Biochemical Pharmacology, Vol. 52, pp. 569–577, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00308-5

Differential Modulation of Protein Kinase C Isozymes in Rat Parotid Acinar Cells

RELATION TO AMYLASE SECRETION

*A. Roger Terzian, Xuejun Zhang and Ronald P. Rubin**

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY, SCHOOL OF MEDICINE AND BIOMEDICAL SCIENCES, STATE UNIVERSITY OF NEW YORK AT BUFFALO, BUFFALO, NY 14214, U.S.A.

ABSTRACT. We investigated the expression, distribution, and activation parameters of protein kinase C (PKC) isozymes in isolated rat parotid acinar cells. By analyzing cellular extracts by western blot analysis and for isozyme-specific RNA, the Ca^{2+}independent PKC-δ, -ϵ, and -ζ were detected in the cytosolic, particulate (plasma membrane), and nuclear fractions of unstimulated cells, whereas the Ca^{2+}-dependent PKC-α was confined to the cytosolic and particulate fractions. The expressed isozymes showed distinct responses to phorbol 12-myristate 13-acetate (PMA), thymeleatoxin, and cell surface receptor agonists with respect to translocation from cytosol to particulate fraction and nucleus, as well as sensitivity to down-regulation caused by prolonged exposure to PMA (3–20 hr). The marked susceptibility to down-regulation displayed by PKC-α and -δ was accompanied by an enhanced secretory response to norepinephrine as compared with control cells. Further, the selective PKC inhibitors Ro 31-8220 and CGP 41 251 also produced a concentration-dependent enhancement of norepinephrine-induced amylase secretion. Our findings suggest that PKC-α or -δ plays a negative modulatory role, rather than an obligatory role, in amylase secretion. Also, the localization and redistribution of PKC-ϵ and -δ to the nucleus by PKC activators imply that one or both of these isozymes may regulate such processes as cellular proliferation and/or differentiation. BIOCHEM PHARMACOL 52;4:569–577, 1996.

KEY WORDS. protein kinase C; parotid acinar cells; amylase secretion; phorbol esters; substance P; protein kinase C inhibitors; isozyme-specific mRNA

The rat parotid gland, like most other tissues, expresses PKC† activity [1, 2]. PKC represents a family of serine/threonine kinases that phosphorylates numerous proteins that modulate diverse cellular processes, including ion fluxes, differentiation, and secretion [3]. The Ca^{2+}-dependent PKCs, designated as cPKCs (PKC-α, -β, and -γ), are activated by Ca^{2+}, DAG, and phosphatidylserine. nPKC isozymes, which exhibit Ca^{2+}-independent activity, include PKC-δ and -ϵ. The so-called atypical PKCs, such as PKC-ζ, display enzyme activity that is not regulated by DAG or phorbol esters [4]. PKC isozymes differ in their tissue and cellular distributions, substrate specificities, and responsiveness to various agonists [5–7]. Differences in the properties of individual isozymes presumably reflect diversity in their respective functions [7].

We previously demonstrated that rat parotid acinar cells express PKC-α, but not PKC-β or -γ [2]. We further showed that agonist stimulation is associated with the Ca^{2+}-dependent redistribution of PKC-α to the particulate fraction [2]. The present study extends this work by demonstrating the expression of the nPKCs -δ and -ϵ and the atypical PKC-ζ in parotid cells. The differential responsiveness of these isozymes to activators of PKC is also compared. Heterogeneity exhibited by PKC isozymes in terms of their subcellular localization, regulation by agonists, and susceptibility to down-regulation may provide a basis for ultimately explaining their differential regulation of cellular processes, including amylase secretion, cell growth, and/or differentiation.

MATERIALS AND METHODS

Materials

PMA, 4α-phorbol, and TX were from LC Services, Waltham, MA. Collagenase was obtained from Worthington Biochemicals, Freehold, NJ. Chemiluminescence reagents were purchased from either the Amersham Corp., Arlington Heights, IL, or DuPont-NEN, Boston, MA, and the supported nitrocellulose membranes were from Schleicher & Schuell, Keene, NH. The monoclonal antibodies for PKC-α, -ϵ, and θ were obtained from Transduction

* Corresponding author: Dr. Ronald P. Rubin, Department of Pharmacology and Toxicology, State University of New York at Buffalo, School of Medicine, 102 Farber Hall, Buffalo, NY 14214. Tel. (716) 829-3048; FAX (716) 829-2801.

† *Abbreviations:* PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TX, thymeleatoxin; NE, norepinephrine; and RT-PCR, reverse transcriptase-polymerase chain reaction; DAG, diacylglycerol; cPKC, Ca^{2+}-dependent PKC; nPKC, Ca^{2+}-independent PKC; and CCh, carbachol.

Received 22 November 1995; accepted 5 March 1996.

Laboratories, Lexington, KY, and the polyclonal antibodies for PKC-δ and -ζ were from either Gibco BRL, Grand Island, NY, or Oxford Biomedical Research, Oxford, MI, respectively. Sheep anti-mouse and goat anti-rabbit antisera were obtained from Amersham, and Kirkegaard & Perry, Gaithersburg, MD, respectively. Substance P was purchased from NovaBiochem, San Diego, CA. Other biochemicals and reagents were purchased from the Sigma Chemical Co., St. Louis, MO. CGP 41 251 and Ro 31-8220 were gifts from Drs. Thomas Meyer (Ciba–Geigy, Basel Switzerland) and G. Lawton (Roche Products Ltd., Welwyn, Garden City, Hertshire, U.K.), respectively.

Preparation of Cell Suspensions

Acinar cells were prepared from parotid glands excised from male Sprague–Dawley rats anesthetized with sodium pentobarbital as described previously [2]. The tissue was dispersed by a 5-min incubation with trypsin, followed by a 5-min incubation with soybean trypsin inhibitor, and then collagenase for 35 min at 37°. The incubation buffer (pH 7.4) had the following composition (mM): NaCl, 137; KCl, 5.4; $MgSO_4$, 0.8; $CaCl_2$, 1.3; NaH_2PO_4, 0.96; $NaHCO_3$, 3; glucose, 5.6; HEPES, 33; plus 1–2% BSA. Acinar cells constitute more than 80% of the tissue mass in rat salivary glands, and the purity of the dispersed acinar cell preparation exceeds 90% [8].

