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Characterization and differential expression of protein kinase C isoforms in PC12 cells

Differentiation parallels an increase in PKC $beta_{II}$

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Nerve growth factor (NGF) treatment of PC12 cells induced a 2.8-fold increase in protein kinase C activity concomitant with differentiation and acquisition of neurites. PKC protein isoforms were separated by sequential chromatography on DEAE-Sephacel/hydroxylapatite. A broad peak of PKC activity eluted which corresponded to the alpha PKC isoform. In control cells, message for all six PKC isoforms was detected and expressed as epsilon>zeta=gamma>delta>beta>alpha. Western blot of whole cell lysates revealed a large increase in the $beta_{II}$, while slight changes were observed for the other five PKC isoforms during treatment (1–14 days) with NGF (50 ng/ml). In parallel, coordinate changes in the expression of the individual transcripts for the six isoforms occurred during NGF treatment. Induction and accumulation of PKC $beta_{II}$ may play a role in maintenance of neuronal morphology.

Protein kinase C; Isoform; Differentiation; Nerve growth factor; PC12

1. INTRODUCTION

The genes which encode the mammalian protein kinase C have recently been cloned, and consist of a highly homologous gene family comprised of 6 members: alpha, $beta_{I,II}$, gamma, delta, epsilon and zeta [1]. The transcripts encoding epsilon and gamma isoforms are most abundant in brain, while alpha seemingly has a wide tissue distribution [2]. The three major forms of protein kinase C can be resolved by hydroxyapatite chromatography [3,4] and the elution pattern of peak I, II and III isozymes corresponds to the encoded genes of gamma, $beta_I$ and $beta_{II}$, and alpha, respectively [5]. Each subspecies shows a slightly differential mode of activation, proteolytic fragment formation, cellular distribution, kinetic properties and substrate specificities [2,6], suggesting differential roles within the cell. In addition, the ontogeny and expression of the various mRNAs appears to be regulated during development [7].

There has been growing evidence from studies conducted in a number of systems suggesting that the expression of the PKC isoforms may be tightly coupled with cellular differentiation [8–10]. PC12 cells can be induced to differentiate to mature sympathetic neurons by nerve growth factor (NGF) [11]. Moreover, it has been demonstrated that treatment of PC12 cells with PKC inhibitors, sphingosine or K252a, diminishes the neurogenic properties of NGF [12,13]. Together these studies implicate PKC as playing a pivotal role in NGF-mediated neuronal differentiation.

Since the enzymatic activity attributed to PKC may reside in more than one isoform protein, it is of importance to critically evaluate the contribution of the individual PKC isoforms in NGF-mediated neurogenesis. The focus of this study was to characterize the expression of PKC isoforms and to examine whether the isoforms present in PC12 cells undergo changes during differentiation induction.

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2. EXPERIMENTAL

2.1. *Cell culture*

Pheochromocytoma cells (PC12) were obtained from the ATCC (Rockville, MD) and cultured as previously described [14]. During long-term treatment with NGF (β 2.5S), the cells were refed every 3 days for maintenance of cell viability.

2.2. *Protein kinase C activity assay*

Cells were harvested from the plates and pelleted by centrifugation. The cell pellets were washed 2× in PBS, pH 7.4, lysed, and the total cellular PKC content determined as previously described by enriching for PKC activity through DEAE-mini column chromatography [15]. PKC activity was measured using histone Type IIIS as previously described [15]. Protein was measured with the Bio-Rad dye binding assay using bovine serum albumin as a standard.

