Signalling from the Plasma Membrane to the Nucleus

**Nuclear localization of protein kinase C**
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### Introduction

A key issue in defining signal transduction pathways is understanding how signals generated at the plasma membrane are communicated to the nucleus. A number of studies have implicated a role for protein kinase C (PKC) in nuclear function. For example, the transcription of many genes, including c-fos and those coding for plasminogen activator, interferon and interleukin-2, is increased by the treatment of cells with phorbol esters, activators of PKC [1-4]. Phosphorylation of a number of nuclear proteins, such as lamin B, matrix proteins and DNA topoisomerase II, is stimulated by phorbol 12-myristate 13-acetate (PMA), suggesting that PKC may be phosphorylating these proteins directly, and thereby regulating their function [5-7].

Using immunological and biochemical analyses, we and others have demonstrated the presence of PKC in the nuclei in liver [8], HL-60 cells [9] and 3T3 cells [10-12]. In some systems, such as the liver, PKC appears to be constitutively expressed in the nucleus [8]. In contrast, in other cell systems, PKC is present in nuclei prepared from stimulated, but not unstimulated cells. Stimulation of nuclear localization results not only from treatment with PMA, but also from incubation of cells with mitogens such as platelet-derived growth factor, insulin-like growth factor-1 and α-thrombin [11-13]. The effect of PMA may result from a direct activation of PKC, but the mechanisms by which mitogens result in increased levels of nuclear PKC are not known.

In this paper we will review our results demonstrating nuclear localization of PKC in two different cell lines, NIH 3T3 cells, and IIC9 cells [10, 13]. Treatment of either cell type with PMA results in increased levels of PKC in the nucleus. In the IIC9 cells, we have also demonstrated that treatment with the physiological agonist α-thrombin stimulates nuclear localization. Furthermore, we have used the IIC9 system to begin to address the mechanisms for α-thrombin-induced PKC nuclear localization, and we have shown that increased nuclear diglyceride production accompanies the increase in nuclear PKC.

### Results and discussion

Using a monoclonal antibody specific for PKCa, we carried out immunofluorescence experiments in fixed and in permeabilized NIH 3T3 cells [10]. In untreated cells, the staining was diffuse and localized in the cytoplasm. In addition, the nuclei were visible due to the absence of staining. After treatment of the cells with 200 nM PMA for 10 min, the staining pattern was markedly altered. The staining covered the entire surface of the cells, and the nuclei were no longer apparent. These results suggest that, in contrast to untreated cells, in PMA-treated cells, the localization of PKC includes the nucleus.

To address this issue directly, we carried out biochemical analyses of isolated nuclei. Since the purity of the nuclei is of paramount importance to the interpretation of the results, we established the purity of our nuclear preparation first. Electron microscopy showed that the preparation contained intact nuclei with no contaminating organelles. Using adenylate cyclase as a marker, plasma membrane contamination was <5% of that in the 100 000 g non-nuclear membrane fraction. Cytosolic contamination, as assessed by superoxide dismutase activity, was not detected.

PKC localization to the nuclear fraction was demonstrated in three ways. Firstly, immunofluorescence of nuclei showed bright, heterogeneous staining in nuclei isolated from PMA-treated cells, but not those from control cells. Secondly, biochemical assays for PKC enzymic activity demonstrated a low specific activity in nuclear lysates from control cells, which was increased 10-20-fold in nuclear lysates from PMA-treated cells. Thirdly, Western blot analysis was performed on nuclear lysates, and the 82 kDa PKC was present only in nuclei from PMA-treated cells. These experiments demonstrated that in untreated 3T3 cells, little or no PKC was present in the nucleus. However, treatment with PMA led to a marked stimulation in the level of nuclear PKC. Kinetic experiments demonstrated that the PMA-induced increase in nuclear PKC occurred within 5 min of phorbol ester treatment, and that the level was maintained for several...
hours. Thus, the nuclear localization appears to be a rapid, primary event following PKC activation.

