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Ca^{2+}-dependent and Ca^{2+}-independent Isozymes of Protein Kinase C Mediate Exocytosis in Antigen-stimulated Rat Basophilic RBL-2H3 Cells

RECONSTITUTION OF SECRETORY RESPONSES WITH Ca^{2+} AND PURIFIED ISOZYMES IN WASHED PERMEABILIZED CELLS*

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Rat basophilic RBL-2H3 cells, which exhibit Ca^{2+}-dependent secretion of granules when stimulated with antigen, contained the Ca^{2+}-dependent α and β and the Ca^{2+}-independent δ, ϵ, and ζ isoforms of protein kinase C. These isoforms associated, to variable extents (*i.e.* δ the most and ζ the least), with the membrane fraction upon antigen stimulation but without external Ca^{2+}; only the Ca^{2+}-independent isoforms did so. Both types of isozymes were probably necessary for optimal responses to antigen as indicated by the following observations. All Ca^{2+}-dependent isozymes were degraded in cells treated with 20 nM phorbol 12-myristate 13-acetate for 6 h, whereas the Ca^{2+}-independent isozymes were not degraded and were retained when the cells were subsequently permeabilized and washed. Cells so treated still exhibited antigen-induced secretion (25–33% of normal) which was suppressed by selective inhibitors of protein kinase C (Ro31-7549 and calphostin C) thereby indicating a possible contribution of the Ca^{2+}-independent isozymes in secretion. Normally, washed permeabilized cells lost all isozymes of protein kinase C and failed to secrete in response to antigen. A full secretory response to antigen could be reconstituted by the subsequent addition of nanomolar concentrations of either β or δ isozymes of protein kinase C (other isozymes were much less effective) but only in the presence of 1 μM free Ca^{2+} to indicate distinct roles for Ca^{2+} and protein kinase C in exocytosis.

The isozymes of protein kinase C, which are important transducers of signals in many, if not all, types of cells, are activated through the binding of diacylglycerol to diacylglycerol binding sites that are present in each of the isozymes. The α, β, and γ isozymes of protein kinase C have, in addition, a Ca^{2+} binding domain that allows Ca^{2+} and diacylglycerol to act synergistically on these isozymes (1). The more recently described δ, ϵ, ζ, and η isozymes lack this Ca^{2+} binding site,[1] but little is known about their reactions *in situ* in response to cell stimuli (1–3).

When cells are activated by physiological stimulants that act via protein kinase C, the α, β, γ (4), and, in more recent reports, Ca^{2+}-independent (*e.g.* 5–7) isozymes shift from the soluble fraction of cell extracts to become tightly associated with the membrane fraction, a process otherwise referred to as translocation. Translocation can be induced also by phorbol esters such as phorbol 12-myristate 13-acetate (5–9), which act by binding to the diacylglycerol binding sites of protein kinase C (10–12). Prolonged exposure to phorbol ester, however, results in degradation of the Ca^{2+}-dependent isozymes in some cells (13, 14).

In this study, we have investigated the role of the individual isozymes of protein kinase C in supporting secretion in antigen-stimulated RBL-2H3 cells. These cells respond to antigen with sustained activation of various phospholipases, including phospholipase C; generation of diglycerides and inositol phosphates; increase in cytosolic Ca^{2+} (15); an increase in membrane-associated protein kinase C activity (16); phosphorylation of myosin at sites phosphorylated by protein kinase C (17); and discharge of secretory granules by a Ca^{2+}-dependent process of exocytosis (15). The cells contain α and β isozymes of protein kinase C (14) which translocate to the particulate fraction when the cells are stimulated with antigen[2] and are degraded when cells are exposed to phorbol ester (14, 18).

Here we show that RBL-2H3 cells also contained three Ca^{2+}-independent isozymes of protein kinase C that resisted degradation when cells were treated with phorbol 12-myristate 13-acetate. Thus we could determine whether antigen-induced secretion still occurred in cells selectively depleted of the Ca^{2+}-dependent isoforms. Because extensive washing of permeabilized cells resulted in the loss of all isozymes of protein kinase C and secretory response to antigen, we investigated, in addition, the role of the individual isozymes in secretion

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[1] The following definitions and abbreviations were used: "Ca^{2+}-dependent isozymes" refers collectively to the α, β, and γ isozymes, and the term "Ca^{2+}-independent isozymes" refers to the δ, ϵ, ζ, and η isozymes of protein kinase C; $[Ca^{2+}]_i$, concentration of free cytosolic calcium; DNP, 2,4-dinitrophenol; DNP-BSA, antigen consisting of 24 molecules of dinitrophenol conjugated with one molecule of bovine serum albumin; PIPES, 1,4-piperazinediethanesulfonic acid.

[2] F. L. Huang, J. R. Cunha-Melo, and M. A. Beaven, unpublished observations.

by adding back recombinant isozymes of protein kinase C to washed, permeabilized cells.

EXPERIMENTAL PROCEDURES

Materials—Materials for the preparation of the individual isozymes of protein kinase C were obtained from Boehringer Mannheim, unless stated otherwise. Other materials were obtained from the following sources: radioactive compounds from Du Pont-New England Nuclear; phorbol 12-myristate 13-acetate from LC Services Corporation, Woburn, MA; reduced streptolysin *O* (catalog MR16) from Burroughs Wellcome Co., Research Triangle Park, NC; dithiothreitol and leupeptin from Schwarz/Mann; rainbow protein molecular weight markers from Amersham Corp.; sodium dodecyl sulfate, alkaline phosphatase color development reagents (5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium), and goat anti-rabbit IgG conjugated with alkaline phosphatase from Bio-Rad; goat anti-mouse IgG conjugated with alkaline phosphatase from Jackson Immuno Research Laboratories Inc., West Grove, PA; Blotto solution from Advanced Biotechnologies Inc., Columbia, MD; 10 and 8% Tris glycine gels from NOVEX, San Diego, CA; BA-S NC nitrocellulose membranes from Schleicher & Schuell; calphostin C from Kamiya Biomedical Co., Thousand Oaks, CA; Ro31-7549 was a gift from Eisai Co., Ibaraki, Japan. The antigen, DNP-BSA, and DNP-specific monoclonal IgE were kindly supplied by Dr. Henry Metzger (NIAMS, National Institutes of Health). Monoclonal anti-protein kinase C β antibody was from UBI, Lake Placid, NY; polyclonal antibodies against other isozymes of protein kinase C and blocking peptides for these antibodies were from GIBCO-BRL. Polyclonal antibodies were also raised in rabbits against synthetic peptides that corresponded to the carboxyl-terminal amino acid sequence of protein kinase C (the 18 carboxyl-terminal amino acids) and ζ (the 16 carboxyl-terminal amino acids) and the amino-terminal sequence (14 amino-terminal amino acid residues) of protein kinase C ζ. Antibody specificity was tested by immunoblotting against purified protein kinase C isozymes expressed in the baculovirus system (see next section).

