Regulation of ATP-induced calcium release in COS-7 cells by calcineurin

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Experiments were conducted to examine the role of calcineurin in regulating Ca^{2+} fluxes in mammalian cells. In COS-7 cells, increasing concentrations (1–10 μM) of ATP triggered intracellular Ca^{2+} release in a dose-dependent manner. Treatment of the cells with calcineurin inhibitors such as cyclosporin A (CsA), deltamethrin and FK506 resulted in an enhancement of ATP-induced intracellular Ca^{2+} release. Measurement of calcineurin-specific phosphatase activity *in vitro* demonstrated a high level of endogenous calcineurin activities in COS-7 cells, which was effectively inhibited by the addition of deltamethrin or CsA. The expression of constitutively active calcineurin (CnAΔCaMAI) inhibited the ATP-induced increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), in both the presence and the absence of extracellular Ca^{2+} . These results suggest that the constitutively active calcineurin prevented Ca^{2+} release from the intracellular stores. In the calcineurin-transfected cells, treatment with CsA

restored the calcineurin-mediated inhibition of intracellular Ca^{2+} release. Protein kinase C-mediated phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ receptor [$\text{Ins}(1,4,5)\text{P}_3\text{R}$] was partly inhibited by the extracts prepared from the vector-transfected cells and completely inhibited by those from cells co-transfected with CnAΔCaMAI and calcineurin B. On the addition of 10 μM CsA, the inhibited phosphorylation of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ was restored in both the vector-transfected cells and the calcineurin-transfected cells. These results show direct evidence that Ca^{2+} release through $\text{Ins}(1,4,5)\text{P}_3\text{R}$ in COS-7 cells is regulated by calcineurin-mediated dephosphorylation.

Key words: endoplasmic reticulum, inositol trisphosphate receptor, FK506-binding protein, phosphorylation, ryanodine receptor.

INTRODUCTION

In non-excitabile cells, Ca^{2+} is released from the endoplasmic reticulum (ER) in response to a stimulus-induced rise in $\text{Ins}(1,4,5)\text{P}_3$. Various stimuli such as hormones, neurotransmitters, neuropeptides and ATP cause the activation of membrane-bound phospholipase C via G-protein-coupled receptors [1–3]. The activation of phospholipase C leads to the generation of $\text{Ins}(1,4,5)\text{P}_3$ via the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$. In the signal transduction cascade, $\text{Ins}(1,4,5)\text{P}_3$ has only a single downstream target, i.e. $\text{Ins}(1,4,5)\text{P}_3$ receptor [$\text{Ins}(1,4,5)\text{P}_3\text{R}$]. The second messenger $\text{Ins}(1,4,5)\text{P}_3$ directly activates $\text{Ins}(1,4,5)\text{P}_3\text{R}/\text{Ca}^{2+}$ release channels and liberates Ca^{2+} from the ER [4,5]. Typically, the release of intracellular Ca^{2+} from the ER causes a Ca^{2+} influx from outside the cells, leading to a further increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), a mechanism called capacitative Ca^{2+} entry [6,7].

ATP serves as a transmitter modulating the function of a great variety of tissues [8,9]. It has been reported that extracellular ATP, via the plasma membrane P_2 purinergic receptor, generates $\text{Ins}(1,4,5)\text{P}_3$ and increases $[\text{Ca}^{2+}]_i$ [10]. ATP is capable of enhancing $[\text{Ca}^{2+}]_i$ in both the presence and the absence of extracellular Ca^{2+} [11]. In the presence of extracellular Ca^{2+} , an ATP-induced global increase in $[\text{Ca}^{2+}]_i$ is a result of both $\text{Ins}(1,4,5)\text{P}_3\text{R}$ -mediated Ca^{2+} release and Ca^{2+} influx by the process of capacitative Ca^{2+} entry [1,2]. The functional $\text{Ins}(1,4,5)\text{P}_3\text{R}/\text{Ca}^{2+}$ release channels exist as homotetramers [12]. Each subunit of the tetramer is associated with one molecule of FK506-binding

protein (FKBP12). When FKBP12 is dissociated from $\text{Ins}(1,4,5)\text{P}_3\text{R}$ by the immunosuppressant FK506, Ca^{2+} efflux from the ER vesicles is induced at lower concentrations of $\text{Ins}(1,4,5)\text{P}_3$ [13,14]. It therefore seems that FKBP12 could have a modulatory effect on $\text{Ins}(1,4,5)\text{P}_3\text{R}$ *in vivo*.

The phosphorylation and dephosphorylation of proteins have been recognized as prime mechanisms in the modulation of various cellular processes. Evidence has suggested that $\text{Ins}(1,4,5)\text{P}_3\text{R}$ activities could be modulated by phosphorylation and dephosphorylation. The phosphorylation of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ takes place *in vitro* at three distinct sites by protein kinase C (PKC), Ca^{2+} -calmodulin (CaM)-dependent protein kinase II (CaMKII) and cAMP-dependent protein kinase (PKA) [15], and could lead to an increase in $\text{Ins}(1,4,5)\text{P}_3\text{R}$ activities [16]. Auto-phosphorylation of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ has also been reported [17]. The putative modulatory domain of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ contains binding sites for various modulators such as Ca^{2+} [18], CaM, ATP and protein kinases [3]. By using cell extracts, it was shown that the activities of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ in rat cerebellum were enhanced when the protein phosphatase calcineurin was inhibited [16]. Calcineurin is the only known serine/threonine protein phosphatase under the control of Ca^{2+} and CaM [19]. Studies have also revealed that calcineurin is physically associated with ryanodine receptor type 2 in rat cerebellum [16] and in cardiac tissues [20]. A recent report from this laboratory demonstrated that treatment of rats with the calcineurin inhibitor cyclosporin A (CsA) altered the characteristics of ryanodine receptor (RyR)/ Ca^{2+} release channels in cardiac tissues [21].