For the down-regulation experiments, parotid cells from two glands were resuspended in 100 mL of a 1:1 mixture of sterile Ham's F-12 and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 5 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. The cell suspension was aliquoted into individual Petri dishes followed by treatment with either PMA or 4α-phorbol in ethanol and incubated in a humidified atmosphere at 37° with 5% CO_2 for the specified time periods. The concentration of ethanol, which did not exceed 0.01%, did not affect amylase secretion. Trypan blue exclusion was used to monitor for cell viability.

Amylase Secretion

Amylase secretion was determined as described previously [9]. Cells were centrifuged through Nyosil oil, and amylase activity was expressed as a percentage of total amylase activity (buffer plus cellular amylase content) after subtraction of basal activity.

Subcellular Fractionation

An aliquot of cells was mixed with ice-cold Solution A containing 0.3 M sucrose (pH 7.35). Solution A contained: 20 mM Tris-HCl; 1 mM EDTA; 1 mM EGTA; 25 μg/mL leupeptin; 0.14 trypsin inhibitory units/mL aprotinin; 50 mM 2-mercaptoethanol; 0.1 mg/mL phenylmethylsulfonyl fluoride. The cells were centrifuged at 4° for 4 min at 500 *g*. The supernatant was removed, and 1 mL of Solution B [solution A containing 50 μM $NaHCO_3$ (pH 7.35)] was added to the pellet. The suspension was vortexed, sonicated, and then centrifuged for 10 min at 500 *g* at 4° to obtain a supernatant and a crude nuclear pellet. The supernatant was diluted to 2 mL with Solution B and centrifuged for 60 min at 100,000 *g* at 4°. This 100,000 *g* supernatant (cytosolic fraction) was removed and placed on ice. The pellet was resuspended in Solution A containing 0.5% Triton X-100 and placed on ice for 60 min with occasional vortexing. The sample was then centrifuged for 60 min at 100,000 *g*, and the resulting supernatant was considered the particulate fraction. The crude nuclear pellet from the initial centrifugation was washed once in Solution B (600 μL) and centrifuged again at 500 *g* for 5 min. The resulting pellet was resuspended in Solution B (600 μL), sonicated, and layered on a discontinuous sucrose gradient (15, 25, 35, 45%) in Solution B. The gradient was centrifuged in a swinging bucket rotor at 1600 *g* for 45 min at 4°. The pellet (nuclear material) was resuspended in Solution A plus 0.5% Triton X-100. In assessing the purity of the subcellular fractions, we found that forskolin-stimulated adenylyl cyclase (an enzyme marker for plasma membrane) was 7-fold higher in the particulate fraction than in the cytosolic fraction, with no detectable activity in the nuclear fraction. The activity of lactate dehydrogenase, a cytosolic marker enzyme, was 6-fold higher in the cytosolic fraction than in the particulate fraction, and below the limits of detection in the nuclear fraction. For down-regulation experiments, parotid cells treated with PMA or 4α-phorbol for various time periods were resuspended in Solution A containing 0.5% Triton X-100, sonicated, placed on ice for 60 min with occasional vortexing, and then centrifuged for 60 min at 100,000 *g*. The supernatant was utilized to determine total amount of cellular isozyme.

Western Blot Analysis

Cellular fractions of equivalent protein concentrations were analyzed by SDS–PAGE as described previously [2, 10]. Following electrophoretic protein transfer from gel to nitrocellulose membranes, immunoblotting was carried out using isozyme specific antibodies (either monoclonal or affinity-purified polyclonal). The immunoblots were then developed with goat anti-rabbit or sheep anti-mouse IgG linked to horseradish peroxidase. Immunoreactivity was detected by chemiluminescence. The amount of each isozyme was quantified by densitometric scanning using an LKB Ultroscan XL. The density of the immunoreactive bands was a linear function of the amount of protein present. The identity of each parotid isozyme was confirmed in every experiment by western blot analysis of corresponding rat brain isozyme.

mRNA Analysis

Total RNA was isolated by ultracentrifugation as described previously [11]. First-strand cDNA was synthesized using

TABLE 1. Specific primers for PKC isozymes

Type		Primer sequences	Fragment size (bp)
α	Forward	5′ CATTCAAGCCCAAAGTGTGC 3′	347
	Reverse	5′ GGGAAGAAAGTTGATCACTT 3′	
β	Forward	5′ TCCATCGCTGAGCCTGTGTGT 3′	209
	Reverse	5′ TTGTACCTCTATCAGTGGGC 3′	
δ	Forward	5′ ACCACGAGTTCATCGCCACCT 3′	275
	Reverse	5′ AAGTGCCACAGTGGTCACAG 3′	
ε	Forward	5′ CCATGGTAGGTTCAATGGCC 3′	405
	Reverse	5′ GATGATCCCGAGAGATCGATG 3′	
ζ	Forward	5′ ATGCCTTGTCCTGGAGAAGAC 3′	242
	Reverse	5′ CCATATGCCTCCTGCAGGTCA 3′	

oligo (dT) primers and reverse transcriptase using an RT-PCR kit (Perkin–Elmer, Norwalk, CT). PKC isozyme-specific primers based on reported sequences from GenBank were formulated as shown in Table 1. PCR was performed using a Perkin–Elmer thermocycler (model 2400) for 35 cycles with 1 U of *Taq* DNA polymerase. The amplification program of denaturation (95°, 1 min), primer annealing (52°, 2 min), and primer extension (72°, 3 min) was utilized. PCR products were analyzed by electrophoresis on 3% agarose gels, and DNA was visualized by ethidium bromide staining.