Stock solutions of Ro31-7549, calphostin C, and phorbol 12-myristate 13-acetate were prepared in dimethyl sulfoxide and diluted to give <0.1% dimethyl sulfoxide (18). All other reagents were dissolved in the PIPES-buffered medium (see below).

Preparation of Isozymes of Protein Kinase C—Preparation of protein kinase C isozymes was performed using the baculovirus expression system from Invitrogen (San Diego, CA). Full-length cDNA's for protein kinase C α, β, δ, and ε isozymes were inserted into pVL1393 baculovirus vector.[3] Insect cell culture, transfection, isolation, and purification of recombinant baculoviruses was performed using standard procedures (19). For protein production, Sf9 insect cells at an initial cell density of 3 × 10^6 cells/ml in a 250-ml spinner flask were infected at a multiplicity of infection of 10 with the different recombinant protein kinase C baculoviruses.

After 54 h of infection, cells were pelleted at 1,000 rpm for 10 min, washed twice with phosphate-buffered saline, and resuspended in a buffer containing 20 mM Tris-Cl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.3% (v/v) 2-mercaptoethanol, 1 μg/ml aprotinin, 20 μg/ml 4-amidinophenylmethanesulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin. Cells were disrupted with 10 strokes in a Potter-Elvehjem homogenizer and centrifuged at 100,000 × *g* for 1 h for recovery of the supernatant (cytosolic) fraction.

The cytosolic extract was loaded onto a DEAE-5PW column (15.0 × 2.0 cm, Tosoh, Tokyo, Japan) previously equilibrated with 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 0.3% (v/v) 2-mercaptoethanol. The column was eluted at a flow rate of 2 ml/min with a 180-ml linear gradient of NaCl (0–300 mM) in the equilibration buffer. The fractions were assayed for protein kinase C activity according to Nakadate *et al.* (20), and those containing high activity (generally eluting around 150 mM NaCl) were pooled and loaded into a Synchropack hydroxylapatite column (10.0 × 2.2 cm, Synchron Inc., Lafayette, IN) that had been preequilibrated with 20 mM potassium phosphate (pH 7.5), 0.5 mM EDTA, 0.067% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol (Sigma). The column was eluted with a 240-ml linear gradient of 20–280 mM potassium phosphate in the equilibration buffer at a flow rate of 0.18 ml/min. Active fractions were pooled, dialyzed overnight against 20 mM Tris-Cl (pH 7.5), 0.5 mM EDTA, 1 mM dithiothreitol, and 50% (v/v) glycerol, and then stored at −70 °C.

Protein kinase C β isozyme was also purified from mouse brain by use of the same purification protocol described above. Protein kinase C β was the second of the three peaks of activity that eluted from the hydroxylapatite column (see Ref. 21). All the purification steps were performed at 4 °C.

The identity of the different protein kinase C isozymes was confirmed by immunoblotting by use of the previously described specific antibodies (data not shown). Quantification of the isozymes was performed by [^{3}H]phorbol 12,13-dibutyrate binding by use of the polyethylene glycol precipitation assay (12). The concentration was calculated from specific maximal binding (B_{max}) as determined from Scatchard analysis, an assumed binding of one molecule of phorbol ester with one of enzyme and the calculated molecular weight. All isozyme preparations were found to have high kinase activity as determined by histone phosphorylation in the presence of phosphatidylserine and phorbol esters. Relative catalytic activities using the protein kinase C pseudosubstrate peptide as substrate were 1, 0.67, 0.62, and 0.28 for protein kinase C α, β, δ, and ε, respectively.

Preparation of Cell Cultures for Experiments—For measurement of secretion of hexosaminidase, cells were plated in 24-well culture plates (2 × 10^5 cells/400 μl of medium/well) or, for studies with recombinant isozymes, in 96-well plates (4 × 10^4 cells/80 μl of medium/well). For immunoblotting procedures, cells were placed in 150-cm^2 tissue culture dishes (20 × 10^6 cells/20-ml medium/dish). These cultures were incubated overnight with DNP-specific IgE and, as required, [^{3}H] inositol in complete growth medium (22). Phorbol 12-myristate 13-acetate (20 nM) was added to the incubation mixture at the designated time. For each experiment, cultures were washed twice, and medium was replaced with a glucose-saline, PIPES-buffered medium (buffer A, Ref. 17) that contained either 1 mM Ca^{2+} (Ca^{2+}-containing medium) or 0.1 mM EGTA instead of Ca^{2+} (Ca^{2+}-free medium). Procedures for the permeabilization of cells with streptolysin *O* have been described (18), and for these experiments, a potassium glutamate, PIPES-buffered medium (pH 7.2, 37 °C) was used. This medium contained 1 mM EGTA and the calculated concentration of Ca^{2+} to give the desired concentration of free Ca^{2+} (18). Where indicated, permeabilized cells were washed twice with the same medium (each wash was left for 60 s) before the final addition of the medium. Reagents, including recombinant isozymes of protein kinase C, were added as indicated.

Measurement of Hexosaminidase and [^{3}H]Inositol Phosphates—Aliquots (10 μl) of medium and cell lysate (in 1 ml of 0.1% Triton X-100) were incubated with 10 μl of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate buffer (pH 4.5) at 37 °C for 1 h. At the end of the incubation, 250 μl of a 0.1 M Na_2CO_3, 0.1 M $NaHCO_3$ buffer (pH 10) was added. Absorbance was measured at 410 nm. Values (mean ± S.E.) were expressed as the actual release (percent of total hexosaminidase) after correction for spontaneous release (2–3%) or as a percent of maximal response. [^{3}H]Inositol phosphates were separated by extraction and use of Dowex formate resin as described previously (18).

Immunoblotting of Protein Kinase C Isozymes—After completion of the experiment, medium was removed for assay of hexosaminidase; separate, identically prepared plates were used for determination of total hexosaminidase. Cells (20 × 10^6/dish) were homogenized with a Dounce homogenizer (100 strokes) in 200 μl of an extraction buffer; 50 mM Tris-Cl (pH 7.4), 2 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM leupeptin. After an initial centrifugation (700 × *g*, 10 min) to remove nuclei, the homogenate was separated into cytosolic and insoluble fractions by centrifugation (105,000 × *g*, 45 min). The insoluble fraction was solubilized by use of the Dounce homogenizer (50 strokes) in 200 μl of the extraction buffer that contained, in addition, 1% of Triton X-100. This solubilized (hereafter referred to as the particulate fraction) and cytosol fractions were assayed for protein with the Bio-Rad protein assay kit.

