The Role of the Calpain-Calpastatin System in Thyrotropin-releasing Hormone-induced Selective Down-regulation of a Protein Kinase C Isozyme, nPKC, in Rat Pituitary GH4C1 Cells*

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We have examined the mechanism for the selective down-regulation of protein kinase C ε (nPKCε) in rat pituitary GH4C1 cells responding to thyrotropin-releasing hormone (TRH) stimulation. Among various low molecular weight protease inhibitors examined, only a cysteine protease inhibitor (calpain inhibitor I, N-acetyl-Leu-Leu-leucinal) blocked the down-regulation of nPKCε. Furthermore, the introduction of a synthetic calpain-potentitve, an exclusively specific inhibitor of calpain, into the cells also reduced the down-regulation, suggesting the involvement of calpain among all the intracellular cysteine proteases in this process. In accordance, we observed TRH-induced translocation of m-calpain from the cytosol to the membrane and the concomitant up-regulation of calpastatin isoforms; presumably, the former represents activation of the protease initiating the kinase degradation, while the latter constitutes a negative feedback system protecting the cells from activated calpain. These results suggest that in GH4C1 cells, TRH mobilizes both protease (m-calpain) and inhibitor (calpastatin) as a strictly regulating system for the nPKCε pathway mediating TRH signals.

Protein kinase C (PKC) isozymes play pivotal roles as major serinethreonine kinases in signal transduction cascades involved in agonist-induced responses of various cells (1). The isozymes can be categorized into three groups: conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC), based on their structural and enzymatic properties (2). Although their distinct tissue distribution has suggested different functional roles for each isozyme (2, 3), their isozyme-specific functions in physiological processes have not yet been fully resolved (4). We have focused our attention on the cellular function of PKC isozymes in the responses elicited by thyrotropin-releasing hormone (TRH), a hypothalamic hormone, in rat pituitary GH4C1 cells and demonstrated that nPKCε provides a major rate-limiting step in the secretion of prolactin (PRL) in response to agonist stimulation (5–8).

GH4C1 cells possess at least six PKC isozymes, i.e. α, βI, cPKC, δ, ε, and γ nPKC, and aPKC γ (5, 8). The suppression of their activities by PKC inhibitors results in a reduction in PRL secretion evoked by TRH (6), indicating a mediator role in stimulus-response coupling. In accordance, TRH induces the translocation of all isozymes except ε from the cytosol to membranes (8). However, only the ε isozyme undergoes subsequent down-regulation (5, 7, 8), indicating that this isozyme is the most extensively involved. Consistently, the introduction of an expression vector carrying nPKCε cDNA into the cells induces an increase in PRL secretion, whereas increasing the amount of other isozymes, α, βI, and δ, has no effect (8). These data suggest that nPKCε among all the PKC isozymes present in GH4C1 cells plays a central role in the secretory process and that down-regulation following translocation is a hallmark for the thorough activation of the enzyme.

Intracellular down-regulation of PKC following activation in general seems to involve facilitated proteolysis rather than transcriptional suppression (9). The protease(s) responsible for this process, however, have not yet been identified, although previous studies on phorbol ester-evoked cPKC down-regulation implies the involvement of calpain (10, 11), serine proteases (12, 13), and/or possibly other proteases (14). Consequently, the physiological significance of this down-regulation remains uncertain. Possibly, the proteolysis of PKC may produce a signal affecting the cellular situation. For instance, an active kinase fragment may be produced from PKC by limited proteolysis (15) as an intermediate product in down-regulation, depending on the protease involved. This kinase that acts independently of cofactors, including phospholipids, would phosphorylate substrates that may not be accessible to intact PKC. Alternatively, a loss of a specific PKC activity may lead to an alteration in cellular functions. Due to our limited knowledge about the mechanism of down-regulation, these possibilities remain obscure.

In the present study, we aimed to elucidate the mechanism of selective nPKCε down-regulation in GH4C1 cells by identifying the responsible protease as an initial step in addressing these questions in this particular system.

