A role for protein kinase C subtypes α and ϵ in phorbol-ester-enhanced K^+ - and carbachol-evoked noradrenaline release from the human neuroblastoma SH-SY5Y

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Protein kinase C (PKC) consists of a family of closely related subtypes which differ in their localization and activation properties. Our previous studies have suggested a role for PKC in the regulation of noradrenaline (NA) release from the human neuroblastoma SH-SY5Y. Here we have used two approaches to characterize the PKC subtypes present in SH-SY5Y cells. Firstly, the PCR was used to show that SH-SY5Y cells contain mRNA encoding PKC subtypes α , β , γ , δ , ϵ and ζ . Secondly, immunoblotting showed that SH-SY5Y cells express PKC subtypes α , ϵ and ζ at the protein level. Prolonged (48 h) exposure of cells to the phorbol ester phorbol 12-myristate 13-acetate (PMA; 100 nM) resulted in a marked decrease in the amounts of PKC- α and PKC- ϵ , with no change in levels of PKC- ζ . Prolonged

PMA treatment had no significant effect on K^+ -evoked NA release from SH-SY5Y cells, whereas carbachol-evoked release was increased 2.2-fold. However, prolonged exposure to PMA completely inhibited the ability of acute (12 min) PMA treatment to enhance both K^+ - and carbachol-evoked NA release. The specific PKC inhibitor RO 31-7459 (10 μ M) was found to inhibit K^+ - and carbachol-evoked release by 27 % and 68 % respectively. RO 31-7549 also completely inhibited the ability of acute PMA treatment to enhance release. These data suggest that PKC- α and/or PKC- ϵ play an essential role in the regulation of PMA-enhanced K^+ - and carbachol-evoked NA release in SH-SY5Y cells.

INTRODUCTION

The protein kinase C (PKC) family of phospholipid-dependent serine/threonine kinases consists of a number of closely related subtypes which differ in their tissue distribution, subcellular location and activation properties (Nishizuka, 1992). The PKC subtypes characterized to date have been classified into three distinct groups (Nishizuka, 1992). The classical PKC subtypes α , β , β_{II} and γ are Ca^{2+} -dependent and activated by the second messenger diacylglycerol (DAG), a product of phosphatidylinositol 4,5-bisphosphate hydrolysis following receptor-mediated activation of phospholipase C. The novel PKC subtypes δ , ϵ , η /L and θ are also activated by DAG but are Ca^{2+} -independent; the atypical PKC subtypes ζ and λ are not activated by either DAG or Ca^{2+} . Acute treatment with the tumour-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA), which mimics the action of DAG, has been shown to activate both classical and novel PKC subtypes (Ryves et al., 1991). Furthermore, prolonged exposure to PMA results in down-regulation of PKC due to increased degradation of the PKC polypeptide (Young et al., 1987). The differential localization and activation properties of the PKC subtypes had prompted studies to determine the roles played by individual PKC subtypes in a number of cellular function, including cell differentiation (Tonini et al., 1991; McSwine-Kennick et al., 1991; Parrow et al., 1992; Wooten et al., 1992; Jalava et al., 1993a,b; Leli et al., 1993) and exocytosis (Naor et al., 1989; Ben-Shlomo et al., 1991; Oda et al., 1991).

Previous studies by our group have shown that the human neuroblastoma clone SH-SY5Y, derived from sympathetic

adrenergic ganglia, releases noradrenaline (NA) in a Ca^{2+} -dependent manner after stimulation with depolarizing agents (Murphy et al., 1991; Vaughan et al., 1993), carbachol, acting at the M_3 muscarinic receptor (Murphy et al., 1991), and the Ca^{2+} ionophore A23187 (Murphy et al., 1991). Pretreatment with PMA for 9 min enhances NA release evoked by these agents (Murphy et al., 1992). Furthermore, addition of the selective PKC inhibitors RO 31-7549 and RO 31-8220 results in inhibition of depolarization- and carbachol-evoked NA release (Murphy et al., 1992). This evidence indicates an important role for PKC in the release of NA from SH-SY5Y cells.

Here we have characterized the PKC subtypes present in human SH-SY5Y neuroblastoma cells and studied the effect of PKC down-regulation on NA release.

MATERIALS AND METHODS

Materials

The SH-SY5Y cell line was kindly provided by Dr. June L. Biedler of the Sloan-Kettering Institute for Cancer Research (Rye, NY, U.S.A.). Foetal-calf serum was purchased from Sera Lab (Crawley Down, Sussex, U.K.), and all other cell-culture reagents were obtained from GIBCO BRL (Paisley, Scotland, U.K.). AMV reverse transcriptase, dNTPs and RNasin were from Boehringer Mannheim (Lewes, Sussex, U.K.), (dT)₁₂₋₁₈ primers were from Pharmacia (Milton Keynes, U.K.) and *Taq* polymerase was purchased from Promega (Southampton, U.K.). Upstream and downstream 20-mer oligonucleotide PCR primers and 36-mer antisense oligonucleotide probes for Southern hybrid-

