Simultaneous visualization of the translocation of protein kinase Cα–green fluorescent protein hybrids and intracellular calcium concentrations

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INTRODUCTION

The advent of quin2 [1] and later fura2 [2] enabled direct monitoring of dynamic calcium changes at high temporal resolution in single cells. Likewise, it is evident that the appearance of green fluorescent protein (GFP) [3] will allow a similar dynamic and detailed characterization of the intracellular localization and movement of proteins, such as cytoskeletal components, phosphatases and kinases. In the case of a subgroup of kinases, movement seems to be a prerequisite to their phosphorylation of target substrates [4]. In such cases, translocation of the proteins can be regarded as diagnostic for their participation in signal transduction. In the present study, we describe for the first time simultaneous monitoring of intracellular calcium concentration ([Ca2+]i) and trafficking of a specific isoform of protein kinase C (PKC) [5,6].

The PKC serine/threonine protein kinase family includes at least 12 different isoforms. They have traditionally been divided into three groups based on their amino acid sequence similarity and dependence upon phospholipid metabolites and calcium. The family comprises: the calcium-dependent conventional PKC (cPKC) isoforms α, β1, βII and γ, the calcium-independent novel PKC (nPKC) isoforms δ, ε, η and θ and the atypical PKC (aPKC) isoforms ι, λ and ζ, which are also calcium independent. In a recently described fourth subclass, the only member is the human μ isoform and its homologue in mice, protein kinase D [7,8]. In addition, cPKCs and nPKCs are regulated by 1,2-diacylglycerol (DAG) and phorbol esters, whereas aPKCs are not [8–10]. The activities of cPKCs, nPKCs and aPKCs are also regulated by phospholipids, in particular by phosphatidylserine (PS) to varying degrees [8].

The tissue distribution of the various PKC isoforms also varies notably. Of the cPKCs, the α isoform is found in most cell types, whereas the βI and βII isoforms are found preferentially in cells of neuronal and haematopoietic lineage [10]. Translocation of the majority of PKC isoforms from the cytoplasm to cellular membranes [9–11] is believed to precede and initiate their activation. For instance, it has been shown, using subcellular fractionation, that stimulation of the plasma membrane bound Gs-coupled muscarinic receptors induces rapid translocation of cPKCs [4,12] as well as fast phosphorylation of target substrates [4]. In this study we have activated the muscarinic receptor type 1 with the acetylcholine analogue carbamylcholine (carbachol), leading to activation of phospholipase Cβ1, which generates DAG and Ins(1,4,5)P3. In turn, Ins(1,4,5)P3 promotes the release of calcium from intracellular stores [13]. Although the membrane lipids DAG and PS also play important roles in the activation of cPKCs, it has been shown that an elevated [Ca2+], alone induces cPKC translocation [14].

GFP offers a novel approach to study protein localization and trafficking in intact living cells [3]. Despite the relatively large size of GFP (238 amino acids), GFP-tagged proteins have been shown to be targeted properly in numerous cases [15]. Recently, GFP-tagged PKCγ has been demonstrated to transiently translocate in a calcium-dependent manner from a cytoplasmic to a plasma membrane localization in response to receptor stimu-
lation [16,17]. To investigate the trafficking of PKCz and its
direct relationship to [Ca\textsuperscript{2+}], in real time in intact living cells, we
tagged the protein kinase with a highly fluorescent variant of
GFP containing F64L and S65T amino acid substitutions
(GFP\textsuperscript{LT}) [18]. We demonstrate here that trafficking of a GFP-
tagged PKCz can be combined with simultaneous measurements of
[Ca\textsuperscript{2+}] using fura2. We conclude that receptor-mediated
PKCz translocation is a very rapid phenomenon closely related to
changes in [Ca\textsuperscript{2+}].

