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POSSIBLE ROLE OF Ca $^{2+}$ -INDEPENDENT PROTEIN KINASE C ISOZYME, nPKC ε, IN THYROTROPIN- RELEASING HORMONE-STIMULATED SIGNAL TRANSDUCTION: DIFFERENTIAL DOWN-REGULATION OF nPKC ε IN GH_4C_1 CELLS *

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ABSTRACT: Protein kinase C (PKC) molecular species of GH_4C_1 cells were analyzed after separation by hydroxyapatite column chromatography. A novel Ca^{2+}-independent PKC, nPKC ε, was identified together with two conventional Ca^{2+}-dependent PKCs, PKC α and βII by analysis of kinase and phorbol ester-binding activities, immunoblotting using isozyme-specific antibodies, and Northern blotting. These PKCs are down-regulated differently when cells are stimulated by outer stimuli; phorbol esters deplete PKC βII and nPKC ε from the cells more rapidly than PKC α, whereas thyrotropin-releasing hormone (TRH) at 200 nM depletes nPKC ε exclusively with a time course similar to that induced by phorbol esters. However, translocation of PKC α and βII to the membranes is elicited by both TRH and phorbol esters. These results suggest that TRH and phorbol ester activate PKC α and βII differently but that nPKC ε is stimulated similarly by both stimuli. Thus, in GH_4C_1 cells, Ca^{2+}-independent nPKC ε may play a crucial role distinct from that mediated by Ca $^{2+}$-dependent PKC α and βII in a cellular response elicited by both TRH and phorbol esters.

Protein kinase C (PKC) is a major component of the transmembrane signaling system. Enzymological and molecular cloning studies have established the existence of two PKC subfamilies, conventional PKCs consisting of four closely related isozymes, PKC α, βI, βII, and γ, and structurally distinct novel PKCs, nPKC δ, ε, and ζ (1-6). Among the latter subfamily, only nPKC ε has been identified at the protein level in brain; the expression of the remainder has been confirmed only at the mRNA level. Types I, II, and III PKC fractions isolated from brains by hydroxyapatite column chromatography contain PKC γ, βI/II and α, respectively (6,7). The four conventional PKCs share similar enzymatic properties as well as phorbol ester-receptor activities (8-11). Recently, we reported that nPKC ε is activated in the same phospholipid-, diacylglycerol (DAG)- (or phorbol ester-) dependent manner as the four conventional PKCs, but that the cofactor dependencies and substrate specificites are clearly different (9-11). One of the most striking differences between nPKC ε and the four conventional PKCs is the Ca^{2+}-independence of membrane association, phorbol ester-binding, and enzyme activation of nPKC ε. This can be ascribed to the absence of the C2 region in nPKC ε (9,12). The presence of these PKC isozymes and their cell type-specific expression (13) strongly suggest that different isozymes have distinct functions within a given cell. However, it is not clear whether various isozymes respond differently to physiological stimuli, although PKC α and/or β has been suggested to participate in cellular responses mediated by phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and phorbol 12,13-dibutyrate (PDBu) (14-17). Unfortunately, nothing is known about the physiological function of nPKC ε.

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The hypothalamic tripeptide, thyrotropin-releasing hormone (TRH), elicits various responses including secretion of prolactin (PRL) and growth hormone (GH) in rat pituitary tumor GH cells such as the GH_4C_1 and GH_3 cell lines. Since phorbol esters mimic some of the actions of TRH in GH cells, it has been suggested that PKC activation is involved in TRH-induced signalling (18,19). Kinetic analysis of PDBu-binding activity in the cytosol fraction of GH_4C_1 cells has revealed the presence of two classes of binding proteins (11,20), one Ca^{2+}-dependent and the other Ca^{2+}-independent. Moreover, some Ca^{2+}-independent cellular responses to phorbol esters and TRH seem to exist in addition to Ca^{2+}-dependent ones (18,21,22). These observations suggest that multiple PKCs, including Ca^{2+}-independent PKCs such as nPKC ε, mediate multiple responses to phorbol esters and TRH in GH cells.

Here, we report that GH_4C_1 cells contain Ca^{2+}-independent nPKC ε in addition to two conventional PKCs, PKC α and βII, and that both TRH and phorbol esters specifically down-regulate nPKC ε, while PKC α and βII are down-regulated only by phorbol esters and not by TRH.