Proteins (20 μg of protein for α isozyme and 40 μg of protein for other isozymes) in the above two fractions were separated by electrophoresis on 8 or 10% sodium dodecyl sulfate-polyacrylamide gels. The proteins were transferred to nitrocellulose filters. Immunoreactive proteins were detected using the isozyme-specific antibodies which were diluted as follows: 5 μg/ml for antibodies against α, δ, ε, and ζ isozymes; 2 μg/ml for antibody against β isozyme; and 1/1,000 dilution for the rabbit antibodies against ζ and η. Specificities of the antibodies were checked by use of antibody-blocking peptides as specified by the manufacturer (GIBCO). The second antibody, either goat anti-rabbit IgG or anti-mouse IgG conjugated with alkaline phosphatase, was used at a 1/2,000 dilution. The bands were quanti-

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[3] M. G. Kazanietz, H. Mischak, J. F. Mushinski, and P. M. Blumberg, manuscript in preparation.

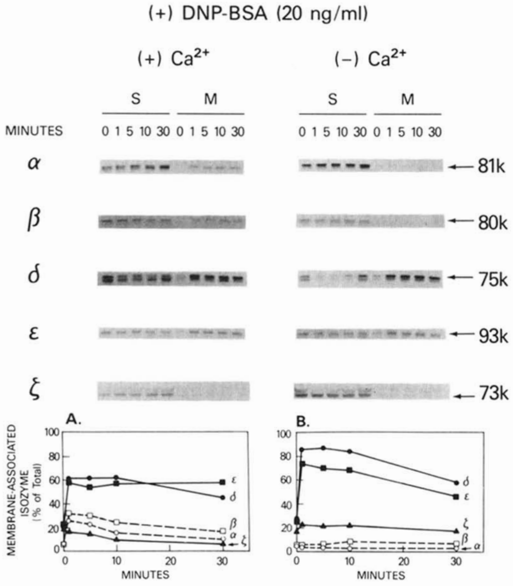

FIG. 1. **Effect of Ca^{2+} on the translocation of protein kinase C isozymes.** The cultures were stimulated with 20 ng/ml DNP-BSA for the indicated times in the presence or absence of Ca^{2+}, and particulate (*M*) and soluble (*S*) fractions were prepared and analyzed for immunoreactivity of protein kinase C isozymes as described under "Experimental Procedures." *Lower panels* (*A* and *B*) indicate the percent of each isozyme (fraction S plus fraction M including the presumed phosphorylated bands; see "Experimental Procedures") associated with the membrane fraction. Results were typical of at least three experiments. Measurements made in the same cultures indicated about 50% release of hexosaminidase in the presence of Ca^{2+} and no detectable release in its absence.

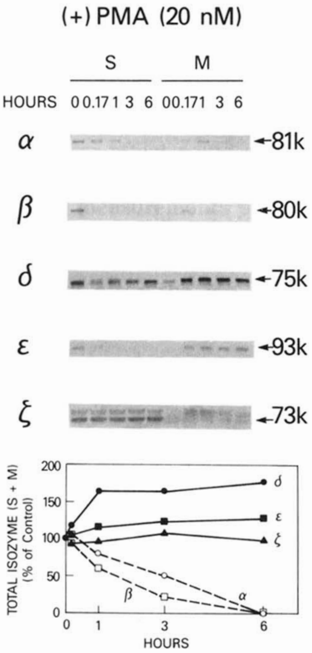

FIG. 2. **Effect of long term exposure to phorbol 12-myristate 13-acetate (20 nM) on immunoreactivity of protein kinase C isozymes.** Cells were exposed for the times indicated in the presence of external Ca^{2+}. Particulate (*M*) and soluble (*S*) fractions were prepared and analyzed as described under "Experimental Procedures." The *lower panel* indicates the total amount (S plus M fractions) of each isozyme expressed as a percent of amount at time 0. Results were typical of three experiments.

fied by use of a scanning densiotometer (ImageQuant Computing Densitometer, Molecular Dynamics, Sunnyvale, CA).

RESULTS

Translocation and Degradation of Protein Kinase C by Antigen and Phorbol Ester—Immunoblotting revealed the presence of α, β, δ, ϵ, and ζ isozymes in the soluble fraction of cell extracts. The γ and η isozymes were not detectable. The amount of protein in the particulate fraction for each isozyme, except ζ, increased significantly after the addition of antigen in the presence of Ca^{2+}, but the extent of this apparent translocation from the soluble to particulate fraction varied from <10% for ζ[4] to >50% for δ and ϵ. Translocation was maximal within 1 min after the addition of antigen. All of the isozymes remained associated with the particulate fraction for at least 30 min (Fig. 1). In the absence of external Ca^{2+}, however, the Ca^{2+}-dependent α and β isoforms did not translocate, whereas the redistribution of the Ca^{2+}-independent forms was unaffected or even enhanced (Fig. 1). Whether this augumented redistribution of δ and ϵ was caused by increased availability of binding sites because of lack of translocation of α and β isozymes or decreased degradation of isozymes will require further investigation. There was, however, no apparent decrease in the total amount (*i.e.* soluble plus particulate immunoreactive protein) of any isozyme over the course of 30 min (as in Fig. 1) or 60 min (data not shown) and, therefore, no indication of down-regulation of any of these isozymes as a result of stimulation under these conditions. There appeared also to be at least two bands of the α, β, δ, and ζ[4] isozymes, the two bands being most obvious for the δ isozyme. In each case, the band of higher molecular weight was the one most prominently associated with the particulate fraction (Fig. 1).[5]

After the addition of 20 nM phorbol 12-myristate 13-acetate[6] virtually all of the α, β, and ϵ isozymes, substantial amounts of the δ isoform, and little or none of the ζ (73-kDa) isozyme became associated with the particulate fraction (Fig.

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[4] Several aspects of the ζ isozyme are still under investigation. The lower band was identified as the ζ isozyme on the basis of its immunoreactivity with all three ζ isozyme-specific antibodies. Also, its immunoreactivity with the GIBCO antibody was blocked selectively by the blocking peptide for this isozyme. This peptide did not interfere in the reactions of the other isozyme-specific antibodies with their respective isozymes (see "Experimental Procedures" for descriptions of reagents). Our conclusions with respect to the ζ isozyme are restricted to changes in this band only. The identity of the upper band (sometimes observed as two bands) was uncertain because it reacted with only two of the three ζ isozyme-specific antibodies (the polyclonal rabbit antibody to the amino-terminal being the exception), although its reaction with the GIBCO antibody was blocked by the blocking peptide of ζ. Antigen induced the redistribution of the upper band (Fig. 1) which could be distinguished from other isozymes on the basis of molecular weight and from the effects of phorbol ester and antigen (Figs. 1 and 2). We suspect that it is a species of the ζ isozyme, possibly identical to that described as *Xenopus* protein kinase C (23), in which the amino-terminal epitope is modified or inaccessible to the one antibody noted above.