MATERIALS AND METHODS

Hybrid cDNAs construction

Hybrid cDNAs encoding N- and C-terminal fusions of murine
PKCz (mPKCz) [19] to GFP\textsuperscript{LT} (mPKCz–GFP\textsuperscript{LT} and GFP\textsuperscript{LT}–
mPKCz) were inserted into the multiple cloning site of the
pZeoS\textsuperscript{G} (Invitrogen Corp., San Diego, CA, U.S.A.) mammalian
expression vector. Briefly, cDNAs encoding mPKCz and GFP\textsuperscript{LT}
were amplified by PCR using the following primers: 5\textsuperscript{\prime}–mPKCz,
TTGGACACAAAGCGTTGACACCCCTAGTGCT-
GACGTTTACCCGGCCAACG; 3\textsuperscript{\prime}–mPKCz, GTCTATTCT-
TCAGGTCATTTCCAGGCGCCCTACGTGACACATTTGCAG-
GATTGTTGTCG–5\textsuperscript{\prime}–GFP\textsuperscript{LT}–TTGGACACAGGCTTGGAC-
ACGCGGCGCCATGAGTAAGAGGAGAAGACTTTTC and
3\textsuperscript{\prime}–GFP\textsuperscript{LT}–GTATCTTCCTACGTGACACTCTTGAGG-
TTGTATAGTCTCATCAGGCCATGT.

HindIII/Ascl-digested mPKCz fragment and Ascl/Xhol-digested
GFP\textsuperscript{LT} fragment were ligated with the HindIII/Xhol-
digested vector, generating the mPKCz–GFP\textsuperscript{LT} fusion construct.
Correspondingly, the GFP\textsuperscript{LT}–mPKCz construct was
constructed by ligating HindIII/Bsa36I-digested GFP\textsuperscript{LT} fragment
and Bsa36I/Xhol-digested mPKCz fragment with the HindIII/Xhol-
digested vector. To generate a similar construct which allowed the
expression of GFP\textsuperscript{LT} alone, GFP\textsuperscript{LT} was inserted in the vector
as a HindIII/Xhol fragment.

Cell cultures

The human muscarinic receptor type 1 (hM1) [20] was stably
expressed in baby hamster kidney cells (BHK/hM1 cells; Novo
Nordisk A/S, Bagsværd, Denmark) and maintained with the
dihydropyrolofuran reductase marker. These cells were transfected
with the vectors containing hybrid cDNA for the mPKCz–GFP\textsuperscript{LT}
or the GFP\textsuperscript{LT}–mPKCz fusion proteins or GFP\textsuperscript{LT} alone using the
calcium phosphate precipitate method in Hepes-buffered saline
[21]. Stable transfectants were selected using 1000 \mu g of
Zeocin\textsuperscript{\textregistered} /ml (Invitrogen) in growth medium [Dulbecco’s modified
Eagle’s medium with 1000 mg of glucose/l and 10% (v/v) fetal
bovine serum (FBS)] purchased from Life Technologies Inc.,
Gaithersburg, MD, U.S.A. The hM1 receptor and mPKCz–
GFP\textsuperscript{LT} were maintained with 500 nM methotrexate and 500 \mu g
of Zeocin\textsuperscript{\textregistered} /ml respectively. For control purposes, the fusion
proteins were stably expressed in BHK-21 cells (A.T.C.C.,
Rockville, MD, U.S.A.) without the hM1 receptor. Stably
transfected cells will subsequently be referred to as, e.g.
BHK/hM1.mPKCz–GFP\textsuperscript{LT} cells.

For fluorescence microscopy, cells were allowed to adhere to
Lab-Tek chambered coverglasses (Nalge Nunc Int., Naperville,
IL, U.S.A.) for at least 24 h and cultured to about 50% confluence.
Before experiments, the cells were cultured overnight
without selection pressure in HAM’s F12 medium with glutamax
(Life Technologies) and 0.3% (v/v) FBS. This medium has low
autofluorescence, enabling fluorescence microscopy of cells
straight from the incubator.