Abbreviations used: DAG, diacylglycerol; HBS, Hepes-buffered saline; NA, noradrenaline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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ization were designed from rat PKC subtype sequences. Upstream primers for PKC subtypes α , β , γ , δ and ϵ ($\alpha\beta\gamma\delta\epsilon$ 5') and for PKC- ζ (ζ 5') had the following nucleotide sequences: $\alpha\beta\gamma\delta\epsilon$ 5' = CCC/TACC/GTTCTGT/CGAT/CCACTG; ζ 5' = GGTGG-ACAGTGAAGGTGACC. Downstream primers for PKC subtypes α , β and γ ($\alpha\beta\gamma$ 3'), PKC subtypes δ and ϵ ($\delta\epsilon$ 3') and PKC- ζ (ζ 5') had the following sequences: $\alpha\beta\gamma$ 3' = TCCCC/TAGC/AACCATG/CAGGAAG; $\delta\epsilon$ 3' = TTGCCAAGCTGCCTT-TGCC; ζ 3' = CACGCCCGATGACTCTGATG. Rat PKC-subtype-specific Southern-hybridization probes had the following oligonucleotide sequences: α = TGTGAGTTTCACCCTGTC-CAGGTTGTTAGATGGCTG; β = GGTCAGTTTCATCCG-GTCCCTGTTGCCATTGTTGTC; γ = AGAGATATGCAG-GCGTCCTGGGCTGGCACCAGAA; δ = ATCTTGCC-ATAGGTCCCGTTGTTGTCTGGGATGTCA; ϵ = CAAGT-TCTTTAGTTCTGTCACAAGGGGAGGTGG; ζ = CC-TGAGAGATTTTGATCCCATCCACCCATCGATGA. Polyclonal rabbit antibodies against PKC subtypes α , γ , δ , ϵ and ζ , and the peptides to which they were raised, were obtained from GIBCO BRL; those for PKC- β_1 and $-\beta_{11}$ were prepared by Dr. N. Groome at the Multiple Sclerosis Synthetic Peptide Laboratory, School of Biological and Molecular Science, Brookes University, Oxford, U.K. Monoclonal anti-(rabbit PKC- β) antibody was from Seikagaku Corp. (Tokyo, Japan). Goat anti-(rabbit IgG)-peroxidase conjugate, 3,3'-diaminobenzidine, carbachol and PMA were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). RO 31-7549 was kindly provided by Roche Products (Welwyn Garden City, Herts., U.K.). [3 H]NA (40 Ci/mmol) and sheep anti-(mouse IgG)-peroxidase conjugate were from Amersham International (Amersham, Bucks., U.K.).

Cell culture

SH-SY5Y cells were cultured in a 1:1 (v/v) mixture of Ham's F12 nutrient mixture and Eagle's minimal essential medium supplemented with 10% foetal-calf serum and non-essential amino acids in an air/CO₂ (49:1) humidified incubator at 37 °C. Cells were harvested in PBS and cultured at a seeding density of 1×10^5 cells/ml.

RNA isolation

Whole-cell RNA was isolated from confluent cultures of SH-SY5Y cells by the method of Chomczynski and Sacchi (1987). After exposure of the cell monolayer to lysis buffer [4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) sarcosyl, 0.7% (v/v) 2-mercaptoethanol], cells were detached with a cell scraper, and 2 M sodium acetate (pH 4.0)/phenol/chloroform (1:10:2, by vol.) was added to the lysate. After incubation at 4 °C for 15 min, the mixture was centrifuged, the aqueous phase collected and the RNA precipitated with propan-2-ol. The resulting pellet was resuspended in sterile water and the RNA sample determined by measuring the A_{260} in a spectrophotometer. Four separate RNA isolations gave a mean yield of 300 ± 37 μ g per 75 cm² flask of SH-SY5Y cells (40 μ g of RNA/ml = 1.0 A_{260} unit).

Reverse transcription

A modification of the reverse-transcription reaction method described by Sambrook et al. (1989) was used to yield cDNA from whole-cell RNA. Before the reaction, 25 μ g of RNA was incubated at 95 °C for 5 min, then cooled on ice. The RNA sample was then added to the reverse-transcription reaction mixture comprising amplification buffer, 1 mM dNTPs, 5 mM

dithiothreitol, 90 units of RNasin, 400 ng of (dT)₁₂₋₁₈ primers and 500 units of reverse transcriptase in a final volume of 90 μ l. The reaction mixture was incubated at 42 °C for 45 min, followed by 5 min at 95 °C to inactivate the reverse transcriptase.

