RACK1 is a protein kinase C (PKC)-binding protein that fulfills the criteria previously established for a receptor for activated C-kinase (RACK). If binding of PKC to RACK anchors the activated enzyme near its protein substrates, then inhibition of this binding should inhibit translocation and function of the enzyme in vivo. Here, we have identified such inhibitors that mimic the RACK1-binding site on βPKC. We first found that a C2-containing fragment, but not a C1-containing fragment, of βPKC, bound to RACK1 and inhibited subsequent βPKC binding. The RACK1-binding site was further mapped; peptides βC2-1 (βPKC(209–216)), βC2-2 (βPKC(186–198)), and βC2-4 (βPKC(218–226)), but not a number of control peptides, bound to RACK1 and inhibited the C2 fragment binding to RACK1. Peptides βC2-1, βC2-2, and βC2-4 specifically inhibited phorbol ester-induced translocation of the C2-containing isozymes in cardiac myocytes and insulin-induced βPKC translocation and function in Xenopus oocytes. Therefore, peptides corresponding to amino acids 186–198, and 209–226 within the C2 region of the βPKC are specific inhibitors for functions mediated by βPKC.

Protein kinase C (PKC) isozymes are phosphatidylserine (PS)- and diacylglycerol (DG)-dependent kinases (1) that translocate from the soluble fraction to the cell particulate fraction following activation (2, 3). Several PKC isozymes are present in each cell type. The isozymes are localized to different subcellular compartments (4–6), and following stimulation, each translocates to distinct intracellular structures (7). The translocation and binding of PKC isozymes to different intracellular structures suggests distinct physiological roles for individual isozymes. We previously demonstrated that inhibition of PKC translocation inhibits its function (8, 9). Therefore, inhibitors that prevent the translocation of specific isozymes, can provide pharmacological tools to determine the function of each isozyme.

The PKC isozymes can be divided into three subfamilies: conventional δPKC, novel nPKC, and atypical αPKC (10). Each PKC isozyme contains unique (V) regions. In addition, there are regions common to all the isozymes. The subfamilies differ from each other in the common (C) regions within their regulatory domains. The regulatory domain of the cPKC isozymes contains two common regions, C1 and C2. The C1 region consists of two cysteine-rich loops that mediate DG and phorbol ester binding (11–13). C1 is also found in the nPKC subfamily and one of the cysteine loops is found in the αPKC subfamily (14–16). The C2 region is present only in the cPKC subfamily and mediates calcium binding; all the C2-containing isozymes require calcium for their activity (10). This region may also serve as a low affinity calcium sensor (17). In addition, the C2 region mediates PS binding (18). However, recent studies indicate that the V1 and C1 regions also mediate calcium and PS binding (19, 20). Finally, the C2 regions of other C2-containing proteins were proposed to mediate direct binding of these translocating proteins to lipids at the plasma membrane (21).

Translocation of PKC to the cell particulate fraction was thought to reflect direct binding of the enzyme to lipids at the plasma membrane. However, data from several laboratories including our own indicate that translocated PKC interacts with proteins at the site of translocation (2, 22–28). We have identified several proteins from the cell particulate fraction that bind PKC only in the presence of its activators (24). Binding of PKC to these proteins was concentration dependent, saturable, and specific, suggesting that these binding proteins are receptors for activated C-kinase, or RACKs (24). Recently, we cloned RACK1, a gene encoding for a 36-kDa homolog of the β subunit of G proteins (accession number U03390) that fulfills the criteria for RACKs (25). RACK1 is neither a PKC substrate nor an inhibitor (25). Rather, it increases PKC phosphorylation of substrates presumably by stabilizing the active form of PKC (25).

The RACK1-binding site on PKC is unknown. If this site is identified, peptides that mimic the binding site could serve as specific inhibitors of PKC translocation and function. Our previous studies suggested that the C2 region of δPKC contains at least part of the RACK-binding site on the enzyme; other C2-containing proteins such as synaptotagmin (29) and phospholipase Cγ (30) also bind to a mixture of RACKs prepared from the cell particulate fraction. In addition, recombinant fragments of synaptotagmin containing the C2 homologous region bind to RACKs and inhibit PKC binding to RACKs (29). However, these studies were carried out with heterologous C2-containing fragments from synaptotagmin and a preparation containing a mixture of RACKs. Here, we used recombinant RACK1, recombinant fragments of βPKC containing the C1 or the C2 regions, and short synthetic peptides derived from the C2 region, to identify the RACK1-binding site on βPKC. We found that the C2 region contains at least part of the RACK1-binding site on PKC and that some C2-derived peptides act as specific inhibitors of hormone-induced translocation and functions of the C2-containing βPKC isozymes.

