The inositol (1,4,5)-trisphosphate 3-kinase of *Xenopus* oocyte is activated by CaMKII and involved in the regulation of $InsP_3$-mediated Ca^{2+} release

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Abstract The effect of Ca^{2+} on inositol (1,4,5)-trisphosphate 3-kinase (3-kinase) activity was measured on *Xenopus* oocyte cytosolic extracts. The Ca^{2+}-evoked elevation in 3-kinase activity appeared to be mediated by calmodulin (CaM) and the calmodulin-dependent protein kinase II (CaMKII). The results observed in vitro were totally retrieved in intact oocytes and tend to demonstrate the involvement of a CaMKII-mediated phosphorylation in the regulation of 3-kinase activity. Finally, electrophysiological recordings of $InsP_3$-elicited chloride current transients in the presence of CaM/CaMKII inhibitors allowed to postulate an involvement of 3-kinase activity in the regulation of $InsP_3$-mediated Ca^{2+} release.

Key words: $InsP_3$ 3-kinase; CaMKII; Ca^{2+} release; *Xenopus* oocyte

1. Introduction

Inositol (1,4,5)-trisphosphate ($InsP_3$) is a well established second messenger that binds to the $InsP_3$ receptor to release Ca^{2+} from intracellular stores [1]. In many tissues, cytoplasmic Ca^{2+} concentration modulate $InsP_3$-mediated Ca^{2+} release (IMCR) in a biphasic manner. At submicromolar concentrations, Ca^{2+} activates the release process whereas at higher concentrations Ca^{2+} is inhibitory. This biphasic effect has been described in *Xenopus* oocytes [2], smooth muscle [3], brain [4] and hepatocytes [5]. Most of the studies concerning IMCR were focused on the release mechanism and more precisely on the $InsP_3$ receptor itself. Ca^{2+} can activate and inhibit IMCR either directly by interacting with an activatory or inhibitory site on the receptor or indirectly through activation or inhibition of the regulating enzymes including $InsP_3$ metabolizing enzymes. The crucial parameters that regulate the formation and termination of the Ca^{2+} signals are the generation and the degradation of the second messenger. Two primary degradative pathways exist for $InsP_3$ but they differ in their relative importance among cell types (for review, see [6]). Several isoforms of the $InsP_3$ 5-phosphatase (5-phosphatase) dephosphorylate $InsP_3$ yielding $Ins(1,4)P_2$ ($InsP_2$). $InsP_3$ is also a substrate for the 3-kinase which phosphorylates $InsP_3$ to form $Ins(1,3,4,5)P_4$ ($InsP_4$). This confers a dual role to the 3-kinase in generating another second messenger as well as decreasing the $InsP_3$ concentration. In rat brain, the 3-kinase has been demonstrated to be activated by cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) through CaM activation [7]. Moreover, Communi et al. [8] demonstrated that a CaMKII-mediated phosphorylation of the rat brain 3-kinase is responsible for the agonist-mediated activation of the enzyme. The conclusions drawn by these latter reports allow to postulate an involvement of the $InsP_3$ metabolism in the Ca^{2+}-dependent regulation of IMCR.

In the present report, we first investigated the effects of Ca^{2+}, CaM and CaM/CaMKII inhibitors on the 3-kinase activity of *Xenopus* oocytes extracts. Our results demonstrated that the Ca^{2+}-evoked positive regulation of 3-kinase depends on CaM either directly or indirectly through a CaMKII-mediated phosphorylation. The effects found with the extracts were consistently retrieved in vivo by the measurement of $InsP_3$ degradation in intact oocytes. Finally, electrophysiological data led to the conclusion that an activation of the 3-kinase may be involved in the Ca^{2+}-dependent down regulation of IMCR.

