F-actin does not modulate the initial steps of the protein kinase C activation process in living nerve cells

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Abstract

Actin is a major substrate for protein kinase C (PKC) and PKC is considered a modulator of the actin network. In addition in vitro studies (Biochemistry 39 (2000) 271) have suggested that all PKC isoforms bind to actin during the process of activation of the enzyme. To test the physiological significance of such a coupling we used living PC12 cells and primary cultures of cerebellar granule cells. When PC12 cells were treated with either latrunculin B, which impairs actin polymerization, or phalloidin, which stabilizes actin filaments, we observed a significant reduction of the \([\text{Ca}^{2+}]_i\) response revealed by Fura-2 fluorescence, while the PKC conformational changes followed by Fim-1 fluorescence were unaffected. The responses induced either by cell depolarization or muscarinic receptor activation were similarly affected by the toxin treatment of PC12 cells. In cerebellar granule cells the \([\text{Ca}^{2+}]_i\) response induced by KCl depolarization was increased by latrunculin treatment, whereas no effect was observed on the PKC response. Latrunculin had no effect on the NMDA-induced responses in these cells. Finally we also show that the response induced by a long-lasting depolarization, which mimics stimulation leading to neuronal plasticity, was not significantly altered by latrunculin or phalloidin treatment of the cells. These results suggest that the actin network is not involved in the initial steps of the PKC activation process in living nerve cells.

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Introduction

The actin cytoskeleton is a dynamic network composed of actin polymers and a large variety of associated proteins. It plays a major role in the cell shape and organizes the cytoplasm in response to cell stimuli. Co-localization of individual PKC isoforms with actin has been described in living cells [1–3]. Several reports have shown that PKC may regulate actin cytoskeleton organization and dynamics [for a review see 4]. Job and Lagnado [5] suggested that stimulation of PKC accelerates growth of F-actin in bipolar cells. Vaughan et al. [6] demonstrated the effect of PKC on rearrangement of the F-actin-based cytoskeleton, and PKC-mediated GAP-43 phosphorylation within the growth cone has been shown to have a direct influence on the structure of the actin cytoskeleton [7]. More specifically, PKC has been shown to induce either F-actin depolymerization in several models [8–10] or G-actin polymerization in other cellular models [5,11,12].

Furthermore Prekeris et al. [13] demonstrated a direct interaction between actin filaments and activated PKCe isoform within intact nerve endings, concluding that actin is the major anchoring protein for PKCe. Depending on the cellular model studied, it has been shown that different specific PKC isoforms associate with actin on cell stimulation [1,14,15]. In vitro studies have also shown that all PKC isoforms can interact with F-actin and that this interaction favors PKC activation [16].

Using imaging techniques involving green fluorescent protein (GFP) fused with PKC isoforms [17–20], recent studies revealed that PKC activation occurs at discrete sites in the cell periphery. Our results obtained by imaging PKC-specific dyes also showed a similar phenomenon in PC12
isoforms were identified when treated with neuronal growth factor (NGF), rat pheochromocytoma [24], which displays a neuronal phenotype. Among other cellular functions, there is growing evidence that these microdomains regulate F-actin cytoskeleton [23] and confine PKC effectors and substrates to discrete sites.

Thus it is of particular importance to decipher the duality between F-actin network as a PKC substrate and F-actin as an anchoring protein for specific PKC isoforms. The aim of this study was to identify the physiological involvement of F-actin in the PKC activation process in stimulated living cells using two fluorescent dyes specific for PKC and Ca$^{2+}$.

We used two models: the PC12 cell line, derived from a rat pheochromocytoma [24], which displays a neuronal phenotype when treated with neuronal growth factor (NGF), and primary cultures of rat cerebellar granule cells. PKC isoforms were identified by immunocytochemistry, and F-actin function was assessed by perturbing the actin cytoskeleton using two toxins, latrunculin B, which chelates G-actin to induce cell differentiation, and phalloidin, which stabilizes actin filaments.

Materials and methods

Materials

Poly-L-ornithine (MW 30,000–70,000), carbachol, phalloidin, rhodamine-conjugated phalloidin, monoclonal antibody against β-actin, and rabbit polyclonal antibodies raised against PKCα, PKCβII, and PKCε were purchased from Sigma (St. Louis, MO, USA). Culture media (RPMI-1640 and DMEM), laminin, murine NGF 2.5 S, and horse serum were purchased from Gibco-BRL (Grand Island, NY, USA). Fetal calf serum was purchased from Dominique Dutscher S.A. (Brumath, France) and type I collagen from rat tail was a product of Becton Dickinson (Pont de Clai, France). Fim-I dipotassium salt was purchased from Teflab (Austin, TX, USA) and Fura-2-AM from Molecular Probes (Eugene, OR, USA). NMDA was purchased from Tocris Cookson (Bristol, UK). Laminin was from Calbiochem (La Jolla, CA, USA).

Cell cultures

PC12 cells were plated on 35-mm glass inserts in plastic Petri dishes (Falcon) coated with collagen and polyornithine (0.1 mg/ml each). They were grown in RPMI-1640 medium containing L-glutamine supplemented with 10% horse serum, 5% fetal calf serum (both decomplemented), 50 U/ml penicillin, and 50 µg/ml streptomycin. They were cultivated for 7 days in a humidified 5% CO$_2$ atmosphere at 37°C. To induce cell differentiation, 2.5 S murine NGF (50 ng/ml) was added to the culture medium 24 h after plating.