EXPERIMENTAL PROCEDURES

Peptides and Reagents—Peptide βC2-1 (KQTKTK1K; βPKC(209–216)), was synthesized at the Biomolecular Resource Center at Univer-
sity of California, San Francisco. βC2-2 (MDPNGLSDPVYKL; βPKC(186–198)), scrambled βC2-1 (TKQKIKIT), and control peptide (LQKAGVDG; βPKC(266–273)) were synthesized at the Protein and Nucleic Acid Facility at Stanford University. βC2-3 (IPDPKSE; βPKC(201–207)) and βC2-4 (SLNPEWDDK; βPKC(218–226)) were a generous gift from Dr. Rafael Nesper, The Hebrew University of jerusalem, Israel. All the peptides used in this study were over 90% pure. Moreover, most of the peptides were made twice, and each batch gave the same biological activities. 4i-Phorbol 12-myristate 1-acetate (PMA) was from L. C. Laboratories Inc. and diacylglycerol and phosphatidylserine were purchased from Avanti Inc. PKC was partially purified from rat brain as described previously (31).

Preparation of RACK1—Recombinant RACK1 was overexpressed in Escherichia coli as a fusion protein with the maltose-binding protein and tagged with a FLAG sequence (DTKDDDDDK; Kodak) down-stream from the Xa proteolysis site. The overexpressed protein was then purified on amylose affinity column (Biolabs Inc.). The 36-kDa RACK1 containing the FLAG sequence was recovered after removal of the maltose-binding protein by incubating with factor Xa (5 μg/ml; Biolabs Inc.) for 48 h at 4 °C.

Recombinant Fragments of βPKC—The plasmids L9 and L10 expressing the C1 (L9) and C2 (L10) regions as fusion proteins with glutathione S-transferase (GST) were a generous gift from Dr. Bernard Weinstein, Columbia University, New York, and the GST-fusion proteins expressed in E. coli were produced as described (20).

Overnight preculture was blotted onto nitrocellulose as described elsewhere (24). Strips of the nitrocellulose sheet (0.5–1 μg RACK1/strip) were incubated in overlay buffer (50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin, 5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 0.1% polyethylene glycol, 0.2 mM NaCl, 0.1 mM CaCl2, and 12 mM β-mercaptoethanol) or preincubated for 30 min at room temperature, in overlay buffer with the indicated peptide (10 μM). L9 or L10 (10 μM) were added in the presence or absence of 50 μg/ml PS, and 1 mM calcium, and the mixture was further incubated for 30 min at room temperature. Where indicated, immobilized RACK1 was first incubated with C2-derived peptides in the absence of PKC activators. PKC fragments in the presence or absence of PKC activators were then added, after which material was washed three times with PBS, and washed three times for 5 min with overlay wash buffer (0.1% polyethylene glycol, 0.2 mM NaCl, 0.1 mM CaCl2, 12 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5). Binding of fragments L9 and L10 to RACK1 was detected with anti-GST polyclonal antibodies (1:5000, a gift from Dr. Richard Scheller, Stanford University). The strips were then incubated with anti-rabbit horseradish peroxidase-linked antibodies diluted 1:1000 (Amersham Life Science) followed by chemiluminescent reaction. Quantitation of L9 and L10 binding was carried out by analyzing autoradiograms using a Microscan 100 gel analyzer (Galai Inc. Israel) at linear range of detection.

Column Assay—An amylose suspension (0.5 ml; Biolabs Inc.) was loaded onto a poly-prep chromatography column (Bio-Rad) and washed with wash buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, and 10 mM β-mercaptoethanol). Crude extracts of RACK1-maltose-binding protein fusion protein (1 ml, 10 μg/ml) were loaded and the column washed extensively (20 column volumes). L9, L10 (10 μM), or partially purified rat brain PKC (containing a mixture of PKC isozymes; 30 nM; 200 units/mg) were then added, in the presence or absence of 50 μg/ml PS, and 1 mM calcium or 50 μg/ml PS, 0.8 μg/ml DG, and 1 mM calcium in overlay buffer. After incubation while shaking for 30 min at room temperature, unbound material was removed, the column was washed with column wash buffer (20 column volumes), the proteins were eluted with 10 mM maltose in column wash buffer, and the amount of bound βPKC and L10 determined by Western blot analysis. To this end SDS-PAGE sample buffer (0.3 mM Tris-HCl, 5% SDS, 50% glycerol, 0.01% bromphenol blue, and 5% β-mercaptoethanol) was added, the samples were boiled for 10 min, and loaded on 10% SDS-PAGE gel. Western blot analysis with anti-FLAG antibodies (Kodak; 1:1000) demonstrated that equal amounts of RACK1 were eluted from all columns by amylose. PKC and L10 binding were determined using labeled βPKC monoclonal antibodies (Seikagaku America, Inc; 1:1000). L9 and L10 binding were also detected with anti-GST antibodies, followed by a chemiluminescent reaction as described above.

RACK1 Binding to the C2-derived Peptides—Peptides (1 nmol/slot) were blotted onto nitrocellulose paper using a slot-blott apparatus (Schleicher & Schuell). Unbound material was removed, and the nitrocellulose was incubated at room temperature (20 mM Tris-HCl, pH 7.5, 3% bovine serum albumin, 0.1% PEG, 0.2 mM NaCl). RACK1 (20 ng) was added in overlay buffer, and the blot was incubated for 30 min at room temperature. Binding of RACK1 to these peptides was determined as described above with anti-FLAG monoclonal antibodies (1:10,000). Binding was quantitated using a Microscan 1000 Gel Analyzer (Galai Inc. Israel).

