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Induction of exocytosis in permeabilized pituitary cells by α- and β-type protein kinase C

(hormone release/permeabilized cells)

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ABSTRACT **Protein kinase C is now recognized to comprise a family of closely related subspecies (PKCs). When cultured rat pituitary cells were permeabilized by digitonin for 5 min in the absence of Ca^{2+}, endogenous PKC activity was decreased by 72%. PKC depletion was also achieved by prior treatment (24 hr) with high concentrations of phorbol 12-myristate 13-acetate (PMA). When purified activated brain PKCs were added for 30 min to PMA-pretreated, digitonin-permeabilized cells, only α- and β- but not γ-type PKC stimulated luteinizing hormone release. Since PKC was implicated as a mediator of gonadotropin secretion, gonadotropin-releasing hormone might utilize α- and β-type PKCs for stimulation of gonadotropin secretion; α- and β-type PKCs might participate also in other exocytotic responses in diverse biological systems in which PKC was implicated.**

Protein kinase C (PKC), which has been regarded as a single enzymatic activity, is now recognized as a family of closely related subspecies (PKCs; refs. 1–12). Three subspecies can be separated from rat brain by biochemical means (called types I, II, and III; ref. 13), corresponding to brain γ-, β-, and α-type PKC (PKCγ PKCβ, and PKCα), respectively, as evident by expression in COS cells transfected with the respective cDNAs (14, 15). Rat brain type I enzyme (697 amino acids) is encoded by the γ-type cDNA sequence. Type II is an unequal mixture of two subspecies determined by βI cDNA and βII cDNA sequences (671 and 673 amino acids, respectively), which differ in the C-terminal end region of about 50 amino acid residues. βI and βII cDNAs result from alternative splicing of a common mRNA transcript. Type III (672 amino acids) is encoded by an α-type cDNA sequence (14, 15). Differential expression of PKCs in various tissues so far examined demonstrated that PKCγ is predominantly present in central nervous tissues, PKCβ is present in most tissues, while PKCα is present in all tissues examined but is the only subspecies present in several cell lines (1, 16–22). The specific role of the PKCs is not yet known.

In this study we attempt to demonstrate direct activation of an exocytotic response [luteinizing hormone (LH) release] by insertion of purified brain PKCs to permeabilized rat pituitary cells that specialize in exocytosis.

MATERIALS AND METHODS

Materials. Phosphatidylserine (bovine brain), calf thymus histone (type III-S), phorbol 12-myristate 13-acetate (PMA), and diolein were from Sigma. [γ-^{32}P]ATP (>5000 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham.

Cellular Studies. Anterior pituitary cells were prepared from Wistar-derived female rats (21 days old) as described (23). For digitonin treatment, the cells (10^7 per tube) were washed with cold Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffer with 0.1% bovine serum albumin, followed by another wash in the same buffer containing also EGTA (1 mM). The cells were then permeabilized with digitonin (16 μM) in high-K^+ buffer (20 mM Hepes/5 mM NaCl/115 mM KCl/5 mM $NaHCO_3$/1 mM KH_2PO_4/1 mM $MgCl_2$/0.05% bovine serum albumin) for various time periods at 37°C, followed by two washes in the same buffer without digitonin. The cells were then homogenized for PKC determination as described below. For down-regulation, pituitary cells (5×10^6 per dish) were cultured for 4 days in medium 199 containing 5% horse serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml, with or without 1 μM PMA for the last 24 or 48 hr of the culture period.

Cells were then washed five times with cold phosphate-buffered saline and transferred (10^7 per tube) by rubber policeman to tubes containing 1 ml of homogenization buffer (20 mM Tris·HCl, pH 7.5/0.25 M sucrose/10 mM EGTA/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/20 μg of leupeptin per ml). The cells were homogenized, and the homogenate was sonicated for 30 s at 4°C and incubated with 0.5% Triton X-100 for 30 min at 4°C to release the membrane-associated PKC. The homogenate was then centrifuged for 10 min at 100,000 × g (Beckman Airfuge, 207 kPa), and the supernatant was applied to a minicolumn of DE-52 cellulose 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). The column was washed with 4 volumes of buffer A and then with 4 volumes of buffer A containing 20 mM NaCl. PKC was eluted with 4 volumes (≈400 μl) of buffer A containing 120 mM NaCl. Samples of 20 μl (≈2 μg of protein) were subjected to the assay of PKC activity as described by Kikkawa *et al.* (15). 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 (type III-S), 2.5 nmol of [γ-^{32}P]ATP ($5–15 \times 10^4$ cpm/nmol), 8 μg of phosphatidylserine per ml, 0.8 μg of diolein per ml, 0.3 mM Ca^{2+}, and the enzyme preparation. Basal activity was measured in the presence of 0.3 mM Ca^{2+} and was subtracted from the total activity. The assay was carried out for 20 min at 30°C. The reaction was stopped by the addition of 25% trichloroacetic acid; the acid-precipitate was collected on membrane filters (0.45 μm), and the samples were assayed by the Cerenkov method.

