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Proteolysis of protein kinase C: mM and μM calcium-requiring calpains have different abilities to generate, and degrade the free catalytic subunit, protein kinase M

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Abstract Limited proteolysis of protein kinase C (PKC) by calpain under cell free conditions cleaves the regulatory and catalytic PKC subunits, generating a free, co-factor independent catalytic subunit, termed PKM. In the present study, we demonstrate distinct differences in the rate, nature, and lipid-sensitivity of PKC and PKM proteolysis by μM and mM calcium-requiring calpain isozymes (μ calpain or m calpain, respectively). PKC is a preferred substrate for m calpain; not even a 100-fold increase in μ calpain was capable of degrading PKC as fast as in calpain. PKM was generated by both m and μ calpains, but was itself rapidly degraded by m calpain and therefore was only transiently detectable. By contrast, PKM was formed but not degraded by μ calpain, and persisted in the presence of μ calpain long after all PKC had been degraded. Phosphatidyl serine (PS) inhibited PKC hydrolysis by m calpain yet enhanced PKC hydrolysis by μ calpain. The ability of either calpain isoenzyme to degrade [^{14}C]azocasein was unaffected by PS, suggesting that the influence of PS was on PKC conformation. These findings point towards distinct roles for μ and m calpain in PKC regulation.

Key words: Protein kinase C; Protein kinase M; Calpain; Calcium-activated proteolysis; Signal transduction; Phosphorylation

1. Introduction

Protein kinase C is a family of Ser/Thr kinases that regulate intracellular signal transduction mechanisms [1]. PKC consists of a catalytic domain and regulatory domain, the latter of which requires activating cofactors including Ca^{2+}, phospholipid, and diacylglycerol (DAG), which induce a conformational change that permits substrate interaction [2]. Limited proteolysis of PKC by calcium-activated proteases cleaves the catalytic and regulatory subunits, generating a cofactor-independent, free catalytic subunit, termed PKM [3–5]. Unlike membrane-bound, intact PKC, PKM has potential access to a distinct set of non-membrane-associated protein substrates [6]. The responsible calcium-activated proteases, calpains, exist as distinct isoforms that are activated in cell-free analyses at micromolar or millimolar Ca^{2+} concentrations (termed 'μ calpain' and 'm calpain', respectively); their respective Ca^{2+} requirements in situ are unknown [7–12].

The respective roles of these two modes of PKC activation remain unclear; however, the inhibition of certain PKC-mediated process by the protease inhibitor leupeptin underscores the potential physiological importance of limited PKC proteolysis [13]. Moreover, some interrelationship may exist between these two modes, since phospholipid-mediated activation of PKC apparently facilitates its hydrolysis by calpain [8,9].

In the present study, we examine PKC proteolysis by μ and m calpain, and the influence of phospholipids on this proces. Portions of this research have been presented in abstract form [14].

2. Materials and methods

2.1. Partial purification of μ and m calpain

m calpain was purified from post-mortem human brain by sequential chromatography on DE-52 cellulose, phenyl sepharose and Ultrogel-AcA-44 as previously described [15]. μ calpain was purified from human erythrocytes by sequential chromatography on DE-52 cellulose followed by phenyl sepharose CL-4B [12]. μ calpain was also purified from bovine brain gray matter by identical methodology [12], followed by immunoprecipitation of residual m calpain with a polyclonal antibody (C_7) directed against m calpain. Calpain activity was assayed against ^{14}C-labeled azocasien as described [15]. One unit (U) of specific activity was defined as that amount of purified m and μ calpain required to degrade 1 μg azocasien/min at 30°C; 1 U corresponded to 1.2 μg of m calpain and 2.1 μg of μ calpain in these analyses.

2.2. Incubation of purified PKC with mM calpain

PKC α from rabbit brain (1 μg; UBI, Inc., Lake Placid, NY) was incubated with 1–100 activity units of either calpain for 0–60 min at 30°C in 50 mM Tris-HCl (pH 7.4) containing 2 mM PMSF and 2 mM $CaCl_2$, after which the reaction was stopped by the addition of 2 × SDS treatment buffer on ice. Additional PKC samples were incubated for 60 min in the absence of calpain, under which no degradation was observed (not shown; see [5]). In some experiments, 0.5 mg/ml phosphatidylserine (PS; Serdary Research Laboratory, Ontario, Canada) was included.