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2.3. *SDS-PAGE/Western blotting*

100 mm plates, washed in PBS, pH 7.4, sonicated in PKC sonication buffer containing: 20 mM Tris, pH 7.4, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 1 mM PMSF, 1 μM leupeptin, 10 mM aprotinin, 100 μM NaF. After sonication 1% NP40 and 10 mM EGTA was added to the homogenate to extract total cellular PKC. The homogenate was mixed end-over-end for 1 h, protein determined and 2× Laemmli sample buffer was added followed by boiling. Polyclonal antisera to PKC isoform peptides alpha, $beta_{I,II}$, gamma were obtained from Dr. T. Saitoh, Department of Neurosciences, University of California, San Diego [16], while antibodies to epsilon, delta and zeta were generated as previously described [17–19]. Typically 100 μg of protein was electrophoresed on a 10% SDS-polyacrylamide gel, blotted to nitrocellulose, blocked with PBS containing 0.1% NP-40 plus 5% non-fat dry milk (NM-PBS) for 2 h at room temperature, and incubated with primary antibody at 1:1,500 for 1 h at 4°C. Blots were then washed with NM-PBS, and incubated with 0.5 μCi/ml [^{125}I]protein A (NEN, Dupont) in NM-PBS for 2 h at room temperature, and then washed and autoradiographed on Fuji X-ray film at −70°C for 1 week. Immunoreactive epsilon, delta and zeta PKC were detected with a similar protocol except that the blots were incubated with HRP-donkey anti-rabbit Ig as the secondary antibody and detected using the Amersham, ECL Western blotting reagents. In this case, blots were exposed to X-ray film for 4 min at room temperature prior to developing. The specificity of each antibody was demonstrated by competition with appropriate PKC peptide 1:1 prior to incubation; in each case this abolished the immunoreactive band.

2.4. *Hydroxylapatite separation of PKC protein isoforms*

All procedures were conducted at 4°C. Cells from five plates (150 mm) were harvested and prepared as described in section 2.3. The homogenate (20 mg protein) was mixed end-over-end for 1 h and loaded onto a 1.5 × 12 cm column packed with DEAE-Sephacel, previously equilibrated with Buffer A (50 mM Tris-HCl, pH 7.5, 50 mM mercaptoethanol, 2 mM EGTA, 20 mM NaCl), washed with 80 ml Buffer A and eluted with 50 ml of Buffer A containing 275 mM NaCl. PKC activity was pooled and dialyzed overnight against Buffer B (10 mM K_2PO_4, 0.5 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, pH 7.5). PKC activity from differentiated and undifferentiated cells were resolved by using two hydroxylapatite (Bio-Rad; Fast flow) columns run simultaneously. The samples were loaded onto the column (10 mg protein/column), followed by washing with 50 ml of Buffer B. PKC isoforms were eluted as previously described using an 80 ml linear gradient from 10–300 mM K_2PO_4 [4]. An aliquot of every other fraction was assayed for PKC activity. In addition, fractions were pooled and the conductivity determined.

2.5. *Isolation, quantitation and blotting of RNA*

On the day of harvest the cells were removed from the plates in media, pelleted by centrifugation and washed 2× with RNase-free DEPC PBS, pH 7.4. RNA was isolated by using RNAozol. Typically 10 μg was blotted and hybridized with [^{32}P]random primed cDNA probe specific for either alpha, beta, gamma (ATCC, Rockville, MD), delta, epsilon or zeta PKC [17–19]. The resulting autoradiograms were scanned using a computer-interfaced gel scanning system (Hoetler Densitometer, Fullerton, CA). Representative experiments are presented as graphs reporting relative peak area (total optical density (corrected for background) of the treatment divided by the intensity of actin as an internal control), in arbitrary units of PKC for each sample. The reliability of these measurements was determined by obtaining multiple autoradiographic exposures to ascertain the peak intensities were within the linear range of the X-ray film.