MATERIALS AND METHODS

GH_4C_1 cells were cultured as described (23). Partial purification of PKC isozymes, using the cytosol from 5 x 10^8 cells, was carried out by chromatography first on DEAE-Sephacel (Pharmacia) and then on a hydroxyapatite (HAP) column (KOKEN, Tokyo) as previously described (10).

PDBu-binding activity was assayed at 0℃ for 16 h in the presence of 50 nM [^{3}H]PDBu (Du Pont-New England Nuclear) and 0.15 mg/ml phosphatidylserine (PS, Serdary Research Laboratories) (9,11). Kinase activity was measured by ^{32}P-incorporation into histone type III (0.5 mg/ml) in the presence of 10 μg/ml PS and 20 μM [γ-^{32}P]ATP with or without 250 nM PDBu at 30℃ for 10 min as described (10).

For down regulation experiments, approximately 10^7 cells in 10 cm dishes were treated with various concentrations of TPA, PDBu, TRH (Sigma), or vehicle (0.15 % dimethylsulfoxide; DMSO alone) in the incubation medium at 37℃ for the indicated times. Whole cell extracts were prepared by treatment of the homogenates from two dishes with 0.3 % Triton X-100 at 0℃ for 1 h, followed by centrifugation at 10^4 x g for 20 min. For translocation experiments, the supernatant and particulate fractions were separated from the treated cells according to Tashjian's procedure (19) except that the cells were disrupted by sonication (11). Whole extracts, supernatants, and Triton X-100 extracts of the particulate fractions were subjected to DEAE-column (300 μl) chromatography. The column was eluted with 150 mM NaCl and 300 μl fractions were collected. Fractions nos. 1 to 4 or pooled fractions were analyzed by immunoblotting.

Immunoblot analysis was done as described (9) using anti-PKC α, anti-PKC βI, and anti-PKC βII antibodies, and anti-nPKC ε antibodies newly raised against a synthesized peptide (C-terminal 28 amino acids). The specificites of the antibodies are clear as shown in a previous report (11) and Fig. 2A.

Northern blots were performed as described (4).

RESULTS

PKC isozymes expressed in GH_4C_1 cells. In order to identify the PKC isozymes present in GH_4C_1 cells, we partially purified PKCs by a two-step purification procedure using DEAE-Sephacel and HAP column chromatographies. Fig. 1 shows representative elution profiles of PKCs from the HAP column. Two Ca^{2+}-dependent peaks (Peaks 1 and 3) for both PDBu-binding (Fig. 1B) and kinase activities (Fig. 1C and D) were observed corresponding to the peaks (Fig. 1A) for Types II and III fractions from rabbit brains, respectively. The Ca^{2+}-dependence of the kinase activity of Peak 1 (Type II) was, however, clear only in the absence of PDBu. Presumably this is due to significant activation of Type II by TPA or DAG alone in the presence of phospholipid and its relatively low Ca^{2+} requirement for full activation as previously described (6,8). In addition to these peaks, a Ca^{2+}-independent peak (Peak 2) was observed for both PDBu-binding and kinase activities. This elution position, immediately after Type II (β), agrees with that of nPKC ε from rabbit brains (Fig. 1A and 2A) and COS cells transfected with nPKC ε-cDNA (10).

