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Biochemical Characterization of a Putative Calcium Influx Factor as a Diffusible Messenger in Jurkat Cells, *Xenopus* Oocytes, and Yeast

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Highly purified high performance thin layer chromatography (HPTLC) fractions containing a putative calcium influx factor (CIF) were prepared from the Jurkat cells and *Xenopus* oocytes in which Ca^{2+} stores were depleted by thapsigargin treatment and from the yeast in which intracellular Ca^{2+} stores were also depleted by genetic means. Microinjection of the fractions has been shown to elicit Ca^{2+}-dependent currents in *Xenopus* oocytes. The nature of the membrane currents evoked by the putative CIF appeared to be carried by chloride ions since the current was blocked by the selective chloride channel blocker 1 mM niflumic acid and its reversal potential was about -24 mV. Injection of the calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N, N, N', N'*-tetraacetic acid (BAPTA) eradicated the current activities, suggesting the current responses are entirely Ca^{2+}-dependent. Moreover, the currents were sensitive to the removal of extracellular calcium, indicating the dependence on calcium entry through the plasma membrane calcium entry channels. CIF activities were insensitive to protease, heat, and acid treatments and to Dische-reaction whereas the activities were sensitive to nucleotide pyrophosphatase and hydrazynolysis. The fraction might have a sugar because it was sensitive to Molisch test and Seliwaniff's resorcinol reaction. From the above results, CIF as a small and stable molecule seems to have pyrimidine, pyrophosphate, and a sugar moiety.

Cytosolic calcium is crucial in the regulation of cell responses in a wide variety of cells (Berridge, 1990; 1993). Multiple mechanisms contribute to regulation of cytosolic calcium levels. The activation of receptors coupled to phosphoinositide hydrolysis releases cytosolic inositol 1,4,5-trisphosphate ($InsP_3$), which discharges calcium from intracellular stores (Berridge, 1993). The subsequent depletion of intracellular calcium stores by $InsP_3$ (Hoth and Penner, 1992; McDonald et al., 1993), or alternative loss of the stores by inhibition of sarcoplasmic reticulum/endoplasmic reticulum (SERCA) type Ca^{2+}-ATPase by selective inhibitors (Zweifach and Lewis, 1993 Petersen and Berridge, 1994) activates calcium influx through a novel calcium entry channel in the plasma membrane. This mode of depletion-activated calcium entry was first recognized by Putney and was termed capacitative calcium entry (Putney, 1986; 1990).

Several hypotheses are currently circulating as potential signaling mechanisms between calcium stores and calcium entry channels (Berridge, 1995; Thomas et al., 1998). Two of them have attracted the greatest interest. One model is that the depletion of calcium stores induces a conformational change of the $InsP_3$ receptor and consequentially leads to a direct interaction with the Ca^{2+} entry channel (Irvine, 1990; Petersen and Berridge, 1996; Thomas et al., 1998). In contrast, the other model suggests that a diffusible messenger, denoted as a calcium influx factor (CIF) is mobilized in response to store depletion and opens the channel (Randriamampita and Tsien, 1993; Berridge, 1995; Thomas et al., 1998). However, the mechanism responsible for the calcium entry remains unresolved (Gilon et al., 1995). My colleagues and I demonstrated that acid extracts prepared from various cell types are able to evoke calcium dependent chloride currents in the *Xenopus* oocytes (Kim et al., 1995; Thomas and Hanley, 1995; Thomas et al., 1998; Csutora et al., 1999; Kim and Hanley, 1999; Kim and Hanley 2000). The membrane currents are due to calcium entry through plasma membrane calcium entry channels.

The data presented here provide structural information

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of the highly purified CIF from *Saccharomyces cerevisiea*, *Xenopu* oocytes, and Jurkat cells. Using *Xenopus* oocytes, I also characterized membrane currents elicited by injection of a putative CIF obtained by a variety of cells.

Materials and Methods

Chemicals

Thapsigargin (TG) was purchased from LC Services. The L-15 medium was from Life Technologies, Inc., the Sep-Pak catridge (C18) was from Millipore, the Bio-Gel P-2 gel was from Bio-Rad, the Microcon-30 was from Amicon, and the HPTLC plate (200 μm layer) was from Whatman International Ltd. Niflumic acid, nucleotide pyrophosphatase (*Crotalus adamaneus* Venom), and all other chemicals were from Sigma Chemical Co.