PKCs were prepared from 30 adult male rat brains as described by Kikkawa *et al.* (15). Immunoblot (Western) analysis with type-specific antibodies confirmed the identity

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Abbreviations: PKC, Ca^{2+}-activated, phospholipid-dependent protein kinase C; PKCα, PKCβ, and PKCγ, α-, β-, and γ-type PKC; PKCs, protein kinase C subspecies; PMA, phorbol 12-myristate 13-acetate; LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone.

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of the various subspecies as described elsewhere (17, 24). The enzymes were kept in small aliquots at −70°C until used.

For PKC insertion, pituitary cells (3×10^5 per dish) were cultured for 4 days as above and treated with or without 1 μM PMA for 24 hr. Cultured cells were then washed twice with medium 199 containing 0.1% bovine serum albumin and left intact or further treated by a wash in ice-cold Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffer with 0.1% bovine serum albumin and a last wash in the same buffer containing also 1 mM EGTA. Cells were then permeabilized with 16 μM digitonin for 5 min at 37°C in high-K^+ buffer as described above and washed twice at 4°C in the same buffer without digitonin. The cultured permeabilized cells were then exposed to PKC subspecies (60 ng in 2.5 μl) or the other stimulants and incubated in glutamate buffer (120 mM postassium glutamate/20 mM NaCl/20 mM Hepes/5 mM glucose/0.1% bovine serum albumin/200 μM Mg ATP/100 nM free Ca^{2+}), pH 7.0, for 30 min at 37°C, and LH released to the medium was determined by using the reagents supplied by the National Institute of Diabetes and Digestive and Kidney Diseases.

RESULTS

When cultured rat pituitary cells were permeabilized by digitonin, a marked loss of endogenous cytosolic and membrane-bound PKC activity was noticed (72% in 5 min, Fig. 1). A similar protocol, albeit for 60 min, was recently described as an effective means for PKC release (80%) from chromaffin cells (25). Since prolonged digitonin treatment (60 min) was not much more effective in our system, a short protocol (5 min) was used. The loss of enzymatic activity was also accompanied by loss of response of the cells to stimulation by PMA (Fig. 2) and gonadotropin-releasing hormone (GnRH), as assessed by LH release (Table 1). Full inactivation of endogenous PKC activity could be obtained upon incubation of normal cells with 1 μm PMA for 24 or 48 hr to down-regulate the enzyme (26) (Fig. 1).

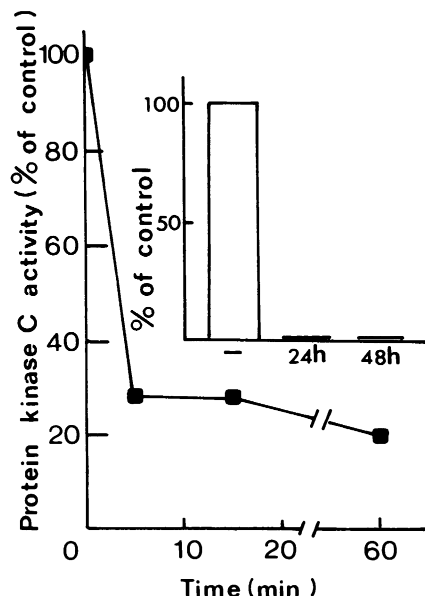

FIG. 1. Loss of PKC activity in pituitary cells permeabilized by digitonin or incubated with 1 μM PMA (*Inset*). Anterior pituitary cells (10^7 per tube) were permeabilized by 16 μM digitonin for the time (in minutes) indicated or cultured for 24–48 hr with 1 μM PMA as described. Cells were then homogenized, and total PKC activity was determined after 0.5% Triton X-100 extraction and DE-52 chromatography as described. Basal activity (100%) amounted to 2.7 pmol of ^{32}P per μg of protein per min.