[5] Treatment of cell extracts with alkaline phosphatase (alkaline phosphatase linked to agarose beads (Sigma), 80 units/ml for 45 min at 37 °C) (24) resulted in the partial disappearance of the upper bands and intensification of the lower bands of α, β, δ, and ζ and unexpectedly the complete disappearance of all bands of ϵ. Therefore serine/threonine phosphorylation of these isozymes is probable and may be necessary for translocation (K. Ozawa, Z. Szallasi, M. G. Kazanietz, P. M. Blumberg, H. Mischak, J. F. Mushinski, and M. A. Beaven, unpublished data).

[6] We used 20 nM phorbol ester because we found in earlier studies that this concentration was sufficient to down-regulate the α and β isozymes and cause partial loss (about 75%) of secretory response to various stimuli (14, 18). We were unaware of the existence of Ca^{2+}-independent isozymes in these early experiments (18, 25).

2). In contrast to antigen stimulation, phorbol ester caused all bands of each isozyme to become associated with the particulate fraction. Consistent with previous observations (14), the β isozyme degraded rapidly and the α isozyme less rapidly. Cells became depleted of both forms by 3–6 h (Fig. 2). The Ca^{2+}-independent δ, ϵ and ζ isozymes, unlike the Ca^{2+}-dependent isozymes, were resistant to degradation in phorbol ester-treated cells for at least 6 h, and the amount of δ actually increased (Fig. 2). The significant finding for the purpose of these studies was the persistence of the Ca^{2+}-independent isozymes in cells exposed to the phorbol ester. The incidental finding of an apparent increase in amount (or immunogenicity) of the δ isozyme was of interest, but a study of the phenomenon was deferred at this time.

Secretory Response after Depletion of Ca^{2+}-dependent Isozymes by Phorbol Ester and the Effects of Inhibitors of Protein Kinase C—Treatment of cells with 20 nM phorbol ester[6] caused progressive, but partial, loss of secretory response to antigen over the course of 6 h. Thereafter a stable, partial response remained (Fig. 3*A*). This residual response varied from 25% (*i.e.* as in Fig. 3*A*) to 33% (mean value for seven experiments, 30%) of the response that was observed in normal cells. To test the possibility that this residual secretory response was dependent on Ca^{2+}-independent isozymes that, as was now apparent (*i.e.* Fig. 2), remained in phorbol ester-treated cells, two inhibitors of protein kinase C were tested, namely calphostin C (26, 27) and compound Ro31-7549 (28). Unlike some inhibitors, these two inhibitors did not inhibit tyrosine phosphorylation of phospholipase Cγ1 and as a consequence hydrolysis of inositol phospholipids in RBL-2H3 cells (29). Both Ro31-7549 (Fig. 3*B*) and calphostin C (Fig. 3*C*) inhibited secretion in cells that had been exposed to phorbol ester for 6 h with undiminished potency when compared with untreated cells (*insets*, Fig. 3, *B* and *C*). If anything, calphostin C had slightly more potent inhibitory action in phorbol ester-treated cells (*inset*, Fig. 3*C*).

In another series of experiments, antigen-induced secretion in untreated (29% release of hexosaminidase) and phorbol ester-treated (8% release of hexosaminidase) cells remained highly dependent on the presence of external Ca^{2+}. Nevertheless, even in the absence of Ca^{2+} a small, but significant, secretory response to antigen (2.0% release of hexosaminidase, $p > 0.025$) was apparent in both untreated and phorbol ester-treated cells. This Ca^{2+}-independent response was blocked completely by 10 μM Ro31-7549 (Fig. 4). Spontaneous release in the absence of antigen (2.5%, subtracted from the above values) was otherwise not significantly affected by the presence of Ro31-7549, the treatment with phorbol ester, or the omission of Ca^{2+} (Fig. 4).

These experiments suggested that secretion was totally dependent on protein kinase C with Ca^{2+}-independent isozymes contributing about 30% of the stimulatory signal for secretion that was mediated by protein kinase C. This conclusion was based on the assumption that down-regulation of α and β isozymes was complete as indicated by the experiments in Fig. 2 (*bottom panel*). Because of the possibility that undetectable traces of α and β isozymes existed in RBL-2H3 cells or that as yet unidentified isozymes were present subsequent experiments tested the validity of this conclusion in different ways. Secretion was also largely dependent on external Ca^{2+}; but in the absence of Ca^{2+}, the release of intracellular Ca^{2+} (30, 31) might have accounted for the 2.5% release noted above.

Loss or Retention of Secretory Response in Permeabilized Cells: Studies with Phorbol Ester—Permeabilization of RBL-2H3 cells with streptolysin *O* resulted in partial loss of secretory response and, after subsequent washing of the permeabilized cells, resulted in a complete loss of secretory response to antigen. The phosphoinositide response to antigen, however, was not impaired and was even enhanced by these procedures (Fig. 5). As noted above, treatment of cells with phorbol ester caused partial loss (to about 30% of normal) of secretory response in intact cells, but permeabilization and washing of these cells caused little further loss of this response. Because Ro31-7549 inhibited secretion (but not hydrolysis of inositol phospholipids) after treatment with phorbol ester in both intact and permeabilized cells (Fig. 5), the retention of secretory response was probably caused by retention of protein kinase C in phorbol ester-treated permeabilized cells. This was indeed the case as demonstrated in additional experiments by immunoblotting techniques (Fig. 6).

The immunoblots revealed that none of the isozymes of protein kinase C was detectable in washed permeabilized cells; that only δ, ϵ, and ζ isozymes of protein kinase C were present in intact cells after treatment with phorbol ester; and that the same isozymes were retained even after subsequent permeabilization and washing of the phorbol ester-treated cells. These retained isozymes, as expected, were largely associated with the membrane fraction, but antigen stimulation did cause small increases (or reduced the losses) in the amounts of membrane-associated isozymes (Fig. 6).