Abbreviations used: AM, acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; CaM, calmodulin; CaMKII, calmodulin-dependent protein kinase II; CnA, calcineurin A; CnB, calcineurin B; CsA, cyclosporin A; ER, endoplasmic reticulum; FKBP12, FK506-binding protein; HA, haemagglutinin; $\text{Ins}(1,4,5)\text{P}_3\text{R}$, $\text{Ins}(1,4,5)\text{P}_3$ receptor; MAP-2, microtubule-associated protein; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; RyR , ryanodine receptor.

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It is thought that calcineurin could regulate two types of Ca^{2+} release channels via the formation of an $\text{Ins}(1,4,5)\text{P}_3\text{R}$ –FKBP12–calcineurin or RyR –FKBP12–calcineurin ternary complex [21,22]. However, the involvement of calcineurin in regulating intracellular Ca^{2+} release channels *in vivo* has not yet been demonstrated. To determine the functional interaction between calcineurin and $\text{Ins}(1,4,5)\text{P}_3\text{R}$, we have examined the effects of calcineurin inhibitors and the expression of calcineurin genes on ATP-induced Ca^{2+} release in kidney fibroblasts, COS-7 cells. Here we show that the inhibition of endogenous calcineurin by exogenously added calcineurin inhibitors enhances Ca^{2+} release in COS-7 cells, whereas the expression of Ca^{2+} -independent constitutively active calcineurin substantially decreased Ca^{2+} release *in vivo*. These results indicate that the ATP-induced release of intracellular Ca^{2+} from the ER in COS-7 cells could be inhibited by calcineurin-mediated dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3\text{R}$.

MATERIALS AND METHODS

Materials

Calcineurin, microtubule-associated protein (MAP-2), cyclophilin, ATP, fura-2 acetoxymethyl ester (AM), bovine brain CaM and the catalytic subunit of PKA were purchased from Sigma Chemicals (St Louis, MO, U.S.A.); CsA, deltamethrin and FK506 were from Calbiochem (La Jolla, CA, U.S.A.). Fluo-3 was purchased from Molecular Probes (Eugene, OR, U.S.A.). Lipofectamine reagents were purchased from Life Technologies (Gaithersburg, MD, U.S.A.). [γ - ^{32}P]ATP was from Amersham Life Science Limited (Little Chalfont, Bucks., U.K.). PKC was purchased from Promega (Madison, WI, U.S.A.).

Cell culture

COS-7 cells were maintained in an air/ CO_2 (19:1) atmosphere in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum and 100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin (Gibco BRL).

Measurement of Ca^{2+} transients by confocal microscopy

The free cytosolic Ca^{2+} concentration was monitored in COS-7 cells using the fluorescent Ca^{2+} indicator, fluo 3/AM (Molecular Probes) as described previously [23]. In brief, cells grown on glass coverslips were loaded with 10 μM fluo-3/AM at 30 °C in Krebs–Ringer solution containing 140 mM NaCl, 5 mM KCl, 0.5 mM MgCl_2 , 5.5 mM Hepes, 10 mM glucose and 2 mM CaCl_2 , pH 7.4. After 60 min, cells were rinsed with the Ringer solution to remove unhydrolysed indicator and transferred to a chamber, where drugs were added as indicated. To exclude the influence of extracellular Ca^{2+} on the release of intracellular Ca^{2+} , cells were stimulated in Ca^{2+} -free Krebs–Ringer solution, which was prepared from standard Krebs–Ringer solution by the omission of CaCl_2 and the inclusion of 100 μM EGTA. Hereafter, ' Ca^{2+} -free medium' indicates Ca^{2+} -free Krebs–Ringer solution. Deltamethrin and FK506 were dissolved in DMSO to make stock solutions of 20 and 2.4 mM respectively. CsA was dissolved in ethanol to make a 16 mM stock solution. Subsequent dilutions were made with Ca^{2+} -free medium. DMSO (0.1%) or ethanol (0.06%) at the highest final concentrations used had no effect by itself on Ca^{2+} release (results not shown). Cells were treated with the above drugs for 10 min before they were subjected to stimulation with ATP. The experiments were performed with a laser scanning confocal microscope (Leica, Heidelberg, Germany). Fluo 3 was excited with light at a wavelength of

488 nm and the emitted fluorescence was measured at a wavelength of 515 nm. Fluo 3 fluorescence was expressed as a normalized increase in fluorescence compared with the resting level (F/F_0). The fluorescence ratio F/F_0 was produced by dividing the fluorescence intensity (F) of each pixel in the original fluorescence image by its intensity at the beginning of the images (defined as F_0), during the time that the cell was assumed to be in the resting state. Data analyses were performed on a workstation with Microsoft Windows NT software.

Quantification of $[\text{Ca}^{2+}]_i$ by ratiometric fluorescence monitoring

Intracellular levels of Ca^{2+} were measured in suspensions of COS-7 cells with the fluorescent Ca^{2+} indicator fura 2/AM, as described previously [24]. In brief, cells were washed with PBS and resuspended in Krebs–Ringer solution containing 0.1% BSA and incubated with 2 μM fura 2/AM for 45 min at 30 °C. At the end of incubation, cells were diluted with an equal volume of the same buffer and centrifuged to remove the excess free dye. Cells were resuspended and stored at room temperature. Just before use, 10^6 cells were centrifuged to wash them further; they were then resuspended in the indicated medium. When experiments were conducted in Ca^{2+} -free medium, cells were washed with Ca^{2+} -free medium. The fluorescence ratio was recorded at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 510 nm with the PTI Deltascan (Photon Technology International, London, Ontario, Canada). The intensity of fluorescence was calculated automatically by computer. R_{max} (the fluorescence ratio of the Ca^{2+} -saturated sample) and R_{min} (the fluorescence ratio of the Ca^{2+} -free sample) were determined by the addition of 40 μl of 10% (v/v) Triton X-100 and 40 μl of 400 mM EGTA respectively. The $[\text{Ca}^{2+}]_i$ levels were calculated from the formula:

$$[\text{Ca}^{2+}]_i = K_d[(R - R_{\text{min}})/(R_{\text{max}} - R)](\text{Sf2/Sb2})$$

where K_d is the effective dissociation constant for the Ca^{2+} /fura 2 complex, and Sf2 and Sb2 are the fluorescence proportionality coefficients obtained at 380 nm under R_{min} and R_{max} conditions, respectively.

