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Degradation of protein kinase Cα and its free catalytic subunit, protein kinase M, in intact human neuroblastoma cells and under cell-free conditions

Evidence that PKM is degraded by mM calpain-mediated proteolysis at a faster rate than PKC

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Abstract

Proteolytic cleavage of protein kinase C (PKC) under cell-free conditions generates a co-factor independent, free catalytic subunit (PKM). However, the difficulty in visualizing PKM in intact cells has generated controversy regarding its physiological relevance. In the present study, treatment of SH-SY-5Y cells with 2-O-tetradecanoylphorbol 13-acetate resulted in complete down-regulation of PKC within 24 h without detection of PKM. By contrast, low levels of PKM were transiently detected following ionophore-mediated calcium influx under conditions which induced no detectable PKC loss. PKM was not detected during rapid cell-free degradation of partially purified SH-SY-5Y PKCα by purified human brain mM calpain. However, when the kinetics of PKC degradation were slowed by lowering levels of calpain, PKM was transiently detected. PKM was also only transiently observed following calpain-mediated degradation of purified rat brain PKCα. Densitometric analyses indicated that, once formed, PKM was degraded approximately 10 times faster than PKC. These data provide an explanation as to why PKM is difficult to observe in situ, and indicate that PKM should not be considered as an 'unregulated' kinase, since its persistence is apparently strictly regulated by proteolysis.

Key words: Protein kinase C; Protein kinase M; Calpain; Phosphorylation; Proteolysis; Signal transduction

1. Introduction

The Ca^{2+}, phospholipid-dependent kinase, protein kinase C (PKC), is a family of related enzymes that mediate transmembrane signaling (for review, see [1]). The different PKC isoforms display differential spatial and temporal localization in neurons [2–5], demonstrate distinct substrate requirements [6] and mediate different functions (e.g. [7–11]. PKC is reversibly activated by 1,2-diacylglycerol (DAG), which is generated by receptor-mediated inositol phospholipid hydrolysis by phospholipase C. DAG stabilizes a complex consisting of PKC and membrane phospholipid. Inositol 1,4,5-trisphosphate, the other product of the phosphoinositide hydrolysis, mobilizes Ca^{2+} from intracellular stores, which associates with the DAG–membrane–PKC complex. In the presence of DAG, the Ca^{2+}- requirement for PKC activation is lowered, resulting in a synergistic effect between these two second messenger molecules. Phosphorylation of a distinct set of proteins then ensues (for reviews of these processes, see [12,13]).

Mobilization of intracellular Ca^{2+} also activates the calcium activated protease, calpain, which translocates to the plasma membrane. Calpain then irreversibly activates PKC by cleavage of the catalytic and regulatory subunits, generating a soluble, Ca^{2+}- and phospholipid-independent form, termed PKM, which migrates on SDS-gels in various studies from 46–50 kDa [12,14–20]. The respective roles of these two methods of PKC activation remain unclear. Some (e.g. [18,19]), but not all (e.g. [21]) PKC-mediated processes are blocked by protease inhibitors such as leupeptin, suggesting that proteolysis may be required for certain PKC functions (for review, see [12]). Since the regulatory subunit, rather than the catalytic subunit, contains the lipid-sensitive calcium-binding domain [22], PKM, unlike the membrane-bound, calcium-dependent native enzyme, has potential access to, and can phosphorylate a distinct set of non-membrane-associated protein substrates [19]. Generation of inappropriate PKM levels as a consequence of hyperactivation of calpain has been considered as potentially contributing to certain neurodegenerative conditions (e.g. see [23,24]).

