An Ins(1,4,5)P₃ receptor in *Paramecium* is associated with the osmoregulatory system

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Summary

In the ciliate *Paramecium*, a variety of well characterized processes are regulated by Ca²⁺, e.g. exocytosis, endocytosis and ciliary beat. Therefore, among protozoa, *Paramecium* is considered a model organism for Ca²⁺ signaling, although the molecular identity of the channels responsible for the Ca²⁺ signals remains largely unknown. We have cloned – for the first time in a protozoan – the full sequence of Ca²⁺ release channels of the inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) receptor from *Paramecium tetraurelia* cells showing molecular characteristics of higher eukaryotic cells. The homologously expressed Ins(1,4,5)P₃-binding domain binds [³H]Ins(1,4,5)P₃, whereas antibodies unexpectedly localize this protein to the osmoregulatory system. The level of Ins(1,4,5)P₃-receptor expression was reduced, as shown on a transcriptional level and by immuno-staining, by decreasing the concentration of extracellular Ca²⁺ (*Paramecium* cells rapidly adjust their Ca²⁺ level to that in the outside medium). Fluorochromes reveal spontaneous fluctuations in cytosolic Ca²⁺ levels along the osmoregulatory system and these signals change upon activation of caged Ins(1,4,5)P₃. Considering the ongoing expulsion of substantial amounts of Ca²⁺ by the osmoregulatory system, we propose here that Ins(1,4,5)P₃ receptors serve a new function, i.e. a latent, graded reflux of Ca²⁺ to fine-tune [Ca²⁺] homeostasis.

Introduction

Increases in the concentration of intracellular Ca²⁺, [Ca²⁺]ᵢ, govern a variety of processes in response to cell stimulation, such as exocytosis and cell contraction. A rise in intracellular Ca²⁺ may be due to Ca²⁺ influx from the outside medium or the activation of stores, such as the endoplasmic or sarcoplasmic reticulum (ER or SR). Stores may comprise Ca²⁺-release channels of the inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] or ryanodine receptor (RyR) type (Berridge et al., 2000). Any latent, i.e. non-stimulated, activity of intracellular stores, and any involvement of such channels and their potential contribution to overall cell function, would be much less amenable to analysis than stimulated responses and, therefore, has so far not been described. We have now found evidence that such problems may occur in the osmoregulatory system (ORS) of *Paramecium*. We unexpectedly found that Ins(1,4,5)P₃ receptors (Ins(1,4,5)P₃;R) are present in the ORS of *Paramecium* and that regulation of their expression depends on the Ca²⁺ concentration in the outside medium, [Ca²⁺]ₒ.

*Paramecium* cells possess a vast system of cortical Ca²⁺-storage compartments, the alveolar sacs (Stelly et al., 1991; Knoll et al., 1993), which are insensitive to Ins(1,4,5)P₃ (Laenge et al., 1995). The alveolar sacs contain Ca²⁺ in concentrations similar to those in skeletal muscle SR and are activated selectively during stimulated exocytosis of dense-core vesicles, which is induced by store-operated Ca²⁺ influx (reviewed in Plattner and Klauke, 2001). In the cell membrane, different types of Ca²⁺ channels have been characterized electrophysiologically (Machemer, 1988) and a Ca²⁺-pump typical of the plasmalemma has been found (Wright and Van Houten, 1990). The vast ER system present throughout the cell has rather low levels of Ca²⁺ and contains a high-capacity, low-affinity Ca²⁺-binding protein which differs from that in the alveolar sacs (Plattner and Klauke, 2001). An SR Ca²⁺-ATPase (SERCA)-type Ca²⁺-ATPase is delivered from the ER to the alveolar sacs, where it is heavily enriched (Hauser et al., 1998; Kissmehl et al., 1998). Both the plasmalemmal as well as the SERCA-type Ca²⁺-pump have low activity (Plattner and Klauke, 2001).

All this is in contrast to results from ⁴⁵Ca²⁺-flux studies in *Paramecium* cells, which revealed considerable basal influx rates without any stimulation (Browning and Nelson, 1976; Kerboeuf and Cohen, 1990; Knoll et al., 1992). In these reports, an important component for the regulation of homeostasis of intracellular Ca²⁺ concentration [Ca²⁺]ᵢ in *Paramecium* cells was still undiscovred. For the following reasons, we assume that the ORS is involved in precisely such activities. Recently, the use of ion-selective electrodes revealed high Ca²⁺ levels in the fluid of the ORS (Stock et al., 2002a; Stock et al., 2002b). The ORS generally consists of two identical units per cell, each composed of a contractile vacuole, with approximately six collecting canals to which a tubular membraneous network is attached (reviewed in Allen and Naitoh, 2002). This network displays a part proximal to the collecting canals that has smooth membranes (smooth spongiome) and a distal part that is studded with V-type H⁺-
ATPase molecules (decorated spongeine). Since the vacuole fluid is expelled by rhythmic vacuole activity, this implies a major contribution of the ORS to \[\text{Ca}^{2+}\] homeostasis in Paramecium cells. The system operates by a \([\text{H}^+]\) gradient, which is formed by the \(\text{H}^+-\text{ATPase} \) (Allen et al., 1990; Allen, 1995; Fok et al., 1995; Tominaga et al., 1998; Wassmer et al., 2005) and which might be coupled not only to the well-established osmotically driven water influx (Grønlien et al., 2002) but possibly also to a hypothetical cation-exchange system (Stock et al., 2002a; Stock et al., 2002b). In the absence of a \(\text{Ca}^{2+}\)-pump, \(\text{Ca}^{2+}\) might, thus, be transported into the ORS. Nevertheless, considering its excretory function, our current finding of \(\text{Ca}^{2+}\)-release channels in ORS membranes was rather surprising.

