Expression of protein kinase C isozymes in hippocampal neurones in culture

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Abstract Several protein kinase C (PKC) isozymes were analyzed by immunoblot and immunocytochemistry in cultures of hippocampal neurones at several stages of differentiation. Our findings reveal the existence of two distinct patterns of expression. Firstly, conventional PKC isozymes α, β and γ, that are expressed at very low levels during the initial stages and then increase continuously with time of culture. Secondly, novel PKC isozymes δ, ϵ and ζ, whose contents increase very early to reach a maximum after three days of culture and then progressively decline. Specific proteolysis for PKC isozymes β and γ was observed throughout the period studied. The developmental profile obtained for the different PKC isozymes is discussed in relation to the differentiation of hippocampal neurones in culture.

Key words: PKC; Isozyme; Neuron; Differentiation; Proteolysis; Hippocampus

1. Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases composed of at least 10 distinct isozymes, including conventional PKC's (α, β_I, β_{II} and γ) and novel PKC's (δ, ε, ζ, η, θ and λ). Their role as mediators of signals generated upon external stimulation by hormones, neurotransmitters and growth factors has been widely and profusely documented (for recent reviews see [1–3]). In the brain, PKC's are highly concentrated and have been implicated in a broad spectrum of neuronal functions, such as modulation of ion channels [4], receptors [5] and neurotransmitter release [6]. PKC activation has also been implicated in the initiation and maintenance of Long Term Potentiation (LTP) [7,8] and Long Term Depression (LTD) [9].

During the last years, increasing evidence has accumulated suggesting a differential involvement of PKC isozymes in specific cellular functions, based on their different expression [10,11], substrate specificity [12], subcellular localization [13] and ability to interact with other cellular components [14–16]. Immunohistochemical studies using isozyme-specific antibodies in the rat brain have revealed that the distribution and subcellular localization of PKC isozymes varies greatly among anatomical regions [17–19] and can be selectively modified by treatments in vivo [20,21]. Very often, changes in the amount and activity of PKC have been implicated in the initiation of neuronal differentiation. However, the contribution of the different PKC isozymes to this process is not well understood. Most of our current knowledge comes from studies performed in cell lines such as PC12 [22,23], NG108–15 [24] and C6 glioma [25], where the expression pattern of PKC isozymes is different from the situation in vivo.

Primary cultures of hippocampal neurones derived from rat embryos constitute an adequate preparation to study neuronal development and function at the molecular and cellular level. They provide a highly homogeneous population of neurones of the pyramidal type which spontaneously display a well characterised program of development during the time of culture [26]. Several authors have stressed the importance of PKC in mediating survival-promoting and neurite outgrowth effects of various growth factors and other treatments on these cultures [27]. In the present work, we analyze the content and localization of several PKC isozymes during the development of hippocampal neurones in culture. Our findings reveal the existence of two distinct patterns of expression. Conventional PKC isozymes (α, β and γ) express very low levels during the initial stages and after two or three days in culture increase in content continuously. On the other side, novel PKC isozymes (δ, ε and ζ) increase early to reach a maximum after three days of culture and then progressively decline. We discuss these results in relation to the process of neuronal differentiation that takes place in these cultures.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibodies against peptide sequences unique to isozymes α, β, γ, δ, ε and ζ of PKC were obtained from Gibco-BRL. Antibody 150 (mouse, IgM) recognizes mode-I phosphorylated Microtubule-Associated Protein 1B (MAP1B) [28]. Antibody AP14 (mouse, IgG) recognizes the high molecular weight isoforms of Microtubule-Associated Protein 2 (MAP2). PMA (phorbol 12-myristate 13-acetate) was from Sigma. Stock solutions of PMA ($\times 100$ and $\times 1000$) were prepared in DMSO and maintained at $-20°$ until use.

2.2. Cell culture and preparation of cell extracts

Cultures of hippocampal neurones were prepared as described first by Banker and Cowan [29] and then modified by Goslin and Banker [30]. In brief, 18 days old rat embryos were used, their hippocampi removed and trypsinized for 15 min. After mechanical disruption in the presence of DNase I and soybean trypsin inhibitor, the cells were counted and seeded on poly-L-lysine coated plastic dishes at a density of 30.000 cells/cm^2 in 10% FCS/DMEM. Three hours later, culture medium was removed and substituted by DMEM-based N2 medium [31], supplemented with 1 mM pyruvate and 1 mg/ml egg albumin. In order to reduce pH fluctuations, sodium bicarbonate was lowered to 0,85 g/l and iso-osmotically substituted by HEPES pH 7,3. One-third of the culture medium was replaced by fresh medium after one week in culture. Cell extracts were prepared in ice-cold buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5% Triton X-100, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin and 1 mM PMSF and homogenised using a sterile syringe with 23-G needle. The homogenates were centrifuged at 100,000 $\times$ g for 30 min at 4° and the supernatants were used directly for analysing the content of PKC isozymes by immunoblot. Total protein content was determined by the BCA method [32].