2. Materials and methods

Adult *Xenopus laevis* were anaesthetized in tricaine methane sulphonate (0.2%; MS 222). Pieces of ovary were surgically removed and placed in ND96 medium of the following composition (mM): NaCl, 96; $CaCl_2$, 1.8; KCl, 2; $MgCl_2$, 2; HEPES, 5, titrated to pH 7.4 with NaOH. Oocytes were treated for 2–3 h with collagenase (2 mg/ml) in Ca^{2+}-free medium in order to remove follicular cells. Stage V and VI oocytes were selected and maintained in ND96 supplemented with gentamycin (50 μg/ml) at 20°C for up to 5 days. The medium was renewed daily.

The procedure for measurement of 3-kinase activity in cytoplasmic extracts was adapted from [9]. Oocytes were homogenized in 200 μl ice cold buffer M (100 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES pH 7.0 with KOH, 50 mM sucrose, 10 μg/ml cytochalasin B, 0.4 mM phenylmethanesulfonylfluoride, 5 μM leupeptin, 20 mM benzamidine, 10 μg/ml calpain inhibitor) using a teflon homogenizer. The crude soluble fraction was obtained by centrifugation at $35\,000\times g$ for 60 min. A 40 μl portion of a further buffer was added to 60 μl of crude soluble fraction to give a concentration of 84 mM HEPES/NaOH, pH 7.5, 1 mg/ml of bovine serum albumin, 1 mM ATP, 20 mM $MgCl_2$, 2.5 mM 2,3-bisphosphoglycerate, 1 mM EGTA, 1 μM $InsP_3$ and 2000 cpm of [^{3}H]-$InsP_3$ (specific activity: 40 Ci/mmol; Amersham, UK). Ca^{2+} was added to adjust the free Ca^{2+} concentration calculated using an apparent affinity constant of EGTA for calcium of 3.17×10^6 M^{-1} at pH 7.5 and Ca^{2+}/CaM was added when needed. After 8 min incubation at 37°C, 1 ml of ice cold 0.1 M formic acid/0.4 M ammonium formate was added to stop the reaction. Samples were diluted with an equal volume of 0.1 M formic acid/0.4 M ammonium formate prior to loading on to a 0.6 ml Dowex 1×8 (formate form; Bio-Rad) column. The column was washed with 6 ml of 0.1 M formic acid/0.4 M ammonium formate. To elute $InsP_3$, 20 ml of 0.1 M formic acid/0.7 M

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Abbreviations: $InsP_1$, D-myo-inositol (1)-phosphate; $InsP_2$, D-myo-inositol (1,4)-biphosphate; $InsP_3$, D-myo-inositol (1,4,5)-trisphosphate; $InsP_4$, D-myo-inositol (1,3,4,5)-tetrakisphosphate; $I_{Cl(Ca)}$, calcium-dependent chloride current; CaM, calmodulin; CaMKII, Ca^{2+}/CaM-dependent protein kinase II; CaMKII(290–309), CaMKII fragment 290–309; RS20, myosin light chain kinase fragment 488–511; LPA, L-α lysophosphatidic acid oleyl; OA, okadaic acid

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ammonium formate were applied to the column and, for elution of $InsP_4$, 8 ml of 0.1 M formic acid/1.2 M ammonium formate were applied. Radioactivity was determined by liquid scintillation counting. Enzyme activity was expressed as nmoles of $InsP_4$ produced/min/mg protein. Protein was determined using a Bradford assay (Bio-Rad). In all experiments, enzyme activity was assayed at 1 μM $InsP_3$.