Cell cultures

Jurkat T lymphocytes were maintained as suspension cultures in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin (100 units) /streptomycin (100 ug/ml). Jurkat cells were passaged by 1:10 dilution every 4 days.

Wild-type (YR98: MAT *ade2 his3- 200 leu2-3, 112 lys2- 201 ura3-52*, called K601) and *pmr1*(YR122: MAT *ade2 his3- 200 leu2-3, 112 lys2- 201 pmr1- 1::Leu2 ura3-52*, called AA542) yeast cells were grown to an OD_{600} of 1.5 in YPD (yeast extract/peptone/dextrose) medium.

Xenopus oocytes

X. oocytes were obtained by ovarectomy as described previously (Kim and Hanley, 1999). Follicular cells were removed from oocytes by treating with collagenase (2 mg/ml, 2 h, 25°C), followed by rolling the oocytes on plastic petri dishes. Defolliculated oocytes were maintained in modified L-15 medium. For the depletion of intracellular calcium stores, *X.* oocytes were incubated (18°C, 2 h) with TG (1 μM) in calcium-free OR2 medium (82 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES, pH 7.4).

Preparation of calcium influx factor (CIF)

Highly purified CIF was prepared from TG-stimulated Jurkat cells and *X.* oocytes and unstimulated K601 and AA542 by a sequence of purification steps; Sep-Pak reverse-phase column, Microcon-30 ultrafiltration, Bio-Gel P-2 gel filtration, and a high performance thin layer chromatography (HPTLC) as previously described (Kim and Hanley, 2000). HPTLC 1-4-1 fractions obtained by HPTLC were used in this study.

Structural characterization of CIF

To identify CIF containing pyrimdine, highly purifiec fraction (HPTLC 1-4-1) was incubated with the same volume of hydrazine hydrate (64%) for 2 h at 90°C. Hydrazine was evaporated under high vacuum and reconstituted to initial volume of 10 mM HEPES, pH 7.0 (Hayes and Hayes-Baron, 1967). Trypsin (20 μg/sample) as a protease was treated for hydrolysis of protein in the HPTLC 1-4-1 at 37°C for overnight. For acid depurination, HPTLC 1-4-1 was added with HCl to give pH 1.6 and then incubated at 37°C for 26 h. The sample was lyophilized and reconstituted with the same buffer as mentioned above (Tamm et al., 1952). Treatment of nucleotide pyrophosphatase was performed that HPTLC 1-4-1 was incubated with 20 units/ml of nucleotide pyrophosphatase containing reaction buffer (150 mM KCl, 6 mM $MgCl_2$, and 10 mM HEPES, pH 7.0) for 30 min at 37°C (Bossuyt and Blanckaert, 1994).

Data acquisition and current analysis

Two-electrode whole cell voltage clamp experiments using *X.* oocytes were performed as described previously (Kim and Hanley, 2000). Briefly, oocytes were voltage-clamped at -60 mV in OR2 medium (82 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES, pH 7.4) with the use of a TEV-200 clamp amplifier (Dagan). Currents were low-pass filtered at 50 kHz using the internal four-pole Bessel filter and digitized at sampling rates of 100 msec (10 Hz) or 500 msec (2 Hz) with the use of the TL-analog/digital converter (Axon Instruments). To obtain current-voltage relations, voltage ramps were run on the activated currents. The ramp data were collected with the use of pCLAMP software (version 5.5) to sample current response to potential changes at a rate of 4 kHz. The ramp protocol consisted of repeated episodes (every 5s) of a -100 mV to +60 mV ramp with an interepisode holding potential of -60 mV.

