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The effects of PMA and TFP and alterations in intracellular pH and calcium concentration on the membrane associations of phospholipid-binding proteins fodrin, protein kinase C and annexin II in cultured MDCK cells

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Annexin II, α-fodrin and protein kinase C (PKC) are associated with the cytoplasmic surface of the plasma membranes. When assayed with liposomes, they show affinity for acidic phospholipids and bind calcium ions. They also respond to or participate in cell signal transduction by altered membrane binding properties. In the present work we have studied the properties of these proteins in epithelial MDCK cells in response to elevated intracellular calcium ion concentration, lowered pH, treatment with tumor promoter phorbol myristoyl acetate (PMA) and calmodulin inhibitor trifluoperazine (TFP). In untreated polarized MDCK cells annexin II was seen both along the lateral walls and membranes of intracellular vesicles, fodrin was located along the lateral walls, whereas PKC was seen in the cytoplasm. There was no observable translocation of these proteins upon elevation of the intracellular calcium concentration using a calcium ionophore A23187. On the other hand, treatment with TFP led to a release of annexin II from the plasma membranes which was accompanied by a transient peak in the intracellular calcium. Treatment with PMA led to a loss of the cubic form of the cells, a slight elevation in the intracellular calcium concentration and a drop in the intracellular pH. Simultaneously fodrin was released from the lateral walls, but still remained insoluble in Triton X-100, PKC became associated with the intracellular membranes and fibers, whereas annexin II remained along the lateral walls. These changes could be prevented by clamping the intracellular pH neutral during PMA treatment. On the other hand, lowering of intracellular pH below 6.5 with the nigericin treatment led to a similar translocation of fodrin and PKC as PMA. This suggests that the protein redistribution is caused by cytoplasmic acidification and is due to an increased hydrophobicity and enhanced protonation of lipids and proteins. In contrast, no changes were seen in the annexin II distribution in response to altered pH. Hence, its release by TFP is presumably due to changes in the cationic properties of the inner phase of the plasma membrane. Thus, proteins which show similar binding properties with liposomes show different characteristics in their association with the intracellular membranes.

Introduction

In virtually all types of cells the interface between the plasma membrane and cytosol comprises a group of proteins which show affinity for acidic phospholipids. These include, e.g., protein kinase C (PKC), annexins and spectrin or spectrin-like proteins, fodrins. In vitro they all show affinity to liposomes or micelles containing anionic phospholipids, especially phosphatidylserine (PS) [1–3]. As to their function these proteins belong to widely diverging categories: PKC is an enzyme catalysing protein phosphorylation [4], annexins act as inhibitors of phospholipase A_2 and link secretory vesicles to the plasma membrane [5,6], whereas fodrin is the major constituent of the membrane skeleton [7]. Besides their location and lipid-binding properties many of these proteins share the property of binding or responding to calcium, an important second messenger in signal transduction.

PKC is the best-known member of a class of proteins which simultaneously interact with calcium and phospholipids. Its activity is in a complex manner dependent on its interactions with the plasma membrane and messengers generated in the lipid phase [4,8,9]. Another group of calcium- and lipid-binding proteins are annexins, a structurally related family of proteins which consists of at least twelve members [10]. One of them, annexin II is a widely distributed cytosolic protein found, e.g., in lung and intestinal epithelial cells. It

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is a substrate of the Rous sarcoma virus pp60$^{v\text{-}src}$-kinase and PKC, associates with the plasma membrane in the presence of micromolar concentrations of calcium and also binds actin and fodrin [11–13]. Also the components of the membrane skeleton, protein 4.1, ankyrin and fodrin interact directly with lipid bilayers [14,15]. Moreover, fodrin is able to bind calcium and calmodulin [16–18].

In a series of studies we have investigated the behaviour of the fodrin-actin skeleton in polarized epithelial Madin-Darby canine kidney (MDCK) cells in order to elucidate its function in the establishment of the cellular polarity and its putative role in signal transduction [19,20]. Upon treating the cells with a tumor promoter phorbol myristoyl acetate (PMA) or lowering the intracellular pH below 6.5, fodrin was released from the lateral walls of the MDCK cells to the cytoplasm in a reversible manner. Moreover, rebuilding of the membrane skeleton by restoring the neutral pH could be prevented by trifluoperazine (TFP).

In view of their anionic phospholipid binding-properties and their involvement in such important and complex phenomena as exocytosis it is important to know what is the parallel behaviour of PKC, annexins and fodrin in response to changes in parameters relevant to these events. Therefore, in the present work we have manipulated the intracellular calcium concentration and pH of MDCK cells, treated them with PMA or TFP, and monitored the distribution and solubility properties of annexin II, fodrin and PKC. The results suggest that the association of PKC and fodrin, but not of annexin II, with the plasma membrane lipids and intracellular structures is regulated mostly by the hydrophobicity and degree of protonation of lipids and proteins. On the other hand, there was no correlation between intracellular pH and the location of annexin II which was released from the plasma membrane by TFP.

Part of this work has been published in an abstract form [21,22].

Materials and Methods

Cell strain and culture conditions. MDCK cells (strain II; kindly provided by Professor Kai Simons, EMBL, Heidelberg, Germany) were grown in Eagle's minimal essential medium with Earle's salts (MEM; Gibco, Gaithersburg, MD, USA), supplemented with 2 mM glutamine, 10% fetal calf serum (FCS), and antibiotics. The cells were grown on collagen-coated glass cover slips for measurements of intracellular pH and calcium and for immunofluorescence microscopy. Confluent monolayers were used in all experiments. Metabolic labelling of the cells with [^{35}S]methionine was carried out as described earlier [19].