Reconstitution of Secretory Response in Washed Permeabilized Cells with Recombinant Preparations of Protein Kinase C Isozymes—The restorative action of the isozymes was tested at low (10 nM) and high (1.0 μM) $[Ca^{2+}]_i$. The latter concentration has been observed in intact antigen-stimulated RBL-2H3 cells (25, 32). The secretory response to antigen could be fully restored, in the presence of 1 μM (Fig. 7*A*) but not 10 nM (Fig. 7*B*) $[Ca^{2+}]_i$, by the addition of a commercial preparation of protein kinase C (rat brain protein kinase C from Calbiochem) which contained only the α, β, and γ isozymes of protein kinase C as determined by immunoblotting techniques and no other protein as determined by Coomassie Blue stain (not shown). The addition of high concentrations of enzyme by itself caused some secretion in the presence of 1 μM $[Ca^{2+}]_i$ (Fig. 7*A*). The secretory response was restored also by the addition of purified preparations of the β and δ isozymes but only weakly so by the addition of the α and ϵ isozymes of protein kinase C. As had been observed with the commercial preparation above, no secretion was observed at 10 nM (Fig. 8*A*) or even 100 nM (Fig. 8*B*) $[Ca^{2+}]_i$, but at high $[Ca^{2+}]_i$ (1 μM) the extent of restoration was dependent on the concentration of β or δ isozyme to give a maximal response that approached that obtained with antigen in intact cells (Fig. 8*C*). Little or no secretion was observed, however, at high $[Ca^{2+}]_i$ in the absence of antigen (Fig. 8*D*). Of particular note in these experiments was that the two preparations of the β isozyme from the baculovirus system and from rat brain were equally potent (Fig. 8*C*) and that restoration of the response to antigen with δ isozyme was dependent on high $[Ca^{2+}]_i$.

The isozymes in combination had additive rather than synergistic actions when tested at concentrations that were less than optimal for secretion (Table I). The concentrations of isozymes that were required to support 50% of the maximal response (EC_{50}) were about 5, 45, and >100 nM for, respectively, β, δ, and the other isozymes (Table II).

The weak actions of α and ϵ isozymes noted above were not caused by degradation of these two isozymes. As will be reported elsewhere, under identical experimental conditions, the α and ϵ isozymes inhibited antigen-induced hydrolysis of inositol phospholipids at nM concentrations, whereas the β

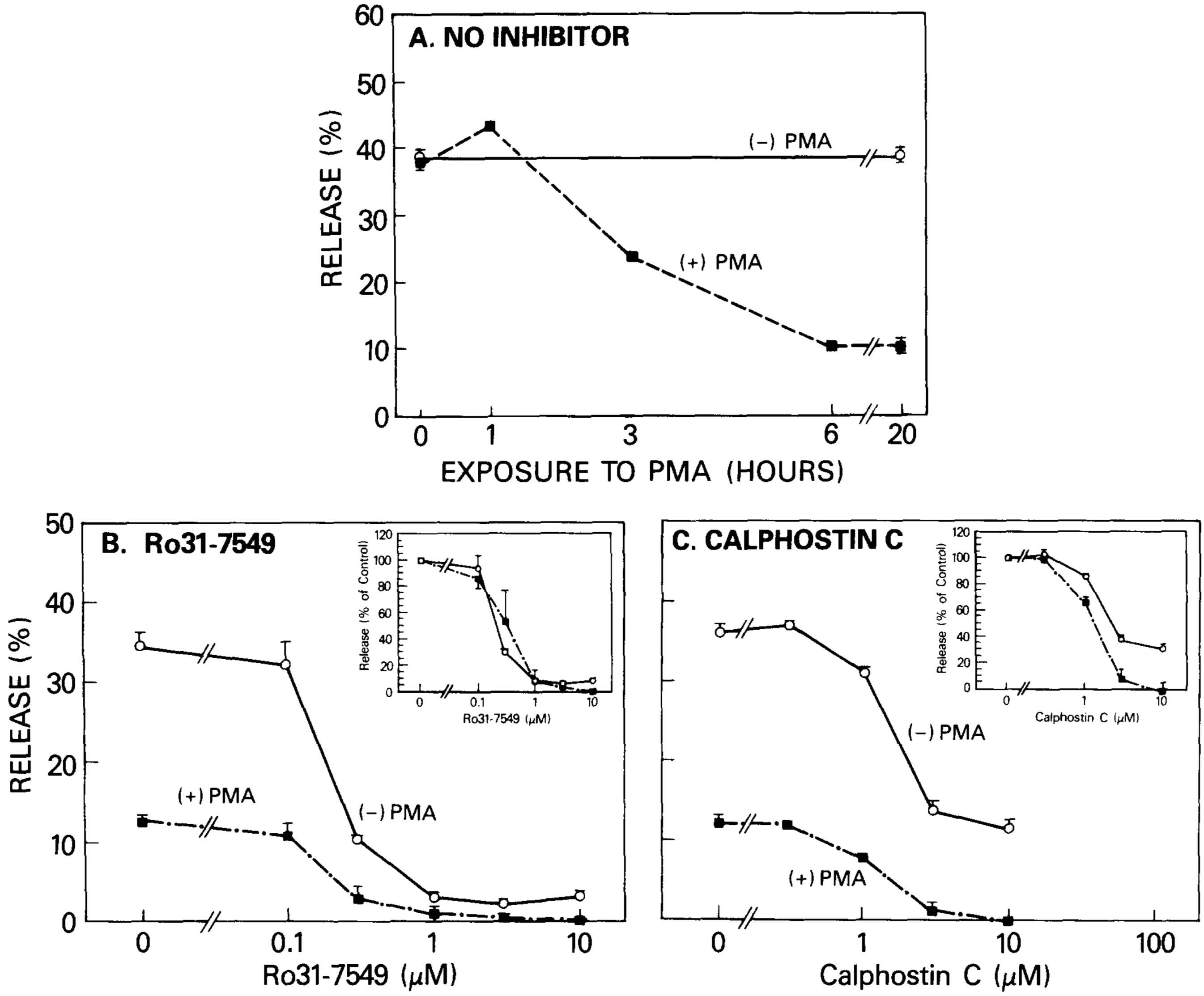

FIG. 3. **Inhibition of antigen-induced exocytosis by protein kinase C inhibitors.** Cells were exposed to vehicle (○) or 20 nM phorbol 12-myristate 13-acetate (*PMA*, ■) to deplete Ca^{2+}-dependent isozymes. In *panel A*, cells were exposed to vehicle or phorbol ester to indicate loss of secretory response to antigen (DNP-BSA, 20 ng/ml) with time. In *panels B* and *C*, cells were first exposed to phorbol ester for 8 h, and the indicated concentrations of Ro31-7549 or calphostin C were then added to the cultures 10 min before stimulation with antigen (20 ng/ml DNP-BSA). Release of hexosaminidase was measured 15 min thereafter. In this and the following figures, the values (mean ± S.E. of values of three cultures) for release of hexosaminidase into the medium were expressed as a percent of intracellular hexosaminidase in matched unstimulated cultures (*i.e.* cells were treated with vehicle, phorbol ester, or inhibitors but no antigen was added) and were corrected for the spontaneous release in unstimulated cultures (4.8 ± 0.4% in normal cells and 2.8 ± 0.2% in phorbol ester-treated cells). In the *insets* (*panels B* and *C*), the release of hexosaminidase was recalculated as a percent of release that was observed without the addition of inhibitor (*i.e.* releases from the controls for untreated and phorbol ester-treated cells were both made equivalent to 100%). Treatment with phorbol ester for 8 and 20 h resulted in, respectively, 6 and 30% reduction in the intracellular content of hexosaminidase.

and δ isozymes were devoid of such activity. Thus, all four preparations of isozymes exhibited distinct types of activities.