Primary cultures of cerebellar granule cells were prepared according to Stewart et al. [27]. Briefly, granule cells were taken from 5-day-old Wistar rats. After decapitation, the cerebellum was dissected and maintained in DMEM. Granule cells were then directly sucked from the superficial external germinative layer and placed in glass-bottom Petri dishes (φ 35 mm) previously coated with polyornithine (0.5 mg/ml) and laminin (10 µg/ml). The cells were cultivated in DMEM containing horse serum (10%), insulin (5.10–7 M), and KCl (25 mM) at 37°C in humidified atmosphere with 5% CO$_2$. They were used after 7 to 14 days in vitro (DIV).

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), for 20 min. After permeabilization for 10 min in PBS containing 0.1% Triton X-100, the cells were incubated for 1 h in 10% normal goat serum in PBS to block nonspecific binding. This step was followed by 2 h incubation with an isoform-specific anti-PKCα antibody (diluted 1/800 in PBS containing 5% normal goat serum) at room temperature. A goat anti-rabbit secondary antibody (1/1000 in 5% goat serum) Alexafluor 488 (Molecular Probes) or indocarbocyanine (Cy3, Jackson)-conjugated was applied for 1 h in PBS. Cultures were finally mounted in Vectashield medium (Vector) to reduce photobleaching. Controls were obtained by omitting the primary antibody in the incubation bath, and no staining of the cells was observed under these conditions.

For F-actin detection, cell fixation was followed by membrane permeabilization for 10 min in 0.1% Triton X-100. Rhodamine-conjugated phalloidin (4 µM final concentration) was then bath applied to the cells for 30 min in PBS.

The labeled cells were finally observed on an epifluorescence inverted microscope (Diaphot TMD, Nikon) or a confocal microscope (LSM 510, Zeiss, Germany).

Immunoprecipitation and Western blots

PC12 cells were grown in 10-cm Petri dishes for 5 days in the presence of NGF. Control dishes were rinsed three time with Krebs solution (106 mM NaCl, 4.5 mM KCl, 1.2 mM MgSO$_4$, 2.5 mM CaCl$_2$, 11 mM d-glucose, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, pH 7.4, equilibrated with 5% CO$_2$) whereas 60 mM KCl was applied for 1 min at room temperature to stimulated cells. The dishes were then placed on ice and the medium was immediately replaced by 2 ml ice-cold buffer (buffer A) including 150 mM KCl, 40 mM Hepes (pH 7.4), 1 mM EDTA, and a cocktail of protease inhibitors. In the case of stimulated cells, this buffer also contained 0.945 mM CaCl$_2$ to ensure a final concentration of 1 µM free Ca$^{2+}$. The cells were lysed under mild sonication for 10 min in an ice-cold water bath. The material
was then scraped, homogenized, and centrifuged for 45 min at 100,000g (TL-100, Beckman Instruments). The pellets were then extracted with 2 ml buffer A (with or without CaCl₂) containing 1% Triton X-100 and centrifuged for 45 min at 100,000g. The Triton extracts were immunoprecipitated according to Hepp et al. [28]. Briefly, 800 μL was incubated overnight at 4°C in the presence of protein A-coupled Sepharose beads for clearance. The supernatants were divided into two aliquots, which were incubated for 2 h at room temperature in the presence of Sepharose beads coupled either to an anti-PKCα–protein A complex or to a rabbit IgG–protein A complex. The beads were washed two times with buffer A (with or without CaCl₂) and four times with a buffer containing 500 mM KCl, 40 mM Hepes (pH 7.2), 1 mM EGTA (with or without CaCl₂). Finally, the beads were suspended in the electrophoresis sample buffer.

Samples were subjected to polyacrylamide gel electrophoresis (7.5% acrylamide) and transferred to a nylon membrane. The blots were subsequently incubated with antiphosphorylated antibodies (1/5000) and revealed with a bioluminescence kit (Tropix).

Simultaneous [Ca²⁺], and PKC activation imaging

Imaging experiments were performed according to Dupont et al. [21]. Briefly, PC12 or cerebellar granule cells were incubated for 30 min at 37°C in Krebs medium containing 2 μM Fura-2-AM. Cells were then washed with Krebs medium and subsequently incubated for 20 min with 0.2 μM Fim-1 dipotassium salt in Krebs solution (extemporaneously prepared from fresh 280 μM stock solution in DMSO containing 60 mM pluronic acid).

After three washes, the cells were placed on an inverted microscope (Axiovert 35M Zeiss, Germany), superfused with Krebs solution (1 ml/min), and alternatively illuminated at 350 ± 10 nm (for Fura-2) or at 490 ± 10 nm (for Fim-1). The fluorescence emission was observed using a dichroic mirror at 510 nm and a long-pass filter at 510 nm. Every 10 s an image for each excitation wavelength (350 nm for Fura-2 and 490 nm for Fim-1) was recorded using an intensified CCD camera (Extended Isis, Photonic Science, UK) and the Fluostar software (Imstar, Paris, France). Drugs were applied by superfusion in Krebs solution for 30 s unless specified.

Image analysis and semiquantification

Two image series, one for each excitation wavelength (350 and 490 nm), were recorded for each experiment. After background subtraction from each image series, another image series was calculated by exponential interpolation to assess the fluorescence baseline during the whole recording period taking into account probe bleaching and possible probe leaks. This interpolation was performed on a pixel-to-pixel basis between the initial recording period and the final recording period. Finally, a third image series was then calculated, dividing experimental images by interpolated baseline images on a pixel-to-pixel basis. This third set of images was called the ratio image series. The normalized ratio values were independent of fluorescence bleaching and probe concentration heterogeneity in the preparation. Image analysis was performed on a DEC-Alpha workstation (Digital Co., Boston, MA, USA) using the Khoros function library (Khoral Research Inc.).