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2.3. Immunoblot analysis

Triton X-100 extracts from hippocampal neurones were denatured in Laemmli solution and subjected to SDS-PAGE in minigels (8% polyacrylamide) [33]. A sample of adult rat brain extract was always included in each blot. Protein was transferred to PVDF membranes for 90 min at 50 V in a semi-dry electrotransfer device. The membranes were incubated successively with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h, with rabbit antibodies to PKC isozymes (α, β, γ, δ, ε and ζ) diluted 1:1000 in PBS, 0.05% Tween 20 (PBS/T20) for 18h and with peroxidase-conjugated anti-rabbit IgG antibody (Affinity) diluted 1:2000 in PBS/T20. Following each incubation, the membranes were extensively washed (× 5) with PBS/T20. The immunoreactive bands were visualised using diaminobenzidine/H_2O_2 in PBS with Nickel–Cobalt enhancement as previously described [34].

2.4. Immunocytochemistry

For immunocytochemistry, hippocampal neurones were plated on poly-L-lysine-coated glass coverslips at 15.000 cells/cm^2. To maintain the viability of the neurones at such low density, coverslips with neurones attached were placed on multiwell dishes with the neurones facing down to the bottom of the well as previously described [35]. At different times of culture, hippocampal neurones were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and washed thoroughly with PBS. All subsequent incubations were done at room temperature. The coverslips were successively incubated with 1 mg/ml sodium borohydride ($NaBH_4$) in PBS (2 × 10 min), with 3% BSA, 0.2% Triton X-100 in PBS (30 min), with 10% horse serum, 0.2% Triton X-100 in PBS (HS Buffer) (30 min), with rabbit antibodies to PKC isozymes (α, β, ε and ζ) diluted 1:100 in HS Buffer (120 min), with biotinylated anti-rabbit antibody (Vector) diluted 1:200 in HS buffer (60 min) and with ABC reagent (Vector) in HS buffer (60 min). Following each incubation, the coverslips were extensively washed with PBS/T20. Development of immunoreactivity was visualised at the light microscope using diaminobenzidine/H_2O_2 in PBS. Negative controls were done by omitting the primary antibody. For immunofluorescence, coverslips were blocked and permeabilized as described above and sequentially incubated with antibody AP14 (1:10), anti-mouse IgG Texas red-conjugated (DAKO), antibody 150 (1:10) and anti-mouse IgM Fluorescein-conjugated (DAKO). Coverslips were mounted in Mowiol.

3. Results

Primary cultures of hippocampal neurones are a suitable preparation to study the expression and subcellular localization of neuronal proteins. Hippocampal neurones obtained from E18 rats readily adhere to the culture substratum and initiate the extension of cytoplasmic processes that will further mature into axon and dendrites. Interestingly, axonal and dendritic outgrowth proceed with different timings and ratios. Thus, axonal elongation starts typically after 36 hours of culture and proceeds very quickly. On the other hand, dendritic outgrowth starts after 4 days in culture and proceeds much more slowly [26]. Fig. 1 illustrates this typical behaviour. Hippocampal neurones cultured for 2 days (T2) are actively extending an axon-like process, as revealed by an antibody that recognizes mode-I phosphorylated MAP1B (T2, MAP1B-P). On the other hand, after ten days of culture (T10), neurones have already develop a network of axons (T10, MAP1B-P) and their dendrites have entered the maturation stage, as revealed by antibodies that recognize high-molecular weight MAP2 (T10, hMAP2).

In order to characterize the presence and timing of expression of PKC isozymes in hippocampal neurones in culture, Triton X-100 extracts obtained at different times of culture (T0–T10) were analyzed by immunoblot (Fig. 2). The levels