Fluorescence microscopy and computer-assisted imaging

Time-lapse recording of mPKCz–GFP\textsuperscript{LT} movement

BHK/hM1.mPKCz–GFP\textsuperscript{LT} cells were cultured in HAM’s F12
medium as described above. The chambers were placed on a temperature-regulated microscope stage and kept at 37°C.
Fluorescence images were captured using an Axiovert 135M
inverted light microscope (Carl Zeiss, Oberkochen, Germany)
equipped with a Fluar 40 x, numerical aperture 1.3 oil immersion
objective (Zeiss) and a cooled (−40°C) CH250 charged
device (CCD) camera (Photometrics Ltd., Tucson, AZ, U.S.A.)
with a KAF4300 1340 x 1037 pixel CCD chip. The microscope
was also equipped with a 470 ± 20 nm excitation filter, a 505 nm
dichroic mirror and a 515 ± 15 nm emission filter (Delta Lys &
Optik, Lyngby, Denmark). The excitation light source was a
100 W HBO arc lamp. The images obtained by time-lapse
recording were then analysed using the IPLab Spectrum software
package (Signal Analytics Corp., Vienna, VI, U.S.A.).
The translocation of the fusion protein was measured by tracking two
regions of interest corresponding to the brightest part of the
perinuclear cytoplasm (covering roughly 20% of the cell area)
and the peripheral 5 \mu m of the cell, i.e. the plasma membrane.
The average fluorescence intensity of these regions, divided by
the average fluorescence intensity of the entire cell, to correct for
bleaching, was calculated for every image in the time-lapse series.
Bleaching was in the range 2–15% for an image series, depending
on exposure time and number of images. The temporal
information thus acquired is expressed throughout as mean±S.D.
High-resolution imaging of mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} distribution before and after PMA stimulation

High-resolution images of single BHK/mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} cells were generated from three-dimensional data sets collected using the microscope set-up as described above. Optical sections were acquired through the entire cell at 0.25 \( \mu \)m focal intervals. Captured images had an effective pixel size of 0.164 \( \mu \)m x 0.164 \( \mu \)m. After acquisition, the data sets were corrected for bleaching and fluctuations in illumination intensity. The out-of-focus information in the images was then removed using iterative, constrained three-dimensional deconvolution (DeltaVision from Applied Precision Inc., Seattle, WA, U.S.A.) based on a theoretically calculated point-spread function.

Simultaneous measurement of mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} movement and changes in [Ca\textsuperscript{2+}]

BHK/hM1, mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} cells, cultured in HAM’s F12 medium as described above, were loaded with 0.5 \( \mu \)M fura2 acetoxyethyl ester (fura2/AM) (Molecular Probes Inc., Eugene, OR, U.S.A.) in Ca\textsuperscript{2+}-Hepes buffer, pH 7.4, containing 10 mM Hepes, 140 mM NaCl, 5 mM KCl, 5.5 mM d-glucose, 1 mM MgSO\textsubscript{4} and 1 mM CaCl\textsubscript{2} for 30 min at 37 \( ^\circ \)C. The fura2-loaded cells were placed on the temperature-regulated stage of a Diaphot300 microscope (Nikon Corp., Tokyo, Japan) equipped with a second generation CXTI intensified video camera (Photonic Science Ltd., East Sussex, U.K.). The cells were illuminated with a Polychrome I monochromator (T.I.L.L. Photonics GmbH., Planegg, Germany). In the light path was a 490 nm dichroic mirror and a 515 ± 75 nm emission filter. To reduce background fluorescence, a 415 ± 75 nm excitation filter was included. GFPL\textsuperscript{T} fluorescence was monitored by exciting at 470 nm, whereas fura2 was excited with alternating 340 and 380 nm light.

The fluorescence ratio between 340 and 380 nm images were converted into [Ca\textsuperscript{2+}], by using the equation [Ca\textsuperscript{2+}] = \( K_i(R - R_{\text{min}})/(R_{\text{max}} - R) \beta/2 \), where the dissociation constant \( K_i \) is 224 nM and \( R \) is the 340/380 fluorescence ratio at each image pixel corrected for background. The internal constants for the system were measured using fura2 pentapotassium salt (Molecular Probes) in solution and found to be \( R_{\text{min}} = 0.15 \), \( R_{\text{max}} = 6.16 \) and \( \beta = 6.27 \). The GFPL\textsuperscript{T} fusion protein gave a small contribution to background fluorescence at 380 nm but none at 340 nm. Fura2 did not contaminate the signal at 470 nm. However, with the temporal resolution of these experiments, there was a significant spill-over from one frame to the next due to relaxation lag of the image intensifier. The implication is that basal calcium values were underestimated and extreme values underestimated. While the effect on the GFP\textsuperscript{T} signal was corrected by dividing by the average fluorescence intensity of the entire cell, we decided to sacrifice precision of the calculated [Ca\textsuperscript{2+}] values to obtain maximal temporal resolution. Importantly, the dynamics of [Ca\textsuperscript{2+}] and mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} translocation remained undisturbed. The average cytoplasmic [Ca\textsuperscript{2+}], was calculated within a region of interest corresponding to the non-nuclear cytoplasm in the cell images, whereas mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} trafficking was followed using the method described in the ‘Time-lapse recording of mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} movement’ section.