3. RESULTS

During the course of differentiation induction, from a proliferating population of chromaffin-like cells to a population of cells comprised primarily of sympathetic neurons (50 ng/ml NGF, 1–14 days), total PKC activity increased approximately 3-fold (500 ± 32 to 1,407 ± 19 pmol/min/mg). In order to examine the relative contribution of each of the PKC isoforms to the total increase noted in PKC activity, the isoforms were separated by DEAE/hydroxylapatetite (Fig. 1). Although some variance was observed in absolute peak heights, no significant differences in PKC isoforms between undifferentiated and differentiated PC12 cell lysates could be detected. Representative fractions, as indicated (Fig. 1), were subjected to Western blotting with isoform specific antibodies to each of the six PKC isoforms. This analysis revealed that the predominant peak separated by this method (140 mM K_2PO_4; fractions 50–55) was comprised of alpha PKC (data not shown). This isoform eluted coincident in conductivity with alpha PKC of rat brain [3–5]. Although minor early peaks of PKC activity were separated by this method and some variance in peak height was observed in PKC activity from the two cell lysates, these peaks were not immu-

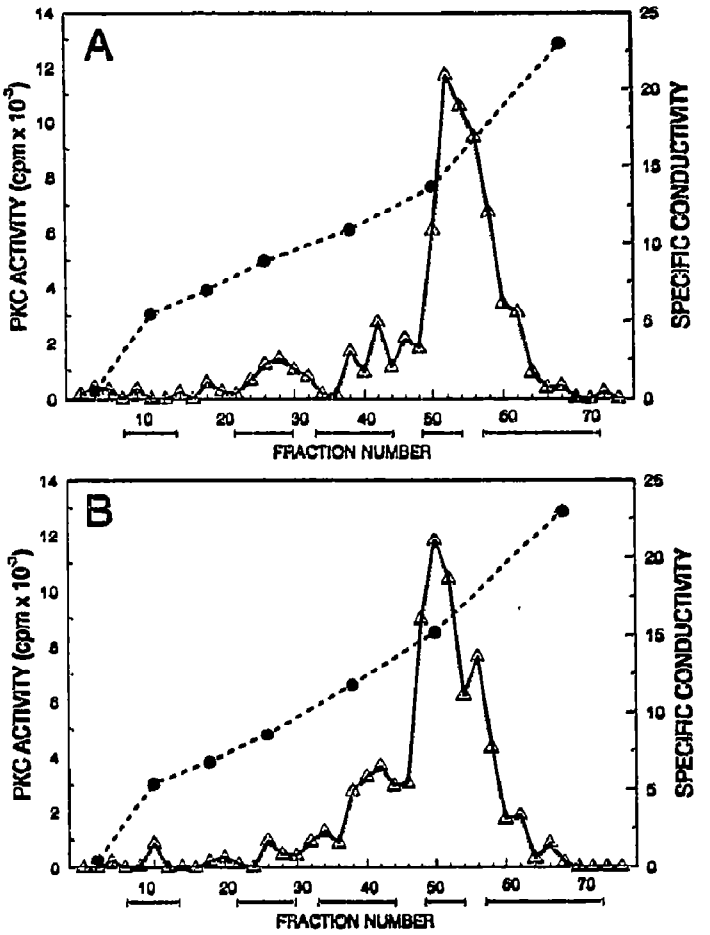

Fig. 1. Fractionation of protein kinase C isoforms by hydroxyapatite chromatography from DEAE-purified extracts. Equivalent units of PKC activity were separated from either (A) undifferentiated or (B) differentiated PC12 cells. Solid bars indicate which fractions were pooled and tested with isoform-specific antibodies. These data are representative of 8 independent experiments.

