

The type 3 inositol 1,4,5-trisphosphate receptor is concentrated at the tight junction level in polarized MDCK cells

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Summary

The subcellular localization of inositol 1,4,5-trisphosphate (InsP₃)-induced Ca²⁺ signals is important for the activation of many physiological functions. In epithelial cells the spatial distribution of InsP₃ receptor is restricted to specific areas, but little is known about the relationship between the receptor's distribution and cell polarity. To investigate this relationship, the best known polarized cell model, MDCK, was examined. This cell line is characterized by a strong expression of the type 3 InsP₃ receptor and the subcellular localization of this receptor was followed during cell polarization using immunofluorescence and confocal analysis. In non-polarized cells, including ras transformed f3 MDCK cells, the type 3 InsP₃ receptor was found to co-localize with markers of the endoplasmic reticulum in the cytoplasm. In contrast, in polarized cells, this receptor was mostly distributed at the apex of the lateral plasma membrane with the markers of tight junctions, ZO-1 and occludin. The localization of the type 3 InsP₃ receptor

in the vicinity of tight junctions was confirmed by immunogold electron microscopy. The culture of MDCK cells in calcium-deprived medium, led to disruption of cell polarity and receptor redistribution in the cytoplasm. Addition of calcium to these deprived cells induced the restoration of polarity and the relocation of the receptor to the plasma membrane. MDCK cells were stably transfected with a plasmid coding the full-length mouse type 1 InsP₃ receptor tagged with EGFP at the C-terminus. The EGFP-tagged type 1 receptor and the endogenous type 3 co-localized in the cytoplasm of non-polarized cells and at the tight junction level of polarized cells. Thus, the localization of InsP₃ receptor in MDCK depends on polarity.

Key words: Inositol 1,4,5-trisphosphate receptor, Tight junction, Polarity, Calcium, Endoplasmic reticulum, InsP₃R-1-EGFP

Introduction

Calcium ions are involved in the control of many physiological functions including muscle contraction, secretion, cell proliferation and cell death. This versatility as an intracellular messenger, is made possible by the variety of mechanisms that can be employed by the cell to generate complex spatio-temporal Ca²⁺ signals (Berridge et al., 2000). A key feature of Ca²⁺ signaling is the initial release of intracellular Ca²⁺ at specific sites, which gives rise to elementary events such as 'blips' or 'puffs' observed in the cytoplasm (Yao et al., 1995). These elementary events can result in the genesis of global events such as Ca²⁺ waves or oscillations which propagate throughout the whole cell (Berridge, 1997).

The localization of these elementary events to specific sites indicate that the Ca²⁺ stores and the proteins responsible for the Ca²⁺ release are highly organized into specific domains (Meldolesi and Pozzan, 1998). Ca²⁺ is actively pumped by the SERCA proteins into endoplasmic reticulum (ER) stores where

it binds to a host of resident proteins in order to buffer the luminal free Ca²⁺. In response to specific signals, Ca²⁺ is quickly released from these intracellular stores through channels. The most ubiquitous intracellular Ca²⁺ channel is the inositol 1,4,5-trisphosphate receptor (InsP₃R) which releases Ca²⁺ in response to InsP₃ produced under agonist stimulation (Berridge, 1993).

Three InsP₃R sub-types have been identified and are expressed at various levels among cell types (Blondel et al., 1993; Furuichi et al., 1989; Maranto, 1994; Südhof et al., 1991). The InsP₃R subtypes assemble into tetramers to form functional channels and are differently distributed throughout the cytoplasm according to cell type (Joseph, 1996; Taylor and Traynor, 1995). In neuronal cells, InsP₃R are associated with the ER membrane and evenly localized throughout the cytoplasm comprising the cell body, the perinuclear area, the dendrites and the axon (Pozzan et al., 1994). Similarly, in oligodendrocytes (Simpson et al., 1997), HeLa cells (Thomas et al., 2000) and rat leukemia cells (Wilson et al., 1998),

the various InsP₃R subtypes show a similar fine reticular distribution throughout the cytoplasm. The formation of receptor clusters dispersed along the ER network has been described in several cell types (Wilson et al., 1998) and may correspond to cell regions that are more responsive to small increases in InsP₃ (Sheppard et al., 1997). In contrast, polarized epithelial cells, in particular exocrine glandular cells or liver cells, are characterized by a distribution of InsP₃R restricted to specific areas. Subcellular fractionation experiments with these cells have shown that the receptor does not always co-purify with ER markers but rather with markers of the plasma membrane (Lièvreumont et al., 1994; Sharp et al., 1992). Immunocytochemical experiments have indicated that the InsP₃R is mainly concentrated at the lateral pole of polarized MDCK cells (Bush et al., 1994; Zhang et al., 2003) and near the apical pole of other polarized cells such as hepatocytes (Dufour et al., 1999; Nathanson et al., 1994a), cholangiocytes (Hirata et al., 2002), pancreatic acinar cells and parotid cells (Lee et al., 1997; Nathanson et al., 1994b; Yule et al., 1997). In pancreatic acinar cells, the three receptor sub-types displayed the same sub-apical localization, indicating that this pattern of localization does not depend on the receptor sub-type. These observations strongly suggest that the subcellular localization of the InsP₃R plays an important part in determining the spatio-temporal characteristics of the Ca²⁺ signal that regulates a particular Ca²⁺-dependent cellular function.

Although the pattern of InsP₃R localization in polarized epithelial cells is now well documented, very little is known about the mechanisms that establish it, and the first step in studying this, was to find an adequate polarized cell model. Such a model must fulfill several criteria: its polarized phenotype must be stable and its polarization easy to follow and quantify; experimental conditions that permit to disturb and restore polarity must be available. Finally, the chosen cells must be easy to transfect, because one of our long term aims, is to perform *in vivo* dynamic studies, using cells stably expressing different versions of GFP tagged InsP₃R. Despite our previous work in liver cells study (Decaens and Cassio, 2001; Lièvreumont et al., 1996) and the fact that the restricted InsP₃R localization in MDCK cells seems to differ from that in other epithelial cells (lateral versus subapical), we have chosen to first investigate this non-tumorigenic cell line (i.e. MDCK) because it readily fulfills all the criteria and is by far the best known polarized cell model. In the present work, the distribution of the InsP₃R type 3 (InsP₃R-3), the most abundant InsP₃R expressed in MDCK cells, was followed during MDCK polarization and also when the polarity was disturbed and restored. We also isolated and examined MDCK cells stably expressing a full-length version of InsP₃R type 1 tagged with EGFP (InsP₃R-1-EGFP). A clear correlation between InsP₃R distribution and polarity was found. In non-polarized cells, endogenous InsP₃R-3 and InsP₃R-1-EGFP were distributed in the whole cytoplasm and co-localized with other markers of the ER. In contrast, in polarized cells, both receptor types are mostly distributed at the apex of the lateral pole with tight junction markers.

Materials and Methods

Antibodies, chemicals and reagents

The characteristics of the primary antibodies used are presented in Table 1. Standard analytical grade laboratory chemicals were from Fisher-Labosi (Elancourt, France), Merck-Eurolab (Illkirch, France),

and Sigma-Aldrich (Saint Quentin Fallavier, France), and U0126 was from Promega (Charbonnières, France).

Cell culture, transepithelial resistance and calcium switch

Madin-Darby canine kidney (MDCK) cells or ras-transformed MDCK f3 cells, (Hordijk et al., 1997) were cultured on plastic culture Petri dishes (Falcon), glass coverslips (CML, France) or inserts (Transwell filters, 30 mm diameter, 0.4 µm pore size, Falcon) in F-12 Coon's modified medium (Sigma-Aldrich) supplemented with 5% FCS (Gibco BRL Invitrogen, Cergy Pontoise, France), 100 units/ml penicillin, 100 µg/ml streptomycin sulfate and 0.25 µg/ml fungizone (Gibco BRL) at 37°C in a humidified atmosphere with 7% CO₂. These cultures were found to be *Mycoplasma* negative using the Mycoplasma PCR ELISA (Roche Applied Sciences, Meylan, France). Cells were confluent, and well-polarized (except f3 cells), 10-12 days after plating at 10⁴ cells/cm². MDCK cells were subcloned at 2-20 cells/cm² with a cloning efficiency of 60-90%. Chromosome content was determined as previously described (Bender et al., 1999).

Transepithelial resistance (TER) was measured with a Millicell apparatus (Millipore). All TER values were obtained using at least three inserts (6-8 measurements per insert at each time) after background subtraction (i.e. filter and solution).

Calcium switch experiments were performed using calcium-free F12 Coon's modified medium (Eurobio, Les Ulis, France), without serum. Well-polarized MDCK cells, grown on glass coverslips or on inserts were rinsed three times and switched in calcium-free medium ([Ca²⁺]_e ≤ 5 µM). During a 2 hour period, TER was recorded and cells fixed for immunostaining at regular intervals. Cells were then switched back to complete medium containing 1.12 mM CaCl₂, and at regular intervals cells were fixed and the TER recorded.