PCR

PCR was performed in a mixture comprising 20 μ l of cDNA and 80 μ l of amplification buffer containing 2 units of *Taq* polymerase and 25 pmol each of the appropriate upstream and downstream oligonucleotide primers (Sambrook et al., 1989). Samples were overlaid with light mineral oil, and PCR was performed using a CETUS thermal cycler (35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min). The resulting PCR products were mixed with 1 μ l of sample buffer [50% (v/v) glycerol, 1 mM EDTA, pH 8.0, 0.25% (w/v) Bromophenol Blue, 0.25% (w/v) xylene cyanol FF] and resolved by 2% (w/v) agarose-gel electrophoresis (Sambrook et al., 1989). Gels were photographed under u.v. illumination after staining with ethidium bromide.

Southern hybridization

PCR products resolved by electrophoresis were transferred by capillary action to nylon blotting membranes for Southern-hybridization studies (Sambrook et al., 1989). Antisense oligonucleotide probes for specific PKC subtypes were labelled with ³²P by the T₄ polynucleotide kinase method (Sambrook et al., 1989). After transfer of PCR products, the nylon membrane was baked for 2 h under vacuum at 80 °C. Membranes were then incubated for 2 h at 42 °C in prehybridization solution [6 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate), 5 $\times$ Denhardt's reagent, 0.1 mg/ml salmon sperm DNA, 50% (v/v) formamide], followed by overnight incubation at 42 °C in hybridization solution [6 $\times$ SSC, 0.5% (w/v) SDS, 0.1 mg/ml salmon sperm DNA, 50% (v/v) formamide] containing ³²P-labelled antisense oligonucleotide probes for specific PKC subtypes. After hybridization, membranes were washed consecutively at room temperature in 2 $\times$ SSC/0.1% SDS, 1 $\times$ SSC/0.1% SDS and 0.1 $\times$ SSC/0.1% SDS and exposed to X-ray film (Amersham International) for 1–2 days at –70 °C.

Immunodetection of PKC subtypes

Whole cell homogenates were prepared from cultures of SH-SY5Y cells by exposing the monolayer directly to Laemmli (1970) sample buffer containing 5 mM EDTA and 25 μ g/ml leupeptin. Rat brain homogenate was prepared by homogenizing whole rat brain in 1% SDS/5 mM EDTA containing 25 μ g/ml leupeptin (10 ml/g of brain), followed by centrifugation to remove debris and dilution of the supernatant 1:4 with Laemmli sample buffer. SH-SY5Y and rat brain samples were heated for 10 min in a boiling-water bath before resolving them by SDS/PAGE (10% gel) (Laemmli, 1970). Electrophoretic transfer of proteins to Immobilon-P poly(vinylidene) difluoride membranes (Millipore, Watford, Herts., U.K.) was carried out in 25 mM Tris base/188 mM glycine (pH 8.3)/20% (v/v) methanol for 2.5 h with a constant current of 400 mA. The membrane was then incubated in blocking solution [5% (w/v) Marvel non-fat dried milk, 0.02% (v/v) Tween-20 in PBS] for 1 h and cut into strips. Membrane strips were incubated for 4 h or overnight in blocking solution containing anti-PKC antibodies (diluted 1:100 for PKC- β , 1:200 for PKC subtypes α , γ , δ , ϵ and ζ , or 1:2000 for PKC subtypes β_1 and β_{11}) in the absence or presence of an excess of the appropriate antigenic peptide. Strips were washed and then incubated for 1 h with peroxidase-conjugated anti-rabbit or

anti-mouse secondary antibody (diluted 1:200 in blocking solution). Immunolabelled bands were detected with 0.05% (w/v) 3,3'-diaminobenzidine/0.01% (v/v) H_2O_2 /50 mM imidazole/50 mM Tris/HCl, pH 7.4.

Measurement of NA release

The release of [^3H]NA from SH-SY5Y cells was measured essentially as described previously (Murphy et al., 1991). Cells were grown in 24-well plates and assayed for NA release after 4 days in culture. Briefly, culture medium was removed and the monolayer washed twice with HEPES-buffered saline (HBS; 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 6 mM glucose, 2.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.6 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 $\mu\text{g}/\text{ml}$ pargyline, 20 $\mu\text{g}/\text{ml}$ ascorbic acid) before replacing it with 0.5 ml of HBS containing 50 nM [^3H]NA. After incubation at 37 °C for 1 h, excess [^3H]NA was removed by washing the cell monolayer with 4×0.5 ml of HBS at 10 min intervals. For measuring [^3H]NA release, the monolayer was washed twice with 0.5 ml of HBS at 4 min intervals, followed by a 4 min incubation with HBS (to measure basal release), elevated K^+ (HBS containing 40 mM NaCl/100 mM KCl) or 1 mM carbachol in HBS. For experiments involving PMA-enhanced release, cells were pre-incubated with 100 nM PMA in HBS for 8 min before addition of releasing agents plus PMA. For studies involving the PKC inhibitor RO 31-7549, cells were pretreated for 8 min with 10 μM RO 31-7549 in HBS before the experiment. Unreleased [^3H]NA in the cell monolayer was extracted with 0.4 M HClO_4 . The amount of [^3H]NA release was expressed as a percentage of the total amount of radioactivity present in the cell monolayer at the beginning of the experiment.