The integrated calcium response or mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} translocation over time was computed in each individual cell as the area between the observed data and a hypothetical baseline created by interpolating the data acquired before agonist addition and after the return of calcium or the fusion protein to basal level. The qualitatively different responses seen with 10 \( \mu \)M ionomycin were not included in this calculation.

RESULTS

GFPL\textsuperscript{T}-tagged mPKC\textsubscript{\text{a}} translocated in parallel with endogenous PKC\textsubscript{\text{a}}

To monitor PKC\textsubscript{\text{a}} trafficking in real time in living cells, either mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} or GFPL\textsuperscript{T}–mPKC\textsubscript{\text{a}} was stably expressed in BHK/hM1 cells. To compare the translocation of the two fusion proteins with that of the endogenous PKC\textsubscript{\text{a}} in response to various agonists, we performed subcellular fractionation on BHK/hM1, mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} cells (Figure 1A), BHK/hM1, GFPL\textsuperscript{T}–mPKC\textsubscript{\text{a}} cells (Figure 1B) and on control BHK/hM1,GFPL\textsuperscript{T} cells (Figure 1C). The cells were stimulated with buffer or 100 nM PMA, separated into soluble and particulate fractions and characterized by Western blot analysis using polyclonal antibodies directed against the C-terminus of PKC\textsubscript{\text{a}}. This experiment indicated that both fusion proteins had the expected molecular mass (Figures 1A and 1B). The two fusion proteins and the endogenous PKC\textsubscript{\text{a}} translocated in parallel from the soluble to the particulate fraction after stimulation with PMA for 15 min (Figure 1). Owing to possible differential recognition of endogenous hamster PKC\textsubscript{\text{a}} and overexpressed mouse fusion proteins by the antibody employed, their expression levels might not be directly comparable. Note that the expression level of mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} might be underestimated, since the C-terminal PKC\textsubscript{\text{a}} epitope recognized by the antibody is masked by the fusion to GFPL\textsuperscript{T}. Most experiments described below have been conducted with both fusion proteins, although for brevity we have chosen to concentrate on the results obtained with mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T}.

Figure 1 Western blots

BHK/hM1 cells expressing either mPKC\textsubscript{\text{a}}–GFPL\textsuperscript{T} (A), GFPL\textsuperscript{T}–mPKC\textsubscript{\text{a}} (B) or GFPL\textsuperscript{T} alone (C) were stimulated for 15 min with buffer (–) or 100 nM PMA. Total lysates were subjected to subcellular fractionation yielding soluble (s) and particulate (p) fractions. The two fusion proteins (f) had the expected molecular mass of 104 kDa. The endogenous PKC\textsubscript{\text{a}} (e) had a molecular mass of 77 kDa.
The subcellular localization of mPKCα–GFP LT was studied before (top) and 15 min after (bottom) stimulation with 100 nM PMA by using digitally deconvolved Z-series of wide-field images. From the deconvolved images, five single slices were merged into 1 μm thick reconstructed slices from the same BHK/hM1,mPKCα–GFPLT cell before and after stimulation and are shown here as two-dimensional representations, the slices being 1.5 μm above the substratum. The images shown are representative for results from three separate experiments.

Response to PMA

The subcellular distribution of mPKCα–GFP LT was studied in high spatial resolution using digitally deconvolved Z-series of wide-field images. Two-dimensional reconstructions, 1 μm thick (based on five 0.25 μm slices), are shown (Figure 2). In unstimulated cells, the fusion protein was distributed fairly homogeneously throughout the cytoplasm, with a somewhat higher concentration in the perinuclear region (Figure 2A).

