Distinct subcellular localization of activated protein kinase C (PKC) isoforms is mediated by their binding to isoyme-specific RACKs (receptors for activated C-kinase). Our laboratory has previously isolated one such protein, RACK1, and demonstrated that this protein displays specificity for PKCβ. We have recently shown that at least part of the PKCε RACK-binding site on PKCε lies within the unique V1 region of this isoyme (Johnson, J. A., Gray, M. O., Chen, C.-H., and Mochly-Rosen, D. (1996) J. Biol. Chem. 271, 24962–24966). Here, we have used the PKCε V1 region to clone a PKCε-selective RACK, which was identified as the COPI coatomer protein, β′-COP. Similar to RACK1, β′-COP contains seven repeats of the WD40 motif and fulfills the criteria previously established for RACKs. Activated PKCε colocalizes with β′-COP in cardiac myocytes and binds to Golgi membranes in a β′-COP-dependent manner. A role for PKC in control of secretion has been previously suggested, but this is the first report of direct protein/protein interaction of PKCε with a protein involved in vesicular trafficking.

Anchoring proteins appear to be essential components of a number of signal transduction pathways (1). In the case of PKC,1 activation of PKC isoforms is associated with their translocation to the particulate fraction, with each activated isoyme localized to distinct subcellular sites (1, 2). This translocation appears to be mediated by the binding of each activated isoyme to specific anchoring proteins we have termed RACKs (receptors for activated C-kinase) (1, 3).

In cultured cardiac myocytes, PKCε translocates from the nucleus to cross-striated structures, the perinucleus, and cell contacts following stimulation with 4β-phorbol 12-myristate 13-acetate or with α1-adrenergic receptor agonists; other PKC isoymes translocate to other distinct intracellular sites (2, 4). Our laboratory has recently demonstrated that the V1 region of PKCε contains at least part of the RACK-binding site in this enzyme. A recombinant PKCε V1 polypeptide (amino acids 2–144; which does not include the pseudosubstrate sequence) selectively inhibits stimulation-induced PKCε translocation and regulation of contraction rate in cardiac myocytes. In contrast, translocation and function of other PKC isoymes are not altered by this fragment (5).

This study describes the use of the PKCε V1 region in an overlay screen to clone a PKCε-selective RACK (6, 7). Using this procedure, we have identified β′-COP, a COPI (coat protein I) coatomer complex protein essential for Golgi budding and vesicular trafficking (8), as a PKCε-selective RACK. Previous studies have linked PKC to control of constitutive membrane trafficking and Golgi function (9–14). However, the mechanism by which PKC exerts this control has not yet been determined. Our finding that β′-COP can also serve as a selective anchoring protein (RACK) for activated PKCε provides a possible mechanism by which PKC regulates Golgi function.

EXPERIMENTAL PROCEDURES

Expression Library Screening—Screening of 2 × 105 plaques with the recombinant PKCε V1-FLAG fragment was as described elsewhere (6, 7).

Isolation of β′-COP 5′-cDNA—The 5′-end of the gene was isolated using a 5′-AmpliFINDER™ RACE kit (CLONTECH). First strand cDNA was synthesized from rat heart mRNA (Sprague-Dawley) using an antisense β′-COP-specific primer, EG36 (TGAGTTCGGTATCAGCTCTC). This cDNA was used as a template for polymerase chain reaction amplification using a primer complementary to the 5′-AmpliFINDER™ anchor and a nested β′-COP-specific primer, EG37 (TTTGTGGACTGGAAGTGAACTTGAAG). This cDNA was used as a template for polymerase chain reaction amplification using a primer complementary to the 5′-AmpliFINDER™ anchor and a nested β′-COP-specific primer, EG37 (TTTGTGGACTGGAAGTGAACTTGAAG) and EcoRI/BamHI digestion. The resulting polymerase chain reaction product was ligated into EcoRI/BamHI-digested pMAL-c2 containing the FLAG adapter. The plasmid pDMD2 encoding the β′-COP C-terminal fragment (amino acids 425–906) was used as a control vector.