For the measurements of $InsP_3$ degradation in intact oocytes, the cells were microinjected with 10 nl of a solution containing 0.1 μCi of lyophilized [^{3}H]-$InsP_3$ (40 Ci/mmol; Amersham) mixed with 1 μl of cold $InsP_3$ (9.75×10^{-5} M). Total calculated concentration of $InsP_3$ (cold and ^{3}H labeled) in the injection pipette was 0.1 mM. Given an injection volume of ~10 nl and an oocyte volume of 1 μl the calculated intracellular concentration was ~1 μM. The exact volume of injectate was determined from the total number of counts contained into the oocyte. The oocytes were incubated at room temperature for the indicated times. At the end of the incubation period, the medium surrounding the oocyte was removed to determine how much of the ^{3}H label has leaked from the oocyte. Cells that lost greater than 10% of the label were excluded. InsP metabolism was arrested by crushing the oocyte in 1 ml of ice cold buffer containing 0.1 M formic acid/0.4 M ammonium formate. The soluble fraction obtained by centrifugation at $35\,000\times g$ for 10 min was loaded on to a 0.6 ml Dowex 1×8 (formate form; Bio-Rad) column. The chromatography was carried out as described above.

Changes in $[Ca^{2+}]_i$ were monitored by electrophysiological recording of the Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) as described elsewhere [10]. Briefly, oocytes were impaled by two microelectrodes filled with 3 M KCl (0.5–1 MΩ) and voltage-clamped to −80 mV using a Geneclamp 500 amplifier (Axon Instruments). Data acquisition and analysis were conducted using the pClamp software package (Axon Instruments). Experiments were carried out at room temperature (18–22°C). Microinjections were performed with a micropipette which tip diameter did not exceed 10 μm.

All chemicals were purchased from Sigma unless otherwise stated. Calmodulin was from Calbiochem.

3. Results and discussion

In a first series of experiments, the 3-kinase activity of the soluble fraction of *Xenopus* oocytes homogenates was measured in the absence of Ca^{2+}, in the presence of Ca^{2+} and in the presence of Ca^{2+} and CaM. Kinase activities measured in the presence of EGTA (control) were always lower as compared to the level reached in the presence of Ca^{2+} (Fig. 1). In the presence of Ca^{2+}, kinase activities were potentiated by addition of CaM (Fig. 1A). Conversely, the presence of the specific CaM antagonist peptide RS20 [11,12] prevented the Ca^{2+}- and Ca^{2+}/CaM-mediated effects on the 3-kinase activity. These results confirm those obtained in rat brain by [7] and are in agreement with [13,14] who guessed that the Ca^{2+} sensitivity of the 3-kinase of *Xenopus* oocyte was mediated by CaM. Moreover, it has been recently reported that phosphorylation of the rat brain 3-kinase A by CaMKII results in 8- to 10-fold enzyme activation and a 25-fold increase in its sensitivity to the Ca^{2+}/CaM complex [8]. Addition of the potent CaMKII inhibitors KN-62 and KN-93 to the extracts prevented the Ca^{2+}/CaM-mediated activation of the 3-kinase (Fig. 1B). The same effects were obtained by microinjecting the highly specific CaMKII inhibitory peptide CaMKII(290–309) prior to cytoplasm extraction. These results allowed us to postulate an involvement of a CaMKII-mediated phosphorylation of the 3-kinase in its own regulation. The involvement of a phosphorylation is further corroborated by the fact that a preincubation of the oocytes with the well known phosphatase inhibitor okadaic acid provided a maximal activation of the 3-kinase (Fig. 1B). A recent report concerning the metabolism of $InsP_3$ and $InsP_4$ in *Xenopus* oocytes [14] led us to think that an alteration in the metabolism of $InsP_3$ may account in the

