Coordinated Movement of RACK1 with Activated βIIIPKC*

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Protein kinase C (PKC) isozymes move upon activation from one intracellular site to another. PKC-binding proteins, such as receptors for activated C kinase (RACKs), play an important role in regulating the localization and diverse functions of PKC isozymes. RACK1, the receptor for activated βIIIPKC, determines the localization and functional activity of βIIIPKC. However, the mechanism by which RACK1 localizes activated βIIIPKC is not known. Here, we provide evidence that the intracellular localization of RACK1 changes in response to PKC activation. In Chinese hamster ovary cells transfected with the dopamine D2L receptor and in NG108-15 cells, PKC activation by either phorbol ester or a dopamine D2 receptor agonist caused the movement of RACK1. Moreover, PKC activation resulted in the in situ association and movement of RACK1 and βIIIPKC to the same intracellular sites. Time course studies indicate that PKC activation induces the association of the two proteins prior to their co-movement. We further show that association of RACK1 and βIIIPKC is required for the movement of both proteins. Our results suggest that RACK1 is a PKC shuttling protein that moves βIIIPKC from one intracellular site to another.

Specific intracellular localization of signaling proteins such as PKC1 is important for the regulation of complex signal transduction cascades (1). PKC is a family of 10 isozymes that are localized to specific intracellular sites in unstimulated cells. Upon activation, each PKC isozyme moves to a different intracellular site (2). Localization of inactive or activated PKC isozymes is mediated, at least in part, by interaction with anchoring proteins (3, 4). For example, inactive PKC isozymes appear to be localized by binding to the scaffolding proteins AKAP-79 and gravin (5, 6). In contrast, activated PKC isozymes appear to be localized by binding to receptors for activated C kinase (RACKs). RACK1 specifically binds the active form of βIIIPKC (7, 8) thereby regulating PKC function (8–12). In vitro, RACK1 binds PKC only in the presence of PKC activators and increases PKC kinase activity, presumably by stabilizing its active conformation (13). The RACK1 binding site on PKC is within the C2 region of the regulatory domain providing a direct protein-protein interaction (8). Indeed, RACK1 belongs to the WD40 family of proteins, and the WD40 motif is implicated in mediating protein-protein interactions (14). Furthermore, peptides derived from either PKC and/or RACK1 can alter PKC activity in vitro and in vivo (8, 12, 15, 16).

Although RACK1 binds activated PKC and is clearly important for PKC function, the mechanism by which RACK1 localizes βIIIPKC to its site after activation is not understood. One prediction is that the anchoring protein RACK1 should always be localized to the same site that accepts βIIIPKC after translocation. We therefore used confocal microscopy to determine whether RACK1 is co-localized with activated βIIIPKC, whether RACK1 is localized to a specific organelle, and whether the intracellular localization of RACK1 changes in response to PKC activation. Here, we provide evidence that RACK1 is localized to different sites in unstimulated and stimulated cells and that PKC activation leads to movement of RACK1. Furthermore, PKC activation induces the association and co-localization of RACK1 with βIIIPKC. Based on these results, we propose that RACK1 is a shuttling protein that localizes βIIIPKC upon activation by shuttling the kinase to its appropriate subcellular site.

EXPERIMENTAL PROCEDURES

Materials

Phorbol 12-myristate 13-acetate (PMA) was purchased from LC Laboratories. ET180CHCl, calphostin C, bisindolylmaleimide HCl (GF-109), chelerythrin chloride and 1,1′-diaminoethane-4-aminooquinardilium chloride (DECA) were purchased from Calbiochem. Diacylglycerol (DAG) and phosphatidylserine were purchased from Avanti. Luminol and p-coumaric acid were purchased from Sigma. The enhanced chemiluminescence plus kit was purchased from Amersham Pharmacia Biotech. The dopamine D2 agonist trihydroxy-N-propyl-noraporphine hydrobromide (NPA) was purchased from RBI. Recombinant human βIIIPKC was purchased from Panvera. Polyclonal anti-βIIIPKC antibodies and the βIIIPKC peptide were purchased from Santa Cruz Biotechnology. Monoclonal anti-mannosidase was purchased from Cappel. Rabbit, Texas Red-conjugated goat anti-mouse (IgM), and Cy5-conjugated goat anti-mouse antibodies (IgM) from Zymed Laboratories. Secondary antibodies were from Transduction Laboratories. The Golgi marker BODIPY FL C6-Ceramide was purchased from Molecular Probes. The secondary antibodies fluorescein isothiocyanate-conjugated goat anti-rabbit, Texas Red-conjugated goat anti-mouse (IgM), and Cy5-conjugated goat anti-mouse antibodies (IgM) were purchased from Cappel.