Calcineurin phosphatase assay

A calcineurin-specific phosphatase assay was performed with phosphorylated MAP-2 as a substrate by following the protocol as described previously [16]. In brief, 10 μg of purified MAP-2 (Sigma) was incubated in reaction buffer [50 mM Tris/HCl (pH 7.4)/100 mM NaCl/10 mM MgCl_2] containing 50 units of PKA (Sigma) and 10 μM [γ - ^{32}P]ATP at 37 °C for 1 h. Samples were warmed to 60 °C in the presence of 10% (w/v) SDS to stop the phosphorylation reactions. Phosphorylated MAP-2 (2 μg equivalent) was then incubated with or without 20 μg of COS-7 cell lysates in reaction buffer containing 5 mM dithiothreitol, 100 μM CaCl_2 and 4 μM bovine brain CaM (Sigma) at 37 °C for 30 min. To test the effect of calcineurin inhibitors, 2 μM deltamethrin or 2 μM CsA/cyclophilin was used in the dephosphorylation reactions. NaF (10 mM) was also used to abolish the phosphatase activity. Reactions were stopped by the addition of 5 $\times$ SDS/PAGE sample buffer. Samples were boiled for 3 min and analysed by SDS/PAGE [6% (w/v) gel]. Gels were stained, destained and dried for autoradiography.

Expression vectors

The cDNA species encoding rat calcineurin A (CnA α isotype [25]) and calcineurin B (CnB) [26] were gifts from Dr T. Kuno and Dr T. Soderling respectively. The calcineurin expression

vectors were prepared by subcloning into mammalian expression vector, pcDNA3 (Invitrogen, Groningen, The Netherlands) with an N-terminal haemagglutinin (HA) tag by using oligonucleotides and PCR techniques. CnA lacking the autoinhibitory domain and the CaM-binding domain (CnAΔCaMAI) were constructed by using PCR to introduce a stop codon at position 1194 of the calcineurin open reading frame, as described previously [27] and were cloned into the *EcoRI* and *XhoI* sites of HA-tagged pcDNA3 vector. The regulatory subunit CnB, encoding the complete open reading frame, was also cloned into the *NotI* and *XbaI* sites of HA-tagged pcDNA3 vector.

Transient transfection

Plasmid DNA (5 µg) was used to transfect 2×10^5 cells on each 60 mm plate with Lipofectamine reagents in accordance with the manufacturer's protocol (Life Technologies). Control cells were transfected with an equal amount of expression vector DNA only. DNA of CnAΔCaMAI (2 µg) and DNA of CnB (3 µg) were used for co-transfection. When cells were transfected with CnAΔCaMAI alone, 3 µg of vector DNA was added to maintain the ratio. For immunofluorescence experiments, COS-7 cells on coverslips were transfected with 2 µg of CnAΔCaMAI or 3 µg of CnB plasmids separately. Because immunofluorescence was conducted with anti-HA antibody and both CnAΔCaMAI and CnB were HA-tagged, the co-transfection experiments were not performed.

Western blotting

Cells were lysed in PBS containing 1% (v/v) Triton X-100 and protease inhibitors (1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin) and centrifuged at 10000 *g* for 15 min at 4 °C. The supernatant was collected to perform immunoblotting with anti-HA antibody (Jackson Immuno-research Laboratories, West Grove, PA, U.S.A.) and anti-Ins(1,4,5) P_3 R antibody (kindly provided by Dr Ilya Bezprozvanny). To check the expression of calcineurin, approx. 15–60 µg of proteins was separated by SDS/PAGE [12% (w/v) gel]; to detect Ins(1,4,5) P_3 R, 100 µg of proteins was analysed by SDS/PAGE [6% (w/v) gel]. The proteins on the gels were then transferred to nitrocellulose membrane. The nitrocellulose membranes were blocked for 2 h with 5% (w/v) non-fat dried milk in TBS-T solution [0.1% (v/v) Tween 20/20 mM Tris base/137 mM NaCl] and incubated overnight with primary antibody at 4 °C. The membranes were washed with TBS-T for 30 min with three changes, then incubated at room temperature with alkaline-phosphatase-conjugated goat anti-mouse or anti-rabbit antibodies for detecting calcineurin and Ins(1,4,5) P_3 R respectively. After 2 h the membranes were washed and immunoreactive bands were developed with Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate.

Immunofluorescence

Transiently transfected cells with truncated calcineurin A (CnAΔCaMAI)/CnB on coverslips were fixed in 4% (w/v) paraformaldehyde in PBS for 10 min and then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min. Cells were immunostained with anti-(rabbit HA) antibody in PBS and washed four times with PBS. Cells were then incubated with 1:100 dilutions of rhodamine-conjugated anti-rabbit IgG (Jackson Immuno-research Laboratories) and mounted on microscope slides with mounting solution. Immunofluorescence was analysed under a Leica DMBRE microscope equipped with a 100× objective lens and filters for epifluorescence.