On the ratio image series, the data were then semiquantified by defining regions of interest usually corresponding to entire cells in the microscopic field. The average of the ratio values inside these regions was calculated and plotted versus the image record time. The curves displayed peaks corresponding to stimulation-induced responses. For a single cell, these peaks were integrated and compared with each other for the same cell. Statistical analysis was performed using a two-tailed Student t test on paired values. The effects of toxin treatments on the cells were evaluated by ANOVA and subsequent Student t test.

Results

Effect of latrunculin B treatment on the structure of the F-actin network and the distribution of PKCα

Using rhodamine-conjugated phalloidin, F-actin was detected inside the cells as a cortical ring along the cellular plasma membrane of PC12 cells (Fig. 1) and cerebellar granule cells (Fig. 2). In both kinds of cells, the structure of the F-actin network did not change noticeably after depolarization induced by a 30-s application of 60 mM KCl.

To impair actin polymerization we used latrunculin B, which penetrates cells and leads to a major alteration in microfilament organization by inhibiting actin polymerization, but has no effect on the microtubular system [25,26,29]. After a 3-h incubation of NGF-treated PC12 cells with 25 μM latrunculin B, the actin network was dramatically disorganized, sometimes displaying scattered thick fibers (Fig. 1). In this case, the F-actin cortical ring was completely disrupted and the shape of the cell body changed from a polygonal aspect to a more spherical aspect (Fig. 1). The efficiency of a shorter latrunculin treatment was also tested and the effects were found to be less consistent.

PKCα was localized in NGF-treated PC12 cells and cerebellar granule cells using an isoform-specific antibody. In NGF-treated PC12 cells, the enzyme appeared in the cytosol in unstimulated cells (Fig. 1). On the other hand, when the cells were fixed after application of 80 mM KCl for 30 s, the immunolocalization of PKCα increased mainly on discrete portions of the plasma membrane in PC12 cells (Fig. 1). At the same time the fluorescence in the cytosol decreased (Fig. 1), indicating that cell depolarization induced a transfer of PKCα from the cytoplasm to the plasma
membrane. The peripheral pattern of translocated PKCα after stimulation suggested that the enzyme was co-localized with cytoskeletal elements.

To verify the pattern of PKCα translocation we also used a 30 μM ATP application for 30 s to stimulate the cells. Again, we observed clear PKCα translocation without any significant change in phalloidin distribution (Fig. 1).

When PC12 cells were treated with latrunculin the distribution of PKCα in resting cells was not altered (Fig. 1). Moreover, most of these treated cells responded to KCl-induced depolarization or ATP stimulation as shown by PKCα translocation to the plasma membrane (Fig. 1), similarly to nontreated cells. In this case, however, PKCα translocation to the cellular plasma membrane occurred in peripheral regions where no actin filament network was detected by phalloidin (Fig. 1).

In granule cells, F-actin distribution appeared more diffuse than in PC12 cells. The labeled phalloidin was observed in cell bodies as well as cell processes, with some decorated fibers at the hillock of the neurites and in varicosities along the neurites (Fig. 2). On latrunculin B treatment of the granule cells these decorated fibers were not observed: a diffuse label was observed in the soma and the neurites of most cells (Fig. 2).

As an index of the effectiveness of the latrunculin B treatment we also examined astrocytes, which were occasionally present in the same primary granule cell cultures. Control astrocytes were polygonal, with numerous decorated actin stress fibers crossing the cytoplasm (Fig. 2). Latrunculin B treatment had a dramatic effect on the actin network of astrocytes, disrupting the stress fibers and leaving only a few short and thick fibers at their anchoring points (Fig. 2). This effect indicated that the latrunculin treatment was effective in these neuronal cell cultures.

In cerebellar granule cells, PKCα was present in the cytosol and in neurites of unstimulated cells (Fig. 2). After KCl-induced depolarization, the enzyme was preferentially localized in clusters on the cell bodies and varicosities of the cell processes (Fig. 2). On latrunculin treatment of the granule cells, there was no apparent change in cytoplasmic PKCα distribution in resting cells (Fig. 2) and PKCα normally translocated to the plasma membrane after cell depolarization (Fig. 2).

In PC12 cells as well as in cerebellar granules cells, the rhodamine-conjugated phalloidin label was not observed when the cell cultures were preincubated with unlabeled phalloidin before fixation and permeabilization (data not illustrated).

We also studies the immunocytochemical localization of PKCβII and PKCε in PC12 cells and cerebellar granule cells. In these cells, none of these isoforms were found to translocate to the plasma membrane on cell stimulation (not illustrated).

Imaging $[Ca^{2+}]$, and PKC activation in stimulated cells

We followed PKC activation in living cells on a time-resolved basis. For this purpose we used the fluorescent probe Fim-1, which specifically interacts with PKC [30].
Indeed the fluorescence yield of Fim-1 increases when PKC conformational changes occur during activation [21,31]. This permits visualization of the PKC conformational changes during the activation process in living cells. Although this fluorescence change occurs regardless of the PKC isoform, it corresponds mostly to cPKC and, more specifically, PKCα conformational changes in the present models [21]. Here, Fim-1 was used simultaneously with Fura-2 to monitor parallel changes in [Ca^{2+}], in response to cell stimulation.