Cell Culture

Chinese hamster ovary (CHO) cells stably expressing the long form of the dopamine D2 receptor (D2L) (17) were seeded and grown in Ham’s F-12 medium containing 10% FBS and 2 mM glutamine. After 48 h, media were replaced with Ham’s F-12 medium containing 5% serum and 25 mM HEPES (pH 7.4), 2 mM glutamine, 50 µg/ml human transferrin, 10 µg/ml oleic acid (complexed with 2 mg/ml fatty acid-free bovine serum albumin), 25 µg/ml bovine insulin, and trace elements at the following concentrations: 0.5 mM MnCl2, 0.5 mM (NH4)2MoO4, 0.25 mM Na2SiO3, 25 mM Na2VO4, 5 mM CdSO4, 0.25 mM NiSO4, 15 mM H2SeO3, and 25 mM Na2SiO3. On day 4, the cells were treated with different concentrations of NPA, trihydroxy-1,1′-decamethylenes-4-aminoquinardilium chloride; DAG, diacylglycerol; NPA, trihydroxy-N-propyl-noraporphine hydrobromide; PH, pleckstrin homology; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

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‡ This abbreviation was used: PKC, protein kinase C; RACK, receptor for activated C kinase; PMA, phorbol 12-myristate 13-acetate; DECA, 1,1′-diaminoethane-4-aminooquinardilium chloride; DAG, diacylglycerol; NPA, trihydroxy-N-propyl-noraporphine hydrobromide; PH, pleckstrin homology; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.
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**Fig. 1. PKC activation induces RACK1 movement.** CHO/D2L cells were treated with either 100 nM PMA for 10 min at 37 °C or with a 500 nM concentration of a D2 receptor agonist, NPA. NG108-15 cells were treated with 100 nM PMA. Cells were washed, fixed, and blocked as described under “Experimental Procedures.” RACK1 localization was assayed by immunostaining with monoclonal anti-RACK1 antibodies (Transduction Laboratories, 1:100). Cells were scanned using a confocal microscope and viewed with a × 60 lens for CHO/D2L and a × 40 for NG108-15 cells. RACK1 staining is represented by false color; intensity is represented by the false color bar on the left, with red indicating the areas with the most intense staining. No change in staining intensity or localization was observed when unstimulated cells were incubated with fresh medium containing 0.001% Me₂SO in the medium or with media containing 0.01% ascorbic for 10 min to 1 h. a, control CHO/D2L cells; b, CHO/D2L cells treated with 100 nM PMA for 10 min; c, CHO/D2L cells treated with 500 nM NPA for 30 min; d, unstimulated CHO/D2L cells stained with anti-RACK1 antibodies (1:100) that were preabsorbed overnight at 4 °C with 5 μg of the recombinant fusion protein maltose-binding protein RACK1; e, unstimulated NG108-15 cells; f, NG108-15 cells treated with 100 nM PMA for 10 min. The images are representative of more than 10 individual experiments for CHO/D2L and 3 experiments for NG108-15. The images shown are individual middle sections of the projected Z-series.

