Preferential release of catecholamine from permeabilized PC12 cells by α - and β -type protein kinase C subspecies

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Protein kinase C (PKC) is now recognized as comprising two groups of closely related subspecies. The PKC γ enzyme is apparently present only in central nervous tissues, and hence was expected to participate in neurotransmitter release. We have utilized a 'depletion-insertion' method to identify the PKCs participating in the exocytotic response. PC12 cells were 'down-regulated' by prior treatment (24 h) with phorbol 12-myristate 13-acetate (PMA; 1 μ M), which nearly abolished endogenous PKC activity. Down-regulated PC12 cells were loaded with [3 H]dopamine, permeabilized with digitonin, and recombinant or purified PKCs were inserted and activated with a low dose of PMA (20 nM). Among group A PKCs, PKC α was the most effective activator of [3 H]dopamine release (215%), followed by β II (185%) and β I (150%). PKC γ had no consistent effect on neurotransmitter release. PC12 cells express PKC α and PKC β , but not PKC γ , as revealed by Northern-blot analysis. We therefore postulate that PKC α and PKC β participate in neurotransmitter release, whereas PKC γ might be involved in other neuronal functions.

INTRODUCTION

Protein kinase C (PKC), a Ca $^{2+}$ /lipid-dependent serine and threonine kinase, plays a pivotal role in cell signalling [1]. PKC is recognized now as two families of closely related subspecies [1–4]. Although Group A (γ , β I, β II and α) contains five variable regions (V $_1$ –V $_5$) and four conserved regions (C $_1$ –C $_4$) and shows Ca $^{2+}$ /lipid-dependency, group B (δ , ϵ , ζ , η) lacks the C $_2$ region and displays only lipid-dependency [1–4]. The differential localization of PKCs suggests specific physiological role for a given subspecies [1,5–14]. Since PKC γ is present only in central nervous tissues [1,5–14], it was expected that the γ -enzyme might be involved in neurotransmission. On the other hand, since PKC α is found in all tissues and cells so far examined [1] and is the only subspecies found in some cell lines [1,15], it was thought that the α -enzyme might be involved in growth and differentiation (see ref. [1] for review). PC12 cells, a cloned rat pheochromocytoma cell line, expresses many of the characteristics of adrenal chromaffin cells and sympathetic adrenergic neurons [16]. PC12 cells are therefore regarded as an appropriate model to study neurotransmitter release. We have therefore utilized a 'depletion-insertion' method [17] to identify the PKCs of group A which might be involved in neurotransmitter release.

MATERIALS AND METHODS

Materials

3,4-Dihydroxy[8- 3 H]phenethylamine ([3 H]dopamine, 27 Ci/mmol; 1 Ci = 37 GBq) and [γ - 32 P]ATP (3000 Ci/mmol) were obtained from New England Nuclear. Phosphatidylserine (PS), calf thymus histone (type III-S), diolein, digitonin, phorbol 12-myristate 13-acetate (PMA; 'TPA') and all other chemical compounds were obtained from Sigma.

Cell culture

PC12 cells were maintained on collagen-coated 140 mm plastic (Sterilin) dishes in RPMI 1640 containing heat-inactivated

(56 °C, 45 min) 10% (v/v) horse serum and 5% (v/v) fetal-calf serum and also 100 units of penicillin/ml and 0.1 mg of streptomycin/ml. Cells [(1–2) $\times$ 10 6 /35 mm dish] were plated for 48 h before the experiment, as recently described [18].

Catecholamine release

PC12 cells were preincubated with [3 H]dopamine (2 μ Ci/ml) in RPMI medium containing 1 mM-ascorbic acid for 2 h at 37 °C. After the labelling, cells were washed four times (2 $\times$ 2 min + 2 $\times$ 20 min) in Ca $^{2+}$ -free buffered physiological saline (BPS), containing 10 mM-Pipes, 130 mM-NaCl, 1.2 mM-K $_2$ HPO $_4$, 2.5 mM-MgSO $_4$ and 11 mM-D-glucose, pH 7.2. After the wash procedure, cells were incubated with stimulants in BPS also containing 1.2 mM-CaCl $_2$ for various time periods, and 0.5 ml samples were collected and the radioactivity was measured. Cells were lysed with 0.1% SDS (1 ml), and 0.2 ml samples were counted for radioactivity for determination of total cell uptake. [3 H]Dopamine release was expressed as percentage of total radioactivity.