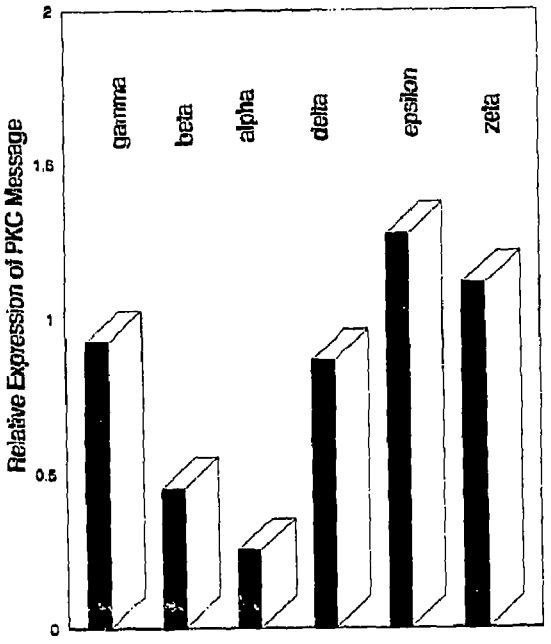

Fig. 2. Relative abundance of PKC transcripts in undifferentiated PC12 cells. RNA was harvested and probed with an equivalent amount of probe (ng/cpm/ml) specific for each PKC transcript as indicated. Six separate blots were washed under identical conditions and exposed for the same length of time (6 days). Shown are the relative levels of transcript expression for each of the six isoforms corrected for the expression of actin. These data are representative of 3 independent experiments.

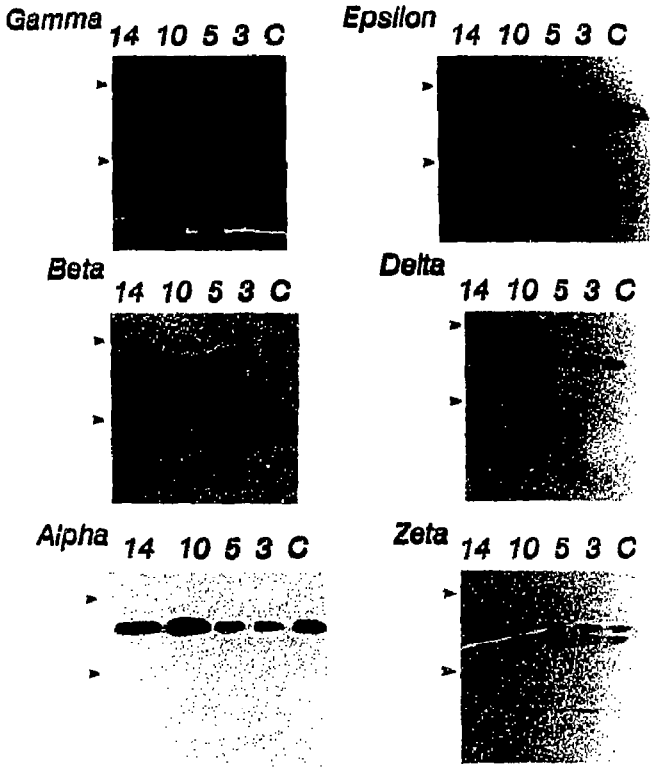

Fig. 3. Immunoblot of whole cell lysates. PC12 cells were treated with NGF (50 ng/ml) for 0–14 days, as indicated. Cells were harvested as described and analyzed by SDS-PAGE/Western blotting with isoform-specific antibodies to gamma, $beta_{II}$, alpha, epsilon, delta or zeta PKC isoforms. Arrows indicate the position of the molecular weight markers phosphorylase b (97 kDa) and bovine serum albumin (68 kDa). These data are representative of 4 independent experiments.

noreactive with any of the isoform-specific PKC antibodies.

An analysis was conducted to determine which individual mRNAs are expressed in control cells (Fig. 2). PC12 cells were shown to express all six PKC transcripts. The relative abundance for the individual isoforms was found to be epsilon>zeta=gamma> delta>beta>alpha. It is possible that due to the labile nature of isoform proteins they were unstable to the prolonged manipulation required for the DEAE/HAP separation, and that this accounts for the lack of specific peaks and diminished immunoreactivity. However, the broad alpha isoform activity peak was detected at the same position and with the same peak height in eight separate analyses.