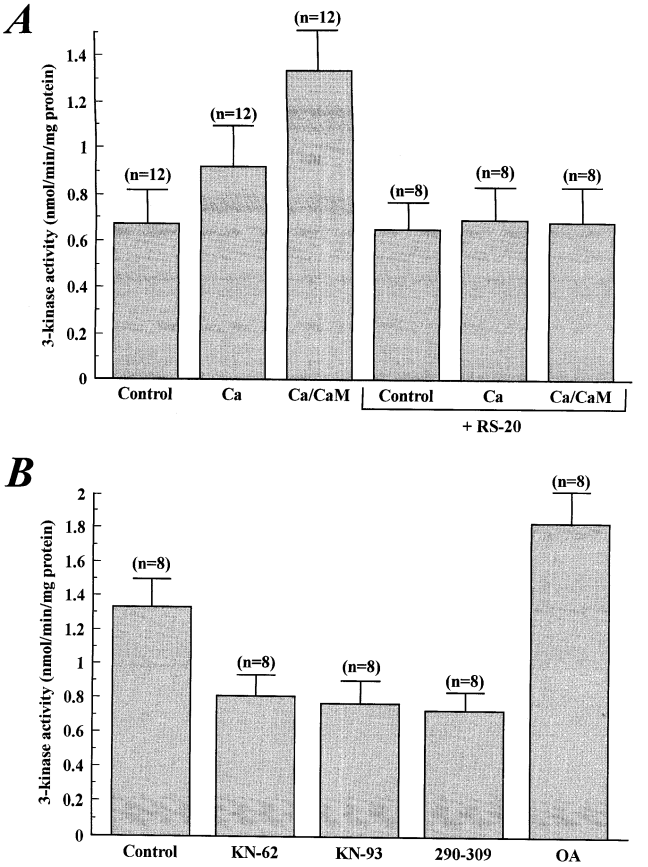

Fig. 1. Regulation of 3-kinase activity in *Xenopus* oocytes cytosolic extracts. The number of cells for each condition is indicated between brackets. Results are presented as activity ± S.E. (indicated by bars). A: The assays were performed in the absence of Ca^{2+}, 10 μM free Ca^{2+} and 10 μM free Ca^{2+} supplemented with 1 μM CaM as indicated on the *x*-axis. The same experiments were performed in the presence of 5 μM of RS20 as indicated. B: All assays were performed in the presence of 10 μM free Ca^{2+} supplemented with 1 μM CaM. The *x*-axis indicates the reagents that were added to the mixture. KN-62, KN-93 and CaMKII(290–309) were used at 1 μM; okadaic acid was used at 30 nM.

regulation of IMCR. Measurements of $InsP_3$ degradation in intact oocytes were conducted to investigate the physiological relevance of the Ca^{2+}/CaM-dependency of the 3-kinase. In that purpose, single oocytes were injected with [^{3}H]-$InsP_3$ to determine its immediate metabolism. The chromatographic method employed in this study (Fig. 2A) allowed the separation of $InsP_{\leq2}$, $InsP_3$ and $InsP_4$. For experimental time points of 5 min or less, the reactions of $InsP_3$ are segregated into two distinct pathways without common metabolites [13,14]. In the 3-kinase pathway, $InsP_4$ accumulates with very little conversion to the lower inositol phosphates and was used as an estimate for the 3-kinase activity. On the other hand, the sum of the concentrations of the products of the 5-phosphatase pathway ($InsP_{\leq2}$) was used to estimate the activity of the 5-phosphatase toward $InsP_3$. Our results confirmed that in vivo, $InsP_3$ is predominantly metabolized by the 3-kinase whereas in the same conditions, $InsP_{\leq2}$ does not accrue to a substantial degree. The measurement of $InsP_3$ degradation has then been carried out on oocytes that had been injected either with Ca^{2+}/CaM or RS20 prior to $InsP_3$ stimulation. Fig. 2Ba shows that an accrued amount of Ca^{2+}/CaM caused an accel-