Expression of Recombinant Protein—All recombinant proteins were expressed as maltose-binding protein (MBP)-FLAG™ epitope (DYKD-DDK) fusion proteins. The PKCε V1 fragment (PKCε amino acids 2–145) was expressed as a MBP-FLAG-PKCE V1 fusion (5). Overexpression and purification were according to the protocol of New England Biolabs Inc. The plasmid encoding the β′-COP C-terminal fragment (amino acids 425–906) was produced as follows. A FLAG adapter (AATT-CGACTACAAAGGACGCACTACAGCTCGTACATCA) was added with or without 1 unit of rat brain PKC, 60 mM 2-mercaptoethanol, 0.1% (v/v) bovine serum albumin, 1% (w/v) polyethylene glycol, 0.5 M NaCl, 0.1% (w/v) bovine serum albumin, 1% (v/v) polyethylene glycol, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml leupeptin) was added with or without 1 unit of rat brain PKC, 60 μg/ml PS, 2 μg/ml DG, and 1 μg/ml CaCl2, as indicated, and the column was sealed and incubated at room temperature for 30 min while shaking.
Unbound material was eluted with 20 column volumes of wash buffer (200 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10 mM 2-mercaptoethanol). The recombinant protein and any associated proteins were eluted with wash buffer containing 20 mM maltose and subjected to SDS-PAGE followed by Western blot analysis using anti-PKC\e antibodies. 

Immunofluorescence—Immunofluorescence studies were carried out as described previously (2). Anti-PKC\e antibodies were obtained from Research and Diagnostics Antibodies, and 23C anti-\b-COP monoclonal antibodies were a generous gift from K. J. Harrison-Lavoie and Dr. K. R. Willison (15). Fluorescein- and rhodamine-conjugated secondary antibodies were obtained from Organon Technika Corp. Adsorption of anti-PKC\e and anti-\b-COP antibodies was carried out in solid phase. Rat brain PKC (0.1 \mu g), recombinant \b-COP (5 \mu g), or 3% normal goat serum was spotted on 0.5-cm² nitrocellulose membrane. After 5 min at room temperature, the membranes were washed and blocked with 3% normal goat serum in phosphate-buffered saline for 1 h. The membranes were then incubated with the appropriate primary antibody overnight at 4 °C, and the unadsorbed material was used for immunostaining. Incubation of either antibody on nitrocellulose spotted with 3% normal goat serum did not reduce any immunostaining seen in Fig. 3 (A and B) (data not shown).

Recombinant PKC Isozyme Binding Experiments—SRI-expressed purified recombinant PKC isozymes were obtained from PanVera Inc. Binding experiments were carried in the presence of 1 mM of the recombinant PKC as described above (see “Column Overlay Assay”). For the classical PKC isozymes (\alpha, \beta, \betaII, and \gamma), binding was carried out in overlay buffer containing PS, DG, and Ca²⁺ as activators. For the novel isozymes (\epsilon and \delta), binding was carried out in overlay buffer containing PS and DG as activators. PKC binding was determined by Western blot analysis of the column eluates using anti-PKC\e, \beta, and \gamma antibodies from Seikagaku America, Inc. and anti-PKC\e and -\delta antibodies from Life Technologies, Inc. For competition binding experiments, an excess of PKC\beta (1 \mu g) was added to the columns in overlay buffer containing PS, DG, and Ca²⁺ and incubated for 30 min at room temperature. After 30 min of preincubation, activated PKC\e was added and incubated for 30 min at room temperature, and then the columns were washed, eluted, and analyzed as described above.