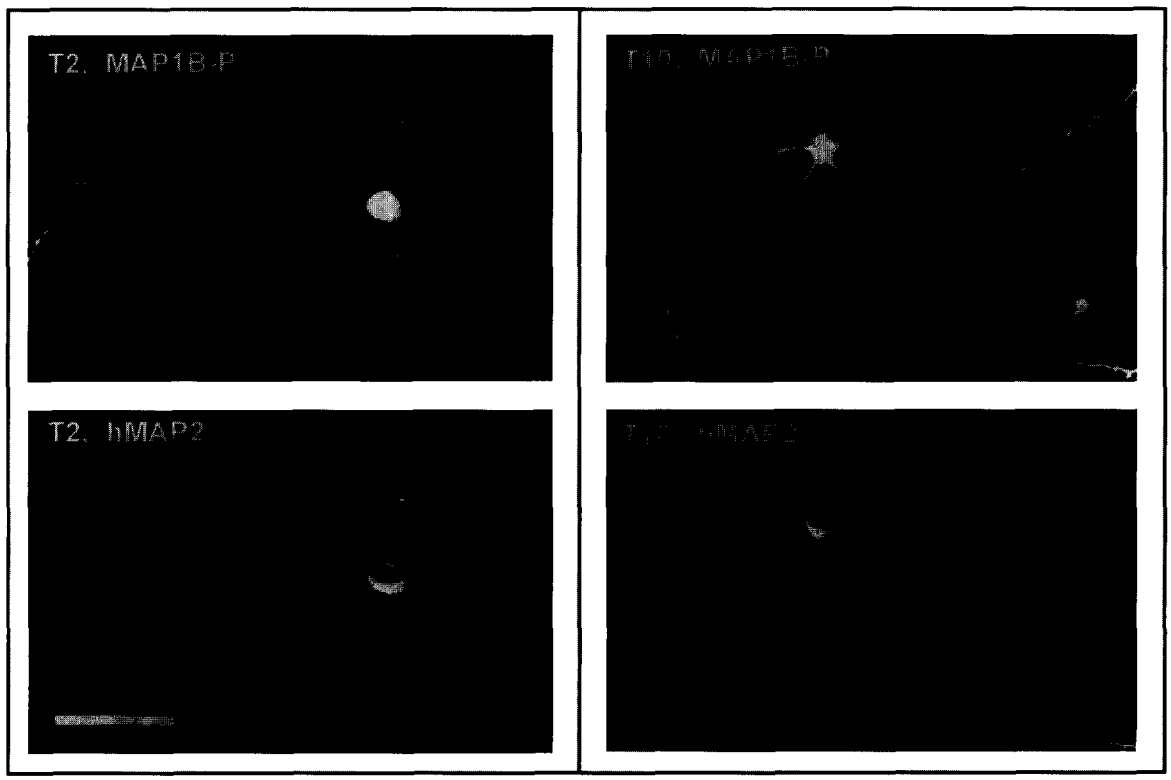

Fig. 1. Characterization of the cultures of hippocampal neurones. Hippocampal neurones grown on coverslips for two days (T2) or ten days (T10) were fixed and processed for double-immunofluorescence with antibody 150 which recognizes mode-I phosphorylated MAP1B (MAP1B-P) and antibody AP14 which recognizes high molecular weight isoforms of MAP2 (hMAP2). Neurites labelled with antibody 150 (MAP1B-P) and not with antibody AP14 (hMAP2) represent axons, whereas neurites labelled with both antibodies AP14 (hMAP2) and 150 (MAP1B-P) represent dendrites. Scale bars = 50 μm.

measured for PKCα were very low and only detectable after 2 days in culture. The relative abundance of this isozyme increased with the time of culture, reaching maximum levels at 10 days. No immunoreactive bands of the molecular weight expected for PKCβ and PKCγ were found. Instead, one band migrating at approximately 68 kDa was encountered for PKCβ and two bands migrating at approximately 72 and 60 kDa were found for PKCγ. In both cases, the amount of immunoreactivity increased with the time of culture. On some occasions, immunoreactive bands with similar migration values (68 kDa for PKCβ and 60 and 72 kDa for PKCγ) were found in rat brain extracts, suggesting that proteolytic degradation could have taken place. To rule out the possibility that proteolysis had occurred during the extraction process, an extraction buffer containing 1% SDS, 1 mM EDTA, 50 mM Tris-HCl, pH 7,4 heated at 90°C was used. Under these conditions, immunoblots for PKCβ and PKCγ were identical to those obtained with Triton X-100 extracts. Novel PKC isozymes ε, δ and ζ exhibited a bell-shaped developmental profile. Their concentration increased since the onset of the cultures to reach maximum levels between two and three days in culture

