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ADRENERGIC AND CALCIUM-MEDIATED SUBCELLULAR REDISTRIBUTION OF PROTEIN KINASE C IN PRIMARY NEURONAL CULTURES

Christopher J. Kalberg and Colin Sumners

Department of Physiology, College of Medicine, University of Florida, Gainesville, Florida 32610

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SUMMARY: Incubation of primary neuronal cultures prepared from the brains of neonatal rats with 50 μM epinephrine resulted in the transient redistribution of protein kinase C from the cytosol to the particulate fraction. This effect occurred after 1 and 5 min of incubation and resulted in a decrease in cytosolic protein kinase C activity with a corresponding increase in particulate protein kinase C of approximately 30% and 15%, respectively. The epinephrine-stimulated translocation of protein kinase C was blocked by 1 μM prazosin indicating the involvement of α_1-adrenergic receptors. Further, inclusion of 0.1 μM Ca^{2+} in the homogenization buffer was found to significantly enhance the binding of protein kinase C to cellular membranes prepared from neuronal cultures. These results indicate that α_1-adrenergic receptors in neuronal brain cell cultures are linked to the activation of protein kinase C and that the mobilization of Ca^{2+} may enhance this effect.

The activation of calcium, phospholipid-dependent protein kinase (protein kinase C) is associated with a shift in the subcellular distribution of this enzyme from a cytosolic localization to a membrane-bound state (1). This translocation of protein kinase C is initiated by the hydrolysis of inositol phospholipids and the generation of diacylglycerol (DG) which is the endogenous activator of protein kinase C (2). The binding of protein kinase C to cellular membranes is apparently promoted in a synergistic manner by DG and Ca^{2+} (3). In peripheral cell types, the stimulation of the protein kinase C translocation by α_1-adrenergic receptor activation has been demonstrated using phenylephrine in pinealocytes and using epinephrine in Madin-Darby canine kidney cells (4,5). However, the α_1-adrenergic receptor-stimulated translocation of protein kinase C has not been demonstrated in neuronal cultures prepared from the brain.

In primary neuronal cultures prepared from the brains of neonatal rats we have associated the short-term activation of α_1-adrenergic receptors with an increase in both the hydrolysis of phosphoinositides and an increase in the number of angiotensin II (Ang II) binding sites (6). Additionally, using phorbol esters, which activate protein kinase C by substituting for DG, protein kinase C was shown to be integrally involved in the regulation of Ang II receptor expression in neuronal cultures (7). Together, these studies implicate the

Abbreviations used: Ang II, angiotensin II; DG, diacylglycerol; DEAE, diethylaminoethyl cellulose; DMEM, Dulbecco's modified Eagle's medium; PDHS, plasma derived horse serum, PMSF, phenylmethylsulfonylflouride; TPA, phorbol 12-myristate-13-acetate.

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involvement of α_1-adrenergic receptors in the activation of protein kinase C. In this study, epinephrine was used to examine the effects of α_1-adrenergic receptor activation on the subcellular distribution of protein kinase C in primary neuronal cultures. Additionally, the effect of Ca^{2+} on the distribution and proteolysis of protein kinase C was analyzed.

MATERIALS AND METHODS

Materials. Epinephrine, ATP, Histone (III-S), PS, diolein, phenylmethylsulfonyl flouride (PMSF), leupeptin, Triton X-100, phorbol 12-myristate-13-acetate (TPA), prazosin and cellulose filters (0.45 μm pore size, 25 mm diam.) were from Sigma Chemical, St. Louis, MO. Diethylaminoethyl cellulose (DEAE 52) was obtained from Whatman BioSystems. The chromatography columns were from Bio-Rad Laboratories. [γ^{32}P]ATP (10-40 Ci/mmol) was from New England Nuclear. Plasma derived horse serum (PDHS) was from Hyclone laboratories and Dulbecco's modified Eagle's medium (DMEM) was from Gibco. All other compounds were of reagent grade.