A KCl application for 30 s induced increases in Fura-2 (λ_{ex} = 350 nm) and Fim-1 (λ_{ex} = 490 nm) fluorescence intensities inside the cells. The time course of the Fura-2 response was similar to the time course of the Fim-1 response as previously described [21]. Additionally both responses were reversed when the stimulus was withdrawn.

As revealed by image analysis, the pattern of [Ca^{2+}], response inside the cells differed from the pattern of PKC activation during depolarization. The [Ca^{2+}], increase occurred in the cytosol, while PKC activation was confined mainly to spots beneath the cellular plasma membrane. These patterns were observed in NGF-treated PC12 cells (Fig. 3A) and in cerebellar granule cell cultures (Fig. 3B), confirming our immunocytochemical observations of PKCα.

Effect of latrunculin B treatment on [Ca^{2+}], and PKC activation in living cells

In NGF-treated PC12 cells KCl application induced a dose-dependent increase in [Ca^{2+}], as revealed by the ratio values of Fura-2 fluorescence (Fig. 4A). It also induced a dose-dependent increase in Fim-1 fluorescence, which indicated PKC conformational changes (Fig. 4B). Preincubation with latrunculin significantly reduced the amplitude of the [Ca^{2+}], response (Fig. 4A, Table 1) without affecting the kinetics of the response. However, the PKC response (Fig. 4B) on KCl application was not significantly affected in latrunculin-treated PC12 cells (Table 1).

NGF-treated PC12 cells express M1 muscarinic receptors [32]. To verify whether this selective effect of latrunculin treatment on [Ca^{2+}], response, but not on PKC activation, was related to the stimulation mode of the cells, we applied carbachol, an agonist for muscarinic receptors, to NGF-treated cells. In control experiments, carbachol induced an increase in [Ca^{2+}], and activated PKC. Pretreatment with latrunculin had an effect on the [Ca^{2+}], response similar to that observed with KCl application (Fig. 4C, Table 1). Moreover, the kinetics and the amplitude of the PKC response were not affected (Fig. 4D, Table 1).

We have previously reported that the quantitative Fim-1 fluorescence intensity changes were higher near the plasma membrane than in the cytoplasm after depolarization or carbachol stimulation of PC12 cells [21]. Here, we performed the same analysis on individual latrunculin-treated cells and control cells. We again found a higher ratio value near the membrane than in the cytoplasm during stimulation, but no statistically significant difference was observed between control and treated PC12 cells.

As observed in PC12 cells, the [Ca^{2+}], response or PKC conformational changes in cerebellar granule cells were dose-dependently sensitive to KCl application (not illustrated). Similarly, latrunculin treatment also significantly increased the amplitude of the [Ca^{2+}], response induced by KCl application on granule cells. This effect could be due to slower rundown of the Ca^{2+} channels in the presence of disrupted actin [33], leading to desensitization of cerebellar granule cells. To verify whether this enhancing effect of latrunculin treatment affected desensitization of the response, we used multiple applications of 80 mM KCl (Fig. 5A). We consistently observed enhancement of the [Ca^{2+}], response in treated cells (Fig. 5A, Table 2). However, the latrunculin-treated granule cells did not display any changes in desensitization (Fig. 5A). Furthermore, the PKC response induced by the same protocol in latrunculin-treated cells was similar to the response of control cells (Fig. 5B).

We also determined if the response of granule cells to latrunculin was related to the mode of stimulation. NMDA application to cerebellar granule cells induced a dose-dependent [Ca^{2+}], increase and PKC conformational change (not illustrated). Using the protocol of repeated stimulation described above for NMDA applications, latrunculin treatment had no effect on either [Ca^{2+}], or PKC conformational change (Figs. 5C and D, Table 2).

Effect of phalloidin treatment on [Ca^{2+}], and PKC activation

The effects of phalloidin, an agent that prevents F-actin depolymerization and stabilizes the actin network [34], were also tested in PC12 cells. Although it has been claimed that phalloidin does not penetrate cells [34], Bernstein et al. [35] showed that phalloidin enters neuronal cells under specific experimental conditions. In agreement we found that phalloidin penetrated differentiated PC12 cells and granular cells and bound F-actin. The rhodamine-conjugated labeled was inhibited by prior treatment of the cells with unlabeled phalloidin (see above). This allowed us to use phalloidin in living cells. PC12 cells were incubated for 3 h in the presence of 5 μM phalloidin before the experiment. The cells were subsequently loaded with Fura-2-AM and Fim-1 before performing sequential depolarization by applying increasing KCl concentrations. The [Ca^{2+}], response to high KCl concentrations was significantly reduced in phalloidin-treated cells (Fig. 6A: 0.63 ± 0.26 in control cells vs 0.37 ± 0.21 in treated cells, P < 0.005). After application of 80 mM KCl, a reduction in the PKC response was also observed (Fig. 6B: 0.25 ± 0.11 in control cells vs 0.16 ± 0.06 in treated cells, P < 0.01).
Effect of latrunculin B or phalloidin on [Ca\(^{2+}\)], and PKC activation induced by prolonged stimulation

Considering the cell integrator function, which has been postulated for PKC [17], Bernstein et al. [35] interestingly reported an effect of phalloidin treatment on the Ca\(^{2+}\) response induced by electrical depolarization in neuronal cells. This effect was restricted to the slow developing component of the response. We thus changed our stimulation protocol and applied a low KCl concentration (30 mM) for a short period (30 s) and for a long period (3 min) on PC12 cells. A low KCl concentration was used to avoid depleting the cells.