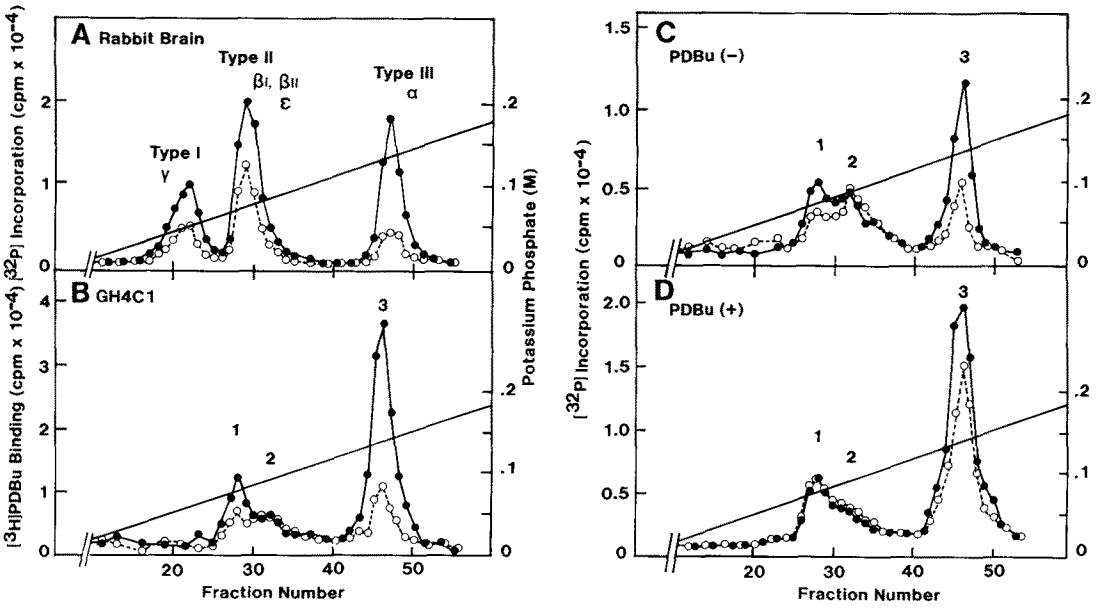

Fig. 1. Elution profiles of PKC isozymes in rabbit brain (A) and GH_4C_1 cell (B, C, and D) extracts from a hydroxyapatite column. Protein kinase and PDBu-binding activities of aliquots (10 and 100 μl, respectively) of 1 ml fractions were determined in the presence of 0.4 mM Ca^{2+} (●) or EGTA (○, 1 and 5 mM for the kinase and binding assay, respectively) as described under "Materials and Methods".

Immunoblot analysis clearly shows that Peaks 1, 2, and 3 are PKC βII, nPKC ε, and PKC α, respectively (Fig. 2A). The expression of the three isozymes in GH_4C_1 cells was confirmed by Northern blotting (Fig. 2B for nPKC ε; data not shown for PKC α and βII).

These results clearly demonstrate that the Ca^{2+}-independent isozyme nPKC ε is expressed in GH_4C_1 cells in addition to Ca^{2+}-dependent PKC α and βII, and that the expression level of PKC α is the highest among the three isozymes. A similar cell line, GH_3, whose original strain is the same as that of GH_4C_1, expresses the same three PKC isozymes, but at lower levels than in GH_4C_1 cells (Fig. 2B for nPKC ε; others not shown). Thus, GH_4C_1 cells were used in this study.

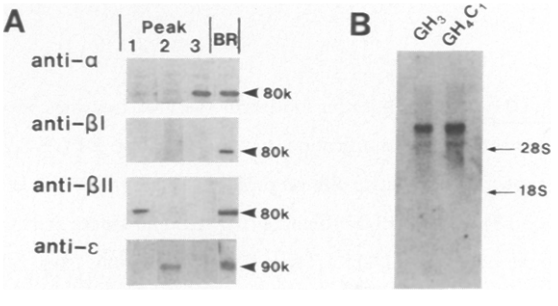

Fig. 2. Western blot (A) and Northern blot (B) analysis of PKC isozymes of GH_4C_1 cells. A: Aliquots (40 μl for nPKC ε and 20 μl for the others) of peaks 1, 2, and 3 HAP fractions shown in Fig. 1 (tube numbers 28, 32, and 46, respectively) were subjected to immunoblot analysis using specific antibodies against PKC α, βI, and βII, and nPKC ε. Types III (PKC α) and II (PKC βI/βII plus nPKC ε) of rabbit brains (RB) were used as standards for anti-α, and for anti-βI, βII and ε, respectively. Arrow heads indicate the position of each PKC isozyme. B: Poly (A) RNAs (equivalent to 100 μg of total RNA) of GH_3 (Lane 1) and GH_4C_1 (Lane 2) were analyzed using an nPKC ε cDNA fragment (nuc. no. 203 to 2168) as a probe. Arrows indicate 28 S and 18 S ribosomal RNAs.

Different modulation of PKC isozymes in GH_4C_1 cells by phorbol esters and TRH. Down-regulation of PKC(s) would be associated with the expression of numerous physiological functions (1). In order to deduce the roles of these PKC isozymes in the signal transduction of GH_4C_1 cells, we examined whether these isozymes are down-regulated by phorbol ester or TRH. As shown in Fig. 3A, exposure of the cells to 160 nM TPA for 24 h causes complete depletion of PKC βII and nPKC ε, but significant loss of PKC α was hardly detectable. Although TRH (200 nM, a concentration eliciting near maximum response in PRL secretion) (18,19) causes the exclusive depletion of nPKC ε after 24 h exposure, no significant decrease in PKC α or βII was detected. The loss of nPKC ε induced by 200 nM TRH is fairly rapid, as for nPKC ε and PKC βII at 100 nM TPA, and a significant decrease (>50 %) was detected within only 30 min (Fig. 3B). These findings suggest that nPKC ε plays an important role in the cellular responses elicited by both phorbol esters and TRH, and that PKC βII also mediates signals in response to phorbol esters.