eration of $InsP_4$ formation. On the contrary, this formation was clearly slowed down in the presence of the CaM antagonist RS20. These modulations of $InsP_3$ degradation appeared to entirely rely on the 3-kinase activity (Fig. 2Ba) whereas the 5-phosphatase activity was not affected by any reagent (Fig. 2Bb). Finally, the data presented on Fig. 2C show that $InsP_4$ formation is definitely affected by an intracellular treatment with KN-93 or CaMKII(290–309). The in vitro experiments indicated that in the presence of RS20 or any CaMKII antagonist, Ca^{2+} and Ca^{2+}/CaM failed to cause an increase in 3-kinase activity. The $InsP_4$ formation measured in vivo in the presence of CaM/CaMKII antagonists seems to result from 3-kinase basal activity. In the absence of inhibitor, the observed elevation of $InsP_4$ formation is liable to be mediated by an increase in 3-kinase activity due to $InsP_3$-evoked elevation in $[Ca]_i$, this effect being further reinforced by addition of Ca^{2+}/CaM. At the light of the experiments conducted with CaMKII inhibitors, it seems likely that CaMKII phosphorylates the 3-kinase resulting in an increase in enzymatic activity as reported for rat brain [8]. In order to assess the involvement of 3-kinase in IMCR, electrophysiological experiments were performed on oocytes that had been pre-injected with either Ca^{2+}/CaM or RS20. Injection of $InsP_3$ gave rise to typical inward Cl^- current transients on oocytes voltage-clamped at −80 mV. Pre-injection of the oocytes with Ca^{2+}/CaM significantly reduced the amplitude of $InsP_3$-evoked Cl^- current transients (413 ± 44 nA, $n = 14$ vs. 699 ± 40 nA, $n = 21$; Fig. 3A, traces b and a). Inversely, intracellular pre-injection of RS20 led to a net increase in the amplitude of the current (1020 ± 137 nA, $n = 9$ vs. 699 ± 40 nA, $n = 21$; Fig. 3A, traces c and a). Alternatively, oocytes were stimulated by the phospholipase C (PLC)-linked agonist lysophosphatidic acid (LPA) which interacts with specific receptors at the plasma membrane of *Xenopus* oocytes [15]. Fig. 3B shows that the response to LPA is partially inhibited by Ca^{2+}/CaM injection (310 ± 62 nA, $n = 7$ vs. 829 ± 112 nA, $n = 7$; Fig. 3B, traces b and a) and greatly potentiated by RS20 treatment (1787 ± 328 nA, $n = 8$ vs. 829 ± 112 nA, $n = 7$; Fig. 3B, traces c and a). Injection of $InsP_3$ into the oocytes or PLC stimulation cause a biphasic increase in $[Ca]_i$: the initial transient phase represents