Immunoprecipitations—Neonatal rat hearts were homogenized in 20 mM Tris-HCl, pH 7.5, containing 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 10 \mu M phenylmethylsulfonyl fluoride, 20 \mu M leupeptin, and 20 \mu M soybean trypsin inhibitor. The homogenate was centrifuged at 100,000 \times g for 40 min at 4 °C, and the pellet was extracted with 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 12 mM 2-mercaptoethanol, and 1% (v/v) glycerol containing the above protease inhibitors and 0.5% Triton X-100 for 30 min on ice. The Triton-soluble extract (supernatant) was separated from the insoluble material following centrifugation at 100,000 \times g for 40 min at 4 °C. For each immunoprecipitation reaction, 2-3 \mu g of primary antibody was added to 50 \mu l of recombinant protein G-agarose (Life Technologies, Inc.) and incubated for 2 h at 4 °C. The protein G-agarose was washed three times with phosphate-buffered saline containing 0.1% Triton X-100. The Triton-soluble fraction of the equivalent of one neonatal heart was added to the protein G-agarose and incubated for 4 h at 4 °C. The protein G-agarose was washed three times with phosphate-buffered saline containing 0.1% Triton X-100. Laemmli sample buffer was added, and the samples were subjected to SDS-PAGE followed by Western blot analysis using anti-PKC\e, 23C anti-\b-COP, and anti-PKC\e antibodies.

PKC\e Binding to Rat Liver Golgi Membranes—Golgi membranes (100 \mu g/ml) from rat liver (16, 17) and cytosol from rat brain (1.5 mg/ml) were incubated in binding buffer (25 mM HEPES-KOH, pH 7, 0.2 mM sucrose, 25 mM KCl, 2.5 mM MgCl₂, 1 mM ATP, 25 mM creatine phosphate, 10 \mu M creatine phosphokinase, and 1 mM dithiothreitol with or without 20 \mu M OGP for the last 10 min) for 25 min at 37 °C. At the end of the incubations, samples were centrifuged at 13,000 rpm for 15 min at 4 °C. The pellet was washed once with binding buffer and resuspended in Laemmli sample buffer. Samples were subjected to SDS-PAGE on 4-15% gradient gels and Western blot analysis using anti-PKC\e and 23C anti-\b-COP antibodies.

RESULTS AND DISCUSSION

We have used the recombinant V1 fragment of PKC\e to screen a rat heart cDNA library for a PKC\e-specific RACK. One clone, pRACK2, contained a partial open reading frame encoding a 481-residue protein homologous to the C-terminal half of human and bovine \b-COP (15, 18). A full-length 906-amino acid open reading frame was obtained by RACE and identified as rat \b-COP (GenBank™ accession number AF002705) as it was found to be 97% identical to human \b-COP at the amino acid level. Therefore, \b-COP protein, one of a seven-protein coatomer complex that forms the coat of COPI-coated transport vesicles (8), binds the V1 region of PKC\e in the overlay cloning procedure. Similar to RACK1 (the RACK for PKC\e), \b-COP contains seven internal repeats of the WD40 motif (Fig. 1, A and B). This motif is also present in the G protein \beta-subunit, which is an anchoring protein for another translocating protein kinase, the G protein receptor kinase, and in other proteins involved in multimeric protein/protein interactions (19).

To determine if \b-COP is a RACK, we tested whether recombinant \b-COP and \b-COP fragments bind PKC\e in vitro. Fragments or full-length \b-COP fused to MBP were immobilized on an amyllose column, and binding of rat brain PKC\e was measured using the column overlay assay (Fig. 1C). Recombinant \b-COP did not bind PKC when the \b-COP was subjected to SDS-PAGE followed by the overlay assay (3), indicating that the native conformation of \b-COP is important for PKC\e binding (data not shown). Because a RACK should bind only activated PKC (3), we determined the effect of PS and DG on PKC binding. As expected for a calcium-independent, novel PKC isozyme, PKC\e binding to \b-COP required PS and DG and was calcium-independent; in fact, calcium inhibited PKC\e binding (Fig. 1C). Furthermore, binding of PKC\e to \b-COP was saturable at ~10 nM PKC\e (Fig. 1D). Finally, binding of PKC\e to \b-COP was not inhibited by an excess of a pseudosubstrate peptide (Fig. 1D, lane 9 versus lane 8), and \b-COP was not a PKC substrate (data not shown). Rather, as in the case of RACK1 (7), phosphorylation of substrate increased in the presence of \b-COP (2). Therefore, \b-COP fulfills the criteria previously established for RACKs (3).