Exposure of most cell systems to the PKC activator 12-O-tetradecanoylphorbol 13-acetate (TPA), a DAG analog that activates PKC by mimicking DAG-induced stabilization of PKC at the plasma membrane, causes

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translocation of the entire PKC content of the cell to the membrane and ultimate down-regulation of the enzyme. Proteolysis has been shown to be an essential factor in this down-regulation [18,25]. However, PKM is not observed under these conditions. An exception is when cells are exposed to calcium ionophore, Ca^{2+} and TPA, which causes the rapid conversion of nearly all PKC to PKM [18–20]. The contrast between the routine observation of PKM under cell-free conditions with the difficulty of PKM detection within intact cells has generated controversy regarding the physiological relevance of PKM. An additional possibility is that PKM may itself be subject to additional proteolytic events, and therefore does not accumulate under physiological conditions (e.g. see [12]). We have attempted to resolve this controversy using SH-SY5Y human neuroblastoma cells, which we have previously shown to express both μM and mM calcium-requiring calpain isoforms and the α, β, δ, e and γ isoforms of PKC [3,8,26–28]. In this paper we provide evidence that PKM derived from PKCα is degraded by mM calpain, and furthermore that, once formed, this free catalytic subunit is degraded at a rate faster than that of the native PKCα.

2. Materials and methods

2.1. Culture conditions and treatment with calpain and kinase inhibitors and activators

SH-SY-5Y cells (originally obtained from the stocks of Dr. June L. Biedler, Memorial Sloan-Kettering Cancer Center, NY) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 10% CO_2. Twenty-four hours later, the medium was replaced with medium containing FCS with either no further additions or TPA (1 μM) for various times from 5 min to 24 h. Influx of calcium into intact cells was achieved by addition of 3 μM calcium ionophore A23187 directly into cultures in DMEM, which contains 1.8 mM $CaCl_2$ [29]. Culture media, FCS, TPA and ionophore were purchased from Sigma Chemical Co. (St. Louis, MO). Cells were dislodged by scraping with a rubber policeman, homogenized and centrifuged to yield particulate and supernatant fractions as previously described [27].

2.2. Partial purification of mM calpain and PKC

mM calpain was purified 950-fold from post-mortem human brain by sequential chromatography on DE-52 cellulose, phenyl Sepharose and Ultrogel-AcA-A4 as previously described [30]. PKC was enriched from the supernatant fraction (above) generated from untreated SH-SY-5Y cells by DE-52 cellulose chromatography as previously described [3].

2.3. Incubation of purified PKC and cellular fractions with mM calpain

Aliquots (containing 100 μg of total protein) of PKC-containing DEAE column fractions from SH-SY-5Y cells or purified PKCα (1 μg; UBI Inc., Lake Placid, NY) were diluted in 50 mM Tris-HCl (pH 7.4) containing 2 mM PMSF and either immediately placed on ice (0 time) or were incubated at 30°C with and without various combinations of 5 mM EDTA, 2 mM $CaCl_2$, and 21.2–85 ng of purified calpain for various time intervals as indicated, after which samples were immediately placed on ice and received Laemlli treatment buffer for subsequent electrophoresis or were immediately assayed for PKM activity as described below.

2.4. Gel electrophoresis and immunoblot analysis

Samples were electrophoresed on SDS 7% polyacrylamide gels and transferred to nitrocellulose in a Hoefer Transphor apparatus as described [31]. 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 1:100 dilution of a commercial monoclonal antibody that specifically recognizes the catalytic domain of PKCα (UBI) followed by reaction with alkaline phosphatase-conjugated goat anti-mouse antibody and visualization as described [31]. This antibody therefore permitted the simultaneous visualization of PKC and PKM. In the present study, we have utilized a monoclonal antibody that specifically recognizes the catalytic domain of PKCα; accordingly, results presented herein are only for that isoform. Since evidence is accumulating that the various PKC isozymes display unique neuronal localizations and sensitivities and mediate distinct functions (see refs. in section 1), it should be considered that the other PKC isoforms [17], and any respective PKM derived from them, may display distinct degradation profiles [32,33]. Due to the difficulty in visualizing PKM in intact cells and fractions, alkaline phosphatase was routinely utilized as chromogen, since it afforded the maximal PKM visualization.