2.3. Gel electrophoresis and immunoblot analysis

Samples were electrophoresed on SDS 7% polyacrylamide gels and transferred to nitrocellulose. The co-migration of calpain (76–80 kDa) and PKC (80 kDa) necessitated the use of immunoblot methodologies to monitor proteolytic degradation of PKC. Nitrocellulose replicas were probed with a 1:300 dilution of a commercial monoclonal antibody that specifically recognizes the catalytic domain of PKC α (UBI), followed by reaction with alkaline phosphatase-conjugated goat anti-mouse antibody and visualization as described [16].

2.4. Densitometric analysis of nitrocellulose replicas

Nitrocellulose replicas were scanned at optimal contrast within a linear range using a Hewlett-Packard ScanJet IIp scanner connected to a Macintosh MacPlus computer. The resulting digitized immunoblot profiles were analyzed with Scan Analysis software (BioSoft, Ferguson, MO) and values were exported into Excel and Cricket Graph software

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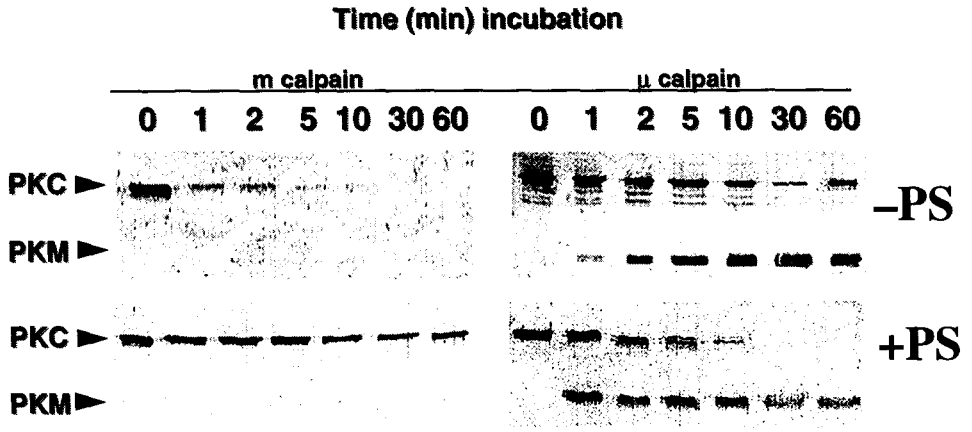

Fig. 1. Proteolysis of PKC by μ and m calpain. PKC (1 μg) was incubated with 0.035 U of m calpain or 4.21 U of μ calpain in the presence of $CaCl_2$ at 30°C for 0–60 min without and with 0.5 mg/ml phosphatidyl serine (PS) as indicated. Note that PKC immunoreactivity was eliminated substantially more rapidly in the presence of m calpain than μ calpain. Note also that PKM was detected only transiently during incubation with m calpain, yet persisted following its generation by μ calpain. Note that PS decreased the rate of PKC degradation by m calpain but increased PKC and PKM degradation by μ calpain.