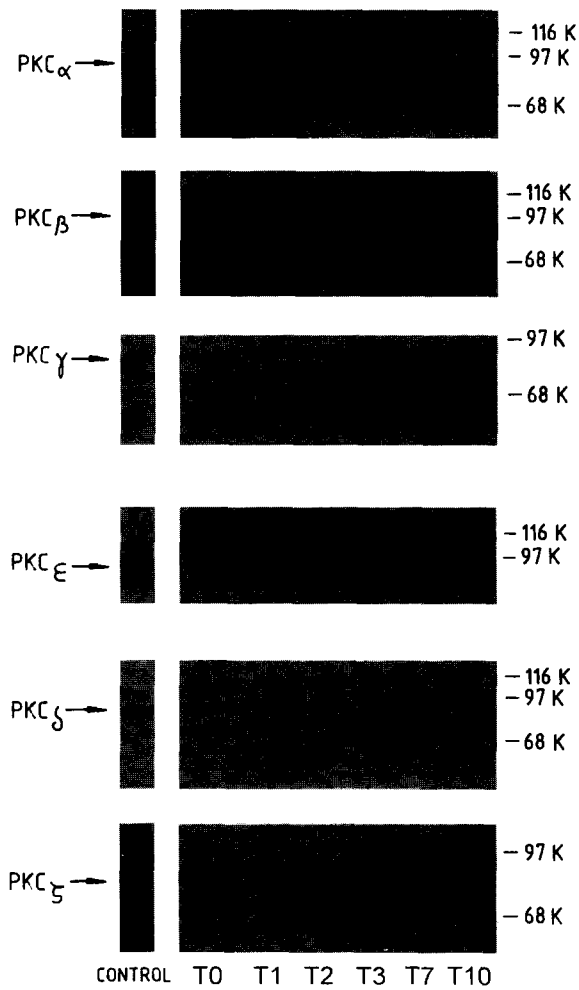

Fig. 2. Immunoblot analysis of PKC isozymes present in hippocampal neurones at several times in culture. Hippocampal neurones were extracted as described in section 2 after 2 hours (T0), one day (T1), 2 days (T2), three days (T3), seven days (T7) and ten days (T10) in culture. Aliquots from adult rat brain extract were run in parallel for each isozyme (CONTROL). 10 μg of total protein was loaded in each lane.

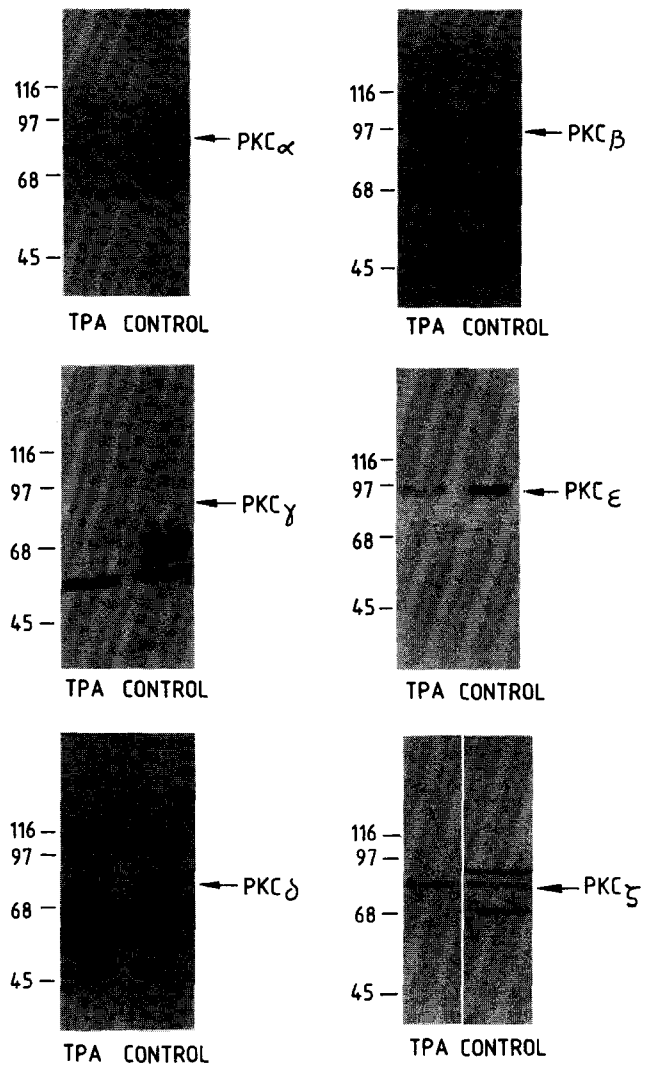

Fig. 3. Down-regulation of PKC isozymes present in hippocampal neurones by phorbol ester treatment. Hippocampal neurones cultured for seven days were treated with 0.5 μM phorbol 12-myristate 13-acetate (PMA) or an equivalent volume of DMSO (CONTROL) for 18 hours and then extracted and analysed by immunoblot as described in section 2. 15 μg of total protein was loaded in each lane. Arrows on the right of the blots indicate the migration position of the corresponding PKC isozyme in an extract from adult rat brain that was run in parallel.

(T2–T3). Then, a progressive decay in their content extended until the latest time studied.

To further characterise the identity of the immunoreactive bands, down-regulation experiments of PKC isozymes were carried out. It is well known that long-term treatment with phorbol esters (PMA) accelerate the proteolytic degradation of PKC isozymes α, β, γ, ε and δ, but not PKCζ [25,36]. Fig. 3 shows that PMA treatment readily down-regulated PKCα, PKCε and PKCδ, but not PKCζ which served as positive control. Both the PKCβ-related 68 kDa band and the PKCγ-related 72 kDa band disappeared after PMA treatment. However, PKCγ-related 60 kDa band did not exhibit down-regulation and probably bears no relationship with PKCγ.