Stimulation with 100 nM PMA caused the cells to round up and the fusion protein to translocate evenly to all parts of the plasma membrane, with a concomitant depletion of the cytoplasm (Figure 2B). The remaining central fluorescence is likely to be associated with the basal plasma membrane since the slice was taken close to the substratum. We cannot, however, based on the present experiments, exclude that a fraction of this fluorescence actually is cytoplasmic.

Wide-field fluorescence micrograph time-lapse series showed that 100 nM PMA induced complete and permanent (cells were followed up to 2 h) translocation of mPKCα–GFP LT within 5 min (Figure 3). Bear in mind that mPKCα–GFP LT, localized to the upper region of the plasma membrane, erroneously gives the impression of fusion protein remaining in the cytoplasm. It should be noted that the reverse GFP LT–mPKCα fusion protein behaved in a similar way in response to PMA (n = 2, results not shown).

Response to carbachol

As shown in Figure 4, 100 μM carbachol caused a fast and transient translocation of mPKCα–GFP LT to the plasma mem-
protein translocation \((n = 2, \text{ results not shown})\) and that GFP\(^{\text{LT}}\) alone, expressed in BHK/hM1 cells, was distributed evenly throughout the cells and neither 50 nM PMA \((n = 2)\) nor 50 \(\mu M\) carbachol \((n = 3)\) had any effect on its diffuse distribution pattern \((\text{results not shown})\).

### Monitoring of PKC\(\alpha\) trafficking and \([Ca^{2+}]\)

The importance of \([Ca^{2+}]\), for the translocation of PKC\(\alpha\) was investigated using several approaches. BHK/hM1,mPKC\(\alpha\)–GFP\(^{\text{LT}}\) cells were pretreated for 5 min with 1 \(\mu M\) thapsigargin (Tg) \([23]\). This treatment inhibited the translocation of mPKC\(\alpha\)–GFP\(^{\text{LT}}\) in response to 100 \(\mu M\) carbachol by 97.5 \(\pm 4\%\) \((n = 8)\), while the accompanying change in \([Ca^{2+}]\), was inhibited by 99.7 \(\pm 0.2\%\) \((n = 5, \text{ separate experiments})\). Similarly, the cells were loaded with 10 \(\mu M\) 1,2-bis-(\(\alpha\)-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid (BAPTA)/AM, a cell permeant non-fluorescent calcium chelator. This treatment inhibited the translocation in response to 100 \(\mu M\) carbachol by 62.5 \(\pm 6\%\) \((n = 7)\), whereas the accompanying change in \([Ca^{2+}]\), was inhibited by 66.8 \(\pm 4\%\) \((n = 6, \text{ separate experiments})\). However, neither Tg treatment \((n = 3)\) nor BAPTA/AM loading \((n = 4)\) changed the mPKC\(\alpha\)–GFP\(^{\text{LT}}\) translocation in response to 100 \(\mu M\) PMA. Under these circumstances no change in \([Ca^{2+}]\), was observed \((\text{Tg: } n = 3, \text{ BAPTA/AM: } n = 6, \text{ separate experiments})\).

To investigate the temporal relationship of changes in \([Ca^{2+}]\), and PKC\(\alpha\) translocation with high time resolution, we loaded BHK/hM1,mPKC\(\alpha\)–GFP\(^{\text{LT}}\) cells with 0.5 \(\mu M\) fura2/AM, a cell permeant fluorescent calcium probe. \([Ca^{2+}]\), and mPKC\(\alpha\)–GFP\(^{\text{LT}}\) were monitored simultaneously by exciting the cells at 340, 380 and 470 nm in rapid succession. Such sets of three images were acquired consecutively at 400 ms intervals. The \([Ca^{2+}]\), was computed based on the images acquired with excitation at 340 and 380 nm, whereas trafficking of mPKC\(\alpha\)–GFP\(^{\text{LT}}\) was followed in the images acquired with excitation at 470 nm (Figure 5).