DISCUSSION

Previous studies have established that the Ca^{2+}-dependent α and β isozymes of protein kinase C are present in RBL-2H3 cells and that they are degraded at different rates in cells treated with phorbol 12-myristate 13-acetate (14). Following the disappearance of these isoforms, antigen-induced hydrolysis of inositol phospholipids and elevation in $[Ca^{2+}]_i$ were no longer inhibited by phorbol ester (18). The present studies have revealed, in addition, the existence of at least three Ca^{2+}-independent isozymes of protein kinase C which are not degraded in phorbol ester-treated RBL-2H3 cells under the conditions of our experiments. The identification of these isoforms (δ, ϵ, and ζ) was based on their immunoreactivity to various antibodies and differences in apparent molecular masses (75, 93, and 73 kDa for the lower bands of δ, ϵ and ζ[4] isoforms, respectively). These apparent molecular masses were similar to the molecular masses (90 kDa for ϵ) as estimated in the literature from sodium dodecyl sulfate gels (5) or as calculated (78, 84, and 68 kDa for the δ, ϵ, and ζ isoforms, respectively) from the deduced amino acid sequences of rat or murine brain cDNA for protein kinase C isozymes (1).

The finding that both Ca^{2+}-dependent and Ca^{2+}-independent isozymes of protein kinase C redistributed in antigen-stimulated cells raised two questions. First, did both types of isozymes contribute stimulatory signals for secretion, and if so, to what extent? Second, did external Ca^{2+}, which was necessary for sustained increases in $[Ca^{2+}]_i$ and secretion (30–32), have a role in secretion other than supporting the redis-

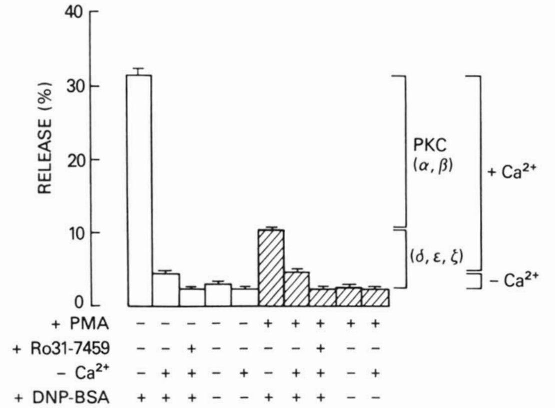

FIG. 4. **Effects of phorbol ester (*PMA*), Ro31-7549, and omission of calcium (−Ca^{2+}) on antigen (DNP-BSA)-induced secretion in intact RBL-2H3 cells.** Cultures were exposed to vehicle (0.1% dimethyl sulfoxide) or 20 nM phorbol ester in complete growth medium for 6 h to deplete cells of the Ca^{2+}-dependent isozymes of protein kinase C (*PKC*). Medium was replaced with the PIPES-buffered medium that contained vehicle, 10 μM Ro31-7549 to inhibit residual protein kinase C, or no Ca^{2+} (with 0.1 mM EGTA) 10 min before the addition of 20 ng/ml DNP-BSA. Secretion was measured 15 min later. The additions are indicated on the *bottom* of the figure. Secretions thus attributed to the Ca^{2+}-dependent and Ca^{2+}-independent isozymes and Ca^{2+} itself are indicated on the *right* of the figure. Values were mean ± S.E. of nine cultures from three separate experiments.

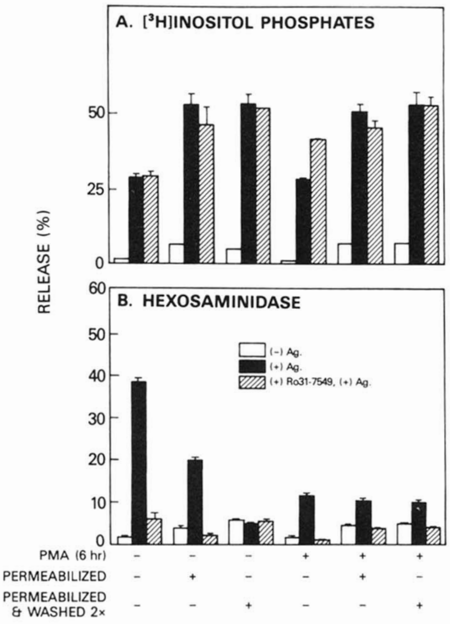

FIG. 5. **Effects of phorbol ester (*PMA*) and Ro31-7549 on antigen (*Ag*)-induced secretion in intact, permeabilized, and washed permeabilized cells.** Cultures were exposed to 20 nM phorbol ester for 6 h as described in Fig. 4 and were then left intact, permeabilized, or permeabilized and washed as indicated in the *bottom* of the figure. A PIPES-buffered medium was used for intact cells, and a potassium glutamate medium ($[Ca^{2+}]_i$ was 1.0 μM) was used for permeabilized cells (see "Experimental Procedures"). Ro31-7549 (10 μM) or vehicle was added 10 min before the addition of 20 ng/ml DNP-BSA; release of inositol phosphates (*panel A*) and secretion (*panel B*) were measured 15 min thereafter. Values were the mean ± S.E. of six cultures from two separate experiments.

tribution and activation of protein kinase C?

Cells depleted of the Ca^{2+}-dependent isozymes with phorbol ester still secreted in response to antigen (about 30% of normal response), but only in the presence of external Ca^{2+}, even though Ca^{2+} was not required for translocation of the residual Ca^{2+}-independent isozymes. Presumably, the additional (*i.e.* 70%) secretory response in normal *versus* depleted cells was caused by the translocation and activation of the Ca^{2+}-dependent isozymes for which Ca^{2+} was clearly required (Fig. 1). Thus, the increase in $[Ca^{2+}]_i$ may have two distinct roles: one is the activation of Ca^{2+}-dependent isozymes, and the other is independent of this action.