The V1 region of PKC\e selectively inhibits PKC\e translocation and function in intact myocytes (5) and therefore should mediate the interaction of PKC\e with \b-COP. In vitro, the V1 fragment of PKC\e bound to the C-terminal fragment, but not the N-terminal fragment of \b-COP (Fig. 2A). In contrast, PKC\e holoenzyme bound to both fragments (Fig. 1C). These data suggest that the PKC\e/\b-COP interaction involves more than one site on either or both molecules. However, the PKC\e V1 fragment inhibited PKC\e holoenzyme binding to \b-COP by 60% (Fig. 2B), indicating that the V1 region of PKC\e contains at least part of the \b-COP-binding site on PKC\e. It is interesting to note that two binding sites on RACK1 for PKC\e were also found (7), suggesting a common theme in PKC/RACK interactions.

To further demonstrate the specificity of PKC\e binding to \b-COP, we compared the binding of purified recombinant PKC isozymes to immobilized \b-COP (Fig. 2C). When using equal amounts of each isozyme, PKC\e binding to \b-COP was at least an order of magnitude better than the binding of the other isozymes. In addition, preincubation of \b-COP with PKC\e (Fig. 2D) or concomitant incubation with both isozymes (data not shown) did not inhibit subsequent binding of PKC\e to \b-COP. Together, these data show preferred binding of PKC\e by \b-COP in vitro.

If \b-COP is a PKC\e-selective RACK, \b-COP should colocalize with activated PKC\e in cells. Indeed, immunofluorescence studies showed that \b-COP colocalized with activated PKC\e to cross-striated structures in cardiac myocytes (Fig. 3B). Immunostaining at the perinucleus and cell/cell contacts was also observed with both anti-\b-COP and anti-PKC\e antibodies. None of the other PKC isozymes or RACKs localize to these structures in either control or 4β-phorbol 12-myristate 13-ace-

\[2\] M. M. Rodriguez and D. Mochly-Rosen, unpublished observation.
FIG. 1. A, a scheme indicating the approximate positions of the WD40 motifs in β'-COP and RACK1. B, alignment of the WD40 motifs from RACK1 (7) and rat β'-COP and the consensus sequence identified by Neer et al. (19). Previously published sequence analyses (15, 18) have not identified WD40 motifs in the C-terminal half of β'-COP, the portion of β'-COP encoded by the original pRACK2 clone. Closer inspection of the sequence in this region revealed a partial WD40 sequence differing in 6 amino acids from the consensus sequence (19). The repeats numbers are indicated by rI through rVII. Numbers on the left indicate the position of the repeat in the sequence. Amino acid sequence is provided using a single-letter code, where X represents any amino acid. Numbers in braces indicate the minimal and maximal numbers of amino acids inserted at
tate-treated cells (2). The specificity of the antibodies used for the immunostaining is shown in Fig. 3 (D and E); no immunostaining was observed after absorption with the corresponding protein. Image analysis indicated that fluorescence intensities for β'-COP and PKCe correlated (p < 0.01). Moreover, using a defined visual threshold to isolate background from signal arising from brighter discrete cellular structures gave a highly significant overlap between β'-COP and PKCe staining (p <