The localization of PKC within the nucleus was examined further in fractionation experiments. Separate fractions containing intranuclear proteins, RNA, DNA and nuclear envelopes were obtained and probed on Western blots with the PKC antibody. Only the nuclear envelope fraction from the PMA-treated cells contained the 82 kDa PKC. As a control in these experiments, the effectiveness of the nuclear separation was tested using antibodies to a nuclear structural protein, to an intranuclear protein and to lamin B, a nuclear envelope protein. These experiments confirmed the efficiency of our fractionation protocol and demonstrated that co-fractionation of PKC with nuclear envelope proteins was not due simply to incomplete separation of the nuclear fractions.

These experiments demonstrated nuclear localization of PKC after treatment with PMA, which activates PKC directly. We were interested in examining whether physiological agonists that activate PKC via the generation of diacylglycerol would also result in the nuclear localization of PKC. For these experiments, we used IIC9 fibroblasts. Our previous work in this system demonstrated that the treatment of these cells with α-thrombin results in increased levels of cellular diacylglycerol (DAG), via the hydrolysis both of phosphoinositides and of phosphatidylcholine (PC) [14–16]. Kinetic analysis showed that two peaks of DAG are generated after treatment of cells with 500 ng/ml α-thrombin. The first peak of DAG occurs at 15 s and results from hydrolysis of phosphatidylinositol 4,5-bisphosphate, while the second DAG peak reaches a maximum at 5 min [17]. Molecular species and head-group analysis has shown that this second peak of cellular DAG results primarily, if not exclusively, from PC hydrolysis. In addition, α-thrombin treatment of IIC9 cells activates PKC rapidly, as demonstrated by an increase in PKC association with the membrane, and stimulates phosphorylation of an endogenous PKC substrate, the myristoylated, alanine-rich C-kinase protein. This PKC activation is temporally related to the formation of DAG from phosphoinositide, but not to PC hydrolysis [14].

Thus, these studies demonstrate that α-thrombin increases cellular diacylglycerol levels and activates PKC. However, the contribution of possible changes in nuclear levels of DAG and/or PKC was not examined. Therefore, using this system we investigated whether α-thrombin treatment induces changes in nuclear phospholipids and whether the nuclear localization of PKC is stimulated.

For these studies, cell fractionation was carried out, and nuclei were isolated [13]. A similar fractionation protocol was used as in the NIH 3T3 cell experiments, except that the isolation was carried out in the complete absence of detergents, to assess changes in nuclear lipids. The purity of the nuclei was established first by electron microscopy. Adenylate cyclase activity measurements demonstrated that plasma membrane contamination of the nuclei was less than 5%. Possible cytoskeletal contamination of the nuclear preparations was also examined. Immunofluorescence experiments demonstrated minimal staining of the nuclei with antibodies to vimentin and tubulin, indicating that the levels of intermediate filament and microtubule contamination were extremely low. These results were confirmed by Western blot analysis of the nuclear lysates using the same antibodies. Thus, these results demonstrate that the nuclei isolated in our procedure were highly pure and structurally intact.

Western blot analysis with isozyme-specific antibodies demonstrated that IIC9 cells express PKCα, -ɛ and -ζ isozymes. Densitometric quantitation of the Western blots demonstrated that in untreated cells PKCα was predominantly cytosolic, while PKCɛ and -ζ were present both in the cytosol and in non-nuclear membrane fractions in approximately equal proportions (Table 1). Nuclear levels of PKCα, -ɛ and -ζ were quite low: 3.2±0.4%, 11±5.1% and 3.2±0.6% respectively, in the untreated cells.

Treatment of cells with 200 nM PMA for 10 min caused a decrease in the level of PKCα and -ɛ in the cytosolic fraction, with a concomitant increase in the membrane PKCα and -ɛ levels. No changes were observed in the localization of PKCζ under any of the treatment conditions, consistent with observations that PKCζ is insensitive to activation by DAG or by phorbol esters [18]. The level of PKCα in the nuclear fraction increased approx. tenfold, to 32±10%. In contrast, nuclear PKCɛ and -ζ remained at control levels.

Consistent with our previous observations [14], treatment of the cells with α-thrombin caused an increase in the level of PKCα in the non-nuclear membrane fraction. In addition, the non-nuclear membrane levels of PKCɛ were increased by α-thrombin treatment. Nuclear PKCα levels
Table 1
PKC isozyme subcellular localization

Densitometric analysis of PKC isozyme Western blots from control (vehicle), PMA (200 nM, 10 min) or α-thrombin (500 ng/ml, 1 min) treated cells was carried out as described in [13]. The results are the average ± S.E.M. of three experiments. Reprinted from [13] with permission.

