The ATP-dependent Membrane Localization of Protein Kinase C α Is Regulated by Ca²⁺ Influx and Phosphatidylinositol 4,5-Bisphosphate in Differentiated PC12 Cells

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Signal transduction through protein kinase Cs (PKCs) strongly depends on their subcellular localization. Here, we investigate the molecular determinants of PKC α localization by using a model system of neural growth factor (NGF)-differentiated pheochromocytoma (PC12) cells and extracellular stimulation with ATP. Strikingly, the Ca²⁺ influx, initiated by the ATP stimulation of P2X receptors, rather than the Ca²⁺ released from the intracellular stores, was the driving force behind the translocation of PKC α to the plasma membrane. Furthermore, the localization process depended on two regions of the C2 domain: the Ca²⁺-binding region and the lysine-rich cluster, which bind Ca²⁺ and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂], respectively. It was demonstrated that diacylglycerol was not involved in the localization of PKC α through its C1 domain, and in lieu, the presence of PtdIns(4,5)P₂ increased the permanence of PKC α in the plasma membrane. Finally, it also was shown that ATP cooperated with NGF during the differentiation process of PC12 cells by increasing the length of the neurites, an effect that was inhibited when the cells were incubated in the presence of a specific inhibitor of PKC α , suggesting a possible role for this isoenzyme in the neural differentiation process. Overall, these results show a novel mechanism of PKC α activation in differentiated PC12 cells, where Ca²⁺ influx, together with the endogenous PtdIns(4,5)P₂, anchor PKC α to the plasma membrane through two distinct motifs of its C2 domain, leading to enzyme activation.

INTRODUCTION

The past two decades have seen an extraordinary expansion in our understanding of the role of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂], both as a direct regulator of membrane and cytoskeletal proteins and as the precursor of key signaling molecules. PtdIns(4,5)P₂ is a critical second messenger that regulates a myriad of diverse cellular activities, including modulation of the actin cytoskeleton, vesicle trafficking, focal adhesion formation, and nuclear events (McLaughlin *et al.*, 2002; Wenk and De Camilli, 2004).

Protein kinase C (PKC) α belongs to the group of classical PKC isoenzymes that share the property of using C1 and C2 domains as membrane-targeting modules. The function of this enzyme is regulated by Ca²⁺, anionic phospholipids, and diacylglycerol (DAG) (Newton, 2001). The literature

describes how the activation of classical and novel PKCs is mainly through the signaling pathway activated by PtdIns(4,5)P₂ hydrolysis mediated by receptor-activated phospholipase C (PLC) (Yang and Kazanietz, 2003). However, whether other different signaling pathways may also lead to an increase in intracellular Ca²⁺ or whether lipids other than diacylglycerol and phosphatidylserine may act as regulators/activators of these isoenzymes has not been established.

Extracellular ATP and its catabolites have a diverse range of biological effects that are mediated by purinoreceptors (Ralevic and Burnstock, 1998). ATP may stimulate the rapid depolarization of cells as well as mediate long-term changes in cellular metabolism. This range of biological actions suggests the involvement of many different classes of purinoreceptors, which are ubiquitously expressed and comprise several distinct subclasses. To date, there has been substantial confusion over the identity and actions of the purinoreceptors, owing largely to the lack of specific agonists or antagonists. Molecular analysis of purinoreceptors and their effectors has now established that ATP acts principally through ionotropic P2X and metabotropic P2Y receptor subclasses. P2X receptors are ATP-gated ion channels, which enable the rapid and selective access of cations such as Na⁺, K⁺, and Ca²⁺ into the cytosol (North, 2002). The P2Y receptors are linked to intracellular signaling events through G protein-coupled pathways, most notably those activating phospholipase C (Ralevic and Burnstock, 1998). Interestingly, both P2X and P2Y receptor subclasses stimulate an increase in intracellular calcium levels, but they accomplish

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Abbreviations used: CFP, cyan fluorescent protein; DAG, diacylglycerol; DiC8, 1,2-*sn*-dioctanoylglycerol; GFP, green fluorescent protein; HBS, HEPES buffer saline; NGF, neural growth factor; PC12, pheochromocytoma cells; PH, pleckstrin homology; PI-PLC, phosphatidylinositol-specific-phospholipase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; PtdIns(4,5)P₂, phosphatidylinositol (4,5)-bisphosphate; YFP, yellow fluorescent protein.

this effect through mechanistically distinct pathways. One of the major unresolved issues surrounding the activation of P2X receptors is how they are linked to intracellular signaling systems that exercise their specific biological effects.

Recent work performed in our laboratory suggests that the C2 domain of PKC α might act in a double mode, depending on the activation mechanism of the enzyme. Thus, if the activation is operated by Ca²⁺/phosphatidylserine (PtdSer), the Ca²⁺-binding region of the C2 domain would be the motif involved in the translocation to the plasma membrane (Bolsover *et al.*, 2003). However, if the activation is driven by Ca²⁺/PtdIns(4,5)P₂, the lysine-rich cluster would be the motif of the C2 domain involved in the enzyme localization (Corbalán-García *et al.*, 2003). Nevertheless, how these types of activation mechanisms are organized in the spatiotemporal physiology of the cell or whether the two motifs act together are unexplored to date.

Advances in the use of green fluorescent protein (GFP) have enabled researchers to investigate the activity of PKC in vivo in intact cells. This technique permits the measurement of the subcellular localization of PKC-GFP in cells transfected with this construct (Oancea *et al.*, 1998; Bolsover *et al.*, 2003). Currently, a variety of biosensors derived from GFP have been designed and can detect most aspects of phosphoinositide signaling. For example, PtdIns(4,5)P₂ microdomains can be detected in the plasma membrane by using the pleckstrin homology (PH) domain of PLC δ 1 (Stauffer *et al.*, 1998), and in a similar way, DAG generation can be studied using the C1a domain of PKC γ (Oancea *et al.*, 1998). These strategies provide us with the means to correlate the changes in each component of the pathway in real time.

We used neural growth factor (NGF)-differentiated pheochromocytoma (PC12) cells as a model system to characterize the membrane localization of PKC α when the cells had been stimulated by extracellular ATP. The present study provides direct evidence that Ca²⁺ influx through the P2X receptors results in the activation of PKC α , the first step being the step that drives the localization of the enzyme in the plasma membrane. Furthermore, cooperation of the PtdIns(4,5)P₂ in the membrane is important for keeping the enzyme bound to this area. The combined use of the PH domain of PLC δ 1 and the C1a domain of PKC α as bioprobes to detect the endogenous PtdIns(4,5)P₂ and DAG, respectively, showed that DAG is not involved in the localization process of the enzyme under these conditions, but PtdIns(4,5)P₂ might participate in the anchorage of PKC α to the plasma membrane. We also observed that there is a correlation between the inhibition of PKC α and the length of the neurite outgrowth promoted by NGF and ATP, suggesting a role for this isoenzyme in the development of the neural phenotype in these cells.