Manipulation of intracellular calcium concentration. Intracellular calcium concentration was elevated with the aid of a calcium ionophore A23187 (Calbiochem, San Diego, USA). It was stored in dimethylsulfoxide (Me_2SO) at a concentration of 1.9 mM and diluted before use with a low calcium medium (S-MEM) containing 0.63 mM $CaCl_2$ (pH 7.2) to a final concentration of 100 nM. The cells were incubated with A23187 for 10 min.

Treatment with TFP. Calmodulin antagonist TFP (Calbiochem) was stored in Me_2SO at a concentration of 12 mM. Before use, it was diluted either with Hanks' balanced salt solution or low pH buffer (140 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM KH_2PO_4, 20 mM Hepes (pH 5.5)) to a final concentration of 120 μM.

Treatment with PMA. PKC-activator phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO, USA) was stored in Me_2SO at a concentration of 5 mg/ml. It was diluted before use with Hanks' balanced salt solution to a final concentration of 20 ng/ml. The cells were incubated with PMA for 60 min. For some PMA experiments 5 μM nigericin was added to the Hanks' balanced salt solution or isotonic KCl buffer (140 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM KH_2PO_4, 20 mM Hepes (pH 7.4)), containing 20 ng/ml PMA to keep the intracellular pH neutral.

Manipulation of extra- and intracellular pH. Acidification of the cells and restoration of the physiological pH (acidification/neutralization cycle) was achieved as described earlier [19]. Briefly, for acidification, the cells were incubated with 5 μM nigericin (Sigma, St. Louis, MO, USA) in isotonic KCl buffer (140 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM KH_2PO_4, 20 mM Hepes) at pH 5.5 at 37°C for 30 min. For neutralization, the acidified cells were incubated in Hanks' balanced salt solution (pH 7.4, Gibco).

The effect of TFP on the recovery from the low pH was studied as follows: the cells were first acidified with nigericin treatment for 30 min as described above. For the last 10 min TFP was added to the acidification medium, whereafter the cells were returned to Hanks' balanced salt solution containing 120 μM TFP [20].

Controls. To control the effect of the solvent, the cells were incubated with 1% (v/v) Me_2SO in Hanks' balanced salt solution.

Immunostaining and fluorescence microscopy. For annexin II and fodrin staining, the cells grown on collagen-coated glass cover slips were fixed in 4% formaldehyde in a cytoskeleton-stabilizing buffer (100 mM Pipes, 5 mM EGTA, 2 mM $MgCl_2$ (pH 6.8)), containing 0.1% Triton X-100 [23]. They were then washed with phosphate-buffered saline (PBS; 145 mM NaCl, 10 mM phosphate (pH 7.4)), and post-fixed in cold methanol for 5 min. For PKC staining cold methanol (−20°C) was used as a fixative. After repeated washings with PBS, the cells were incubated

with 10% FCS to saturate non-specific protein binding sites. This was followed by incubation with a primary antibody at +4°C for 30 min, and then with FITC-conjugated antimouse antibody (Dakopatts, Glostrup, Denmark). The cells were mounted in Shandon mounting liquid (Immu-Mount, Pittsburg, PA, USA), and viewed under Olympus BH2 or Zeiss Axiovert 405M microscope. Kodak TMAX 3200 ASA film was used for photography.

The monoclonal anti-α-fodrin antibody (AA6101, cross-reacting with mammalian α-fodrin; [24]) was a kind gift of Professor Ismo Virtanen, Department of Anatomy, Helsinki, Finland. A monoclonal antibody against α- and β-species of PKC was purchased from Amersham International (clone MC5, Amersham, UK). A monoclonal antibody against bovine lung annexin II was purchased from Zymed Laboratories (clone Z014, San Francisco, CA, USA).

Measurement of intracellular Ca^{2+}. Fura-2/AM (acetoxymethyl ester of fura-2), pluronic F-127 and 4-bromo-A23187 were from Molecular Probes (Eugene, OR, USA). Just before use fura-2/AM was dissolved in 50 μl Me_2SO and 50 μl FCS containing 0.025% pluronic F-127 to obtain 0.5 mM fura-2/AM stock solution. This mixture was diluted to a final concentration of 5–8 μM fura-2/AM in an appropriate medium and the cells were incubated for 30–60 min. After loading with the fluorophore, the cells were washed to remove extracellular fura-2/AM and the coverslips were placed in a flow-through perfusion chamber that allowed the change of the medium.

Intracellular free Ca^{2+} concentration was measured with an image analysis system (Imaging Research) consisting of Nikon phase contrast microscope, Nikon Fluor 40 Ph3DL objective, Videoscope International amplifier, MTI CCD72 camera and Intel 486 microcomputer. Measurements were carried out using a fluorescence ratio method [25]. The cells were excited alternately with light of wavelengths of 340 and 380 nm by computer-controlled switching of Omega filters. Emitted light was passed through a 400 nm dichroid mirror and was collected through a Nikon 510 nm