Cell permeabilization

PC12 cells were washed twice in BPS as above (2 $\times$ 2 min), followed by a wash in Dulbecco's phosphate buffer (PBS; 117 mM-NaCl, 2.7 mM-KCl, 6 mM-Na $_2$ HPO $_4$, 1.5 mM-KH $_2$ PO $_4$, pH 7.2) and another wash with PBS+1 mM-EGTA, both at 4 °C. Cells were then transferred to a high-K $^+$ medium (115 mM-KCl, 5 mM-NaCl, 5 mM-NaHCO $_3$, 1 mM-KH $_2$ PO $_4$, 20 mM-Hepes, 0.05% BSA, 1 mM-MgCl $_2$ and 20 μ M-digitonin) for 7 min at room temperature. Cells were then washed twice in high-K $^+$ buffer without digitonin at 4 °C (2 $\times$ 1 min). For further incubations, cells were transferred to a potassium glutamate buffer (KG), containing 120 mM-potassium glutamate, 20 mM-NaCl, 20 mM-Hepes, 0.1% BSA, 100 nM-Ca $^{2+}$, 200 μ M-MgATP, 5 mM-D-glucose, pH 7.0. The cells were incubated with various PKC subspecies in the presence of PMA (20 nM) for 15 min at 37 °C. At the end of the incubation period, samples were taken for determination of [3 H]dopamine release.

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate ('TPA'); PS, phosphatidylserine; BPS, buffered physiological saline; PBS, phosphate-buffered saline (Dulbecco's).

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Preparation of PKC subspecies

Group A PKCs were prepared by using recombinant baculovirus expression vector according to Summers & Smith [19]. Recombinant viruses containing the cDNAs for bovine α and γ and human β I and β II PKCs were constructed as recently described [20]. Viruses were used to infect insect cell suspension cultures. Recombinant proteins were purified as described elsewhere [20], dialysed into 50 mM-Tris/HCl (pH 7.5)/2 mM-EDTA/1 mM-dithiothreitol/0.02% Tween 20/50% (v/v) glycerol and stored at -80°C [20]. Group A PKCs (γ , β and α) were also partially purified from 30 adult male rat brains as previously described [17,21].

Preparation of PKC

Cultures were washed three times with cold PBS, followed by addition of homogenization buffer (1 ml/90 mm plate): 20 mM-Tris/HCl, pH 7.5, containing 0.25 M-sucrose, 10 mM-EGTA, 2 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride and 20 μg of leupeptin/ml. The cells were scraped from the plates with a rubber policeman, transferred to Eppendorff tubes [(4–5) $\times 10^6$ cells/tube] and sonicated for 30 s at 4°C . The sonicated preparations were incubated with Triton X-100 (0.5%) for 30 min at 4°C with shaking to release the membrane-associated PKC. The samples were centrifuged for 15 min at 10000 g and the supernatant was applied to a mini-column of DEAE-cellulose (DE-52) equilibrated with 20 mM-Tris/HCl, pH 7.5, containing 0.5 mM-EGTA, 0.5 mM-EDTA and 10 mM-2-mercaptoethanol (Buffer A). After loading the samples, the columns were washed with 4 vol. of Buffer A, and then with 4 vol. of Buffer A containing 20 mM-NaCl. PKC was eluted with 4 vol. of Buffer A containing 120 mM-NaCl. Samples (30 μl ; ~ 2 –3 μg of protein) were assayed for PKC activity as previously described [17,21].

PKC assay

The reaction mixture (0.25 ml) contained 5 μmol of Tris/HCl, pH 7.5, 1.25 μmol of magnesium acetate, 50 μg of histone (III-S), 2.5 nmol of [γ - ^{32}P]ATP [(5–15) $\times 10^4$ c.p.m./nmol], 8 μg of PS/ml, 0.8 μg of diolein/ml, 0.3 mM- Ca^{2+} and enzyme preparation. The assay was carried out for 20 min at 30°C . The reaction was stopped by adding 25% trichloroacetic acid and collecting the acid precipitate on membrane filters (0.45 μm pore), followed by counting the samples for radioactivity by the Čerenkov method. Basal activity was measured in the presence of 0.3 mM- Ca^{2+} and was subtracted from the total activity.

Isolation of RNA

Total cellular RNA was extracted from cells by vigorous homogenization in 5 mM-guanidinium thiocyanate/50 mM-Tris/HCl (pH 7.5)/10 mM-EDTA/8% 2-mercaptoethanol. Subsequently RNA was isolated by direct precipitation with 2.5 M-LiCl.