MATERIALS AND METHODS

cDNA Constructions

N-terminal fusions of rat PKC α and the different mutants to enhanced green fluorescent protein (EGFP) were generated by inserting cDNAs into the multiple cloning site of the pEGFP-N3 (BD Biosciences Clontech, Palo Alto, CA) mammalian expression vector. Briefly, cDNAs encoding PKC α and its mutants D187N/D246N/D248N and K197A/K209A/K211A were amplified by PCR by using the following primers: 5'-ATTCTCGAGCTATGGCTGACGTT and 3'-CCGGGTACCTACTGCACTTTGCAAGAT.

XhoI/KpnI-digested PKC α and mutated fragments were ligated with the XhoI/KpnI-digested vector to generate the different fusion constructs. To generate the PKC α -enhanced yellow fluorescent proteins (EYFP) constructs, the cDNAs were amplified by PCR by using the primers 5'-CTCAAGCTTATGGCTGACGTTTAC and 3'-GCGGTACCGTTACTGCACTTTGCAAGAT and inserted into the HindIII/KpnI sites of the pEYFP-N1 mammalian expression

vector (BD Biosciences Clontech), which was modified to introduce the mutation A206K to reduce the intrinsic homoaffinity of all GFPs and preclude intermolecular fluorescence resonance energy transfer by enhanced cyan fluorescent protein (ECFP)-EYFP dimerization (Zacharias *et al.*, 2002). Further details concerning site-directed mutagenesis to generate the different mutants were reported by Conesa-Zamora *et al.* (2001) and Rodríguez-Alfaro *et al.* (2004). All constructs were confirmed by DNA sequencing (Sistemas Genómicos, Valencia, Spain). The stability and viability of the mutated proteins was studied by using specific activity measurements. It was demonstrated that the mutants can be activated in a PtdSer-dependent manner, although to a reduced extent compared with the wild-type protein (Conesa-Zamora *et al.*, 2001; Rodríguez-Alfaro *et al.*, 2004). Moreover, previous studies have shown that a C-terminal GFP tag does not affect the catalytic activity or the cofactor dependence of PKC α (Valentin *et al.*, 2000).

The C1a domain of PKC α was amplified by PCR by using the primers 5'-GAAGCTTAAGAACGTCATGAG and 3'-GGGGTACCTTAGTCAGGTC-CCTTATC before being cloned into the HindIII/KpnI restriction sites of the pEYFP-N1 (BD Biosciences Clontech) mammalian expression vector. This vector was previously modified by inclusion of the ECFP oligonucleotide sequence in the BglII/HindIII restriction sites. Note that the oligonucleotide of the 3' end contains a stop codon to prevent the EYFP expression.

The cDNA corresponding to the PH-PLC δ domain was kindly provided by Dr. Tobias Meyer (Stanford University, Stanford, CA) and was characterized by Raucher *et al.* (2000). This domain was amplified by PCR by using the primers 5'-ACGGTACCGATGGACTCGGGCCGG and 3'-GCTCTAGACTG-GATGTTGAGCTCCTT and introduced into the KpnI/XbaI restriction sites of the pECFP-N1 mammalian expression vector. Both ECFP and EYFP contained the mutation A206K, as stated above.

Cell Culture and Transfections

PC12 cells (European Collection of Cell Cultures, Salisbury, Wiltshire United Kingdom) were cultured on poly-ornithine-coated culture coverslips in DMEM supplemented with 10% heat inactivated horse serum and 5% fetal bovine serum (FBS). To promote cell differentiation, the medium was changed to DMEM supplemented with 5% heat inactivated horse serum, 2.5% FBS, and 100 ng/ml mouse submaxillary NGF (Alomone Labs, Jerusalem, Israel). The cells were kept under differentiating conditions for 2–3 d, and the differentiation medium was replaced every 24 h. Cells were transfected with 2 μ g of DNA/6 cm plate with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. The cells were analyzed 10–16 h after transfection.

To analyze the cells by confocal microscopy, the coverslips were washed with 3 ml of extracellular HEPES buffer saline (HBS) (120 mM NaCl, 2.5 mM glucose, 5.5 mM KCl, 3 mM CaCl₂, 1 mM MgCl₂, and 20 mM HEPES, pH 7.2). All added substances were dissolved or diluted in HBS. 1,2-Dioctanoyl-*sn*-glycerol (DiC8) was dissolved in dimethyl sulfoxide (DMSO) and diluted to the final concentration with extracellular buffer shortly before the experiment. During the experiment, the cells were not exposed to DMSO concentrations higher than 1%. All the experiments were carried out at room temperature, and, unless otherwise stated, on at least four different occasions. In each experiment, recordings were obtained for two to six cells. When used, the phospholipase inhibitors U73122 (10 μ M) and 1-butanol (50 mM) were present for 20 min before stimulation with 100 μ M ATP.

Confocal Imaging and Data Analysis of Intracellular Calcium ([Ca²⁺]_i) and GFP Variants

[Ca²⁺]_i was measured using Fura-Red (Molecular Probes, Eugene, OR). Stock solutions (2 mM) of the acetoxymethyl ester (AM)-ester form of the fluorescent Ca²⁺ indicator were made using a solution of 2.5% (wt/vol) Pluronic F-127 in absolute DMSO. This stock was diluted 1000-fold in growth medium and applied to cells for 30 min at 25°C and 5% CO₂. After incubation, cells were washed with HBS and analyzed using a Leica TCS SP2 confocal system (Leica, Heidelberg, Germany) using a Nikon PLAN APO-CS 63 $\times$ 1.4 numerical aperture oil immersion lens. During imaging, cells were stimulated with different compounds as indicated in the text. Confocal images were obtained by excitation with a laser Ar/ArKr at 488 nm and by using a reflection short pass (RSP) 500 filter. Emission wavelengths were detected at 640–700 nm for Fura Red. To detect the different EGFP-expressing constructs the cells were washed with HBS and examined under the same conditions stated above. Confocal images were obtained by excitation at 488 nm, by using an RSP500 filter and emission wavelengths at 510–525 nm for EGFP. Constructs expressing ECFP were excited at 458 nm, an RSP460 filter, and emission wavelengths at 470–490. Constructs expressing EYFP were excited at 514 nm, a double dichroic filter DD458/514 and emission wavelengths at 530–580. Confocal images of simultaneous detection of ECFP and EYFP were obtained by excitation at 458 and 514 nm, a double dichroic filter DD458/514, and emission wavelengths recorded in two independent channels at 470–490 and 540–580. It was tested that under these conditions there was no bleed-through of the CFP into the YFP emission. Series of 30–60 confocal images were recorded for each experiment at time intervals of 10 s.