Figure 1 Extracellular ATP-induced Ca²⁺ release in COS-7 cells

COS-7 cells plated on coverslips were loaded with the Ca²⁺-sensitive dye fluo 3. Coverslips were washed and transferred to a chamber containing Ca²⁺-free medium as described in the Materials and methods section. The chamber was then placed on the stage of a confocal microscope and the cells were stimulated with different concentrations (1–10 µM) of ATP [arrow in (B)]. (A) Confocal images of fluo-3-loaded cells after different durations of stimulation with ATP. (B) Representative traces of Ca²⁺ transients in individual COS-7 cells. Experiments were conducted three times.

Immunoprecipitation and phosphorylation of Ins(1,4,5) P_3 R

COS-7 cell extracts solubilized in Triton X-100 (0.1%, v/v) were used as a starting material to immunoprecipitate Ins(1,4,5) P_3 R for phosphorylation experiments. Approx. 800 µg of protein was incubated with 5 µg of anti-[rabbit Ins(1,4,5) P_3 R] antibody for 4 h at 4 °C; Protein A–Sepharose was used to precipitate the antibodies. Immunoprecipitates were washed sequentially with 0.5% (v/v) Triton X-100 in PBS, 0.5 M NaCl in PBS, and PBS alone. Protein A–Sepharose-bound Ins(1,4,5) P_3 R immunoprecipitate was used to check PKC-dependent phosphorylation.

Figure 2 Calcineurin inhibitors potentiate ATP-induced Ca^{2+} release in COS-7 cells

(A) The extracellular ATP-induced Ca^{2+} release in COS-7 cells was measured as described in the legend to Figure 1. The fluo-3-loaded cells were incubated for approx. 10 min in the absence (con) or presence (del) of 20 μM deltamethrin and monitoring of the fluorescence intensity was started (origin of the graph). The cells were stimulated with 5 μM ATP (arrow). (B, C) Quantification of ATP-induced Ca^{2+} release in COS-7 cells was performed in the absence or presence of calcineurin inhibitors by ratiometric fluorescence monitoring. A 2 ml sample of 2 μM fura-2 loaded cells (10^6) were incubated in the absence (con) or presence (del) of 20 μM deltamethrin, 10 μM CsA, 10 μM FK506 or 10 μM rapamycin (rap) for approx. 10 min and then transferred to the cuvette. The cell suspensions in the cuvette were placed in the sample

To assess the effect of endogenously as well as exogenously expressed calcineurin on $\text{Ins}(1,4,5)\text{P}_3\text{R}$ phosphorylation, vector-transfected and CnAΔCaMAI plus CnB co-transfected COS-7 cell extracts were added to the reaction mixture as follows. The Protein A–Sepharose-bound $\text{Ins}(1,4,5)\text{P}_3\text{R}$ immunoprecipitates were phosphorylated with 100 μM [$\gamma\text{-}^{32}\text{P}$]ATP in 50 mM Tris/HCl, pH 7.4, containing 100 mM NaCl, 10 mM MgCl_2 and 100 μM CaCl_2 in the absence (control) or presence of 10 units of PKC (Promega), in combination with lysates (20 μg of protein) prepared from vector-transfected and CnAΔCaMAI plus CnB co-transfected COS-7 cells at 37 °C for 30 min. The effect of calcineurin inhibitor was examined by adding CsA and cyclophilin, as indicated, to the reaction mixture. Phosphorylation reactions were stopped by adding 1% (v/v) 5×SDS/PAGE sample buffer and heating to 90 °C for 3 min. Samples were resolved on SDS/PAGE [6% (w/v) gel]. The gels were stained, destained and dried for autoradiography.

RESULTS

ATP-induced Ca^{2+} release in COS-7 cells

To detect intracellular Ca^{2+} release induced by extracellular ATP in individual COS-7 cells, Ca^{2+} imaging by confocal microscope was performed. Because ATP also caused capacitative Ca^{2+} entry in the presence of extracellular Ca^{2+} [2], Ca^{2+} -free medium was used to monitor Ca^{2+} release from intracellular stores, as described previously [1,2,11,24,28]. As shown in Figure 1, the intensity of fluorescence in fluo-3-loaded COS-7 cells remained unchanged when treated with 1 μM ATP. In response to 5 and 10 μM ATP, the fluorescence intensity increased approx. 4-fold and 7-fold respectively (Figure 1B). Representative images show a clear area of COS-7 cells with high fluorescence in response to stimulation with 5 and 10 μM ATP (Figure 1A). These results are in agreement with ATP-induced Ca^{2+} transients in other cell types [1,2,11].

Effects of calcineurin inhibitors on $[\text{Ca}^{2+}]_i$

To investigate whether calcineurin modulates the release of intracellular Ca^{2+} , ATP-induced Ca^{2+} release in COS-7 cells was measured in the absence and the presence of calcineurin inhibitors, deltamethrin and CsA [29–32]. These experiments were conducted in the absence of extracellular Ca^{2+} to examine only the release of Ca^{2+} from the ER through $\text{Ins}(1,4,5)\text{P}_3\text{R}$. As shown in Figure 2(A), the intensity of fluorescence in fluo-3-loaded cells was substantially increased in response to 5 μM ATP in the presence of 20 μM deltamethrin. A similar increase in the fluorescence intensity in response to 5 μM ATP was also found in the presence of 10 μM CsA (results not shown).

The effect of calcineurin inhibition on ATP-induced Ca^{2+} release was examined further by quantifying Ca^{2+} release in cell suspensions by ratiometric fluorescence monitoring with a PTI machine. Because 5 μM was found to be the minimum dose of ATP leading to a significant elevation of fluorescence intensity in individual COS-7 cells (Figure 1), the effect of calcineurin

compartment of a PTI Deltascan and stirred at 200 rev./min at room temperature. A dose of 5 μM ATP was added to the chamber as indicated by the arrow in (B). The alteration in fluorescence intensity was measured and quantified as described in the Materials and methods section. Representative traces of Ca^{2+} transients in response to 5 μM ATP in control (con) and deltamethrin (del)-treated cells are shown in (B). The summary data of ATP-induced intracellular Ca^{2+} release in the presence of various calcineurin inhibitors are shown in (C). *Significant difference from the control (con) at $P < 0.05$ ($n = 4$).