Treatment and the Preparation of Cellular Fractions. Neuronal cultures were prepared from the whole brains of one-day-old Sprague-Dawley rats as described previously (7). The cells suspended in DMEM containing 10% PDHS were plated at a concentration of 18 X 10^6 cells per dish on 100 mm Falcon tissue-culture dishes and were used after 14-21 days of culture. Neuronal cultures were treated with epinephrine for various times by adding the compounds directly into the growth media, after which the growth media was aspirated off and the cells were washed twice with 3.0 ml of ice-cold (4°C) homogenization buffer A (20 mM Tris HCl, pH 7.5, 2.0 mM EDTA, 0.5 mM EGTA, 0.25 M sucrose, 0.2 mM PMSF, and 2.0 μg/ml leupeptin), rapidly scraped from the dish in 1.0 ml of homogenization buffer A (4°C), homogenized in a dounce homogenizer (15-20 strokes), and centrifuged at 20,000 RPM (~43,000 X g) for 45 min at 4°C. The supernatant was used as the cytosolic fraction and the pellet was resuspended in 1.0 ml of homogenization buffer A (4°C) containing 0.1 % Triton X-100 for 30 min at 4°C. The suspension was centrifuged at 20,000 RPM (~43,000 X g) for 45 min at 4°C and the supernatant was used as the particulate fraction. The cytosol and particulate fractions were partially purified by DEAE cellulose chromatography. Disposable columns with a 12 ml volume were packed with 0.6 ml of DEAE 52 equilibrated with homogenization buffer B (20 mM Tris HCl, pH 7.5, 2.0 mM EDTA, 0.5 mM EGTA, 0.2 mM PMSF and 1.0 μg/ml leupeptin). The packed columns were washed with 2.0 ml of homogenization buffer B, the respective cytosolic and particulate samples were applied, and the columns were washed with 2.0 ml of homogenization buffer B. Protein kinase C was eluted from the columns by a single-step elution with 5.0 ml of homogenization buffer B containing 0.15 M NaCl.

Protein Kinase C assay. The enzyme activity was determined by measuring the incorporation of γ^{32}P from [γ^{32}P]ATP into lysine rich histone (type III-S). The final assay mixture (250 μl) contained 20 mM Tris HCl, pH 7.5, 5.0 mM $MgSO_4$, 0.5 mM $CaCl_2$, 50 μg histone, 6 μg phosphatidylserine (PS), 0.4 μg diolein, 10 μM [γ^{32}P]ATP (500 cpm/pmol) and 1-5 μg protein. After 3 min of incubation, the reaction was stopped by the addition of 1.0 ml of 25% trichloroacetic acid (4°C). The precipitates were collected by vacuum filtration onto 0.45 μM cellulose filters and washed 4 times with 2.0 mls of 5% trichloroacetic acid (4°C). Additionally, kinase activity was determined in the absence of lipids and with 1.0 mM EGTA substituted for $CaCl_2$. The filters were placed in scintillation vials to which 10 ml of liquiscint was added and the vials were analyzed for radioactivity present. Protein kinase C activity was expressed as nmol ^{32}P/min/mg protein.

Protein determination. The protein content from the cellular fractions collected for the protein kinase C assay were determined by the method of Lowry et al (8).

RESULTS

The effect of epinephrine on the distribution of protein kinase C was analyzed using a single-step elution procedure. Using this elution method, the translocation of protein kinase C from the cytosol to the particulate (membrane) fraction is readily apparent in cells treated with the phorbol ester, phorbol 12-myristate-13-acetate (TPA) (Fig 1). After 15 min of incubation with TPA (0.8 μM), the activity of protein kinase C in the cytosol fraction significantly decreased by 65%. This was associated with a significant 41% increase in the protein kinase C activity in the particulate fraction. The translocation of protein kinase C was followed by the downregulation of protein kinase C after 1 hr of treatment with TPA. The downregulation of protein kinase C by phorbol esters is characteristic of prolonged treatment with these drugs (9).