Plasmid construction, transfection and isolation of stable transfectants

The expression plasmid coding for the InsP₃R-1-GFP chimera was constructed using the full-length cDNA of the mouse InsP₃R type 1 (Furuichi et al., 1989). This cDNA was first subcloned from pRC/CMV vector (supplied by D Ogden, NIMR, Mill Hill, London, UK) into the *Xba*I site of pBluescript SK (Stratagene). The stop codon at the end of the InsP₃R-1 coding sequence was removed by cutting with *Avr*II and *Sal*I, followed by Mung Bean nuclease treatment to create blunt ends and then these ligated together. The *Xba*I-*Xho*I cDNA fragment of this vector was inserted into the *Nhe*I-*Xho*I site of pEGFP-N1 (Clontech) to create pInsP₃R-1-EGFP. The authenticity of the plasmid was verified by automated nucleotide sequencing using a Perkin Elmer machine (Genome Express, France). pInsP₃R-1-EGFP codes for a chimera that is composed of the full-length mouse InsP₃R-1 attached, via a 22 amino acid linker at its C-terminal, to EGFP.

For transfection, 5×10² cells/cm² were seeded the day before in 6-well plates (Falcon) and transfected with 1-2 µg DNA using Superfect reagent (Qiagen). MDCK cell populations expressing InsP₃R-1-EGFP or the host plasmid pEGFP-N1 were selected with 600 µg/ml G418 (Gibco BRL) 3 days after transfection, and subcloned 3 weeks later at 2 cells/cm². Independent colonies (39 and 15 from the cell populations transfected with pInsP₃R-1-EGFP and the empty pEGFP-N1, respectively) were isolated, and characterized. A few colonies well expressing the transfected gene were retained, frozen two months after transfection and studied. They were always cultured with 600 µg/ml G418, and their chromosomal content as the absence of mycoplasma were tested.

SDS-PAGE and immunoblot analysis

Cells were grown in 10 cm Petri dishes. At appropriate times, cells from one dish were counted and cells from the remaining ones were rinsed three times in cold PBS and lysed on ice by scraping with a

Table 1. Characteristics of antibodies used in this study

Target	Antibody	Immunogen	Origin
InsP₃R			
Type 1 (InsP ₃ R-1)	Polyclonal (rabbit)	Synthetic peptide: amino acids 1829-1848 from the human protein	Affinity Bioreagent (Coger, Paris, France)
Type 2 (InsP ₃ R-2)	Polyclonal (rabbit)	Synthetic peptide: last C-terminal 16 amino acids (2686-2701) of the rat protein	CovaLab (Oullins, France), Picard et al., 2000
Type 3 (InsP ₃ R-3)	Monoclonal (mouse)	Recombinant polypeptide: amino acids 22-230 from the N-terminal part of the human protein	Transduction Laboratories (Interchim, Montluçon, France)
	Polyclonal (rabbit) AP75	Synthetic peptide: amino acids 2655-2670 from the C-terminal part of the rat protein	A. H. Sharp (Case Western Reserve University, Ohio, USA), Sharp et al., 1999
Endoplasmic reticulum			
Protein disulfide isomerase (PDI)	Monoclonal (mouse)	Synthetic peptide: amino acids 499-509 from the rat protein	Stressgen (Tebu, Le Perray en Yvelines, France)
Bulk of resident proteins of endoplasmic reticulum	Polyclonal (rabbit)	Membrane fraction	D. Louvard (Institut Curie, Paris), Louvard et al., 1982
Tight junction			
Zonula occludens-1 (ZO-1)	Monoclonal (rat)	Hybridoma culture medium	B. R. Stevenson (University of Alberta, Edmonton, Canada), Stevenson et al., 1986
Occludin	Polyclonal (rabbit)	Chicken occludin cytoplasmic domain	M. S. Balda (University College, London), Balda et al., 1996
Membrane markers			
erbB-2 (Neu C-18)	Polyclonal (rabbit)	Synthetic peptide: C-terminal part of the human Neu gp185	Santa Cruz Biotechnology (Tebu, Le Perray en Yvelines, France)
E-cadherin	Monoclonal (mouse)	Protein fragment: amino acids 735-833 from the human protein	Transduction Laboratories (BD Biosciences, Interchim, Montluçon, France)
Pan-cadherin	Monoclonal (rat): DECMA 1 clone	Mouse embryonal carcinoma cell line PCC4 aza R1	Sigma Aldrich, France (Cat. No. U-3254)
	Polyclonal (rabbit)	Synthetic peptide from C-terminal of chicken N-cadherin	Sigma Aldrich, France (Cat. No. C-3678)
gp-114	Polyclonal (rabbit)	Apical membrane fraction	A. Le Bivic (CNRS, Université de la Méditerranée, Marseille, France), Le Bivic et al., 1990
Caveolin	Polyclonal (rabbit)	N-terminal from the human protein	Transduction Laboratories (BD Biosciences, Interchim, Montluçon, France)
Miscellaneous			
GFP	Monoclonal (mouse)	Not available	BD Biosciences Clontech, France (Cat. No. 8371-1)
sec6	Monoclonal (mouse)	Recombinant His-tagged rat sec6 protein	Stressgen (Tebu, Le Perray en Yvelines, France)

rubber policeman in either ice-cold PBS or in 10 mM Tris-HCl (pH 7.5), 5 mM EDTA containing 5 µg/ml antipain, 5 µg/ml aprotinin, 2 mM benzamide, 1 mM DTT, 10 µg/ml leupeptin, 5 mM NEM, 10 µM pepstatin A, 5 µM 1, 10-phenanthroline, 1 mM phenylmethylsulfonyl fluoride, 50 µg/ml soybean trypsin inhibitor, and 0.02% sodium azide. The protein concentration of the lysates was determined using BC Assay Uptima (Interchim, France). 30-50 µg protein/lane were subjected to SDS-PAGE (Laemmli, 1970). Positive controls for type 1 and type 3 inositol 1,4,5-trisphosphate receptors were rat cerebellum membranes (Lièvreumont et al., 1994) and HeLa cell lysate (Transduction Laboratories), respectively.

Immunoblotting was performed following a standard method. Briefly, the proteins were electrophoretically transferred from the gel onto a Hybond-C extraTM nitrocellulose membrane (Amersham Pharmacia Biotech) in a Tris-glycine buffer with 20% methanol and 0.04% SDS. The membrane was saturated with PBS (pH 7.5) containing 10% (w/v) nonfat milk and 0.2% Tween 20 for 1 hour at 37°C, then incubated overnight at 4°C with the appropriate dilutions of the primary antibody in PBS-milk-Tween. This was followed by washing and incubation with a horseradish-peroxidase-conjugated secondary antibody (donkey anti-mouse or anti-rabbit Ig, Amersham Pharmacia Biotech) (goat anti-rabbit Ig or sheep anti-mouse Ig, Pasteur, Sanofi, France). The membranes were developed using the ECLTM system (Amersham Pharmacia Biotech) on Biomax ML films

(Kodak). Prestained SDS-PAGE Standards (BioRad) were used to calibrate molecular mass.

Immunofluorescence and confocal microscopy

Cells grown on glass coverslips, were rinsed three times with cold PBS (without Ca²⁺ and without Mg²⁺, pH 7.4), and generally fixed on ice with 2% formaldehyde in PBS for 1 minute and methanol at 4°C for 10 minutes (Cassio et al., 1991), except for occludin (30 minutes in 100% ethanol at 4°C and acetone at -20°C for 1 minute) and sec6 detection (1% Triton X-100 on ice for 10 minutes and 100% methanol at -20°C for 10 minutes). Cells were then incubated with single primary antibodies or a mixture of them for 45 minutes at 37°C, washed three times in PBS and incubated (20 minutes at 37°C) with the appropriate Alexa-conjugated secondary antibodies (Molecular Probes). After three PBS washes, the coverslips were mounted in PBS-glycerol mounting medium (Sigma) and examined with a Axioskop fluorescence microscope (Zeiss, Germany).

Confocal microscopy was performed using either a BioRad 600 or a Zeiss 510 confocal microscope and in both cases using a ×63 Plan-Apochromatic objective (NA=1.4), oil immersion. Optical sections were collected at 0.2-0.3 µm intervals and the images were processed using Photoshop 5.5 (Adobe) software.