Figure 3 Detection of calcineurin phosphatase activities in COS-7 cells

Cells were cultured in a 100 mm dish for 3 days in the presence of 10% (v/v) fetal bovine serum in Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin as described previously. Cells were scraped off and homogenized in PBS containing protease inhibitors. The calcineurin substrate MAP-2 was phosphorylated with [γ - 32 P]ATP in the presence of PKA. Phosphorylated MAP-2 was then incubated in the absence (lane 1) or presence of COS-7 cell lysates without (lane 2) or with 2 μ M deltamethrin (lane 3), 2 μ M CsA/cyclophilin (lane 4), 10 mM NaF (lane 5) or 1 μ g of purified calcineurin (lane 6) for 30 min at 37 °C and the samples were prepared for autoradiography as described in the Materials and methods section. The arrow indicates the position of MAP-2 on the gel. The experiment was repeated four times with extracts prepared from different batches of cells.

inhibitors on ATP-induced Ca^{2+} release was examined at 5 μ M ATP. As shown in Figure 2(B), the resting $[\text{Ca}^{2+}]_i$ (mean $\pm$ S.E.M.) was 16.8 ± 0.2 nM. $[\text{Ca}^{2+}]_i$ increased to 31.4 ± 1.1 nM in response to 5 μ M ATP (Figure 2C). The ATP-induced $[\text{Ca}^{2+}]_i$ remained unchanged when the cells were pretreated with 1 μ M CsA (32.8 ± 1.0 nM), 1 μ M deltamethrin (31.5 ± 3.3 nM) or 1 μ M FK506 (32.2 ± 0.8 nM). It was significantly ($P < 0.05$) increased when the cells were pretreated with 5 μ M CsA (39.0 ± 3.6 nM), 5 μ M deltamethrin (38.5 ± 0.6 nM) or 5 μ M FK506 (40.0 ± 2.5 nM). However, the same dose of ATP further elevated the $[\text{Ca}^{2+}]_i$ to 58.0 ± 6.7 , 46.8 ± 3.0 and 54.8 ± 3.0 nM when the cells were preincubated with 10 μ M CsA, 10 μ M deltamethrin and 10 μ M FK506 respectively (Figures 2B and 2C). The magnitude of the ATP-induced Ca^{2+} release was raised to 43.2 ± 2 nM when the cells were pretreated with 10 μ M rapamycin. Figure 2(C) depicts the results obtained with highest doses of calcineurin inhibitors. The present results indicate that an inhibition of endogenous calcineurin leads to a greater ATP-induced Ca^{2+} release from the ER.

Endogenous phosphatase activity of calcineurin in COS-7 cells

We have detected a conserved 62 kDa calcineurin A and a 19 kDa CnB in COS-7 cell lysates by immunoblotting with anti-(rabbit calcineurin) antibody (results not shown). To ascertain the presence of calcineurin activity in COS-7 cells, phosphatase activity was measured with phosphorylated MAP-2 substrate. Initially, phosphorylation of MAP-2 was performed in the presence of PKA and [γ - 32 P]ATP as described previously. Phosphorylated MAP-2 (MAP-2-P) was incubated without or with cell lysates (20 μ g of protein) in the presence or absence of CsA/cyclophilin, deltamethrin or NaF. Dephosphorylation of MAP-2-P was also examined in the presence of 1 μ g of purified

Figure 4 Expression of a deletion mutant of CnA with or without CnB in COS-7 cells

(A) Diagrams of full-length CnA, truncated, constitutively active CnA (CnAΔCaMAI) and full-length CnB. Full-length CnA contains a catalytic domain, CnB and CaM-binding domains and an autoinhibitory domain (AI). (B) Total cell lysates (15 μ g) of COS-7 cells transfected with control vector alone (5 μ g; lane 1), CnAΔCaMAI (2 μ g CnAΔCaMAI plus 3 μ g of control vector; lane 2) or CnAΔCaMAI and CnB (lane 3) were separated by SDS/PAGE, transferred to nitrocellulose membrane and probed with anti-HA antibody to detect truncated CnA (48 kDa) and full-length CnB (21 kDa). Because CnB is smaller protein, it was detected separately when 60 μ g of protein of CnAΔCaMAI and CnB co-transfected cell lysates (lane 4) was resolved by SDS/PAGE [15% (w/v) gel] and the proteins were transferred to nitrocellulose membrane for 1 h for immunoblotting. The positions of molecular mass markers (lane M) are indicated (in kDa) at the left; the positions of the truncated CnA and full-length CnB are indicated by arrows. The other bands on the gel could be due to non-specific reactions with the anti-HA polyclonal antibody. (C) COS-7 cells untransfected (left panel), transfected with CnAΔCaMAI (middle panel) or transfected with CnB (right panel) were detected by immunofluorescence cytochemistry with anti-HA antibody as described in the text.

calcineurin. As shown in Figure 3, MAP-2-P (lane 1) was significantly dephosphorylated in the presence of cell lysates (lane 2); this was partly blocked by deltamethrin (lane 3), CsA (lane 4) or NaF (lane 5). The addition of purified calcineurin completely dephosphorylated MAP2-P (Figure 3, lane 6). These results indicate that COS-7 cells display a high degree of endogenous calcineurin activity that can be inhibited by the calcineurin inhibitors.