Further evidence for the involvement of both Ca^{2+}-dependent and Ca^{2+}-independent isozymes was the inhibition of secretion in normal as well as depleted cells with Ro31-7549 and calphostin C. These two compounds act at different sites on the protein kinase C molecule, Ro31-7549 at the ATP binding site (28) and calphostin C at the phorbol ester/

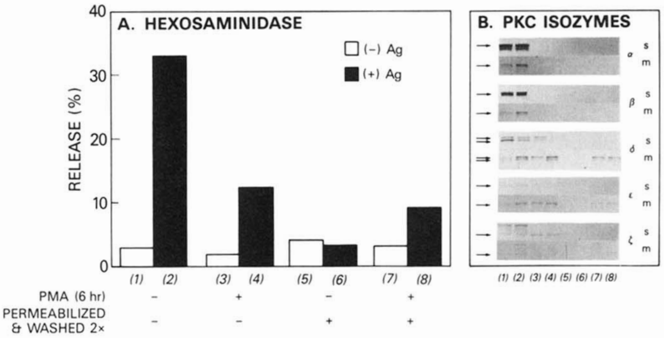

FIG. 6. **Retention of secretory response and Ca^{2+}-independent isozymes of protein kinase C (*PKC*) in washed, permeabilized cells when cells were treated with phorbol ester (*PMA*) before permeabilization.** Cultures were prepared in large culture dishes for immunoblotting as described under "Experimental Procedures." Otherwise the cells were exposed to 20 nM phorbol ester or vehicle for 6 h, left intact, or permeabilized and washed and then stimulated with 20 ng/ml DNP-BSA for 15 min exactly as described in Fig. 5 and as indicated in the *bottom* of *panel A*. For these experiments $[Ca^{2+}]_i$ was buffered at 1.0 μM. Samples of medium were removed for assay of hexosaminidase; additional cultures were used for measurement of intracellular hexosaminidase to determine percent release (*panel A*). Extracts of cells were prepared from the same cultures for detection of the isozymes by immunoblotting (*panel B*). The individual cultures were designated by the *italicized numbers* to indicate the corresponding data in each *panel*.

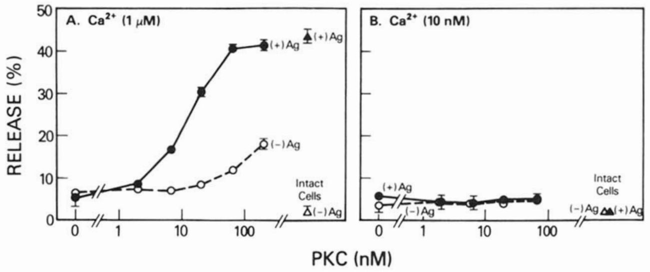

FIG. 7. **Reconstitution of secretory response by the addition of protein kinase C (*PKC*) isozymes to washed permeabilized cells.** Cultures in 96-well plates (40,000 cells in 40 μl/well) were left intact (△, ▲) in a glucose-saline PIPES medium (1 mM Ca^{2+}) or permeabilized with streptolysin *O* (0.2 units/ml in 200 μl) and washed twice with 200 μl of medium before the final addition of 40 μl of medium (○, ●). The medium used for studies with permeabilized cells was a potassium glutamate medium (18) in which $[Ca^{2+}]$ was buffered at 1.0 μM (*panel A*) or 10 nM (*panel B*). A commercial preparation of protein kinase C that contained α, β, and γ isozymes was added to give the indicated concentrations of protein kinase C (see "Experimental Procedures"). The cultures were incubated for 15 min before addition of DNP-BSA (10 μl, 100 ng/ml) (●, ▲) or vehicle (○, △). The cultures were incubated for an additional 15 min before terminating the reaction for measurement of release of hexosaminidase as described in previous legends. Values were mean ± S.E. of three cultures. *Ag*, antigen.

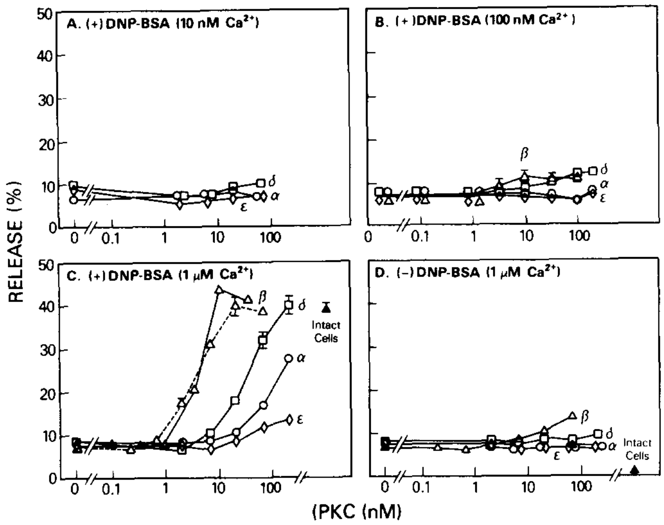

FIG. 8. **Reconstitution of secretory response by addition of purified preparations of protein kinase C to washed permeabilized cells.** The preparations included recombinant isozymes (α, β, δ, and ϵ) of protein kinase C from the baculovirus expression system (*solid lines*) and a purified preparation of the β-isozyme from rat brain (*dashed line*). Experiments were performed with cultures in 96-well plates exactly as described in the legend of Fig. 7. Intact (▲) or permeabilized, washed (O, △, □, ◇) cells were incubated with the indicated concentration (see "Experimental Procedures") of each isozyme of protein kinase C (α, O; β, △; δ, □; ϵ, ◇) for 15 min in the presence of the indicated concentration of free Ca^{2+}. Intact cells (*solid symbols, panels C* and *D*) were kept in the glucose-saline PIPES medium (1 mM Ca^{2+}). Cells were then incubated for an additional 15 min in the presence (*panels A, B,* and *C*) or absence (*panel D*) of DNP-BSA (100 ng/ml). Values were the mean ± S.E. of three cultures.

TABLE I

Secretory response of washed, permeabilized cells to antigen (DNP-BSA, 100 ng/ml) in the presence of different isozymes of protein kinase C

Results are the mean values of three experiments. The standard error of the means was <5% of mean value except for α, *viz.* 9.9 ± 0.9%. Experiments were performed as described in the legend of Fig. 8. Isozymes were tested either separately (first column) or in combination with each of the other isozymes (final three columns). Calculated values for additive responses are indicated in parentheses.