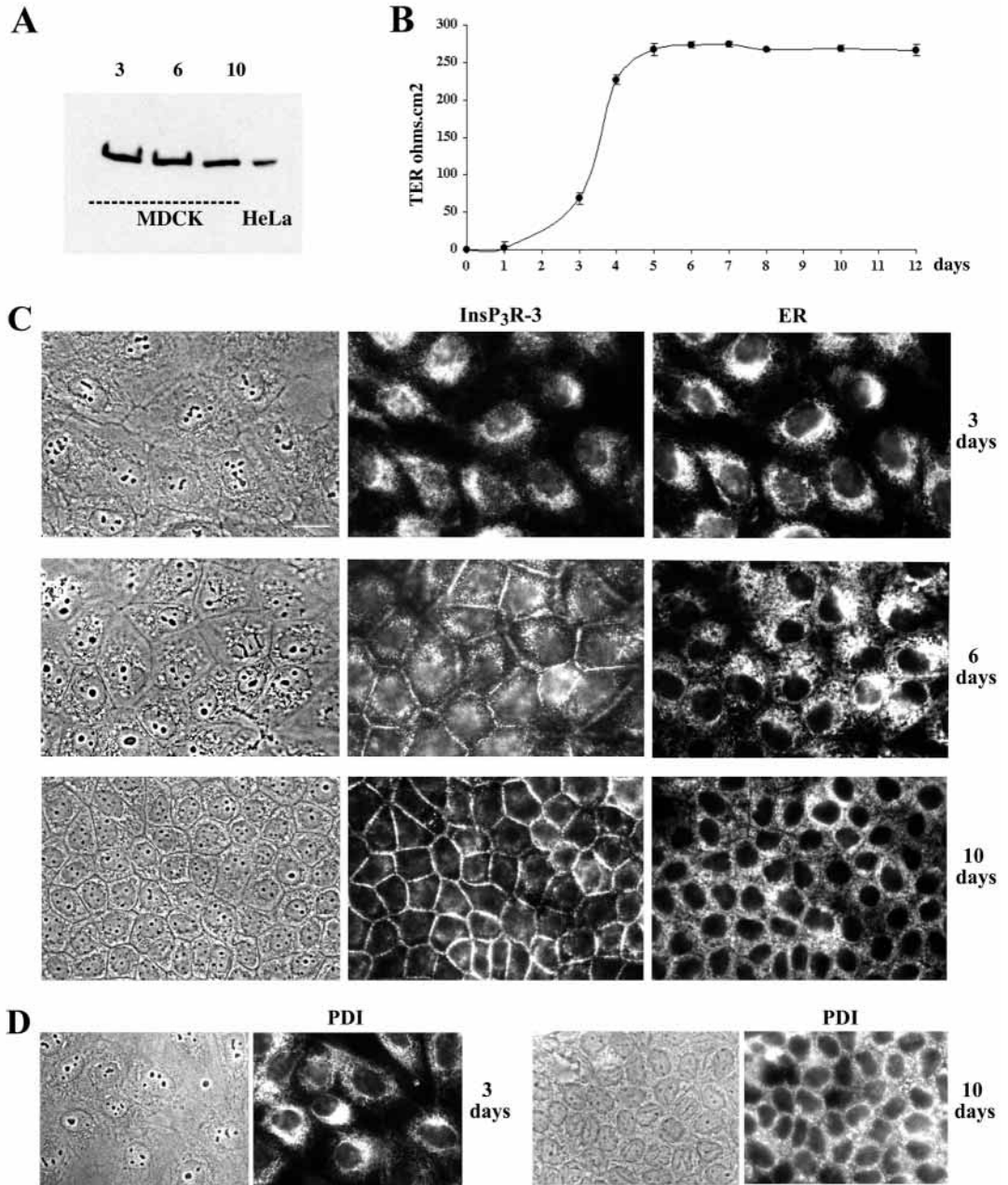


Fig. 1. Expression and localization of InsP₃R-3 during the polarization of MDCK cells. MDCK cells, plated at 10^4 cells/cm² were cultured over a period of 12 days. (A) Western blot: 50 µg of protein of cell lysates, corresponding to $1.2\text{--}1.4 \times 10^5$ cells from cultures of 3, 6 and 10 days, were loaded on each lane; the last lane was loaded, as a positive control, with a sample of HeLa cell lysate. Proteins were subjected to 5% (w/v) SDS-PAGE and the western blotting was carried out using a primary antibody directed against the type 3 InsP₃R. (B) TER. (C) Localization of InsP₃R-3 and proteins of the endoplasmic reticulum (ER) in MDCK cells cultured for 3, 6 and 10 days; in each case the corresponding phase contrast images are shown on the left. (D) Localization of another marker of the endoplasmic reticulum, the protein PDI, in cultures of 3 and 10 days, with the corresponding phase contrast image on the left. Bar, 10 µm.

Immunogold electron microscopy

For immunoelectron microscopy, ultrathin frozen sections of MDCK cells were obtained and processed as described for Caco-2 cells and human colon (Saito et al., 2001). Ultrathin cryosections were incubated overnight at 4°C with anti-InsP₃R-3 monoclonal antibody diluted 1:15 or 1:20 in 5% goat serum in PBS. Primary antibodies were revealed by incubation for 1 hour with colloidal gold 6 nm-conjugated goat anti-mouse IgG diluted 1:25 in 5% goat serum in PBS. Sections were examined under a Leo 912 electron microscope.

Results

The localization of InsP₃R-3 changes during MDCK polarization

The expression of InsP₃R was first analyzed in confluent polarized MDCK by Western blotting, using several antibodies

specific for the three types (Table 1). We found that MDCK cells were characterized by a strong expression of the type 3 InsP₃R (Fig. 1A, penultimate lane), whereas the type 1 was barely detectable (as illustrated later in Fig. 7) and the type 2 undetectable (results not shown). These data are in good agreement with a previous work (Bush et al., 1994) showing that the type 3 mRNA is by far the most abundant in MDCK cells. In view of these results and our own, our study was focused on InsP₃R type 3 (InsP₃R-3).

The localization of this protein was studied during the polarization of MDCK cells. For this purpose MDCK cells were plated at 10^4 cells/cm², and the establishment of polarity was monitored by measuring the transepithelial resistance (TER), as shown in Fig. 1B. InsP₃R-3 and other ER proteins were localized during this process by indirect

immunofluorescence (Fig. 1C,D). For InsP₃R-3, a mouse monoclonal antibody was used and for ER proteins two different antibodies were used: one polyclonal directed against a bulk of ER resident proteins (Louvard et al., 1982), that permits to double stain MDCK cells for ER and InsP₃R-3 (Fig. 1C) and one monoclonal antibody specifically directed against protein disulfide isomerase (PDI) (Fig. 1D). As shown in Fig. 1C, the intracellular distribution of InsP₃R-3 was different, according to the polarity state of the cells. In early cultures, when cells were sparse and not yet polarized (day 1-3, TER less than 60 ohm cm²), InsP₃R-3 displayed a cytoplasmic distribution similar to that of ER proteins. In contrast, in late cultures (day 10 and later), when cells were confluent and well-polarized (stabilization of TER at 260 ohm cm² and formation of domes), InsP₃R-3 was mostly concentrated near the plasma membrane (PM). This change in the localization of InsP₃R-3 was progressive, as illustrated in Fig. 1C by the comparison of MDCK cells after 3, 6 and 10 days in culture. This change was specific for InsP₃R-3, and was not observed for the bulk of ER proteins (Fig. 1C), or PDI (Fig. 1D).

In parallel to the localization of InsP₃R-3, we evaluated the cellular content of InsP₃R-3 at different times during the polarization of MDCK cells. Fig. 1A shows that the amount of InsP₃R-3 (expressed either by mg of protein or by number of cells) was similar at day 3, 6 and 10 suggesting that only the cellular distribution and not the level of InsP₃R-3, is modified during MDCK polarization. To examine whether this change affected all MDCK cells, several hundred colonies were examined at different times after cloning. InsP₃R-3 was detected in the cytoplasm of all the cells of each colony very soon after cloning, but after a few days it had in all cases become concentrated near the PM. These results indicate that the change in localization of InsP₃R-3 during polarization is a general trait of MDCK cells.

InsP₃R-3 is concentrated at the tight junction level in polarized MDCK cells

The distribution of InsP₃R-3 in polarized MDCK cells was further analyzed by confocal microscopy. The protein was first localized using two antibodies: the monoclonal antibody used above (Fig. 1C), directed against the N terminal part (aa 22-230) of human InsP₃R-3 and an affinity purified polyclonal antibody (Sharp et al., 1999), directed against a synthetic peptide corresponding to the C-terminal portion of rat InsP₃R-3 (aa 2655-2670). An identical staining was obtained with each antibody. This is illustrated in Fig. 2, particularly in the merged image, that shows the distribution of InsP₃R-3 in polarized cells at day 13: as previously shown in Fig. 1C, the receptor was detected in clusters in the cytoplasm, and already concentrated along the PM. This distribution was observed in all cells and was homogeneous over all the cell population. For the fraction of InsP₃R-3 present in the vicinity of the PM, the staining was not continuous but patchy, and in some cases one can distinguish a double row of patches (Fig. 2), that probably corresponds to InsP₃R-3 molecules present on both sides of the cell-cell contacts.