[Ca²⁺]_i was calculated as described by Bolsover *et al.* (2003). The images were analyzed using ImageJ NIH software (<http://rsb.info.nih.gov/ij/>), 1997–

2004). The individual analysis of protein translocation for each cell was performed by tracing a line intensity profile through an area that crosses the membrane and part of the cytosol of the cell. The relative increase in plasma membrane localization is expressed as the ratio $R = (I_{mb} - I_{cyt})/I_{mb}$, where I_{mb} is the fluorescence intensity at the plasma membrane and I_{cyt} is the average cytosolic fluorescence intensity (Oancea *et al.*, 1998). Mean values are given $\pm$ SE of the mean.

Measurements of Neurite Outgrowth

The response of cultures to NGF and ATP was quantified by counting neurite-bearing cells. Neurite-bearing cells were scored under phase optics and only processes greater than 10 μ m in length were considered neurites. A positive score requires the presence of at least one neurite per cell. For each culture condition, at least 100 cells from ten randomly chosen fields were scored. The program used to measure the length of neurites was NeuronJ, which is included in the plug-ins of the ImageJ NIH software (<http://rsb.info.nih.gov/ij/>, 1997–2004).

RESULTS

PKC α Translocates to the Plasma Membrane When Differentiated PC12 Cells Are Stimulated with Extracellular ATP

Translocation from the cytosol to the plasma membrane is a key event of classical PKCs activation in somatic cells. Here, we monitor the dynamics of fluorescently labeled PKC α in NGF-differentiated PC12 cells (dPC12) during stimulation with extracellular ATP. This construct has been successfully used in RBL-2H3 cells to study PKC α activation and was found to retain the catalytic and regulatory properties of the native protein (Conesa-Zamora *et al.*, 2001; Bolsover *et al.*, 2003). In unstimulated cells, the protein was evenly distributed through the cytosol (Figure 1A), whereas stimulation with ATP resulted in its localization in the plasma membrane although unequally distributed (Figure 1A). When the time course of this event was analyzed, PKC α translocation to the plasma membrane resulted in maximal localization 20 s after ATP stimulation. This persisted for 50 s and initiated a slow separation that took 132 s to reach half-maximal dissociation (Figure 1B).

When intracellular Ca^{2+} concentrations were measured in these cells, a maximum Ca^{2+} peak was observed 10 s after ATP stimulation, rapidly reaching a half-maximal decrease at 23 s (Figure 1C). The results obtained analyzing individual cells pointed to the great variability of intracytosolic Ca^{2+} concentration peaks, which ranged from 1.3 to 4 μ M, and all of them correlated with PKC α translocation to the plasma membrane. Close examination of the kinetics of the PKC α -EGFP and Ca^{2+} signals indicated that protein localization in the plasma membrane started within the first 10 s of the intracytosolic Ca^{2+} peak, suggesting that translocation to the membrane was a Ca^{2+} -driven process. However, the protein remained localized in the plasma membrane, whereas the Ca^{2+} concentration in the cytosol corresponded to basal levels (compare the time profiles of Figure 1, B and C), suggesting that other ligands besides Ca^{2+} might be involved in protein anchorage to the plasma membrane.

Ca^{2+} Influx Elicited by ATP Stimulation Is Needed to Localize PKC α in the Membrane

As stated in Introduction, extracellular ATP leads to the stimulation of at least two types of receptor: ionotropic P2X and metabotropic P2Y. Due to the lack of specific agonists or inhibitors for each subtype of these receptors, it is very difficult to discern their individual contribution to a specific effect (Ralevic and Burnstock, 1998). In addition, the nature of the Ca^{2+} dependency of classical PKCs to be activated makes it difficult to establish whether stimulation of only one of these receptors or both is needed to generate the Ca^{2+}

signal that promotes protein translocation to the plasma membrane. One way to study this is to use UTP because it specifically stimulates several subtypes of P2Y receptor (Ralevic and Burnstock, 1998). Thus, to study their contribution to the effect observed above, dPC12 cells were transfected with PKC α -EGFP and stimulated with 100 μ M UTP. Strikingly, no protein translocation to the plasma membrane was observed (Figure 2A) and when the intracytosolic Ca^{2+} concentration was measured in these cells, it ranged from 240 to 500 nM (Figure 2B). These results suggest that the Ca^{2+} liberated from the intracellular stores by P2Y receptor stimulation is not enough to promote the translocation of PKC α to the plasma membrane of these cells. Furthermore, the high intracytosolic Ca^{2+} concentration observed is more likely to depend on the Ca^{2+} influx generated by the direct activation of P2X receptor stimulated by ATP. The possibility of a Ca^{2+} influx caused by the capacitative Ca^{2+} entry (through store-operated Ca^{2+} channels in the plasma membrane) can be discarded, because the UTP stimulation of dPC12 cells did not generate an intracytosolic Ca^{2+} peak similar to that obtained after ATP stimulation.

To confirm the nature of the elevation of intracytosolic Ca^{2+} concentration obtained, we designed another experiment, in which dPC12 cells transfected with PKC α -EGFP were incubated in a buffer in the absence of extracellular Ca^{2+} (in the presence of EGTA) and stimulated with 100 μ M ATP; in this way, the effect of Ca^{2+} influx in the cells would be avoided, and the only Ca^{2+} released would be that from the intracellular stores. The results were very similar to those obtained when the cells were stimulated with UTP and no PKC α membrane localization was observed (Figure 3). Furthermore, the intracytosolic Ca^{2+} levels measured were very low with a maximum concentration of 500 nM (Figure 3). Importantly, when the same cells were washed with Ca^{2+} -containing buffer and stimulated again with 100 μ M ATP, PKC α -EGFP translocated to the plasma membrane and the concentration of intracytosolic Ca^{2+} ranged again from 1.3 to 4 μ M (Figure 3). These results clearly showed that the Ca^{2+} influx produced by ATP stimulation is a key event leading to PKC α membrane localization in dPC12 cells.

Together, these unexpected data suggest that the ATP-dependent Ca^{2+} influx is the driving force responsible for the localization of PKC α in the plasma membrane of dPC12 cells, most probably through the stimulation of P2X receptors, and the P2Y receptor subfamily does not seem to play a key role in the generation of intracellular Ca^{2+} signals that regulate PKC α localization under these conditions.