**Fig. 2. PKC activation induces βIIPKC movement to the Golgi apparatus in CHO cells.** CHO/D2L cells were treated with 100 nM PMA for 10 min at 37 °C. Cells were washed, fixed, and blocked as described under “Experimental Procedures.” Cells were then stained with both polyclonal anti-βIIPKC antibody (1:100) (a–d) and monoclonal anti-mannosidase antibodies (1:5000) (e) and visualized with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (1:500) for βIIPKC and Texas Red-conjugated goat anti-mouse (1:500) antibodies for mannosidase. Cells were scanned using a confocal microscope and viewed with a × 40 lens. No change in staining intensity or localization was observed when unstimulated cells were incubated with fresh media, with 0.001% Me₂SO in the medium (because PMA stock solution is dissolved in Me₂SO), or with 0.01% ascorbic acid in the medium (because dilution of NPA is done in medium containing 0.01% ascorbic acid) for 10 min to 1 h. CHO/D2L cells incubated in the absence (a and c) or presence of 100 nM PMA (b, d–e). Specificity of the anti-βIIPKC antibody was confirmed by staining the cells with antibody preabsorbed to the immunizing βIIPKC peptide (e). Panel f represents a merged image of d and e performed with image analysis as described under “Experimental Procedures.” The images are representative of three individual experiments. The images shown are individual middle sections of the projected Z-series.
Recombinant RACK1 (125 ng) was blotted onto nitrocellulose membrane (Schleicher & Schuell) using a slot blot apparatus (Schleicher & Schuell). Unbound material was removed, and the membrane was incubated in overlay block (0.2 mM NaCl, 50 mM Tris-HCl, pH 7.5, 3% bovine serum albumin, and 0.1% polyethylene glycol) for 1 h at room temperature. In a separate tube, βIIPKC (1 μg of purified recombinant SF9-expressed protein) was incubated in the presence of 50 μg/ml phosphatidylserine, 0.8 μg/DAG, and 1 μg/ml calcium in overlay buffer (50 mM Tris-HCl, pH 7.4, 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) and in the presence or absence of PKC inhibitors (12 nM bisindolylmaleimide HCl, 10 μM DECA, 50 mM calphostin C, and 660 mM chelerythrin chloride). The mixture was incubated for 15 min while being rotated at room temper-
FIG. 4. PKC activation induces movement of βIIIPKC and RACK1 from different sites to the same site. a–c, CHO/D2L cells treated as described in Figs. 1 and 2 with either PMA or NPA. The cells were stained with both monoclonal anti-RACK1 antibodies and polyclonal anti-βIIIPKC antibodies (1:100) that were visualized with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (1:500) for βIIIPKC and Cy5-conjugated goat anti-mouse (IgM) antibodies for RACK1 (1:500). RACK1 staining is red, βIIIPKC staining is green, and the merged image of the two colors is yellow. Images were viewed with a × 40 lens. a, double staining of βIIIPKC and RACK1 in control cells; b, double staining of βIIIPKC and RACK1 in PMA-treated cells; c, double staining of βIIIPKC and RACK1 in NPA-treated cells. Images are representative of six unstimulated experiments, six PMA experiments, and four NPA experiments. The images shown are individual middle sections of the projected Z-series. d, quantification of the data presented in A–C. Data were analyzed using NIH Image as described under “Experimental Procedures.” The percentage of βIIIPKC and RACK1 colocalization is defined as (the pixels value of the merged image/pixel value of RACK1 staining) × 100. Data are mean ± S.D. of unstimulated and PMA n = 6, NPA n = 4. Data were analyzed by Student’s t test. *, p < 0.01. e–g, CHO/D2L cells treated as described in Figs. 1 and 2 with either PMA or NPA. The cells were stained with both monoclonal anti-RACK1 antibodies and visualized with Texas Red-conjugated goat anti-mouse (IgM) (1:500) together with the Golgi marker BODIPY FL C5-ceramide (1:200). RACK1 staining is red, Golgi staining is green, and the merged image of the two colors is yellow. Images were viewed with a × 60 lens. g, double staining of RACK1 and the Golgi marker. f, RACK1 staining. g, Golgi staining. Images are representative of two experiments. The images shown are individual middle sections of the projected Z-series.