The failure to detect down-regulation of PKC α (and βII) in response to TPA or TRH suggest that these isozymes may not be involved in transmitting signals of phorbol ester- or TRH-stimulation. However, as shown in Fig. 4, both PKC α and βII in the membranes clearly increases after incubation of the cells with 1 μM PDBu or TPA for 15 min or with 200 nM TRH for 15 sec in the presence of 0.5 mM Ca^{2+}, although significant amounts of both PKC α and βII still remain in the cytosol in the case of TRH treatment. Under these conditions of high concentration (1 μM) of phorbol esters and short exposure (15 min), PKC βII is rapidly depleted from the cells; TPA is more effective than PDBu, reflecting the biological potencies of these agents. Moreover, the total amount of PKC α present in the cytosol and membranes decreases, indicating that PKC α is also down-regulated by short treatment with phorbol esters. On the other hand, neither phorbol esters nor TRH elicits the translocation of PKC α and βII when the intracellular Ca^{2+} is depleted by preincubation with 100 μM EGTA for 30 min (data not shown). This is in significant contrast to nPKC ε which associates with membranes in a Ca^{2+}-independent manner after stimulation by phorbol ester (11).

These results on the biochemical properties, down-regulation, and translocation of PKC isozymes indicate that the multiple responses elicited by phorbol esters- or TRH-stimulation in GH_4C_1 cells involve Ca^{2+}-independent nPKC ε in addition to Ca^{2+}-dependent PKC α and βII.

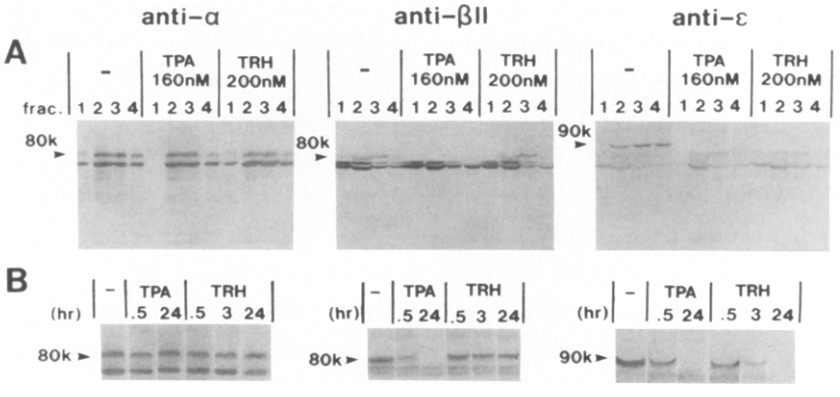

Fig. 3. A: Different modes of down-regulation of PKC isozymes present in GH_4C_1 cells in response to phorbol ester and TRH. Cells incubated for 24 h with 160 nM TPA, 200 nM TRH or vehicle (-, DMSO alone). B: Time courses of specific down-regulation of PKC isozymes in cells incubated with 100 nM TPA, 200 nM TRH, or DMSO (-). Aliquots [5, 20, and 40 μl of each of DEAE fraction (A) or pooled fraction (B)] were analyzed for PKC α and βII, and nPKC ε, respectively. Bands corresponding to each PKC isozyme are marked by arrow heads. Other cross-reacting bands are unidentified. See "Materials and Methods" and legend to Fig. 2.

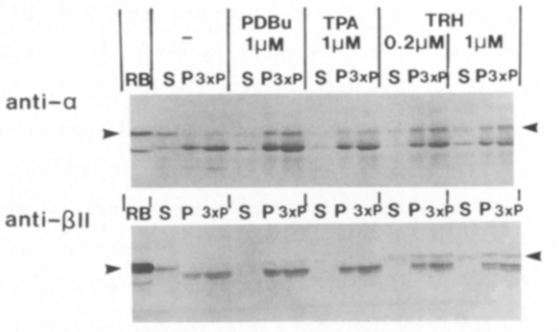

Fig. 4. TRH-induced translocation of PKC α and βII in GH_4C_1 cells. The cells were treated with phorbol esters or TRH at the indicated concentrations in the presence of 0.5 mM Ca^{2+} as described under "Materials and Methods". The amounts of PKC α or βII in the cytosol (S) and particulate (P and 3xP) fractions corresponding to 5 or 12 x 10^5 cells ("3xP" represents 3 fold amounts of "P") for PKC α or βII, respectively, were analyzed by immunoblotting. See legends to Fig. 2 and 3.