The relative contribution of each of the PKC isoforms to the increases in total PKC activity was examined using whole cell lysates prepared from NGF-treated cells. This design was employed to rapidly prepare cells without possible protease degradation of the isoforms. Equivalent amounts of total protein obtained from either controls or cells undergoing differentiation induction revealed a substantial increase in the $beta_{II}$ isoform protein (Fig. 3). Slight changes (increases) were observed in the expression of the other PKC isoforms. While all the isoforms remained elevated, gamma, alpha and zeta declined slightly after 10 days.

To investigate possible changes in the PKC transcripts themselves, the mRNA for each of the encoded transcripts was analyzed. With respect to all six encoded transcripts, NGF (50 ng/ml) increased the abundance of each of the transcripts (Fig. 4); however, expression of beta and gamma declined after 7 days, alpha remained elevated until 10 days of treatment and epsilon, delta and zeta remained relatively stable once induced.

4. DISCUSSION

Increased expression of each PKC transcript did not directly parallel increased accumulation of the respective protein isoform; therefore, it can be concluded that some of the isoforms are more rapidly turned over while others remain relatively stable and accumulate. These data suggest that protein stabilization of the isoform during differentiation, rather than transcriptional induction, may account for the primary increase observed for the isoform protein. However, this study does not exclude the possibility that the PKC isoforms undergo