We here give the first thorough analysis of the gene encoding the Ins(1,4,5)\(P_3\)R in a protozoan. This was possible by having access to partial genomic sequences obtained by an international Paramecium genome project (Dessen et al., 2001; Sperling et al., 2002), based on a genomic library (Keller and Cohen, 2000). The derived protein structure shows characteristics of an Ins(1,4,5)\(P_3\)R and we named the protein \(\text{PtIP}_3\text{R}_\text{N}\). Antibody (Ab) labeling shows specific localization of \(\text{PtIP}_3\text{R}_\text{N}\) in the ORS and, moreover, when \([\text{Ca}^{2+}]\) is reduced, transcription of \(\text{PtIP}_3\text{R}_\text{N}\) is downregulated. We propose a role of \(\text{PtIP}_3\text{R}_\text{N}\) in the homeostasis of cytosolic \([\text{Ca}^{2+}]\), based on spontaneous \([\text{Ca}^{2+}]\) fluctuations seen along the ORS and the effect – although variable – uncaging Ins(1,4,5)\(P_3\) has on these fluctuations. This putative function is new and might be considered the cellular equivalent of kidney function on a systemic level.

Results
Cloning of the gene encoding \(\text{PtIP}_3\text{R}_\text{N}\)
A partial sequence resembling that of the Ins(1,4,5)\(P_3\) receptor, M24E11u(rc), was isolated in a pilot genome project of Paramecium (Dessen et al., 2001; Sperling et al., 2002). In order to clone the gene, we screened an indexed genomic library (Keller and Cohen, 2000) in the laboratory of Jean Cohen (CNRS, Gif-sur-Yvette, France). By using probes designed from sequences of M24E11u(rc), four positive clones (28c10, 55d24, 113e9, 118e7) were identified and sequence analysis was extended to the original clone M24E11u(rc) by covering the 5' region of the gene and reaching in the 3' region up to bp 5171. A further extension of 1558 bp was done by primer walking using a genomic \(\lambda\)ZAPII library of \(P.\) tetraurelia (Hauser et al., 1998). Based on a current Paramecium genome project initiated by the Groupement de Recherches Européen (GDRE) and coordinated by Jean Cohen and Linda Sperling (CNRS, Gif-sur-Yvette, France) in collaboration with the Genoscope (Evry, France), sequences of the whole gene including flanking regions were obtained (Fig. 1A). cDNA sequences of the entire gene were amplified and cloned, revealing that the gene is expressed. Sequence analysis resulted in an open reading frame of 8670 bp coding for a protein of 2890 aa and a calculated molecular mass of 321 kDa. A comparison of the genomic sequence with their cDNA equivalent revealed six introns of 22 bp, 23 bp, 25 bp, 27 bp, 28 bp and 29 bp (Fig. 1A), a length typical for Paramecium (Russell et al., 1994; Sperling et al., 2002). The gene was named \(\text{IP}_3\text{R}_\text{N}\) (accession number CR932323).

Molecular structure of \(\text{IP}_3\text{R}_\text{N}\)
Ins(1,4,5)\(P_3\) receptors are composed of an N-terminal ligand-binding domain, a central modulatory domain and a C-terminal channel domain with six membrane-spanning helices (reviewed in Bezprozvanny, 2005). The \(\text{IP}_3\text{R}_\text{N}\) protein possesses these size and topology characteristics. Deducing the amino acid sequence from \(\text{IP}_3\text{R}_\text{N}\) and performing a BLAST search using the NCBI database, the protein showed strongest similarity to the Ins(1,4,5)\(P_3\) receptor type 3 (R3) from rat (Fig. 2) throughout its length, with an overall identity of 19% and similarity of 34% (Fig. 1B). Alignment and comparison of \(\text{IP}_3\text{R}_\text{N}\) with metazoan Ins(1,4,5)\(P_3\) receptor sequences (Fig. 1E) show a relatively low degree of conservation of the Paramecium sequence in contrast to the close relationship of these proteins among metazoans.

The N-terminus of Ins(1,4,5)\(P_3\) receptors (residues 224 to 579 of the mouse Ins(1,4,5)\(P_3\)R)1) contains the crucial region for Ins(1,4,5)\(P_3\) binding (Yoshikawa et al., 1996). Using the NCBI database, BLAST analysis of the corresponding region of the \(\text{IP}_3\text{R}_\text{N}\) gene product found 39% sequence identity and 49% similarity compared with rat Ins(1,4,5)\(P_3\)R3 (Fig. 1B). Of ten residues forming a basic pocket that interacts with the negatively charged phosphate groups of Ins(1,4,5)\(P_3\) four are conserved in Paramecium (Fig. 2), including the three residues essential for specific binding in type 1 Ins(1,4,5)\(P_3\) receptors (Yoshikawa et al., 1996). To get more evidence of whether this domain is able to bind Ins(1,4,5)\(P_3\) we used the SWISS MODELL server for a 3D-alignment of the region with the published crystal structure of the mouse \(\text{R1 Ins(1,4,5)P}_3\)-binding domain (Bosanac et al., 2002). The model of the Paramecium Ins(1,4,5)\(P_3\)-binding domain (Fig. 1C) shows some differences from that of the mouse receptor structure but the central core appears quite similar.

Furthermore, a second domain, the RyR- and Ins(1,4,5)\(P_3\)-homology (RHH) domain, was described that may provide a binding site for Ins(1,4,5)\(P_3\) (Pointing, 2000). The RHH domain is found in the RyR and the Ins(1,4,5)\(P_3\R and spans a region of 203 residues, starting in Ins(1,4,5)\(P_3\R approximately at residue 1200. The conserved-domain database of the NCBI shows that this domain is also found in the Ins(1,4,5)\(P_3\R sequence of Paramecium starting at residue 1313.