<table>
<thead>
<tr>
<th>PKC isozyme content (%)</th>
<th>α</th>
<th>ε</th>
<th>ζ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>84 ± 5.0</td>
<td>54 ± 13</td>
<td>54 ± 1.2</td>
</tr>
<tr>
<td>Non-nuclear membrane</td>
<td>13 ± 5.0</td>
<td>35 ± 15</td>
<td>43 ± 1.8</td>
</tr>
<tr>
<td>Nuclear lysate</td>
<td>3.2 ± 0.4</td>
<td>11 ± 5.1</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>PMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>15 ± 1.2</td>
<td>2.7 ± 2.0</td>
<td>49 ± 12</td>
</tr>
<tr>
<td>Non-nuclear membrane</td>
<td>54 ± 10</td>
<td>83 ± 9.0</td>
<td>48 ± 12</td>
</tr>
<tr>
<td>Nuclear lysate</td>
<td>32 ± 10</td>
<td>1.9 ± 0.3</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>α-Thrombin</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cytosol</td>
<td>63 ± 9.2</td>
<td>9.6 ± 5.0</td>
<td>48 ± 3.3</td>
</tr>
<tr>
<td>Non-nuclear membrane</td>
<td>26 ± 7.0</td>
<td>87 ± 3.5</td>
<td>50 ± 2.1</td>
</tr>
<tr>
<td>Nuclear lysate</td>
<td>10 ± 3.0</td>
<td>5.7 ± 3.0</td>
<td>1.9 ± 1.1</td>
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</tbody>
</table>

Table 2
PKC activity in IIC9 cell extracts

Cells were incubated with vehicle (control), in 200 nM PMA (10 min) or 500 ng/ml α-thrombin (1 min) and subcellular fractions were prepared. The membrane and nuclear fractions were extracted with 1% (w/v) NP-40 and PKC activity in all the fractions was measured after DEAE chromatography as described in reference 13. Results are the average ± S.E.M. of triplicate determinations from one representative experiment. Reprinted from [13] with permission.

<table>
<thead>
<tr>
<th>PKC activity (nmol/mg per min)</th>
<th>Control</th>
<th>PMA</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000 g Supernatant</td>
<td>3.76 ± 0.27</td>
<td>0.17 ± 0.093</td>
<td>2.12 ± 0.13</td>
</tr>
<tr>
<td>100,000 g Non-nuclear membrane</td>
<td>0.68 ± 0.039</td>
<td>7.01 ± 0.097</td>
<td>2.35 ± 0.28</td>
</tr>
<tr>
<td>Nuclear lysate</td>
<td>0.21 ± 0.14</td>
<td>5.53 ± 0.12</td>
<td>2.09 ± 0.11</td>
</tr>
</tbody>
</table>

increased approx. threefold in the α-thrombin-treated cells, while nuclear PKCe and ζ levels did not change. These results indicate that treatment either with PMA or with α-thrombin leads to a differential localization of PKCa, but not PKCe or ζ, to the nucleus.

Kinetic experiments demonstrated that the nuclear localization of PKCa after α-thrombin treatment occurred rapidly. Increased levels of nuclear PKCa were apparent at 30 s, the earliest time-point tested. At 1 min, nuclear PKC levels were still elevated, but by 5 min, the level was decreased, and nuclear PKC was undetectable at 10 min.

The presence of PKC in the nucleus of IIC9 cells was further demonstrated in enzymic activity assays. To measure nuclear kinase activity, purified nuclei were extracted with 1% (w/v) Triton X-100, and PKC activity in the extracts was measured after DEAE chromatography (Table 2). In control cells, the specific activity of the nuclear fraction was low, at 0.21 nmol/mg per min. The activity in the
nuclear fraction increased approx. 20-fold after treatment with 200 nM PMA for 10 min. Similarly, in nuclei isolated from α-thrombin-treated cells, the specific activity was increased.

Phosphorylation of endogenous nuclear proteins was also stimulated by PMA and by α-thrombin. To carry out these experiments, nuclear lysates isolated from 32P-labelled cells were analysed by two-dimensional PAGE, and quantified by densitometry. Increased phosphorylation of at least six nuclear proteins was observed in nuclei from PMA- and α-thrombin-treated cells. The proteins were approx. 67–77 kDa in molecular mass, with pI values of approx. 6.5–7.0. The identity and function of these proteins is not known, but they indicate that the stimulated levels of nuclear PKC result in phosphorylation of specific nuclear proteins.