RESULTS

In order to determine whether RACK1 is co-localized with activated translocated (moved) βIIIPKC, we first established the localization of RACK1 in CHO cells using confocal microscopy. As shown in Fig. 1a, RACK1 was localized to a perinuclear structure in unstimulated cells. Next, we determined the localization of βIIIPKC in CHO cells. As shown in Fig. 2a, βIIIPKC is localized to the cytoplasm in unstimulated cells. Activation by a phorbol ester (PMA) induced βIIIPKC to move from the cytoplasm (Fig. 2a) to a site that resembles the Golgi apparatus (Fig. 2b) and not the perinuclear structure where RACK1 is found (Fig. 1a). This structure was identified as the Golgi apparatus by double-staining of cells with anti-βIIIPKC antibodies (Fig. 2d) and anti-mannosidase antibodies (Golgi marker) (Fig. 2c). Colocalization of βIIIPKC with the Golgi marker was confirmed by performing image analysis on the merged image as described under “Experimental Procedures.” βIIIPKC (Fig. 2d) co-localized with mannosidase (Fig. 2e) as shown in the merged image (Fig. 2f). Because RACK1 in un-
stimulated cells is localized to the perinuclear (Fig. 1a) and is not localized to the Golgi apparatus, where translocated (moved) activated βIIIPKC is found (Fig. 2b), RACK1 is not always localized to the same site as βIIIPKC.

We next determined whether RACK1 is always localized to a specific organelle. Specifically, we asked whether RACK1 is always localized to perinuclear structures in CHO cells, regardless of the activation state of the cell. CHO cells that stably express the dopamine D2L receptor (CHO/D2L) were treated with either PMA or with the dopamine D2 agonist NPA, and RACK1 localization was determined. PKC activation either directly with PMA or by NPA activation of the D2 receptor induced the movement of RACK1 from perinuclear structures (Fig. 1a) to a different site (Fig. 1b and c). In another cell line, NG108-15 neuroblastoma x glioma cells, RACK1 was also localized to different intracellular sites before (Fig. 1d) and after PKC activation (Fig. 1e). In NG108-15 cells PKC activation induced RACK1 to move to yet unidentified cytosolic structures and to neurites (Fig. 1f). Therefore, RACK1 is not localized to a specific organelle, and activation of PKC leads to movement of RACK1.

If RACK1 movement is dependent on PKC activation via phosphatidylinositol-derived second messengers, then a PLC inhibitor should inhibit RACK1 movement induced by activation of the D2L receptor. Indeed, movement of both RACK1 (Fig. 3a) and βIIIPKC (Fig. 3b) was inhibited when CHO/D2L cells were pretreated with the PLC inhibitor ET180CH3 prior to activation with NPA. As expected, ET180CH3 did not inhibit PMA-induced movement of βIIIPKC or of RACK1 (Fig. 3c), because phorbol esters bypass PLC signaling and directly activate PKC. Therefore, RACK1 movement is dependent on PLC activation, suggesting that under physiologic conditions, the generation of second messengers is required not only for the movement of βIIIPKC but also for movement of RACK1.

PKC activation induced movement of both RACK1 and βIIIPKC (Figs. 1 and 2). We therefore determined whether RACK1 and βIIIPKC become co-localized to the same site after PKC activation. CHO/D2L cells were incubated in the absence and presence of PMA or NPA and stained for both RACK1 and βIIIPKC. The images were merged in order to detect co-localization, and image analysis was performed. As shown in Fig. 4, a and d, in unstimulated cells, approximately 60% of RACK1 and βIIIPKC were not co-localized. Upon PKC activation, RACK1 and βIIIPKC moved to the same site, and their intracellular staining patterns merged to more than 70% (Fig. 4, b–d). In CHO cells, PKC activation induced βIIIPKC to move to the Golgi apparatus (Fig. 2, d–f). To confirm that RACK1 also localized to the Golgi apparatus upon PKC activation, we stained cells with anti-RACK1 antibodies together with the specific Golgi marker BODIPY FL C6- ceramide. As shown in Fig. 4, e and f, RACK1 co-localized with the Golgi marker after PKC activation. Thus, PKC activation induces the co-localization of RACK1 and βIIIPKC and the movement of both proteins from different sites to the same locations.