2.5. 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, and resulting digitized immunoblot profiles were compared with the Scan Analysis densitometric program (BioSoft, Ferguson, MO) and values were exported into Statworks and Cricket Graph software as described [34]. The value 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. The relative rates of decrease in purified PKC and PKM immunoreactivity between 2 and 10 min was obtained by subtraction of each data set (i.e. (relative densitometric value at 2 min) − (densitometric value at 10 min)) from individual densitometric analyses, and the mean ± S.E.M. was then determined for the grouped differences; this procedure was carried out separately for PKC and PKM. Representative nitrocellulose replicas are presented, and accompanying graphs present values obtained from the respective nitrocellulose replica; all experiments were carried out at least twice with identical profiles. 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 not known 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.

2.6. PKC activity assay

PKC activity in supernatant fractions derived from untreated and ionophore-treated SH-SY-5Y cells, and in purified PKC samples following incubation with calpain was assayed using a commercially-available PKC assay system (Gibco, New York) based on incorporation of [γ-^{32}P]ATP (DuPont-NEN, Boston, MA) into a synthetic peptide corresponding to a fragment of myelin basic protein ($MBP_{4\text{-}14}$) in the absence of lipids, $CaCl_2$ and diolein according to the manufacturer's instructions (e.g. see [35]). In some SH-SY-5Y supernatant fractions, 5 mM EDTA was added in these assays to chelate any free $CaCl_2$.

3. Results

Treatment of intact SH-SY-5Y cells with TPA results in the rapid translocation of PKC to the plasma membrane, followed by complete down-regulation within 24 h (Fig. 1). PKM immunoreactivity was not detected during this translocation and down-regulation (Fig. 1), nor was it transiently detected at earlier time points (e.g. 1–30 min) during TPA treatment (not shown). By contrast,

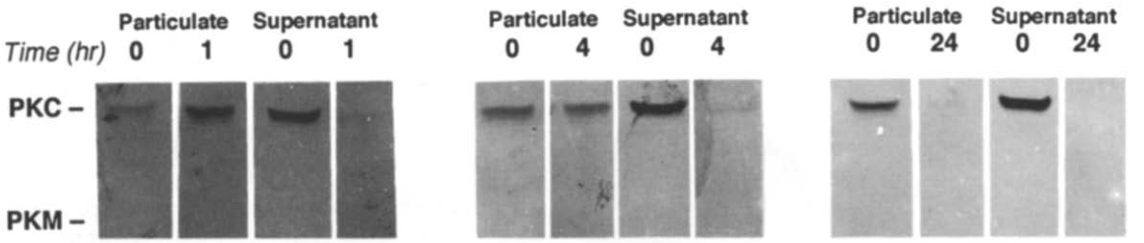

Fig. 1. Nitrocellulose replica of SH-SY-5Y cellular fractions immunostained with an anti-PKC monoclonal antibody that recognizes the catalytic domain of PKCα. Cells were exposed to 1 μM TPA in culture medium and incubated for 0, 1, 4 and 24 h as indicated, after which cells were homogenized and subjected to centrifugation to yield particulate and supernatant fractions as described in section 2. The migratory positions of PKC (80 kDa) and PKM (46 kDa) are indicated. Note the translocation of virtually all of the PKC content of the cell to the particulate fraction within 1 h of TPA treatment, and the subsequent down-regulation of PKC over 24 h. Note also the lack of PKM immunoreactivity before and during this treatment.