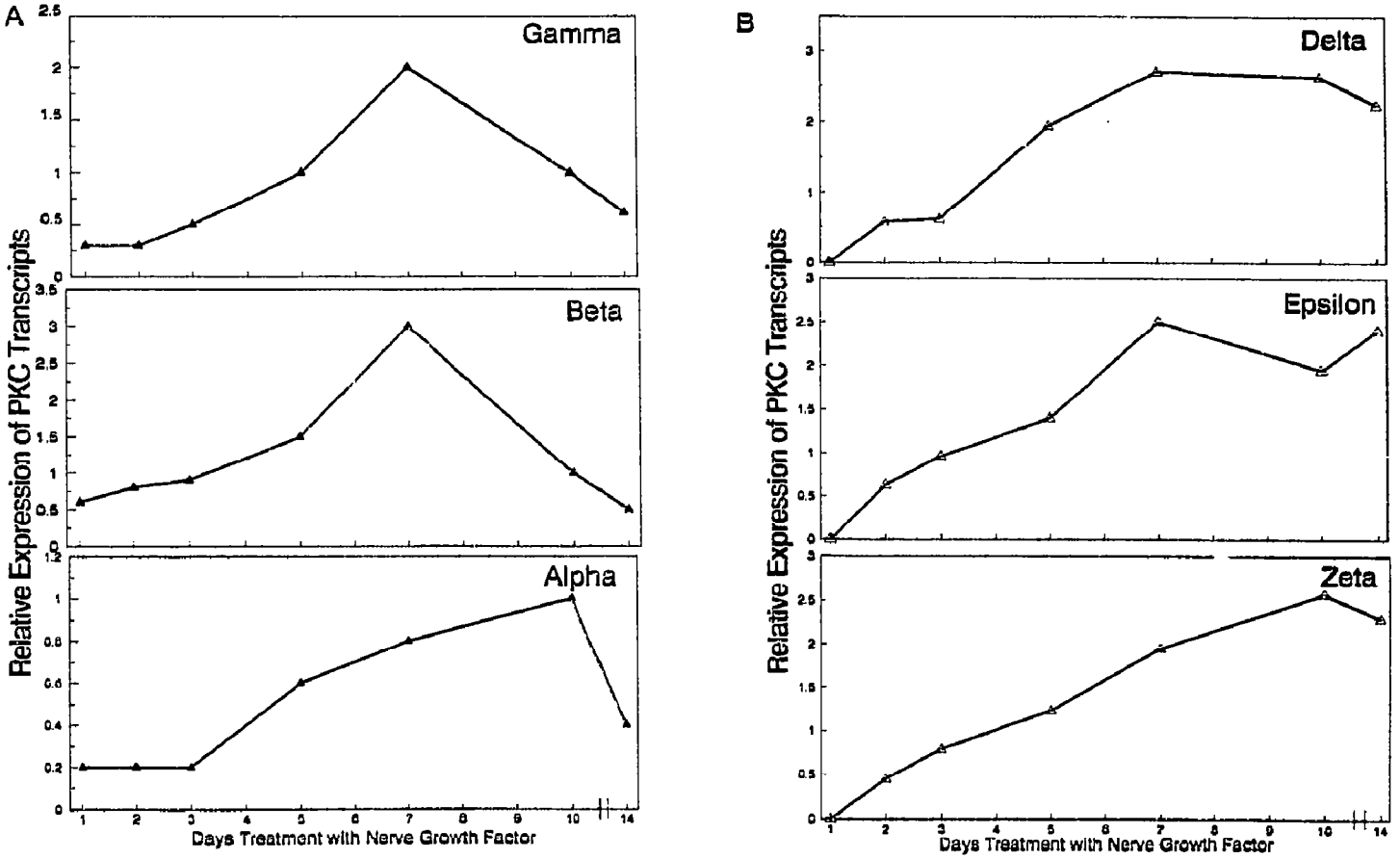

Fig. 4. Relative expression of PKC isoform-specific transcripts. PC12 cells were treated with NGF (50 ng/ml) for 0–14 days, as indicated. Total RNA (10 μg) was probed for either the (A) classical or (B) non-classical PKC transcripts. These data are representative of 3 independent experiments.

changes at the transcriptional level. In fact, Obeid et al. [20] have demonstrated that both beta and alpha PKC display changes in the rate of transcription during differentiation induction of HL60 cells with vitamin D_3 treatment.

As a function of activity, these cells express alpha PKC to a greater extent than the other isoform activities. A similar finding has been reported by Koda et al. [21] in a brief report. In control cells the epsilon transcript is the most abundant class of message present, however all transcripts undergo the same relative changes in expression. PKC epsilon has been previously documented in neurons [22]; therefore, it is not surprising to demonstrate expression of this isoform in PC12 cells which are also of neural crest origin. Discordance between activity, protein and mRNA has recently been documented for PKC isoforms in two other systems [23,24]. Taken together, the data herein, as well as other studies, demonstrate that predictions on amount of isoform protein cannot be made in relation to either the amount of specific message or activity. Other factors such as protein turnover and changes in isoform affinity for a particular substrate must also be taken into account as regulators of PKC [24].

With respect to protein, PKC $beta_{II}$ accumulates at a rate concomitant with acquisition of elongated neurites and thus may play a role in differentiation. Increased levels of PKC beta have also been documented in parallel with increased differentiation rates of MEL cells [9]. In addition, introduction of purified PKC beta protein by permeabilization potentiates differentiation [25]. A recent study of human pheochromocytoma [26] documented increased amounts of PKC beta, thus suggesting that the proportion of this isoform may play a critical role in differentiation. It is of interest to note that a well characterized PC12-PKC substrate, GAP43/B-50, found in the growth cones of neurites, is a PKC beta-preferred substrate [27,28]. Increased amounts of the $beta_{II}$ PKC isoform may play a crucial role in maintenance of neuronal morphology through substrate specific phosphorylation.