As previously observed a short application of 30 mM KCl induced a transient increase in [Ca\(^{2+}\)] (Figs. 7A and C) and a simultaneous change in PKC conformation (Figs. 7B and D). When KCl was applied for a longer period, the [Ca\(^{2+}\)] reached a plateau value within 1 min. This [Ca\(^{2+}\)] value was maintained during KCl application and returned to baseline after KCl withdrawal. The PKC response assessed by Fim-1 fluorescence intensity followed the same kinetics, although the plateau value of Fim-1 fluorescence appeared less stable than that of [Ca\(^{2+}\)]. Additionally, the long KCl application did not alter the amplitude of the response subsequently induced by KCl for 30 s (Fig. 7).

Neither in latrunculin-treated PC12 cells (Figs. 7A and B) nor in phalloidin-treated cells (Figs. 7C and D) were the integral values of [Ca\(^{2+}\)], and PKC responses affected when this protocol was applied, indicating that F-actin was not directly involved in modulating PKC activation induced by a long stimulation of the cells. Moreover the subsequent application of KCl for 30 s induced an unaltered response in NGF-treated cells.

PKC\(\alpha\) immunoprecipitation

To further assess the interaction between PKC\(\alpha\) and actin we immunoprecipitated PKC\(\alpha\) from Triton X-100 extracts of PC12 cells. Since PKCo–actin interactions may depend on intracellular [Ca\(^{2+}\)] [36] and, more generally, on ionic concentrations, special care was taken during the extraction and the immunoprecipitation procedure to ensure physiological concentrations of Ca\(^{2+}\) and K\(^{+}\) in the extraction buffer. Although actin was present in the Triton X-100 extracts, no actin was detected in Triton X-100 extracts immunoprecipitated with an antibody against PKCo in either resting or stimulated cells (Fig. 8A). Interestingly, a second PKCo immunoreactive band with an apparent molecular mass around 45 kDa was revealed in stimulated cells only and probably corresponds to PKM formation due to PKC hydrolysis during stimulation.

Discussion

The major PKC isoform in NGF-treated PC12 cells and cerebellar granule cells is PKC\(\alpha\) [21,36]. This isoform is a member of the conventional group and has been well characterized [37]. Its activation requires the membrane lipid phosphatidyserine, calcium, and diacylglycerol. Our immunocytochemical data clearly showed that KCl-evoked depolarization of either PC12 cells or granular cells induces a PKC\(\alpha\) translocation from the cytoplasm to clusters near the plasma membrane. In contrast, no translocation of PKC\(\beta\)II and PKCc was observed by immunocytochemistry. We conclude that PKC\(\alpha\) is apparently the major isoform that displays this translocation pattern after depolarization in our cellular models. However, it should be mentioned that the PKC\(\alpha\) antibody is directed against the C-terminal domain and hence also recognizes PKM\(\alpha\), which is derived from PKC\(\alpha\) by hydrolysis of the hinge region between the catalytic and regulatory domains of the protein. Thus there is ambiguity in the immunolocalization as PKC\(\alpha\) cannot be distinguished from PKM\(\alpha\). Our immunoprecipitation experiments suggest that both proteins were interacting with the membrane after depolarization since they were present in a Triton X-100 extract. Additionally this rapid formation of PKM\(\alpha\) during depolarization of the cells could explain the desensitization of the Fim-1 response, which we have previously described [21].

To explore whether PKC\(\alpha\) translocation was dependent on actin filament integrity, we used two toxin, latrunculin B and phalloidin, that respectively disrupt and stabilize actin filaments. In our models latrunculin B disrupted the F-actin network as demonstrated with rhodamine-conjugated phalloidin.

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Fig. 2. Effect of latrunculin B treatment on PKC\(\alpha\) distribution and F-actin network organization in resting or depolarized cerebellar granule cells and in astrocytes. Immunofluorescence confocal micrographs of cultured cerebellar granule cells incubated with an anti-PKC\(\alpha\) antibody (A–D) or with rhodamine-conjugated phalloidin (E, F). The cells were fixed before stimulation (A, C) or immediately after depolarization (80 mM KCl application for 30 s: B, D) and incubated with an antibody raised against PKC\(\alpha\) (1/800 dilution). Control cells (A, B, E) are compared with cells pretreated with latrunculin B before the experiment (C, D, F). In resting cerebellar granule cells (A), PKC\(\alpha\) appears mostly diffuse or cytosolic, while after depolarization immunofluorescence increases along the plasma membrane (arrowheads in B) and in varicosities along the nerve processes indicating PKC\(\alpha\) translocation. Latrunculin-treated cells (C, D) do not display PKC\(\alpha\) immunofluorescence changes at rest (C) and PKC\(\alpha\) translocation after depolarization (D) is similar to that of control cells. In control cerebellar granule cells (E) actin filaments revealed by phalloidin were diffuse in the cell body and a dense network was observed under the plasma membrane, especially at the neurite hillock (arrowhead) and in neurite varicosities (arrows). This dense actin network was not observed in latrunculin B-treated granule cells (F). As an index of the effectiveness of latrunculin B treatment, astrocytes, which eventually grew in cerebellar granule cell cultures, were also observed (G, H). In control astrocytes (G) actin filaments are organized in stress fibers. In contrast, these fibers were completely disrupted into short thick fibers in latrunculin-treated astrocytes (H). Bars = 20 \(\mu\)m.
The immunocytochemical distribution of PKCα was not affected by latrunculin treatment in either PC12 cells or cerebellar granule cells at rest. This suggests that F-actin does not interact with nonactivated PKC. Moreover, the fact that enzyme localization after 30 s stimulation was not altered by latrunculin treatment also suggests that activated