however, ionophore-mediated calcium influx into intact cells did generate detectable PKM, but only in minor amounts (Fig. 2). PKM immunoreactivity was first detected in particulate fractions after 5 min, followed by a rapid decrease and loss from this fraction within 30 min. This decrease coincided with the transient appearance of PKM at 15 min within the supernatant fraction. PKM immunoreactivity also decreased within the supernatant compartment by 30 min. No significant translocation of PKC to the plasma membrane, nor any overall decrease in PKC immunoreactivity, was apparent during this 30 min incubation with ionophore; densitometric analysis (not shown) confirmed the lack of decrease PKC immunoreactivity. It should be noted that the nitrocellulose replica presented in Fig. 2 represents a favorable image, and in many instances, significantly less PKM was observed. Low but significant levels of co-factor independent phosphorylation of the specific PKC substrate MBP_{4-14} were observed when this fragment was incubated in cell-free assays with SH-SY-5Y supernatant fractions, providing additional evidence for at least some PKM generation within SH-SY-5Y cells. In the absence of added PKC co-factors or activators (phosphatidyl serine, DAG, TPA) and in the presence of 5 mM EDTA, 1277.3 ± 157.0 cpm were incorporated into filter-precipitable material within 5 min in supernatants to which MBP_{4-14} was added versus a 'background' of 876.8 ± 65.1 cpm incorporated into phosphocellulose-bound material in supernatants not receiving MBP_{4-14}; Since levels of phosphorylation of supernatants from cells not treated with ionophore were defined as baseline for each condition, this difference (approx. 400 cpm) therefore represents PKM-mediated phosphorylation of MBP_{4-14}.

The transient appearance of PKM following ionophore-mediated calcium influx into intact cells and the only a modest increase in MBP_{4-14} phosphorylation suggested that PKM may also be subjected to rapid proteolytic degradation within intact cells. To examine this possibility, PKC enriched from the soluble fraction of SH-SY5Y cells was exposed to purified human brain mM calpain, which rapidly degraded nearly all of the PKC. By contrast, no significant loss of PKC immunoreactivity, nor any increase in PKM immunoreactivity, was observed when these fractions were incubated for as long as 24 h in the absence of calpain and only

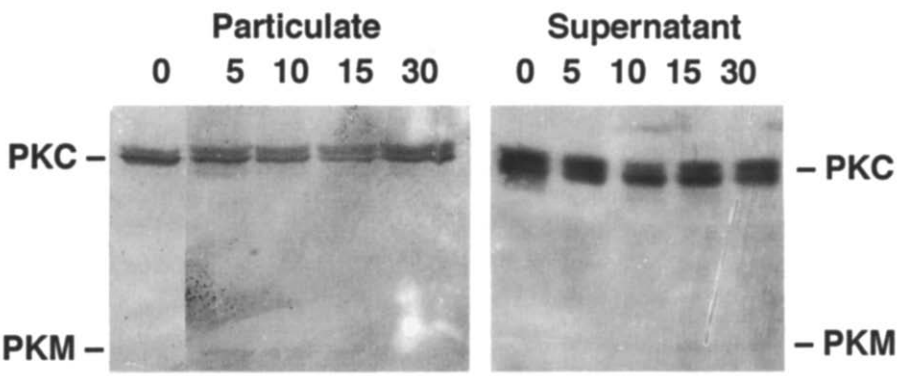

Fig. 2. Nitrocellulose replica of SH-SY-5Y cellular fractions immunostained with an anti-PKC monoclonal antibody that recognizes the catalytic domain of PKCα. Cells were exposed to 3 μM calcium ionophore A23187 in culture medium and incubated for 0–30 min as indicated, after which cells were homogenized and subjected to centrifugation to yield particulate and supernatant fractions as described in section 2. The migratory positions of PKC (80 kDa) and PKM (46 kDa) are indicated. Note the appearance of PKM immunoreactivity in particulate fractions after 5 min of incubation, the subsequent decrease and loss from this fraction within 30 min, the transient appearance of PKM at 15 min within the supernatant, and the loss of PKM from the supernatant was evident by 30 min. Note also the lack of significant translocation of PKC to the plasma membrane, nor any overall decrease in PKC immunoreactivity.

