the injection pipette). (B) Current amplitude was measured at the average time for the peak appearance (6 s) as well as at the end of the Ca^{2+} pulse (2 min) before and after peptide injection. Oocytes were injected either with MLCK(488–511) (a) or with CaMKII(290–309) (b). The graphs show that the main part of the effect is supported by the delayed phase of the response.

traces there seemed to be a deceleration of the signal before it reached a higher peak. The emergence of this point of inflexion was not always as clear as on the traces in Figure 3, but it caused

us to consider that the signal switches from a fast waveform to a slower one. Thus current amplitude was measured at 6 s (average time for the peak's appearance) as well as at the end of the Ca²⁺ pulse (2 min) in both control and peptide-injected conditions. Figure 3(B, a) shows that no significant changes in the Cl⁻ transients amplitude were observed in response to MLCK(488–511) when measured at 6 s (I/I_{\max} 0.13 compared with 0.14), whereas at 2 min it was increased 11-fold (I/I_{\max} 0.09 compared with 1). Injection of CaMKII(290–309) revealed the same kind of results (I/I_{\max} at 6 s, 0.14 compared with 0.13; at 2 min, 0.11 compared with 1), as shown in Figure 3(B, b). These results suggest that the inhibition of CaMKII activity increases the amplitude of the capacitative Ca²⁺ entry, apparently by blocking the onset of the inactivation process.

DISCUSSION

In the present study the Ca²⁺-activated Cl⁻ channels located in the plasma membrane were used as an index to monitor I_{CRAC} . Using fluorescent indicators, Parker and Yao [7] demonstrated that the Cl⁻ current magnitude is approximately proportional to the rate of increase in [Ca²⁺]_i, rather than to its absolute level. The ability of the Cl⁻ conductance to behave as a high-pass filter might account for the kinetics we observed on the Ca²⁺-evoked Cl⁻ transients (Figure 1). On-line intracellular injection of CaM/CaMKII inhibitory peptides clearly increased the amplitude of the Cl⁻ currents induced by Ca²⁺ readmission on thapsigargin-treated oocytes (Figure 1B). In Figure 1 the traces have been selected to depict the most meaningful effects of MLCK(488–511) and CaMKII(290–309) and therefore correspond to the maximal increase in $I_{\text{Cl(Ca)}}$ amplitude. In Figure 2 the data were averaged from experiments performed on the same batches of oocytes and present the relative effects of both peptides on the Cl⁻ current transients. With these latter experiments, we arrived at the conclusion that CaMKII(290–309) was more potent than MLCK(488–511) in triggering an increase in the capacitative Ca²⁺ entry process. Moreover, the effects we obtained with the CaM/CaMKII inhibitory peptides can be considered to be specific because nothing was observed with the nonsense peptide SC(290–309). Thus we postulate that at least a partial inhibition of I_{CRAC} can be mediated by CaM and/or CaMKII. Although we observe the same effects with a CaMKII inhibitor as with a CaM antagonist, we cannot totally exclude an involvement of CaM itself in this regulatory process. In effect, both Ins(1,4,5)P₃ receptors [18] and the transient receptor potential gene product [the molecule that shares the best functional similarities with the Ca²⁺-release-activated Ca²⁺ (CRAC) channel] sequences present CaM-binding domains, but their modulatory effects on the physiological activity of the two proteins remain unknown [19]. This proposal tacitly implies the existence of a CaMKII activity in immature oocytes, which has not been investigated. In contrast, it has been shown that in mature *Xenopus* oocytes CaMKII activates ubiquitin-dependent cyclin degradation in cytosolic extracts and mediates the effects of Ca²⁺ at fertilization in inactivating M-phase promoting factor and cytosolic factor activities [15]. Indeed, biochemical assays allowed us to demonstrate that a CaMKII activity can be triggered by activating the capacitative Ca²⁺ entry process. This suggests that capacitative Ca²⁺ entry can mobilize the CaMKII pathway, which could account for its own Ca²⁺-mediated regulation.

Analysis of the Cl⁻ current transients at high resolution of time revealed that Ca²⁺ entry describes a marked biphasic pattern; a very quick onset is followed by a rapid reversal, both being

monitored by similar variations in $I_{\text{Cl(Ca)}}$ (Figure 3). This biphasic response can be explained by reference to the well-known bell-shaped activation curve, as summarized by Berridge [20]. Rapid activation is ensured by the positive feedback effect due to channel opening. As [Ca²⁺]_i rises, negative feedback occurs and down-regulates the entry. The effects we observed by blocking CaMKII clearly resemble those reported by Petersen and Berridge [8,21] in response to various kinase activators. These authors demonstrated that PKC exerts opposite effects on capacitative Ca²⁺ entry, depending on its degree of activation. Low levels of activation of PKC potentiate Ca²⁺ influx by blocking the inactivation process, whereas high levels of activation simply inhibit capacitative Ca²⁺ entry. Ca²⁺ would therefore regulate its own entry by forming a positive and a negative feedback loop through PKC activation. In that context, our results might indicate that CaMKII could also participate in the inactivation process of I_{CRAC} that occurs at high cytosolic Ca²⁺ concentration. Nevertheless the point of inflexion that constitutes the transition between the two phases of the signal might convey an inhibitory effect of the peptides, which in turn could reflect a feed-forward effect of CaMKII as well as a feedback. These latter statements imply that one of the molecules involved in the capacitative Ca²⁺ entry can be phosphorylated by CaMKII.

A number of reports indicate a role for protein phosphorylation in the regulation of capacitative Ca²⁺ entry and it is difficult to know which kinase phosphorylates a given component of the entry mechanism. For instance, CaMKII has been shown to phosphorylate the Ins(1,4,5)P₃ receptor [22], which might facilitate its Ca²⁺ release properties [9]. It has been proposed that Ins(1,4,5)P₃ receptors might have two different functions: they might either release Ca²⁺ from the endoplasmic reticulum or utilize their cytosolic heads to transfer signals to the plasma membrane channel according to the conformational coupling model [20]. Moreover, separate isoforms of the Ins(1,4,5)P₃ receptor have been reported [23–25] and it is not excluded that the two signalling functions might be attributed to different receptors. The development of capacitative Ca²⁺ entry when the stores are emptied through the Ca²⁺-ATPase blockade seems to discount the involvement of the type I Ins(1,4,5)P₃ receptor in conformational coupling. Berridge [20] mentions that conformational coupling might be mediated by the type III receptor, the activation of which has no effect on Ins(1,4,5)P₃-mediated Ca²⁺ release but markedly enhances Ca²⁺ entry. However, no evidence was found for the presence, in *Xenopus* oocytes, of other Ins(1,4,5)P₃ receptor isoforms than type I or of other types of intracellular Ca²⁺ release channels [24].

To summarize, type III Ins(1,4,5)P₃ receptor would sense when the store is empty and then communicate the information to the CRAC channel through conformational changes. These two proteins are likely targets for CaMKII-mediated phosphorylation and might account for the negative regulation that we observed.

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