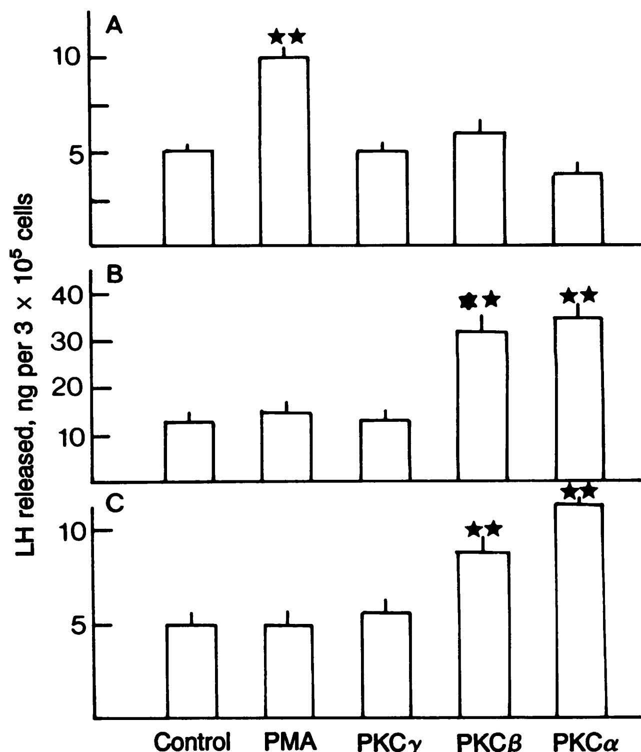

FIG. 2. Effect of PKC subspecies on LH release from permeabilized cultured pituitary cells. PKC subspecies were prepared from adult male rat brains as described. For insertion studies the cultured cells (3×10^5 per dish) were treated with 1 μM PMA at 37°C (*C*) or without PMA for 24 hr (*A* and *B*). The cultured cells were then left intact (*A*) or permeabilized by 16 μM digitonin treatment for 5 min (*B* and *C*) at 37°C in high-K^+ buffer as described. The cultured permeabilized cells were then washed and further incubated in glutamate buffer. The normal cells (*A*) were incubated in medium 199 containing 0.1% bovine serum albumin. The stimulants were PMA (100 ng/ml) and PKC subspecies (2.5 μl; ≈60 ng of enzyme activated by PMA at 100 ng/ml). Incubation was continued for 30 min at 37°C, and medium was then collected and stored at −20°C until assayed for LH by RIA. Results are expressed in terms of the RP-2 rat reference preparation of the National Institute of Diabetes and Digestive and Kidney Diseases. Results shown here (mean ± SEM) are taken from five experiments performed in triplicate. **, $P < 0.01$.

To identify the exocytotic PKC subspecies in our system, we used PMA-pretreated (1 μM for 24 hr), digitonin-permeabilized (5 min) cells. When purified brain PKCs (Table 2) were added to digitonin-permeabilized cells (Fig. 2*B*) or to down-regulated, digitonin-permeabilized cells (Fig. 2*C*) and the PKCs were activated by PMA at 100 ng/ml, only PKCα and PKCβ but not PKCγ stimulated LH release from the permeabilized cells ($P < 0.01$) whether or not they were down-regulated. Similar results were obtained when equivalent total stimulated activities of the various PKCs were

Table 1. Effect of GnRH on LH release in normal and digitonin-permeabilized cultured pituitary cells

Treatment	LH released, ng per dish	
	Normal cells	Digitonin-treated cells
Control	3.95 ± 0.17	5.5 ± 0.7
GnRH (100 nM)	16.3 ± 2.8**	4.6 ± 0.6

Cultured pituitary cells (3×10^5 cells per dish) were left intact (normal cells) or permeabilized by 16 μM digitonin (5 min at 37°C) as described. The cultured cells were washed and further incubated with 100 nM GnRH for 30 min at 37°C in medium 199 containing 0.1% bovine serum albumin (normal cells) or in glutamate buffer (digitonin-treated cells). Medium LH content was determined by RIA.
**$P < 0.01$.

Table 2. Protein kinase C subspecies activity

Treatment	Protein kinase C activity, cpm		
	Type I (γ)	Type II (β)	Type III (α)
Ca^{2+}	7,685	3,630	2,392
Ca^{2+} + PS + DG	14,531	29,628	10,392

PKC subspecies were prepared from adult male rat brains. Samples of 2.5 μl ($\approx$60 ng) were used for PKC activity determination in the presence of Ca^{2+} alone (0.3 mM) or in the presence of Ca^{2+} (0.3 mM), phosphatidylserine (PS; 8 μg/ml), and diolein (DG; 0.8 μg/ml). The assay was carried out for 20 min at 30°C as described in Fig. 1.