Statistical Analysis

Paired comparisons were made from at least three independent experiments using the two-tailed Student's *t*-test. The PKC distribution data were analyzed by one-way analysis of variance followed by Duncan's test for multiple comparisons. Data are expressed as means ± SEM, and differences were considered significant at the $P < 0.05$ level.

RESULTS

Identification and Localization of nPKCs in Unstimulated Parotid Cells

We previously observed by western blot analysis that rat parotid cells express PKC-α as the sole Ca^{2+}-dependent PKC isozyme [2]. In the present study, the possibility that parotid cells also express Ca^{2+}-independent PKC isozymes was investigated in cellular extracts. Western blot analysis was performed using a monoclonal antibody recognizing PKC-ε and polyclonal antibodies reactive with PKC-δ and -ζ (Fig. 1). The expression of PKC-ε and -δ in the cytosolic and particulate fractions of parotid cells was observed as protein bands at 93 and 72 kDa, respectively. The identity of each isozyme was confirmed by comparison with characterized immunoreactive proteins extracted from rat brain (data not shown). In addition, when antibodies for PKC-δ and -ζ were mixed with their corresponding peptide, the appearance of the appropriate band was blocked (data not shown). PKC-δ was generally detected as a doublet (Fig. 1). In addition to the nPKCs that were identified, the atypical isozyme PKC-ζ was also detected by western blotting (Fig. 1). Parotid cells did not express PKC-θ (data not shown).

In further experiments, the nuclear fraction was investigated as a possible locus for PKC in unstimulated parotid cells. Figure 1 shows that the nuclear fraction expressed PKC-ε, -δ, and -ζ, while deficient in PKC-α. To substantiate the presence of the respective isozymes, the presence of mRNA for individual isozymes was determined by RT-PCR. Analysis of DNA fragments generated by RT-PCR revealed the presence of transcripts for PKC-α, -δ, -ε, and -ζ (Fig. 2). Transcripts for PKC-β (Fig. 2) and -γ (data not shown) were not detected.

Differential Regulation of PKCs by PKC Activation

To determine whether Ca^{2+}-dependent and -independent isozymes are differentially responsive to PKC activators, the effects of PMA and TX on the cellular distribution of PKC-α and -ε were evaluated. TX is a daphnane ester sec-

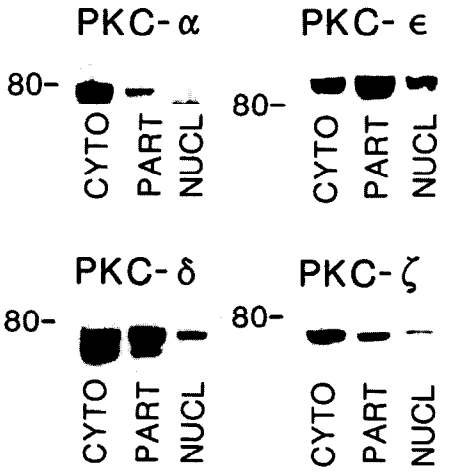

FIG. 1. Western blot identification of PKC-α, -δ, -ε, and -ζ immunoreactive proteins in subcellular fractions of parotid cells. The cytosolic, particulate (plasma membrane), and nuclear fractions were analyzed by SDS–PAGE and immunoblotting. Equal amounts of protein were loaded in each lane (15 μg protein/lane). A molecular mass marker for each isozyme is shown at the left.

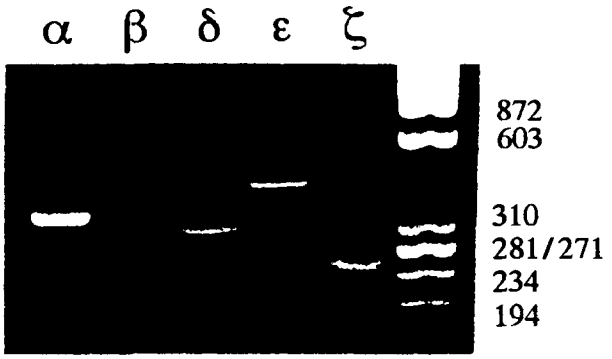

FIG. 2. RT-PCR analysis with specific primers showing the expression of PKC-α, -δ, -ε, and -ζ. Total RNA from isolated parotid cells was reverse transcribed into cDNA and amplified by PCR using isozyme-specific primers as described in Materials and Methods. The PCR products were processed by electrophoresis and the DNA stained with ethidium bromide. Numbers on the right-hand side represent DNA size markers with base pairs indicated.

ondary stage tumor promoter that also activates PKC [12]. Representative immunoblots of such experiments are depicted in Fig. 3A, and a quantitative analysis is provided in Fig. 3B.

In addition to the decline in the level of cytosolic PKC-α and the corresponding increase in the particulate fraction following phorbol ester stimulation [2] (Fig. 3, A and B), PMA induced the appearance of PKC-α in the nuclear fraction (Fig. 3, A and B). PMA also caused the level of cytosolic PKC-ε to decline markedly, while a greater proportion of the isozyme was detected in the nuclear fraction (Fig. 3, A and B). PMA failed to induce a significant accumulation of PKC-ε in the particulate fraction (Fig. 3, A and B). PKC-δ behaved similarly to PKC-ε in that this isozyme was translocated to the nuclear fraction but not to the particulate fraction by PMA treatment (data not shown).

TX, like PMA, decreased cytosolic PKC-α and produced a modest but statistically significant rise in PKC-α immunoreactivity in both the particulate and nuclear fractions (Fig. 3, A and B). The increase in the nuclear fraction was almost equivalent to that caused by PMA (Fig. 3B). Despite its ability to translocate PKC-α, TX failed to decrease PKC-ε immunoreactivity in the cytosol (Fig. 3, A and B) and did not augment the amount of immunoreactive isozyme in either the particular or nuclear fraction (Fig. 3, A and B). TX also failed to alter the amount of PKC-δ and -ζ immunoreactivity in either the particular or nuclear fraction (data not shown).