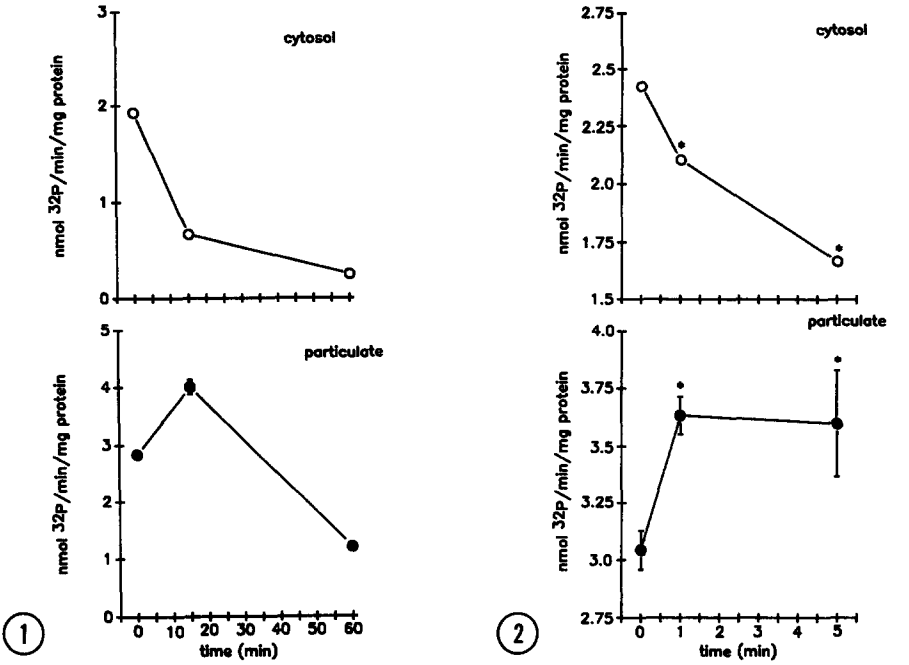
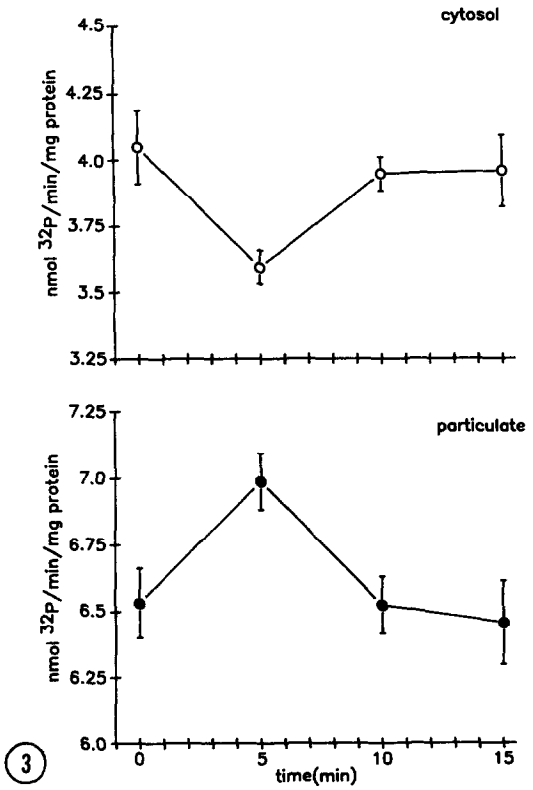


Fig. 1: Effect of 0.8 μM TPA on the subcellular distribution of protein kinase C in neuronal cultures. The cytosol (O) and particulate (●) fractions were prepared as described in the Methods after treatment of the neuronal cultures with TPA for the indicated times. The data are the means ± SEM of triplicate assays and the results are representative of one additional experiment.

Fig. 2: Effect of epinephrine on the subcellular distribution of protein kinase C. Neuronal cultures were treated with 50 μM epinephrine for 1 and 5 min. Unstimulated control values are shown at time zero. Protein kinase C activity was determined in the cytosolic (O) and particulate fractions (●). Data are means ± SEM of triplicate assays and are representative of three additional experiments. ANOVA 1, $p<0.05$ for both cytosol and particulate fractions. * significantly different from controls as determined by Newman-Keuls post-hoc analysis.

The effect of epinephrine on the distribution of protein kinase C was determined next. In unstimulated control cells, the activity of protein kinase C in the cytosol and particulate fractions represented 44% and 56%, respectively, of the total protein kinase C activity (Fig 2). Treatment of the neuronal cultures for 1 and 5 min with 50 μM epinephrine significantly decreased the activity of protein kinase C in the cytosol fractions by 23% and 31%, respectively (Fig 2). This was associated with corresponding significant increases of 16% and 15% in the amount of protein kinase C activity in the particulate fraction of cells treated for 1 and 5 min, respectively. This experiment is a representative experiment which was repeated three times with similar percent changes in protein kinase C activity occurring each time. A translocation of protein kinase C was not observed in neuronal cultures treated with epinephrine for less than 1 min (data not shown).

An extended time course of epinephrine treatment is shown in Fig 3. A translocation of protein kinase C in cells treated with 50 μM epinephrine was detected after 5 min of incubation. This effect was transient as the levels of protein kinase C in the cytosol and particulate fractions returned to control levels after 10 and 15 min of incubation with epinephrine. Fig 3 shows a representative experiment which was repeated twice with similar results.