RESULTS

Detection of mRNAs encoding PKC subtypes by using the PCR

Whole-cell RNA was isolated from SH-SY5Y cells and used in a reverse-transcription reaction to yield cDNA. The PCR was used to amplify specific cDNA sequences corresponding to short regions of mRNA encoding human PKC subtypes α , β ($\beta_1 + \beta_{11}$), γ , δ , ϵ and ζ . The pairs of oligonucleotide PCR primers used were complementary to 20-base sequences within the cDNA encoding the C1 and C3 conserved regions of rat PKC subtypes. Primers were designed to flank regions of differing length for each PKC subtype to facilitate identification of PCR fragments by nature of their size. Furthermore, PCR fragments contained a complete variable region allowing identification of individual subtypes by Southern hybridization using subtype-specific ^{32}P -labelled 36-mer antisense oligonucleotide probes. The use of rat PKC PCR primers and Southern-hybridization probes for studying human PKC subtype cDNAs exploited the high degree of similarity between rat and human nucleotide sequences for PKC subtypes; the oligonucleotide primers exhibited 85–100% similarity to human PKC nucleotide sequences (deposited in the EMBL/GenBank database), whereas the Southern-hybridization probes were 81–97% similar to the human sequences.

PCR fragments produced by amplification of SH-SY5Y-cell cDNA were separated according to size by agarose-gel electrophoresis (Figure 1). The $\alpha\beta\gamma\delta\epsilon$ 5' + $\alpha\beta\gamma$ 3' pair of primers was designed to amplify cDNA for PKC subtypes α , β and γ with sizes of 706 bp, 715 bp and 745 bp respectively. A doublet of 710 bp and 750 bp was visible on ethidium bromide-stained gels after PCR with these primers (Figure 1a). Southern hybridization of these PCR products showed that the 710 bp fragment bound both the PKC- α and PKC- β antisense oligonucleotide probes (Figures 2a and 2b), indicating that the 710 bp band comprised

a combination of PCR fragments for PKC- α (706 bp) and PKC- β (715 bp). The 750 bp fragment bound only the PKC- γ Southern-hybridization probe (Figure 2c), confirming that this was the PKC- γ PCR fragment (745 bp). The $\alpha\beta\gamma\delta\epsilon$ 5' + $\delta\epsilon$ 3' pair of primers, designed to produce PCR fragments for PKC- δ (359 bp) and PKC- ϵ (506 bp), yielded fragments of 350 bp and 510 bp (Figure 1b), which were confirmed by Southern hybridization to be PCR products for PKC subtypes δ and ϵ respectively (Figures 2d and 2e). An anomalous 740 bp fragment was also produced by this pair of primers, and this was found to

Figure 1 Ethidium bromide-stained agarose gels of SH-SY5Y-cell PCR fragments

SH-SY5Y-cell PCR fragments were resolved by 2% (w/v)-agarose-gel electrophoresis, and gels were stained with ethidium bromide. DNA size markers (1353, 1078, 872, 603, 310 and 276 bp from top to bottom) are shown at the left. Lanes: (a) $\alpha\beta\gamma\delta\epsilon$ 5' + $\alpha\beta\gamma$ 3' primers; (b) $\alpha\beta\gamma\delta\epsilon$ 5' + $\delta\epsilon$ 3' primers; (c) ζ 5' + ζ 3' primers.

Figure 2 Southern hybridization of SH-SY5Y-cell PCR fragments

PCR fragments were transferred from agarose gels to nylon blotting membrane for Southern-hybridization studies using rat PKC subtype-specific ^{32}P -labelled antisense oligonucleotide probes. The positions of DNA size markers (bp) are indicated at the left. Lanes: (a) PKC- α probe; (b) PKC- β probe; (c) PKC- γ probe; (d) PKC- δ probe; (e) PKC- ϵ probe; (f) PKC- ζ probe.

cross-react with the PKC- β Southern hybridization probe (results not shown). Studies of the nucleotide sequence of PKC subtypes revealed that the $\delta\epsilon$ 3' PCR primer could bind to a region 735 bp from the $\alpha\beta\gamma\delta\epsilon$ 5' primer-binding region within the PKC- β nucleotide sequence, therefore producing a 735 bp PCR fragment. The resultant PCR fragments of the ζ 5' + ζ 3' pair of oligonucleotide primers, designed to yield a 602 bp PCR fragment for PKC- ζ , gave rise to intense general ethidium bromide staining after electrophoretic separation (Figure 1c). No distinct band in the region of 600 bp was visible; however, Southern hybridization of the gel with the PKC- ζ probe revealed the presence of a single 600 bp band (Figure 2f). These data confirm that mRNA encoding PKC subtypes α , β , γ , δ , ϵ and ζ can be detected in SH-SY5Y cells by the PCR.