The Ca^{2+} -binding Region of the C2 Domain Is Responsible for the ATP-induced Membrane Translocation of PKC α

It was striking to observe that the stimulation of dPC12 cells with ATP induced the release of Ca^{2+} from the intracellular pools that was not enough to promote enzyme localization in the plasma membrane. Nevertheless, when the same construct has been studied in somatic cells, it translocated to the plasma membrane at lower Ca^{2+} concentrations ($\sim$ 400 nM), which were released from the intracellular reservoirs (Oancea and Meyer, 1998; Bolsover *et al.*, 2003). Because of this, we examined the minimum Ca^{2+} influx able to promote enzyme localization in the membrane. For this, dPC12 cells were transfected with PKC α -EGFP and stimulated with 100 μ M ATP in HBS containing several different free Ca^{2+} concentrations: 2, 1, 0.75, and 0.5 mM. When protein localization

Figure 1. Protein kinase C α translocates to the plasma membrane of dPC12 cells via ATP stimulation. (A) Confocal images of dPC12 cells expressing protein kinase C α -EGFP and stimulated with 100 μ M ATP. The frames shown in the figure correspond to 0 and 20 s after ATP stimulation. Protein kinase C α -EGFP fluorescence intensity was measured in the cytosol and in the plasma membrane in every frame of the time series (1 frame every 10 s). (B) The protein localization was measured by a line profile (pixel density) traced in each frame as indicated in *Materials and Methods* procedures section and analyzed with the program ImageJ NIH. The resulting net change in protein kinase C α localization is expressed as the Imb-Icyt/Imb ratio (R) and is represented versus time ($n = 24$ cells). The dotted line represents an average of the time course of [Ca²⁺]_i after ATP stimulation, which has been taken from part C of this figure and inserted here to facilitate the interpretation of the data. (C) Time course of [Ca²⁺]_i fluctuations were monitored with Fura Red, the figure shows different profiles representative of the wide range of the [Ca²⁺]_i observed ($n = 125$ cells). It is important to note that all of them correlated with protein kinase C α translocation to the plasma membrane after ATP stimulation.

was examined, it was found that the extracellular Ca²⁺ concentration of 0.75 mM (Figure 4A) was still compatible with PKC α localization in the plasma membrane after ATP stimulation at levels similar to those obtained with 3 mM CaCl₂ (Figure 1). The intracellular Ca²⁺ concentrations, measured by loading the cells with the indicator Fura Red, ranged from 1.125 to 2.9 μ M. However, a concentration of extracellular Ca²⁺ of 0.5 mM was not able to promote enzyme localization in the plasma membrane and, when the intracellular Ca²⁺ concentrations were measured, they ranged from 229 to 830 nM (Figure 4B). Together, these data suggest a threshold value of [Ca²⁺]_i of ~ 1 μ M for PKC α translocation. Furthermore, it is important to observe how the Ca²⁺ peaks leading to membrane localization of PKC α

last for a longer time than those not inducing the localization process. The higher [Ca²⁺]_i peaks remained for at least 20 s (Figures 1C and 4A), whereas the lower [Ca²⁺]_i peaks remained < 10 s (Figures 2B and 4B).

To further examine the role of the Ca²⁺-binding region of the C2 domain, we also studied the behavior of a PKC α -EGFP mutant, with a triple substitution of the key aspartate residues by Asn, which has previously been demonstrated to be unable to bind Ca²⁺ or PtdSer (Conesa-Zamora *et al.*, 2001; Bolsover *et al.*, 2003). In this case, the mutant protein did not translocate to the plasma membrane when the cells were stimulated with 100 μ M ATP, confirming that the Ca²⁺-binding region of C2 domain of PKC α is also the motif responsible for the membrane localization of PKC α after Ca²⁺ influx (Figure 5).

Figure 2. UTP stimulation does not induce protein kinase $C\alpha$ localization in the plasma membrane of dPC12 cells. (A) Confocal images corresponding to dPC12 cells treated with $100\ \mu\text{M}$ UTP at 0 and 10 s after stimulation. (B) Time course of $[Ca^{2+}]_i$ variations in dPC12 cells loaded with Fura Red-AM ester and stimulated with $100\ \mu\text{M}$ UTP. The profiles represented in the figure are representative of the cells analyzed ($n = 32$) and none of them correlated with protein kinase $C\alpha$ translocation to the plasma membrane after UTP stimulation.

The Diacylglycerol Generated by Phosphatidylinositol (PI)-specific-PLC Seems Not to Be Involved in PKC α Membrane Localization

It is important to note that PKC α remained bound to the plasma membrane independently of the Ca^{2+} peak originated by ATP stimulation (Figure 1B), suggesting the possible contribution of other additional factors in the localization of the enzyme. Among these, DAG could be a good candidate, because the addition of extracellular ATP also stimulates P2Y receptors, which in turn, activate the PI-PLC that hydrolyzes PtdIns(4,5) P_2 into DAG and inositol-1,4,5-trisphosphate (Ins P_3) (Ralevic and Burnstock, 1998). To test this hypothesis, the transfected cells were preincubated with $10\ \mu\text{M}$ U73122, which is a specific PI-PLCs inhibitor (Smallridge *et al.*, 1992); however, PKC α -EGFP translocation to the membrane was observed after ATP stimulation (Figure 6A). Furthermore, when the time profile of the PKC α localized in the plasma membrane was examined, the protein remained there for a long time, the half-maximal dissociation being 222 s (Figure 6B), compared with the 132 s observed in the absence of inhibitor.

Another source of DAG, in response to cell stimulation, is generated by the activation of phospholipase D (PLD), which hydrolyzes phosphatidylcholine (PtdCho) to produce phosphatidic acid (PtdOH) and choline, in a second step a

phosphohydrolase catalyzes the synthesis of DAG (Wakelam, 1998). One way to inhibit this reaction is by using 1-butanol, which restrains PtdOH synthesis. The transfected cells were preincubated with 50 mM 1-butanol, and they were activated with ATP afterward. Analysis of the time profile of the PKC α localized in the plasma membrane revealed a half-maximal dissociation time of 133 s (Figure 6C), which is very similar to that exhibited in the absence of 1-butanol, suggesting that PLD might not be involved in this localization process. We also tested the effect of the preincubation with U73122 and 1-butanol together on dPC12 cells, and the results were very similar to those obtained when U73122 was used alone (our unpublished data).

Together, these results show that the inhibition of PI-PLCs increase the permanence of PKC α in the plasma membrane, and consequently PtdIns(4,5) P_2 could mediate the anchoring of PKC α to the membrane, rather than the DAG generated in the reactions catalyzed by the phospholipases.

To further study whether DAG is involved in the membrane targeting of PKC α after ATP stimulation, we monitored the subcellular localization of the isolated C1a domain of PKC α , which it has been demonstrated is responsible for the DAG-dependent membrane translocation of the enzyme (Medkova and Cho, 1999). To study this, the C1a domain of PKC α was fused to ECFP to produce a fluorescence probe able to sense the endogenous DAG generated under cell stimulation (Oancea *et al.*, 1998). The dPC12 cells transfected with the ECFP-C1a construct were stimulated with $100\ \mu\text{M}$ of either ATP or UTP, and no localization of the C1a domain in the plasma membrane was observed (Figure 7A). These results suggest that neither ATP nor UTP stimulation induces a downstream pathway to generate sufficient DAG to produce the effect expected, or in lieu, the C1a domain of PKC α is not able to bind to the DAG generated in the plasma membrane under these conditions. To gain further insight into this, we performed an analysis of the time course of C1a domain membrane localization depending on the extracellular addition of DiC8, a cell-permeable analogue of diacylglycerol. In this case, the ECFP-C1a domain responded to DiC8 and started to localize in the plasma membrane at extracellular concentrations of $2\ \mu\text{g/ml}$, with an apparent half-maximal [DiC8] of $\sim 5\ \mu\text{g/ml}$, suggesting that the endogenous DAG concentration generated under ATP or UTP stimulation is lower than this and not sufficient to drive the plasma membrane localization of the domain in a DAG-dependent manner (Figure 7B).