Cell Culture—Primary cultures of ~90% pure cardiac myocytes were prepared from hearts of 1-day-old rats by gentle trypsinization at room temperature as described (7). Cells were cultured for 4 days in Lab-Tek chamber slides (Nunc Inc.), pre-coated with laminin (1 μg/ml) in the presence of Dulbecco’s modified Eagle’s medium, with 5% fetal bovine serum. The culture medium was then replaced with serum-free medium containing transferrin (10 μg/ml) and insulin (5 μg/ml) for 2 days. Bromodeoxyuridine (0.1 μg/ml) was used through the third day of culture to keep the level fibroblasts at 10% or lower (7).

Permeabilization of Cardiac Myocytes and PKC Translocation: Immunofluorescence Studies—The cells were washed once with PBS and treated with cold permeabilization solution (10 mM EGTA, 140 mM KCl, 20 mM HEPES, 50 μM saponin, 5 mM sodium azide, and 5 mM potassium diatolate, pH 7.4) together with the indicated peptide (10 μM) for 10 min at room temperature. Permeabilization does not affect the viability of the cells nor does it alter the rate of spontaneous and stimulated contraction, basal or hormone-induced expression of c-fos, and stimulation-induced hypertrophy, indicating that complex cell function is not altered by the permeabilization (7).

PMA-induced translocation of PKC isozymes was determined by immunofluorescence studies as described before (32). The cells were then washed with PBS, fixed with cold acetone for 3 min, and washed twice with cold PBS. The cells were incubated for 1 h with 1% normal goat serum in PBS containing 0.1% Triton X-100 followed by overnight incubation with anti-P1, β1, or βPKC polyclonal antibodies (Research and Diagnostic Antibodies; 1:100), or anti-RACK1 polyclonal antibodies (Santa Cruz Biotechnology; 1:100) or anti-RACK1 (Transduction Laboratories; 1:100) diluted in PBS containing 0.1% Triton X-100 and 2 mg/ml bovine serum albumin. The cells were washed three times with PBS containing 0.1% Triton, incubated for 2 h with fluorescein-conjugated anti-rabbit IgG antibodies (to detect binding of anti-PKCI antibodies; Organon Teknika; 1:1000) or anti-mouse IgM antibodies (to detect binding of anti-RACK1 antibodies; Boehringer Mannheim; 1:100) washed again three times with PBS containing 0.1% Triton. After mounting with Miowiol 4–88 (Calbiochem), the slides were viewed with a Zeiss IM35 microscope using a 40X water immersion objective. Multiple fields of cells for each treatment group and each PKC isozyme were monitored, and the number of cells showing the localization of activated isozyme (32) was recorded. Data are presented as the percentage of cells having the tested isozyme at the activated site. Images of RACK1 and PKC localization from the Zeiss microscope were recorded on Kodak TMax 400 film, and the exposure time was 30 s for these micrographs.

Xenopus Oocyte Maturation Assay—Microinjection of Xenopus oocytes was carried out by Wu and associates (Berkeley, CA) as described previously (8). Oocytes were injected with 50 nl of the indicated peptide 1 h before insulin treatment (8.25 μg/ml). Insulin-induced oocyte maturation was then determined by monitoring the appearance of a white spot in the animal pole of the oocyte that is indicative of germinal vesicle breakdown and maturation. Ten to 15 oocytes were included in each treatment, and oocytes were scored for up to 35 h after insulin treatment.

Analysis of βPKC Distribution in Xenopus Oocytes—One hour after microinjection of the indicated peptide to the oocytes (100 oocytes/group), the oocytes were incubated for an additional hour with or without insulin and then hormone treatemnts were proceeded as described (8). Samples were diluted in homogenization buffer (500 μl; 20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 20 mM EDTA, 0.25 mM sucrose, and 20 μg/ml of each soybean trypsin inhibitor, leupeptin, and aprotinin), and centrifuged at 100,000 x g for 30 min at 4 °C. The supernatant (cytosolic fraction) was removed, and the pellet (particulate fraction) was dissolved in homogenization buffer (500 μl). After addition of sample buffer, these samples were loaded onto 10% SDS-PAGE, and the amounts of βPKC in both fractions were determined by Western blot analysis using anti-βPKC antibodies (Seikagaku America 1:1000) followed by IgG rabbit anti-mouse (Zymed Inc. 1:2000) and 32P-labeled protein A.

1 J. A. Johnson, K. E. Eadie, C. E. Hertler, and S. L. Mullins, manuscript in preparation.

2 J. A. Johnson, M. O. Gray, and D. Mochly-Rosen, manuscript in preparation.
Translocation Inhibitors of C2-containing Protein Kinase C