The channel domain of \(\text{PtIP}_3\text{R}_\text{N}\) (residues 2447-2733) shares closest homology to type 3 RyRs. This is interesting, regarding the hypothesis that these channels represent a kind of hybrid between RyRs and Ins(1,4,5)\(P_3\)Rs, as postulated for intracellular \(\text{Ca}^{2+}\)-release channels in the related unicellular parasite Toxoplasma gondii (Lovett et al., 2002). We found that the close relationship of the channel domain in \(\text{IP}_3\text{R}_\text{N}\) and RyRs might be due to a loop between transmembrane region 5 and the pore region present in the rat Ins(1,4,5)\(P_3\R3 (residues V2398-A2453), which is missing in the Paramecium sequence as well as in RyRs. Moreover, analysis of the deduced amino acid sequence and hydrophobicity analysis (Fig. 1D) shows that the C-terminus of \(\text{IP}_3\text{R}_\text{N}\) contains six membrane-spanning helices that have the pore region lying between transmembrane domain 5 and 6, which is characteristic of all Ins(1,4,5)\(P_3\) channels. There is also a high degree of sequence identity (up to 50%) in the transmembrane regions 5 and 6 and in the pore-forming region (Fig. 2).

On the basis of the overall size and topology, we propose that the \(\text{IP}_3\text{R}_\text{N}\) is related to intracellular \(\text{Ca}^{2+}\)-channels of the
An Ins(1,4,5)P₃ receptor in *Paramecium* 3707

**Fig. 1.** Molecular characterization of *PtIP₃RN*. (A) Schematic representation of macronuclear sequences of the *PtIP₃RN* gene from *P. tetraurelia* (*Pt*): The *PtIP₃RN* gene is flanked upstream by a gene (*PtHKG*) homologous to hemoglobin of *Paramecium tetraurelia* (Yamauchi et al., 1995), accession number S60032, and downstream by a gene (*PtSNF1 PK*) homologous to a putative SNF1-related protein kinase [Zagulski et al., 2004], accession No YP_054292]. Start (+1) and stop codons (+8825) of *IP₃RN* were determined by RT-PCR, likewise introns, which are shown as triangles. The positions of the introns are indicated at nucleotide level at 5′ (intron 5′) and 3′ end (intron 3′). (B) Domain structure of the *Paramecium* Ins(1,4,5)P₃ receptor *IP₃RN*. Results of sequence analysis of single domains are summarized in the table. (C) Modeling of the Ins(1,4,5)P₃-binding domain using the Swiss-Model homology-modeling server (Peitsch and Jongeneel, 1993). (Right) Published structure of the Ins(1,4,5)P₃-binding region of mouse Ins(1,4,5)P₃ receptor type 1 (Bosac et al., 2002); (left) model of the *Paramecium* Ins(1,4,5)P₃-binding region. (D) Hydrophobicity analysis of the channel domain of *IP₃RN* reveals six transmembrane regions. (E) Evolutionary relationship of the *Paramecium* Ins(1,4,5)P₃ protein. Predictions from multiple sequence alignments are shown in a neighbor-joining tree with 1000 bootstrap replicates generated with the MEGA version 3.0 program. Sequences representing the three different types of mammalian Ins(1,4,5)P₃ receptors were from *Mus musculus* (*MmIP₃R* type 1, P11881), *Homo sapiens* (*HsIP₃R* type 2, Q14571) and *Rattus norvegicus* (*RnIP₃R* type 3, AAA44146). Other metazoan *IP₃R* sequences were from *Aplysia californica* (AcIP₃R, AB626080), *Osopleura dioica* (OcIP₃R, AAT47836), *Caenorhabditis elegans* (CeIP₃R1-Ifa, AA306668), *Xenopus laevis* (XIP₃R, BAA03304), *Drosophila melanogaster* (DmIP₃R, BAA14399), *Panulirus argus* (PaIP₃R, AAC61691) and *Asterina pectinifera* (ApIP₃R, BAB84088). Bootstrap support values are given above the branches and evolutionary distances are indicated by the scale bar below.

Determination of Ins(1,4,5)P₃ binding to the putative Ins(1,4,5)P₃-binding domain of *IP₃RN*

Because the putative Ins(1,4,5)P₃-binding domain of *IP₃RN* is less conserved than in other Ins(1,4,5)P₃ receptors of metazoans, we examined Ins(1,4,5)P₃ binding to this domain. To avoid mutating all deviant 23 *Paramecium* glutamine codons to universal glutamine codons, this region was expressed directly in *Paramecium*. Therefore, we constructed a GFP-fusion protein, in which GFP was fused to the C-terminus of residues T267-L657 of *IP₃RN*, using the pPXV-GFP vector. The fusion construct or the GFP-vector alone (control) were microinjected into the macronucleus of *Paramecium* cells and overexpression was monitored by GFP-fluorescence (Fig. 3A). As expected, overexpression of GFP alone leads to a fluorescent signal throughout the cell, including the macronucleus; overexpression of GFP-IP₃BD leads to a fluorescent signal only in the cytosol, in agreement with the calculated mass (~72 kDa) of GFP-IP₃BD. Transformed cell clones were propagated and purification of GFP and GFP-IP₃BD proteins was performed by immunoprecipitation using Abs against GFP. To confirm a successful precipitation, one-third of precipitated proteins were analyzed by immuno-blotting (Fig. 3B). As shown in Fig. 4B, GFP-specific Abs efficiently precipitated the recombinant proteins GFP and GFP-IP₃BD (Fig. 3B, lanes 2 and 4), whereas control IgGs did not (Fig. 3B, lanes 1 and 3).