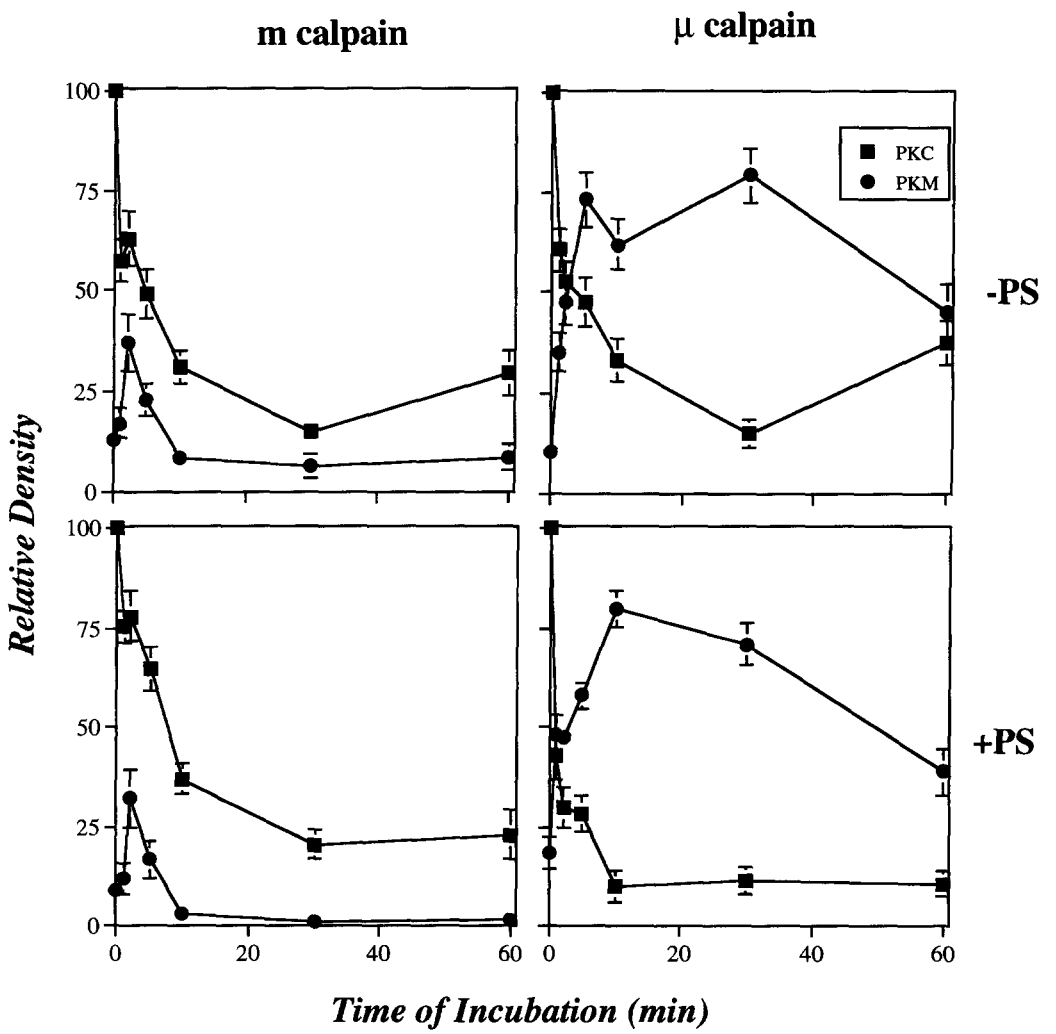

Fig. 2. Quantitation of PKCα degradation profiles by μ and m calpain. Nitrocellulose replicas of PKC degradation profiles by calpain in the presence and absence of PS were digitized and quantitated as described in section 2. Values are presented as the mean ± standard error of the mean obtained from 3 separate experiments.