**RESULTS**

Previous studies suggested that at least part of the RACK-binding site on βPKC lies within the C2-region of the enzyme (29). In addition these studies suggested that the activators of PKC (PS, DG, and calcium) are required to expose the RACK-binding site on βPKC rather than for the interaction of the C2 region with RACKs (29). If the RACK1-binding site on βPKC is within the C2 region, a C2-containing fragment of βPKC should bind to RACK1 in a PS-, DG-, and calcium-independent manner. We used two fragments of βPKC expressed as fusion proteins with GST. One of the fragments, L9, includes the V1 region, the pseudosubstrate sequence, and the first cysteine repeat from the C1 region, as well as the entire C2 and V3 regions (amino acids 3–182) (20). The second fragment, L10, includes the V1 region, the pseudosubstrate sequence, and the first cysteine repeat from the C1 region, as well as the entire C2 and V3 regions (amino acids 3–76 and 143–339) (20). In an overlay assay, L10, the C2-containing fragment, but not L9, the C1-containing fragment, bound to RACK1 (Fig. 1). Saturation of binding of L10 to RACK1 was observed at 1 μM. In contrast, the binding of L9 to RACK1 (Fig. 1, lanes 2 and 4) was minimal, not saturable and similar to the nonspecific binding of GST carrier protein alone (not shown). The V1 region, the pseudosubstrate site, and the first cysteine-loop of C1 are present in both L10 and L9. However, L10 also contains the C2 and V3 regions of PKC. Therefore, these results suggest that the C2 and/or the C3 regions bound to RACK1. In addition, PKC activators PS and calcium did not raise the binding of L10 to RACK1 (Fig. 1, lanes 1 versus 3). These results are consistent with our previous studies (29) suggesting that the PKC activators are required only to expose the RACK-binding site in the intact PKC and that this site is already exposed in the C2-containing fragment L10.

If the RACK1-binding site is within the C2 and/or V3 regions of βPKC, then L10 (which contains these regions) should inhibit the binding of intact βPKC to RACK1. RACK1 was immobilized on an amylose column, and βPKC binding in the presence of PS and calcium and L10 or L9 was determined (Fig. 2). In the presence of L10 (Fig. 2, lanes 2 versus 1), but not L9 (Fig. 2, lane 3), βPKC binding to RACK1 was completely inhibited. Similar results were also obtained when the effect of the fragments on βPKC binding to RACK1 was determined using the overlay assay (not shown). Since L10 inhibited βPKC binding to RACK1, the C2 region and/or the V3 regions, present in L10 and not in L9, are likely to contain at least part of the RACK1-binding site on βPKC.

We found that synaptotagmin fragments that contain the C2 homologous region bind to purified RACKs (29) and to recombinant RACK1 (n = 3, data not shown) and inhibit PKC binding to RACKs (29). We have therefore reasoned that homologous sequences within the C2 region of βPKC and synaptotagmin may mediate their binding to RACK1. Three βPKC-derived peptides derived from the homologous sequences of βPKC and synaptotagmin were prepared (Fig. 3A): βC2–1 (KQKTKTIK) (βPKC(209–216)), βC2–2 (MDPNGLSDPVYKL) (βPKC(186–198)), and βC2–4 (SLNPEWNET) (βPKC(218–226)). In addition, another C2-derived peptide, βC2–3 (IPDPKSE) (βPKC(201–207)), that shares no homology with synaptotagmin (Fig. 3A), was also synthesized. As seen in Fig. 3B, immobilized peptides βC2–1, βC2–2, and βC2–4, but not βC2–3, bound directly to RACK1. Similar binding of these peptides to RACK1 in the absence of PKC activators was observed (not shown). An additional control peptide corresponding to βPKC (amino acids 266–273 in the C2 domain) did not bind to RACK1 (data not shown; n = 3).

If the βC2–1, βC2–2, and βC2–4 peptides represent the binding site for RACK1 in the C2 region, these peptides should inhibit C2 binding to RACK1. As expected, using the overlay assay, we found that when RACK1 was preincubated with βC2–1, βC2–2, and βC2–4 subsequent binding of L10 to RACK1 was inhibited (Fig. 3C). However, minimal or no inhibition of this binding occurred by incubation with βC2–3 or scrambled βC2–1 (Fig. 3C). Therefore, amino acids 186–198 and 209–226 of βPKC appear to be at least part of the RACK1-binding site in the C2 region.

Using primary rat neonatal cardiac myocytes in culture and with antibodies to both activated PKC (βIPKC) with RACK1 was present in cardiac myocytes. We have previously demonstrated that activation of PKC by PMA or by norepinephrine causes isozyme-specific translocation to distinct subcellular sites (7, 32). If these C2-derived peptides mimic the RACK1-binding site on the C2-containing isozymes, they should inhibit stimulation-induced translocation and binding of these isozymes to their RACKs, but not the translocation of the C2-less isozymes. To test this prediction, we first determined whether RACK1 is present in cardiac myocytes. Using anti-RACK1 antibodies, we found RACK1 immunostaining at perinuclear structures and diffusely in the cytosol (Fig. 4A). RACK1 localization was not altered by PMA or norepinephrine (3–100 nM and 2 μM, respectively; not shown). However, following activation with norepinephrine or PMA, βIPKC immunoreactivity translocated and was co-localized with RACK1 (Fig. 4, A and B, respectively, and not shown). Partial co-localization of activated βPKC with RACK1 was also noted (not shown, see also Ref. 32). In contrast, RACK1 immunoreactivity did not co-localize with inactive or activated C2-less isozymes, δ or εPKC (see in the following and Ref. 32), suggesting that RACK1 may be a specific anchoring protein for activated βPKC in cardiac myocytes.
We next determined whether the C2-derived peptides, that inhibit βPKC binding to RACK1 in vitro, inhibit activation-induced translocation of these C2-containing isozyymes. The peptides were introduced into the cells by transient permeabilization with saponin (50 μg/ml), which has been successfully used to introduce various peptides and other compounds into different cell types (33–35). As was demonstrated in cardiac myocytes⁵ and other cells (33, 36–38), permeabilization with saponin does not affect the viability of the cells nor other cellular functions including contraction rate, gene expression, and cell growth. After permeabilization, the subcellular localization of different PKC isozyymes following stimulation with PMA was determined by immunofluorescence as described previously (32).