Results and Discussion

My collogues and I have previously demonstrated that partially purified CIF from TG-stimulated Jurkat cells and *X.* oocytes induces calcium entry upon its microinjection into *X.* oocytes (Kim et al., 1995, Csutora et al., 1999; Kim and Hanley, 1999; Kim and Hanley, 2000). In *S. cerevisiae*, depletion of Ca^{2+} from Golgi appratus achieved by mutation of the Ca^{2+}-ATPase encoded by *PMR1* (Rudolph et al., 1989), results in an increase in cytosolic Ca^{2+} concentration. Even though, without treatment of TG, extracts prepared from *PMR1* mutant, AA542, also elicit calcium entry because the strain genetically depleted of intercellular organellar Ca^{2+} produces a CIF that elicits Ca^{2+} influx in *X.* oocytes (Csutora et al., 1999). This

elucidates that AA542 contains a large content of CIF in the normal condition, suggesting that AA542 is a good model system for understanding the action mechanism of CIF and studying the structure of CIF.

First of all, I observed that a putative CIF, called HPTLC 1-4-1 obtained from TG-stimulated Jurkat cells evoked membrane currents (peak current, 367±109 nA, solid traces in Fig. 1A). Moreover, the currents were almost eradicated by removal of the extracellular calcium as expected (peak current, 20±9 nA, dotted traces in Fig. 1A). Microinjection of the CIF fraction from TG-activated *X.* oocytes also elicited current responses (peak current, 294±31 nA, solid traces in Fig. 1B), which were eliminated by removal of the extracellular calcium, too (peak current, 11±2 nA, dotted traces in Fig. 1B). When the normalized CIF activities were compared to putative CIF fractions between stimulated cells and unstimulated cells, the current responses were drastically reduced in the unstimulated Jurkat cells and *X.* oocytes (Table 1). These results suggest that the HPTLC 1-4-1 fractions from both TG-treated Jurkat cells and *X.* oocytes contain solely authentic CIF. Several previous reports have

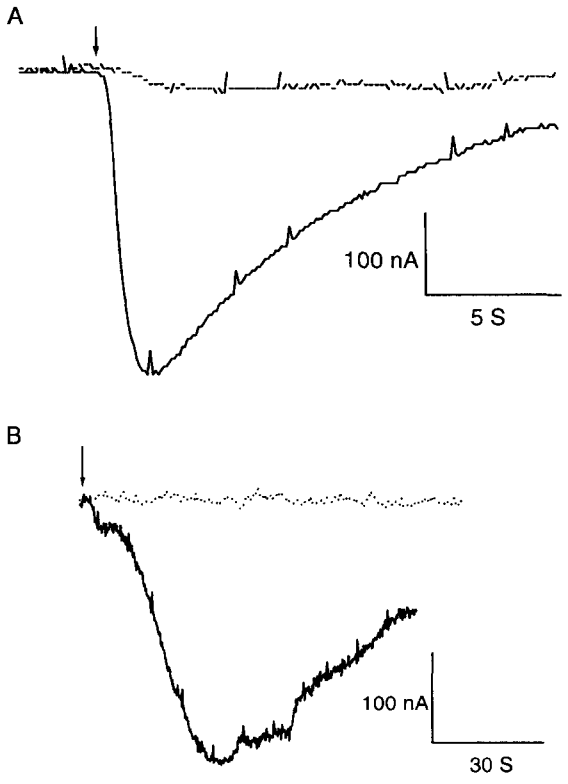

Fig. 1. Microinjection of putative CIFs obtained from TG-treated Jurkat cells (A) and *Xenopus* oocytes (B) elicited membrane currents into *Xenopus* oocytes. The maximal currents induced by Jurkat cells and *Xenopus* oocytes were 367±109 nA (solid traces, n=22) and 294±81 nA (solid traces, n=18), respectively. Removal of extracellular Ca^{2+} (Ca^{2+}-free OR2 containing 0.1 mM EGTA) inhibited the current response evoked by microinjections of HPTLC1-4-1 from Jurkat cells (20±9 nA, dotted traces, n=16) and *Xenopus* oocytes (11±2 nA, dotted traces, n =8). The arrow denotes time of the injection.