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REFERENCES

[1] Nishizuka, Y. (1988) Nature 334, 661–665.
[2] Farago, A. and Nishizuka, Y. (1990) FEBS Lett. 268, 350–354.
[3] Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U. and Nishizuka, Y. (1988) Biochem. Biophys. Res. Commun. 151, 973–981.
[4] Huang, K.-P., Nakabayashi, H. and Huang, F.L. (1986) Proc. Natl. Acad. Sci. USA 83, 8538–8539.
[5] Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K. and Nishizuka, Y. (1987) Science 236, 1116–1120.
[6] Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C., Schaap, D., Stabel, S. and Webster, C. (1989) Mol. Cell. Endocrinol. 65, 1–11.
[7] Sposi, N.M., Bottero, L., Cossu, G., Russo, G., Testa, U. and Peschle, C. (1989) Mol. Cell. Biol. 9, 2284–2288.
[8] Makowske, M., Ballester, R., Cayre, Y. and Rosen, O.M. (1988) J. Biol. Chem. 263, 3402–3410.
[9] Melloni, E., Pontremoli, S., Viotti, P.L., Patrone, M., Marks, P.A. and Rifkind, R.A. (1989) J. Biol. Chem. 264, 18414–18418.
[10] Wada, H., Ohno, S., Kubo, K., Taya, C., Tsuji, S., Yonehara, S. and Suzuki, K. (1989) Biochem. Biophys. Res. Commun. 165, 533–538.
[11] Greene, L.A. and Tischler, A.S. (1976) Proc. Natl. Acad. Sci. USA 73, 2424–2428.
[12] Hall, F.L., Fernyhough, P., Ishii, D.N. and Vulliet, P.R. (1988) J. Biol. Chem. 263, 4460–4466.
[13] Koizumi, S., Conrearas, M.L., Matsuda, Y., Hama, R., Lazarovici, P. and Guroff, G. (1988) J. Neurosci. 8, 715–721.
[14] Wooten, M.W. and Ewald, S.J. (1991) Brain Res. 550, 333–339.
[15] Wooten, M.W. (1991) Exp. Cell. Res. 193, 274–278.
[16] Masliah, E., Cole, G., Shimohama, S., Hansen, L., DeTeresa, R., Terry, R.D. and Saitoh, T. (1990) J. Neurosci. 10, 2113–2124.
[17] Olivier, A. and Parker, P.J. (1991) Eur. J. Biochem. 202, 805–810.
[18] Schaap, D., Parker, P.J., Bristol, A., Kriz, R. and Knopf, J. (1989) FEBS Lett. 243, 351–357.
[19] Ways, K. (1991) J. Biol. Chem. (submitted).
[20] Obeid, L.M., Okazaki, T., Karolak, L.A. and Hannun, Y.A. J. Biol. Chem. 265, 2370–2374.
[21] Koda, Y., Hobayashi, H., Mizuki, T., Okazaki, M., Uezono, Y., Yanagihara, N., Wasa, A. and Izumi, F. (1990) Brain Res. 518, 334–336.
[22] Heidenreich, K.A., Toledo, S.P., Brunton, L.L., Watson, M.J., Daneil-Issakani, S. and Strulovici, B. (1990) J. Biol. Chem. 265, 15076–15082.
[23] Abraham, I., Sampson, K.E., Powers, E.A., Mayo, J.K., Ruff, V.A. and Leach, K.L. (1991) J. Neurosci. Res. 28, 29–39.
[24] Ways, E., Riddle, R., Ways, M. and Cook, P. (1991) J. Biol. Chem. 266, 1258–1264.
[25] Melloni, E., Pontremoli, S., Sparatore, B., Patrone, M., Grossi, F., Marks, P.A. and Rifkind, R.A. (1990) Proc. Natl. Acad. Sci. USA 87, 4417–4420.
[26] Koda, Y., Uezono, Y., Kobayashi, H., Izumi, F., Inatomi, H., Yamada, Y. and Okamura, T. (1991) Neurosci. Lett. 127, 31–33.
[27] Sheu, F.-S., Marais, R.M., Parker, P.J., Bazan, N.G. and Routtenberg, A. (1990) Biochem. Biophys. Res. Commun. 171, 1236–1243.
[28] Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. and Nishizuka, Y. (1991) Proc. Natl. Acad. Sci. USA 88, 5149–5153.