Northern blotting and hybridization

Total RNA (25 μg /lane) was fractionated on 1%-agarose gel containing 6.5% formaldehyde and 1 μg of ethidium bromide/ml, as described elsewhere [18]. Gels were treated with 50 mM-NaOH for 30 min, followed by treatment for 45 min with 1.5 M-NaCl/0.5 mM-Tris/HCl (pH 7.5) and blotted on to Gene Screen (New England Nuclear) by using 10 $\times$ SSC buffer (1 $\times$ SSC = 150 mM-NaCl/15 mM-sodium citrate). The positions and intensities of the 18 S and 28 S rRNAs were directly observed on the filter under u.v. after transfer. These bands served as internal

molecular-mass standards. Membranes were prehybridized at 42°C in a solution containing 50% (v/v) formamide, 6 $\times$ SSC, 1 $\times$ Denhardt's solution, 50 mM-Tris/HCl (pH 7.5), 1% SDS and 200 μg of salmon sperm DNA/ml. Blots were then hybridized overnight, at 42°C with ^{32}P -labelled nick-translated probes (sp. radioactivity approx. 5×10^8 c.p.m./ μg) in the same solution. The membranes were washed in 2 $\times$ SSC/0.1% SDS at room temperature, followed by two washes in 2 $\times$ SSC/1% at 68°C and two washes in 0.2 $\times$ SSC/0.2% SDS at 68°C . The blots were exposed to Agfa Curix RP2 X-ray films at -80°C with intensifying screens for 18 h.

DNA probes

That for PKC α is a 1300 bp *Eco*RI fragment derived from clone PKC α -7, that for PKC β a 1700 bp *Eco*RI fragment derived from clone PKC β -15, and that for PKC γ is a 1400 bp *Eco*RI fragment derived from clone PKC γ -6. The probes were obtained from A.T.C.C./N.I.H. (American Type Culture Collection, Rockville, MD, U.S.A.).

RESULTS

Effects of PMA and K^+ on [^3H]dopamine release

Prelabelled PC12 cells were incubated with increasing concentrations of PMA, a known PKC activator. Maximal [^3H]dopamine release was noticed at about 100 nM-PMA. Dose-response studies for K^+ evoked release revealed maximal response at 50 mM. Both stimuli exerted significant and consistent exocytotic responses at 20–30 min of incubation (results not shown).

Synergistic action of PMA and K^+ on [^3H]dopamine release

To evaluate the relative contributions of Ca^{2+} and PKC to the exocytotic response, PC12 cells were incubated with K^+ (50 mM) or with PMA (100 nM) (Fig. 1). Both stimuli on their own were able to elicit a significant response (PMA 1.5–2-fold, $P < 0.01$;

Fig. 1. Synergistic action of PMA and K^+ on dopamine release from PC12 cells

[^3H]Dopamine-preloaded cells were washed as described in the Materials and methods section and incubated for 30 min in BPS (Ca^{2+} , 1.2 mM) in the absence or presence of PMA (100 nM) or K^+ (50 mM) or both. Results (mean $\pm$ S.E.M.) represent percentage of content: ** $P < 0.01$ (Student's t test).

Fig. 2. Down-regulation of PKC by PMA in PC12 cells

Cells were incubated for 24 h in the presence of increasing concentrations of PMA. Cells were then washed three times in cold PBS, transferred to homogenization buffer, and total PKC activity was determined after extraction with 0.5% Triton X-100 and DE-52 chromatography of each sample as described in the Materials and methods section. Results are means of duplicate determinations.

Fig. 3. Effect of digitonin on PC12-cell permeabilization

PC12 cells were permeabilized by digitonin (20 µM) in high-K⁺ buffer at room temperature for the time points indicated. At the end of each incubation, cells were washed twice in high-K⁺ buffer without digitonin at 4 °C, and the degree of permeabilization was determined by Trypan Blue exclusion. ▲, Intact cells; ●, cells which took up Trypan Blue and were permeabilized. Results are means of duplicates.

K⁺ 2.5-fold, $P < 0.01$) (Fig. 1). However, the combined treatment with both K⁺ and PMA elicited a synergistic response (6-fold) (Fig. 1). Hence the simultaneous activation of the bifurcating inositol lipid pathway seems to be required for maximal activation of neurotransmitter release from PC12 cells.

'Down-regulation' of endogenous PKC activity by PMA

To remove the contribution of endogenous PKC activity, PC12 cells were incubated for 24 h with increasing concentrations of PMA. As shown in Fig. 2, concentrations above 500 nM-PMA induced near-complete loss of total PKC activity in PC12 cells

Fig. 4. Effect of digitonin on PKC release from PC12 cells

Cells were permeabilized by digitonin (20 µM) in high-K⁺ buffer at room temperature for the time indicated. At the end of the incubation period, the medium and cells were separately transferred to homogenization buffer for PKC activity determination as described in the Materials and methods section. PKC activity was determined after partial purification of each sample on DE-52 minicolumns. The PKC assay was carried out in the presence of Ca²⁺ alone (0.3 mM) or in the presence of Ca²⁺ (0.3 mM), PS (8 µg/ml) and diacylglycerol (0.8 µg/ml) as described in the Materials and methods section. Results from duplicate dishes represent the difference between the two activities (Ca²⁺/PS/diacylglycerol – Ca²⁺). ▲, PKC activity in the medium (right ordinate); ●, total cellular activity after Triton X-100 extraction and chromatography (left ordinate).