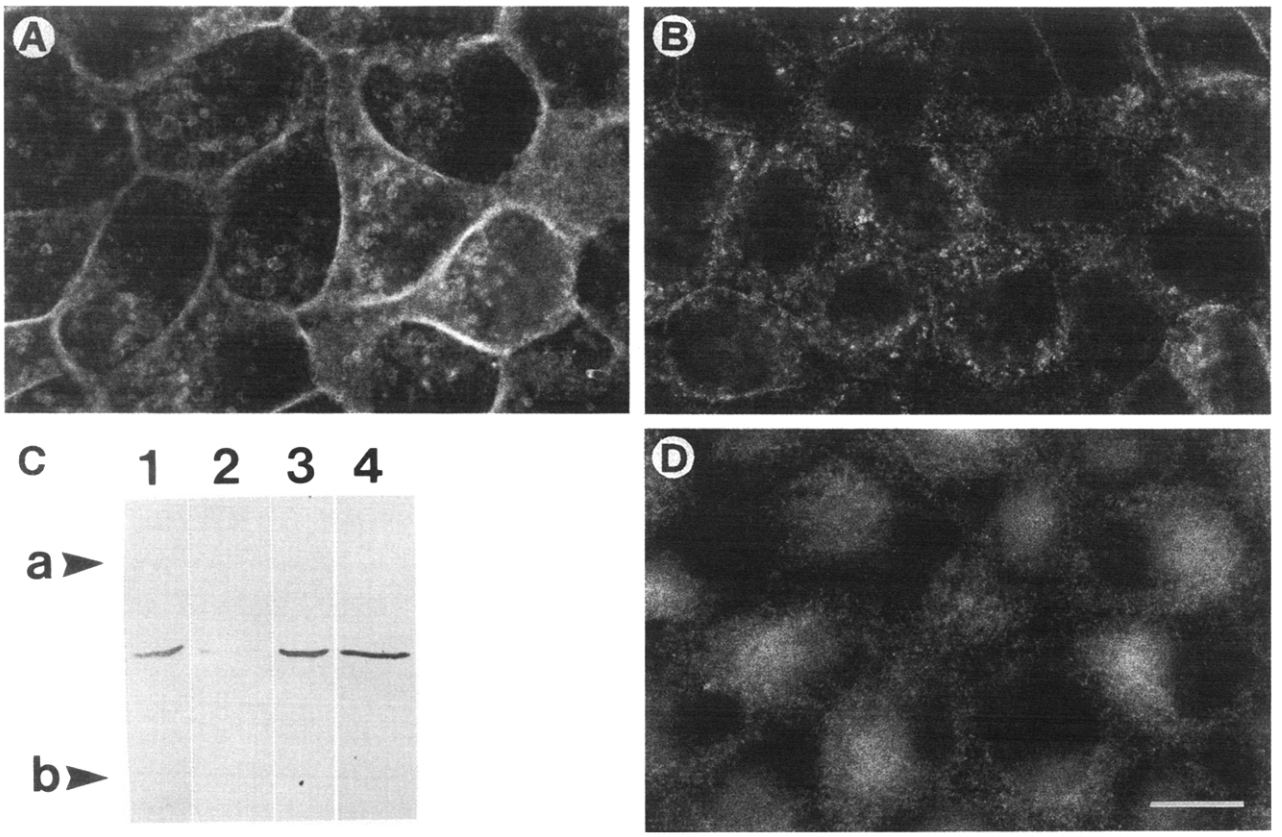

Fig. 1. Fluorescence micrographs showing the localization of annexin II in untreated MDCK cells (A), and in MDCK cells after treatment with 120 μM TFP produced in Hanks' balanced salt solution for 10 min (B). Immunoblotting with annexin II antibodies of MDCK cells (C): Lane 1: Triton-soluble fraction of untreated cells. Lane 2: Cytoskeletal fraction of untreated cells. Lane 3: Triton-soluble fraction of TFP-treated cells. Lane 4: Cytoskeletal fraction of TFP-treated cells. The arrows show protein standards ovalbumin (M_r 45000; a) and carbonic anhydrase (M_r 29000; b). Fluorescence micrograph showing the localization of annexin II in MDCK cells acidified to pH 5.5 for 20 min without TFP, 10 min with 120 μM TFP and then returned to Hanks' balanced salt solution containing 120 μM TFP for 30 min, bar: 10 μm (D).

barrier filter. Fluorescence intensities were measured and the ratio F_{340}/F_{380} was obtained pixel by pixel to produce a ratio image of the microscope field.

Intracellular free Ca^{2+} was determined from the relation [25]

$$[Ca^{2+}]_i = K_d\{(R - R_{min})/(R_{max} - R)\} \times \beta \quad (1)$$

where K_d = dissociation constant for Ca^{2+} binding to fura-2, $R = F_{340}/F_{380}$, F_{340} = fluorescence intensity at 340 nm excitation, F_{380} = fluorescence intensity at 380 nm excitation, $R_{max} = R$ at saturating levels of Ca^{2+}, $R_{min} = R$ at zero Ca^{2+}, and $\beta = F_{380}$(zero Ca^{2+})$/F_{380}$ (saturating Ca^{2+}). R_{max} was achieved by perfusing the cells in Hanks' buffer solution containing 1.24 mM Ca^{2+} and 2 μM 4-bromo-A23187. R_{min} was achieved by perfusing the cells in the same buffer containing 10 mM EGTA and 2 μM 4-bromo-A23187. K_d values were obtained from Lattanzio et al. [26]. At low pH, the calcium binding capacity of fura-2 is decreased and the levels of the K_d values were changed accordingly: at pH 7.3 K_d is 130 nM and at pH 5.5 K_d is 1000 nM. For each pH value the K_d values were estimated from the data of Lattanzio et al. [26].

Measurement of intracellular pH. Membrane permeant fluorescent pH indicator BCECF/AM (acetoxymethyl ester of indicator 2',7'-bis(carboxymethyl)-5(6)-carboxyfluorescein) was from Molecular Probes. BCECF/AM was stored as 1 mM stock solution in Me_2SO. Intracellular pH was measured with the same equipment as Ca^{2+} after loading the cells with 1 μM BCECF/AM. After 30-min incubation the cells were washed with an appropriate medium and the coverslips were placed in a flow-through perfusion chamber.