By using GFP- or GFP-IP₃BD-coupled protein-A agarose beads, [³H]Ins(1,4,5)P₃ binding experiments were carried out (Fig. 3C). We found a two- to threefold enrichment of [³H]Ins(1,4,5)P₃ bound to GFP-IP₃BD beads compared with GFP-coupled beads. The binding specificity of GFP-IP₃BD beads has been shown by competition with 10 μM non-radiolabeled Ins(1,4,5)P₃, which reduced [³H]Ins(1,4,5)P₃ binding close to background level (Fig. 3C right panel).

**Immunofluorescence localization and western blots**

To analyze the subcellular distribution of *IP₃RN*, we raised a polyclonal antiserum to a recombinant polypeptide corresponding to *IP₃RN* residues R896-Q1001 (Fig. 2). As shown in Fig. 4A, affinity-purified anti-
Fig. 2. Sequence analysis of IP₃RN. Alignment of PtIP₃RN and rat Ins(1,4,5)P₃R₃ (RnIP₃R₃) using the ‘Blast 2 Sequences’ tool (Tatusova and Madden, 1999). Sequences are shown in single-letter code and are numbered on the left side. Residues that are identical are shaded black, similar residues are shaded gray. The putative binding region for Ins(1,4,5)P₃ is boxed in yellow. Amino acids shaded blue are involved in Ins(1,4,5)P₃-binding, those bordered in dark blue are essential for Ins(1,4,5)P₃-binding (Yoshikawa et al., 1996). The homology domain for Ins(1,4,5)P₃Rs and RyRs (RIH-domain) is boxed in green, the channel domain in light blue. The six transmembrane regions are highlighted in red, the pore region in yellow. The antigenic region used to raise a polyclonal Ab is boxed in gray. Rat Ins(1,4,5)P₃R₃ sequences (accession number L06096) are published by Blondel et al. (Blondel et al., 1993).
IP₃RN Abs recognize the polypeptide with high affinity in western blots. To ensure a specific interaction with IP₃RN, the same Abs were used to investigate insoluble fractions (100,000-g pellet) of whole-cell homogenates. In immunoblots anti-IP₃RN-Abs recognize a high-molecular-mass band of ~250 kDa (Fig. 4B). An additional band of 37 kDa is probably a degradation product of IP₃RN, because the ratio of the two bands changes depending on the protease inhibitor concentration applied during preparation (data not shown). The detected proteins were completely extracted when 100,000-g pellets were treated with 1.5% (data not shown) or 2% Triton X-100 (Fig. 4B) as usual for membrane proteins like Ins(1,4,5)P₃ (Wassmer et al., 2006) is not influenced (Fig. 6A) under these conditions. Since an [Ca²⁺]ₒ at 1 μM is the limiting concentration for our cells to survive, we examined whether the ORS activity is affected when cells were incubated at varying [Ca²⁺]ₒ. However, this does not effect the pumping activity of the ORS, because no significant differences in contraction periods were observed under the varying conditions and their maximal diameter of ~9 μm remained unaffected (data not shown).

Effects of exposure to LiCl
Li⁺ interferes with the phospho-inositol cycle by inhibiting phospho-inositol-monophosphatases (Hallcher et al., 1980; Gee et al., 1988), leading to reduced formation of Ins(1,4,5)P₃. Since several reports had indicated that Paramecium possesses targets for LiCl (Beisson and Ruiz, 1992; Wright et al., 1992), we examined whether Li⁺ has an effect on IP₃RN.

LiCl (25 mM) was added to growing populations of P. tetraurelia for 2, 3 and 4 hours, followed by immunofluorescence analysis. We found significant changes in the labeling of cells stained with IP₃RN-specific Abs; and the extent of these changes depended on [Ca²⁺]ₒ (Fig. 7A). Although Ab labeling is decreased and/or redistributed to a speckled pattern in aliquots incubated with Li⁺ (Fig. 7A; left panel), control cells incubated with Na⁺ show the same staining as untreated cells. After exposure to Li⁺, we could not detect any changes in IP₃RN mRNA levels (data not shown). Therefore, we assume that (in contrast to the observations with varying [Ca²⁺]ₒ), Li⁺ mainly causes IP₃RN redistribution rather than affecting the levels of IP₃RN. The effect of Li⁺ is restricted specifically to IP₃RN because the staining pattern of the ORS with Abs against V-type H⁺-ATPase does not change (Fig. 7A; right panel). Furthermore, these experiments indicate that the decorated spongiome remains attached to the organelle.

Li⁺ also clearly affected the activity of contractile vacuoles independently of [Ca²⁺]ₒ. Incubation of P. tetraurelia in 25 mM

Fig. 3. Expression and [³H]Ins(1,4,5)P₃-binding activity of the putative Ins(1,4,5)P₃-binding domain of PrIP₃RN. (A) Overexpression of GFP alone (top) and GFP fused to the putative Ins(1,4,5)P₃-binding domain (IP₃BD) of IP₃RN (bottom) in Paramecium 7S cells. (B) Western blot analysis of immuno-precipitated GFP-IP₃BD fusion protein or GFP alone with GFP-specific Ab. (C) [³H]Ins(1,4,5)P₃- ([³H]IP₃) binding assay using agarose beads coupled to protein A either with GFP alone or with GFP-IP₃BD fusion protein. Inhibition of specific [³H]Ins(1,4,5)P₃-binding was measured in the presence of 10 μM cold Ins(1,4,5)P₃. The graph represents one out of five experiments.
LiCl for 3 hours decreased vacuolar activity significantly (Fig. 7B). When cells were allowed to recover for 3 hours in culture medium without Li⁺, contraction periods returned to normal values, indicating that the Li⁺ effect was reversible. Although the Li⁺ effect was not investigated in more detail, our data suggest that ORS activity is under latent control of IP₃RN activity.