To better localize the fraction of InsP₃R-3 that is concentrated near the PM in well-polarized MDCK cells, double localizations were performed for InsP₃R-3 and proteins specific for different PM domains. We first studied the distribution of

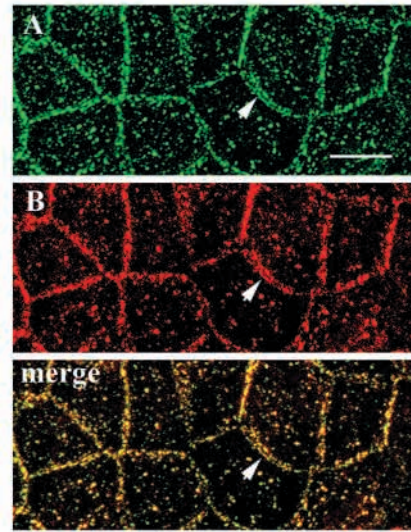


Fig. 2. Localization of InsP₃R-3 in polarized MDCK cells using two different specific antibodies: confocal analysis. MDCK plated at 10⁴ cells/cm² were cultured for 13 days and double stained using a monoclonal antibody (A) and a polyclonal antibody (B) specific for InsP₃R-3. The images shown correspond to a complete compiled z series of 30 xy sections taken in 0.2 μm steps. Note the patchy staining along the plasma membrane with, in some cases, a double row of patches (arrow). Bar, 10 μm.

the tight junction-associated protein ZO-1 and InsP₃R-3 (Fig. 3A). We found that InsP₃R-3 was present near the PM, in the same location as that of ZO-1, namely at the frontier between the apical and the basolateral pole. In contrast, the fraction of InsP₃R-3 that is not concentrated near the PM was distributed in the whole cytoplasm (Fig. 3A). Concerning the staining near the PM, xy and xz sections shows a clear co-localization of InsP₃R-3 and ZO-1, the InsP₃R-3 staining being in some cases a little more laterally spread. However InsP₃R-3 was never found all-over the lateral domain. To confirm the presence of InsP₃R-3 at the tight junction level, polarized MDCK cells were double stained for InsP₃R-3 and for the intrinsic tight junction protein, occludin. Fig. 3B shows that the fraction of InsP₃R-3 present near the PM co-localized with occludin. Finally, double staining of InsP₃R-3 with lateral or apical markers, ErbB2 and gp-114, were also performed (Fig. 3C,D) and once more showed that when MDCK cells were well-polarized, the InsP₃R-3 was highly concentrated at the restricted part of the PM corresponding to the tight junction zone.

To determine the precise localization of InsP₃R-3 in polarized MDCK cells immunogold-electronmicroscopy was used and Fig. 4 shows two representative micrographs. In all cases InsP₃R-3 was concentrated in a restricted intracellular location, at the upper part of the cells and in the vicinity of tight junctions. We did not observe gold particles on the apical surface of the PM, nor along the lateral or the basal one. These results strongly suggest that InsP₃R-3 in polarized cells is mostly present in a subcompartment of the ER very close to the tight junctions.

InsP₃R-3 is not concentrated near the PM when MDCK polarity is disturbed

The relationship between MDCK polarization and the

localization of InsP₃R-3 was analysed in two different cases where MDCK polarity was disturbed. We first examined cells of the ras transformed MDCK cell line f3, that have been shown to be unpolarized and deficient in E-cadherin expression (Chen et al., 2000). As shown in Fig. 5A, the localization of InsP₃R-3 was similar in early and late cultures; a cytoplasmic staining was observed with no accumulation of InsP₃R-3 near the PM, even in late confluent cultures. Moreover, as expected, ZO-1 was not detected and E-cadherin was not expressed (Fig. 5A). Chen et al. have reported that the formation of tight junctions and the reexpression of E-cadherin can be induced in

f3 cells, by inhibiting the mitogen-activated protein kinase kinase (MEK1) (Chen et al., 2000). Therefore f3 cells were treated with the MEK1 inhibitor U0126 (Favata et al., 1998) (Fig. 5B). ZO-1 was detected at the PM of a few percent of cells after 10 hours of treatment and of the majority of cells after 24 hours. E-cadherin was re-expressed later but only in a fraction of treated cells (10 and 30% after 24 and 72 hours, respectively). No change in the localization of InsP₃R-3 occurred, except in a minority (less than 5%) of E-cadherin re-expressing cells, in which InsP₃R-3 was detected near the PM (Fig. 5B). It should be noted that after this treatment, the TER

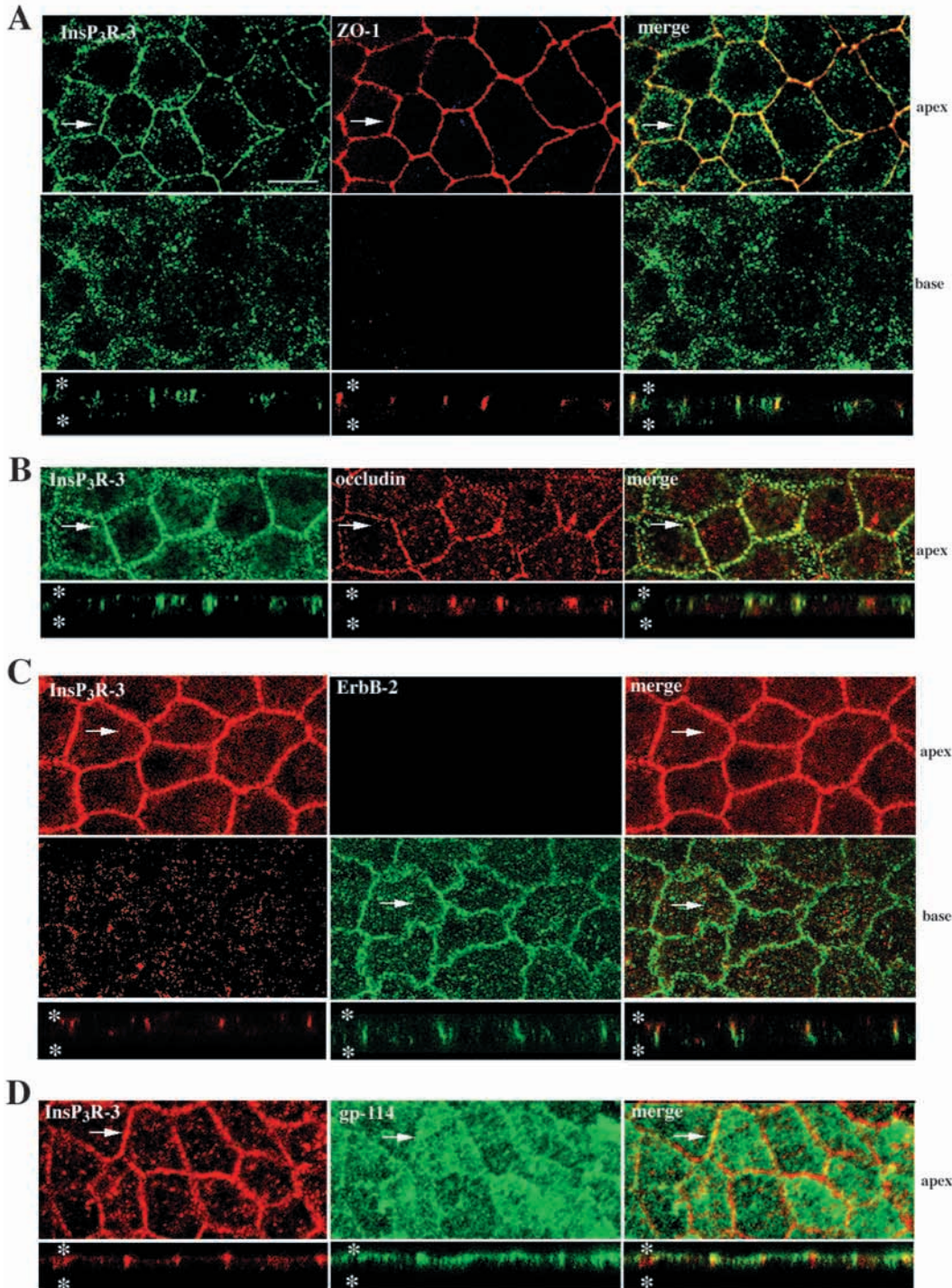


Fig. 3. Double staining of InsP₃R-3 and polarity markers in polarized MDCK cells: confocal analysis. MDCK plated at 10^4 cells/cm² were cultured for 10–12 days and double stained using a monoclonal antibody anti-InsP₃R-3 and antibodies directed against the tight junction associated protein ZO-1 (A), the tight junction protein occludin (B), the lateral marker ErbB-2 (C) and the apical protein gp-114 (D). The immunolocalization obtained for each protein is presented, in addition to the merged image. In A and C, two xy sections taken at the apex and at the base of the cell layer were shown with an xz section taken at the level indicated by the arrow on the corresponding xy sections. In B and D, one apical xy section was shown with an xz section taken at the level indicated by the arrow on the corresponding xy section. The top and the bottom of the cell layer shown in the xz sections are indicated by asterisks (*). In each case a restricted distribution of InsP₃R-3 was observed in the tight junction zone, which corresponds to the frontier between the lateral and the apical pole. This restricted distribution is particularly visible in the xy and xz sections stained for InsP₃R-3 and the lateral marker ErbB-2 shown in C. Bar, 10 μm.

value, initially of 40-50 ohm cm², reached only 120 ohm cm² (Fig. 5B) versus 270 ohm cm² for non-transformed cells (Fig. 1B).