Immunodetection of PKC subtypes

Immunoblot analysis of SH-SY5Y-cell homogenate was carried out with monoclonal antibodies raised against rabbit PKC- β or polyclonal antibodies raised against peptides corresponding to short regions at the N-terminal (PKC subtypes α and γ) or C-terminal (PKC subtypes β_1 , β_{11} , δ , ϵ and ζ) of the rat PKC molecule (Henrich, 1991). Incubation of membrane strips with specific polyclonal antibodies resulted in the detection of multiple bands. However, incubation in the presence or absence of antigenic peptides allowed identification of PKC subtypes by the occurrence of immunolabelled bands which were not observed when antigenic peptide was present. PKC subtypes α , β_1 , β_{11} , γ , δ , ϵ and ζ are all reported to be present in brain (Nishizuka, 1992). In order to confirm the specificity of the anti-PKC antibodies, immunoblotting of rat brain homogenate was carried out alongside that of SH-SY5Y-cell homogenate, and the mean molecular mass ($\pm$ S.E.M.) of relevant bands was estimated from 3–4 gels (typical results appear in Figure 3). Incubation of membrane strips with the anti-PKC- α antibody resulted in the detection of an 80.3 ± 1.5 kDa protein in rat brain and a 78.0 ± 1.5 kDa protein in SH-SY5Y-cell homogenate. A second protein, of size 60 kDa, was also detected in SH-SY5Y-cell homogenate. Immunolabelling with the anti-PKC- β_1 antibody detected a protein of 78.0 ± 0.6 kDa in rat brain, but not in SH-SY5Y cells. The anti-PKC- β_{11} antibody labelled two proteins in rat brain (79.3 ± 0.9 kDa and 50 kDa) and in SH-SY5Y cells (87.0 ± 0.6 kDa and 50 kDa); however, only a single 77.8 ± 0.8 kDa protein was detected in rat brain, but not in SH-SY5Y cells, with the anti-PKC- β monoclonal antibody. A 79.0 ± 0.6 kDa band was detected in rat brain, but not in SH-SY5Y-cell homogenate, with the anti-PKC- γ antibody. PKC- δ was detected as a 70.7 ± 1.2 kDa and 75.7 ± 0.9 kDa doublet in rat brain, but was not detected in SH-SY5Y cells, whereas PKC- ϵ was detected in large amounts as a doublet in both rat brain (89.2 ± 3.0 kDa and 95.0 ± 2.5 kDa) and SH-SY5Y-cell homogenate (86.6 ± 1.8 kDa and 90.2 ± 1.8 kDa). The anti-PKC- ζ antibody detected two major proteins (77.2 ± 1.0 kDa and 71.2 ± 1.1 kDa), as well as two other proteins (95 kDa and 55 kDa) in rat brain, whereas only a 68.2 ± 1.1 kDa band was observed for SH-SY5Y cells. These data confirm that the antibodies used can detect PKCs α , β_1 , β_{11} , γ , δ , ϵ and ζ in rat brain and show that only the α , ϵ and ζ subtypes can be detected in SH-SY5Y-cell homogenate.

Effect of prolonged PMA treatment on [3 H]NA release from SH-SY5Y cells

SH-SY5Y cells were grown in the absence (control cells) or presence (48 h-PMA-treated cells) of the phorbol ester PMA (100 nM) for 48 h before measuring [3 H]NA release (Table 1).

Figure 3 Immunoblotting of SH-SY5Y-cell homogenate with PKC-subtype-specific polyclonal antibodies

SH-SY5Y-cell homogenate (SH; 360 μ g of protein/lane) and rat brain homogenate (RAT; 95 μ g of protein/lane) were resolved by SDS/PAGE (10% gels), and proteins were electrophoretically transferred to Immobilon-P membranes. Membrane strips were incubated with rabbit polyclonal antibodies raised against PKC subtypes α , β_1 , β_{11} , γ , δ , ϵ and ζ , as labelled, in the presence (+) or absence (–) of antigenic peptide, or with mouse monoclonal antibody against PKC- β (BETA). After incubation with peroxidase-conjugated secondary antibody, immunolabelled bands were detected with 3,3'-diaminobenzidine. Relevant bands for PKC isoenzymes detected with polyclonal antibodies, marked with arrows, are those detectable in the absence of antigenic peptide but not detectable in the presence of antigen. The positions of molecular-mass markers (kDa) are indicated at the left.

Table 1 Effect of prolonged PMA treatment on [3 H]NA release from SH-SY5Y cells

Control SH-SY5Y cells or cells exposed to 100 nM PMA for 48 h were stimulated with 100 mM K^+ or 1 mM carbachol (CARB), after pretreatment for 8 min with or without 100 nM PMA, and the amount of [3 H]NA released from the cells was measured. Results are expressed as percentage release, from which basal release (i.e. release in the presence of HBS, approx. 3.5%) has been subtracted, and are mean values $\pm$ S.E.M. of three separate experiments, each carried out in quadruplicate.