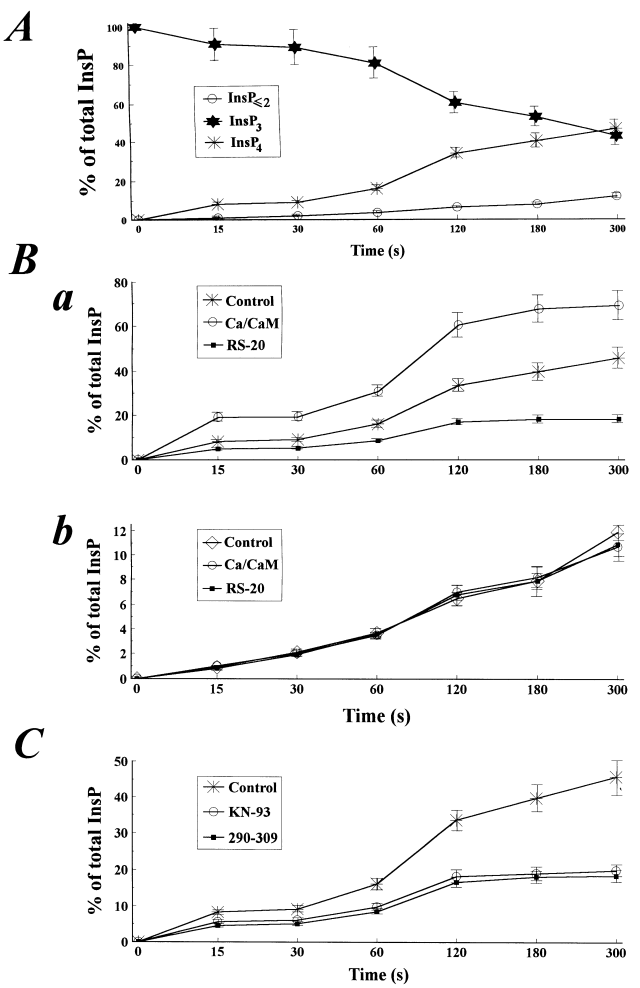

Fig. 2. Measurement of $InsP_3$ metabolism in intact oocytes. Radioactivity specific to each InsP was expressed as a percentage of total injected dpm. Data were averaged from eight cells for each point. A: The schematic of $InsP_3$ metabolism is valid for incubation times of 5 min. Value recorded at each time point represented mean ± S.E. of radioactivity attributable to each InsP as indicated in the inset. B: Ca^{2+}/CaM (Ca^{2+} 2 μM/CaM 1 μM final estimated concentration) and RS20 (5 μM final concentration) modulate 3-kinase activity but do not affect 5-phosphatase. a: $InsP_4$ formation is measured as a function of time in control conditions or in the presence of CaM or RS20 as indicated in the inset. b: No modulation of $InsP_{\leq 2}$ formation could be elicited by either CaM or RS20 (see inset) when injected into the oocytes. C: Experiments identical to those described in B were performed with CaMKII specific inhibitors (used compound indicated in inset; their final intraoocyte concentration was 1 μM). $InsP_4$ formation appears to be clearly diminished by CaMKII inhibitors.

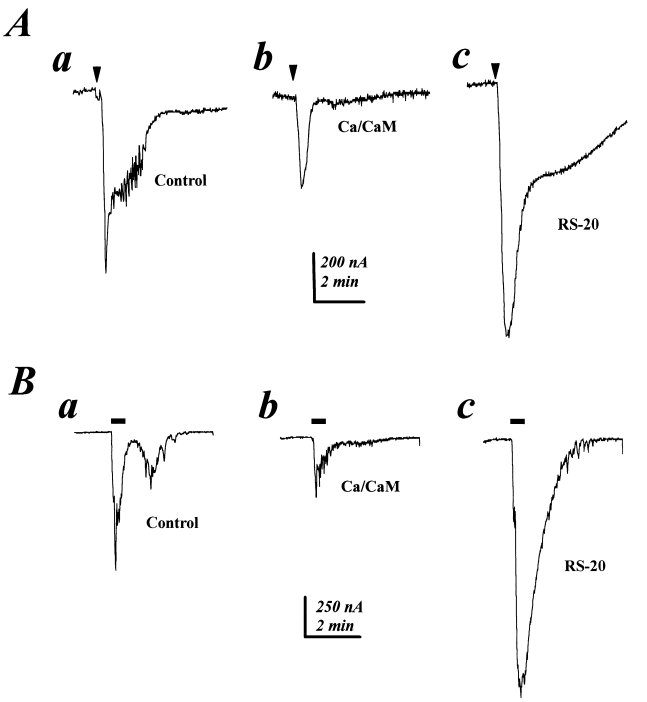

Fig. 3. Effects of Ca^{2+}/CaM complex or RS20 microinjection on $I_{Cl(Ca)}$ transients elicited by either $InsP_3$ injection or LPA superfusion. *Xenopus* oocytes were voltage-clamped at −80 mV and bathed in ND96 medium. A: The oocytes were injected with $InsP_3$ (1 μM, final concentration, ▼). Pretreatment of the oocytes with Ca^{2+}/CaM (Ca^{2+} 2 μM/CaM 1 μM) reduced the amplitude of the $InsP_3$-evoked Cl^- current transient whereas it was potentiated by RS20 (5 μM, final concentration) pre-injection. B: The same results were obtained when the Cl^- current transient was triggered by LPA superfusion (1 μM, 30 s, black box).