Table 1. Comparison of CIF activity induced by putative CIF from untreated and TG-treated Jurkat cells and *Xenopus* oocytes and from K601 and AA542

Treatment	CIF activities (nA)[a]	Fold
Jurkat cells (1×10^4 cells)		
Untreated	17.6± 4	1
TG-treated	184.0± 29.6	10.5
Xenopus oocytes (2 cells)		
Untreated	18.4± 4	1
TG-treated	307.2± 52.8	16.7
S. cerevisiae (1×10^6 cells)		
K601	16.5± 8.3	1
AA542 (YPD)	377.2± 19.7	22.9
AA542 (YPD+Ca^{2+})	32± 4.2	1.9

[a]CIF activity=currents at 2 mM $[Ca^{2+}]_o$-current at 0 mM $[Ca^{2+}]_o$ containing 0.1 mM EGTA. $[Ca^{2+}]_o$ indicates extracellular calcium ion concentration and currents were measured Ca^{2+} dependent Cl^- currents by injection of the each sample into *Xenopus* oocytes. Values are means ± standard deviation of five or six independent experiments from different batches of oocytes.

described that partially purified CIF may be detected upon calcium depletion in Jurkat cells (Kim et al., 1995; Thomas and Hanley, 1995), neutrophils (Davies and Hallett, 1995), and *Xenopus* oocytes (Kim and Hanley, 1999). Here, I provide information that highly purified fraction containing an active component has a calcium entry activity in Jurkat cells and *X.* oocytes (Fig. 1 and Table 1).

Microinjection of HPTLC 1-4-1 from AA542 elicited current responses (peak current, 136±26 nA, solid traces in Fig. 2A) in the presence of 2 mM extracellular Ca^{2+} (see Table 1). The currents were completely abolished by removal of extracellular Ca^{2+} (Fig. 2B). However, the same fraction prepared from K601 strain had little current responses (Table 1). Interestingly, HPTLC 1-4-1 fraction from AA542 cells grown under the condition of YPD medium containing 5 mM Ca^{2+} drastically reduced CIF activities by about 12 fold (Table 1), indicating that CIF production is dependent upon concentration of calcium in the growth medium.

To estabilish the nature of the membrane currents elicited by the putative CIF fractions, I examined the initial I-V relationship and effects of several pharmacological reagents on the currents. The reversal potential for the each fraction-activated currents was approximately -24 mV, elucidating that the currents may be carried by Cl^- (Fig. 3 and also see Barish, 1983). This conclusion was further supported by the observation that fractions-activated currents were substantially reduced by the perfusion of 1 mM niflumic acid, a blocker of Cl^- channels (data not shown). Injection of BAPTA (1 mM final concentration) eradicated all current activities elicited by the fractions, indicating that the responses are completely calcium dependent (data not shown). Using the oocytes, I indirectly examined CIF activities by measuring the

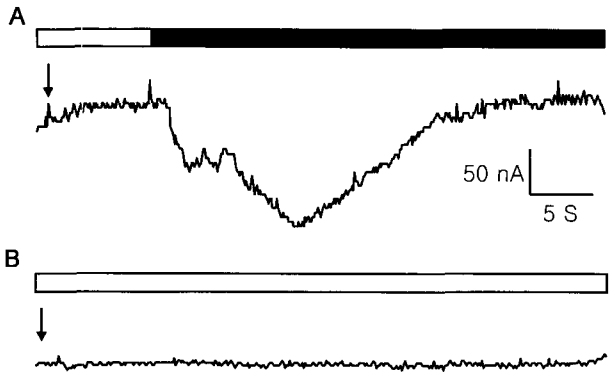

Fig. 2. Microinection (10 nl) of HPTLC 1-4-1 obtained from yeast AA542 strain evoked maximal current (136±26 nA, n=21) in the presence of 2 mM extracellular Ca^{2+} concentration (A). Under the free extracellular Ca^{2+} condition, the current was entirely abolished (B). Open bar and solid bar indicate free and 2 mM calcium concentration in the extracellular medium, respectively. The arrow denotes time of the injection.

Ca^{2+}-dependent Cl^- currents using the voltage clamp techniques. The *X.* oocytes have many Ca^{2+}-activated Cl^- channels in the plasma membrane which have been used as an amplification system to detect calcium signals (Petersen and Berridge, 1994). This result, taken together with above results, suggest that the current are due to the Ca^{2+} influx through calcium entry channels in the *Xenopus* oocytes, indicating that this HPTLC 1-4-1 enriched fractions contain an authentic CIF.