Expression of HA-tagged CnAΔCaMAI and CnB in COS-7 cells

To assess the effects of exogenous calcineurin on Ca^{2+} release from the ER, we constructed an HA-tagged constitutively active form of CnA (HA-CnAΔCaMAI [27,33]) and its regulatory subunit, CnB [34,35], as schematically presented in Figure 4(A). Immunoblot analysis of COS-7 cell lysates (Figure 4B) with anti-HA antibody revealed the expression of a 48 kDa CnACaMAI in the cells transfected with CnACaMAI (lane 2) and co-transfected

Figure 5 Expression of constitutively active calcineurin suppresses ATP-induced Ca^{2+} release from the ER

COS-7 cells transfected for 48 h were loaded with $2 \mu M$ fura 2/AM for 45 min at $30^\circ C$. After washing, a sample of 10^6 cells was transferred to the cuvette for measurement of fluorescence as described earlier. (A) Representative traces of Ca^{2+} transients in the cells transfected with vector alone (vector) or vector plus CnAΔCaMAI (CnA) after stimulation with $10 \mu M$ ATP (arrow) are shown. (B) Average values are shown of the resting Ca^{2+} levels of the vector-transfected cells before ATP stimulation (basal) and the maximum levels of $[Ca^{2+}]_i$ due to stimulation with $10 \mu M$ ATP in the cells transfected with vector alone (vector), vector plus CnAΔCaMAI (CnA) or CnAΔCaMAI plus CnB (CnAB) in Ca^{2+} -free medium. To see the effects of CsA, the co-transfected cells (CnAB) were pretreated with $10 \mu M$ CsA (CnABCsA) for 10 min.

with CnB (lanes 3 and 4). In contrast, COS-7 cell lysates transfected with vector alone did not show the 48 kDa band (Figure 4B, lane 1). Because CnB is a low-molecular-mass (19 kDa) protein, the expression of HA-tagged CnB was detected in a separate gel when an excess amount of the sample was loaded (Figure 4B, lane 4). The 34 kDa band present in the cell lysates seems to be a non-specific band. The successful expression of HA-tagged CnAΔCaMAI and CnB in COS-7 cells was also confirmed by the immunological localization of cells with anti-HA antibody (Figure 4C).

Effects of constitutively active calcineurin on the release of Ca^{2+} from the ER

To examine the effects of Ca^{2+} -independent constitutively active calcineurin on intracellular Ca^{2+} release from the ER, ATP-induced Ca^{2+} release was measured in transfected cells in Ca^{2+} -free medium. The profile of Ca^{2+} transients shows a decrease in ATP-induced Ca^{2+} release in CnAΔCaMAI (Figure 5A), or CnAΔCaMAI and CnB co-transfected cells (results not shown) in comparison with the vector-transfected cells. As shown in Figure 5(B), the stimulation of vector-transfected cells with $10 \mu M$ ATP evoked a sharp rise in $[Ca^{2+}]_i$ to 88 ± 3.33 nM. The peak level of $[Ca^{2+}]_i$ was significantly ($P < 0.05$) decreased to 54.8 ± 2.1 and 42.8 ± 19.3 nM in the cells transfected with CnAΔCaMAI and co-transfected with CnAΔCaMAI and CnB respectively. On treatment with $10 \mu M$ CsA, ATP-induced intracellular Ca^{2+} release was elevated to 71.33 ± 0.9 nM in CnAΔCaMAI and CnB co-transfected cells (Figure 5B). These results indicate that the expression of constitutively active calcineurin mutant inhibits intracellular Ca^{2+} release from the ER.

The effect of expression of constitutively active calcineurin on the ATP-stimulated increase in $[Ca^{2+}]_i$ was also measured in cell suspensions in the presence of extracellular Ca^{2+} . Figure 5(C) shows a similar trend of inhibition of ATP-induced $[Ca^{2+}]_i$ transients in cells transfected with CnAΔCaMAI or co-transfected with CnAΔCaMAI and CnB.

Phosphorylation of $Ins(1,4,5)P_3R$

It is well known that $Ins(1,4,5)P_3R$ is the only single downstream effector of $Ins(1,4,5)P_3$ that is produced in response to the extracellular application of ATP [4,5]. Various studies have indicated that the activities of $Ins(1,4,5)P_3R$ could be modulated by phosphorylation and dephosphorylation processes *in vivo* [13–18]. To examine whether the expression of constitutively active calcineurin effected the PKC-mediated phosphorylation of $Ins(1,4,5)P_3R$ in COS-7 cells, a $Ins(1,4,5)P_3R$ phosphorylation assay was conducted *in vitro*. Triton X-100-solubilized cell lysates were immunoprecipitated with anti-[rabbit $Ins(1,4,5)P_3R$] antibody and phosphorylation of $Ins(1,4,5)P_3R$ was conducted in the presence of PKC as described in the Materials and methods section. In the absence of PKC, phosphorylation of $Ins(1,4,5)P_3R$ was negligible (Figure 6A, lane 1). In contrast, the addition of PKC led to the substantial phosphorylation of $Ins(1,4,5)P_3R$ (Figure 6A, lane 2). The phosphorylation of $Ins(1,4,5)P_3R$ was partly and completely inhibited when COS-7 cell lysates prepared from vector-transfected (Figure 6A, lane 3) and calcineurin co-transfected (lane 4) cells respectively were present in the reaction mixture. The ability of COS-7 cell lysates to prevent $Ins(1,4,5)P_3R$

(C) The same experiment was conducted in the presence of 2 mM extracellular Ca^{2+} . *Significantly different at $P < 0.05$ ($n = 3$) from vector-transfected cells; **significantly different at $P < 0.01$ ($n = 3$) from co-transfected cells without CsA.