The cell-assosiated BCECF fluorescence intensity was imaged as for intracellular Ca^{2+} measurements described above. The cells were excited by alternating the 440 nm and 495 nm excitation filters. The emitted fluorescence light was passed through a 510 nm dichroid mirror and 515 nm barrier filter and the fluorescence ratio F_{495}/F_{440} was measured. Because the amount of the fluorophore varied for every experiment, the intensity ratios had to be normalized as follows: at the end of the experiment the cells were

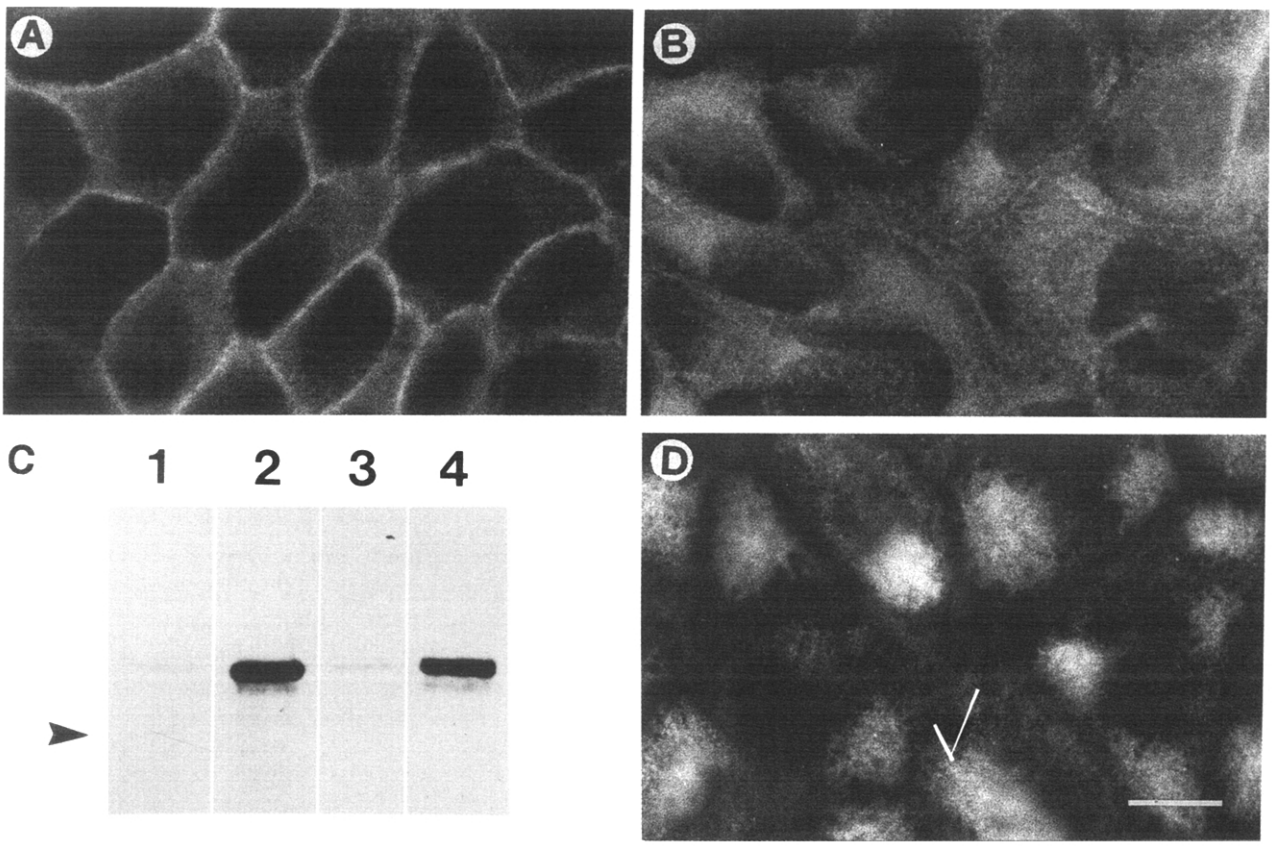

Fig. 2. Fluorescence micrographs showing the localization of α-fodrin in untreated MDCK cells (A), in MDCK cells after treatment with PMA (20 ng/ml produced in Hanks' balanced salt solution) for 60 min (B). Immunoprecipitation with α-fodrin antibodies of metabolically labelled MDCK cells (C): Lane 1: Triton-soluble fraction of untreated cells. Lane 2: Cytoskeletal fraction of untreated cells. Lane 3: Triton-soluble fraction of PMA-treated cells. Lane 4: Cytoskeletal fraction of PMA-treated cells. The arrow shows a protein standard myosin (M_r 200000). Fluorescence micrograph showing the localization of α-fodrin in MDCK cells acidified to pH 5.5 for 20 min without TFP, 10 min with 120 μM TFP and then returned to Hanks' balanced salt solution containing 120 μM TFP for 30 min, bar: 10 μm (D).

exposed to a medium containing 140 mM KCl and 10 μM nigericin at pH 7.0 and the fluorescence ratio was measured. The ratio value obtained in the experiment was divided by the ratio value obtained at pH 7.0. This normalized ratio value was then converted to an intracellular pH value with the aid of a normalized calibration curve. The calibration curve was obtained by exposing the cells to the 140 mM KCl media containing 10 μM nigericin at pH values 6.15–7.5, dividing the measured intensity ratios by the ratio at pH 7.0 and plotting a calibration curve $R(pH_i)/R(pH\ 7.0) = f(pH_i)$, $pH_i = 6.15$ to 7.5 [27].

Solubility assays, immunoprecipitation and immunoblotting. The solubility properties of fodrin in PMA-treated cells were investigated by immunoprecipitating [^{35}S]methionine-labelled cells as described earlier [19,20].

The solubility properties of annexin II were studied by immunoblotting. The proteins of the Triton-soluble and -insoluble fractions of the unlabelled MDCK cells were separated in SDS-PAGE (12.5%) and transferred to nitrocellulose membrane. They were then incubated with anti-annexin II antibodies, followed by biotinylated rabbit antimouse immunoglobulins (Dakopatts, Glostrup, Denmark) and avidin-biotinylated horseradish peroxidase complex (AB-HRP-Complex, Dakopatts). Immunoblots were developed with 3,3-diaminobenzidine tetrahydrochloride (DAB, Fluka AG, Buchs, Switzerland) in the presence of 0.01% hydrogen peroxide and 0.03% $NiCl_2$.