Fig. 3. Imaging \([\text{Ca}^{2+}]_i\) and PKC activation in PC12 cells on 80 mM KCl application for 30 s. The living cells were loaded with Fura-2-AM and Fim-1 and the changes in \([\text{Ca}^{2+}]_i\) and PKC conformation were simultaneously recorded. Pseudo-color ratio images indicate a homogeneous increase in Fura-2 fluorescence inside the cell bodies induced by depolarization of PC12 cells while PKC activation occurred predominantly in patches near the plasma membrane (A). A similar pattern was observed in cerebellar granule cells (B). Bar = 50 μm.
PKC/\(H_9\)251 has no direct or indirect interaction through anchoring proteins with F-actin. But at this point we have no information on how F-actin modulates the kinetics of PKC activation and conformational changes. This was achieved by cellular imaging.

Using these two cellular models, we have shown that latrunculin treatment affects Fura-2\(fluorescence changes induced by a cellular depolarization, indicating that the intracellular Ca\(^{2+}\) concentration was modulated by F-actin integrity. Surprisingly, opposing responses were observed

Fig. 4. Calcium response and PKC activation induced by depolarization or carbachol application on control (○) or latrunculin B-treated (■) PC12 cells. Fura-2- and Fim-1-loaded cells were sequentially stimulated with increasing concentrations of KCl (40, 60, and 80 mM in A and B) or carbachol (10, 100, and 1 000 \(\mu\)M in C and D) as indicated by solid bars in the graphs and the value of the fluorescence ratio (see Materials and Methods) was plotted against time. The \([\text{Ca}^{2+}]_i\) response and the PKC conformational change were simultaneously recorded in the same cells (A and B or C and D). Each curve is the average from \(n\) cells recorded during three separate experiments at least (in A and B, \(n = 21\) for control cells and \(n = 27\) for treated cells; in C and D, \(n = 10\) for control cells and \(n = 7\) for treated cells). Latrunculin treatment induced a significant decrease in the \([\text{Ca}^{2+}]_i\) response without affecting the PKC response.

PKC\(\alpha\) has no direct or indirect interaction through anchoring proteins with F-actin. But at this point we have no information on how F-actin modulates the kinetics of PKC activation and conformational changes. This was achieved by cellular imaging.

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Table 1
Effect of latrunculin treatment on the \([\text{Ca}^{2+}]_i\) response and PKC conformational change induced by KCl or carbachol application on PC12 cells

<table>
<thead>
<tr>
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<th>([\text{Ca}^{2+}]_i) response</th>
<th>PKC response</th>
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<tr>
<td></td>
<td>40 mM</td>
<td>60 mM</td>
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<tr>
<td>KCl</td>
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<tr>
<td>Control, (n = 21)</td>
<td>0.28 ± 0.14</td>
<td>0.46 ± 0.27</td>
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<tr>
<td>Treated, (n = 27)</td>
<td>0.12 ± 0.10</td>
<td>0.27 ± 0.16</td>
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<td>(P &lt; 0.1%)</td>
<td>(P &lt; 5%)</td>
<td>(P &lt; 5%)</td>
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<td></td>
<td>10 (\mu)M</td>
<td>100 (\mu)M</td>
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<tr>
<td>Carbachol</td>
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<tr>
<td>Control, (n = 10)</td>
<td>0.20 ± 0.04</td>
<td>0.22 ± 0.05</td>
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<tr>
<td>Treated, (n = 7)</td>
<td>0.05 ± 0.02</td>
<td>0.13 ± 0.02</td>
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<td>(P &lt; 0.1%)</td>
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\(^{a}\) The fluorescence ratio was integrated and expressed in arbitrary units ± SEM. The statistical analysis was performed by ANOVA and subsequent Student \(t\) test (ns, non significant).
in the two models: the Ca\textsuperscript{2+} response decreased in PC12 cells and increased in granule cells. At present, F-actin regulation of Ca\textsuperscript{2+}/H\textsubscript{11001} channels is ambiguous. Nakamura et al. [38] showed that L-type Ca\textsuperscript{2+}/H\textsubscript{11001} channels were inhibited by actin filament disruption in smooth muscle cells. The partial inhibition of the [Ca\textsuperscript{2+}]\textsubscript{i} response in PC12 cells may reflect such a dependence as L-type channels account at least for 35% of the [Ca\textsuperscript{2+}]\textsubscript{i} increase on cell depolarization (Geeraert et al., unpublished results). F-actin depolymerization has also been reported to slow the desensitization rate of volt-

![Fig. 5. Calcium response and PKC activation induced by depolarization or NMDA application on control (○) or latrunculin B-treated (■) cerebellar granule cells. Fura-2- and Fim-1-loaded granule cells (DIV 14) were sequentially stimulated with 80 mM KCl (A, B) or 100 µM NMDA (C, D) as indicated by bars in the graphs. The [Ca\textsuperscript{2+}]\textsubscript{i} response and the PKC conformational change were simultaneously recorded in the same cells (A and B or C and D). Each curve is the average of n cells recorded during at least three separate experiments (in A and B, n = 7 for control cells and n = 14 for treated cells; in C and D, n = 14 for control cells and n = 9 for treated cells). Latrunculin treatment induced a significant increase in [Ca\textsuperscript{2+}]\textsubscript{i} response when the cells were depolarized by KCl. However, no significant effect was observed on PKC response. Additionally, there was no change in the cellular response of treated cells when they were stimulated by NMDA.]