The distribution and subcellular localization of PKC isozymes α, β, ε and ζ in cultures of hippocampal neurones was

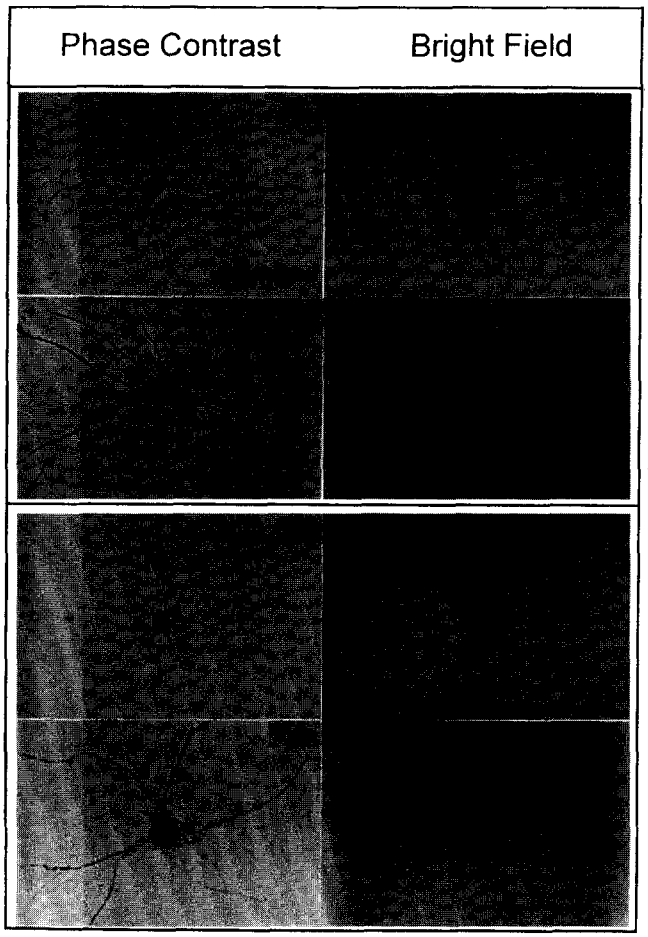

Fig. 4. Immunolocalization of PKCα and PKCβ in cultured hippocampal neurones. Hippocampal neurones grown on coverslips for two days (T2) or ten days (T10) were fixed and processed for immunocytochemistry with antibodies to PKCα (upper panel) or PKCβ (lower panel). Phase contrast micrographs, showing neuronal morphology, are presented on the left side of the panels and the corresponding bright field micrographs, showing only immunostaining, are presented on the right side of the panels. Scale bar = 50 μm.

analyzed by immunocytochemistry. PKCγ and PKCδ were excluded of this study since the available antibodies recognised more than one band by immunoblot. No evidence could be obtained in support of a differential distribution of PKC isozymes into the axonal and/or dendritic compartments of fully developed neurones. Figs. 4 and 5 show representative samples chosen from hippocampal neurones cultured for 2 days (T2) and 10 days (T10). It must be emphasized that, for most isozymes, the highest degree of staining was found in the perinuclear region. This pattern of staining is totally absent during the first hours of culture, only partially present at day 1 of culture and especially evident at later stages of culture (Fig. 4 – T10, PKCβ). PKCα and PKCβ are more clearly detected in neurones after 10 days of culture (Fig. 4 – T10, PKCα and PKCβ). The immunoreactivity concentrates mostly in the perinuclear region – especially PKCβ – with less but still visible staining in the neurites. Both isozymes, but more particularly PKCβ, showed very low levels of immunostaining which made difficult to confirm their presence in the thinner, axon-like, neurites, during the early stages of development (Fig. 4 – T2, PKCβ). PKCε and PKCζ immunoreactivity is better visualized at 2 days of culture, being clearly present in all neurites, either axon-like or dendrite-like ones (Fig. 5 – T2, PKCε and PKCζ). Later on, the immunoreactivity for these isozymes decreases in the neurites and restricts more to the cell bodies (Fig. 5 – T10, PKCε and PKCζ). These observations may reflect the different timing and rate of expression among PKC isozymes rather than a compartmentalization phenomenon.