Results

Five types of experiments were carried out with MDCK cells: (1) treatment with A23187; (2) treatment with TFP; (3) treatment with PMA; (4) acidification/neutralization cycle; and (5) treatment with TFP during acidification/neutralization cycle. The location of annexin II, fodrin and PKC was registered by immunofluorescence microscopy. Intracellular pH and intracellular free calcium ion concentration were measured by fluorometry.

Annexin II. In untreated polarized MDCK cells annexin II was seen both along the lateral walls and membranes of intracellular vesicles (Fig. 1A). When the cells were incubated in Hanks' balanced salt solution with 120 μM TFP for less than 10 min the membrane fluorescence disappeared, while the vesicular

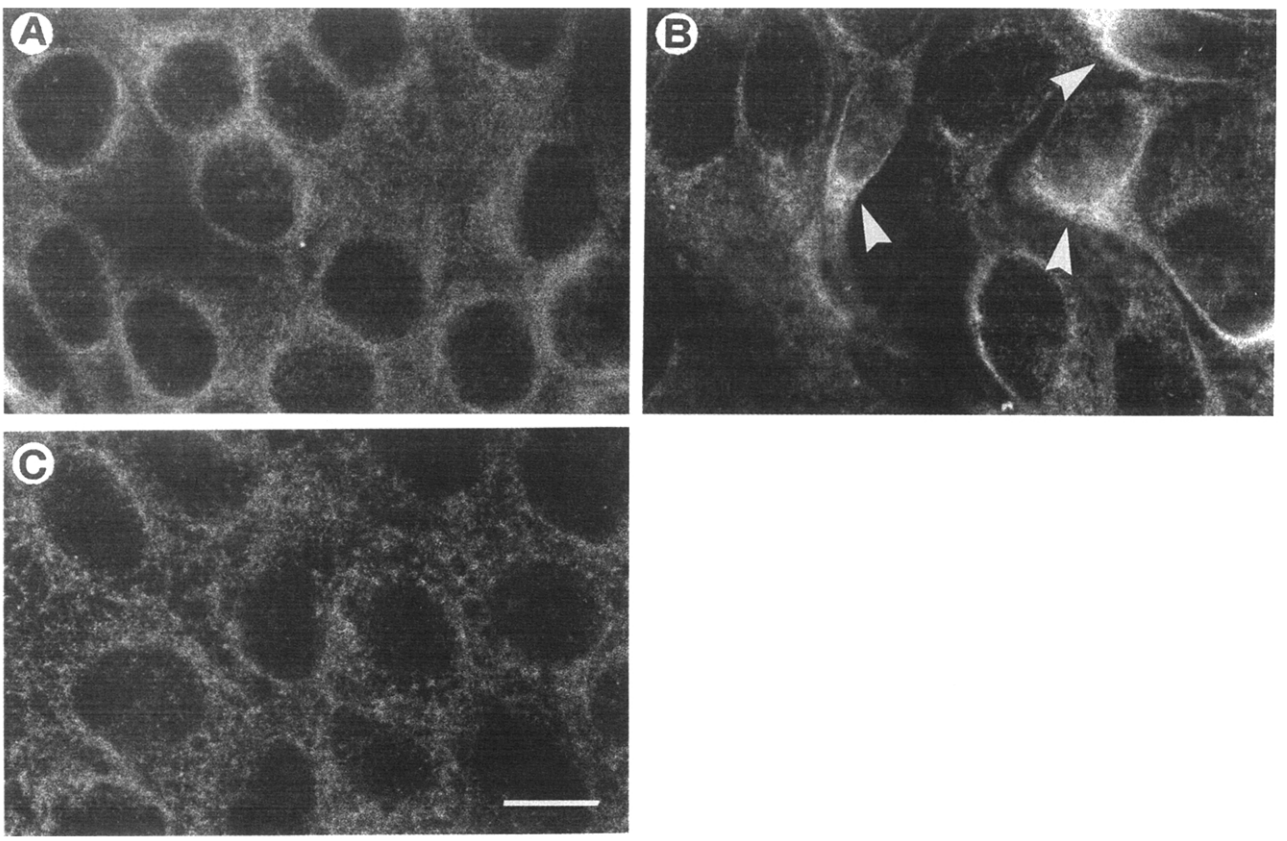

Fig. 3. Fluorescence micrographs showing the localization of PKC in untreated MDCK cells (A), in MDCK cells after treatment with PMA (20 ng/ml produced in Hanks' balanced salt solution) for 60 min (B), in MDCK cells after treatment with PMA (20 ng/ml produced in 140 mM KCl buffer containing 5 μM nigericin at pH 7.4) for 60 min, bar: 10 μm (C). Arrowheads show binding of PKC into cytoskeletal fibers upon PMA treatment (B).

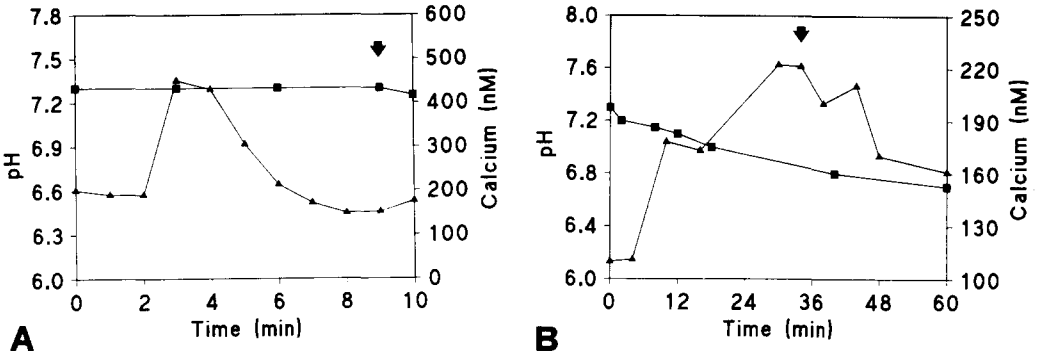

Fig. 4. Intracellular pH (■) and free Ca^{2+} (▲) of MDCK cells as a function of time during perfusion of the cells with TFP solution (A). The total volume of the measuring chamber was changed in one minute after starting the perfusion. An arrow denotes the time point for the release of annexin II from the plasma membrane. Intracellular pH (■) and free Ca^{2+} (▲) of MDCK cells as a function of time during perfusion of the cells with PMA solution (B). An arrow denotes the time point for the release of fodrin from the plasma membrane and for the association of PKC to cytoskeletal elements.