The chemical structure of CIF was characterized by employing several chemical and biochemical methodologies (Table 2). CIF seemed small and stable molecule but not peptide or proteins because treatment with heat or protease has not reduced its activity. The fraction was insensitive to Dische-reaction for detecting nucleic acid, indicating that CIF might be not DNA or RNA (data not shown). The small size of CIF (about 500 dalton) was

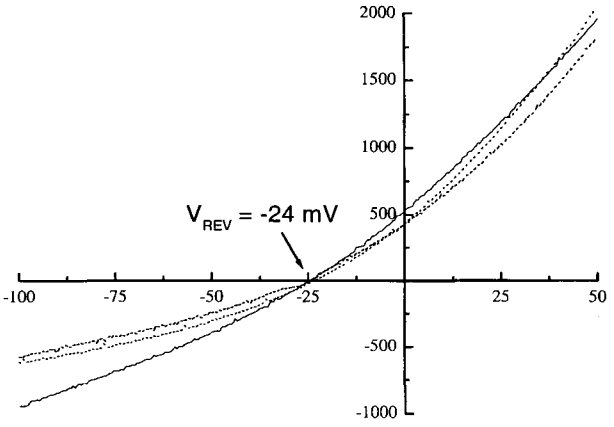

Fig. 3. The I-V curves of the currents activated by injection of HPTLC 1-4-1 obtained from Jurkat cells (solid traces), *Xenopus* oocyes (dotted traces) and AA542 (dashed traces). Each reversal potential was -24 mV. Results are representative of three independent experiments.

Table 2. Characterization of CIF from Jurkat cells, *Xenopus* oocytes, and *S. cerevisiae*

Treatment	Jurkat cell	*X.* oocytes	*S. cerevisiae*
	Activity (%)		
Control	100	100	100
Heat (2 h at 100°C)	80±7	88±17	88±18
Trypsin (20 μg, 37°C, overnight)	89.6±3	89.7±2	91.1±4
Hydrazinolysis	9±2	12±8	8±1
Acid depurination	83±6	95±18	91±19
Pyrophosphatase, nucleotide	9±2	20±7	11±3
UV-irradiation (5 nm, 220-300 nm)	11±4	15±4	7±5

Values are means±standard deviation of four independent experiments from different batches of oocytes

determined by 500 cutoff filter and gel filtration chromatography through Bio-Gel P-2 polyacrylamide. The CIF activities drastically reduced after treatment with nucleotide pyrophosphatase, UV, and hydrazine hydrate (Table 2). The sensitivity to pyrophosphatase, UV-irradiation, and hydrazinolysis suggests that CIF might have pyrimidine and pyrophosphate. However, activity was insensitive to acid treatment, which should not contain purine ring or non-essential (Table 2). The fraction might also have sugar moieties since the fraction was sensitive to Molisch test and Seliwaniff's resorcinol reaction (data not shown). These results suggest that CIF seems to have pyrimidine, pyrophosphate, and a sugar. Tsien and his co logues elucidate that CIF might have hydroyls on adjacent carbons, a phosphate, and a small molecule (Randriamampita and Tsien, 1993). The chemical structure of CIF that was demonstrated here might be similar to that of the small molecule previously described by Tsien (Randriamampita and Tsien, 1995). However, Ellis and his collogues previously demonstrated that 5,6-epoxyeicosatrienoic acid may be a component of CIF and may participate in regulation of cerebral vascular tone (Rzigalinski et al., 1999). At this point, the chemical structure of CIF is obscure. Therefore, further studies will provide to us about the information of the chemical structure.

I hereby report that the highly purified HPTLC fraction obtained from Jurkat cells and *Xenopus* oocytes after the depletion of calcium stores, and *PMR1* mutant, AA542, contains an authentic CIF and its structural information. CIF might contain pyrimidine-sugar conjugate, for example CDP-sugar or UDP-sugar and it is a diffusible messenger for the activation of capacitative calcium entry and is ubiquitous in a wide variety of cells. However, the exact chemical structure of CIF remains speculative.

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