The translocation of mPKC\(\alpha\)–GFP\(^{\text{LT}}\) in response to carbachol observed under these conditions had a similar temporal profile \((\text{Table } 1)\) as described in the previous section. With the temporal resolution of these experiments we were unable to separate the initial calcium rise after carbachol or ionomycin stimulation from the onset of PKC\(\alpha\) translocation to the plasma membrane. Both responses were initiated within the same 400 ms interval. However, it is evident from Table 1 and Figure 5 that, in the case of both carbachol and ionomycin, the calcium response preceded the translocation of PKC\(\alpha\). There was a tendency to a slower PKC\(\alpha\) translocation in response to carbachol in these experiments compared with the cells not loaded with fura2. In particular, high-maximal translocation was reached after about 8 s \((\text{Table } 1)\) in fura2-loaded cells stimulated with 100 \(\mu M\) carbachol, whereas the time required in cells not loaded with the indicator was 2.9 \(\pm 0.4\) s.

The degree of PKC\(\alpha\) translocation in response to carbachol correlated with the increase in \([Ca^{2+}]\), \((\text{Figure } 5A–5C)\). The most pronounced increase in \([Ca^{2+}]\), was observed with 100 \(\mu M\) carbachol, coincident with the highest degree of PKC\(\alpha\) translocation, whereas 1 \(\mu M\) carbachol gave a reproducible, although small, increase in \([Ca^{2+}]\), but no detectable PKC\(\alpha\) translocation. At 10 \(\mu M\) carbachol the increase in \([Ca^{2+}]\), and the degree of PKC\(\alpha\) translocation were intermediate. For each cell, the integrated mPKC\(\alpha\)–GFP\(^{\text{LT}}\) translocation is plotted directly against the integrated calcium response in Figure 5(D), regardless of carbachol dose. Similarly, stimulation with ionomycin also showed correlation between changes in \([Ca^{2+}]\), and the degree of PKC\(\alpha\) translocation \((\text{Figure } 5E–5G)\): 10 \(\mu M\) ionomycin gave a sustained increase in \([Ca^{2+}]\), coincident with a sustained trans-
Figure 5  Simultaneous monitoring of mPKCα–GFPLT and [Ca2+]i

Fura2-loaded BHK/hM1,mPKCα–GFPLT cells were stimulated with three different concentrations of carbachol (A–D) or ionomycin (E–H). [Ca2+]i (A and E) was monitored simultaneously with movement of mPKCα–GFPLT, as seen by the change in relative fluorescence intensity (arbitrarily set to 1 at the beginning of each experiment) of the perinuclear cytoplasm (B and F) and the plasma membrane (C and G). As indicated in the graphs, cells were stimulated with either 1 μM, 10 μM or 100 μM carbachol or with 0.1 μM, 1 μM or 10 μM ionomycin. Arrows indicate the addition of agonist. The integrated responses over time were computed as described in the Materials and methods section on the basis of graphs similar to those shown in (A and B) and (E and F). The integrated mPKCα–GFPLT translocation away from the cytoplasm is plotted directly against the corresponding integrated calcium responses to evaluate the correlation of these parameters independent of carbachol or ionomycin dose. Such results from 5–8 cells from three independent experiments for each carbachol concentration are shown in (D) while results from 4–7 cells from two independent experiments for each ionomycin concentration are shown in (H). Results from addition of 10 μM ionomycin have been omitted from (H) as they are qualitatively different. Integrated calcium and translocation measurements are highly correlated with Rank correlations of 0.65, P < 0.003 (D) and 0.79, P = 0.004 (H) respectively.

Table 1  Time from initiation of a response to half-maximal [Ca2+]i, and mPKCα–GFPLT translocation (t1/2 max)

The results were extracted from curves such as those shown in Figures 5(A)–5(C) and 5(E)–5(G). All t1/2 max values are given as means ± S.D. and are based on 4–8 cells from 2–3 independent experiments for each agonist concentration.