Table 2
Effect of latrunculin treatment on the [Ca\textsuperscript{2+}] response and PKC conformational change induced by repeated KCl or NMDA applications on cerebellar granule cells

<table>
<thead>
<tr>
<th></th>
<th>1st appl.</th>
<th>2nd appl.</th>
<th>3rd appl.</th>
<th>PKC response</th>
<th>1st appl.</th>
<th>2nd appl.</th>
<th>3rd appl.</th>
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<tr>
<td><strong>KCl (80 mM repeated)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Control, n = 7</td>
<td>0.67 ± 0.15</td>
<td>0.31 ± 0.14</td>
<td>0.51 ± 0.18</td>
<td>0.21 ± 0.07</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.09</td>
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<tr>
<td>Treated, n = 14</td>
<td>1.14 ± 0.14</td>
<td>0.78 ± 0.14</td>
<td>0.87 ± 0.15</td>
<td>0.13 ± 0.07</td>
<td>0.17 ± 0.08</td>
<td>0.22 ± 0.07</td>
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<tr>
<td>P &lt; 0.1%</td>
<td>P &lt; 0.1%</td>
<td>P &lt; 0.5%</td>
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<th></th>
<th>1st appl.</th>
<th>2nd appl.</th>
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<th>PKC response</th>
<th>1st appl.</th>
<th>2nd appl.</th>
<th>3rd appl.</th>
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<td><strong>NMDA (100 µM repeated)</strong></td>
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<tr>
<td>Control, n = 14</td>
<td>0.38 ± 0.11</td>
<td>0.48 ± 0.09</td>
<td>0.47 ± 0.11</td>
<td>0.15 ± 0.05</td>
<td>0.15 ± 0.03</td>
<td>0.16 ± 0.03</td>
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</tr>
<tr>
<td>Treated, n = 9</td>
<td>0.44 ± 0.08</td>
<td>0.49 ± 0.07</td>
<td>0.51 ± 0.12</td>
<td>0.10 ± 0.04</td>
<td>0.12 ± 0.05</td>
<td>0.12 ± 0.05</td>
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*a The fluorescence ratio was integrated and expressed in arbitrary units ± SEM. The statistical analysis was performed by ANOVA and subsequent Student t test.
age-dependent Ca\textsuperscript{2+} channels in neurons \[33\] and this could explain our observation in granule cells. The discrepancy between these two cellular models may thus reflect differences in F-actin regulating properties of voltage-dependent Ca\textsuperscript{2+} channels.

PC12 cells also differed from cerebellar granule cells in the fact that the carbachol-induced [Ca\textsuperscript{2+}]\textsubscript{i} response in PC12 cells was inhibited by F-actin depolymerization while the NMDA-induced response in granule cells was unaffected. Accordingly, NMDA-induced Ca\textsuperscript{2+} entry into cerebellar granule cells has been previously reported to be independent of F-actin regulation \[39,40\]. In contrast, the muscarinic receptor, unlike the NMDA receptor, is coupled to phospholipase C. Thus disruption of actin filaments could have a direct effect on the spatial relationship between phospholipase C and inositol triphosphate receptors \[41\]. This may explain the different responses of the two cell models.

Aside from this modulation of the Ca\textsuperscript{2+} response after latrunculin treatment, no detectable change in Fim-1 fluorescence intensity was induced by latrunculin B treatment. The PKC spatial distribution was not altered between cytoplasm and plasma membrane in either stimulated PC12 cells or granular cells as revealed by Fim-1 fluorescence. This suggests that PKC activation is not strictly controlled by the changes in the [Ca\textsuperscript{2+}]\textsubscript{i} response induced by the toxin treatment or that the local Ca\textsuperscript{2+} concentration necessary for PKC activation was not affected by latrunculin B treatment. In fact, the [Ca\textsuperscript{2+}]\textsubscript{i} requirement for PKC\textsubscript{\alpha} activation has been reported to be lower than that of other PKC isoforms \[42\]. Our finding suggests that [Ca\textsuperscript{2+}]\textsubscript{i} is above the threshold necessary for PKC\textsubscript{\alpha} activation both before and after latrunculin B treatment.

Slater et al. \[16\] have shown in vitro that PKCs, especially conventional PKC isoforms (cPKC), are activated by F-actin. cPKCs translocate to F-actin in response to an elevation in calcium levels. The authors hypothesized that PKC mediates a functional coupling of intracellular Ca\textsuperscript{2+} fluctuations with F-actin-mediated processes. Additionally, they suggested the possibility that an interaction with F-actin is a general property of all PKC isoforms and may be induced by an increase in intracellular Ca\textsuperscript{2+} only. Our results, however, argue against this functional coupling in living cells, at least in the first steps of PKC\textsubscript{\alpha} activation process. Two reasons might explain this difference: the functional coupling described in vitro between F-actin and
PKCa requires high intracellular Ca\(^{2+}\) concentrations (about 100 \(\mu\)M) [16]. Additionally, the authors performed their experiments in hypotonic buffer which may allow electrostatic interactions not relevant under physiological conditions. Alternatively one could argue that Fim-1 is not sensitive to F-actin–PKC interaction since its fluorescence changes reveal discrete PKCa conformational changes. However, the immunoprecipitation results do not support an actin–PKC interaction even in the absence of latrunculin.