Transient permeabilization of these cells in the absence of any peptide did not affect the localization of β₁, β₁I, δ, or εPKC isozyymes before or after stimulation. β₁PKC in non-stimulated cells was found on cytosolic structures. After exposure to 100 nM PMA for 15 min, antibodies against this isozyyme showed localization to perinuclear and intranuclear structures in ~80% of the cells (Fig. 5, vehicle). β₁IPKC was also cytosolic before stimulation, and in ~80% of the cells it translocated to perinuclear structures after PMA treatment (Fig. 5, vehicle). In contrast, permeabilization in the presence of peptides βC2–1, βC2–2, or βC2–4 (10 μM extracellular concentration) resulted in inhibition of the PMA-stimulated translocation of the β₁ and β₁IPKC isozyymes by 65–95%, with βC2–4 causing the largest inhibition (Fig. 5). Other peptides, including scrambled βC2–1, a control peptide derived from the C2-region outside the synaptotagmin-C2 homology region (βPKC (266–272)), and the C2-derived peptide βC2–3 that did not inhibit L10 binding to RACK1 (Fig. 3C) did not affect PMA-induced translocation of β₁ and β₁IPKC in cardiac myocytes (Fig. 5).

Because the C2 region is present in βPKC, but not in δ or εPKC, for example (1, 16), the C2-derived peptides should only affect the translocation of the C2-containing isozyymes, but not that of the C2-less isozyymes. Similar to non-permeabilized cells, we found that treatment with 100 nM PMA resulted in the translocation of εPKC from the nucleus to cross-striated structures in 80% of the cells, whereas δPKC translocated from the nucleus to perinuclear and fibrillar cytosolic structures in 90% of the cells. Moreover, as predicted, the translocation of these C2-less isozyymes was not affected by introduction of any of the C2-derived peptides into the cells (Fig. 5). These results indicate that the βC2–1, βC2–2, and βC2–4 peptides are specific inhibitors of translocation for the C2-containing εPKC isozyymes such as β₁ and β₁IPKC, but not for the C2-less nPKC isozyymes such as δ and εPKC.

If translocation of βPKC is required for its function, peptides that inhibit βPKC translocation should also inhibit βPKC-mediated function. The function of βPKC in cardiac myocytes has not yet been determined. Therefore, we used another assay system, insulin-induced maturation of Xenopus oocytes. We previously demonstrated that oocyte maturation is mediated in part by βPKC; insulin treatment results in translocation of βPKC (but not other PKC isozyymes) from the cytosol to the cell particulate fraction (9) and maturation is delayed by the PKC-specific inhibitor pseudosubstrate peptide (8, 9). Furthermore, this insulin-induced response is also inhibited when PKC translocation is blocked by injection of purified RACKs (8) or a peptide corresponding to the PKC-binding site on RACKs (9). Therefore, inhibition of translocation inhibits PKC-mediated function.

If C2-containing isozyymes regulate oocyte maturation, microinjection of C2-derived peptides that inhibit the transloca-

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**Fig. 3.** A, amino acid alignment of homologous sequences within the C2-region of βPKC and synaptotagmin (p65). Alignment of part of the C2 region was carried out according to Clark et al. (21). Boxed areas are the peptides βC2–1, βC2–2, βC2–3, and βC2–4. Capital letters denote conserved amino acids; lowercase letters denote unique sequences; and — denotes a gap. Numbers on the right are the position of the carboxy amino acid in the protein. The percent homology for synaptotagmin and βPKC in the area indicated is 67%. B, selective RACK1 binding of the C2-derived peptides. The C2-derived peptides βC2–1, βC2–2, βC2–3, and βC2–4 (1 mM/mg) were immobilized on nitrocellulose with a slot-blot apparatus, and the immobilized peptides were overlaid with RACK1 (10 nM). Bound RACK1 was detected using anti-Flag antibodies (1:10,000). Shown are average results of four independent experiments for βC2–1 and βC2–2 and two independent experiments for βC2–3 and βC2–4. C, selective inhibition of L10 binding to RACK1 by the C2-derived peptides. The peptides (10 μM) were incubated with immobilized RACK1 as described under "Experimental Procedures" and subsequent binding of L10 to RACK1 determined in an overlay assay. Binding is expressed as percent of that obtained in the absence of peptide and is representative of two independent experiments.
The distribution of C2-containing isozymes should delay insulin-induced oocyte maturation. The C2-derived peptides were microinjected into intact oocytes, βC2-1, βC2-2, and βC2-4 (5 μM–500 μM) significantly delayed oocyte maturation in a dose-dependent manner (Fig. 6, A and B, and not shown). When 50% of the vehicle-injected oocytes reached maturation only 3–8% of the oocytes injected with βC2-1, βC2-2 and βC2-4 have responded (Fig. 6C). In contrast, microinjection of βC2-3 (Fig. 6, B and C), or a number of other control peptides including scrambled βC2-1 (Fig. 6C), did not affect insulin-induced oocyte maturation (see also Ref. 8). Therefore, βC2-1, βC2-2, and βC2-4 peptides derived from the RACK1-binding site on βPKC specifically inhibited insulin-induced regulation of oocyte maturation.