Experiments were also carried out using substance P, which is a natural ligand for the neurokinin 1 (NK_1) receptor [13]. The cellular mechanism underlying the action of this agonist, like that of muscarinic and α-adrenergic agonists, involves a phosphoinositide-linked receptor to generate DAG and inositol trisphosphate [14, 15]. Densitometric analysis of the representative data depicted in Fig. 4 revealed that a 1-min exposure to substance P produced a modest (20%) loss of PKC-α in the cytosolic fraction, with larger reductions for PKC-δ and -ε (70 and 80%, respectively) (N = 2). Only PKC-α increased in the particulate fraction (58%) (a 1.6-fold increase) (Fig. 4). The most prominent feature of substance P action was its ability to mimic the effect of PMA in increasing PKC-δ and -ε immunoreactivity in the nuclear fraction (Fig. 4). PKC-δ and -ε immunoreactivity increased in the nuclear fraction by an average of 158% (2.6-fold) and 150% (2.5-fold), respectively, following a 1-min incubation with substance P (N = 2). However, unlike PMA, the peptide did not cause a detectable redistribution of PKC-α to the nucleus (Fig. 4). Even after 5 min, substance P failed to cause the migration of PKC-α to the nucleus (data not shown). Similar to PMA, substance P did not alter the cellular redistribution of PKC-ζ (data not shown). In another experiment, a 1-min exposure to 10 μM CCh caused an increase in PKC-δ and -ε immunoreactivity in the nuclear fraction of 1.8- and 1.9-fold, respectively. No increase in PKC-α immunoreactivity could be detected in the nuclear fraction by densitometry after exposure to CCh for up to 5 min (data not shown).

Differential Down-Regulation of PKC Isozymes by PMA

The extent and kinetics of PMA-induced down-regulation of isozymes expressed in parotid cells were characterized. Representative immunoblots depicting a complete time-course of PMA treatment are shown in Fig. 5, and a quantitative assessment after a 20-hr exposure to PMA is provided in Fig. 6A. A prolonged exposure to PMA (3–20 hr) resulted in a progressive decrease in total cellular PKC-α levels (Fig. 5). Immunoreactivity decreased to 60% of control levels by 6 hr and to approximately 25% by 20 hr (Figs. 5 and 6A). Down-regulation of PKC-δ initially proceeded at a slower rate in that after 6 hr immunoreactivity fell to 75% of control values (Fig. 5); by 20 hr the total levels of this isozyme decreased to 26% of control values (Figs. 5 and 6A). PKC-ε was unaffected by PMA treatment after 6 hr (Fig. 5); however, after 20 hr immunoreactivity fell to 62% of control values (Figs. 5 and 6A). PKC-ζ immunoreactivity remained relatively constant over the 20-hr exposure to PMA (115% of control) (Figs. 5 and 6A), indicating that this isozyme is resistant to down-regulation. No detectable decrease in the levels of the expressed isozymes was observed following a 20-hr exposure to the inactive 4α-phorbol; in fact an increase in PKC-α and -δ immunoreactivity was observed (Fig. 5).

Effects of Prolonged PMA Pretreatment and PKC Inhibitors on Amylase Secretion

To determine whether down-regulation of PKCs leads to an altered expression of cellular function, evoked amylase release was measured in cells following prolonged exposure to PMA. Cells that had been exposed to 1 μM PMA for 20 hr exhibited an almost 2-fold greater capacity to respond to 10