Stimulus	NA release (%)	
	Control	+ PMA (48 h)
K^+	13.2 ± 1.4	11.8 ± 1.5
K^+ + PMA	23.6 ± 0.1	11.2 ± 1.7
CARB	2.4 ± 0.7	5.4 ± 0.2
CARB + PMA	11.0 ± 1.2	4.6 ± 0.7

Prolonged PMA treatment had no significant effect on K^+ -evoked NA release relative to control cells; however, carbachol-evoked release increased 2.2-fold under these conditions. In control cells, acute treatment with 100 nM PMA for 12 min gave rise to a 1.8-fold and 4.6-fold increase in K^+ - and carbachol-evoked release respectively. This enhancement of NA release by acute exposure to PMA was completely inhibited in 48 h-PMA-treated cells.

Effect of prolonged PMA treatment on SH-SY5Y-cell PKC subtype expression

SH-SY5Y cells were cultured in parallel with those used in [3 H]NA release assays and treated identically with or without 100 nM PMA for 48 h. Cell homogenates were prepared and immunoblotting was carried out to detect PKC subtype ex-

Figure 4 Effect of prolonged PMA treatment on immunodetection of PKC subtypes in SH-SY5Y-cell homogenate

Homogenates were prepared from SH-SY5Y cells after 48 h incubation in the presence (+ PMA) or absence (– PMA) of 100 nM PMA. Immunoblot analysis was performed as described for Figure 3. All labelling is as for Figure 3.

Table 2 Effect of RO 31-7549 on [3 H]NA release from SH-SY5Y cells

[3 H]NA release from SH-SY5Y cells was measured after stimulation with K^+ (100 mM) or carbachol (1 mM) after 8 min pretreatment with or without 100 nM PMA. Cells exposed to RO 31-7549 (10 μ M) were pretreated with the inhibitor for 8 min. Results are expressed as in the legend for Table 1 and are mean values $\pm$ S.E.M. of three separate experiments, each carried out in quadruplicate.

Stimulus	NA release (%)	
	Control	+ RO 31-7549
K^+	20.1 $\pm$ 1.4	14.6 $\pm$ 0.9
K^+ + PMA	31.0 $\pm$ 1.2	21.6 $\pm$ 1.3
CARB	4.1 $\pm$ 0.6	1.3 $\pm$ 0.4
CARB + PMA	14.1 $\pm$ 0.3	1.6 $\pm$ 1.1

pression (Figure 4). A marked decrease in PKC- α and PKC- ϵ was observed in 48 h-PMA-treated cells relative to control cells. The expression of PKC- ζ was not affected by prolonged PMA treatment. PKC subtypes β , γ and δ were not detected in either control cells or 48 h-PMA-treated cells (results not shown).

Effect of the PKC inhibitor RO 31-7549 on [3 H]NA release from SH-SY5Y cells

The release of [3 H]NA from SH-SY5Y cells was measured after pretreatment with or without the PKC inhibitor RO 31-7549 (Table 2). Control SH-SY5Y cells exhibited K^+ - and carbachol-evoked NA release which was enhanced 1.5-fold and 3.4-fold respectively after acute exposure to 100 nM PMA. Pretreatment of cells for 8 min with 10 μ M RO 31-7549, the dose shown previously to give maximum inhibition of release (Murphy et al., 1992), resulted in 27% and 68% inhibition of K^+ - and carbachol-evoked release respectively. Cells exposed to RO 31-7549 exhibited complete inhibition of the acute PMA-enhanced portion of NA release. PMA-enhanced carbachol-evoked release was inhibited by RO 31-7549 to the same level as for carbachol-evoked release. In contrast, PMA-enhanced K^+ -evoked release was not inhibited to the same level as K^+ -evoked release.

DISCUSSION

In this study, two methods for detecting PKC subtypes in the human neuroblastoma clone SH-SY5Y have been used. The PCR detected mRNAs encoding PKC subtypes, whereas immunoblotting with PKC-subtype-specific antibodies detected PKC subtypes at the protein level. The use of rat PCR primers and Southern-hybridization probes for amplification and de-

tection of human PKC subtype cDNAs exploited the high degree of similarity between rat and human nucleotide sequences for PKC subtypes. The identification of PCR fragments by nature of their size and the ability of Southern-hybridization probes to bind to them confirmed the presence of PCR fragments for PKC subtypes α , β , γ , δ , ϵ and ζ in SH-SY5Y cells (Figures 1 and 2). Northern-hybridization studies by Parrow et al. (1992) have shown that mRNA encoding PKC- α can be detected in SH-SY5Y cells, but not mRNA for PKC subtypes β or γ . This is not inconsistent with our results showing detection of all three of these PKC subtypes, as the PCR technique is known to be many times more sensitive than Northern hybridization (Scharf et al., 1986).