added. Also, similar results were obtained when PKCα and PKCβ but not PKCγ were stimulated with GnRH. The stimulatory effect of PKCα and PKCβ upon LH release was dose-dependent both for the amount of added enzyme and for PMA (data not shown). PMA at 100 ng/ml stimulated LH release from normal cells (Fig. 2*A*; $P < 0.01$) but not from digitonin-permeabilized cells or from down-regulated cells, ruling out the possibilities that the effect observed with activated PKCα and PKCβ was induced by PMA alone or contributed by endogenous residual PKCs. When brain PKCs were added to normal cells, no stimulation of LH release was observed (Fig. 2*A*). When the enzymes were added with PMA, the stimulation observed was due to PMA (Fig. 2*A* and data not shown). Addition of the PKCs to the permeabilized cells in the absence of PMA showed no consistent stimulatory effect upon gonadotropin secretion.

DISCUSSION

Although PMA pretreatment down-regulated endogenous PKC activity better than digitonin (Fig. 1), exocytosis induced by GnRH was not abolished by PMA, providing the cells were not treated further with digitonin (Table 1 and data not shown). On the other hand, PMA pretreatment abolished PMA-induced LH release, and digitonin permeabilization abolished both PMA- and GnRH-induced LH release (Table 1 and Fig. 1). The results imply that digitonin permeabilization is accompanied by loss of exocytotic components in addition to PKC. Furthermore, GnRH stimulation of gonadotropin secretion is most likely mediated by PKC (27–32) and by other messenger molecules such as Ca^{2+} and arachidonic acid and/or its metabolites as discussed elsewhere (33–37). However, since the exocytotic response elicited by PMA in normal cells (Fig. 2*A*) is later restored in its magnitude by exogenous PKCs (Fig. 2 *B* and *C*), the results suggest that the ability of PKCs to cause LH secretion is not dependent on the presence of other cellular components lost during digitonin permeabilization. This demonstrates direct activation of an exocytotic response elicited by PKCs.

In view of the proposal that PKCγ is present predominantly in central nervous tissues (1, 16–19, 21, 22) and the lack of response observed here, it is possible that the "brain PKC" specializes only in neuronal functions. However, this assumption seems now to be complicated because overproduction of PKCγ in transfected NIH 3T3 cells enhanced tumorigenicity and reduced dependence on serum for sustained cell growth (38). Nevertheless, the altered cell growth regulation observed in the transfectant lines were much less pronounced as compared with *ras*-transformed cells, suggesting that PKCγ does not specialize in growth regulation and tumorigenicity. Also, although PKCγ is not detectable in the human promyelocytic leukemia cell line HL-60, exposure of the cells to dimethyl sulfoxide or retinoic acid, which are known to cause differentiation in this cell line, resulted in the appearance of the γ subspecies (39). The results suggest that PKCγ, which is found in abundance in the brain, might be expressed under certain conditions in some other cell types during differentiation. More surprising is the finding reported here that PKCα can elicit exocytotic response in pituitary cells. Several cell lines express only PKCα (1, 20); therefore, the enzyme was implicated in growth and differentiation. Indeed PKCβ was found here to stimulate LH release as expected, but its effect was less marked and less consistent as compared with PKCα. In this regard, it was recently reported that overproduction of βI-type PKC in rat fibroblasts conferred multiple growth abnormalities but was not sufficient to achieve the typical characteristic of malignantly transformed rodent fibroblasts (40). The type II enzyme (PKCβ) prepared from brain is a mixture of βI (12%) and βII (88%) (17). The two enzymes are encoded by a single gene via alternative splicing of a common RNA transcript, and they differ only in a short range of about 50 amino acid residues at the C-terminal region (1, 5, 6, 11, 14). Since the pituitary most likely expresses only the βII and PKCα subspecies (24), it is possible that the exocytotic response observed here was elicited by the βII enzyme.

Our results presented here and the transfection studies reported recently by others (38–40) argue that a physiological or a pathological task might be executed by more than one subspecies. Therefore, it is possible that specific activation of a given PKC subspecies is achieved by alternative biochemical pathways such as phospholipase C, phospholipase A_2, and others not yet clarified. Indeed, PKCγ can be markedly activated by low and possibly physiological concentrations of arachidonic acid in the absence of Ca^{2+}, phosphatidylserine, and diolein (24). The results also imply that the specific role of the various PKCs should be separately examined in each biological system. The combination of PMA and digitonin treatment described here can be applied to other models of exocytosis.

Interestingly, whereas in the brain PKCα is a relatively minor enzyme, representing about 25% of total PKC activity (17), in the pituitary it is the most abundant subspecies (60%) (24). It is possible that PKCα is overexpressed in the pituitary because exocytosis is the main task of the gland.

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