cytoplasmic staining remained visible (Fig. 1B). Concomitantly with the translocation, it became less soluble in 0.5% Triton X-100 and part of it was found in the Triton-insoluble cytoskeletal fraction (Fig. 1C).

There was no alteration in the distribution of annexin II in the cells treated with A23187 or PMA. Neither did acidification of the cytoplasm alter the membrane localization of annexin II (data not shown). However, it began to form a cytoplasmic cluster within 30 min upon returning the cells from an acidic medium to neutral in the presence of TFP (Fig. 1D).

Fodrin. In untreated polarized MDCK cells fodrin

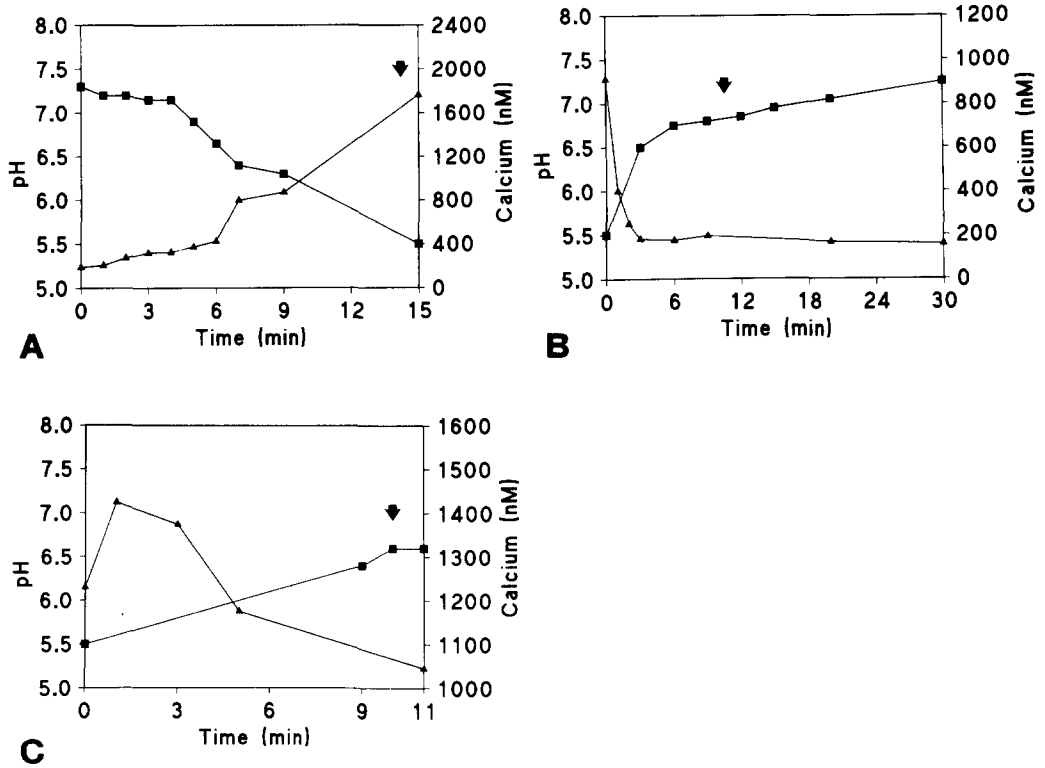

Fig. 5. Intracellular pH (■) and free Ca^{2+} (▲) of MDCK cells as a function of time during acidification of MDCK cells by perfusion with 140 mM KCl containing 5 μM nigericin, pH 5.5 (A). The lowest measured pH value is 6.3 after 9 min perfusion. The final value, pH 5.5, is based on the presumption that extra- and intracellular pH are at equilibrium. An arrow denotes the time point for the release of fodrin from the plasma membrane and for the association of PKC to cytoskeletal elements. Intracellular pH (■) and free Ca^{2+} (▲) of MDCK cells first acidified to pH 5.5 for 30 min and then returned to Hanks' balanced salt solution having pH 7.4 (B). The graphs show pH and calcium as a function of time from the start of perfusion of the acidified cells with Hanks' balanced salt solution. An arrow denotes the time point for the relocation of fodrin and PKC. Intracellular pH (■) and free Ca^{2+} (▲) of MDCK cells first acidified to pH 5.5 for 20 min without TFP, 10 min with 120 μM TFP and then returned to Hanks' balanced salt solution containing 120 μM TFP (C). The graphs show pH and calcium as a function of time from the start of the perfusion of the acidified cells with Hanks' balanced salt solution containing 120 μM TFP. An arrow denotes the time point for the formation of α-fodrin cluster.

was located along the lateral walls (Fig. 2A). No effect on the fodrin distribution was observed by the treatment with A23187 or TFP (data not shown). After 30-min incubation with PMA at a concentration of 20 ng/ml the cells acquired a more elongated morphology and fodrin was released from the lateral walls into the cytoplasm (Fig. 2B, see also Huotari et al. [20]). It still remained insoluble in 0.5% Triton X-100 (Fig. 2C). When the PMA treatment was carried out in the presence of nigericin at neutral pH, there was no change in the cell morphology and fodrin remained in its normal residence (data not shown).