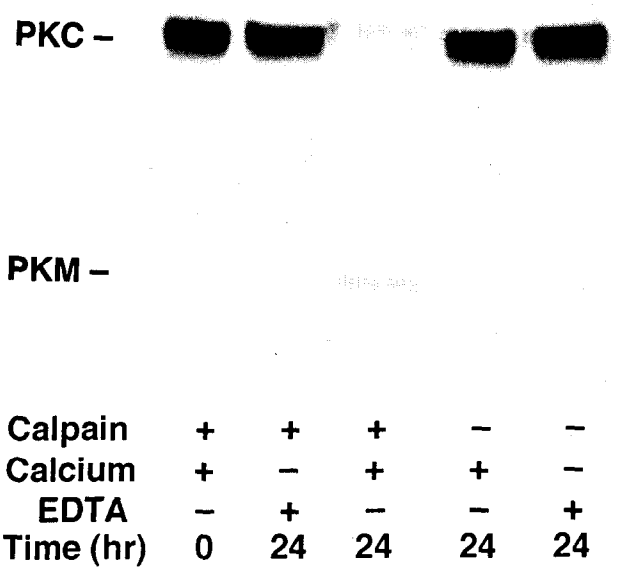

Fig. 3. Nitrocellulose replica of PKC-enriched DEAE column fractions obtained from supernatant fractions of untreated SH-SY-5Y cells and incubated with purified mM calpain for 0 or 24 h as described in section 2. Replicas were immunostained with an anti-PKC monoclonal antibody that recognizes the catalytic domain of PKCα. The migratory positions of PKC (80 kDa) and PKM (46 kDa) are indicated. Note the loss of PKC immunoreactivity only following incubation with calpain and $CaCl_2$.

slight loss was observed after 24 h in the presence of calpain but in the absence of $CaCl_2$ (Fig. 3, see also [28]). These data demonstrated the absence of any protease activity directed against PKC in these fractions, and further confirmed the ability of human brain calpain to degrade PKC isolated from SH-SY-5Y cells. Despite proteolysis of nearly all of the PKC, the lack of significant accumulation of PKM immunoreactivity (Fig. 3) suggested that PKM was also undergoing proteolysis. To examine this phenomenon more closely, we altered the kinetics of PKC degradation by exposing a constant amount of PKC-enriched column fractions from SH-SY-5Y cells to varying amounts of mM calpain for 0–15 min and assayed for the presence of PKC and PKM by immunoblot analysis under otherwise identical incubation conditions (Fig. 4). Incubation of PKC-enriched fractions with 85 ng of mM calpain resulted in the degradation of approximately 75% of the PKC within 15 min; PKM was not detected at any time under these conditions. Decreasing the calpain concentration in the incubation to 65 ng substantially lessened the extent of PKC degradation; only 25% of the PKC immunoreactivity was lost by 15 min. However, transient low levels of PKM immunoreactivity were now detected, with a peak at 5 min of incubation. Decreasing the calpain to 42.5 ng resulted in loss of only approximately 10% of the PKC immunoreactivity, yet yielded relatively more PKM than did 65 ng of calpain, although PKM was still transient and was lost by 15 min. Finally, PKM was not observed, and no PKC immunoreactivity was lost, when calpain was decreased to 21.2 ng. Increased but transient observation of PKM following the progressive reduction in calpain activity suggests that, once formed, PKM may represent a preferred substrate for mM calpain than intact PKC.

These findings in intact cells and cellular fractions were substantiated by similar analyses with purified PKCα. As with cellular fractions (above), no loss of purified PKCα, nor increase in PKM, was observed during a 60 min incubation in the absence of calpain (not shown). However, PKCα was rapidly degraded following incubation with 65 ng mM calpain in the presence of $CaCl_2$ (Fig. 5); densitometric analysis revealed an approximate 80% decrease in PKC immunoreactivity within 2 min of incubation. This loss of PKC was not accompanied by PKM accumulation; rather, it was accompanied by the transient appearance of PKM immunoreactivity and activity, as demonstrated by immunoreactivity at 46 kDa and the corresponding transient appearance of co-factor independent phosphorylation of MBP_{4-14}, respectively (Fig. 5). Densitometric analyses of PKM immunoreactivity revealed a time course similar to that of PKM-mediated MBP_{4-14} phosphorylation. These data confirm that human brain mM calpain can generate PKM from PKC by limited proteolysis, and that PKM is also subsequently degraded by mM calpain. The failure of PKM to have accumulated at larger levels following 2 min of incubation with calpain, during which approximately 80% of the PKC immunoreactivity was lost, coupled with the subsequent transient observation of PKM further suggested that, once formed, PKM is degraded at least as fast, if not faster, than PKC; shorter incubation times (e.g. 30 s–1 min) did not reveal a transient higher level of PKM (not shown). Therefore, in order to examine further the relative degradation rates of PKC and PKM, we separately compared the difference in densitometric values obtained for PKC and PKM at 2 min (i.e., the observed peak of PKM immunoreactivity and activity) and 10 min (the last point examined in these an analyses) of incubation in 6 separate scans. PKM immunoreactivity between 2 and 10 min decreased by 9.6 ± 1.3% (mean ± S.E.M.), yet PKC immunoreactivity did not decrease further during this time (+0.4 ± 1.2); it should be noted that the decrease in PKM represents a conservative estimate, since at least some PKM may theoretically still be generated by PKC degradation during the 10 min incubation. These comparisons of densitometric data of purified proteins indeed suggest that PKM is degraded by mM calpain at a faster rate than PKC.