4. Discussion

We have investigated the localization and abundance of several PKC isozymes in hippocampal neurones during their development in culture. Distinct development profiles have been observed for conventional (Ca^{2+}-dependent) and novel (Ca^{2+}-independent) PKC isozymes during neuronal differentiation. PKC isozymes α, β and γ increased in content continuously during the whole period of culture. However, PKC isozymes ε, δ and ζ increased in content to reach a maximum at about two or three days of culture and then declined until the 10th

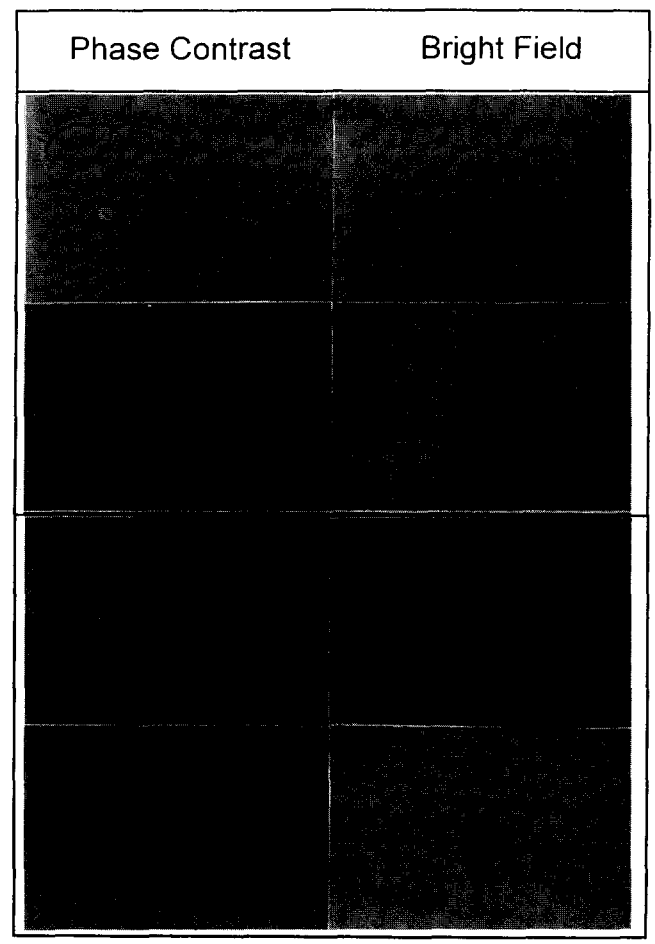

Fig. 5. Immunolocalization of PKCε and PKCζ in cultured hippocampal neurones. Hippocampal neurones grown on coverslips for two days (T2) or ten days (T10) were fixed and processed for immunocytochemistry with antibodies to PKCε (upper panel) or PKCζ (lower panel). Phase contrast micrographs, showing neuronal morphology, are presented on the left side of the panels and the corresponding bright field micrographs, showing only immunostaining, are presented on the right side of the panels. Scale bar = 50 μm.

day of culture. Immunocytochemical studies with PKC isozymes $\alpha, \beta, \varepsilon$ and ζ did not show specific compartmentalization either in the axon or the dendrites.

The search for a correlation between neuronal differentiation and PKC activity has long deserved particular attention in the literature. However, despite the efforts that have been made, no clear agreement has been reached onto whether an increase or a decrease of PKC activity is associated to neuronal differentiation. Down-regulation or inhibition of PKC activity has been associated to initiation of neuronal differentiation in N2A neuroblastoma cells [37] and SH-SY5Y neuroblastoma cells [38–40]. Also, dibutyryl cAMP-induced differentiation of NG108-15 cells is accompanied by a decrease in PKCα [24]. On the other hand, PKC activity present in LAN-5 cells increased when differentiated with γ-interferon [41] and differentiation of NT2/D1 cells by retinoic acid is accompanied by an increase in PKC [42]. Lastly, PC12h cells differentiated with dibutyryl cAMP showed increased levels of PKCβ and decreased levels of PKCα [43] whereas PKCα content of PC12 cells is unchanged by treatment with nerve growth factor (NGF) [36]. It must be considered that all of these studies have been done in cell lines of neural origin and that neuronal differentiation was achieved by the addition of exogenous factors or compounds.

Given the different developmental profiles observed for conventional and novel PKC isozymes, it would be tempting to speculate that axonal development is associated to the activity of novel PKC isozymes and dendritic maturation is associated with the activity of conventional PKC isozymes. We had previously demonstrated that the third day of culture is a critical time for the development of hippocampal neurones in vitro, since a change in the phosphorylation pattern of microtubule associated protein 2 (MAP2) – a PKC substrate – occurs at that time [44]. In the present study, most PKC isozymes did not show specific subcellular compartmentalization during in vitro development. Only PKCβ showed such low levels of immunostaining that its presence in thinner axon-like neurites could not be confirmed. So, if this is the case, one could suggest that isozyme compartmentalization is not a relevant feature for PKC action during neuronal differentiation in vitro, but rather the timings and levels of expression for each isozyme. However, this in vitro observation contrasts with the in vivo situation of the adult hippocampus, where a defined subcellular localization exists for each PKC isozyme [17–19,45]. Similar puzzling results have been obtained before in primary cultures of rat cerebellum where neither the expression of different isozymes to different cell populations nor the subcellular localization observed in adult cerebellum is achieved in vitro [46]. It may be that additional signals, probably of extracellular origin, are needed to determine PKC isozyme subcellular localization.