Immunoblotting techniques were employed to determine the PKC subtypes expressed in SH-SY5Y cells. Parallel studies with rat brain homogenate detected PKC subtypes α , β_1 , β_{II} , γ , δ , ϵ and ζ (Figure 3), thus confirming the validity of the methodology used. SH-SY5Y cells were found to express only PKC subtypes α (78 kDa), ϵ (87–90 kDa) and ζ (68 kDa) at the protein level (Figure 3). No proteins were detected in SH-SY5Y-cell homogenate by using antibodies raised against PKC subtypes β_1 , γ or δ . An 87 kDa SH-SY5Y-cell protein immunolabelled with the anti-PKC- β_{II} antibody was determined not to be PKC- β_{II} because of its higher molecular mass than rat brain PKC- β_{II} (79 kDa) and also its lack of immunoreactivity with the anti-PKC- β monoclonal antibody. The molecular masses of PKC- α and PKC- ϵ detected in SH-SY5Y cells are in agreement with those expected (Henrich, 1991). PKC- ζ has been detected previously as two proteins with sizes of 70 kDa and 80 kDa (Henrich, 1991), both of which were detected in rat brain in this study. However, only the lower-molecular-mass form of PKC- ζ was observed in SH-SY5Y cells. Immunoreactive proteins with molecular masses higher or lower than those expected for PKC subtypes α , β_{II} and ζ may correspond to proteins other than PKC which show amino acid sequence similarity to the peptide to which the antibodies were raised. The distribution of PKC subtypes in SH-SY5Y cells is consistent with previous reports (reviewed by Nishizuka, 1992) which indicate that PKC subtypes α and ζ are present in many tissues and that PKC- ϵ is less widespread, being present mainly in tissues and cells of neuronal origin. The central-nervous-system-specific PKC- γ was not detected in SH-SY5Y cells, which are derived from the peripheral nervous system. The detection of only three of the PKC subtypes at the protein level in SH-SY5Y cells contrasts with the studies using PCR, which detected mRNA for all six of the PKC subtypes. This supports the observation that PKC protein and mRNA levels do not necessarily correlate (Wooten, 1992). The detection of mRNA for PKCs β , γ and δ , not observed previously in SH-SY5Y cells (Parrow et al., 1992), may be a result of the extremely high sensitivity of the PCR technique for detecting small amounts of mRNA (Scharf et al., 1986). Previous reports on the immunodetection of PKC subtypes in SH-SY5Y cells have indicated the presence of PKC subtypes α (Parrow et al., 1992; Jalava et al., 1993b; Leli et al., 1993), β (Jalava et al., 1993b; Leli et al., 1993), ϵ (Jalava et al., 1993b; Leli et al., 1993) and ζ (Jalava et al., 1993b) in cell homogenates. Leli et al. (1993) also report the presence of an 86 kDa protein, which may be PKC- γ , although this was not detected by Jalava et al., (1993b). PKC- δ has not been observed in crude SH-SY5Y-cell homogenates; however, a weak immunoreactive band (80 kDa) has been detected with an antibody against PKC- δ after a partial purification of SH-SY5Y-cell PKC (Leli et al., 1993). Our observations that PKC subtypes α , ϵ and ζ are readily detected by immunoblotting of SH-SY5Y-cell homogenate, but that PKC subtypes γ and δ are not observed by this technique, are therefore in agreement with previous reports (Parrow et al.,

1992; Jalava et al., 1993b; Leli et al., 1993). There is little agreement from previous studies on the expression of PKC- β in SH-SY5Y cells. Leli et al. (1993) report that PKC- β_1 is the most abundant PKC subtype in SH-SY5Y cells, whereas Jalava et al. (1993b) observed that 3–4 times more protein was required to detect PKC- β than the other PKC subtypes. In addition, Parrow et al. (1992) report the absence of mRNA encoding PKC- β in SH-SY5Y cells. In this study we were unable to detect PKC- β in SH-SY5Y-cell homogenate using three different antibodies under normal immunoblotting conditions (Figure 3) or even using an enhanced detection method (ECL, Amersham; results not shown). It has been suggested previously that differences in PKC-subtype expression in SH-SY5Y cells may be a result of differing cell-culture conditions (Jalava et al., 1993b), and it is noteworthy that SH-SY5Y cell-culture conditions reported by Parrow et al. (1992), Leli et al. (1993) and Jalava et al. (1993b), and those employed by our group, are all different.

Previous studies by our group have shown that acute exposure to the phorbol ester PMA enhances K⁺- and carbachol-evoked NA release from SH-SY5Y cells (Murphy et al., 1992), implicating a role for PKC in exocytosis (see also Dekker et al., 1991). Furthermore, PKC inhibitors have been shown to inhibit a proportion of depolarization-evoked release in a number of cell types (for review see Dekker et al., 1991), whereas prolonged exposure to PMA, which down-regulates PKC, has been shown to decrease depolarization-evoked exocytosis in PC12 pheochromocytoma cells (Matthies et al., 1987) and bovine adrenal chromaffin cells (Burgoyne et al., 1988).