Experiments were then performed on MDCK cells, in order to disrupt cellular junctions by lowering the external calcium concentration. Polarized MDCK cells were placed in calcium-deprived medium for several hours and then placed again in calcium containing-medium. During this calcium switch treatment, the TER was measured and the localization of InsP₃R-3 and ZO-1 followed (Fig. 6). A rapid delocalization of InsP₃R-3 was found in cells deprived of calcium, the protein no longer being concentrated near the PM, after 30 minutes of treatment. ZO-1 was also internalized but less rapidly: after two hours in calcium-deprived medium, some ZO-1 staining was still present at the PM, although these cells, compared to control ones, were less cohesive, rounded up and had a very low TER (50 ohm cm²). When cells deprived of calcium were switched into calcium-containing medium, polarity was re-established (Fig. 6). This re-establishment is a relatively slow phenomenon (TER was completely restored only after 24 hours) and is accompanied by a relocalization at the PM level of both ZO-1 and InsP₃R-3. Whereas the relocalization of ZO-1 occurred rapidly, that of InsP₃R-3 was more progressive; most of this protein was present near the PM, only after 8 hours in calcium containing medium, when cells exhibit a TER of 200 ohm cm² (Fig. 6).

Stably transfected type 1 InsP₃R-EGFP behaves as the endogenous InsP₃R-3 during MDCK polarization

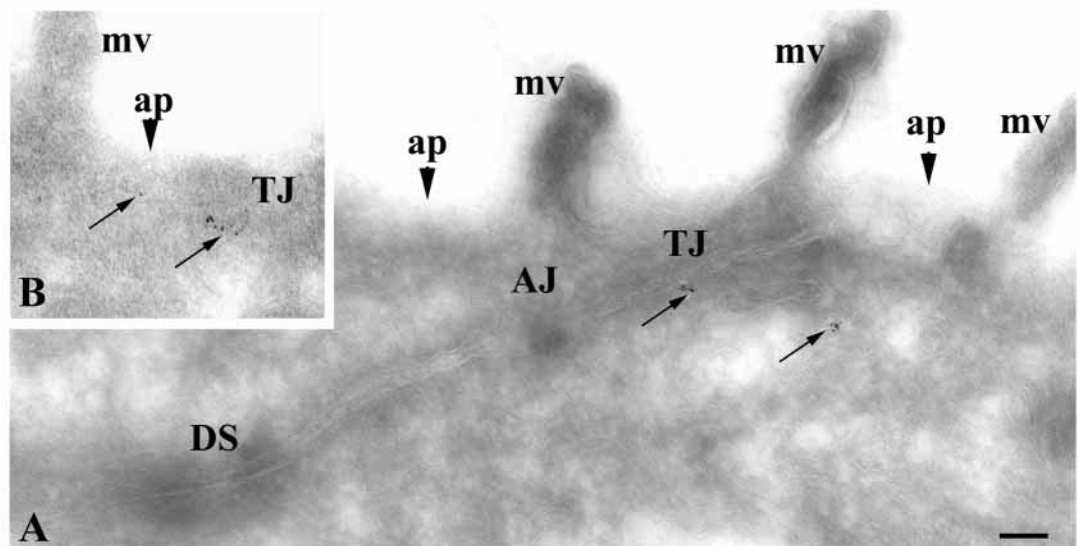
To investigate whether other subtypes of InsP₃-R locate to the tight junction, MDCK cells were transfected with pInsP₃R-1-EGFP, a plasmid coding for the full-length mouse type 1 InsP₃R tagged with EGFP at the C-terminus. Some forty stable transfectants were isolated. In most of these clones, only a minor fraction (5-10%) of cells expressed the transfected receptor. One stable clone called 10B was retained for further analysis: 70-80% of its cells expressed the transfected InsP₃R-

1 and, according to the fluorescence intensity, the protein was expressed at a similar level in the positive cells. The chromosomal content of 10B cells was similar to that of parental MDCK cells (mean chromosome number 74 for both lines with a range of 60-80 and 66-77 for 10B and MDCK, respectively). MDCK cells were also transfected with pEGFP-N1, and the stable transfectant clone 1E was analyzed to investigate the effect of EGFP on its own. EGFP was strongly expressed by all cells of clone 1E, with a broad localization (cytoplasm and nucleus) whatever the polarity state of the cells (results not shown).

The type and the level of the InsP₃R expressed by the two stable transfectants and their MDCK parent was analyzed by western blot using antibodies against InsP₃R-1, GFP and InsP₃R-3, respectively (Fig. 7A). InsP₃R-3 was present in similar amounts in lysates of each clone. Using an antibody anti-type 1 receptor a robust band migrating at the same level as the cerebellar type 1 receptor, was detected only in 10B lysate (Fig. 7A). When blots were probed using an anti-GFP antibody a similar high molecular weight band was present only in 10B cells whereas, as expected, a band with an apparent molecular weight of 30 kDa was detected for the 1E lysate (Fig. 7A). This data shows that the isolated transfected MDCK clones, correctly express the EGFP exogenous proteins introduced by transfection.

The endogenous InsP₃R-3 and the transfected InsP₃R-1-EGFP were localized during the polarization of 10B cells. As shown in Fig. 7B, both InsP₃R displayed the same localization. In 3-day cultures, when cells are non-polarized, a cytoplasmic staining was observed, as previously shown in non-transfected MDCK cells for InsP₃R-3 (Fig. 1). In late cultures (10 days), when cells are well-polarized, both types of InsP₃R were concentrated near the cell PM (Fig. 7B). This change in localization was observed in all 10B cells expressing type 1 InsP₃R. Confocal analysis was performed to refine the localization of the transfected InsP₃R-1-EGFP (Fig. 8). In non-polarized cells (Fig. 8A), this protein was distributed in the cytoplasm in a similar fashion to that of ER proteins, with a slightly more intense perinuclear localization for the InsP₃R-

Fig. 4. Immunoelectron microscopy analysis. InsP₃R-3 was immunostained (6 nm gold particles, arrows) in ultrathin cryosections of polarized MDCK cells. Panel A shows a section passing through the different junctions present along the contact zone between two adjacent cells. The image shown in the inset B is centered on the upper part of the cells, at the limit between the apical pole and the lateral one. InsP₃R-3 labeling was nearly exclusively seen in the vicinity of the tight junction (TJ). AJ, adherens junction; DS, desmosome; ap, apical surface; mv, microvilli. Bar, 100 nm.