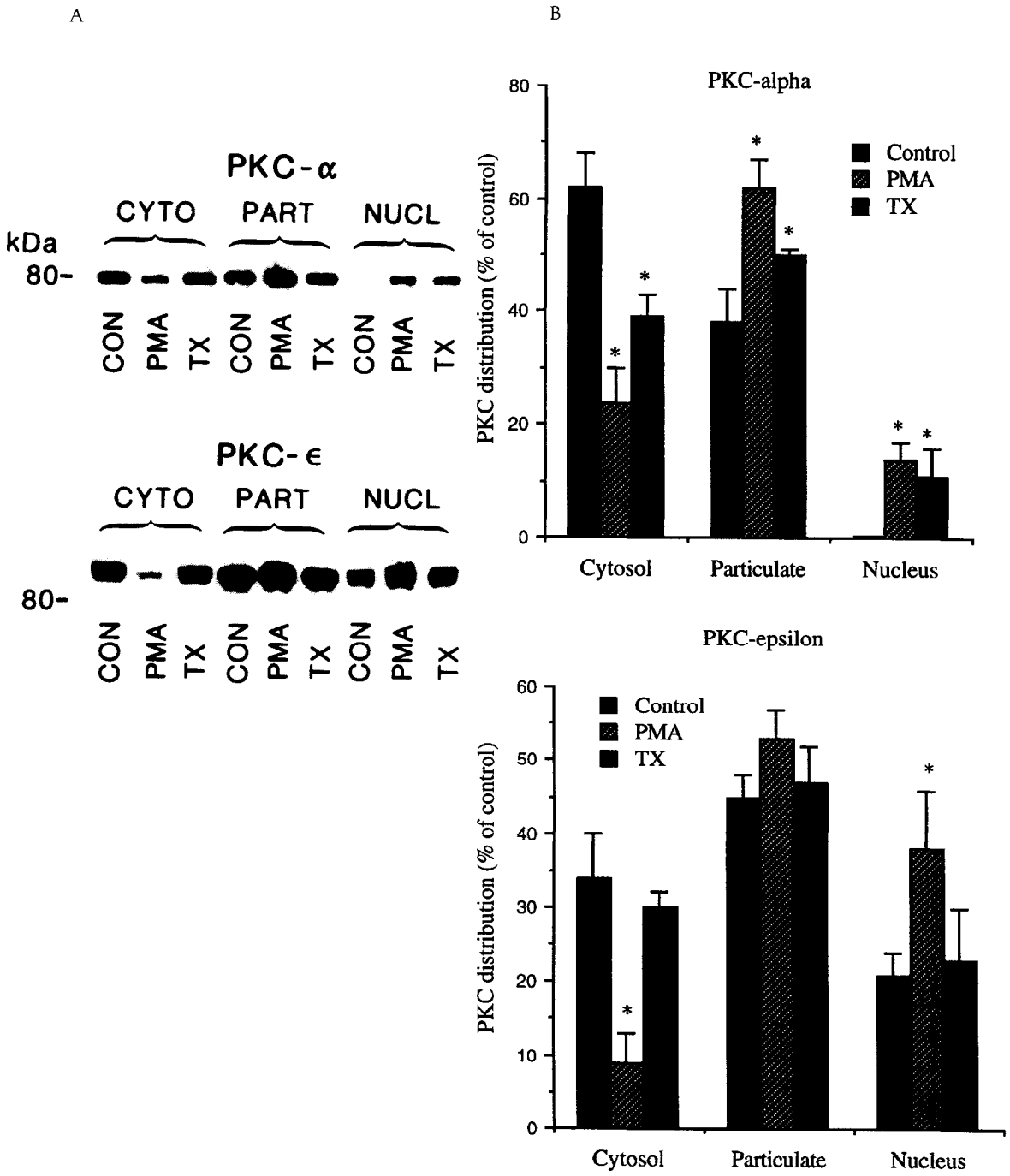

FIG. 3. Western blot analysis of PKC isozymes in subcellular fractions from parotid cells treated with either PMA or TX. Subcellular fractions were processed for western blot analysis using PKC isozyme-specific antibodies. (A) Representative immunoblots of each subcellular fraction derived from cells exposed to vehicle, 100 nM PMA, or 100 nM TX for 10 min. (B) Results (means ± SEM) obtained after densitometric analysis of autoradiograms of 3–4 independent experiments. Results are expressed as a percentage of the total amount of isozyme present in all fractions. Key: (*) significantly different from control as assessed by one-way analysis of variance plus Duncan's test for multiple comparisons ($P < 0.05$).

μM NE than cells treated for the same time period with the inactive 4α-phorbol (14.6 vs 8.2%) (Fig. 6B). In contrast, the secretory response to NE following a 20-hr exposure to 4α-phorbol was not significantly different (8.2 ± 0.8%) compared with the secretory response of freshly prepared cells incubated in serum-enriched medium (9.9 ± 0.5%) (N = 3).

The possible involvement of PKC in the regulation of evoked amylase secretion was also investigated using two selective inhibitors of PKC, Ro 31-8220 and CGP 41 251

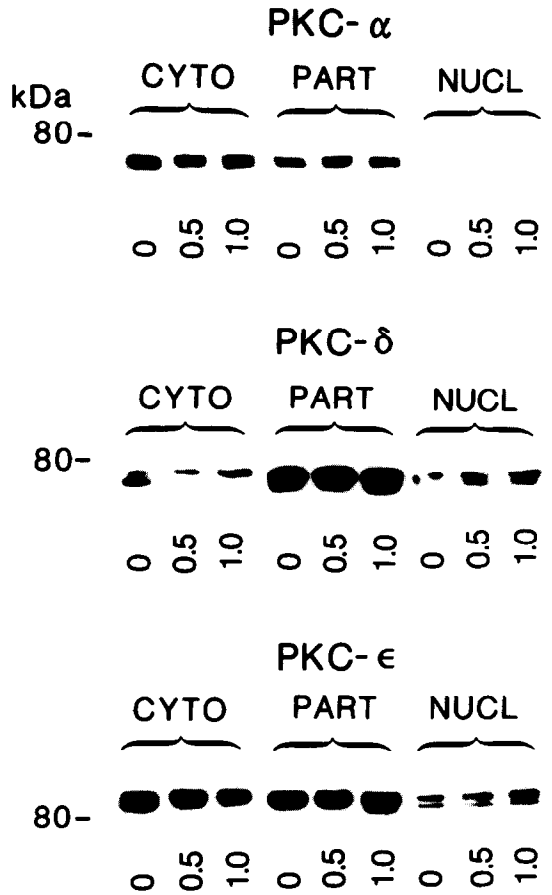

FIG. 4. Western blot analysis of PKC isozymes in subcellular fractions of parotid cells treated with substance P. Parotid cells were incubated under control conditions or with 1 μM substance P for 0.5 or 1 min. Immunoblot analysis was then carried out on subcellular fractions. The data are representative of those from two independent experiments.

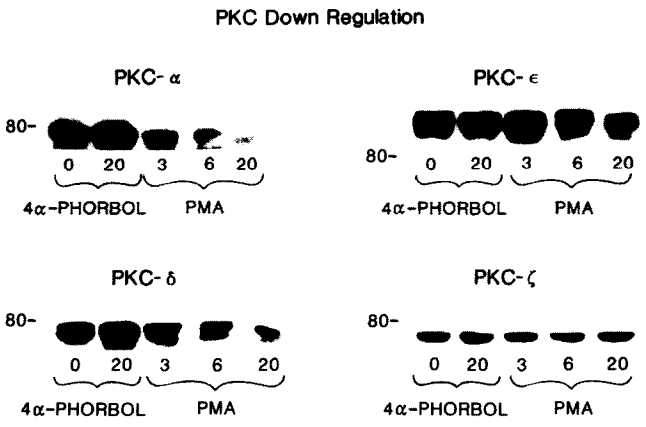

FIG. 5. Differential effects of PMA on the down-regulation of PKC isozymes. Cells were exposed to 1 μM PMA for 3–20 hr. Samples of the Triton X-100 soluble cell fraction were analyzed by SDS–PAGE and immunoblotting. Representative immunoblots of the total amount of each isozyme present in cells incubated in the presence of PMA or 4α-phorbol are shown.