Ca²⁺ imaging studies
A functional Ins(1,4,5)P₃R is usually determined by significant Ca²⁺ release in response to formation of Ins(1,4,5)P₃ after stimulation. To visualize Ca²⁺ release, we used high-affinity dextran-coupled Fluo-4, a derivative that, due to its size, stays in the cytosol. This was either used to monitor spontaneous [Ca²⁺]ᵢ oscillations near the ORS or combined in injections with NPE-caged Ins(1,4,5)P₃. The ability of our microscopical set-up to activate caged compounds by UV-light was tested with DMNB-caged fluorescein-coupled dextran (10,000 kDa), which can be uncaged efficiently (data not shown). Thus dextran-coupled Fluo-4 was injected with or without NPE-caged Ins(1,4,5)P₃. As soon as the fluorochrome was evenly distributed in the cell, we started recording (Figs 8-10) in different locations of the cell, including regions of the ORS containing the spongiome, where IP₃RN was localized by immuno-EM (Fig. 5).

**Fig. 4.** Characterization of polyclonal antibodies against IP₃RN. (A) Affinity-purified anti-IP₃RN Abs recognize the polypeptide corresponding to IP₃RN residues R896-Q1001 (AG) with high affinity in immuno-blots (second and third lanes), whereas the preimmunserum (PIS) does not show any interaction (fourth lane). The first lane, C, shows 2 μg of the purified AG (Coomassie Blue-stained) used for immunization. (B) Western blot analysis using anti-IP₃RN Abs. 100,000-g pellet of whole Paramecium cell homogenate (left lane) was extracted with 2% Triton X-100 and insoluble proteins (middle lane) were separated from soluble proteins (right lane). (C) Subcellular distribution of IP₃RN in Paramecium cells. Immunofluorescence analysis shows that Abs against IP₃RN stain the ORS.

**Fig. 5.** Immuno-gold EM localization of PtIP₃RN. (A,B) Dense labeling (gold grains) occurs in the layer around the collecting canals (CC) and represents the smooth spongiome (SS) (A), whereas the decorated spongiome (DS) shows only few gold grains (B). (C) Labeling also occurs directly adjacent to the lumen of the collecting canal. Bars, 0.1 μm.
First, spontaneous Ca\(^{2+}\) sparks were seen along parts of the ORS when \(\text{Ins}(1,4,5)P_3\) was not uncaged (Fig. 8). These signals were superimposed by Ca\(^{2+}\) oscillations, one such wave is shown in Fig. 8. Such spontaneous Ca\(^{2+}\) oscillations, with periods of approximately 8-20 seconds, were frequently observed in baseline recordings before uncaging with UV (Figs 9, 10). The maximum of these Ca\(^{2+}\) signals was detected anywhere between the systolic phase of the contractile vacuole and the diastolic phase, thus the recorded fluorescence differences cannot be simply due to a change in volume. Also, periods of ORS contraction activity and Ca\(^{2+}\) signals were not strictly identical.

In addition to the Ca\(^{2+}\) oscillations, we found spontaneous Ca\(^{2+}\) signals traveling along the radial arms of the ORS (Fig. 8A, supplementary material Movies 1 and 2). This observation was confirmed by the ratio of evaluated line tracings of distinct cellular regions (Fig. 8B). Traces obtained from spots in close proximity to the ampullae (traces b, c) or the radial arms (trace a) show additional Ca\(^{2+}\) peaks compared with trace d, obtained from a region more distant to the ORS. Trace d represents the large Ca\(^{2+}\) signal of an oscillation wave, enhancing the small additional Ca\(^{2+}\) sparks visualized in traces a to c. This finding agrees with a localization of IP\(_3\)RN to the smooth spongiome (Fig. 5). Enhancement of the small, locally confined Ca\(^{2+}\) signals (Fig. 8) may result in larger, eventually oscillating signals. This supports the regulation of localized [Ca\(^{2+}\)]\(\text{i}\) via the ORS, by sequestration and partial reflux.

To test the involvement of \(\text{Ins}(1,4,5)P_3\) in these Ca\(^{2+}\)-dynamics, we raised the concentration of intracellular \(\text{Ins}(1,4,5)P_3\) by uncaging \(\text{Ins}(1,4,5)P_3\) in the cytosol. In Figs 9 and 10, respectively, we present Ca\(^{2+}\) oscillations before and after the release of \(\text{Ins}(1,4,5)P_3\), followed by evaluation of different cell regions. In both cases, a change in Ca\(^{2+}\) oscillations after UV treatment is seen. Results are similar at
the anterior and posterior pole (Fig. 10), when analyzed over larger cell areas. More scrutinized analysis of sites closer to and further away from the ORS showed maximal effects at sites close to the ORS (Fig. 9). In Fig. 9, fluorescence signals were also evaluated from an area of the anterior and posterior part of the cell outside the reach of the corresponding contractile vacuole (Fig. 9, blue and green areas). These signals did not show such a distinctive Ca\textsuperscript{2+} peak as the one close the ORS, and seem to be similar to the spontaneous Ca\textsuperscript{2+} oscillations observed in baseline recordings. Some experiments suggest that the frequency of these signals is influenced by uncaging Ins(1,4,5)P\textsubscript{3} (Fig. 10). Thus, significant changes in the amplitude were seen only in regions where the spongiome is attached to a collecting canal (Fig. 9, red area). The effect of uncaging Ins(1,4,5)P\textsubscript{3} varied from cell to cell as to be expected for a stochastic fine-tuning activity, involving a compartment moderately enriched with Ca\textsuperscript{2+} (see Discussion).