The co-localization of RACK1 and βIIIPKC after PKC activation, observed by immunofluorescence (Fig. 4), suggests that the two proteins associate with each other in cells. To explore this possibility, we determined whether the two proteins can be co-immunoprecipitated and whether PKC activation is required for their association. βIIIPKC was immunoprecipitated from unstimulated, PMA-treated, or NPA-treated cells using anti-βIIIPKC antibodies, and we determined whether RACK1 was co-immunoprecipitated. Anti-βIIIPKC antibodies co-immunoprecipitated RACK1 in CHO-D2L cells (Fig. 5a, lanes 5 and 6) and in NG108-15 cells (Fig. 5b, lane 2), and anti-RACK1 antibodies also co-immunoprecipitated βIIIPKC (data not shown), indicating that RACK1 and βIIIPKC do associate in cells. Furthermore, the association between RACK1 and βIIIPKC was increased by PKC activation with PMA or NPA (Fig. 5a, lanes 5 and 6 compared with lane 7 for CHO/D2L cells and Fig. 5b, lane 2 compared with lane 1 for NG108-15 cells). Anti-βIIIPKC antibodies, which were used as control antibodies, did not immunoprecipitate RACK1 (data not shown), indicating that the association between βIIIPKC and RACK1 is specific. Western blot analysis of RACK1 (30 kDa) and βIIIPKC (80 kDa) show that the amount of the detected protein does not significantly change with the experimental conditions (Fig. 5, a, lanes 1–3, and b, lanes 5 and 6), and no cross-reactivity with either antibody was observed (data not shown). Taken to-
Fig. 6. NPA-induced RACK1 and βII PKC movement and co-localization as function of time. a, CHO/D2L cells were treated with 500 nM NPA at 37 °C. Cells were washed, fixed, and blocked at different time points as described under “Experimental Procedures.” Results are presented as mean ± S.D. of three experiments. The percentage of movement was defined as (the number of cells in which RACK1 or βII PKC was translocated (moved)/total number of cells) × 100. Data are the mean ± S.D. of three experiments. b, CHO/D2L cells were treated as in a, and the data were analyzed using the computer program NIH Image 1.61 as described under “Experimental Procedures.” The percentage of βII PKC and RACK1 co-localization is defined as (the pixels value of the merged image/the pixel value of RACK1 staining) × 100. Data are the mean ± S.D. of three experiments. c, CHO/D2L cells were treated with 500 nM NPA for 30 s to 5 min as described in Fig. 1. RACK1 staining is red, βII PKC staining is green, and a merged image of the two colors is yellow. Images were viewed with a × 60 lens. Image is a representative of three different experiments. The images shown are individual middle sections of the projected Z-series.

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gether, our data indicate that activation of PKC causes RACK1 and βII PKC to associate with each other.

We next determined whether RACK1 and βII PKC move together or whether the movement of one precedes the other. We therefore compared RACK1 and βII PKC movement (Fig. 6a) and co-localization (Fig. 6b and c) as a function of time. As shown in Fig. 6a, the time courses of movement for both RACK1 and βII PKC were very similar, indicating that it is unlikely that one protein moves prior to the other. In contrast, the time course of co-localization (Fig. 6b and c) indicates that the two proteins co-localize prior to their movement. At 1 and 5 min, more than 70% of RACK1 was co-localized with βII PKC, but only 25% of both proteins had reached the Golgi apparatus at that time (Fig. 6a and b). Because co-localization was detected before RACK1 and βII PKC reached the Golgi apparatus (Fig. 6c, compared with Figs. 1, 2, and 4), it is possible that the two proteins associate prior to their movement.

If prior association of βII PKC and RACK1 is required for movement, then inhibition of RACK1 and βII PKC association should prevent movement. Recently, the PKC inhibitor dequalinium (DECA) has been shown to inhibit PKC movement by interacting with the RACK1 binding site on PKC (1). We determined whether DECA inhibits the interaction of βII PKC with RACK1 and compared the results with the effect of other PKC inhibitors. The regulatory domain inhibitor calphostin C and the kinase domain inhibitors bisindolylmaleimide HCl and chelerythrine chloride were used at concentrations equal to their IC_{50} values. Fig. 7a presents an overlay assay of βII PKC binding to immobilized RACK1 in the presence of activators and in the presence of DECA, calphostin C, and bisindolylmaleimide HCl. DECA, as well as calphostin C, reduced the binding of βII PKC to RACK1 (Fig. 7a). On the other hand, bisindolylmaleimide HCl did not affect the interaction between βII PKC and RACK1 (Fig. 7a). Similar effects were obtained with chelerythrine (data not shown due to high background). We next determined whether PKC inhibitors would inhibit the movement of both RACK1 and βII PKC. All inhibitors were used at concentrations equal to their IC_{50} values. DECA inhibited movement of βII PKC (Fig. 7b) in CHO/D2L cells. Interestingly, DECA also inhibited the movement of RACK1 (Fig. 7b). These data suggest that activation-induced binding of βII PKC to RACK1 is a prerequisite for the movement of both proteins. Furthermore, the regulatory domain inhibitor calphostin C inhibited NPA-induced movement and, to a lesser degree, PMA-induced movement (Fig. 7b). Because calphostin C is a competitive inhibitor for the DAG binding site, these results are another indication that suggests that generation of second messengers is required for the interaction and movement of both proteins. On the other hand, the kinase domain inhibitors bisindolylmaleimide HCl and chelerythrin did not significantly
inhibit the movement of βIIPKC and RACK1, indicating that PKC kinase activity is not involved in the movement of both proteins (Fig. 7b).