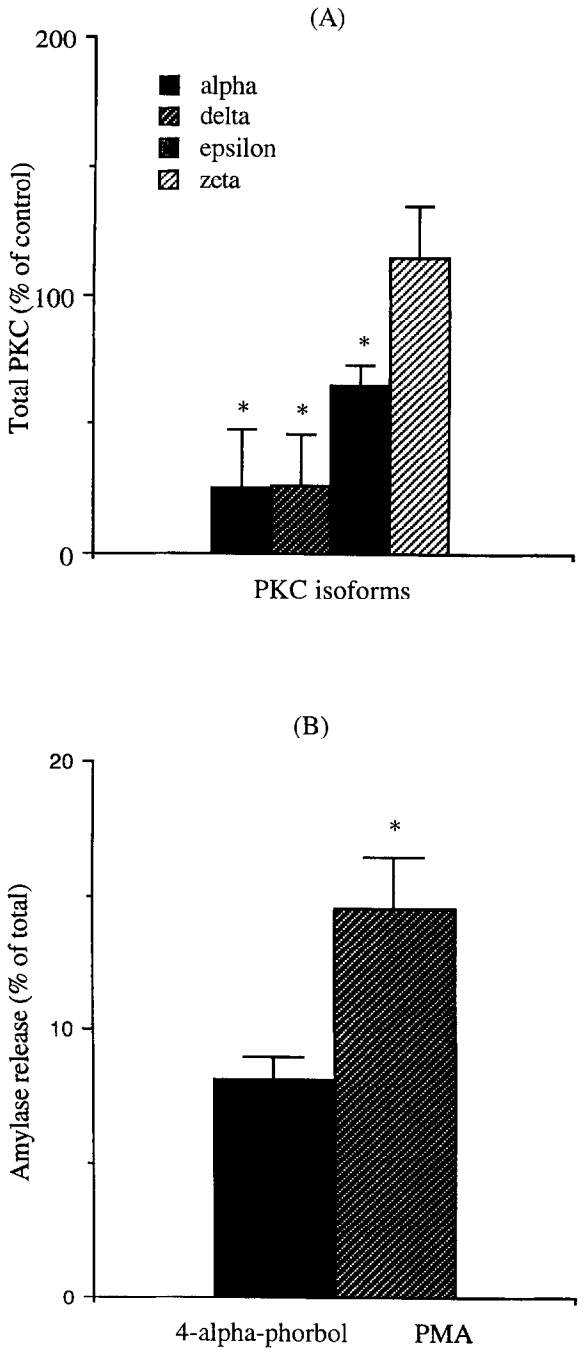

FIG. 6. Comparison of the effects of prolonged exposure to PMA on the down-regulation of PKC isozymes and NE-evoked amylase secretion. (A) Quantitative representation of the results shown in Fig. 5 after densitometric analysis of the immunoblots (means ± SEM of 3–4 separate experiments). Data are expressed in arbitrary units as a percentage of the amount of isozyme present in cells treated with 4α-phorbol. Key: (*) significantly different from control values, as assessed by one-way analysis of variance ($P < 0.05$). (B) Representation of NE-induced amylase secretion (means ± SEM of 4-5 separate experiments). Cells incubated in serum-enriched medium and treated for 20 hr with either 1 μM PMA or 4α-phorbol were washed, exposed to 10 μM NE for 30 min, and analyzed for amylase release. Secretion is expressed as a percentage of total cellular amylase activity. Key: (*) significantly different from control values, as assessed by the paired Student's *t*-test ($P < 0.05$).

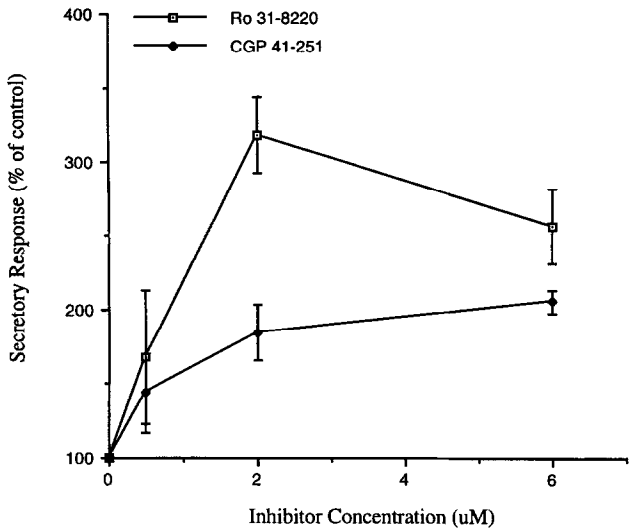

FIG. 7. Stimulatory effect of PKC inhibitors on evoked amylase release. Cells were exposed to 10 μM NE for 15 min after being pretreated for 5 min with various concentrations of Ro 31-8220, CGP 41 251, or with vehicle (dimethyl sulfoxide). Each value for amylase secretion represents the mean ± SEM from 3–5 independent experiments. Secretion was normalized as a percentage of the corresponding evoked output from cells incubated in the absence of inhibitor. Neither Ro 31-8220 nor CGP 41 251 had any discernible effect on basal amylase secretion.

[16–19]. The staurosporine analog Ro 31-8220 in the concentration range of 0.5 to 6 μM potentiated amylase secretion elicited by NE (Fig. 7). Potentiation was maximal with 2 μM Ro 31-8220 where NE-induced secretion increased more than 3-fold. CGP 41 251 also produced a graded enhancement of the secretory response to NE (Fig. 7). CGP 41 251 was less efficacious than Ro 31-8220, although both agents appeared comparable in potency (Fig. 7).

DISCUSSION

The present study has combined immunochemical and molecular biological techniques to identify three Ca^{2+}-independent PKC isozymes in rat parotid acinar cells, namely PKC-ε, -δ, and -ζ. The identification of PKC-δ and -ζ in parotid cells is consistent with their relatively wide distribution in peripheral rat tissues [4]. On the other hand, expression of PKC-ε may signify a role for this isozyme in a specialized function since there is limited distribution of this isozyme in rat tissues [4]. Our inability to detect PKC-θ in parotid cells is consistent with previous findings that confine this isozyme to skeletal muscle [4].