The Endogenous PtdIns(4,5) P_2 Cooperates in the Plasma Membrane Localization of PKC α

Overall, the above-mentioned results indicate that the DAG generated in the membrane of ATP-stimulated dPC12 cells plays little part in the membrane targeting of PKC α , and instead PtdIns(4,5) P_2 might take part in this process. A good candidate for this function in PKC α would be the lysine-rich cluster of the C2 domain that is localized in the $\beta 3$ - $\beta 4$ strands of the domain and that has been demonstrated to specifically interact with PtdIns(4,5) P_2 *in vitro*, leading to activation of the enzyme (Corbalán-García *et al.*, 2003). To study the endogenous levels of PtdIns(4,5) P_2 in the plasma membrane of dPC12 cells, we used the PH domain from PI-PLC $\delta 1$ as a PtdIns(4,5) P_2 biosensor in the plasma membrane, because this probe has been extensively demonstrated to constitutively localize in PtdIns(4,5) P_2 -rich areas of the plasma membrane of different cell lines (Stauffer *et al.*, 1998). Furthermore, stimuli received by

Figure 3. ATP evoked Ca²⁺ influx is necessary to drive protein kinase C α to the plasma membrane. Time course of [Ca²⁺]_i variations of dPC12 cells loaded with Fura Red-AM ester and stimulated with 100 μ M ATP. The cells were incubated with extracellular medium containing Ca²⁺ free HBS (from 0 to 150 s) and stimulated with ATP at the time indicated by the arrow. At 150 s of the recording, the cells were washed with 4 ml of HBS containing 3 mM CaCl₂, and stimulated again with ATP, which now induced a Ca²⁺ peak, ranging from 1.3 to 4 μ M. Confocal images (a–c) of dPC12 cells transfected with protein kinase C α -EGFP, taken at the times indicated by the corresponding letters a–c in the [Ca²⁺]_i time profile.

receptors in the membrane, which are sufficiently strong to generate a decrease in the concentration of PtdIns(4,5)P₂, produce a transient dissociation of the probe from the plasma membrane (Stauffer *et al.*, 1998; Raucher *et al.*, 2000).

Thus, we generated a probe consisting of the PH domain of PLC δ fused to ECFP (ECFP-PH-PLC δ). Figure 8A shows the localization of the ECFP-PH-PLC δ domain expressed in dPC12 cells. The protein spread unevenly through the plasma membrane in areas that would correspond to areas enriched in PtdIns(4,5)P₂, as expected. However, after ATP stimulation of the cells transfected with the ECFP-PH-PLC δ domain, it did not dissociate from the plasma membrane, suggesting that PtdIns(4,5)P₂ hydrolysis is not noticeably stimulated in these conditions and indirectly confirming the data reported above that demonstrated that the phosphoinositide-dependent generation of DAG might be very low or nonexistent. Similar results were obtained when the cells were stimulated with 100 μ M UTP (our unpublished data).

Afterward, dPC12 cells were cotransfected with the ECFP-PH-PLC δ domain and PKC α -EYFP to obtain further information on the colocalization of both proteins. In resting conditions, the ECFP-PH-PLC δ domain was localized in the

plasma membrane and PKC α -EYFP was localized unevenly throughout the cytosol (Figure 8B). Strikingly, when the cells were stimulated with 100 μ M ATP, it was observed that the localization of PKC α in the plasma membrane correlated with a dissociation of the ECFP-PH-PLC δ domain from the membrane (Figure 8, B and C). Moreover, when PKC α returned to the cytosol, the PH-PLC δ domain translocated again to the plasma membrane (Figure 8C). It is important to note that this seems to be a direct effect related to PKC α localization in the membrane because no similar results were observed when the cells were transfected only with the PH-PLC δ domain and stimulated with ATP. Such observations imply an important role for the endogenous PtdIns(4,5)P₂ in the membrane localization of PKC α , and to further corroborate this hypothesis, we used a PKC α mutant that has been demonstrated not to bind to PtdIns(4,5)P₂ in vitro (Corbalán-García *et al.*, 2003) and that has two Lys residues substituted by Ala in the β 4 strand of the C2 domain (PKC α K209A/K211A-EYFP). The mutant protein was cotransfected with the ECFP-PH-PLC δ domain, and the cells were stimulated with ATP. In this case, it was observed

Figure 4. Threshold value of $[Ca^{2+}]_i$ for translocation of protein kinase C α in dPC12 cells. The cells were loaded with Fura Red-AM ester and preincubated with HBS containing different concentrations of extracellular Ca^{2+} . (A) Time course of $[Ca^{2+}]_i$ variations when the cells ($n = 32$) were incubated with HBS containing $750 \mu M$ $CaCl_2$ and stimulated with ATP. The panels show dPC12 cells transfected with protein kinase C α -EGFP in a representative experiment in resting conditions (a) and 20 s after ATP stimulation (b). (B) Time course of $[Ca^{2+}]_i$ variations when the cells ($n = 30$) were incubated with HBS containing $500 \mu M$ $CaCl_2$ and stimulated with ATP. No protein kinase C α -EGFP membrane localization was observed in these conditions. Note that the $[Ca^{2+}]_i$ is a transient peak that only reached 830 nM, which seems insufficient to promote translocation of protein kinase C α to the plasma membrane. The concentration of free Ca^{2+} was estimated from total concentration of Ca^{2+} and EGTA by using computer software developed by Fabiato (1988).

that the former translocated partially to the plasma membrane. Thus, only 58% of the mutant PKC α localized in the membrane and the half-maximal dissociation was of 97 s compared with 132 s exhibited by the wild-type protein. In this case, the PH-PLC δ domain did not break away from the membrane (Figure 9), suggesting that the smaller affinity of mutant PKC α reverses the competitive effect observed in the case of the wild-type PKC α .