that position; numbers in parentheses indicate the number of amino acids of the motif that deviate from the consensus sequence and are marked in the motif with a lower-case boldface letter. (Alignment was carried out by eye.) C, PKCe binds to N-terminal, C-terminal, and full-length β-cOP in vitro. N-terminal (amino acids 1–449), C-terminal (amino acids 425–906), and full-length β'-COP-MBP fusion proteins were immobilized on amylase affinity columns and subjected to the column overlay assay (see “Experimental Procedures”). The recombinant protein and any associated proteins were eluted with wash buffer containing 20 mM maltose and subjected to SDS-PAGE followed by Western blot analysis using anti-FLAG antibodies (IBI). These antibodies gave one major band of 21 kDa that disappeared when FLAG antibodies were immunosorbed with FLAG peptide or another FLAG-containing recombinant protein (data not shown). The PKCe V1 fragment does not appear to bind C-terminal β'-COP. Results are representative of three independent experiments, and averaged data from these three experiments are provided below the figure. B, the PKCe V1 fragment inhibits binding of rat brain PKC to full-length β'-COP. Full-length β'-COP-MBP fusion protein was immobilized on amylase columns. Overlay buffer with or without 1 μM PKCe V1 fragment was added, and the columns were incubated for 15 min at room temperature prior to addition of rat brain PKC and PS/DG as described under “Experimental Procedures.” The recombinant protein and associated proteins were eluted with wash buffer containing 20 mM maltose and subjected to SDS-PAGE followed by Western blot analysis using anti-PKC antibodies. Results are representative of three independent experiments, and averaged data from these three experiments are provided below the figure. C, activated PKCe binds to β'-COP better than other PKC isoforms. Purified recombinant PKCe isoforms (1 μg each) expressed in Sf9 cells were activated as described under “Experimental Procedures” and applied to full-length β'-COP-MBP fusion protein immobilized on amylase columns. Binding was determined with isozyme-specific antibodies as described for B, except that only one-fourth of the eluate was loaded on SDS-polyacrylamide gel for analysis. As standard for comparison, increasing amounts of recombinant isoforms (indicated in ng above each lane) were also included in the Western blot analysis (left lanes in each panel). Using these standards, we found that >250 ng of PKCe bound to β'-COP as compared with 25 ng of PKCe and <1 ng of the other isoforms. Hence, PKCe binding to β'-COP was at least an order of magnitude better than the binding of the other isoforms. D, activated PKCe does not compete for PKCe binding to β'-COP. The experiment was carried out as described for C, except that activated PKCe was added after preincubation of β'-COP with activated PKCe (1 μg).

Fig. 2. A, the PKCe V1 fragment binds N-terminal and full-length β'-COP. N-terminal (amino acids 1–449), C-terminal (amino acids 425–906), or full-length β'-COP-MBP fusion proteins were immobilized on amylase columns, and column overlay assays were carried out with 1 μM PKCe V1-FLAG fragment in the absence of activators. The recombinant protein and any associated proteins were eluted with wash buffer containing 20 mM maltose and subjected to SDS-PAGE followed by Western blot analysis using anti-FLAG antibodies (IBI). These antibodies gave one major band of 21 kDa that disappeared when FLAG antibodies were immunosorbed with FLAG peptide or another FLAG-containing recombinant protein (data not shown). The PKCe V1 fragment does not appear to bind C-terminal β'-COP. Results are representative of three independent experiments, and averaged data from these three experiments are provided below the figure. B, the PKCe V1 fragment inhibits binding of rat brain PKC to full-length β'-COP. Full-length β'-COP-MBP fusion protein was immobilized on amylase columns. Overlay buffer with or without 1 μM PKCe V1 fragment was added, and the columns were incubated for 15 min at room temperature prior to addition of rat brain PKC and PS/DG as described under “Experimental Procedures.” The recombinant protein and associated proteins were eluted with wash buffer containing 20 mM maltose and subjected to SDS-PAGE followed by Western blot analysis using anti-PKC antibodies. Results are representative of three independent experiments, and averaged data from these three experiments are provided below the figure. C, activated PKCe binds to β'-COP better than other PKC isoforms. Purified recombinant PKCe isoforms (1 μg each) expressed in Sf9 cells were activated as described under “Experimental Procedures” and applied to full-length β'-COP-MBP fusion protein immobilized on amylase columns. Binding was determined with isozyme-specific antibodies as described for B, except that only one-fourth of the eluate was loaded on SDS-polyacrylamide gel for analysis. As standard for comparison, increasing amounts of recombinant isoforms (indicated in ng above each lane) were also included in the Western blot analysis (left lanes in each panel). Using these standards, we found that >250 ng of PKCe bound to β'-COP as compared with 25 ng of PKCe and <1 ng of the other isoforms. Hence, PKCe binding to β'-COP was at least an order of magnitude better than the binding of the other isoforms. D, activated PKCe does not compete for PKCe binding to β'-COP. The experiment was carried out as described for C, except that activated PKCe was added after preincubation of β'-COP with activated PKCe (1 μg).
immunoprecipitates (bodies to another coatomer protein, between the two molecules. Using commercially available anti-