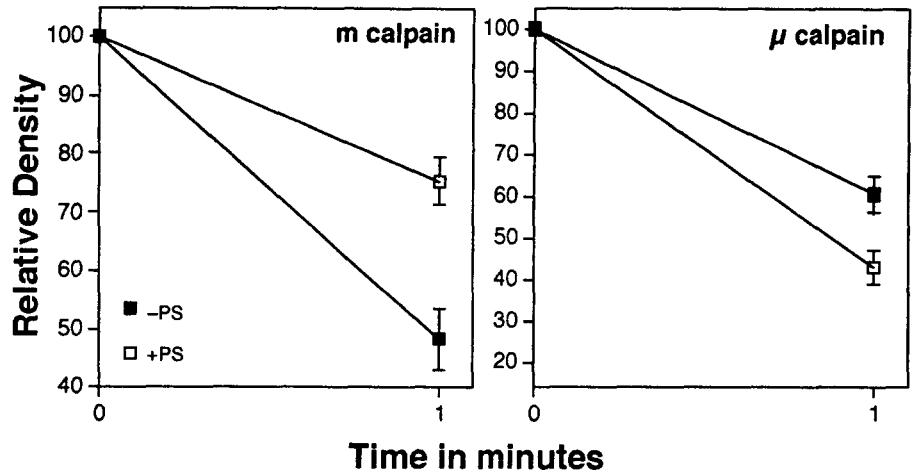

Fig. 3. Degradation rates of PKC. Regression analyses of linear portions of PKC degradation profiles were calculated as described in section 2. PS decreased PKC degradation by m calpain by 47%, and enhanced PKC degradation by μ calpain by 45%.

as described [17]. Staining intensity obtained for PKC at 0 incubation time for each nitrocellulose replica was defined as 100%, and all additional immunoreactive PKC and PKM bands on each respective replica were calculated as percentages relative to their respective PKC value at time 0.

Representative nitrocellulose replicas are illustrated, and corresponding graphs represent the mean ± the standard error of the mean derived from analysis of replicas from 3 separate experiments. It should be noted that the relative affinity of this monoclonal antibody for the native enzyme (PKC) versus the proteolytically-cleaved catalytic domain (PKM) is unknown and therefore cannot be compared. Calculation of PKM as a percentage of PKC at time 0 serves only as a reference point for construction of graphs, since in many instances there is no detectable PKM at time 0. In this study, no attempt is made to compare the absolute amount of PKM immunoreactivity with that of PKC; the relative presence of PKM or PKC are separately evaluated by their respective immunoreactivities during incubation with calpain. PKC degradation rates were calculated by plotting the mean ± standard error of the initial decay rates (0–1 min), and best-fit lines were generated by Cricket Graph software. The percentage change in rate of PKC degradation in the presence of PS was determined by the formula (mean degradation rate with PS/mean degradation rate without PS) × 100.

3. Results

PKCα was degraded following incubation with either μ or m calpain in the presence of $CaCl_2$ (Fig. 1). This loss of PKC was accompanied by the appearance of PKM immunoreactivity at 46 kDa. PKC was a preferred substrate for m calpain as compared to μ calpain. While incubation of PKC with 0.035 U of m calpain rapidly depleted PKC immunoreactivity (Fig. 1), the identical activity U of μ calpain did not degrade PKC (not shown); moreover, even a 100-fold excess in μ calpain activity U did not degrade PKC as fast as m calpain (Figs. 1 and 2). Consistent with previous studies [5], PKM was only weakly and transiently detected during PKC hydrolysis by m calpain. By contrast, PKM was generated by μ calpain but apparently did not undergo further degradation (Figs. 1 and 2). That the 46 kDa PKC-immunoreactive polypeptide generated in these reactions was, in fact PKM, was previously confirmed by its ability to phosphorylate a peptide corresponding to amino acid resi-