Inhibition of Endogenous PKC α Decreases the Neuritogenic Effect of Extracellular ATP

Overall, the results obtained in this work suggest a distinct mode of action of PKC α in dPC12 cells that depends directly on the Ca^{2+} influx activated by ATP and the PtdIns(4,5) P_2 present in the plasma membrane. Due to the novelty of these findings, we decided to study the possible biological implications of this type of PKC α activation. Thus, we investi-

PKC α -D187N/D246N/D248N-EGFP

Figure 5. The Ca²⁺-binding region of the C2 domain is a key motif in the membrane localization of protein kinase α . Confocal images of dPC12 cells expressing protein kinase α -D187N/D246N/D248N-EGFP mutant before (a) and 20 s (b) after ATP stimulation are shown ($n = 21$ cells). The protein kinase α mutant is cytosolic in unstimulated cells and does not translocate to the plasma membrane after the addition of ATP in any of the frames analyzed. Confocal images were recorded every 10 s for 10 min.

gated whether the activation of PKC α by ATP plays a role in the differentiation process of PC12 cells. One of the key features of NGF biological actions is neuritogenesis and, in our study, NGF-treated PC12 cells became increasingly neuronal in morphology with branching neurites being observed after 24–48 h (compare Figure 10, A and C). In addition, it has been demonstrated that extracellular ATP improves the neuritogenic effect of NGF (D'Ambrosi *et al.*, 2000), and accordingly, we designed an experiment in which ATP was added to the differentiation medium in the absence and in the presence of NGF, measuring both neuronal morphology and neurite length after 48 h of incubation with various differentiation media. Our results showed that $71 \pm 6\%$ of the PC12 cells stimulated by NGF displayed neurites with a length of $28 \pm 3 \mu\text{m}$ (Figure 10C). When ATP was added to the differentiation media in the absence of NGF, only $21 \pm 3\%$ of the cells demonstrated a very low grade of neuronal morphology and the neurites in these cells were shorter ($13 \pm 2 \mu\text{m}$) than those in the NGF-treated cells (compare Figure 10, B and C), suggesting that ATP stimulation by itself is not able to induce neural cell differentiation. However, when NGF and ATP were added together to the differentiation medium, $75 \pm 10\%$ of the cells were differentiated, and most importantly, they exhibited very long and branched neurites ($47 \pm 10 \mu\text{m}$) compared with those displayed by the cells treated only with NGF ($28 \pm 3 \mu\text{m}$) (compare Figure 10, C and D). In general, these results confirm that ATP complements the neuritogenic effect exerted by NGF in this cell line.

To study whether PKC α was involved in the differentiation process, we used bisindolylmaleimide XI (BIM XI), which, at a low concentration of 20 nM, acts as a specific inhibitor of this isoenzyme (Wilkinson *et al.*, 1993). Thus, the cells were incubated with NGF, ATP, or NGF plus ATP in the presence of BIM XI, and the results were analyzed after 48 h of treatment. As shown in Figure 10, F and G, the inhibitor had no effect on the degree of differentiation or the length of the neurites whether it was incubated with NGF or ATP. However, the inhibitor was observed to have a strong effect on the cells differentiated in the presence of NGF and ATP together. In this case, the PC12 cells exhibited a new phenotype and showed up as large, round cells with sym-

A PKC α -EGFP + 10 μM U73122

Figure 6. Inhibition of PI-PLC increases the localization time of protein kinase α in the plasma membrane. (A) dPC12 cells expressing protein kinase α -EGFP were pretreated with 10 μM U73122 for 20 min before ATP stimulation. Translocation of the protein to the plasma membrane started 10 s after ATP addition. (B) Time profile of the effect of U73122 on the protein kinase α -EGFP membrane localization that is expressed as relative plasma membrane translocation, R ($n = 15$ cells). (C) Time profile of the effect of preincubation for 20 min with 50 mM 1-butanol on the protein kinase α -EGFP membrane localization, R ($n = 21$ cells).

metrical spreading and forming clumps (Figure 10E), whereas no neurites were developed. Only $28 \pm 7\%$ of the cells showed the differentiated phenotype, but importantly, the length of the neurites was shorter ($21 \pm 4 \mu\text{m}$) than in the

Figure 7. Neither ATP nor UTP stimulation induces the ECFP-C1a domain translocation to the plasma membrane. (A) The C1a domain of protein kinase α was fused to ECFP and expressed in dPC12 cells after ATP (top) or UTP (bottom) stimulation. Confocal images were collected every 10 s for 10 min. Observe that the domain localized both in the cytosol and in the cells nucleus, as described previously by Oancea and Meyer (1998). (B) To confirm the functionality of the cloned C1a domain, the transfected cells were incubated with increasing concentrations of extracellular DiC8 and the maximal relative membrane localization (R) was calculated for each of the DiC8 concentrations used. The apparent half-maximal [DiC8] was calculated graphically.

cells differentiated in the absence of inhibitor (compare Figure 10, D and E). These findings are consistent with the mediation of PKC α in the ATP/NGF differentiation process, and more specifically, they provide evidence for a critical role of this enzyme in the development of the neurite outgrowth.

DISCUSSION

PKC α is an isoenzyme that belongs to the PKC family and responds to effectors such as Ca^{2+} , DAG, and PtdSer. Recent studies suggest that PtdIns(4,5) P_2 also interacts with the C2 domain of this enzyme, leading to its activation (Corbalán-García *et al.*, 2003). The present work reveals a series of findings that enable us to propose a new model for the plasma membrane translocation of PKC α when differentiated PC12 cells are stimulated with extracellular ATP.

ATP-provoked Ca^{2+} Influx Is Responsible for the Membrane Localization of PKC α

PC12 cells have a long history as model cells for the study of ATP responses because they resemble sympathetic neurons under the influence of NGF. It has been shown that ATP acts as a fast transmitter or cotransmitter in autonomic and sensory nerves, mostly through the activation of ionotropic P2X receptors but also through metabotropic P2Y receptors (Cunha and Ribeiro, 2000). Moreover, the Ca^{2+} that enters through P2X receptors initiates a broad variety of downstream signaling functions, for example, the activation of mitogen-activated protein kinases (Swanson *et al.*, 1998) or the stimulation of transforming growth factor- β 1 mRNA expression (Wang *et al.*, 2003). Our work shows that the increase in $[\text{Ca}^{2+}]_i$ after ATP stimulation is responsible for driving PKC α to the membrane, through its C2 domain, with a threshold of 1 μ M $[\text{Ca}^{2+}]_i$ and whose duration is at least 20 s. A very surprising discovery was the fact that the ATP-induced Ca^{2+} influx in the cells is the main motor driving the membrane localization process of PKC α , rather than the Ca^{2+} released from the intracellular stores, which is operated by InsP_3 . The literature points to few cases where Ca^{2+} influx may induce the translocation of PKC isoenzymes, one example being insulin-producing cells through the activation of their voltage-dependent Ca^{2+} channels (Pinton *et al.*, 2002; Mogami *et al.*, 2003). The new results obtained in our study provide the first evidence that a single intracellular Ca^{2+} pulse originated by stimulation of ionotropic P2X receptors is able to induce PKC α membrane localization, thus increasing the possibility that classical PKCs act in signaling pathways other than those described to date. It is important to note that in this process additional factors besides Ca^{2+} are involved in anchoring the enzyme to the membrane because it was observed that the dissociation of PKC α from the plasma membrane was very slow compared with the rapid fall in the intracytosolic Ca^{2+} concentration exerted by the Ca^{2+} buffering systems of the cell.