(50% inhibition at ~100 nM-PMA). A minimum of 12–24 h of incubation with PMA was required to achieve effective 'down-regulation'.

Effect of digitonin on cell membrane permeability

To render the plasma membrane permeable to proteins, we treated PC12 cells with digitonin, which was shown by others to permeabilize PC12 cells [22,23]. About 90% of control PC12 cells excluded Trypan Blue, and the number decreased to 10% after 7 min of treatment with digitonin (20 µM; Fig. 3). In parallel, the number of cells stained with this low-molecular-mass charged nuclear stain increased as the cells became permeable (Fig. 3).

Effect of digitonin on endogenous PKC

A second parameter for plasma-membrane permeabilization by digitonin was the release of endogenous PKC. As shown in Fig. 4, the release of endogenous PKC in digitonin-permeabilized cells occurs in a time-dependent manner similar to the time course described for chromaffin cells [24], but different from cultured rat pituitary cells, where the process was faster [17]. Nevertheless PKC activity recovered in the supernatant of the permeabilized cells represented only a fraction of that lost, suggesting degradation of the released enzyme.

Insertion of PKC subspecies to permeabilized cells

Down-regulated digitonin-permeabilized PC12 cells were then used for evaluating the exocytotic response of various PKCs (Fig. 5). Recombinant PKCs expressed in insect cells were inserted at similar activities into the permeabilized cells, activated with low concentration of PMA, and [³H]dopamine release was monitored. Addition of PMA (20 nM) on its own to the permeabilized cells exerted no stimulatory effect, indicating the lack of active endogenous PKCs in the preparation. PKCα was the most effective activator of [³H]dopamine release (215%, $P < 0.01$), followed by PKCβII (185%, $P < 0.01$) and PKCβI (150%, $P < 0.05$). PKCγ showed no consistent effect. The stimulatory

Fig. 5. Effect of group A PKC subspecies on dopamine release from down-regulated digitonin-permeabilized PC12 cells

The recombinant PKC subspecies were prepared by a baculovirus insect cell expression system. For insertion studies the cells were first down-regulated for 24 h with PMA (1 μ M) as described above. Cells were then loaded with [³H]dopamine for 2 h and washed several times as described in the Materials and methods section. Cells were then permeabilized by digitonin (20 μ M, 7 min, room temperature) and further washed twice in high-K⁺ buffer without digitonin at 4 °C. Cells were then incubated in KG buffer with the various PKC subspecies (similar activities were added, about 0.4–1 unit of PKC of each) in the presence of PMA (20 nM). Incubation was carried out for 15 min at 37 °C, and [³H]dopamine release was determined as percentage of content. * P < 0.05, ** P < 0.01 (treatment versus PMA alone); α versus β II, P < 0.01; β II versus β I, P < 0.01; PMA versus control, not significant.

Fig. 6. Dose-response of PKC-subspecies-induced dopamine release from down-regulated digitonin-permeabilized PC12 cells

The PKC subspecies were prepared from rat brain as described in the Materials and methods section. Radioactivity added was 6725, 13580 and 6823 c.p.m./10 μ l of enzyme for PKC- α , - β and - γ respectively. Depletion and insertion were carried out as described in Fig. 5 legend.

PKCs had no effect on [³H]dopamine release from intact PC12 cells, and no stimulatory effect was noticed in down-regulated permeabilized cells in the absence of PMA (results not shown). To rule out the possibility that the rank order resulted from the fact that the enzymes were expressed in insect cells, we prepared PKCs from rat brains as previously described [17,21]. A mixture of PKC β I and PKC β II was used, since they are eluted together from the hydroxyapatite column [21]. A dose-response study of

Fig. 7. Northern-blot analysis of group A PKC subspecies in PC12 cells

mRNA was extracted from PC12 cells and from rat brain for control. Blot analysis was performed as described in the Materials and methods section. Autoradiogram from a representative experiment is shown here.

[³H]dopamine release induced by partially purified PKCs is shown in Fig. 6. As with the recombinant enzymes, PKC α was a more effective activator of exocytosis compared with a mixture of PKC β I and PKC β II from rat brain, whereas PKC γ was inactive (Fig. 6).