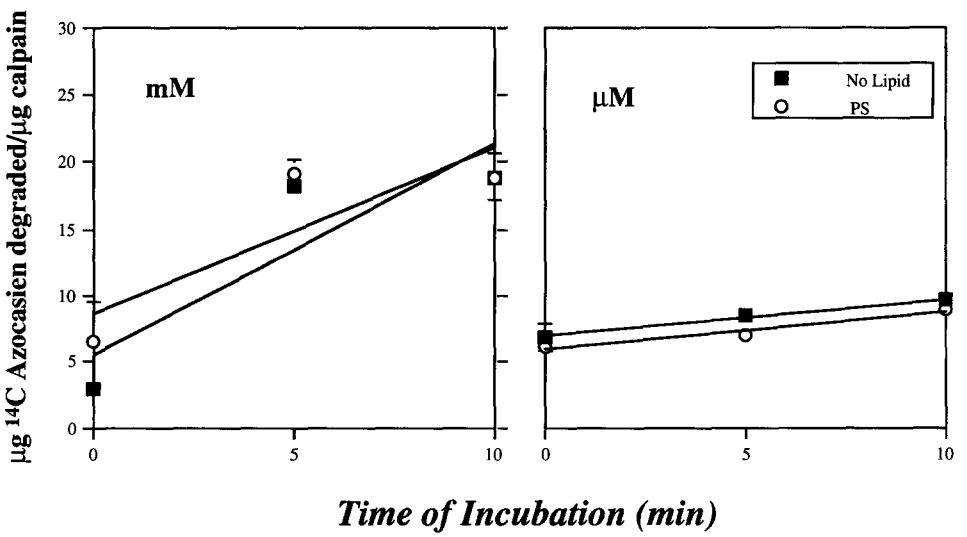

Fig. 4. Effect of PS on μ and m calpain activity. The degradation of 100 μg [^{14}C]azocasien by 2.5 μg or 1.0 μg of m and μ calpain, respectively, was performed as described [13] without and with 0.5 mg/ml PS. Values presented represent the mean ± standard error of the mean for 3 separate experiments. Note that the rate of azocasein degradation by either calpain was not affected by these phospholipids.

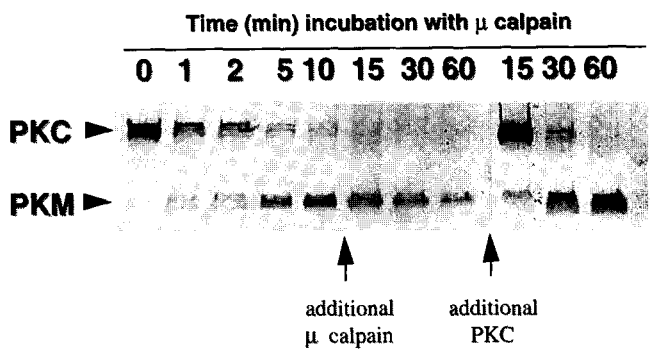

Fig. 5. Relative resistance of PKM to μ calpain-mediated proteolysis. PKC (1 μg) was incubated from 0–60 min with 4.21 U of μ calpain, 0.5 mg/ml PS and 2 mM $CaCl_2$ at 30°C. After 14 min, some samples received an additional 4.21 U of μ calpain or an additional 1 μg of PKC as indicated and reactions were continued. Note the persistence of PKM following introduction of additional μ calpain, and the rapid proteolysis of additional PKC.

dues 4–14 of myelin basic protein in the absence of PKC-specific cofactors [5].

We next examined the impact of PS on calpain-mediated PKC hydrolysis, which activates PKC by inducing a conformational change in the regulatory subunit [2]. PS slowed m calpain-mediated PKC degradation by approximately 47% yet increased the rate of PKC degradation by μ calpain by approximately 45% (Figs. 1–3). PKM underwent degradation by μ calpain in the presence of PS (Figs. 1 and 2). Degradation of [^{14}C]azocasein by either calpain was unaffected by PS (Fig. 4), suggesting that the influence of PS on PKC degradation was not due to a direct effect of PS on either calpain.

This possibility remained that the accumulation of PKM was a reflection of rapid depletion of μ calpain activity during the first few minutes of PKC degradation. However, introduction of additional μ calpain at 14 min (by which time all PKC had been hydrolyzed) resulted in an insignificant loss of PKM (Fig. 5). Moreover, additional PKC introduced into the reaction after 14 min was rapidly degraded, resulting in further PKM accumulation (Fig. 5). These findings confirm that PKM accumulation was not a result of rapid depletion of μ calpain activity during the initial phases of PKC hydrolysis in our assay systems, and confirm that the accumulation of PKM is indeed a reflection of it being a poorer μ calpain substrate than is intact PKC. The possibility that the relative inability to degrade PKM represented a unique characteristic of erythrocyte μ calpain was eliminated by the observation of identical patterns of PKC hydrolysis and PKM accumulation following incubation with μ calpain from bovine brain (Fig. 6).

4. Discussion

In the present study we observed differential rates of proteolysis of PKC and the resultant free catalytic subunit PKM by μ and m calpain. PKC underwent rapid proteolysis by m calpain in the absence of lipids forming transiently detectable PKM, but in the presence of lipids, m calpain-mediated proteolysis of PKC is inhibited. By contrast, μ calpain hydrolyzed PKC faster in the presence of lipids than without lipids producing PKC both cases. Once formed, PKM was not further degraded by μ calpain under these conditions.