4. Discussion

While numerous studies in cell-free systems have demonstrated that limited proteolysis of PKC generates a

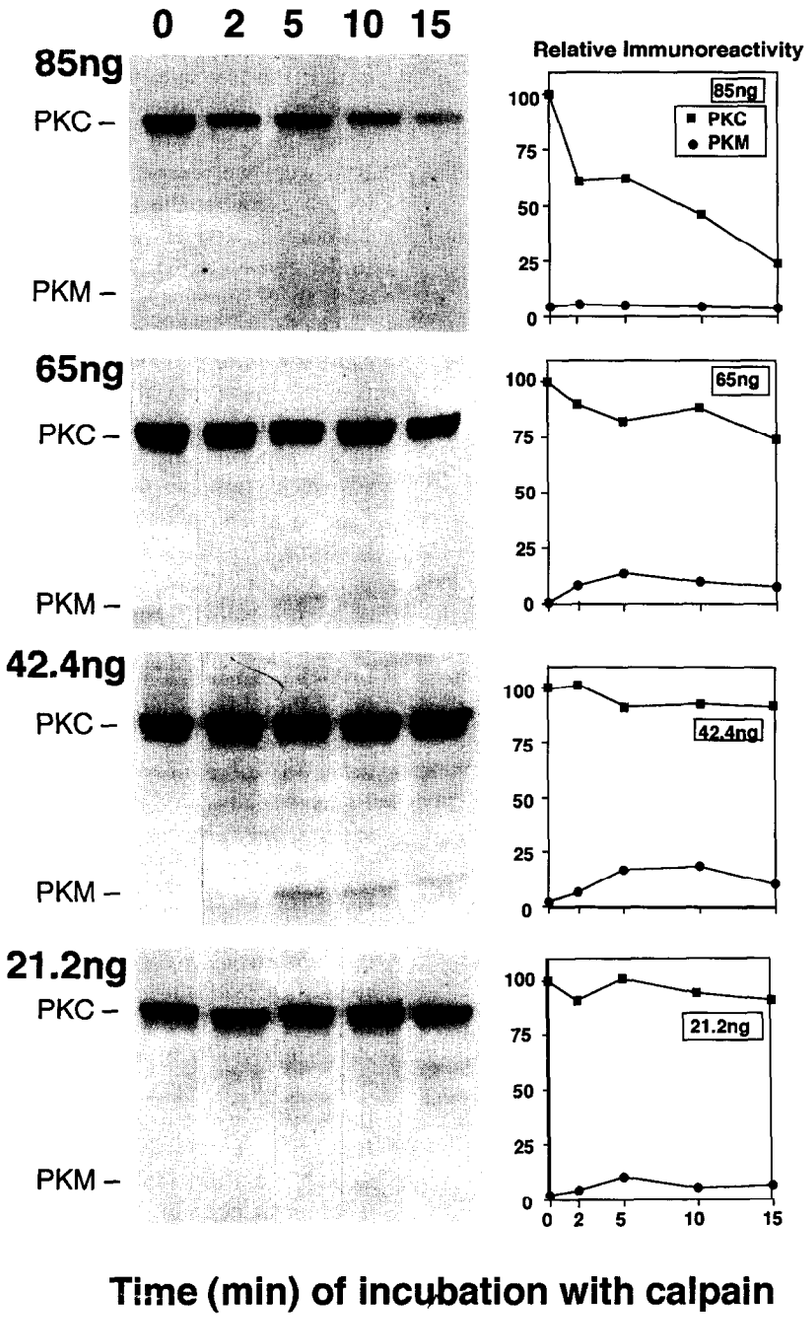

Fig. 4. Nitrocellulose replica of PKC-enriched DEAE column fractions obtained from supernatant fractions of untreated SH-SY-5Y cells and incubated with various concentrations of purified mM calpain as described in section 2. Replicas were immunostained with an anti-PKC monoclonal antibody that recognizes the catalytic domain of PKCα. The migratory positions of PKC (80 kDa) and PKM (46 kDa) are indicated. The graph accompanying each replica presents corresponding numerical values obtained by densitometric analysis of the respective replica. Note the following: the loss of approximately 75% of the PKC immunoreactivity within 15 min, and the lack of PKM immunoreactivity, following incubation with 85 ng calpain; the loss of approximately 25% of the PKC immunoreactivity and the transient appearance of PKM immunoreactivity following incubation with 65 ng calpain; the loss of approximately 10% of the PKC immunoreactivity and a transient appearance of PKM immunoreactivity following incubation with 42.5 ng calpain; and finally, the lack of PKC loss of PKC immunoreactivity, and lack of appearance of PKM immunoreactivity, following incubation with 21.2 ng calpain. Note also the apparent increase in the amount of PKM transiently obtained following incubation of cellular fractions with 42.5 ng calpain versus that obtained with 65 ng calpain.

co-factor-independent, free catalytic subunit ('PKM'), the difficulty to demonstrate the presence of PKM in intact cells has generated controversy concerning the physiological relevance of this process [12]. 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, since PKM can theoretically act on additional classes of substrates that are