In fact, the absence of F-actin involvement in the PKC translocation has also been observed in another cellular model. After baby hamster kidney cells expressing the fusion protein PKCa–GFP were incubated with cytochalasin D, Almholt et al. [20] observed cells with an altered shape but failed to detect any PKCa distribution change, even after application of ionomycin. However, overexpressed PKC–GFP could saturate the binding sites on the actin network, thus masking any signal change. Here, using different cellular models and different methods of detection for PKC, which avoid such a bias, we confirm Almholt’s observation [20]. In pituitary cells stimulated with either phorbol ester or thyrotropin-releasing hormone Vallentin et al. [43] showed that PKCa was able to interact with actin filaments. The kinetics of this interaction, however, were slow and required a 1-h stimulation to develop. During short-term stimulation PKCa translocation to the plasma membrane was not regulated by F-actin. We also conclude that F-actin has no effect on the conformational change of PKC revealed by Fim-1 since the integrated intensity change of Fim-1 fluorescence during stimulation was unaltered by latrunculin treatment in either PC12 cells or granule cells.

Phalloidin treatment of PC12 cells decreased the integrated responses of \([\text{Ca}^{2+}]\), and PKC induced by high K\(^+\) concentrations. This effect of phalloidin on the \([\text{Ca}^{2+}]\), response may be related to the interaction of voltage-operated Ca\(^{2+}\) channels with actin filaments (see above). However, partial inhibition of the \([\text{Ca}^{2+}]\), response induced by latrunculin treatment showed no alteration of the PKC response. Hence this slight effect on PKC response must be phalloidin-specific. Without excluding an inhibition of the PKC response mediated by phalloidin stabilization of actin filaments, it is worthwhile noting that phalloidin has several targets in cellular functions. Besides its effect on actin filaments, phalloidin has been shown to induce cell death, which is not the case for actin-depolymerizing drugs [34], and to greatly alter phosphoinositide metabolism in hepatocytes [44]. This alteration is likely to explain the observed inhibition of the PKC response.

Bernstein et al. [35] showed that prolonged electrical depolarization leads to a decrease in vesicular transport at synaptic terminals of phalloidin-treated neurons. Considering the integrative role of PKC in the cellular response [17], it was also interesting to observe how a long stimulation could modulate the PKC activation process and how the actin network was involved in such situations. Although our stimulation protocol involved a 30 mM KCl concentration to avoid cellular exhaustion, it induced a saturating \([\text{Ca}^{2+}]\), response, as revealed by a plateau, and PKC showed a similar response. No significant difference was observed in either latrunculin- or phalloidin-treated cells. Hence if a long stimulation has any effect on vesicular transport through actin network alteration [35], this effect probably occurs downstream of PKC activation since actin network modification has no direct effect on the initial steps of the PKC activation process.

In conclusion, an alteration of F-actin dynamics in both cellular models does not modify the stimulation-induced conformational change of PKCa, which is the initial step of PKC activation detected with the fluorescent dye Fim-1 in living cells. Moreover, F-actin does not seem to play a role in the topography of PKCa activation sites. This does not exclude that minor PKC isoforms may interact with F-actin in our models as described in another cell type [15,45].

Fig. 7. Effect of latrunculin B or phallolidin treatment on the calcium response and PKC activation induced by a sustained depolarization of PC12 cells. Fura-2- and Fim-1-loaded cells were depolarized by 30 mM KCl applications for 30 s, 3 min, and 30 s sequentially as indicated by solid bars in the graphs. Shown are the effects of latrunculin treatment on \([\text{Ca}^{2+}]\), (A) and PKC (B) responses. No significant difference was observed between control (○, \(n = 29\)) and treated (●, \(n = 19\)) cells. Similarly, phallolidin treatment did not significantly affect either \([\text{Ca}^{2+}]\), (C) or PKC response (D) in control (○, \(n = 26\)) or treated (●, \(n = 23\)) cells.

Fig. 8. Western blot analysis of PC12 cell extracts immunoprecipitated with an anti-PKCa antibody. (A) Immunoblots revealed either with an anti-PKCa antibody (lanes 1–3) or with an anti-actin antibody (lanes 4–6). (B) Parallel control experiment in which samples were immunoprecipitated with an anti-IgG to visualize co-precipitated IgG during the sample preparation procedure. In each panel lanes 1 and 6 correspond to crude cell extracts, lanes 2 and 4 to immunoprecipitated material from resting cells, and lanes 3 and 5 to immunoprecipitated material from stimulated cells (60 mM KCl for 1 min). Note the appearance of a second PKC-immunoreactive band around 45 kDa in stimulated cells. No actin was co-precipitated with PKCa antibodies in either resting or stimulated cells.
Additionally we can also conclude that PKC activation sites were present before F-actin network remodeling in our cellular models. The remaining question is the nature of the microdomains involved in the activation of PKCα. If they are not regulated by the F-actin cortical network, they might be defined by the lipid distribution inside the plasma membrane. Such domains, also called rafts, have been described [22]. One subtype of raft, so-called cholesterol-dependent, seems particularly relevant in our case since (1) it is located in the inner leaflet of the cellular membrane, (2) it contains large amounts of phosphatidylserine and phosphatidylcholine [46], an essential effector for PKC activation, and several proteins including MARKS, GAP43, and CAP23 which are able to bind PKC and are PKC substrates. These domains are crucial for regulating membrane structure, cytoskeleton dynamics, and signal transduction system [23]. Alternatively, membrane proteins displaying discrete binding sites for activated PKC isoenzymes may be involved in defining the activation sites for PKCα. Such proteins have been described: RACK1 [47], PICK1 [48], AKAP79 [49].

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References