EXPERIMENTAL PROCEDURES

Materials—Leupeptin and casein were purchased from Microbial Research Institute and Merck, respectively. TRH was from Sigma. E-64d was generously provided by Dr. K. Hanada (Taisho Pharmaceutical). Other reagents including protease inhibitors were purchased from Wako Pure Chemicals, Nacalai Tesque, or Sigma. Antipeptidic antibodies to the carboxyl-terminal sequence of nPKCε (8), to the amino-terminal sequence (22-mer) of m-calpain (16), and to the amino-terminal sequence (18-mer) of m-calpain (17) were previously described.
An anti-calpastatin antibody was raised against a synthetic 16-mer peptide, CTIELDLISWCLFSLV, conjugated to keyhole limpet hemocyanin (Calibiochem) as described previously (16). This sequence corresponds to the carboxyl-terminal portion of rat calpastatin (18). A 27-mer synthetic calpastatin peptide (CS peptide, acetyl-DPMSTSTYIEEL-KGREVFPKRELLA-NH2) corresponding to the minimum inhibitory isozyme antibodies. The localization of calpastatin was examined in a similar manner except that “homogenization buffer” was employed in place of “lysis buffer.” The cytosol and membrane pellets were collected as described previously (16). An anti-calpastatin antibody was raised against a synthetic 16-mer peptide, CTIELDLISWLCFSVL, conjugated to keyhole limpet hemocyanin (Calibiochem) as described previously (16). A 27-mer synthetic calpastatin peptide (CS peptide, acetyl-DPMSTSTYIEEL-KGREVFPKRELLA-NH2) corresponding to the minimum inhibitory concentrations of inhibitor giving 50% inhibition was about 5 

### RESULTS

**Effect of Various Protease Inhibitors on the TRH-induced Down-regulation of nPKCe—Upon stimulation of GH4C1 cells with TRH, the amount of nPKCe decreased indicating down-regulation (Fig. 1, lanes 1 and 2), as previously reported (5, 7). To identify the class of protease(s) involved in this catabolic process, we examined the effects of various protease inhibitors.**

**Inhibition of Down-regulation by Calpastatin Peptide—** Besides the inhibitory effect of ALLNal, originally developed as a cell-permeable cysteine protease inhibitor (24), blocked the down-regulation of nPKCe (lane 9). Phosphoramidon, pepstatin, Nα-tosyl-L-lysyl chloromethyl ketone, N-tosyl-L-phenylalanyl chloromethyl ketone, leupeptin, and E-64 had no effect. O-Phenanthroline seemed to be toxic to cells at the concentration used, causing cell damage and resulting in the apparent promotion of down-regulation. These results indicate that a cysteine protease, not a metallo, serine, or aspartic protease, is involved in the down-regulation of nPKCe. Lysosomal cysteine proteases do not seem to be involved since leupeptin had no effect (25). We do not know why E-64, another cell-permeable cysteine protease inhibitor, did not inhibit the down-regulation. We presume that it may have been quickly degraded extra- or intracellularly and failed to reach sufficient concentrations to be inhibitory in the cytosol. This is because E-64 needs to enter cells in the ester form and is then hydrolyzed by an intracellular esterase to yield E-64 (26).

The effect of ALLNal was dose dependent (Fig. 2A). The concentration of inhibitor giving 50% inhibition was about 5 μM, consistent with previous studies (27, 28). The possibility that ALLNal influences the translocation of nPKCe from the cytosol to membranes that occurs prior to down-regulation was excluded because the inhibitor had no effect on the amount of nPKCe in the membrane fraction of TRH-treated cells (Fig. 2B).

**FIG. 1. Effect of various protease inhibitors on the TRH-induced down-regulation of nPKCe in GH4C1 cells.** Cells cultured in serum-free medium were preincubated for 1 h with vehicle (lanes 1 and 2), 100 μM phenanthrolone (lane 3), 10 μM phosphoramidon (lane 4), 100 μM pepstatin (lanes 5), 10 μM Nα-tosyl-L-lysyl chloromethyl ketone (TLC) (lane 6), 10 μM Nα-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) (lane 7), 100 μM leupeptin (lane 8), 10 μM ALLNal (lane 9), or 100 μM E-64 (lane 10), followed by stimulation with (+) or without (−) 300 nM TRH for 6 h in the presence of the inhibitors. The cell extracts were analyzed by Western blot analysis using anti-nPKCe antibody. The arrow indicates the relative molecular weight of nPKCe, 90,000, as confirmed using recombinant nPKCe expressed in GH4C1 cells (8). The lower panel shows the densitometric quantification of the Western blot results.
specific for calpain (24, 31). We therefore employed a more specific method to inhibit intracellular calpain activity using a synthetic CS peptide corresponding to the inhibitory segment of calpastatin (Fig. 3). A scramble peptide with an identical amino acid composition and a random sequence was employed as a negative control.