This study has also shown that the PKC isozymes expressed in parotid cells exhibit a differential ability to respond to various agents. The ability of PMA to translocate PKC-α, but not PKC-ε, to the plasma membrane, constitutes evidence supporting differential effects of putative PKC activators on PKC isozymes in parotid cells. The additional findings that TX translocated PKC-α to the plasma membrane and nuclear fractions but failed to redistribute PKC-ε further documents agonist specificity. The differential sensitivity exhibited by PKC isozymes in response to agonists and the translocation of PKC-α, -δ, and -ε to the nucleus which has been demonstrated in parotid cells are in accord with findings in other cell systems [7, 20–23].

The redistribution of PKC-ε and -δ to the nuclear region produced by substance P or CCh was not accompanied by a similar translocation of PKC-α. Thus, another key feature distinguishing PKC-α from the nPKCs is the observed absence of this Ca^{2+}-dependent isozyme from the nuclear region of cells exposed to agonists that cause phosphoinositide breakdown. This finding may be viewed as another example of the diverse mode of activation of the nPKCs compared to the Ca^{2+}-dependent (conventional) PKCs [24]. However, in light of the ability of PMA and TX to cause the redistribution of PKC-α to the nucleus, one must also consider the possibility that PKC-α is present in the nuclei of quiescent and receptor agonist-stimulated parotid cells at levels that are below the limits of detection with the present methodology. Thus, additional experiments are planned to firmly establish whether parotid cell activation by cell surface receptor agonists that promote phosphoinositide hydrolysis is associated with the redistribution of PKC-α to the cell nucleus.

Another property of specific PKC isozymes which has functional implications is susceptibility to down-regulation [7]. The observations that in parotid acinar cells PKC-α was highly susceptible to PMA-induced down-regulation, whereas PKC-δ was somewhat less responsive in terms of time–course, and PKC-ε somewhat resistant, are compatible with findings in certain other cell types [25–27]. The demonstrated down-regulation of both Ca^{2+}-dependent and Ca^{2+}-independent PKC isozymes in parotid cells suggests that this process does not involve altered Ca^{2+} levels. Our observations that PKC-ε is not translocated significantly to the plasma membrane and is down-regulated only modestly by PMA support the concept that redistribution to the plasma membrane is a prelude to proteolytic degradation leading to down-regulation [7]. Our inability to detect down-regulation of PKC-ζ is also in accord with previous work [27] and is presumably due to the absence of one zinc finger that renders this isozyme incapable of binding phorbol esters [28].

The marked down-regulation of PKC-α elicited by a 20-hr exposure to PMA permits a comparison of diminished isozyme activity and altered secretory function, despite the varied depletion of Ca^{2+}-independent isozymes. The fact that marked down-regulation of PKC-α was accompanied by an enhanced secretory response to NE suggests that this isozyme serves to negatively modulate secretion. This line of reasoning may extend to PKC-δ which also exhibited only 25% of normal immunoreactivity after a 20-hr exposure to PMA. The additional fact that the secretory response was amplified by concentrations of the highly selective PKC inhibitors CGP 41 251 and Ro 31-8220, which

maximally reduce protein phosphorylation in other test systems [16–19], substantiates the notion that PKC (more specifically PKC-α and/or -δ) serves as a negative modulator of salivary amylase secretion. The observed amplification of the secretory response to NE after PKC inhibition may be attributed to the well-documented negative feedback regulation by PKC of phospholipase C-mediated Ca^{2+} signaling which has been observed in acinar exocrine cells [29–31]. The observed inhibition of evoked amylase secretion elicited by the putative PKC inhibitor H-7 in a previous study [1] may be ascribed to the lack of selectivity of this agent.

One cannot exclude the possibility that PKC-ε, which was only partially down-regulated, and PKC-ζ, which was unaffected by prolonged PMA treatment, may also serve some role in regulating exocytotic amylase secretion. In this connection, our previous study which demonstrated that diacylglycerol decreases the Ca^{2+} requirement for amylase release from permeabilized parotid cells also implicates PKC as a positive modulator at a distal step in the secretory process [32].

In conclusion, our findings reveal that parotid cells contain PKC-α and three Ca^{2+}-independent isozymes that display varied time-courses and sensitivities to translocation and down-regulation in response to various putative activators of PKC. Utilizing these distinct properties, we have demonstrated that PKC-α and possibly PKC-δ may serve as negative modulators of amylase release. Another major outcome of this study derives from the finding that the nucleus appears to be a key locus of action of PKC-ε, -δ, and possibly -α in parotid cells. In this context, there is an increasing body of evidence that PKC isozymes regulate cell proliferation and differentiation in various cell types [22]. The attribution of PKC-dependent responses to specific isozymes represents a particularly intriguing aspect of cell regulation, and this work comprises an important first step in elucidating the nature of the cellular events regulated by PKC isozymes in parotid acinar cells. Further exploitation of these isozyme-specific properties should clarify the functional relevance of the expression, subcellular distribution, and regulation of PKC isozymes in parotid gland.

The authors thank Jennifer Cohen and Richard Smiehorowski for technical assistance during certain phases of this study. This work was supported by USPHS Grant DE-05764 (NIDR).