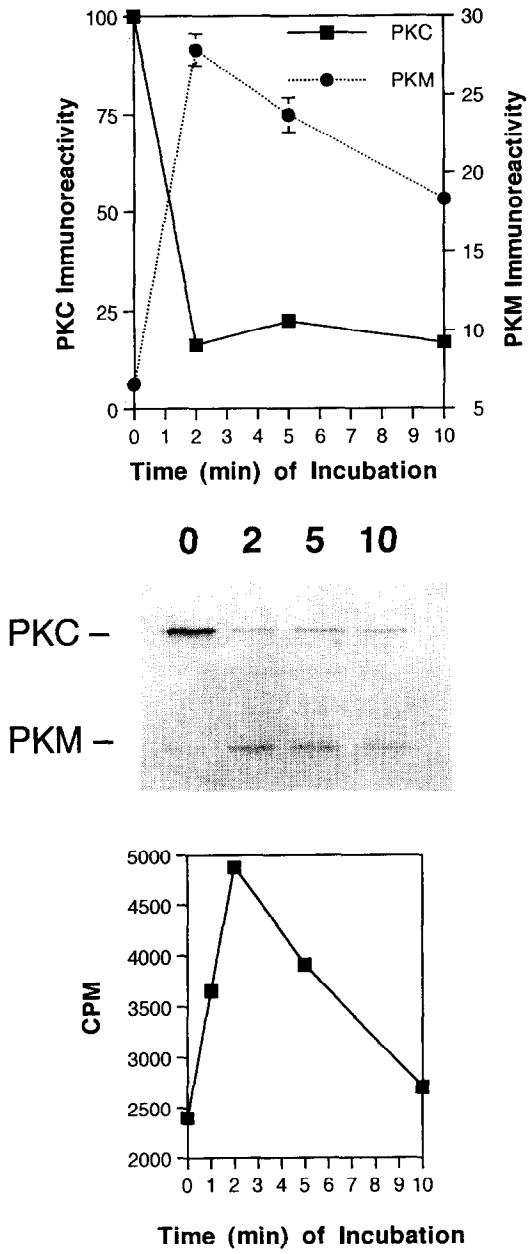

Fig. 5. Analysis of purified PKCα incubated for 0–10 min with purified mM calpain as described in section 2. Nitrocellulose replicas were immunostained with an anti-PKC monoclonal antibody that recognizes the catalytic domain of PKCα. A representative replica, with the migratory positions of PKC (80 kDa) and PKM (46 kDa) indicated, is presented in the center panel. The top panel presents densitometric analysis of immunoblots. Values are presented as the mean ± S.E.M. for 6 separate scans; data points in which error bars are not detected indicates that the S.E.M. is contained within the respective graph point. The bottom panel presents co-factor-independent (i.e. PKM-mediated) incorporation of [γ-^{32}P]ATP into a synthetic MBP peptide fragment obtained following incubation of this fragment with the above calpain-treated PKC samples as described in section 2.

inaccessible to its membrane-associated, co-factor-dependent parent enzyme [19]. The findings of the present study indicate that PKM is indeed formed in intact neuronal cells as a consequence of limited mM calpain-mediated proteolysis of PKC. Our findings further indicate that a major factor in the difficulty of visualizing PKM is that the free catalytic subunit is degraded at a faster rate than the native enzyme; indeed, only by slowing down the kinetics of mM calpain-mediated PKC proteolysis were we reproducibly able to visualize PKM even in cell extracts; the transient observation of PKM immunoreactivity in neutrophils, and furthermore only under certain experimental conditions [20], is consistent with these findings.

The findings of the present study substantiate the transient formation of PKM in neuronal cells; the observation of PKM initially within the particulate fraction, followed by its loss from that fraction and appearance in the supernatant fraction indicates, as previously suggested by others (for review, see [12]) that PKM is formed at the plasma membrane and, since it lacks a lipid-binding domain, migrates into the cytosol. Our visualization of PKM was likely to be facilitated by the use of a monoclonal antibody that recognizes an epitope within the catalytic subunit; a similar experimental approach, i.e. using a monoclonal antibody that recognizes both PKC