Figure 6 Effects of constitutively active calcineurin on phosphorylation and expression of Ins(1,4,5)P₃R

(A) Immunoprecipitation of Ins(1,4,5)P₃R was conducted with anti-[rabbit Ins(1,4,5)P₃R] antibody and the phosphorylation of immunocomplex was performed as described in the Materials and methods section. Phosphorylated Ins(1,4,5)P₃R was detected by autoradiography. Lane 1, immunocomplex without PKC; lane 2, immunocomplex with PKC; lane 3, immunocomplex with PKC in the presence of crude extracts prepared from vector-transfected cells; lane 4, immunocomplex plus PKC in the presence of crude lysates prepared from CnAΔCaMAI and CnB co-transfected cells; lane 5, as lane 3 but in the presence of 2 μM CsA/cyclophilin; lane 6, as lane 4 but with 2 μM CsA/cyclophilin; lane 7, as lane 3 but in the presence of 10 μM CsA/cyclophilin; lane 8, as lane 4 but in the presence of 10 μM CsA/cyclophilin. Phosphorylation of Ins(1,4,5)P₃R was repeated with the cells prepared from three different transfection experiments. (B) Western blot analysis of extracts prepared from untransfected COS-7 cells (lane 1) or COS-7 cells transfected with vector alone (lane 2), vector plus CnAΔCaMAI (lane 3) or CnAΔCaMAI and CnB co-transfected (lane 4) by using anti-[rabbit Ins(1,4,5)P₃R] antibody. The arrow indicates the position of Ins(1,4,5)P₃R.

phosphorylation was blocked when it was pretreated with 2 μM CsA/cyclophilin (Figure 6A, lane 5). However, 2 μM CsA/cyclophilin failed to restore completely the phosphorylation of Ins(1,4,5)P₃R by lysates prepared from CnAΔCaMAI and CnB co-transfected cells (Figure 6A, lane 6). When the vector or CnAΔCaMAI and CnB co-transfected cell lysates were pretreated with 10 μM CsA/cyclophilin, phosphorylation of Ins(1,4,5)P₃R immunoprecipitate was significantly restored (Figure 6A, lanes 7 and 8). Thus it seems that co-transfection with CnAΔCaMAI and CnB resulted in the highest level of phosphatase activity in COS-7 cells. These results show directly that the inhibition of endogenous calcineurin activity enhances phosphorylation of Ins(1,4,5)P₃R [16].

Effect of expression of calcineurin on the level of Ins(1,4,5)P₃R expression in COS-7 cells

It has been recently demonstrated that the inhibition of calcineurin by FK506 blocked Ins(1,4,5)P₃R expression in cerebellar granule cells [36], suggesting another role for calcineurin in regulating the activity Ins(1,4,5)P₃R. To examine whether the overexpression of calcineurin in COS-7 cells influences the expression of Ins(1,4,5)P₃R, an immunoblot analysis was performed with the untransfected and transfected cell extracts. As shown in Figure 6(B), the expression level of Ins(1,4,5)P₃R remained unchanged in the cells transfected with the vector, CnAΔCaMAI, or co-transfected with CnB, suggesting that the inhibitory effect of calcineurin expression on cytoplasmic Ca²⁺ was not due to the altered levels of expression of Ins(1,4,5)P₃R.

DISCUSSION

Several reports have demonstrated that calcineurin has an important role in the transduction of Ca²⁺ signals to regulate various cellular processes [37–40]. For instance, the calcineurin-

regulated growth-factor-induced transcription of several genes is crucial for T-cell activation [29,30]. Calcineurin also functions in Ca²⁺-activated cell death in mammalian cells [41], in hippocampal long-term depression [42] and in the migration of neutrophils [43]. Studies *in vitro* have suggested that calcineurin could regulate the activity of Ins(1,4,5)P₃R by dephosphorylation of the phosphorylated receptors in rat cerebellum [16]. The present studies were aimed at examining the physiological role of calcineurin in regulating ATP-induced Ca²⁺ release through Ins(1,4,5)P₃R in mammalian cells. Our results demonstrate that intracellular Ca²⁺ release through Ins(1,4,5)P₃R induced by ATP is enhanced by the inhibition of endogenous calcineurin and is inhibited by the expression of constitutively active calcineurin *in vivo*.

Extracellular ATP evokes many physiological effects such as platelet aggregation, inflammation and muscle contraction in numerous cell types [10]. These various effects of ATP are mediated via plasma membrane P₂ purinergic receptors [44]. As an initial event, the stimulation of purinergic receptor by ATP causes an increase in [Ca²⁺]_i by the generation of Ins(1,4,5)P₃ via the phospholipase C β pathway [10]. The ATP-induced increase in [Ca²⁺]_i is therefore considered a direct effect of Ins(1,4,5)P₃R activation. Here we have shown that exogenous ATP resulted in a Ca²⁺ release in COS-7 cells in a dose-dependent manner in the absence of extracellular Ca²⁺ (Figures 1A and 1B). The COS-7 cells show a tendency to a larger Ca²⁺ release in response to ATP when endogenous calcineurin is inhibited by calcineurin inhibitors (Figure 2). Because confocal experiments produce only relative intensities of fluorescence, the quantification of Ca²⁺ transients was attempted by ratiometric fluorescence monitoring (Figures 2B and 2C). The tendency to a larger Ca²⁺ release from the internal stores by the calcineurin inhibitors is clearly seen (Figures 2B and 2C) as in the experiments with the confocal microscope (Figure 2A).