Northern-blot analysis of PKCs in PC12 cells

Northern-blot analysis was then used to identify the members of group A PKCs which are expressed in PC12 cells. The blot analysis confirmed the presence of the PKC α and PKC β subspecies in this cell line (Fig. 7). No mRNA for PKC γ was found, in agreement with the claim that the γ -enzyme is brain-specific [1].

DISCUSSION

Although PKC has been regarded as a single entity, the isolation of its cDNAs unravelled a large family of closely related subspecies (see [1–14,25–28]).

The monomeric multifunctional PKC consists of five variable regions (V₁–V₅) and four conserved regions (C₁–C₄). Group A (α , β I, β II, γ) contains all the regions mentioned above and shows variable responsiveness to the classical activators of PKC, namely Ca²⁺, PS, diacylglycerol and phorbol esters such as PMA [1–4]. Group B PKC subspecies (δ , ϵ , ζ , η) do not contain the C₂ region, and subspecies ζ contains only the first cysteine-rich tandem of two such sequences present in the C₁ region of the N-terminal regulatory domain [1–3]. Members of group B do not show Ca²⁺-dependence, suggesting that the C₂ regulatory region contains a Ca²⁺-binding site. It is the members of group A PKCs that were examined here for their potential involvement in neurotransmitter release.

The use here of the baculovirus expression system [19,20,26,27] has enabled us to study the effect of pure PKCs, since conventional purification methods of group A PKCs might also include some members of group B [28]. Nevertheless, some key experiments were repeated with purified enzymes, to avoid the possibility that the PKCs were modified by the insect cells.

Several investigators have utilized PC12 cells as a model for exocytosis (e.g. [16,18,22–24,29,30]). Data were presented demonstrating that both Ca²⁺ and PKC were involved in catecholamine release from the cells. Furthermore, both messengers seem to be acting in a synergistic fashion [30]. Thus the present paper supports previous findings and extends the studies to address the problem of PKC subspecies. Digitonin-permeabilized PC12 cells were also used previously to demonstrate Ca²⁺-induced exocytosis of catecholamine [23]. Furthermore, when chromaffin cells were permeabilized with

digitonin, about 80 % of endogenous PKC activity was lost after 60 min of incubation [24]. The above observations prompted us to adopt a 'depletion-insertion' method [17] to examine the role of specific PKCs in catecholamine secretion from PC12 cells. Since PKC γ is present only in central nervous tissues [1–14,31,32], it was expected that the brain PKC might specialize in neurotransmitter release. On the other hand, several cell lines express only PKC α [1,15], hence it was expected that PKC α would be involved in growth and transformation. Therefore the findings reported here are surprising, since PKC α was found to be the best candidate to reconstitute the exocytotic response elicited by PMA. The physiological relevance of the data might be applied to activation of muscarinic receptors by acetylcholine. On the other hand, PKC γ had no consistent and significant effect on catecholamine secretion, and therefore might be involved in other neuronal functions. Interestingly, PKC α -overexpressing cell lines did not display a transformed phenotype, but exhibited enhanced growth rates under low serum conditions and decreased epidermal-growth-factor (EGF) receptor numbers [33]. Also, point mutations in the primary structure of PKC α have been recently found in a murine u.v.-induced fibrosarcoma cell line [34]. Expression of the mutated PKC α in 3T3 fibroblasts induced cell transformation capable of producing solid tumours when inoculated into nude mice [34]. It is therefore possible that normal PKC α is involved in exocytosis or other functions such as activation of phospholipase A₂ [35], whereas mutation of the enzyme is required for transformation. Overexpression of PKC γ and PKC β in NIH/3T3 and Rat1 cells showed some enhanced tumorigenicity and decreased dependence on serum for sustained growth, but was not sufficient to achieve the typical characteristic of malignantly transformed rodent fibroblasts [36,37]. Also overexpression of PKC β in C3H/10T1/2 cells failed to induce growth in soft agar or to induce tumours in nude mice [38]. Thus, although overexpression of PKCs might be associated with some tumours, the collective data suggest that this is not sufficient to induce transformation.

Diversity of responses, which is the nature of the PKC family, might be achieved by differential expression of PKC subspecies in a tissue or in specific cell types [1–15]. Alternatively, the regulatory control mechanism might differ for the various PKCs, such as activation of PKC γ by arachidonate [21,27,39,40] and the insensitivity of group B PKCs to Ca²⁺ [1–3,26]. The present paper exemplifies a third mechanism, namely that specific PKCs activate different cellular pathways.

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