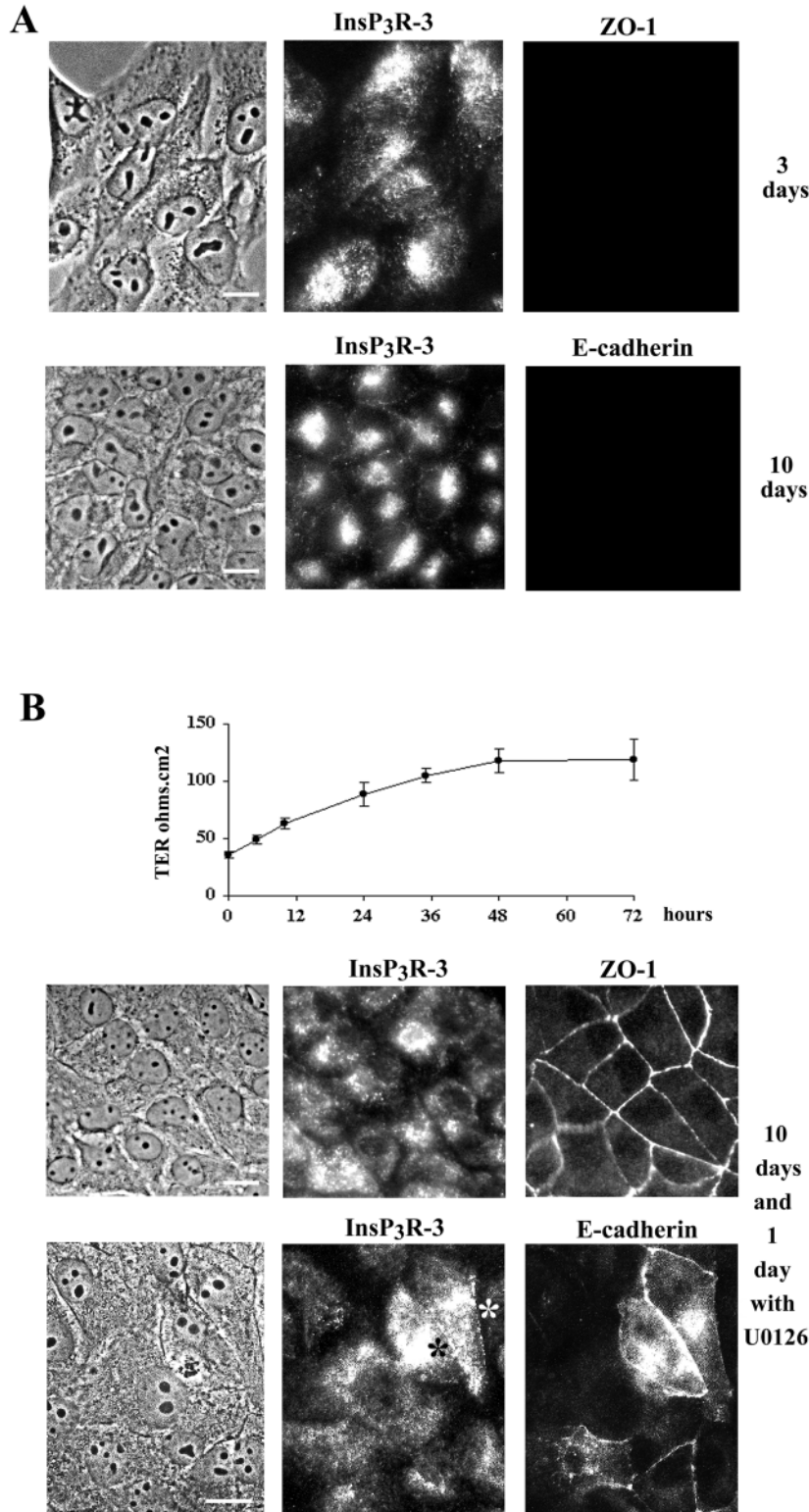


Fig. 5. InsP₃R-3 in non-polarized ras transformed MDCK f3 cells. (A) f3 cells were plated at 10⁴ cells/cm² and examined for the localization of InsP₃R-3 and ZO-1 or E-cadherin after 3 and 10 days, respectively. (B) After 10 days in culture, cells were treated with the MEK inhibitor U0126 (25 μM): TER was measured and cells were double stained for InsP₃R-3 and ZO-1 or InsP₃R-3 and E-cadherin, at regular intervals during this treatment. After 1 day with U0126, ZO-1 was detected at the PM of most cells; E-cadherin was re-expressed in only a fraction of treated cells and in a small minority of those cells InsP₃R-3 was present not only in the cytoplasm, but also at the plasma membrane (*). Bar, 10 μm.

with ER markers (Fig. 8C), with lateral markers such as cadherin(s) recognized by the pan cadherin antibody (Fig. 8D) or with the exocyst component sec6 (Fig. 8E), known to be recruited at the tight junction in MDCK cells (Grindstaff et al., 1998). Moreover the concentration of the transfected type 1 InsP₃R-EGFP in the tight junction area was also seen in living polarized 10B cells (Fig. 8F). These results demonstrate that the presence of InsP₃R at the tight junction level in polarized MDCK cells is a general feature, at least for the type 3 and EGFP-type 1.

Discussion

An increase in the cytosolic Ca²⁺ concentration regulates many of the dedicated functions of polarized cells such as exocytosis and electrolyte and fluid transport. The polarized function of epithelial cell requires specialized organization of signaling complexes and, accordingly, InsP₃R are heterogeneously distributed in different epithelial cell types. In the present work, we have investigated the distribution of the InsP₃R in MDCK cells in relation to the polarity state of the cells.

Changes of InsP₃R localization and MDCK polarity

Western blotting experiments indicated that the type 3 InsP₃R is easily detected in MDCK cells whereas the type 1 and the type 2 receptors are barely detectable. We focused our study on InsP₃R-3 and found that it was redistributed during the development of MDCK polarity. In non-polarized cells, InsP₃R-3 was detected in the cytoplasm and co-localized with different markers of the ER. In contrast, in polarized cells InsP₃R-3 was highly concentrated near the PM. Confocal

microscopy allowed us to clearly show that InsP₃R-3 localized with tight junction markers and not with lateral markers such as ErbB-2 (Fig. 3) cadherins or caveolin (not shown). This localization was confirmed by immunoelectron microscopy. To our knowledge, this is the first description of such a distribution of InsP₃R. The tight junction-like localization has been

1-EGFP (visible when both images were merged). However, in polarized cells most of InsP₃R-1-EGFP was concentrated at the periphery of the cells (Fig. 8A), where it co-localized with the endogenous type 3 receptor at the tight junction level (Fig. 8B). The localization in the tight junction zone of InsP₃R-1-EGFP in 10B polarized cells was confirmed by localizing this protein

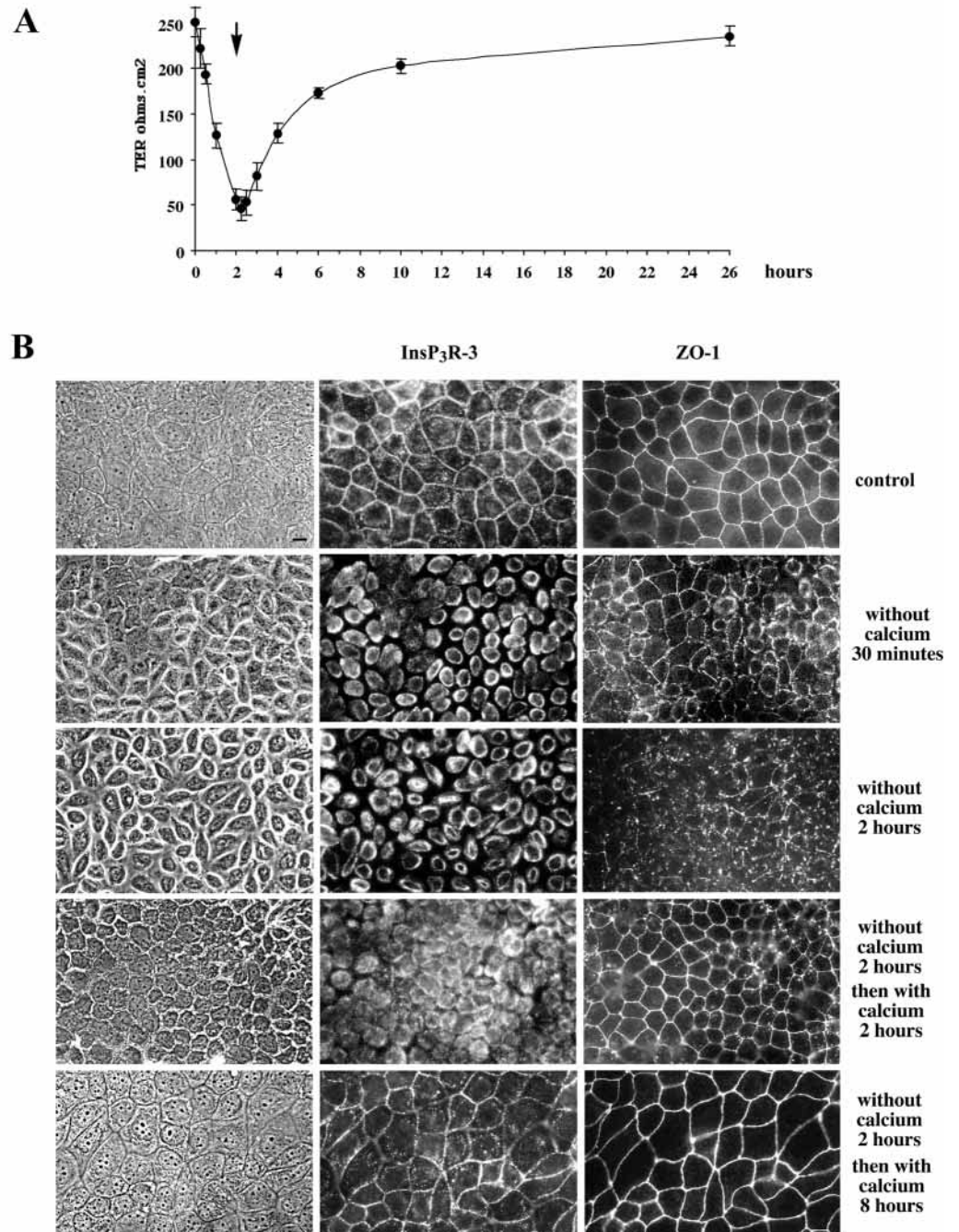


Fig. 6. Changes in InsP₃R-3 and ZO-1 localization in MDCK cells during calcium switch. Polarized MDCK cells were incubated for 2 hours in medium deprived of calcium and then incubated in a calcium-containing medium for 24 hours. (A) TER measurement during this treatment; the arrow indicates the time when the cells were renewed with calcium-containing medium. (B) InsP₃R-3 and ZO-1 localization. Bar, 10 μ m.

observed with two different antibodies directed against different epitopes of the InsP₃R-3 and furthermore, a full-length InsP₃R-1-EGFP fusion protein stably expressed in MDCK cells displayed the same distribution.