PtdIns(4,5) P_2 -dependent and DAG-independent Membrane Localization of PKC α upon ATP Stimulation of dPC12 Cells

The results of this work demonstrate that the concentration of DAG generated by ATP stimulation is very low and not relevant to PKC α membrane localization through its C1a domain. Three important findings clearly demonstrate this: 1) the inhibition of PI-PLC increases the time that PKC α remains anchored to the plasma membrane, suggesting that the PtdIns(4,5) P_2 molecule itself might be responsible for this effect rather than the DAG resulting from its hydrolysis; 2) of the two bioprobes tested, one, the C1a domain of PKC α , which has been demonstrated to be responsible for the DAG binding of the enzyme (Medkova and Cho, 1999) and which is able to bind to the endogenous DAG generated by the PI-PLC hydrolysis of PtdIns(4,5) P_2 (Oancea *et al.*, 1998), does not bind to the plasma membrane of dPC12 cells after ATP stimulation; and 3) the second bioprobe tested was the PH domain of PLC δ , which also has been demonstrated to bind to the plasma membrane of the cells, in which it is expressed, through its interaction with PtdIns(4,5) P_2 (Stauffer *et al.*, 1998). In this case, the domain was bound to the plasma membrane and did not dissociate after ATP stimulation, indirectly confirming the results obtained previously using the C1a domain. This suggests that the rate of PtdIns(4,5) P_2 hydrolysis is very low and practically nonexistent under these conditions. Further evidence supporting that PtdIns(4,5) P_2 can directly activate PKC α in the absence of DAG has been shown *in vitro* by using phospholipid ves-

Figure 8. The membrane localization of protein kinase Cα after ATP stimulation induces the dissociation of the PH domain of PLCδ. (A) Ca²⁺ influx driven by ATP stimulation does not induce ECFP-PH-PLCδ membrane dissociation. Confocal images of dPC12 cells expressing ECFP-PH-PLCδ domain (0 s), which localizes heterogeneously in areas of the plasma membrane of dPC12 cells. The PH-PLCδ domain did not exhibit a transient dissociation from the plasma membrane after ATP stimulation (30 s), as might be expected if this stimulation induced the hydrolysis of the endogenous PtdIns(4,5)P₂. (B) dPC12 cells were cotransfected with ECFP-PH-PLCδ (left) and protein kinase Cα-EYFP (right) constructs. Confocal images of simultaneous double detection of ECFP and EYFP were obtained by excitation at 458 and 514 nm, a double dichroic filter DD458/514 and emission wavelengths recorded in two independent channels at 470–490 and 540–582 nm. It was tested that under these conditions there was no bleed-through of the CFP into the YFP emission channels. A series of 30–60 confocal images were recorded for each experiment at time intervals of 10 s. Top, localization of each construct in resting conditions. Bottom, localization of each construct 30 s after the ATP stimulation that induced protein kinase Cα translocation to the plasma membrane and a simultaneous dissociation of the PH domain. (C) Time course of the relative membrane localization of both constructs: ECFP-PH-PLCδ (○) and protein kinase Cα-EYFP (■). The maximal relative membrane localization was expressed as R and calculated as described in *Materials and Methods*.

cles containing PtdCho and PtdIns(4,5)P₂ to determine the catalytic activity of the enzyme, which only decreased 24%

compared with that obtained in the presence of DAG (Corbalán-García *et al.*, 2003).

Figure 9. ATP stimulation induces a transient membrane localization of a lysine-rich cluster protein kinase α mutant. (A) dPC12 cells were cotransfected with the ECFP-PH-PLC δ domain, a PtdIns(4,5) P_2 biosensor (left) and with the protein kinase α K209A/K211A-EYFP mutant (right). The images represent cells in resting conditions (top) and 30 s after stimulation with 100 μ M ATP (bottom). Partial plasma membrane localization of the protein kinase α mutant was observed, which correlated with a lack of dissociation of the PH domain from the plasma membrane. Confocal images were collected in a similar way to that used in the experiment depicted in Figure 8. (B) Time course of the relative membrane localization of protein kinase α K209A/K211A-EYFP mutant (■). The maximal relative membrane localization was expressed as R and calculated as described in *Materials and Methods*.

Strikingly, when PKC α was localized in the plasma membrane, it was able to induce the dissociation of the PtdIns(4,5) P_2 -sensing PH domain (PLC δ -PH domain), suggesting that both proteins compete for PtdIns(4,5) P_2 , with PKC α exhibiting a higher affinity for the ligand. Binding experiments performed with PtdIns(4,5) P_2 -containing vesicles and isolated PH domain have shown an apparent dissociation constant of 1.66 μ M (Lemmon *et al.*, 1995). In the isolated C2 domain, the apparent dissociation constant calculated was 0.6 μ M in the presence of Ca^{2+} (Corbalán-

García *et al.*, 2003). Hence, the higher affinity exhibited by the C2 domain is in agreement with the hypothesis that PKC α might be able to displace the PH domain during its translocation to the plasma membrane.

It is important to take into account that Ins P_3 , which is the product of PtdIns(4,5) P_2 hydrolysis, competes very effectively with PtdIns(4,5) P_2 for the PH domain (Lemmon, 2003) and might be considered to produce the dissociation effect observed; however, this possibility can be ruled out because ATP stimulation of dPC12 cells transfected only with the PH domain did not induce its detachment from the plasma membrane. Further evidence confirming that the direct localization of PKC α in the plasma membrane is responsible for the effect observed in the dissociation of the PH domain was obtained when the lysine-rich cluster-protein kinase α mutant was cotransfected with the PH domain, in which case a partial and transient membrane localization of the PKC α mutant was found and no dissociation of the PH domain was observed, and this correlates well with the loss of affinity for PtdIns(4,5) P_2 in the PKC α mutant. However, the possibility of an intermediate event between protein kinase α localization in the plasma membrane and dissociation of the PH domain cannot be discarded, although the association/dissociation profiles of both proteins fit perfectly with no delay between them, suggesting a more direct effect.

These results also confirm the importance of the lysine-rich cluster, in the Ca^{2+} /PtdIns(4,5) P_2 -dependent localization of protein kinase α , suggesting that the two motifs of the C2 domain might be related in the localization and activation process of the enzyme. Previous studies in our laboratory have demonstrated *in vitro* that mutations in the lysine-rich cluster affect the PtdIns(4,5) P_2 -dependent membrane interaction and activation of protein kinase α (Corbalán-García *et al.*, 2003). A sequential mechanism for protein kinase α interaction with PtdIns(4,5) P_2 -containing membranes was proposed due to the observation that Ca^{2+} needs to bind to the Ca^{2+} -binding region before the lysine-rich cluster can interact with its ligand. This hypothesis is confirmed in this work because the protein kinase α mutant of the Ca^{2+} -binding region could not translocate to the plasma membrane in spite of having an intact lysine-rich cluster motif. Additional evidence that the two regions are necessary to anchor the C2 domain in the membrane of dPC12 cells is provided by the results obtained when using the lysine-rich cluster protein kinase α mutant, which localizes to a lesser extent than the wild-type protein in the plasma membrane, despite of having an intact Ca^{2+} -binding region.