Upon returning the neutral pH in the presence of TFP, fodrin, like annexin II started forming a cytoplasmic cluster (Figs. 1D and 2D).

PKC. Upon PMA treatment, PKC, which was normally seen in the cytoplasm became associated with the cytoskeletal structures (Figs. 3A, B). This association could be prevented when PMA treatment was carried out in the presence of a proton ionophore, nigericin in a neutral KCl buffer (Fig. 3C).

Controls. No staining was seen in immunofluorescence microscopy when the primary antibodies were omitted.

Intracellular pH and Ca^{2+}. In untreated polarized MDCK cells intracellular pH was 7.2 and free calcium concentration 170 nM. Upon TFP treatment intracellular calcium increased transiently, while pH remained normal (Fig. 4A). PMA caused a slight increase in the intracellular calcium concentration which began to normalize, however, after 45 min even in the presence of PMA. During the same period intracellular pH was decreased to 6.7 (Fig. 4B).

It is known from our previous studies that fodrin is released from the lateral walls of MDCK cells within 15 min, when intracellular pH is lowered below 6.5 [19]. The rate of the drop in pH, after exposing the cells to an acidic KCl buffer containing nigericin (pH 5.5) is shown in Fig. 5A. Upon acidification intracellular free calcium increased drastically, especially when pH dropped below 6.5 (Fig. 5A). The kinetics of the normalization of pH during the latter part of the acidification/neutralization cycle is shown in Fig. 5B: the cytoplasmic pH returned above 6.5 in 3–4 min and the calcium level was normalized within 3 min upon removal of nigericin and returning the cells to Hanks' buffer (Fig. 5B). TFP, on the other hand prevented the normalization of pH and intracellular calcium level during the acidification/neutralization cycle (Fig. 5C).

Discussion

Studies on the lipid–protein interactions are usually carried out in vitro utilizing lipid vesicles or micelles and purified protein preparations. This approach gives accurate and definitive answers to questions concerning factors necessary for the membrane binding of proteins or in the case of enzymes for their activation. It lacks, however, the abundance of different molecules always present in the living cells and, therefore, important correlations and indirect effects may remain unobserved. MDCK cells is a favorite target for studies on protein–lipid interactions in vivo due to its well-polarized membrane domains and well-characterized sorting and transport systems [28]. In this work we used MDCK cells to study the distribution of the lipid- and surface-associated proteins, annexin II, fodrin and PKC in response to agents which modify the intracellular calcium, calmodulin, pH and protein phosphorylation.

Calcium as a modulator of protein–lipid interactions

α-Fodrin has two EF-hand structures from which at least one is able to bind calcium [17]. Thus, it belongs to the family of calcium-regulated proteins with the 'EF-hand' domain which includes calmodulin and troponins. The function or significance of the calcium-binding domain of α-fodrin is, however, unknown. The second group of calcium-binding proteins is annexins which have a different type of calcium-binding structure: they have four or eight repeats of 70 amino acids in length which are involved in calcium-binding and arranged on the same side of the annexin molecule as its membrane-binding site [29,30]. PKC represents still another type of calcium-binding proteins as it forms a complex with calcium and PS [4,8].

In the present study we could see membrane-associated annexin II in untreated MDCK cells which have intracellular free Ca^{2+} concentration of 170 nM. This is in accordance with previous data showing that annexin II binds to the plasma membrane lipids at Ca^{2+} concentrations of about 150 nM [2]. Further elevation of intracellular Ca^{2+} by calcium ionophore A23187, acidification and/or PMA treatment did not affect this association. Also fodrin and PKC remained in their normal location upon A23187 treatment. Thus, it seems that a static elevation of intracellular free calcium concentration per se without specific stimuli is not sufficient to induce microscopically observable mobilization of these three proteins in MDCK cells.

Effect of trifluoperazine and/or calmodulin

TFP treatment led to a transient elevation of the intracellular calcium, but had no effect on the intracellular pH. The rapid increase in the intracellular free calcium ion concentration could be due to the inhibitory action of TFP towards calmodulin, an important regulator of the intracellular calcium-dependent processes. The cells seemed to recover, however, rather rapidly and reestablished their normal cytoplasmic calcium ion level within 5–6 min after exposure to TFP.

In TFP-treated cells fodrin and PKC remained in situ. Annexin II was, on the other hand, released from

the lateral walls and became associated with the Triton X-100-insoluble cytoskeletal fraction. We think that the exclusion of annexin II from the membrane is due to the basic nature of TFP [31], which upon binding to membrane lipids alters their cationic properties and prevents annexin association with the charged lipid head groups. In line with this, Shadle and Weber [32] have shown that trifluophenothiazine prevents association of annexin IV (protein II) with PS liposomes. TFP has also been shown to inhibit activation of PKC probably by perturbing the association of basic proteins with the lipid membranes [33]. It is also known that tamoxifen, another basic drug, prevents binding of annexin I to the plasma membrane by interacting with serine residues of lipids [34].

Distribution of fodrin remained unchanged in TFP-treated cells. Thus, the behaviour of fodrin and annexin II is not similar, although there are reports on the binding of annexin II to fodrin [11]. Our finding is in concert with the observations that the membrane location of these two proteins is not tightly linked. For instance, microinjection of an antibody to the p11 light chain of annexin II resulted in simultaneous patching of the light and heavy (36000) chain of annexin II, whereas fodrin was not enriched in these patches [35].

Effect of PMA

Upon PMA treatment MDCK cells acquired a spindle-like morphology. In these cells PKC associated with membranes and cytoskeletal structures. Fodrin, on the other hand, was released from the lateral walls to the cytosol. These translocations coincided with a drop in the intracellular pH and elevation in the intracellular calcium concentration.