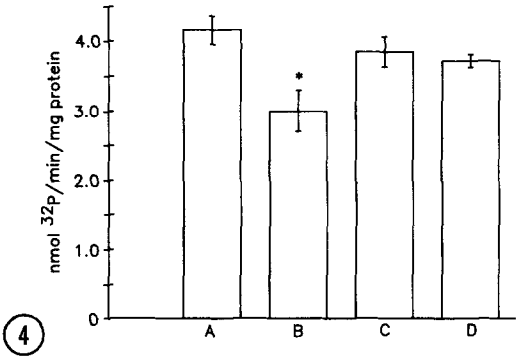


Fig. 3: Extended time course showing the subcellular distribution of protein kinase C in epinephrine-treated neuronal cultures. Cells were treated with 50 μM epinephrine for the indicated times and the protein kinase C activity was determined in isolated cytosol (O) and particulate (●) fractions. The data are means ± SEM and are representative of two similar experiments.

Fig. 4: Effect of prazosin on the amount of protein kinase C activity associated with the cytosol fraction of epinephrine-treated neuronal cultures. Neuronal cultures were treated for 5 min with (A), phosphate buffered saline (vehicle solution); (B), 50 μM epinephrine; (C), 50 μM epinephrine + 1 μM prazosin; (D), 1 μM prazosin. The data are means ± SEM of three separate experiments. ANOVA 1, $p<0.05$. * significantly different from controls as determine by Newman-Keuls post-hoc analysis.

The specific α_1-adrenergic receptor antagonist prazosin blocked the translocation of protein kinase C in cultures treated with epinephrine (Fig 4). The activity of protein kinase C in the cytosol fraction of unstimulated control cells was 4.160 ± 0.12 nmol ^{32}P/min/mg protein (n=3 experiments). Treatment with epinephrine (50 μM, 5 min) significantly decreased the protein kinase C activity in the cytosol fraction to 3.00 ± 0.17 nmol/min/mg protein (n=3 experiments). The levels of protein kinase C in cells co-treated with prazosin (1μM) and epinephrine (50 μM, 5 min) were not significantly different from control levels (3.847 ± 0.12 nmol ^{32}P/min/mg protein; n=3 experiments). Prazosin (1μM) alone had no effect (3.723 ± 0.09 nmol ^{32}P/min/mg protein; n=3 experiments). A similar inhibition of epinephrine-stimulated increases in the membrane-associated protein kinase C was found in cells treated with prazosin (data not shown).

To assess the effects of Ca^{2+} on the subcellular distribution of protein kinase C, neuronal cultures were homogenized in either a buffer containing Ca^{2+} chelators (buffer A) or a buffer containing 0.1 μM Ca^{2+} (20 mM Tris HCl, pH 7.5, 0.1 μM $CaCl_2$, 0.2 mM PMSF and 2.0 μg/ml

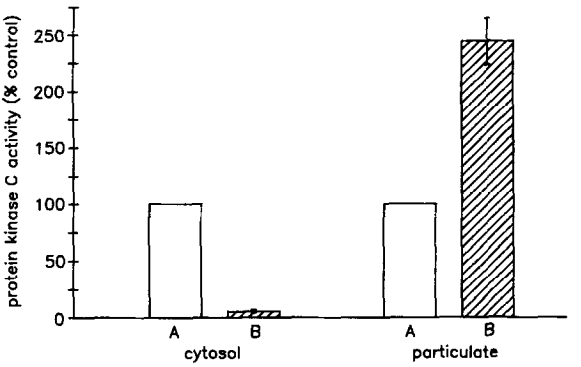


Fig. 5: Effect of Ca^{2+} on the subcellular distribution of protein kinase C. Neuronal cultures were homogenized in the presence of Ca^{2+}-chelators (A) or with 0.1 μM $CaCl_2$ (B). Data are means $\pm$ SEM two separate experiments.

leupeptin). The homogenization of cells with 0.1 μM $CaCl_2$ resulted in a 96% reduction in the activity in the protein kinase C activity associated with the cytosol fraction and a 244% increase in the protein kinase C in the particulate fraction. The total activities (i.e. cytosol + particulate fraction) of cells homogenized with Ca^{2+} chelators or with Ca^{2+} were not significantly different and had respective values of 1.29 $\pm$ 0.03 and 1.32 $\pm$ 0.1/ nmol ^{32}P/min/mg protein. This indicates that the homogenization of neuronal cultures in the presence of Ca^{2+} did not result in the degradation of protein kinase C by Ca^{2+}-dependent proteases such as calpain (10). (See Fig. 5.)