An important question here is whether the C2 domain can accommodate its two motifs in the plasma membrane at the same time. Previous studies regarding the orientation of the domain by electron paramagnetic resonance and x-ray diffraction suggested that the domain docks in a nearly parallel orientation with respect to the plasma membrane surface (Verdaguer *et al.*, 1999; Kohout *et al.*, 2003), which is compatible with the possibility that the C2 domain may dock in the plasma membrane through its two motifs, and it would be in agreement with the results obtained in this work (Figure 11). There is recent evidence that other C2 domain-containing proteins, such as synaptotagmin, need to interact with PtdIns(4,5) P_2 , leading to an increase in the speed of the synaptotagmin response to the ion by steering its membrane penetration activity toward the plasma membrane (Bai *et al.*, 2004). However, synaptotagmin interacts with PtdIns(4,5) P_2 before binding Ca^{2+} , which is the opposite order to that observed in protein kinase α .

Figure 10. Extracellular ATP induces neurite growth through protein kinase C α signaling. PC12 cells were differentiated for 48 h in a medium containing DMEM supplemented with 5% heat inactivated horse serum, 2.5% FBS in the presence of either 100 μ M ATP (B) or 100 ng/ml NGF (C). (D) Image representative of a PC12 cell culture differentiated with 100 μ M ATP and 100 ng/ml NGF simultaneously. (E) PC12 cells differentiated in the presence of ATP, NGF, and 20 nM BIM XI, which is a specific protein kinase C α inhibitor. All the images correspond to phase contrast micrographs taken after 48 h. Bar, 40 μ m. The response of PC12 cells to the different incubation conditions was quantified by two criteria: number of neurite-bearing cells (F) and length of neurites (G), at least 100 cells from 10 randomly chosen fields were scored. Control cells (A) were kept for 48 h under the same conditions except that NGF or ATP were not added to the differentiation media.

Protein Kinase C α Is Involved in the Neuritogenic Effect Induced by Extracellular ATP

The results obtained in this work confirm that ATP stimulation of PC12 accelerates the neural differentiation process initiated by NGF stimulation as described by D'Ambrosi *et al.* (2000). Furthermore, the use of BIM XI, a selective inhibitor for protein kinase C α , strongly suggested that the enzyme is involved in the ATP-dependent component of this development, because the effect of the drug was only observed when ATP and NGF were used together to promote the differentiation process. The

presence of BIM XI impeded the proper differentiation of PC12 cells by producing a phenotype of rounded cells with symmetrical spreading and no neurite process formation. These data are consistent with the notion that protein kinase C α might be involved in the regulation of the progress of neurite outgrowth stimulated by addition of ATP and NGF; however, how the Ca²⁺/PtdIns(4,5)P₂/protein kinase C α complex is connected to downstream pathways or whether other isoforms of protein kinase C are involved in the process are not known and further work needs to be done.

Figure 11. Model for plasma membrane translocation of protein kinase $C\alpha$ in dPC12 cells stimulated with ATP. The ATP stimulation of dPC12 cells induces a Ca^{2+} influx through P2X receptors, this elevation of intracytosolic Ca^{2+} provides the ions that bind to the Ca^{2+} -binding region (CBR) in the C2 domain (represented by three yellow balls), which act as a bridge between the five aspartate residues of this region and the PtdSer in the membrane (Verdaguer *et al.*, 1999). Besides its interaction with Ca^{2+} /PtdSer, the C2 domain interacts with PtdIns(4,5) P_2 through the lysine-rich cluster (LRC) region, which is located in the $\beta 3$ - $\beta 4$ strands (Corbalán-García *et al.*, 2003), thus leading to a longer stay of protein kinase $C\alpha$ in the plasma membrane and probably locating the enzyme close and in the proper orientation to its downstream targets. The program used to represent the C2 domain structure was Swiss-Pdb Viewer 3.7 by GlaxoSmithKline (Guex and Peitsch, 1997). The PDB code is 1DSY.

It is now well established that PtdIns(4,5) P_2 is a key co-factor in signaling to the actin cytoskeleton and in vesicle trafficking (Caroni, 2001; Wenk and De Camilli, 2004) and that these processes directly regulate the development of neurite outgrowth. A hypothetical relationship between the Ca^{2+} /PtdIns(4,5) P_2 /protein kinase $C\alpha$ complex and downstream targets could be, for example, the GAP-43 family of proteins, which potentiates the assembly of the actin cytoskeleton in a process that requires their accumulation in PtdIns(4,5) P_2 -containing rafts (Laux *et al.*, 2000). Furthermore, two members of this family, GAP-43 and MARCKS, are the main substrates of protein kinase C in brain (Aderén, 1995), and thus it is possible that the interaction of protein kinase $C\alpha$ with PtdIns(4,5) P_2 could serve as a scaffold to facilitate enzyme localization close to its targets. Our data suggest a new role for PtdIns(4,5) P_2 , which could serve as a link between Ca^{2+} /protein kinase C and probably the GAP-43 family, thus providing a local environment at the membrane that promotes the corecruitment and activation of signaling components. Whether and how these and other proteins are connected to the Ca^{2+} /PtdIns(4,5) P_2 /protein kinase C complex is still unclear, and more work is needed to establish the spatiotemporal organization of PtdIns(4,5) P_2 , protein kinase $C\alpha$ and other downstream targets in the mechanism of neural differentiation.

Conclusions

This study contributes to our knowledge of the molecular mechanism that controls the spatiotemporal localization of protein kinase $C\alpha$ in model neurons and suggests a new mode of membrane localization and activation of protein kinase $C\alpha$. Contrary to the conventional mechanism of activation, the ATP stimulation of dPC12 cells induces a Ca^{2+} influx through P2X receptors, which is the driving force to translocate the enzyme to the plasma membrane. Besides its interaction with Ca^{2+} , the enzyme needs to bind PtdIns(4,5) P_2 , leading to a longer stay in the plasma membrane. Both interactions are executed through two distinct motifs located in the C2 domain: the Ca^{2+} -binding region

and the lysine-rich cluster, which bind Ca^{2+} /PtdSer and PtdIns(4,5) P_2 , respectively (Figure 11). More surprising are the findings that DAG is not playing a key role to localize protein kinase $C\alpha$ in the plasma membrane, which might reflect an alternative way of action of protein kinase $C\alpha$ in scenarios where no DAG is available.

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