Partitioning of PKC to the particulate fraction of the cells and activation by PMA takes place relatively rapidly: a 550% and 225% increase in the membrane-associated PKC activity has been observed within 10 and 30 min, respectively, after exposing MDCK cells to PMA [36]. The disappearance of PKC activity is due to down-regulation by an enzyme calpain which cleaves the enzyme to the regulatory and catalytic domains [37]. The PKC antibody MC5 used in this study recognizes the hinge domain between the regulatory (N-terminal) and catalytic (C-terminal) domains (residues 312–323 of bovine PKCα) which belongs to the regulatory domain of PKC after cleavage of PKC by calpains [37]. Thus, the immunodetectable PKC represents the membrane- and PMA-binding part of PKC whether intact or cleaved. Fiber-associated PKC observed in MDCK cells after 60 min treatment with PMA most likely represents the PKC molecules or fragments passed through the activated and catalytic phases in their life cycle and waiting and susceptible to down-regulation.

In cells growing attached to the substratum PMA treatment brings about translocation of PKC to the plasma membrane and to intracellular structures: In NIH 3T3 cells, for instance, treatment with PMA led to a rapid redistribution of type III (α) PKC to the nuclear envelope [38]. Moreover, upon activation by norepinephrine or PMA, PKC translocated to myofibrils in cardiac myocytes and to microfilaments in fibroblasts [39]. It has been suggested that there are intracellular receptor proteins that bind PKC in a phosphatidylserine- and calcium-dependent manner [40] or even that the insoluble PKC exists in at least two states, one in association with phospholipid and calcium, and the other in association with phospholipid, Zn and cytoskeletal proteins [41].

There was a dramatic translocation of fodrin in response to PMA. It seems to be due to PMA-induced pH drop. This is evident on two accounts: Firstly, release of fodrin was preceded by a drop of the intracellular pH to 6.7. Secondly, the cells preserved their normal morphology and fodrin and PKC distribution if the PMA treatment was done with cells having intracellular pH clamped neutral. Analogously to the acidified cells [19], also in PMA-treated cells fodrin was insoluble in Triton X-100. Contrary to fodrin and PKC, annexin II was not removed from the lateral walls by PMA treatment.

There are several studies on the effect of PMA on the pH of cells grown in a suspension and of substratum-attached nonepithelial cells. However, to our knowledge, there is no previous data on the effect of PMA on the intracellular pH and calcium levels in MDCK cells. In lymphocytes PMA causes slight alkalinization of the cytoplasm, but no increase in intracellular calcium [42]. In neutrophils PKC activates a proton channel leading to cytoplasmic alkalinization [43]. Interestingly, the increase in pH by PMA/PKC seems to depend on the adhesion of the cells: the pH of round C3BH10T1/2 cells increased and that of spread cells slightly decreased after a 20–30-min incubation with PMA [44]. Thus, the tight substratum adhesion of MDCK cells may explain the acidification of the cytoplasm brought about by PMA. It is also noteworthy that fodrin location in non-anchored lymphocytes is quite different from that seen in MDCK cells. In resting T lymphocytes fodrin is soluble and its distribution can vary considerably from membrane-associated to focal accumulations or a single large aggregate [45,46]. In lymphocytes, by PMA treatment, fodrin remained in intracellular aggregates and co-localized with PKC [46,47].

Effect of intracellular pH

Acidification of the cell cytoplasm by the nigericin treatment brought about a dramatic translocation of fodrin and PKC, whereas annexin II remained immo-

bile. The mechanisms of these translocations are unknown. The pH threshold for the mobilization of fodrin and PKC, pH 6.5 is the same at which endo- and exocytosis are stopped [19,20] suggesting that some changes at the cytoplasmic face of the plasma membrane lies behind these phenomena. One example of these changes is the large increase in the intracellular free calcium ion level due to the drop in pH below 6.5. The origin of these calcium ions may be either the plasma membrane or intracellular organelles. The calcium ions bound to the lipid head groups might be released upon acidification, since it has been shown that, e.g., phosphatidylethanolamine binds more calcium at higher pH values [48]. Acidification may also inactivate intracellular calcium pumps and in this way cause the release of calcium from the intracellular stores.

Effect of TFP during acidification / neutralization cycle

Rebuilding of the membrane skeleton of the acidified cells could be prevented by the calmodulin antagonist TFP. In these circumstances intracellular pH remained low (6.6–6.8), calcium high (over 1000 nM), fodrin formed a big cluster in the cytoplasm and PKC remained associated with the intracellular fibers. The most likely explanation for these alterations is that TFP either inactivates the energy production necessary for restoring the intracellular pH or directly inhibits the plasma membrane pumps involved in the normalization of intracellular pH and calcium concentration [49]. The failure of the restoration of normal pH could prevent fodrin and PKC from returning to their normal residence and contribute to the formation of the fodrin aggregate. α-Fodrin has a calmodulin binding site with an unknown function [16]. From the present results we could speculate that calmodulin prevents self-aggregation of fodrin as long as it exists as a dimer in the cytoplasm and is not yet bound to the membrane skeleton. Annexin II was also seen in intracellular fodrin clusters in 30 min after the cells were returned to neutral pH in the presence of TFP. This difference in the kinetics of accumulation supports the idea that a possible association of annexin II to fodrin is not due to covalent bonds, but to electrostatic attraction [11,36].

The present experiments clearly show different responses of annexin II on one hand, and PKC and fodrin on the other hand, to changes in intracellular pH or treatment with PMA and TFP. The results suggest that annexin II is sensitive to the cationic content of the membrane lipids and PKC and fodrin to the hydrophobicity and the degree of protonation in their membrane interactions. This difference in the lipid binding properties might reflect the fact that in living cells annexin II is involved in bringing together intracellular transport vesicles and plasma membrane, whereas fodrin and PKC interact with one component at a time, either with the plasma membrane or cytoskeletal structures.

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