We first examined the ability of the CS peptide to inhibit purified calpain activity and to permeate into cells. In test tubes, the CS peptide inhibited the proteolytic activity of purified calpain with high affinity; 50% inhibition was achieved at a peptide concentration of 20 nM (panel A). In contrast, approximately 10^3-fold more scramble peptide was necessary to suppress calpain activity. The permeability of the synthetic peptide into cells was confirmed using a rhodamine-labeled peptide (panel B); fluorescence was observed only in cells incubated with the peptide. Although this observation does not quantify the actual concentration of CS peptide inside GH_4C_1 cells, it indicates that at least a fraction of the extracellularly administered CS peptide entered the cells and remained in cytoplasm.

Confirming the potent inhibitory effect of the CS peptide on calpain and its cell permeability, we examined its effect on intact cells (panel C). The CS peptide significantly reduced the down-regulation of nPKCε, whereas the control scramble peptide had no effect. Because no proteases other than calpain have been shown to be inhibited by calpastatin or calpastatin peptide to our knowledge (32), these data suggest that it is indeed calpain, among various intracellular cysteine proteases, that is involved in the proteolytic process.

Calpain and Calpastatin Activities in GH_4C_1 Cells—Although the experiments using protease inhibitors indicated the involvement of calpain in the down-regulation of nPKCε in GH_4C_1 cells, previous studies have identified neither calpain nor calpastatin in either enzymatic or immunochemical terms in these cells. We therefore analyzed the calpain and calpastatin activities of GH_4C_1 cells by anion exchange HPLC (Fig. 4) since cellular calpain activities are measurable only after sep-
The proteolytic activities eluted at the NaCl concentrations of 0.15 and 0.4 M were identified as \( \mu \)-calpain and m-calpain, respectively (panel A), as indicated by Western blot analysis using human isozyme-specific antibodies (panel C). GH4C1 cells seem to have comparable \( \mu \)- and m-calpain activities. These data, in turn, confirm that the antibodies employed are specific enough to distinguish between rat \( \mu \)- and m-calpain.

The activity to inhibit calpain (panel B) and the anti-calpastatin antibody immunoreactivity showing 100–110-kDa bands on Western blotting (panel C) eluted identically, i.e. at 0.25 M NaCl, confirming the specificity of the antibody. Notably, the calpastatin activity present in the cells exceeded the sum of the calpain activities, suggesting that the protease activity is regulated rather stringently in these cells as previously reported in KB cells (22).

**DISCUSSION**

In the present study, we have shown that calpain inhibitors with broad and narrow specificities, ALLNal and CS peptide, inhibit the TRH-induced down-regulation of nPKC\(e\) in GH4C1 cells and that TRH causes both the translocation of m-calpain and an increase in the calpastatin level. These observations indicate that the calpain-calpastatin system is a component in TRH-induced signal transduction and that calpain is the major

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**Fig. 4.** Calpain and calpastatin activities in GH4C1 cells. Panel A, calpain activity in cells. Cell extracts were fractionated by ion exchange HPLC, and the enzyme activity was assayed as described under “Experimental Procedures.” The dotted line indicates the NaCl concentration gradient used for elution. Panel B, calpastatin activity in the cells. The assay was performed as described under “Experimental Procedures.” Note the scale of activity is greater than in panel A. Panel C, Western blot analysis of HPLC fractions using anti-\( \mu \)-calpain, anti-m-calpain, and anti-calpastatin antibodies as indicated. The bars to the right indicate the positions of marker proteins (Bio-Rad) and their molecular weights (phosphorylase b, 106,000; bovine serum albumin, 80,000).