Additional isozyme	Release of hexosaminidase (%)			
	None	α (50 nM)	δ (25 nM)	β (5 nM)
50 nM ϵ	5.7	18.2 (15.6)	22.2 (22.9)	17.4 (21.3)
50 nM α	9.9		26.4 (27.1)	24.0 (25.5)
25 nM δ	17.2			32.2 (32.8)
5 nM β	15.6			

diglyceride binding site (26). These compounds also do not inhibit, unlike staurosporine and other serine/threonine kinase inhibitors, the tyrosine phosphorylation and the activation of phospholipase C γ1 in RBL-2H3 cells (29).

Reconstitution of the secretory responses with either β or δ isozymes in washed permeabilized cells provided the most direct evidence that both the Ca^{2+}-dependent and -independent isozymes can mediate antigen-induced secretion. The studies point to the possibility that different isozymes of protein kinase C might have discrete functions within the cell. Certainly β and, to a lesser extent, δ were uniquely potent in restoring the secretory response. We have proposed previously that the recruitment of protein kinase C by antigen resulted in weak feedback regulation of phospholipase C (25) because, in comparison, low concentrations of phorbol ester markedly inhibited the activation of phospholipase C (25) and the increase in $[Ca^{2+}]_i$ (33) in antigen-stimulated cells. Loss of this inhibitory action during prolonged exposure to phorbol ester was correlated with the disappearance of the α isozyme of protein kinase C (18). Also, as previously mentioned, our studies with purified isozymes in permeabilized cells indicated that both α and ϵ isozymes have such inhibitory activity.

TABLE II

Concentration of each isozyme of protein kinase C that was required for restoration of 50% of the maximal secretory response (EC_{50}) to antigen in washed, permeabilized RBL-2H3 cells

Calculations were based on the apparent molecular weights depicted in Fig. 1. The estimated concentration of protein kinase C in RBL-2H3 cells was 90 nM as determined by the number of phorbol 12,13-dibutyrate binding sites (60,000/cell) and based on an assumed molecular mass of 80 kDa, one binding site/molecule, and a cell volume of 1.1 pl (K. Ozawa, unpublished data).

Isozyme	EC_{50}[a]
	nM
α	175
β	5
δ	45
ϵ	>250

[a] Concentration required to achieve 50% of maximal antigen-induced response in intact cells.

It should be noted that similar approaches have been used previously to investigate the role of Ca^{2+}-dependent isozymes of protein kinase C in exocytosis. For example, when rat pituitary cells were treated with 1 μM phorbol 12-myristate 13-acetate for 24 h and then permeabilized with digitonin, before the addition of purified brain α, β, or γ isozymes of protein kinase C, only the α and β isozymes restored the secretory response (release of luteinizing hormone) to phorbol ester (34 and studies cited therein). Although the possibility of tissue differences cannot be excluded, the apparent stimulatory action of α isozyme of protein kinase C in pituitary cells was not necessarily at variance with its weak stimulatory action in RBL-2H3 cells. We note, for example, that high unphysiological concentrations of α isozyme of protein kinase C will support same secretion in RBL-2H3 cells (Fig. 8*C*). The relative potencies or actual concentrations of α and β isozymes of protein kinase C that were employed in the studies with pituitary cells could not be assessed because of insufficient description of experimental details in the original paper (34). We note also that phorbol 12-myristate 13-acetate caused virtually complete translocations of α isozyme of protein kinase C to the membrane fraction, whereas antigen caused only modest redistribution (~20%; Fig. 1, *inset A*) of isozyme in RBL-2H3 cells. Therefore, valid comparisons cannot be made between RBL-2H3 cells and pituitary cells because the stimulants for these studies were quite different, namely, the phorbol ester for pituitary cells and antigen for RBL-2H3 cells.

We have not yet measured the amounts of the individual isozymes in RBL-2H3 cells, but estimation of the number of $[^3H]$phorbol-12,13-dibutyrate binding sites indicates an intracellular concentration of protein kinase C of approximately 90 nM (60,000 binding sites/cell) and the translocation of about 20% of these sites after antigen stimulation. Therefore the concentrations of protein kinase C β and possibly δ that were required for restoration of secretion in permeabilized cells (Table II) appeared to be physiologically relevant. Also of physiological significance, secretion was not restored at 0.1 μM $[Ca^{2+}]_i$, but it was fully restored at 1 μM $[Ca^{2+}]_i$ (Fig. 8). These concentrations correspond to these observed, in respectively, unstimulated and stimulated RBL-2H3 cells (25, 32).

Antigen-induced secretion in mast cells and related cell lines such as the RBL-2H3 cell is thought to be dependent on the simultaneous activation of protein kinase C and an increase in $[Ca^{2+}]_i$ (15). This premise, however, is based on indirect evidence. Secretion occurs when both events are induced pharmacologically by the combination of low concentrations of Ca^{2+}-ionophores and phorbol ester (25), but this does not indicate that the responses to antigen are mediated necessarily or exclusively through these two events. Moreover, because of the residual secretory response after prolonged exposure of cells to phorbol ester, the mechanism for this residual response was unclear (15, 18). The present work indicates that the residual response is probably caused by the persistence of the δ isozyme in these cells. We have not investigated the effects of high concentrations of phorbol ester (*i.e.* > 20 nM), which might conceivably down-regulate the Ca^{2+}-independent isozymes, and our conclusion is based on the particular protocol used in this and our previous work (18).

Of the critical factors that are necessary for exocytosis, it would appear that the isozymes of protein kinase C are readily lost by washing permeabilized cells. Other factors, which do not appear to be lost upon permeabilization, include presumably phospholipase C, the proteins regulated by Ca^{2+}, and substrates for protein kinase C. The only well studied substrates in RBL-2H3 cells are the light and heavy chains of myosin. The phosphorylation of these chains by protein kinase C is temporally correlated with the secretory response to antigen (17). Beyond this little is known about the secretory mechanism in RBL-2H3 cells, but the present studies suggest that washed, permeabilized cells may provide a simpler model for studies of secretory mechanism than intact cells. At the least, the use of individual isozymes might simplify the search for the critical substrates of protein kinase C for exocytosis. Also, the reconstitution of the secretory response with the Ca^{2+}-independent δ isozyme alone might facilitate studies of the possible site(s) of action of the Ca^{2+} that are necessary for exocytosis.

We conclude that antigen-induced secretion is dependent on the presence of certain protein kinase C isozymes (principally β and δ) and free Ca^{2+}. Because Ca^{2+} is not required for translocation of the δ isozyme (Fig. 1), a second Ca^{2+}-activated process, other than the Ca^{2+} activation of protein kinase C, is required for secretion. The data do not exclude, however, the existence of another necessary pathway as, for example, activation of tyrosine kinases (35, 36) if this pathway remains intact in permeabilized cells.

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