Immunoreactive bands of 68 kDa for PKCβ and of 72 and 60 kDa for PKCγ were detected by immunoblot of hippocampal neurones extracts. We propose that 68 kDa peptide (PKCβ) and 72 kDa peptide (PKCγ) represent proteolytic fragments of PKCβ and PKCγ. These peptides are recognized by isozyme-specific antibodies, down-regulated by phorbol ester treatment and detected occasionally on immunoblots of adult rat brain extracts. Further, previous studies had already reported that PKCβ and PKCγ exhibit a greater sensitivity to proteolysis than other PKC isozymes [47]. The question now arises on to whether proteolysis occurs in situ or during the extraction procedure. We favour the former possibility for several reasons. Firstly, a cocktail of proteases inhibitors was used throughout the extraction procedure. Secondly, proteolysis did not extend to other PKC isozymes assayed in the same extracts. Lastly, direct extraction with hot buffers containing SDS did not improve the recovery of intact PKCβ and PKCγ.

Proteolytic degradation of PKC isozymes by calpains has been shown to render a fragment of 36 kDa containing the regulatory element of the kinase and a second fragment of 45–48 kDa containing the catalytic moiety, which is referred as PKM [48]. In hippocampal neurones extracts, immunoreactive bands for PKCβ and PKCγ exhibited higher molecular weights than those expected for calpain fragments. Nonetheless, we can not exclude the possibility that they may represent true PKM fragments since the migration values that we obtained for intact PKCβ and PKCγ from rat brain are in the range of 90–100 kDa, slightly higher than those reported in the literature [2]. Alternatively, they could represent intermediate products of protease degradation. These products would contain the catalytic domain which is down-regulated by phorbol ester treatment, the V3 region -the antibodies used were raised against peptides of the V3 region- and probably the C2 domain which confers Ca^{2+}-dependence. Proteolytic activation and degradation of PKC isozymes has been widely discussed as a physiological mechanism to regulate the activity and localization of PKC isozymes. Tryptic activation of PKCε increased the kinase activity towards histone about 10-fold [49]. Also, Suzuki et al. [50] have shown that PKCβ and PKCγ isozymes are selectively associated with postsynaptic densities obtained from rat hippocampus and suggest that proteolytic activation of these isozymes could be a step in postsynaptic signal processing. It may be therefore possible that PKCβ and PKCγ present in hippocampal neurones in culture are subject to specific regulation by in vivo proteolysis.

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References

[1] Azzi, A., Boscoboinik, D. and Hensey, C. (1992) Eur. J. Biochem. 208, 547–557.
[2] Hug, H. and Sarre, T.F. (1993) Biochem. J. 291, 329–343.
[3] Tanaka, C. and Nishizuka, Y. (1994) Annu. Rev. Neurosci. 17, 551–567.
[4] Shearman, M.S., Sekiguchi, K. and Nishizuka, Y. (1989) Pharmacol. Rev. 41, 211–237.
[5] Huganir, R.L. and Greengard, P. (1990) Neuron 5, 555–567.
[6] Robinson, P.J. (1992) Mol. Neurobiol. 5, 87–130.
[7] Malinow, R., Schulman, H. and Tsien, R.W. (1989) Science 245, 862–866.
[8] Sacktor, T.C., Osten, P., Valsamis, H., Jiang, X.L., Naik, M.U. and Sublette, E. (1993) Proc. Natl. Acad. Sci. USA 90, 8342–8346.
[9] Linden, D.J. and Connor, J.A. (1991) Science 254, 1656–1659.
[10] Mischak, H., Goodnight, J., Kolch, W., Martinybaron, G., Schaechtle, C., Kazanietz, M.G., Blumberg, P.M., Pierce, J.H. and Mushinski, J.F. (1993) J. Biol. Chem. 268, 6090–6096.
[11] Clemens, M.J., Trayner, I. and Menaya, J. (1992) J. Cell Sci. 103, 881–887.
[12] Kazanietz, M.G., Areces, L.B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J.F. and Blumberg, P.M. (1993) Mol. Pharmacol. 44, 298–307.