The panel demonstrates that β'-COP is also located at cross-striated structures, the perinucleus, and cell/cell contacts (white arrows). B, anti-PKC binding is detected with rhodamine-conjugated anti-rabbit secondary antibodies. The panel demonstrates that PKCε is located at cross-striated structures, the perinucleus, and cell/cell contacts (white arrows). C, A and B are combined. Colocalization of the β'-COP and PKCε antibodies is indicated in yellow. Results are representative of three independent experiments. D, no specific staining is obtained after incubation of the anti-β'-COP antibody with 5 µg of β'-COP. E, no specific staining is obtained after incubation of the anti-PKCε antibody with 0.1 µg of rat brain PKC (2, 5). F, anti-PKCε antibody (Ab; but not control immunoglobulins) immunoprecipitates (IP) β'-COP along with PKCε from neonatal heart. Similarly, PKCε (but not PKCβ) co-immunoprecipitates with β'-COP using anti-β'-COP antibodies. A representative result of three independent experiments is shown.

0.001). Therefore, activated PKCε is in close proximity to β'-COP in cardiac myocytes, suggesting a direct association between the two molecules. Using commercially available antibodies to another coatomer protein, β'-COP (Sigma), we observed a faint perinuclear staining, but no cross-striated staining. These data may suggest that β'-COP, but not the full coatomer complex, localizes to cross-striated structures. However, we cannot rule out the possibility that the antibody recognition site on β'-COP is hidden by an associated protein when the coatomer complex is present on the cross-striated structures. Future studies with multiple anti-coat protein antibodies will elucidate this question.

In addition, if β'-COP is a PKCε-selective RACK, β'-COP should co-immunoprecipitate with PKCε-specific antibodies and PKCε should co-immunoprecipitate with anti-β'-COP antibodies. This is shown in Fig. 3F. β'-COP was co-immunoprecipitated with anti-PKCε antibodies, but not with control immunoglobulin. (Immunoprecipitating anti-PKCβ antibodies are currently not available and therefore could not be used in the assay.) In addition, although there is a similar amount of PKCβ immunoreactivity compared with PKCε immunoreactivity in this preparation, some of PKCε, but no PKCβ, co-immunoprecipitated with anti-β'-COP antibodies. This is not seen with control immunoglobulin. These data suggest that at least some of the cellular β'-COP associates with PKCε even after extensive dilution, homogenization, and cell fractionation. β'-COP assembles as part of the coatomer complex and binds to Golgi membranes in a GTP-dependent manner. To further demonstrate the interaction between β'-COP and PKCε, we used cell systems where β'-COP/Golgi interaction has been characterized. Using purified rat liver Golgi membranes and rat brain cytosol in binding experiments, we tested if PKCε bound to Golgi membranes and determined whether association of PKCε with Golgi membranes was dependent on β'-COP binding to this structure (Fig. 4). GTPγS stimulates binding of ADP-ribosylation factor (ARF), and thus COPI coatomer binding, to Golgi membranes (10). Here, we show that the increased binding of β'-COP to the Golgi membranes seen in the presence of GTPγS was associated with a corresponding increase in PKCε binding (Fig. 4). Furthermore, treatment with brefeldin A (BFA), a fungal metabolite that inhibits GTPγS-stimulated coatomer binding to Golgi membranes, resulted in reduced β'-COP association and a corresponding reduction of PKCε association with the Golgi membranes (Fig. 4). Although 4β-phorbol 12-myristate 13-acetate treatment augments PKCε binding to Golgi membranes (data not shown), PKCε binding was seen without the addition of PKC activators. A plausible explanation for this is that DG or other fatty acid activators...
were generated on the Golgi membrane as a result of GTPγS activation of phospholipase D, for example. Nevertheless, these results show that the level of PKCe bound to Golgi membranes is dependent on factors that affect β'-COP/Golgi association. Furthermore, these data are in agreement with our hypothesis that PKCe associates with β'-COP and suggest that PKCe may have a role in coatamer function and vesicular transport.