Discussion

We have identified, for the first time on a molecular level an Ins(1,4,5)P\textsubscript{3}R in its full length in a protozoan. Since the immunolocalization of IP\textsubscript{3}RN to the ORS was unexpected, we compared its structure in some detail with Ins(1,4,5)P\textsubscript{3}Rs of other cells and provide information of its potential role in establishing [Ca\textsuperscript{2+}]i homeostasis.

Molecular properties of IP\textsubscript{3}RN compared with Ins(1,4,5)P\textsubscript{3}Rs from other cells

Investigations of intracellular Ca\textsuperscript{2+} signaling in other protozoa imply the presence of Ins(1,4,5)P\textsubscript{3}Rs and RyRs in these organisms. For example, in Dictyostelium disruption of the iplA gene, encoding an Ins(1,4,5)P\textsubscript{3}-receptor-like protein, abolishes Ca\textsuperscript{2+} entry stimulated by ATP or folic acid (Traynor et al., 2000). The relationship of the IplA protein to Ins(1,4,5)P\textsubscript{3}Rs is based on homologous regions corresponding to the channel domain and two regions of approximately 200 amino acid residues flanking the Ins(1,4,5)P\textsubscript{3}-binding domain. Despite the evidence that Ins(1,4,5)P\textsubscript{3} can cause the release of Ca\textsuperscript{2+} from internal stores in Dictyostelium (Flandt et al., 1993), biochemical evidence that IplA is an Ins(1,4,5)P\textsubscript{3} receptor is still lacking. Furthermore, homologous sequences are also present in the genomes of parasitic protozoa, but so far they have not been cloned. Based on functional analysis in Toxoplasma gondii, a parasite and close relative of Paramecium, a mixed-type Ca\textsuperscript{2+}-release channel has been postulated (Lovett et al., 2002). In Paramecium, one might think of such a mixed type, but despite the described similarity of the IP\textsubscript{3}RN to RyR in its channel region, the overall molecular characteristics are clearly in favor of an Ins(1,4,5)P\textsubscript{3}R.

Fig. 7. Li\textsuperscript{+} affects subcellular distribution of IP\textsubscript{3}RN. (A) Immunofluorescence analysis of cells grown in media with 1 \mu M [Ca\textsuperscript{2+}], and incubated with 25 mM LiCl for the times indicated, followed by immuno-labeling with IP\textsubscript{3}N-specific Abs (left panels, \alpha-IP\textsubscript{3}RN) or Abs against V-type H\textsuperscript{+}-ATPase (right panels, \alpha-vATPase). The IP\textsubscript{3}RN is selectively affected, with a maximal outcome after 3 hours, resulting in reduced ORS-staining and increased diffuse background fluorescence. As a control, cells were treated with NaCl for 3 hours (bottom panels) with no remarkable effect. (B) Contraction periods of contractile vacuoles of cells treated with LiCl for 3 hours are significantly prolonged (black bar) when compared to control cells (white bar, P<0.001) or to cells treated with NaCl (light gray bar, P<0.001). Contraction periods of cells incubated with NaCl are only slightly prolonged in comparison to control cells, with weak significance (P=0.014 for NaCl to untreated control). Three hours after treatment with LiCl, contraction periods return to control levels when cells are transferred to culture medium (dark gray bar).
An Ins(1,4,5)P₃ receptor in *Paramecium*

**Appraisal of different effects on IP₃Rs expression**

We observed the downregulation of IP₃Rs in the ORS when [Ca²⁺]₀ was greatly reduced. This might imply that, in the absence of significant Ca²⁺ influx into the cell, no Ca²⁺ is sequestered into the ORS and, therefore, no Ca²⁺ is recycled into the cytosol. Experiments with LiCl yielded similar results. From yeast (Navarro-Avino et al., 2003) to mammals (Berridge et al., 1989; Parthasarathy and Parthasarathy, 2004), Li⁺ is known to inhibit, though not exclusively, biosynthesis of Ins(1,4,5)P₃ precursors. These data lend further support to a role of Ins(1,4,5)P₃Rs in Ins(1,4,5)P₃-mediated [Ca²⁺]ᵢ homeostasis. Along those lines, in *Paramecium*, positive chemotactic responses (Wright et al., 1992) that are normally accompanied by Ca²⁺ signals as well as surface pattern formation (Beisson and Ruiz, 1992) are inhibited by LiCl. It is not surprising that, under such conditions of latent activity, no Ins(1,4,5)P₃ formation has been reported in *Paramecium* up to now.

**Possible implications for [Ca²⁺]ᵢ homeostasis**

Implications for [Ca²⁺]ᵢ homeostasis were analyzed by manipulating [Ca²⁺]₀ and [Ca²⁺]ᵢ, based on the fact that [Ca²⁺]ᵢ in *Paramecium* is rapidly adjusted to levels of Ca²⁺ available in the medium (Browning and Nelson, 1976; Kerboeuf and Cohen, 1990; Erxleben et al., 1997). The general assumption was that the ORS in *Paramecium* not only serves the adjustment of internal hydrostatic pressure but, necessarily, also of the internal ionic milieu. This interplay might be complicated because the H⁺-ATPase located in the decorated spongiome (Allen et al., 1990; Fok et al., 1995; Naitoh et al.,

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*Fig. 8.* Recordings of Ca²⁺ signals in close proximity to the ORS. (A) Elevated by a large Ca²⁺ signal traveling through the whole cell, small Ca²⁺ sparks localized close to the ampullae and radial arms (RA) can be observed (arrows) These spontaneous subcellular Ca²⁺ signals move along the ORS (CV, contractile vacuole). (B) Line tracings of the different spots marked by colored arrows in A. Trace d represents the large Ca²⁺ signal which elevates the small additional Ca²⁺ sparks visualized in traces a-c.
Tominaga et al., 1998) produces electrogenic force not only for organellar water uptake (Grønljen et al., 2002; Stock et al., 2002a; Stock et al., 2002b), but might also be coupled to a secondary active ion transport by exchangers. Among them, one may envisage a H+-Ca\textsuperscript{2+}-based or a similar Ca\textsuperscript{2+}-based exchanger, as occurring in acidocalcisomes of some parasitic protozoa (Docampo and Moreno, 2001) and in plant cell vacuoles (Hetherington and Brownlee, 2004). What might be the relative contribution of such a mechanism to overall Ca\textsuperscript{2+} homeostasis?