Other works using indirect immunofluorescence with antibodies directed against InsP₃R purified from rat cerebellum or against a peptide sequence of mouse InsP₃R-1 indicated that this receptor type localized to the basolateral domain of the plasma membrane of polarized MDCK cells (Bush et al., 1994; Zhang et al., 2003). This suggests that InsP₃R-1 and InsP₃R-3 translocate to different domains of the plasma membrane during the polarization of the MDCK cells. However, in the present work, we have found that InsP₃R-1-EGFP co-localizes

with InsP₃R-3 at the tight junction level. The discrepancy between our results and those previously published could be due to differences between the analysed cell populations which would differently express the protein(s) required for InsP₃R-1 translocation. Alternatively, it is possible that the presence of EGFP at the C-terminal end of the InsP₃R-1 could inhibit the translocation of the receptor to the basolateral membrane and this would be sufficient to permit receptor recruitment at the tight junction level, suggesting that InsP₃R-1 and InsP₃R-3 have common determinant(s) for translocation to the tight junction.

The fact that the localization of InsP₃R-3 depends on the cell polarity state was confirmed by studying MDCK cells

presenting polarity perturbations: calcium-deprived cells and unpolarized f3 ras transformed ones. In both cases InsP₃R-3 was distributed in the cytoplasm. Calcium addition to calcium-deprived cells induces the re-establishment of polarity and the relocalization of the receptor at the tight junction level. However this relocalization was slow and occurred only when the polarity was well re-established (TER > 200 ohm cm²). The addition of MEK1 inhibitor, U0126, to f3 cells allows a partial recovery of TER, the relocalization of ZO-1 at the PM, but not that of InsP₃R-3. These results suggest that the restricted distribution of InsP₃R-3 at the frontier of the lateral and apical pole requires the formation of fully constituted tight junctions. Thus, InsP₃R-3 recruitment would be a late event in the scaffolding of tight junctions and InsP₃R-3 could be a peripheral component of tight junctions rather than an intrinsic one. This hypothesis is in agreement with the observation that InsP₃R-3 is very rapidly redistributed in the cytoplasm when polarized cells are switched in calcium-deprived medium. The InsP₃R-3 redistribution during the Ca²⁺ switch and the immunoelectron microscopy pictures showing that, in polarized cells, this receptor is localized intracellularly rather than in the plasma membrane itself, strongly suggest that during the polarization of the cells, the InsP₃R concentrated in a subdomain of the ER closely associated to the tight junctions. Owing to the generation of MDCK clones expressing fluorescent InsP₃R versions, as the 10B clone described in this report, it will be possible to follow in living cells the localization changes of InsP₃R and thus to approach the mechanisms implicated in these changes.

Interactions of InsP₃R with proteins

The tight junction-like restricted distribution of InsP₃R in polarized MDCK cells opens several questions. The first one concerns this peculiar localization compared to the subapical distribution observed in other epithelial cells. In fact similar results were previously obtained for the sec6/8 complex. This complex is localized at the apical pole of pancreatic acinar cells (Shin et al., 2000), whereas in the case of MDCK, it is found in the cytosol of non-polarized cells and at the apex of the lateral membrane in polarized ones (Grindstaff et al., 1998). Moreover the sec6/8 complex behaves exactly as InsP₃R in MDCK cells submitted to the calcium shift (Grindstaff et al., 1998). This similarity could reflect a close interaction between the two systems. The next question concerns the association and the interactions of InsP₃R with other proteins that would form an ER subdomain in close association with tight junctions. What is the molecular basis of this association? What are the proteins that interact with this receptor? These questions will be approached by determining the regions of InsP₃R implicated in its recruitment in the tight junction area and by looking for possible interactions in polarized cells between InsP₃R and several proteins, in particular tight junction associated ones or tight junction components.

Interactions between InsP₃R and several proteins have been previously described. InsP₃R has been found to be closely associated with the actin network in different cell types and to interact with ankyrin allowing a linkage with the cytoskeleton (Joseph and Samanta, 1993; Rossier et al., 1991). A study with ankyrin-B-deficient mice indicated that ankyrin-B is required for intracellular targeting of Ca²⁺ homeostasis proteins

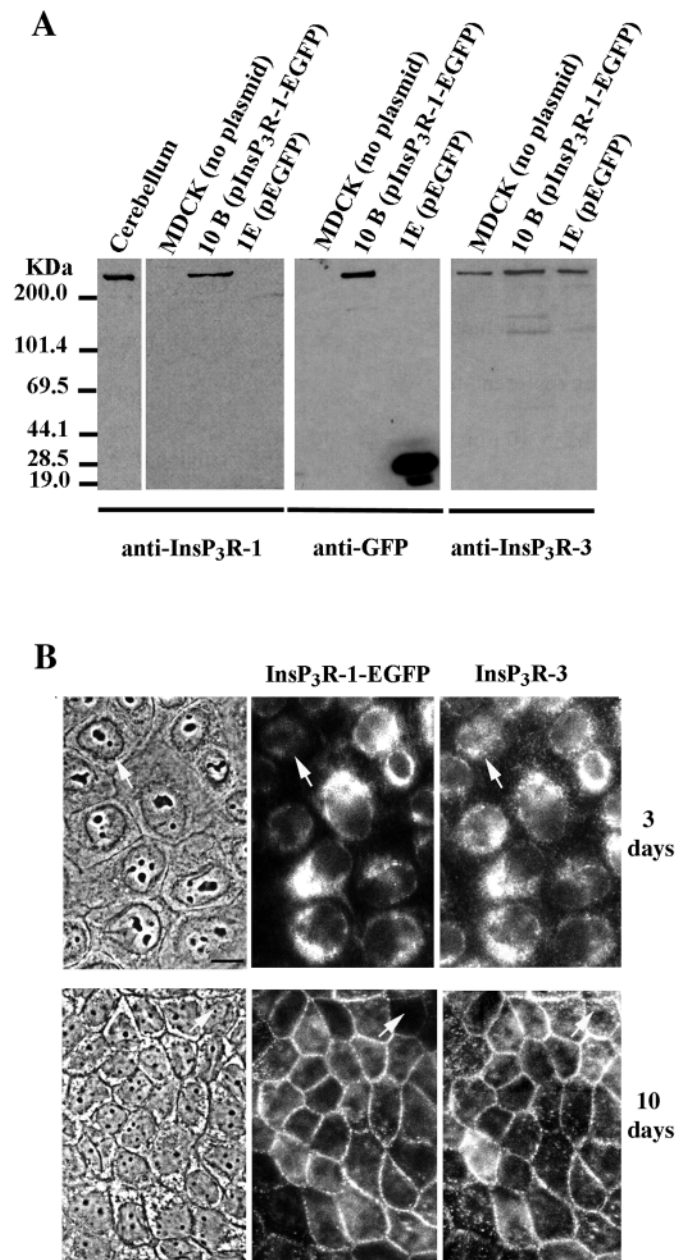
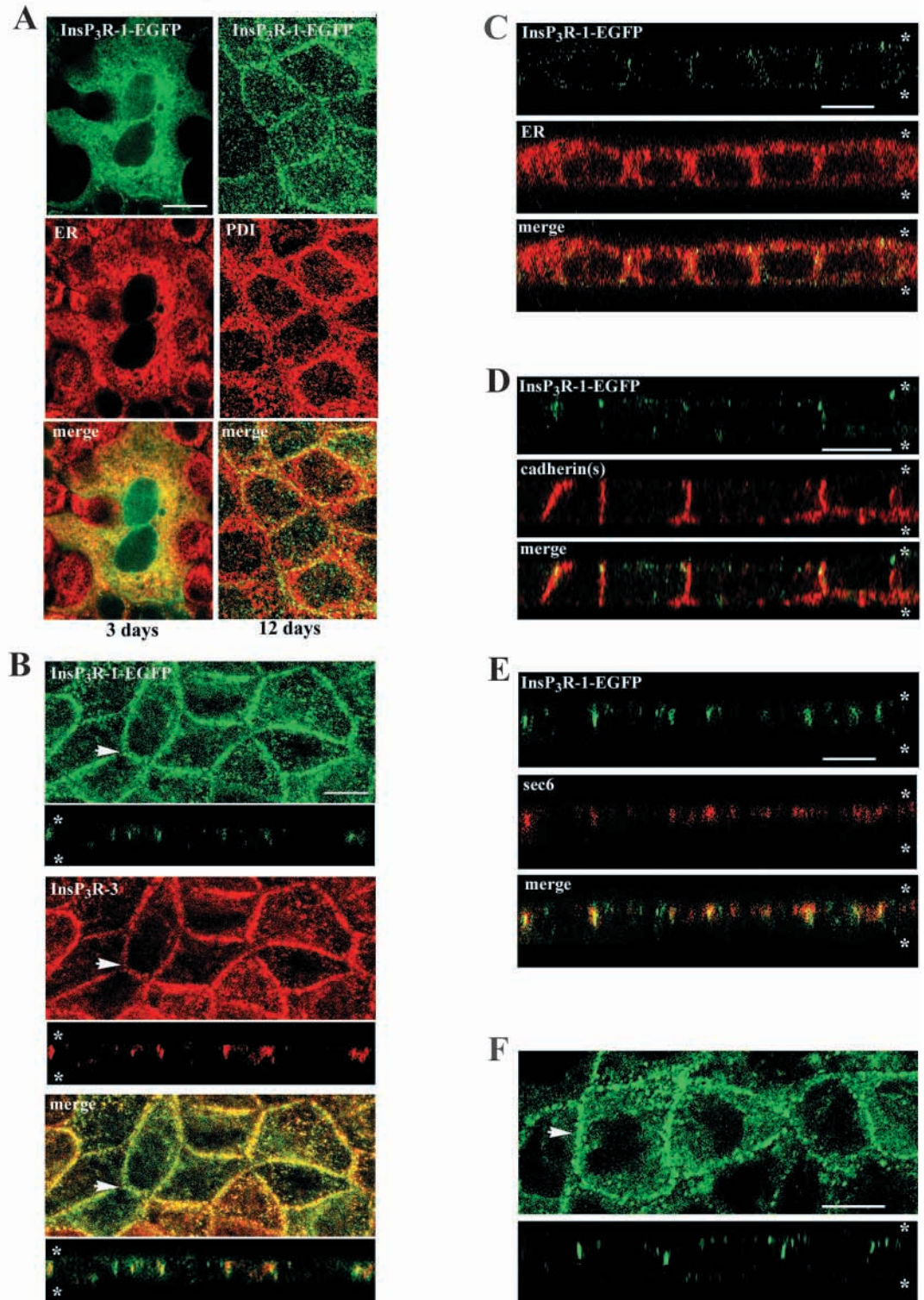