We then determined whether this inhibition of PKC function in oocytes was due to prevention of insulin-induced βPKC translocation to the cell particulate fraction. Since immunofluorescence studies in Xenopus oocytes are not possible, we determined βPKC translocation by cell fractionation. The distribution of βPKC between the soluble and particulate fractions of oocytes (100,000 × g supernatant and pellet, respectively) was determined in oocytes injected with vehicle or βC2-1 using anti-βPKC antibodies (Fig. 7). Microinjection of βC2-1 to non-stimulated oocytes did not affect βPKC distribution (not shown) and was similar to control non-stimulated oocytes (Fig. 7, lanes 1 and 2). In vehicle-injected oocytes, insulin treatment resulted in a decrease in the level of the 80-kDa βPKC from the cytosol and a corresponding increase in the particulate fraction level (Fig. 7, lanes 4 and 3 versus 2 and 1). However, no insulin-induced translocation of βPKC was observed following microinjection of βC2-1; rather, there was a decrease in the βPKC level in the particulate fraction (Fig. 7, lanes 5 versus 1), suggesting degradation of βPKC. Similar results were also observed following microinjection of βC2-2 (not shown). Therefore, βC2-1 and βC2-2 inhibition of PKC-mediated function following insulin-induced stimulation appears to be due to inhibition of βPKC translocation.

**DISCUSSION**

Using the L9 and L10 recombinant fragments of βPKC and short peptides derived from the C2 region, we have mapped at least part of the RACK1-binding site on βPKC to amino acids 186–198 and 209–226 within the C2 region. Furthermore, peptides corresponding to these sequences inhibited the translocation of C2-containing isozymes but not the translocation of C2-less isozymes in neonatal cardiac myocytes. Finally, these peptides inhibited PKC-mediated function in Xenopus oocytes. Since RACK1 immunoreactivity was found in cardiac myocytes (Fig. 4A) and Xenopus oocytes,
cellular site (39), it is likely that isozyme unique sequences (e.g. V1, V3, and V5) also contain isozyme-specific RACK-binding sites in addition to the site within the common C2 region. Other studies suggested that binding of PKC to proteins different from RACK1 is mediated by the pseudosubstrate sequence (via a phospholipid bridge (40)) or by the catalytic domain of PKC (41, 42). However, the role of the interaction of PKC with these PKC-binding proteins in vivo has not yet been determined.

Very recently, the role of the C1 region in localizing ePKC to the Golgi apparatus has been reported (43). Golgi functions were inhibited by overexpression of both intact ePKC and the C1 fragment of ePKC, leading the authors to suggest that the C1 region may mediate subcellular localization. Our studies with the C1 fragment of βPKC cannot exclude the possibility that this domain may also participate in localizing the enzyme. However, the combined in vitro and in vivo studies indicate that the C2 domain is required for this interaction; inhibitors of the C2 domain binding to RACK1 prevent βPKC translocation and function.

The C2 region of other translocating enzymes also appears to be required for their translocation and function. Recent data indicate that the C2 region of cytosolic phospholipase A2 associates with membranes, whereas a mutant of this lipase lacking the C2 region does not (44). In addition, a fusion protein containing 43 amino acids from the C2 region of Ras GTPase (GAP) confers calcium-dependent interaction with cellular membranes, whereas a GAP mutant lacking this region does not (45). Finally, the binding of synaptotagmin to membranes is abolished by protease treatment of the membrane (46), and peptides derived from the C2 region of synaptotagmin inhibited calcium-induced neurotransmitter release from the giant squid axon (47). Therefore, the C2 region appears to mediate translocation for a number of translocating proteins.

It appears that the inhibitory effects of the C2-derived peptides presented here are due to the inhibition of translocation of PKC rather than of other C2-containing translocating proteins. PMA is not thought to induce translocation of phospholipase Cγ or GTPase activating protein, and synaptotagmin immunoreactivity was not found in cardiac myocytes (not shown). Similarly, insulin treatment does not induce translocation of phospholipase Cγ or GTPase activating protein in Xenopus oocytes, nor is there synaptotagmin immunoreactivity in oocytes (data not shown). In addition, progesterone-induced oocyte maturation that does not involve PKC activation (8, 48) was not affected by the C2-derived peptides (n = 3, data not shown). Therefore, the effects of the βC2–1, βC2–2, and βC2–4 peptides are most likely specific for PKC. Because we do not have antibodies that distinguish between β1 and β1PKC for Western blot analysis, we could not determine whether one or both mediate the insulin-induced effect.