Assuming, that a Paramecium cell has two contractile vacuoles, each releasing a volume of ~100 femtoliters second\textsuperscript{-1} (Grønljen et al., 2002), i.e. 6 picoliters minute\textsuperscript{-1}, a total cell volume of 0.7 \mu l (Erxleben et al., 1997), the ORS would discharge 8.6% of the cell volume per minute. Release of a total equivalent of the cell volume would, thus, require 11.6 minutes. Under standard conditions of [Ca\textsuperscript{2+}]\textsubscript{OR}= 1 mM, a [Ca\textsuperscript{2+}]\textsubscript{ORS}= 2.5 mM was found by impaling Ca\textsuperscript{2+}-selective microelectrodes (Stock et al., 2002a; Stock et al., 2002b). Then, 0.29 mM l\textsuperscript{-1} would be released by the ORS per minute. Latent Ca\textsuperscript{2+} influx under similar conditions, as determined by \textsuperscript{45}Ca\textsuperscript{2+}-flux measurements with unstimulated cells, is ~2 pM second\textsuperscript{-1} per 1\textsuperscript{3} cells (Kerboeuf and Cohen, 1990). Considering the given cell volume, this amounts to an influx of 1.7 mM l\textsuperscript{-1} minute\textsuperscript{-1}, which implies that Ca\textsuperscript{2+} expulsion via the ORS requires only 5.9 minutes to compensate for the latent Ca\textsuperscript{2+} influx, disregarding any other extrusion mechanisms. In comparison, Ca\textsuperscript{2+} expulsion via the pumps is known to operate rather sluggishly (Plattner and Klauke, 2001). This makes the ORS an interesting key-player in the regulation not only of cell volume and hydration, but unexpectedly also in [Ca\textsuperscript{2+}] homeostasis.

We therefore expected some effect of [Ca\textsuperscript{2+}]\textsubscript{o} on the function of the Ins(1,4,5)\textsubscript{P}\textsubscript{3}R. We altered the [Ca\textsuperscript{2+}]\textsubscript{o} levels down to 1 \mu M – a level just above the minimum levels tolerated by Paramecium cells over some time (Kerboeuf and Cohen, 1990) and observed that lowering [Ca\textsuperscript{2+}]\textsubscript{o} to threshold values greatly reduces the expression of IP\textsubscript{3}RN.

In aggregate, all these findings strongly support our hypothesis that, in Paramecium, Ins(1,4,5)\textsubscript{P}\textsubscript{3}Rs serve [Ca\textsuperscript{2+}],...
homeostasis. As in how this might work, one has to consider several aspects. (1) Substantial Ca$^{2+}$ secretion is executed by the ORS, as determined by ion-selective electrodes (Stock et al., 2002a; Stock et al., 2002b). (2) The [Ca$^{2+}$]$_i$ level actually available depends on the Ca$^{2+}$ influx. (3) This rapidly adjusts to levels of [Ca$^{2+}$]$_i$. (Browning and Nelson, 1976; Kerboeuf and Cohen, 1990; Erxleben et al., 1997). Based on these arguments it is, therefore, plausible to postulate a counter-acting efflux mechanism operating at the ORS for fine-tuning of [Ca$^{2+}$]$_i$. Remarkably, this is what happens, on an organismic level, in the kidney nephrons.

Implication of Ca$^{2+}$ signals for the function of IP$_3$R

Our system does not provide the common Ins(1,4,5)P$_3$-induced Ca$^{2+}$-response as it is known from mammalian systems, i.e. a large, long-lasting peak. Ca$^{2+}$-signals induced by uncaging Ins(1,4,5)P$_3$ seem to be concentrated to the specific region of the cell where the ORS harbors the smooth spongiome with the IP$_3$RN we identified in this study. Regarding the IP$_3$RN we identified in this study. Regarding the [H]Ins(1,4,5)P$_3$-binding domain from mouse Ins(1,4,5)P$_3$ receptor type 1 (Bosanac et al., 2002) was amplified by PCR using primers pBD-f (5' gattctataagcaatataaactcat-3') and pBD-rev (5' ggcttattgcagattctg-3'). Amplified cDNA fragments were directly cloned in the pCRII-TOPO cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Plasmid DNA was extracted from bacteria according to standard protocols and was analyzed by sequencing.