<table>
<thead>
<tr>
<th>Agonist (μM)</th>
<th>[Ca2+]i</th>
<th>mPKCα–GFPLT</th>
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<tr>
<td></td>
<td>(s×nM)/1000</td>
<td></td>
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<tr>
<td>Carbachol</td>
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<tr>
<td>100</td>
<td>0.8 ± 0.5</td>
<td>8.0 ± 1.0</td>
</tr>
<tr>
<td>10</td>
<td>1.2 ± 0.7</td>
<td>5.2 ± 1.7</td>
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<tr>
<td>1</td>
<td>11.5 ± 6.3</td>
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<tr>
<td>Ionomycin</td>
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<tr>
<td>10</td>
<td>1.0 ± 0.3</td>
<td>4.6 ± 2.8</td>
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<tr>
<td>1</td>
<td>1.3 ± 0.1</td>
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<td>0.1</td>
<td>8.7 ± 2.7</td>
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location of PKCα to the plasma membrane; 1 μM ionomycin gave a substantial calcium transient and a transient PKCα translocation; and 0.1 μM ionomycin gave a significant, although small, increase in [Ca2+]i, but no detectable PKCα translocation. For each cell, the integrated mPKCα–GFPLT translocation is plotted directly against the integrated calcium response in Figure 5(H) for ionomycin doses 0.1 and 1 μM. Rank correlation of the results in Figures 5(D) and 5(H) indicated a high probability for a positive correlation between PKCα translocation and [Ca2+]i, (P < 0.01) in response to carbachol or ionomycin. In contrast, the translocation of mPKCα–GFPLT in response to 100 nM PMA observed under these conditions was not accompanied by any change in [Ca2+]i (Figure 6).

Possible influence of the cytoskeleton

The cells rounded up in response to 10 μM ionomycin. The onset of this morphological change was about 1 min after addition of
agonist, i.e. long after fusion protein translocation was complete. No significant morphological changes were observed with carbachol or the lower concentrations of ionomycin.

To assess whether the cytoskeleton influences or controls the redistribution of PKCs we stimulated cells with 10 nM ionomycin, after 20 min preincubation with 1 μg/ml colcemid (n = 4), to depolymerize microtubules, or with 1 μM cytochalasin D (n = 6) to block the formation of F-actin. Both drugs caused some rounding of the cells at the concentrations used, but did not by themselves cause any redistribution of mPKCz–GFP\textsuperscript{LT} beyond confinement within the altered shape of the cells. The subsequent addition of ionomycin caused a permanent redistribution of fusion protein, much like the response seen in cells not treated with anticytoskeletal drugs.

**DISCUSSION**

The aim of the present study was to follow the trafficking of PKCz in real time in intact living cells with high spatial resolution. In addition, we wanted to correlate this process with intracellular calcium changes. To achieve this, we generated N- and C-terminal fusion proteins of mPKCz to a highly fluorescent variant of GFP. The following observations suggest that both fusion proteins behave like the endogenous kinase with regard to localization and trafficking. We found GFP\textsuperscript{LT}–mPKCz and mPKCz–GFP\textsuperscript{LT} in the same subcellular fraction as the endogenous kinase, before and after stimulation with phorbol ester. The partition of PKCz between soluble and particulate fractions agrees with previous studies using this fractionation method [22]. Furthermore, the intracellular localization of fusion protein in BHK/hM1, mPKCz–GFP\textsuperscript{LT} cells, before and after stimulation with PMA or carbachol, was very similar to the localization of endogenous PKCz in smooth muscle cells before and after stimulation with phenylephrine respectively, as visualized by immunocytochemistry [24]. Thus, it is reasonable to presume that PKCz trafficking can be studied in an isoform-specific manner using either of the described fusion proteins.

The time course of PMA-induced translocation described herein is comparable with results from a previous study in smooth muscle cells [25] where a fluorescently tagged phorbol ester, used both to track and activate PKCs, caused their translocation after about 2 min. It should be stressed that treatment of cells with Tg or loading with the calcium chelator BAPTA did not noticeably affect the fusion protein translocation in response to PMA, nor did PMA in itself cause a change in [Ca\textsuperscript{2+}]. Thus the PMA-induced translocation seems to be independent of an increase in [Ca\textsuperscript{2+}]. According to a previous study, PKCz translocated to the endoplasmic reticulum as well as to the plasma membrane after PMA stimulation [11]. The reason for this discrepancy is not clear, but our results are strengthened by the fact that individual cells were followed dynamically during the entire process of translocation. Although the translocation of fusion protein in response to PMA is accompanied by a rounding up of cells, we have no reason to suspect that this major morphological change has any influence on the partition of fusion protein between the cytoplasm and plasma membrane, since a very similar redistribution was observed in response to carbachol without any morphological changes. Furthermore, a previous study [25] has elegantly shown that PMA-inducible PKC translocation clearly precedes cell contraction in smooth muscle cells, although they both occur within about 2 min.