and PKM also demonstrated the transient appearance of PKM in human neutrophils [20]. These data do not confirm the physiological significance of PKM, which would require the demonstration 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 in the present study, nor following TPA treatment of neutrophils [20]. While it has been shown that proteolysis of PKC is a necessary component of TPA-mediated PKC down-regulation [18,25], it remains to be determined whether or not significant levels of PKM are transiently generated during this process. Finally, it is yet unclear whether or not μM calpain, mM calpain or both are responsible for in situ proteolysis of PKC, although both calpain isoforms readily degrade PKC and PKM in cell-free studies (Shea et al., unpublished).

An important implication of the findings of the present study is that PKM, although co-factor-independent and not restricted to the plasma membrane, should not be considered to be an 'unregulated' kinase; in fact, our data showing that PKM is degraded faster than PKC demonstrate that PKM is actually under tight regulation by calpain-mediated proteolysis. The same reasoning holds in other cell systems where PKM may not be degraded faster than, but rather a rate similar to that of, PKC. Accordingly, rapid elimination of PKM by calpain and/or additional proteases would normally circumvent excessive phosphorylation of potentially inappropriate

substrates. An additional interpretation of the present findings is that disregulations in proteolytic activities that may accompany certain pathophysiological conditions may give rise to the persistence of excessive PKM [23,24]. Related studies in SH-SY-5Y cells indicate that ionophore-mediated calcium influx across the plasma membrane selectively activates membrane-associated, but not cytosolic, μM calpain [28]. Accordingly, calcium influx and consequent hyperactivation of plasma membrane-associated calpain at the plasma membrane could rapidly generate PKM in amounts exceeding that which membrane-associated calpain could degrade further. 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 excessive PKM generation or persistence in the pathogenesis of certain conditions remains an important area of investigation.

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