The lower effectiveness of rapamycin in stimulating ATP-induced Ca²⁺ release (Figure 2C) could be due to the fact that rapamycin is not an inhibitor of calcineurin but disrupts the interaction between FKBP12 and Ins(1,4,5)P₃R. Earlier studies revealed that the dissociation of FKBP12 from Ins(1,4,5)P₃R resulted in Ca²⁺ leakage and an increase in sensitivity to Ins(1,4,5)P₃ [45]. Owing to the partial depletion of stored Ca²⁺ by the removal of FKBP12 from Ins(1,4,5)P₃R, the net increase in [Ca²⁺]_i owing to the high dose of ATP (100 μM) was less when cow tracheal epithelial cells were treated with 10 μM FK506 [46]. However, in our studies, no significant difference in the amount of Ca²⁺ efflux was observed when FK506-treated COS-7 cells were stimulated with 100 μM ATP in comparison with the control (results not shown). Thus the dissociation of FKBP12 from Ins(1,4,5)P₃R might not affect the resting level of the ER luminal [Ca²⁺] in COS-7 cells. It is likely that FKBP12 modulates stimulus-induced Ca²⁺ efflux from internal stores, preventing cytosol from flooding with excess Ca²⁺. Because calcineurin is a ubiquitous protein, endogenous phosphatase activity of COS-7 cells is found to be substantially high but severely inhibited by calcineurin inhibitors (Figures 3 and 6A). We propose that calcineurin phosphatase activities functionally regulate Ins(1,4,5)P₃R and thereby prevent ER from emptying Ca²⁺ storage *in vivo*.

Although COS-7 cells have substantially high intrinsic calcineurin phosphatase activity (Figure 3) and thus the role of calcineurin can be deduced by treatments with calcineurin inhibitors, the evidence still remains indirect. We therefore directly tested the hypothesis that calcineurin regulates Ca²⁺ release in mammalian cells by transfecting COS-7 cells with constitutively active calcineurin mutant (CnAΔCaMAI). The

experiments in the Ca^{2+} -free medium were necessary to eliminate the Ca^{2+} influx pathways such as capacitative Ca^{2+} entry in COS-7 cells (Figure 5). In agreement with the results shown in Figure 2, transfection of COS-7 cells with the truncated CnA Δ CaMAI led to a significant decrease in Ca^{2+} release (Figure 5A). Co-transfection of CnA Δ CaMAI with CnB seems to decrease the Ca^{2+} release further (Figure 5B), in agreement with increased activity of CnA Δ CaMAI in the presence of CnB [26,27,41]. These results could provide direct evidence that the constitutively active calcineurin inhibits Ca^{2+} transients in COS-7 cells. It is interesting to note that the decrease in intracellular Ca^{2+} transients by transfection with CnA Δ CaMAI and CnB was restored significantly ($P < 0.01$) by $10 \mu\text{M}$ CsA. The incomplete restoration of the intracellular Ca^{2+} transients by $10 \mu\text{M}$ CsA could be due to the decreased sensitivity of constitutively active calcineurin mutant to CsA [33]. In the presence of external Ca^{2+} , the peak $[\text{Ca}^{2+}]$ induced by $10 \mu\text{M}$ ATP in the presence of external Ca^{2+} was approx. 3-fold that measured in the absence of external Ca^{2+} (Figure 5C). The general tendencies to inhibition of Ca^{2+} release by transfection, and recovery by CsA, were similar between the two types of experiments (Figures 5B and 5C), suggesting that the influx of Ca^{2+} through the plasma membrane is roughly proportional to the amount of Ca^{2+} released from the internal stores.

Under physiological conditions, endogenous calcineurin could be spontaneously inhibited to maintain Ca^{2+} homeostasis by its autoinhibitory domain [26,47] but the exogenous truncated form of CnA (CnA Δ CaMAI) is constitutively active when expressed in mammalian cells [27,33]. Expression of this mutant alone or in combination with the regulatory subunit, CnB, is expected to ensure a sustained level of phosphatase activity *in vivo* [30,33]. Thus constitutively active CnA might dephosphorylate various substrates in COS-7 cells. It is important to note that the treatment of CnA Δ CaMAI and CnB co-transfected cells with CsA led to a substantial recovery from the inhibition by the constitutively active CnA (Figures 5B and 5C). This result indicates that the inhibition of ATP-induced Ca^{2+} release in the cells transfected with constitutively active CnA was due not to any non-specific or permanent changes in the cells but to dephosphorylation of one or more components directly associated with the ATP-induced Ca^{2+} release process. In view of the well-defined ATP-induced Ca^{2+} release process *in vivo* [1,2,8–11] or in studies *in vitro* [16,22], we examined Ins(1,4,5) P_3 R as a primary target for the regulation by calcineurin. In agreement with the earlier observations [16,22], the phosphorylation of Ins(1,4,5) P_3 R was found to be inhibited by the extracts prepared from the cells transfected with CnA Δ CaMAI and CnB (Figure 6A). The results suggest that as a result of decreased phosphorylation (Figure 6A), the functional Ins(1,4,5) P_3 R could be decreased.

The degree of inhibition by the constitutively active mutant of calcineurin (50–60%) was similar to the 55–60% transfection efficiency of COS-7 cells as judged by β -galactosidase staining of the cells transfected with the *LacZ* expression vector (results not shown). These results suggest that in most of the transfected cells the Ca^{2+} release from the ER was completely blocked. Though some reports have already suggested the involvement of phosphorylation–dephosphorylation processes in Ca^{2+} fluxes [16,48–52], our results specifically show a link between calcineurin and ligand-dependent intracellular Ca^{2+} release *in vivo*. We suggest that Ca^{2+} -activated calcineurin is important in the regulation of intracellular Ca^{2+} release. This might create a feedback inhibitory system to terminate the Ca^{2+} efflux *in vivo*. In light of the evidence that FKBP12 bridges calcineurin with Ins(1,4,5) P_3 R or RyR [20,22], further studies are necessary for an

understanding of the functional role of FKBP12 in the calcineurin-mediated dephosphorylation of Ins(1,4,5) P_3 R.

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