The temporal profile of carbachol-induced translocation reported here extends the conclusions of previous studies, based on subcellular fractionation, where the earliest time points are 15 s. These reports show rapid and transient translocation from a soluble to a particulate fraction of PKCz [4,26] and of PKCs in general [12]. We followed PKCz translocation with a higher temporal resolution and found that the initial calcium rise preceded the onset of translocation by less than 400 ms. The calcium increase in these cells was very steep, reaching half maximum after about 1 s in response to high or intermediate doses of carbachol or ionomycin, whereas PKCz translocation under the same conditions was half-maximal after 4–8 s. The fusion protein returned to the same localization as before stimulation within 1 min. Carbachol did not induce PKCz translocation in cells pretreated with Tg and translocation was attenuated in cells loaded with the calcium chelator BAPTA. This suggests that an elevated [Ca\textsuperscript{2+}], is necessary for receptor-induced translocation in our system. Furthermore, these observations imply that an increased DAG concentration alone was insufficient for PKCz translocation. We also observed that ionomycin induced either a transient or permanent PKCz translocation. Taken together these observations may indicate, as reported earlier [14], that an elevated [Ca\textsuperscript{2+}], is sufficient for PKCz translocation, without a concomitant increase in the concentration of DAG or PS. It has previously been proposed that there is a threshold value of [Ca\textsuperscript{2+}], for translocation of PKCz [24]. Simultaneous monitoring of mPKCz–GFP\textsuperscript{LT} and [Ca\textsuperscript{2+}], supports this notion, since small increases in [Ca\textsuperscript{2+}], did not produce any detectable PKCz translocation. However, due to the imprecision of the calculated [Ca\textsuperscript{2+}], values, we are reluctant to give an absolute value for this threshold.

With regard to the molecular mechanism behind PKC translocation, it is tempting to implicate the cytoskeleton, based on previous work where an association between PKC isoforms and cytoskeletal components was reported [11,27,28]. However, in parallel with a previous study on PKC\textgamma translocation [16], our observation that disturbance of microtubules or actin filaments did not influence the distribution or calcium-dependent translocation of fusion protein does not point to a direct involvement of the cytoskeleton in the mechanism of PKC translocation. In fact, although the translocation reported here is very rapid, it does not necessarily require an active transport process. We can estimate a diffusion coefficient for the 104 kDa fusion protein in the cytoplasmic matrix under the assumption that Stokes’ Law, describing the movement of a sphere with a constant velocity through a viscous liquid, can be applied to globular proteins. With a diffusion coefficient of GFP in water of 8.7 × 10\textsuperscript{-7} cm\textsuperscript{2}/s
[29,30], and a roughly 5-fold higher viscosity for translational diffusion in the cytoplasm compared with water [30,31]. We calculate a diffusion coefficient for mPKCα–GFP314 in cytoplasm of 1.1 × 10⁻² cm²/s. The implication of this value is that cytoplasmic fusion protein on average travels about 10 μm by diffusion alone within 4.5 s. This timing is compatible with our observation of maximal and half-maximal translocation of mPKCα–GFP314 after 14.7 ± 3.4 s and 2.9 ± 0.4 s respectively.

The present observations are compatible with a model for PKC translocation previously proposed by Mochly-Rosen [32]. In that model there is a weak interaction in unstimulated cells between PKC and an anchoring component in the target compartment. When the signal is present, it increases the affinity of the kinase for the target molecule, which shifts the equilibrium of PKC to favor the bound form. Applied to the present results, the diffusional equilibrium of PKCα would shift towards the plasma membrane bound form upon signal generation.

In conclusion we demonstrate that the trafficking of a GFP-tagged specific isoform of PKC can be followed simultaneously with measurements of [Ca²⁺], in single living cells. Our results suggest that a definite increase in [Ca²⁺], is necessary for receptor-mediated PKCα translocation. The present findings give no support for a direct role for the cytoskeleton in PKCα translocation. Indeed, the timing of this event is such that it may be explained by mere diffusion of the protein to the plasma membrane from its apparent cytoplasmic localization.

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