**Fig. 5.** Effect of TRH on the cellular localization of m- and \( \mu \)-calpains in GH4C1 cells. Panel A, translocation of m-calpain from the cytosol to membrane upon stimulation with TRH. Cells stimulated with TRH for the indicated periods were fractionated in Ca\(^{2+}\)/EGTA buffer (pCa of 7) and subjected to Western blot analysis using anti-m-calpain antibody as described under “Experimental Procedures.” Panel B, localization of \( \mu \)-calpain analyzed using anti-\( \mu \)-calpain antibody as in panel A. The arrowheads show the 80-kDa subunit of each isozyme. Panel C, the proportion of membrane-associated calpains. The Western blot results shown in panel A (m-calpain, closed circles) and B (\( \mu \)-calpain, open circles) were densitometrically quantified.
protease involved in down-regulation. This is the first demonstration of calpain involvement in PKC down-regulation associated with physiological stimulus-response coupling in contrast to previous studies employing phorbol ester or calcium ionophore to induce down-regulation (10, 11).

The present results, however, do not exclude the possible involvement of other intracellular proteases in down-regulation (12–14). Because the effect of the CS peptide was only partial (Fig. 3), a secondary protease other than calpain may participate in the proteolytic process; a candidate is proteasome, which was recently shown to be inhibited by ALL NaI (31, 35). The presence of a secondary protease is also implied by the fact that we do not observe an active nPKCe fragment corresponding to the catalytic fragment (Fig. 1) produced in vitro by calpain-catalyzed limited proteolysis (30). Apparently, degradation of the active fragment proceeds faster than its production, as shown previously in the case of cPKCa (36). Our observations also indicate that nPKCe, which is enzymatically independent of calcium (30), could be regulated by the calcium signaling cascade through the proteolytic action of calpain. Whether or not proteolysis of nPKCe bears any specific physiological signals in these cells remains to be elucidated.

The data showing the translocation of m-calpain, but not μ-calpain, from the cytosol to membranes in response to TRH treatment (Fig. 5) and suggesting the involvement of m-calpain in nPKCe down-regulation were rather unexpected because μ-calpain, which requires lower calcium concentrations for activation in vitro, has been considered as the more probable candidate for intracellular proteolytic phenomena (37). However, the present observation agrees with our previous reports, demonstrating that phorbol ester induces the synthesis and translocation of m-calpain in COS and K562 cells (38, 39). Possibly, the mobilization of m-calpain is controlled by the PKC pathway. It is therefore likely that in some cases, m-calpain is more involved in cellular stimulus-response coupling than μ-calpain. There may exist an unknown cellular factor associated with membranes that contributes to the specific activation of m-calpain.

A possible reason for the selective down-regulation of nPKCe among other PKC isozymes (see the Introduction) may lie in the difference in the time course of translocation to membrane. In contrast to cPKCa and βII, which are translocated transiently and are rapidly dissociated from the membrane within 1 min (data not shown), the majority of nPKCe remains associated with the membrane in a more sustained manner favoring colocalization with m-calpain. Furthermore, this membrane association indicates that PKC exists under activating conditions, in which the kinase is more susceptible to proteolytic attack (15, 40). Presumably, other PKC isozymes escape from calpain action due to their relatively short periods of association with membranes.

The up-regulation of calpastatin isoforms, particularly of the high molecular weight form in the membrane fraction, that is induced by TRH treatment (Fig. 6) seems to be part of the cellular negative feedback system countering activated calpain and protecting cellular components. Because the excessive and random degradation of proteins associated with membranes and the cytoskeleton would be highly toxic to cells and because the too rapid proteolysis of calpain substrates including nPKCe might interfere with cellular functions, the strict regulation of calpain activity by calpastatin should be vitally important. The three isoforms with different relative molecular weights probably represent different phosphorylation levels (41) or differential alternative splicing (42); each isoform may possess distinct properties affecting cellular localization and interaction with calpain. Each might provide specific protection to a group of proteins that would otherwise be proteolyzed by calpain. Both qualitative and quantitative changes in calpastatin expression may play important roles, as shown for differentiation and secretion in other cells (43–45). The precise role of calpastatin in PRL secretion from GH3 cells remains to be elucidated. In conclusion, we propose that physiologically stimulated nPKCe is strictly regulated at the cell membrane by the calpain-calpastatin system.

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