The inhibitory effects of the βC2 peptides were sequence-specific. βC2–1, βC2–2, and βC2–4, but not βC2–3 or a number of control peptides, inhibited translocation of βPKC. Since βC2–1 is highly basic (Fig. 3A), it was previously proposed to

![Figure 6. Xenopus oocyte maturation after microinjection of the C2-derived peptides.](image1)

Time course of insulin-induced Xenopus oocyte maturation at the indicated times after microinjection of vehicle (control, 20 mM NaCl) (■), βC2–1 (□), βC2–2 (○) (50 μM). Xenopus oocyte maturation at the indicated time after microinjection of vehicle (control, 20 mM NaCl) (■), βC2–3 (△) and βC2–4 (●) (50 μM). In each experiment, 10–15 oocytes were microinjected. Results are expressed as percentage of oocytes that reached germinal vesicle breakdown and are representative of at least three independent experiments. Percentage of oocytes that reached germinal vesicle breakdown and are representative of at least three independent experiments. Percentage of oocytes reaching maturation after microinjection of tested peptides at a time that 50% of vehicle-injected oocytes reached maturation (indicated by a dashed line in A and B). Results are expressed as average ± S.E. n denotes the number of independent experiments.

![Figure 7. Effect of βC2-1 microinjection on the subcellular distribution of βPKC in Xenopus oocytes.](image2)

Oocytes were microinjected with vehicle (20 mM NaCl) (lanes 1–4) or βC2–1 (50 μM; lanes 5 and 6) and the distribution of βPKC in the particulate (p) and cytosolic (c) fractions was determined 60 min after incubation without (lanes 1 and 2) or with insulin (lanes 3–6). The cell particulate fraction (lanes 1, 3, and 5) and cytosolic fractions (lanes 2, 4, and 6) of the oocytes were prepared as described under “Experimental Procedures,” using 100 oocytes for each treatment and PKC was detected using anti-βPKC antibodies (1:1000) in Western blot analysis. The antibodies reacted with an ~80-kDa protein that corresponds with βPKC. The identity of the two other immunoreactive bands in the particulate fraction of control and insulin-treated oocytes is unknown. (This antibody recognizes both β1 and β1PKC isozymes. However, only β1PKC appears to translocate on insulin treatment in these cells). The figure is a representative of results obtained in three independent experiments.
mediate direct binding of the C2-containing proteins to the negatively charged PS in the membrane (21). However, we found that its inhibitory activity cannot be attributed to charge only, since a scrambled βC2–1 peptide was inactive in inhibiting either the translocation of C2-containing βPKC isoforms (Fig. 5) or their function (Fig. 6).

Why was there a decrease in the levels of βPKC in activated oocytes injected with peptides βC2–1 or βC2–2? Activated PKC isozymes may reflect multiple binding sites on relatively large surface pockets. In contrast, the interaction of two proteins in the cell mediate specific cellular functions in cells in which multiple interaction surfaces between PKC and RACK1 are constituted only by the translocation of C2-containing PKC isozymes, most likely βPKC, mediate this function in oocyte maturation. Since in cardiac myocytes, inhibition of translocation of C2-less isoforms was not observed, the inhibitory peptides can be used as tools to identify the PKC isoforms that mediate specific cellular functions in cells in which multiple isoforms are activated by a single stimulus. The role of C2-containing isoforms in the PMA-induced regulation of cardiac myocyte function is currently under investigation using these peptides.

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REFERENCES
1. Nishizuka, Y. (1988) Nature 334, 661–665
2. Wolf, M., and Sahyoun, N. (1986) Biol. Chem. 261, 13327–13332
3. Zalewski, P. D., Forbes, I. J., Valente, L., Apostolous, S., and Hurst, N. P. (1988) Biochem. Pharmacol. 37, 1415–1417
4. Halsey, D. L., Girard, P. R., Kudt, J. F., and Bladshere, P. J. (1987) J. Biol. Chem. 262, 2324–2343
5. Chen, Z. Z., McGuire, J. C., Leach, K. L., and Cambier, J. C. (1987) J. Immunol. 138, 2345–2352
6. Marnoufi, A., Labourdette, G., Mersel, M., Huang, F. L., Huang, K.-P., Vincenton, G., and Malviya, A. N. (1989) J. Biol. Chem. 264, 1172–1179
7. Mochly-Rosen, D., Heinrich, C. J., Cheeuer, L., Kaner, H., and Simpson, P. C. (1990) Mol. Biol. Cell 1, 705–706
8. Smith, B. L., and Mochly-Rosen, D. (1992) Biochim. Biophys. Res. Commun. 188, 1235–1240
9. Ron, D., and Mochly-Rosen, D. (1994) J. Biol. Chem. 269, 21165–21178
10. Halsey, D. L., Girard, P. R., Kudt, J. F., and Bladshere, P. J. (1987) J. Biol. Chem. 262, 2324–2343
11. Burns, D. J., and Bell, R. M. (1990) J. Biol. Chem. 265, 18380–18388
12. Okuyama, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U., and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 4868–4871
13. Quest, A. F. G., Bardes, E. S. G., and Bell, R. M. (1994) J. Biol. Chem. 269, 2695–2709
14. Kikkawa, U., Kishimoto, A., and Nishizuka, Y. (1989) Annu. Rev. Biochem. 58, 31–44
15. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1988) J. Biol. Chem. 263, 6927–6932
16. Ohno, S., Akita, Y., Konno, Y., Imajoh, S., and Suzuki, K. (1988) Cell 53, 731–741
17. Nehr, E., and Penner, R. (1994) Nature 367, 316–317
18. Perin, M. S., Fried, V. A., Mignery, G. A., jahn, R., and Sudhof, T. C. (1990) Nature 345, 260–263
19. Mosher, D. M., and Mason, S. (1991) Biophys. J. 60, 149–159
20. Luo, J.-H., Kahn, S., O’Driscoll, K., and Weinstein, I. B. (1993) J. Biol. Chem. 265, 3715–3719
21. Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, G. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) Cell 65, 1043–1051
22. Gopalakrishna, R., Barsky, S. H., Thomas, T. P., and Anderson, W. B. (1986) J. Biol. Chem. 261, 16438–16445
23. Wolf, M., and Baggiolini, M. (1990) Biochem. J. 269, 723–728
24. Mochly-Rosen, D., Moyo, H., and Lopez, J. J. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 3997–4000
25. Ron, D., Chen, C.-H., Caldwell, J., Jameson, L. J., Orr, E. R., and Mochly-Rosen, D. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 839–843
26. Jaken, S., and Jakes, J. W. (1992) in Protein Kinase C (Current Concepts and Future Perspectives (Lester, D. S., Ed.) pp. 237–273, Ellis Horwood Ltd., W. Sussex, United Kingdom
27. Hyatt, S. L., Liao, L., Chaplin, C., and Jaken, S. (1994) Biochemistry 33, 1223–1228
28. Chaplin, C., Ramsay, K., Kluk, T., and Jaken, S. (1993) J. Biol. Chem. 268, 6885–6881