The results indicate that PKM is a good substrate for m calpain (see also [5]), but a poor substrate for μ calpain. One possible implication of these findings is that limited PKC proteolysis by μ calpain may be part of a mechanism to induce relatively localized PKM-specific phosphorylation, e.g. of transmembrane membrane proteins such as MARKCS and other integins, and several membrane-skeletal proteins [1,19]. Although PKM is thought to be formed at the plasma membrane, it is free to migrate into the cytosol it is liberated from the lipid-binding region within the regulatory domain of PKC [13]. PKM formed as a result of μ calpain activity may be eliminated by m calpain following its diffusion away from the inner membrane. This possibility is consistent both with the preferential localization of μ, but not m, calpain at the plasma membrane and with our data showing a preference of μ calpain for the active form of PKC and m calpain for the native conformation. In this regard, PKM, although co-factor independent and not restricted to the membrane, should not be considered to be 'unregulated' kinase; in fact, our data showing that PKM is degraded faster than PKC demonstrate that PKM is actually under tight regulation by m calpain-mediated proteolysis [5].

Another possible implication of the present findings is that disregulation of calpains accompanying certain pathophysiological conditions may rise to the persistently high levels of PKM [19,20]. Calcium influx at a concentration sufficient to induce hyperactivation of μ, but not m calpain could rapidly generate PKM, which μ calpain may not degrade further, or may do so only relatively slowly. Consequently, excessive amounts of PKM may enter the cytosolic compartment and initiate phosphorylation of inappropriate substrates, and/or hyperphosphorylation of otherwise normal substrates. The potential role of abnormally high levels of PKM activity in the pathogenesis of certain conditions remains an important area of investigation.

While numerous studies in cell-free systems have demonstrated that limited proteolysis of PKC generates a co-factor independent, free catalytic subunit ('PKM'), the difficulty in demonstrating the presence of PKM in intact cells has generated controversy concerning the physiological relevance of this process [13]. Whether or not PKM indeed exists within intact cells is of considerable importance in understanding the full nature and extent of the roles of PKC in situ, because PKM can theoretically act on additional classes of substrates that are inaccessible to its membrane-associated, cofactor-dependent

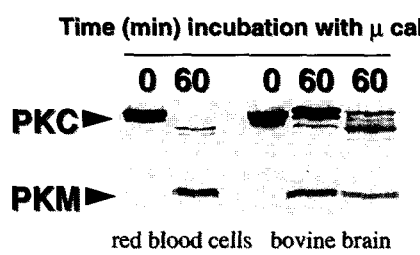

Fig. 6. Comparison of PKC degradation and PKM accumulation by μ calpain from human erythrocytes and bovine brain. PKC α was incubated for 0 min or 60 min in the presence of $CaCl_2$, 4.21 U of erythrocyte μ calpain ('red blood cells'), 4.21 U ('bovine brain', first lane incubated for 60 min) or 8.42 U ('bovine brain', second lane incubated for 60 min) bovine brain μ calpain. Note PKC hydrolysis and PKM accumulation with μ calpain from both sources.

parent enzyme [11]. The findings of the present study provide insight into the nature of limited proteolysis of PKC by calpain, and point towards potentially different physiological roles for μ and m calpain in the regulation of this enzyme. Moreover, these findings present the first documentation for potential functional differences between the calpain isozymes. These data, however, do not confirm the physiological significance of PKM, which would require the demosntration of PKM-specific substrate phosphorylation in situ. In addition, these studies do not address whether or not additional proteases are active against PKC and/or PKM, and whether or not alternative methods of PKC activation, or alternative cleavage patterns can result in PKC down-regulation without generating PKM. Indeed, PKM was not visualized in SH-SY-5Y cells during TPA-mediated PKC activation and ultimate down-regulation, nor following TPA treatment of neutrophils [21]. While studies have implicated proteolysis of PKC as a necessary component of TPA-mediated PKC down-regulation [5,7,10,22], it remains to be determined whether or not significant levels of PKM are transiently generated during this process. Although the results of the present study confirm that both calpain isoforms readily degrade PKC in cell-free studies, it is yet unclear whether or not μ calpain, m calpain or both are responsible for in situ proteolysis of PKC. Finally, in the present study, we have examined the calpain-mediated degradation of the alpha isoform of PKC and results presented herein are therefore potentially unique for that isoform. Since evidence is accumulating that the various PKC isozymes display unique neuronal localizations and sensitivities and mediate distinct functions, it should be considered that the other isoforms [8], and any respective PKM derived from them, may display distinct degradation profiles [23,24].