DISCUSSION

The present results provide the first evidence for the modulation of a novel Ca^{2+}-independent, phospholipid-dependent protein kinase, nPKC ε, not only by phorbol esters but also by a physiological hormone, TRH. This suggests that Ca^{2+}-independent nPKC ε plays a crucial role in TRH-stimulated signal transduction. Moreover, we have demonstrated that three PKCs, PKC α and βII, and nPKC ε expressed in GH_4C_1 cells are subjected to different specific modulation in response to phorbol esters and TRH; TPA and PDBu preferentially deplete PKC βII and nPKC ε, whereas TRH exclusively and dramatically depletes nPKC ε, although both stimuli induce the redistribution of PKC α and βII. Down-regulation of PKC α is clear after only a short treatment with phorbol ester. The different spectra of down-regulation in response to TRH and phorbol ester suggest that the molecular mechanisms mediated by PKC α and βII are different for the two types of stimuli.

Since membrane-associated active forms of PKCs are preferentially proteolyzed or degraded (15,24) and a point mutant of PKC α lacking kinase activity is resistant to phorbol ester-induced down-regulation (25), the depletion of PKC isozymes by treatment of cells with phorbol esters, hormones, or other biologically active compounds appears to reflect the different abilities of these stimuli in the translocation and activation of various isozymes. It has been reported that TRH-induced redistribution of 80 kDa PKCs in GH cells (presumably PKC α and βII on the basis of our results) is transient because of a limitation (transient increase) of DAGs in the plasma membrane, but that the phorbol ester-induced redistribution is sustained (26). The present observation that TRH, unlike phorbol esters, fails to down-regulate PKC α and βII may reflect an apparent resistance of PKC α and βII to proteolysis because of their rapid and transient translocation. Whether TRH stimulated association of nPKC ε with the membranes is sustained, as in the case of phorbol esters (11), remains to be clarified. In this respect, investigating whether sustained accumulation of DAGs in intracellular membranes of TRH-stimulated GH cells (26) is responsible for the activation and degradation of nPKC ε would be interesting. The selective persistence of PKC α observed after long term exposure to phorbol esters could be explained by the relatively large pool of this isozyme (Fig. 1 and 2), by postulating that the rates of synthesis of PKC α is greater than those of other isozymes during long term treatment (27), and/or by its resistance to proteolysis (15).

The specific down-regulation of PKC isozymes in response to biologically active stimuli may be a phenomenon common to many cell types, but the susceptibility of each species appears to differ among cell lines (14-17). PKC α is susceptible, but β (βII) is resistant to down-regulation in BC3H-1, U937, and MDCK-D1 cell lines; whereas in KM3 and RBL-2H3 cell lines, PKC α is resistant, but β (βII) is susceptible to down-regulation by phorbol esters. GH_4C_1 cells behave like the latter group upon stimulation by phorbol esters but, in the case of TRH-stimulation, their PKC α and βII are resistant while nPKC ε is specifically susceptible to down-regulation.

PKC α and βII, and nPKC ε all seem to mediate some multiple cellular responses to phorbol esters or TRH in GH_4C_1, such as secretion of PRL and GH and morphological changes. Further experiments are necessary to define the role of each isozyme in the regulation of various responses. We have recently observed that TRH- or TPA-stimulated PRL secretion is significantly retained, even in Ca^{2+}-depleted cells, and is inhibited by H7 or staurosporine, specific inhibitors of nPKC ε (22) as well as other conventional PKC isozymes. Therefore, it is highly likely that nPKC ε is involved in the Ca^{2+}-independent secretory response in GH_4C_1 cells. However, the consequences of the activation of nPKC ε by DAGs or phorbol esters and its rapid inactivation by proteolysis in the secretory process remain to be clarified. Further precise analysis of the specific down-regulation of nPKC ε is important to reveal the Ca^{2+}-independent pathway mediated by nPKC ε.

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