There are substantial data indicating that some PKC isozymes (including PKCe) are localized on the Golgi complex and that PKC plays a role in constitutive membrane trafficking in general and in Golgi function in particular (10–14). The mechanisms responsible for such localization and for the regulation of secretory function by PKC are unknown. We propose that a direct interaction between PKCe and β'-COP is one of the mechanisms sustaining the Golgi-specific localization of some PKC isoforms. In NIH 3T3 cells overexpressing PKCe, PKCe binds to the Golgi apparatus, at least in part, via the C1 region of PKCe and appears to regulate Golgi function (20, 21). It has not yet been determined whether the C1 region of PKCe also binds β'-COP. However, these data are in agreement with our findings in Fig. 1, which demonstrate that the V1 fragment contains only part of the β'-COP/RACK-binding site on PKCe.

The mechanism by which PKCe exerts its action on secretion remains to be resolved, but a number of possibilities are raised by the direct interaction of PKC with a COPI protein. Activation of PKCe enhances binding of ARF and β'-COP to Golgi membranes and stimulates vesicle formation (10). Furthermore, it has been suggested that Golgi-associated PKCe may control the activation of ARF (12). However, the PKCe isozyme(s) responsible for the effects seen in these studies was not determined. This may suggest a model in which ARF, by promoting COPI binding to the Golgi complex and hence sustaining PKCe localization and activity on it, initiates a positive feedback loop that reinforces its own binding to these membranes. In addition, the COPI coatamer subunits β-COP and δ-COP appear to undergo regulated phosphorylation (22). It is therefore possible that phosphorylation of these COPI components by PKCe may result in different COPI conformation. Such COPI conformation changes could result in a shift in the bidirectional interaction, allowing it to engage with its receptors on Golgi membranes, and produce a shift in the anterograde versus retrograde membrane flux along the secretory pathway (23). The essential role of DG in vesicle formation (9) further supports the involvement of PKCe in control of budding. A further possible target of PKCe relevant to its role in regulating Golgi function could be phospholipase D, which is enriched on Golgi membranes. Phospholipase D activity is stimulated by ARF and appears to be involved in vesicle formation (24). Moreover, it has been known for some time that phospholipase D is also activated by PKCe (25), although the isozyme responsible is a matter of some debate (26, 27). A noncatalytic role for PKCe has been proposed (28), and noncatalytic involvement of PKCe in porcine brain phospholipase D activation, PKCe has also been implicated in a number of studies (26) including one using cardiac myocytes (30).

As shown in Fig. 3, β'-COP was also found on cross-striated structures in cardiac myocytes. Since these structures are not characteristic Golgi structures, we wanted to determine the significance of this finding. Several cell types, including cardiac myocytes, appear to be resistant to BFA treatment, and hence, β'-COP association with Golgi and cross-striated structures could not be dissected. This still leaves the question of β'-COP/PKCe complex function at these structures. We previously found that PKCe mediates inhibition of contraction rate (negative chronotropy) (5) and resistance to ischemia-induced cell death (31). Together with the finding that β'-COP is the anchoring RACK for activated PKCe, the data suggest that regulation of these functions may be mediated by secretion of autocrine factors. Alternatively, localization in cardiac myocytes of β'-COP to cross-striations in addition to Golgi membranes may suggest a novel role for β'-COP in this cell type, i.e., anchoring PKCe close to substrates that are not involved in COPI function. Localization of β'-COP and PKCe at cross-striated elements may bring PKCe into contact with contractile proteins such as troponin (32) and/or with sarcoplasmic reticulum PKC substrates such as phospholamban (33). Finally, the complex may actually lie on T-tubules, plasma membrane invaginations that penetrate deep into the myofibrils close to the Z-lines (34).

In summary, we have shown that β'-COP fulfills the criteria for a PKCe-selective RACK: it binds activated PKCe better than any other isozyme, and PKC binding is dose-dependent and saturable, β'-COP co-immunoprecipitates and colocalizes with activated PKCe in cardiac myocytes. Finally, β'-COP mediates PKCe binding to Golgi membranes. The significance of β'-COP as a PKCe-selective RACK remains to be fully determined, but the study of PKCe/β'-COP interaction should lead to new insights into PKCe-specific functions.

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