Taken together, these results clearly demonstrated that the physiological agonist α-thrombin increased nuclear localization of PKCα. Given our previous observations demonstrating that α-thrombin induced changes in cellular DAG levels [14], we investigated whether changes in nuclear DAG also occurred. Mass levels of DAG were assayed using a DAG kinase assay, after nuclear isolation and Bligh–Dyer extraction [13]. Nuclear DAG levels were increased two–fourfold after α-thrombin treatment of IIC9 cells (Figure 1). The response was rapid, and peak levels occurred within 2–5 min, and remained elevated for at least 30 min.

Further experiments were carried out to examine the potential phospholipid pool(s) from which the nuclear DAG was derived. We demonstrated previously that short-term labelling of IIC9 cells with [3H]myristate preferentially labels the PC pool in whole cells [19]. Accordingly, cells were labelled for 2 h with 5 μCi/ml of [3H]myristic acid and then incubated with 500 ng/ml α-thrombin. Within 5 min of treatment, an increase in the level of radiolabelled nuclear DAG was observed. These results indicate that the stimulated nuclear DAG may be generated, at least in part, from PC hydrolysis. While these results indicate that the stimulated nuclear DAG may be derived from PC, increases in nuclear DAG mass were observed before an increase in radiolabelled DAG was detected [13]. Furthermore, examination of radiolabelled nuclear phospholipids indicated that other phospholipids, in addition to PC, were labelled significantly (K. I.

**Figure 1**

**Time-course of DAG production in IIC9 nuclei**

IIC9 cells were treated with 500 ng/ml α-thrombin (•) or with α-thrombin vehicle (○), and, at the indicated times, nuclei were prepared as described in [13]. The results presented represent two independent experiments each performed in duplicate, and are representative of at least six (main graph) or two (inset) independent experiments. Error bars are present on all data-points and indicate the range of values observed. Reprinted from [13] with permission.
Leach and D. M. Raben, unpublished work). Recent studies of the molecular species of induced nuclear diglycerides and phospholipids demonstrate that PC is the primary source of the induced nuclear diglycerides at all times (M. B. Jarpe, K. L. Leach and D. M. Raben, unpublished work).

Overall, these results demonstrate that in two different cell systems, PMA treatment leads to an increase in nuclear PKC levels. In the IIC9 cells, the physiological agonist α-thrombin also stimulates nuclear PKC. The nuclear localization in the IIC9 cells was isozyme-specific, since only the α isozyme was observed in nuclear lysates. Such a differential activation of specific PKC isozymes has been observed in other studies. For example, lysophosphatidylcholine potentiates the activation of PKCα, -β, and -γ, but inhibits the activation of PKCδ and -ζ [20]. In nuclear studies, Hocevar and Fields have demonstrated that in HL-60 cells, bryostatin treatment causes the nuclear localization of PKCβ, but not of PKCα [9]. These results are all consistent with the hypothesis that PKC isozymes can be activated differentially by cellular agonists. In addition, the differential localization of isozymes to various subcellular compartments, including the nucleus, may be a key regulator of PKC function.

The PMA- and α-thrombin-stimulated nuclear localization of PKC was rapid, suggesting that it may be an early step in PKC activation. In all our studies, we observed a concomitant increase in PKC levels both in the nuclear and in the non-nuclear membrane fractions following stimulation. Thus, activated PKC may associate with more than one membrane compartment.

The mechanism(s) by which α-thrombin stimulated nuclear PKC levels is not known. However, a marked increase in nuclear DAG levels accompanied the rise in nuclear PKC levels. DAGs derived from plasma-membrane-stimulated phospholipid hydrolysis have been shown clearly to activate PKC, and an analogous mechanism may exist in the nucleus. Our results support the idea that changes in nuclear DAG levels may be the determinant of PKC nuclear localization, by promoting the association of specific PKC isozymes with the nuclear membrane. Clearly, additional study is necessary to identify the mechanisms by which thrombin stimulates DAG, and how they may act to regulate PKC activity.

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