[13] Rosenberg, U., Shakibae, M. and Buchner, K. (1995) Biochem. J. 305, 269–275.
[14] Ozawa, K., Yamada, K., Kazanietz, M.G., Blumberg, P.M. and Beaven, M.A. (1993) J. Biol. Chem. 268, 2280–2283.
[15] Otte, A.P. and Moon, R.T. (1992) Cell 68, 1021–1029.
[16] Dekker, L.V. and Parker, P.J. (1994) Trends Biochem. Sci. 19, 73–77.
[17] Saito, N., Itouji, A., Totani, Y., Osawa, I., Koide, H., Fujisawa, N., Ogita, K and Tanaka, C (1993) Brain Res. 607, 241–248.
[18] Kose, A., Ito, A., Saito, N. and Tanaka, C. (1990) Brain Res. 518, 209–217.
[19] Tanaka, C. and Saito, N. (1992) Neurochem. Int. 21, 499–512.
[20] Yokota, M., Peterson, J.W., Kaoutzanis, M and Kowall, N.W. (1992) Brain Res. 587, 123–129.
[21] Shimohama, S., Saitoh, T. and Gage, F.H. (1993) Hippocampus. 3, 43–56.
[22] Wooten, M.W. (1992) Exp. Cell. Res. 199, 111–119.
[23] Roivainen, R., McMahon, T. and Messing, R.O. (1993) Brain Res. 624, 85–93.
[24] Battaini, F., Garbillo, G., Bergamaschi, S., Parenti, M., Wetsel, W.C., Govoni, S and Trabucchi, M. (1994) Biochem. Biophys. Res. Commun. 201, 135–142.
[25] Chen, C-C. (1993) FEBS Lett. 332, 169–173.
[26] Dotti, C.G., Sullivan, C.A. and Banker, G.A. (1988) J. Neurosci. 5, 2119–2142.
[27] Louis, J.C., Magal, E., Gerdes, W. and Seifert, W. (1993) Eur. J. Neurosci. 5, 1610–1621.
[28] Ulloa, L., Díez-Guerra, F.J., Avila, J. and Díaz-Nido, J. (1994) Neuroscience 61, 211–223 (1994).
[29] Banker, G.A. and Cowan,WM (1977) Brain Res. 126, 397–425.
[30] Goslin, K. and Banker, G. (1991) in: Culturing Nerve Cells (Banker, G. and Goslin, K., Eds.) pp. 251–282, MIT Press, Cambridge, MA.
[31] Bottenstein, J.E. and Sato, G.H. (1979) Proc. Natl. Acad. Sci. USA 76, 514–519.
[32] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[33] Laemmli, U.K. (1970) Nature *227*, 680–685.
[34] De Blas, A.L. and Cherwinski, H.M. (1983) Anal. Biochem. 133, 214–219.
[35] Brewer, G.J. and Cotman, C.W. (1989) Brain Res. 494, 65–74.
[36] Wooten, M.W., Seibenhener, M.L., Soh, Y., Ewald, S.J., White, K.R., Lloyd, E.D., Olivier, A. and Parker, P.J. (1992) FEBS Lett. 298, 74–78.
[37] Miñana, M.D., Felipo, V. and Grisolía, S. (1990) Proc. Natl. Acad. Sci. (USA) 87, 4335–4339.
[38] Heikkilä, J.E., Akerlind, G. and Akerman, K.E.O. (1989) J. Cell. Physiol. 140, 593–600.
[39] Leli, U., Parker, P.J. and Shea, T.B. (1992) FEBS Lett. 297, 91–94.
[40] Leli, U., Shea, T.B., Cataldo, A., Hauser, G., Grynspan, F., Beermann, M.L., Liepkalns, V.A., Nixon, R.A., Parker, P.J. (1993) J. Neurochem. 60, 289–298.
[41] Ponzoni, M., Lucarelli, E., Corrias, M.V. and Cornagliaferraris, P. (1993) FEBS Lett. 322, 120–124.
[42] 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.
[43] Uehara-Kunugi, Y., Shimohama, S., Kobayakawa, H., Tamura, H. and Taniguchi, T. (1994) J. Neurochem. 63, 125–132.
[44] Díez-Guerra, F.J. and Avila, J. (1993) Neuroreport. 4, 419–422.
[45] Tsujino, T., Kose, A., Saito, N. and Tanaka, C. (1990) J. Neurosci. 10, 870–884.
[46] Shimohama, S. Ueharakunugi, Y., Terai, K., Taniguchi, T., Kimura, J. and Saitoh,T (1991) J. Neurosci. Res. 29, 261–270.
[47] Huang, F.L., Yoshida, Y., Cunha-Melo, J.R., Beaven, M.A. and Huang, K-P. (1989) J. Biol. Chem. 264, 4238–4243.
[48] Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S-I., Tominaga, M., Kuroda, T. and Nishizuka, Y. (1989) J. Biol. Chem. 264, 4088–4092.
[49] Schaap, D. and Parker, P.J. (1990) J. Biol. Chem. 265, 7301–7307.
[50] Suzuki, T., Okumuranoji, K., Tanaka, R., Ogura, A., Nakamura, K., Kudo, Y. and Tada, T. (1993) Brain Res. 619, 69–75.