References

[1] Hug, H. and Sarre, T.F. (1993) Biochem. J. 291, 329–343.
[2] House, C. and Kemp, B.E. (1987) Science 238, 1726–1728.
[3] Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 1156–1164.
[4] Al, Z. and Cohen, C.A. (1993) Biochem. J. 296, 675–683.
[5] Shea, T., Beermann, M.L., Griffin, W.R. and Leli, U. (1994) FEBS Lett. 350, 223.
[6] Luo, J.-H., Kahn, S., O'Driscoll, K. and Weinstein, J.B. (1993) J. Biol. Chem. 268, 3715–3719.
[7] Alonso, M.T., Sanchez, A. and Herreros, B. (1989) FEBS Lett. 244, 407–410.
[8] 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.
[9] Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610–7616.
[10] Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Salamino, F., Sparatore, B. and Horecker, B.L. (1986) J. Biol. Chem. 261, 8309–8313.
[11] Pontremoli, S., Melloni, E., Michetti, M., Sparatore, B., Salamino, F., Sacco, O. and Horecker, B.L. (1987) Proc. Natl. Acad. Sci. USA 84, 3604–3608.
[12] Vitto and Nixon. (1986) J. Neurochem. 47, 1039–1051.
[13] Murray, A.W., Fournier, A. and Hardy, S.J. (1987) Trends Neurol. Sci. 12, 53–54.
[14] Lang, D., Hauser, G.H., Cressman, C.M., Mohan, P.S., Nixon, R.A. and Shea, T.B. (1995) Trans. Am. Soc. Neurochem., in press.
[15] Nixon, R.A., Quackenbush, R. and Vitto, A. (1986) J. Neurosci. 6, 1252–1263.
[16] Shea, T.B., Paskevich, P.A. and Beermann, M.L. (1993) J. Neurosci. Res. 35, 507–521.
[17] Shea, T.B. (1994) Biotechniques 16, 1126–1128.
[18] Yasuda, I., Kishimoto, A., Tanaka, S.-I., Tominaga, M., Sakurai, A. and Nishizuka, Y. (1990) Biochem. Biophys. Res. Commun. 166, 1220–1227.
[19] Nixon, R.A. (1989) in: Calcium, Membranes, Aging, and Alzheimer's Diseases. Ann. N.Y. Acad. Sci. 568, 198–208.
[20] Saitoh, T. and Masliah, E. (1993) in: (Corain, B., Iqbal, K., Nicolinin, M., Winblad, B., Wisniewski, H. and Zatta, P., Eds.) Alzheimer's disease: Advances in Clinical and Basic Research, Wiley, pp. 349–353.
[21] Pontremoli, S., Michetti, M., Melloni, E., Sparatore, B., Salamino, F. and Horecker, B.L. (1990) Proc. Natl. Acad. Sci. USA 87, 3705–3707.
[22] Pontremoli, S., Melloni, E., Damiani, G., Salamino, F., Sparatore, B., Michetti, M. and Horecker, B.L. (1988) J. Biol. Chem. 263, 1915–1919.
[23] Pontremoli, S., Melloni, E., Sparatore, B., Michetti, M., Salamino, F. and Horecker, B.L. (1990) J. Biol. Chem. 265, 706–712.
[24] Saido, T.C., Mizuno, K. and Suzuki, K. (1991) Biochim. Biophys. Acta 50, 485–489.