B. L. Smith and D. Mochly-Rosen, unpublished result.
29. Mochly-Rosen, D., Miller, K. G., Scheller, R. H., Khaner, H., Lopez, J., and Smith, B. L. (1992) Biochemistry 31, 8120–8124
30. Disatnik, M.-H., Hernandez-Stornayor, S., Jones, G., Carpenter, G., and Mochly-Rosen, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 559–563
31. Mochly-Rosen, D., and Koshland, D. E., Jr. (1987) J. Biol. Chem. 262, 2291–2297
32. Disatnik, M.-H., Buraggi, G., and Mochly-Rosen, D. (1994) Exp. Cell Res. 210, 287–297
33. Lohse, M. J., Leffkowitz, R. J., Caron, M. G., and Benovic, J. L. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3011–3015
34. Liu, S.-H., Chu, J. C. J., and Ng, S.-Y. (1993) Nucleic Acids Res. 21, 4005–4010
35. Padfield, P. J., Balch, W. E., and Jamieson, J. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1658–1660
36. Jacob, M. C., Favre, M., and J.-C, B. (1991) Cytometry 12, 550–558
37. Yanai, S., Sugiyama, Y., Iga, T., Fuwa, T., and Hanano, M. (1991) Pharm. Res. 8, 357–362
38. Erusalimsky, J. D., Brooks, S. F., Herget, T., Morris, C., and Rozengurt, E. (1991) J. Biol. Chem. 266, 7073–7080
39. Disatnik, M.-H., Winnier, A. R., Mochly-Rosen, D., and Arteaga, C. L. (1994) Cell Growth Diff. 5, 873–880
40. Liao, L., Hyatt, S. L., Chapline, C., and Jaken, S. (1994) Biochemistry 33, 1229–1233
41. James, G., and Olson, E. N. (1992) J. Cell Biol. 116, 863–874
42. Staudinger, J., Zhou, J., Burgess, R., Elledge, S. J., and Olson, E. N. (1995) J. Cell Biol. 128, 263–271
43. Lehel, C., Olah, Z., Jakab, G., and Anderson, W. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1406–1410
44. Naefski, E. A., Sultzman, L. A., Martin, D. M., Kriz, R. W., Towler, P. S., Knopf, J. L., and Clark, J. D. (1994) J. Biol. Chem. 269, 18239–18249
45. Gawler, D., Zhang, L.-J., Reedik, M., Tung, P., and Moran, M. (1995) Oncogene 10, 817–825
46. Brose, N., Petrenko, A. G., Sudhof, T. C., and Jahn, R. (1992) Science 256, 1021–1025
47. Bernert, K., Charlton, M. P., DeBello, W. M., Chin, G. J., Betz, H., and Augustine, G. J. (1993) Neuron 10, 219–230
48. Smith, L. D. (1989) Development 107, 685–699
49. Kishimoto, A., Kajikawa, N., Shiotani, M., and Nishizuka, Y. (1983) 258, 1156–1164
50. Ford-Hutchinson, A. W. (1991) Trends Pharmacol. Sci. 12, 68–70
51. Mochly-Rosen, D., Khaner, H., Lopez, J., and Smith, B. L. (1991) J. Biol. Chem. 266, 14866–14868
52. Sutton, R. B., Davletov, B. A., Berghuis, A. M., Sudhof, T. C., and Sprang, S. R. (1995) Cell 80, 929–938
53. Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995) Nature 375, 554–560