References

1. Shimomura H, Terada A, Hashimoto Y and Soderling TR, The role of protein kinase C on amylase secretion from rat parotid gland. *Biochem Biophys Res Commun* **150:** 1309–1314, 1988.
2. Terzian AR and Rubin RP, Translocation of the α-isozyme of protein kinase C during stimulation of rat parotid acinar cells by phorbol ester and carbachol. *Arch Oral Biol* **38:** 1051–1056, 1993.
3. Nishizuka Y, Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258:** 607–614, 1992.
4. Graber R, Sumida C and Nunez EA, Fatty acids and cell signal transduction. *J Lipid Mediat Cell Signal* **9:** 91–116, 1994.
5. Dekker LV and Parker PJ, Protein kinase C—A question of specificity. *Trends Biochem Sci* **19:** 73–77, 1994.
6. Evans FJ, Parker PJ, Olivier AR, Thomas S, Ryves WJ, Evans AT, Gordge P and Sharma P, Phorbol ester activation of the isotypes of protein kinase C from bovine and rat brain. *Biochem Soc Trans* **19:** 397–402, 1991.
7. Hug H and Sarre TF, Protein kinase C isoenzymes: Divergence in signal transduction? *Biochem J* **291:** 329–343, 1993.
8. Putney JW Jr, Inositol lipids and cell stimulation in mammalian salivary glands. *Cell Calcium* **3:** 369–383, 1982.
9. Bernfeld P, Amylases, α and β. *Methods Enzymol* **1:** 149–158, 1955.
10. Laemmli UK, Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
11. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning; A Laboratory Manual*, Book 1, Chap. 7. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
12. Brooks G, Evans AT, Aitken A and Evans FJ, Tumour-promoting and hyperplastic effects of phorbol and daphnane esters in CD-1 mouse skin and a synergistic effect of calcium ionophore with the non-promoting activator of protein kinase C, sapintoxin A. *Carcinogenesis* **10:** 283–288, 1989.
13. McLean S and Lowe JA, Agonist and antagonist receptor binding. In: *The Tachykinin Receptors* (Ed. Buck SH), pp. 67–69. Humana Press, Totowa, NJ, 1994.
14. Putney JW Jr, Receptors and the inositol phosphate-calcium signaling system. In: *The Tachykinin Receptors* (Ed. Buck SH), pp. 257–283. Humana Press, Totowa, NJ, 1994.
15. Taylor CW, Merritt JE, Putney JW Jr and Rubin RP, A guanine nucleotide-dependent regulatory protein couples substance P receptors to phospholipase C. *Biochem Biophys Res Commun* **136:** 362–368, 1986.
16. Kojima I, Kawamura N and Shibata H, Rate of calcium entry determines the rapid changes in protein kinase C activity in angiotensin-stimulated adrenal glomerulosa cells. *Biochem J* **297:** 523–528, 1994.
17. Meyer T, Regenass U, Fabbro D, Alteri E, Rosel J, Muller M, Caravatti G and Matter A, A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and in vitro anti-proliferative as well as *in vivo* anti-tumor activity. *Int J Cancer* **43:** 851–856, 1989.
18. Raffaniello RD and Raufman J-P, Carbachol activates protein kinase C in dispersed gastric chief cells. *Biochem J* **300:** 21–24, 1994.
19. Walker TR and Watson SP, Synergy between Ca^{2+} and protein kinase C is the major factor in determining the level of secretion from human platelets. *Biochem J* **289:** 277–282, 1993.
20. Disatnik M-H, Winnier AR, Mochly-Rosen D and Arteaga CL, Distinct responses of protein kinase C isozymes to c-erbB-2 activation in SKBR-3 human breast carcinoma cells. *Cell Growth Differ* **5:** 873–880, 1994.
21. Duyster J, Schwende H, Fitzke E, Hidaka H and Dieter P, Different roles of protein kinase C-β and -δ in arachidonic acid cascade, superoxide formation and phosphoinositide hydrolysis. *Biochem J* **292:** 203–207, 1993.
22. Olson EN, Burgess R and Staudinger J, Protein kinase C as a transducer of nuclear signals. *Cell Growth Differ* **4:** 699–705, 1993.
23. Ryves WJ, Evans AT, Olivier AR, Parker PJ and Evans FJ, Activation of the PKC-isotypes α, β_1, γ, and ε by phorbol esters of different biological activities. *FEBS Lett* **288:** 5–9, 1991.
24. Ohno S, Mizuno K, Adachi Y, Hata A, Akita Y, Akimoto K, Osada S, Hirai S and Suzuki K, Activation of novel protein kinases Cδ and Cε upon mitogenic stimulation of quiescent rat 3Y1 fibroblasts. *J Biol Chem* **269:** 17495–17501, 1994.

25. Borner C, Guadagno SN, Fabbro D and Weinstein B, Expression of four protein kinase C isoforms in rat fibroblasts. *J Biol Chem* **267:** 12892–12899, 1992.
26. Huwiler A and Pfeilschifter J, A role for protein kinase C-α in zymosan-stimulated eicosanoid synthesis in mouse peritoneal macrophages. *Eur J Biochem* **217:** 69–75, 1993.
27. Szallasi Z, Smith CB, Pettit GR and Blumberg PM, Differential regulation of protein kinase C isozymes by bryostatin 1 and phorbol 12-myristate in NIH 3T3 fibroblasts. *J Biol Chem* **269:** 2118–2124, 1994.
28. Ono Y, Fuji T, Ogita K, Kikkawa U, Igarashi K and Nishizuka Y, Protein kinase C ζ subspecies from rat brain: Its structure, expression, and properties. *Proc Natl Acad Sci USA* **86:** 3099–3103, 1989.
29. Bird G St.J, Rossier MF, Obie JF and Putney JW Jr, Sinusoidal oscillations in intracellular calcium requiring negative feedback by protein kinase C. *J Biol Chem* **268:** 8425–8428, 1993.
30. Gromada J, Jorgensen TD and Dissing S, Role of protein kinase C in the regulation of inositol phosphate production and Ca^{2+} mobilization evoked by ATP and acetylcholine. *Pflugers Arch* **429:** 578–586, 1995.
31. Willems PHGM, Smeets RLL, Bosch RR, Garner KM, Van Mackelenbergh MGH and DePont JJHHM, Protein kinase C activation inhibits receptor-evoked inositol trisphosphate formation and induction of cytosolic calcium oscillations by decreasing the affinity-state of the cholecystokinin receptor in pancreatic acinar cells. *Cell Calcium* **18:** 471–483, 1995.
32. Rubin RP and Adolf MA, Cyclic AMP regulation of calcium mobilization and amylase release from isolated permeabilized rat parotid cells. *J Pharmacol Exp Ther* **268:** 600–606, 1994.