Table 1
Comparison of Cl^- current transients amplitude and 3-kinase activity elicited by $InsP_3$ injection in control and CaM/CaMKII inhibitors injected oocytes

Condition	I_{max}		Activity (15 s)	
	(nA)	% of control	nmol/min/mg	% of control
Control	699 ± 40	100	0.93 ± 0.09	100
RS20	950 ± 73	136	0.63 ± 0.07	68
KN-93	998 ± 140	143	0.55 ± 0.05	59
CaMKII(290–309)	1020 ± 137	146	0.49 ± 0.05	53

Voltage-clamped *Xenopus* oocytes were injected with $InsP_3$ (1 μM final intraoocyte concentration) and the maximal amplitude of the first phase of the response was considered as an indicator of the Ca^{2+} release magnitude. Values are expressed as current amplitude and percentage of control. The 3-kinase activity measured 15 s after $InsP_3$ injection is reported in the table in control conditions as well as in the presence of RS20 (5 μM), KN-93 (1 μM) or CaMKII(290–309) (1 μM). Values are indicated as enzyme activity and percentage of control.

the release of Ca^{2+} from intracellular stores and the more sustained phase that follows is referred to as capacitative Ca^{2+} entry [16]. The presence of an accrued amount of Ca^{2+}/CaM into the oocytes caused an important reduction of the amplitude of both phases of $[Ca]_i$ increase whereas the opposite effect was observed with RS20. To further establish the link between 3-kinase activity and IMCR, we directly compared the maximal amplitude of the first phase of the current elicited by $InsP_3$ injection into Ca^{2+}/CaM-, RS20-, KN-93- and CaMKII(290–309)-treated oocytes to its corresponding 3-kinase activity. Results summarized in Table 1 show a definitive correlation between the current amplitude and the 3-kinase activity. Basically, the current reaches its maximal amplitude (146% of control) for the lowest value of 3-kinase activity (53% of control) which was elicited by microinjection of CaMKII inhibitory peptide into the oocyte (Table 1). It seems therefore likely that the level of 3-kinase activity directly regulates the amount of $InsP_3$ and consequently modulates the amplitude of the Ca^{2+} release process. Furthermore, our data tend to indicate that the 3-kinase activation process in *Xenopus* oocytes may arise from its phosphorylation by CaMKII. It has been reported that IMCR is activated by CaMKII in a Ca^{2+}-dependent manner and inhibited by phosphatase 2B [17]. In this context, IMCR should have been enhanced by Ca^{2+}/CaM and instead it was inhibited. The same paradox was raised by the CaM/CaMKII inhibitors which caused an increase in the release process although they should have been inhibitory. These data led us to put forward the proposal that the modulation of IMCR we observed may arise from $InsP_3$ metabolism through 3-kinase activation or inhibition. An increase in 3-kinase activity could be responsible for a significant reduction of $InsP_3$ concentration. Conversely, inhibiting the CaM/CaMKII pathway would prevent any Ca^{2+}-evoked increase in 3-kinase activity resulting in a higher $InsP_3$ concentration as compared to control conditions. This interpretation is corroborated by the fact that the effects of both CaM and inhibitors are already perceptible at the mean time of appearance of the peak inward current (10–15 s). Because of the use of a permeabilized cell system on which $InsP_3$ was added extracellularly, it is possible that Zhang et al. [17] did not observe the effects of $InsP_3$ metabolism. One may reasonably propose that the 3-kinase of *Xenopus* oocytes could be regulated by the Ca^{2+}/CaM complex either directly or indirectly through a CaMKII-mediated phosphorylation. The fact that the effects of CaM and CaM/CaMKII inhibitors on IMCR could be elicited using a physiological way to produce $InsP_3$ tend to demonstrate that the 3-kinase may play a key role in regulating signals arising from the PLC-related transduction system. Finally, as theoretically supposed by [18] the regulation of 3-kinase by Ca^{2+} through CaM might represent an additional pathway to support the Ca^{2+}-mediated down regulation of IMCR.

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