Quantification of RNA transcription level

Reverse transcription of 0.5 µg total RNA was performed using Transcriptase reverse transcriptase (Roche, Mannheim, Germany). One-tenth of cDNA samples were amplified by PCR (35 cycles) with the Advantage 2 PCR Enzyme System (Clontech, Palo Alto, CA) using act8-specific primers (act8-f: 5' ggtcttattgcagattctg-3' and act8-rev: 5' ctgctgcagatgtcaacatcttggaaaattaatctt-3') as control or IP$_3$R-specific primers (ef (see above) and ew2: 5' atcgaagatccttttgctaactac-3') as control or IP$_3$R-specific primers (ef (see above) and ew2: 5' atcgaagatccttttgctaactac-3'). Amplified cDNA fragments were directly cloned in the pCRII-TOPO cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Computational analysis

BLAST searches were performed at the NCBI database (Altschul et al., 1997). Protein alignments were performed with CLUSTAL W (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were performed using MEGA version 3.0 (Kumar et al., 2004). Modeling of protein structures was done using the SWISS-MODEL server (http://swissmodel.expasy.org) for automatic comparative modeling (Peitsch et al., 1993). For the IP$_3$R Ins(1,4,5)P$_3$-binding domain the alignment mode was chosen and the structurally known Ins(1,4,5)P$_3$-binding domain of mouse Ins(1,4,5)P$_3$-binding domain, receptor type 1, 160S, receptor type 1 (Bosgen, 5' act8-specific primers (act8-f: 5' ggtcttattgcagattctg-3' and act8-rev: 5' ctgctgcagatgtcaacatcttggaaaattaatctt-3') as control or IP$_3$R-specific primers (ef (see above) and ew2: 5' atcgaagatccttttgctaactac-3'). Amplified cDNA fragments were directly cloned in the pCRII-TOPO cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Expression and immuno-precipitation of GFP-fusion protein

Sequences encoding the Ins(1,4,5)P$_3$-binding domain of IP$_3$R$_b$ (S268-L658) were amplified by PCR using primers pBD-f (5' gctgctgcagatgtcaacatcttggaaaattaatctt-3') and pBD-rev (5' gctgctgcagatgtcaacatcttggaaaattaatctt-3'). Amplified cDNA fragments were directly cloned in the pCRII-TOPO cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Plasmid DNA was extracted from bacteria according to standard protocols and was analyzed by sequencing.

Materials and Methods

**P. tetraurelia** strains and cultivation

*P. tetraurelia* wild-type stocks 7S and d4-2 derived from stock 5JS (Sonneborn, 1974) were cultured as previously described (Kissmehl et al., 2004). For Ca$^{2+}$-imaging we used the trichocyst non-discharge strain H11032 (Lefort-Tran et al., 1981). Genomic-library screening

A genomic library of *P. tetraurelia* macronuclear sequences was screened according to Keller and Cohen (Keller and Cohen, 2000). Specific probes were generated by PCR using IP$_3$R-specific primers p6 5' aataaatggaaataaatcaaaat-3' and p8 5' aataaatggaaataaatcaaaat-3'. Sequencing

Sequencing was done by the MWG Biotech (Ebersberg, Germany) custom-sequencing service. DNA sequences were aligned by CLUSTAL W, integrated in DNASTAR Lasergene software package (Madison, WI).
Electron microscopy

The method applied was as indicated by Kissmehl et al. (Kissmehl et al., 2004). Briefly, cells were injected into 5% formaldehyde + 0.1% glutaraldehyde, pH 7.2, 4°C, using a quenched-flow machine and processed by the ‘progressive lowering of the temperature’-method. This involved stepwise reduction of the temperature, with increasing ethanol concentrations, followed by LR Gold methacrylate resin-embedding and UV polymerization at −35°C. Anti-IP3RN Abs have been used for immuno-gold localization by protein-A-gold conjugated to 5-nm gold (Au) in a Zeiss electron microscope, EM10.

Functional analysis with varying [Ca2+]o and with Li+

Paramecium cells (stock 4H-2) were centrifuged (2 minutes, 180 g) and suspended in the experimental solution, with two changes. Different [Ca2+]o concentrations were added by adjusting 2 mM, 1 mM or 0.85 mM CaCl2 to 5 mM Pipes pH 7.1 or 1 mM KCl, 1 mM EGTA. Free [Ca2+]o was calculated according to Patton et al. (Patton et al., 2004) using the MaxChelator program Winmaxc v.2.40. Experiments with LiCl have been carried out as described by Beisson and Ruiz (Beisson and Ruiz, 1992). A 2 M LiCl stock solution was diluted to 25 mM in an exponentially growing culture, where the number of cells was adjusted to 106 cells per ml culture media supplemented with 1 mM EGTA and 0.85 mM Ca2+ to get a final concentration of 1 mM [Ca2+]o. Cells were incubated with LiCl for the times indicated and then analyzed by immuno-labeling (see above). The contraction periods of contractile vacuoles were measured in cells contained in a microfluidic overlad with paraffin oil.

[Ca2+]o fluorochrome measurements

P tetrarella (strain nr6) cells were isolated in microdrops of PIPES-buffer with 0.2% BSA added and covered with paraffin oil. After cautious reduction of the centrifugation buffer to immobilize them they were injected using the Eppendorf injection system consisting of the Injectman N2, Femtotjet and Femtotips I (Eppendorf, Hamburg, Germany). As a Ca2+-fluochrome we used the high-affinity dextran-coupled Fluo-4 (10,000 kDa, Molecular Probes). The volume injected was ~5-10% of the cell volume. For injection 17 mg/ml dextran-coupled Fluo-4 was mixed with one or together with 670 μM NPEI-caged Ins(1,4,5)P3. Molar Probes) both dissolved in 10 mM Tris-HCl pH 7.2. After injection, cells were flooded for a recovery period of 15-30 minutes. Then cells were immobilized again and Fluo-4 signals were recorded with a 40x×-plan Neofluor objective, NA 0.75, on an Axiovert 200 M microscope equipped with an Axiocam MRm digital camera (Carl Zeiss). Excitation light (50-65% intensity) was selected from a 100 W HBO lamp.

Fluorescent Ca2+-signals were recorded using the Axiovision 4.3 Software (Carl Zeiss). For uncaging of Ins(1,4,5)P3 cells were locally illuminated for ~1 second with UV light which was selected by filterset 49 with excitation at 365 nm and emission at 445 nm. Recordings were done in a 2×2 binning mode, with an illumination time of 150 milliseconds, pictures were taken every 360 milliseconds.

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