DISCUSSION

The present study demonstrates that the incubation of neuronal cultures with epinephrine results in the redistribution of protein kinase C in neuronal cultures prepared from the brains of neonatal rats. A decrease in the protein kinase C levels of approximately 20-30% was detected in the cytosol fraction of cells treated for 1 and 5 min with epinephrine as compared to unstimulated control cells. This effect was associated with a 10-15% increase in the protein kinase C activity in the particulate fraction of neuronal cultures treated with epinephrine. The translocation of protein kinase C was transient and was not detected after 10 or 15 min of incubation with epinephrine. The percent changes in the protein kinase C activity in the cytosol and particulate fraction were not equal due, in part, to the greater amount of protein kinase C in the particulate fraction of unstimulated control cells.

Prazosin was shown to inhibit the translocation of protein kinase C in neuronal cultures treated with epinephrine. This indicates that the activation of α_1-adrenergic receptors is involved in the translocation of protein kinase C in cultures treated with epinephrine. A similar α_1-adrenergic receptor-mediated translocation of protein kinase C by epinephrine was reported in MDCK-D1 cells (5). However, in MDCK-D1 cells, the hydrolysis of phosphatidylcholine was associated with increased DG production and the translocation of protein kinase C. The hydrolysis of phosphatidylcholine by α_1-adrenergic receptors has not been characterized in primary neuronal cultures. However, the activation of α_1-adrenergic receptors produces a large stimulation of phosphoinositide hydrolysis in primary neuronal cultures (6,11,12).

Compared with epinephrine, the phorbol ester TPA induced a more pronounced translocation of protein kinase C. This is not surprising as phorbol esters are not readily metabolized as opposed

to DG which is rapidly produced and inactivated (1). This may contribute to both the smaller degree and the transient nature of protein kinase C translocation in epinephrine-treated cells. Further, TPA may stimulate the translocation of all of the isozymes of protein kinase C which are located in the cytosol as opposed to epinephrine which may affect only a single isozyme or a subpopulation of isozyme(s) (13).

In previous experiments, the incubation of neuronal cultures with epinephrine or norepinephrine for 15-60 min at concentrations which maximally stimulate the hydrolysis of phosphoinositides (5-50 μM) has been shown to increase the number of Ang II binding sites by a mechanism specific for α_1-adrenergic receptors (6). Further, protein kinase C is known to be integrally involved in the increased expression of Ang II receptors in neuronal cultures (7). These findings have led to the hypothesis that the activation of α_1-adrenergic receptors leads to the hydrolysis of phosphoinositides and the subsequent activation of protein kinase C which acts to increase the expression of Ang II receptors in primary neuronal cultures prepared from the brains of neonatal rats. The present study furthers this hypothesis by showing that α_1-adrenergic receptor activation elicited by epinephrine stimulated the translocation of protein kinase C in neuronal cultures. However, it is not assumed that epinephrine is the actual endogenous neurotransmitter which acts at α_1-adrenergic receptors to regulate Ang II receptors in the brain. It is likely that norepinephrine fulfills this role and in preliminary experiments norepinephrine was found to stimulate the translocation of protein kinase C in neuronal cultures (data not shown). However, the involvement of epinephrine cannot be ruled out as some epinephrine cell groups are found in the medullary region of the brainstem which send projections anterioventrally to the hypothalamus (14). Further, epinephrine has a greater efficacy for phosphoinositide hydrolysis and produces a greater fractional release of inositol phosphates in the brain than either norepinephrine or phenylephrine (15,16).

The ability of Ca^{2+} to affect the subcellular distribution of protein kinase C has both physiological and experimental implications. Physiologically, agents which act to mobilize calcium may cause the translocation and activation of protein kinase C independent of the production of DG or act to facilitate the DG-mediated translocation of protein kinase C. Wolf et al., (3) found that Ca^{2+} at concentrations between 100 nm and 10 μM increases the binding of protein kinase C to erythrocyte cell membranes. These levels of Ca^{2+} are near steady-state levels and are reached during the stimulation of Ca^{2+} mobilization (17). Experimentally, the amount of Ca^{2+} present during homogenization has an important bearing on the reported subcellular distribution of protein kinase C. For example, Farrago et al. (18) found an 80% increase in the amount of protein kinase C in the particulate fraction when adrenal glomerulosa cells were homogenized in the presence of Ca^{2+} compared to cells homogenized in the presence of Ca^{2+}-chelators. Thus, the protein kinase C recovered in the particulate fraction in the presence of Ca^{2+}-chelators represents chelator-stable protein kinase C and may not reflect the true subcellular distribution of protein kinase C.

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