Fig. 7. Analysis of stable transfectant MDCK cells expressing InsP₃R-1-EGFP. (A) Western blot: 50 μg of protein of cell lysates from parental MDCK, clone 10B and clone 1E were loaded on a gradient 4–10% polyacrylamide minigel (BioRad MiniPROTEAN system). The gradient minigel allowed both the small molecular weight of the GFP and the high molecular weight of the InsP₃R to be visualized on the same blot. After transfer, western blotting was performed using antibodies against InsP₃R-1, GFP and N-terminus InsP₃R-3, respectively. (B) Localization of InsP₃R-3 and InsP₃R-1-EGFP in MDCK clone 10B, which stably expressed InsP₃R-1-EGFP. The tagged InsP₃R-1 is expressed by most cells of this clone, except a few (arrows), whereas all cells express InsP₃R-3. Bar, 10 μm.

including InsP₃R (Tuvia et al., 1999). In vivo and in vitro interactions between the type 1 InsP₃R and 4.1N, a homolog of the erythrocyte membrane cytoskeleton protein 4.1 have been recently reported (Zhang et al., 2003). InsP₃R is also

Fig. 8. Confocal analysis of MDCK 10B cells expressing InsP₃R-1-EGFP.
 (A) Localization of InsP₃R-1-EGFP and proteins of the endoplasmic reticulum in non-polarized (3 days) and polarized (12 days) 10B cells. For the 3-day culture an xy section taken in the middle of the cell layer is presented; note the more intense perinuclear staining of InsP₃R-1-EGFP, particularly visible in the merged image. For the 12-day culture a complete compiled z series of 27 xy sections taken in 0.3 μm steps is shown.
 (B) Localization of both types of InsP₃-R in polarized 10B cells. The staining obtained for each protein is presented in addition to the merged images. The images shown correspond to a complete compiled z series of 30 xy sections taken in 0.3 μm steps with an xz section taken at the plane indicated by the arrow on the corresponding compilation.
 (C) Localization of InsP₃R-1-EGFP and ER proteins in polarized 10B cells: an xz section of cells stained with a polyclonal antibody directed against a bulk of ER proteins is shown. The staining for each protein and the merge image are presented. InsP₃R-1-EGFP is mostly present at the upper part of the lateral pole, whereas ER proteins are widely distributed around the nucleus, in the whole cytoplasm.
 (D) Localization of InsP₃R-1-EGFP and cadherin(s) in polarized 10B cells: an xz section of cells stained with anti-pan cadherin is shown, the staining obtained for each protein being presented in addition to the merged image. Note the restricted distribution of InsP₃-R-1-EGFP at the top of the lateral pole.
 (E) Localization of InsP₃R-1-EGFP and sec6 in polarized 10B cells: an xz section is shown with the staining for each protein and the merged image.
 (F) Localization of InsP₃R-1-EGFP in living polarized 10B cells. An xy section and an xz one taken through the cells at the plane indicated by the arrow are presented. The top and the bottom of the cell layer shown in all the xz sections are indicated by asterisks (*). Bars, 10 μm.



involved in communication between the ER and proteins of the plasma membrane. There is evidence that Homer proteins form a physical tether linking metabotropic glutamate receptors with the InsP₃R; this link could contribute to the regulation of

intracellular calcium and long-term neuronal plasticity (Tu et al., 1998). InsP₃R also interacts with TRP Ca²⁺ channel and this interaction plays an important role in the stimulation of Ca²⁺ entry from the extracellular medium (Kiselyov et al.,

1998). Moreover it has been found that InsP₃R, like several Ca²⁺ signaling proteins associates with the Sec6/8 complex in neuronal cells and in pancreatic acinar cells (Shin et al., 2000)

Role of InsP₃R at the tight junction

The presence of the Sec6/8 complex in the proximity of the tight junctions of polarized MDCK cells has been associated with exocytosis and the delivery of vesicles to the basolateral membranes. The co-localization of the InsP₃R and proteins of the Sec6/8 complex asks about a putative role of Ca²⁺ on this delivery. In other epithelial cell types the initiation site of the Ca²⁺ wave, found at the luminal pole, has been associated to a higher sensitivity of this region to InsP₃R linked to a higher density of InsP₃R (Kasai et al., 1993; Lee et al., 1997; Nathanson et al., 1994b; Thorn et al., 1993). It can be assumed that the proximity of this Ca²⁺ release channel will provide the increase in the intracellular Ca²⁺ concentration necessary for the fusion of vesicles with the plasma membrane. This could participate in the establishment and maintenance of the cell polarity.

Another putative function of intracellular Ca²⁺-release channel in the tight junction area is the regulation of the paracellular permeability. In MDCK cells, intracellular Ca²⁺ may be necessary for intermediate sorting and biochemical stabilization steps in tight junction and desmosome biogenesis and therefore may regulate tight junction permeability (Stuart et al., 1996). On the other hand, a Ca²⁺-dependent phosphorylation of myosin light-chain could regulate the contraction of the peri-junctional actomyosin ring and, thus, the permeability of tight junctions. In monolayers of MDCK cells expressing the catalytic domain of the myosin light-chain kinase (MLCK), the increase in phosphorylation of myosin light chain was correlated with a decrease of TER (Hecht et al., 1996). It can be assumed that a local increase of Ca²⁺ concentration could be a key event allowing the regulation of the MLCK (Turner, 2000).

Finally, tight junctions may regulate the availability of signal transduction molecules and participate in the regulation of cell growth and differentiation. Such a feature has been described for the transcription factor ZONAB which interacts with ZO-1 and regulates ErbB-2 expression in proliferating MDCK cells. The complex is recruited at the tight junctions in polarized cells and therefore is no longer expressed in the nucleus (Balda and Matter, 2000). It is interesting to note that in proliferating non-polarized MDCK cells, InsP₃R is found in the cytoplasm and particularly around the nucleus. This could provide the Ca²⁺ necessary for the regulation of gene expression and proliferation of the cells. During the polarization of the cells, InsP₃R is concentrated at the tight junctions level, away from the nucleus and thereby possibly limiting cell proliferation.

The data presented in this paper demonstrate that there is a redistribution of InsP₃R during the polarization of MDCK cells independently of the type of receptor expressed. The establishment of the 10B MDCK clone, that stably expresses InsP₃R-1-EGFP, provides a nice tool for studying in living cells the receptor subcellular localization, mobility and redistribution during events such as cell polarization.

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