The role of PKC subtypes in the regulation of NA release was investigated by comparing the effects of prolonged PMA treatment on NA release and PKC subtype expression. Prolonged treatment (48 h) with 100 nM PMA had no significant effect on K⁺-evoked release of NA from SH-SY5Y cells; however, carbachol-evoked release was increased more than 2-fold (Table 1). The stimulatory effects of acute PMA treatment were inhibited in 48 h-PMA-treated cells (Table 1), suggesting that PKC subtypes down-regulated by prolonged PMA treatment are involved in PMA-enhanced release, but not in normal depolarization- or carbachol-evoked release. These results correlate with studies on bovine adrenal medullary cells (Isosaki et al., 1991) and rat brain synaptosomes (Oda et al., 1991), whereas Burgoyne et al. (1988), also using bovine adrenal chromaffin cells, and Matthies et al. (1987), using PC12 cells, have shown that prolonged PMA exposure also inhibits release in the absence of acute PMA treatment.

The expression of PKC subtypes α and ϵ was markedly decreased after 48 h exposure of SH-SY5Y cells to 100 nM PMA. No change was observed in levels of PKC- ζ (Figure 4). These observations suggest a role for PKC- α and/or PKC- ϵ in PMA-enhanced K⁺- and carbachol-evoked NA release from SH-SY5Y cells. Previous studies on the regulation of exocytosis have suggested a role for PKC subtypes α and β . Naor and colleagues report that, of the four classical Ca²⁺-dependent PKC subtypes, PKC- α is most effective in reconstituting exocytosis in PMA-down-regulated pituitary and PC12 cells; in addition, PKC subtypes β_1 and β_{11} , but not PKC- γ , were able to reconstitute exocytosis to a lesser extent (Naor et al., 1989; Ben-Shlomo et al., 1991). Oda et al. (1991) also report that PKC- γ is not involved in the regulation of neurotransmitter release and that PKC- α and/or PKC- β may play a role in the regulation of PMA-enhanced K⁺-evoked NA release from synaptosomes.

Down-regulation of PKC subtypes by prolonged PMA treatment has been observed previously in studies on SH-SY5Y-cell differentiation. Leli et al. (1993) report that prolonged treatment with 10 nM PMA, the concentration that gives optimum differ-

entiation of SH-SY5Y cells, results in down-regulation of both PKC- α and PKC- ϵ . However, both Parrow et al. (1992) and Jalava et al. (1993a) have reported that there is no change in the expression of PKC- α under these conditions, but that prolonged exposure to 300 nM PMA (Jalava et al., 1993a) or 1.6 μ M PMA (Parrow et al., 1992), which results in poor cell differentiation, does lead to PKC- α down-regulation. Differentiation of human and mouse neuroblastoma cells with agents other than PMA has also been shown to result in decreased levels of mRNA encoding PKC- α (Wada et al., 1989; Tonini et al., 1991) and PKC- ϵ (Wada et al., 1989). A number of groups have studied expression of classical Ca²⁺-dependent PKC subtypes and shown that protein levels of PKC- α fall after chronic PMA exposure in human promyelocytic-leukaemia U937 cells (Strulovici et al., 1989), human leukaemic T cells (Isakov et al., 1990) and rat glioma 328 cells (Young et al., 1987). In addition, studies on WEHI-231 B lymphoma cells (Mizuguchi et al., 1988) and a human HL-60 cell sub-line (McSwine-Kennick et al., 1991) have shown that both PKC- α and PKC- β were down-regulated after prolonged PMA exposure.

Further studies on the role of PKC in NA release from SH-SY5Y cells were carried out using the selective PKC inhibitor RO 31-7549 (Davis et al., 1989; compound 2). Our initial results using this inhibitor suggested that both K⁺- and carbachol-evoked NA release were inhibited by 10 μ M RO 31-7549 (Murphy et al., 1992). Here we report that RO 31-7549 inhibits K⁺- and carbachol-evoked release in the absence of PMA by 27% and 68% respectively, and completely inhibits the enhancement of NA release by acute treatment with PMA (Table 2). These results indicate that PKC plays a major role in carbachol-evoked NA release, but that K⁺-evoked release is largely PKC-independent. These data also confirm that PMA-enhanced release is entirely due to activation of PKC. Previous reports have shown that the PKC inhibitors 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7), staurosporine and polymyxin B all inhibit phorbol-ester-enhanced depolarization-evoked neurotransmitter release in a variety of neuronal preparations (for review see Dekker et al., 1991). However, inhibition of depolarization-evoked release in the absence of phorbol esters varies considerably from 0 to 100% inhibition with these inhibitors, depending on the cell type and the PKC inhibitor used (Dekker et al., 1991).

In conclusion, our data on the effects of prolonged PMA treatment on PKC subtype expression and NA release, together with that on the inhibitory effects of RO 31-7549 on NA release, suggest that either or both of the PKC subtypes α and ϵ mediate PMA-enhanced K⁺- and carbachol-evoked NA release from SH-SY5Y cells.

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