**DISCUSSION**

PKC anchoring proteins determine the localization of different activated PKC isozymes (3, 4). However, the mechanism by which PKC anchoring proteins localize PKC isozymes to specific sites after movement is not well understood. Here, we provide evidence that RACK1, the anchoring protein for activated βIIPKC, also moves upon activation of PKC. RACK1 moves in response to PKC activation and localizes to the same sites as activated βIIPKC. The PLC inhibitor ET18OCH₃ blocked dopamine D₂ receptor-induced movement of RACK1, and calphostin C, an inhibitor that competes with DAG, interfered with the interaction and movement of RACK1 and βIIPKC. These results suggest that generation of second messengers needed for the activation of PKC is also necessary for movement of RACK1. On the other hand, our results with PKC kinase inhibitors suggest that PKC kinase activity per se is not involved in the binding or is required for the movement of the two proteins. These findings are in line with previous data showing that RACK1 itself is not a substrate for PKC (13).

We further show that RACK1 and βIIPKC co-localize prior to their movement and that the association of the two proteins appears to be required for their simultaneous movement. Based on these findings, we propose that RACK1 is a PKC shuttling protein. When βIIPKC is activated, it binds to RACK1. RACK1 then moves together with βIIPKC to bring the enzyme in close proximity to its appropriate substrate.

The association between activated βIIPKC and RACK1 in situ was detected by co-immunoprecipitation. These results are in agreement with previous *in vitro* studies showing that the association of RACK1 and βIIPKC occurs only in the presence of the PKC activators phosphatidylserine, DAG, and calcium (13). The early time points of co-localization between RACK1 and βIIPKC (30 s to 5 min) could be detected with confocal microscopy but could not be confirmed by immunoprecipitation. Labeling each protein with a different fluorescence tag may allow us to follow the movement and co-localization of RACK1 and βIIPKC at early time points in live cells.

Although it is possible that movement of βIIPKC is responsible for movement of RACK1, we consider this possibility unlikely for several reasons; RACK1 belongs to the WD40 family of proteins that regulate (via protein-protein interaction) the localization and/or activity of various signaling proteins. For example, the β-adrenergic receptor kinase is localized by the WD40-containing protein Gβ (the β subunit of GTP-binding protein) (19); the transforming growth factor-β receptors interact with a subunit of phosphatase 2A (a WD40-containing protein) (20); cytosolic phospholipase A2 binds to the WD40-containing protein PLAP (21), and ePKC is localized by yet another WD40-containing RACK, RACK2 (22). Furthermore, PKC-mediated functions are inhibited when the association between RACK1 and PKC is disrupted by peptides (8, 16). Therefore, it is most likely that RACK1 is directing activated βIIPKC to a specific site.

Furthermore, PKC activation induces βIIPKC to move to different sites in different cells. For example, in NIH3T3 cells, activated βIIPKC is found in cytoskeletal elements (23); in cardiac myocytes, activated βIIPKC is localized to perinuclear structures; and in human leukemic cell lines, βIIPKC moves to the nuclear membrane, where it phosphorylates lamin B (24). Activation induces βIIPKC to move to cytoplasmic filaments (25) in human endothelial cells, and to the plasma membrane in HEK 293 cells (26). In addition, different stimuli cause βIIPKC to move to different intracellular sites in the same cell (2, 25). Therefore, it is not surprising that we detected βIIPKC movement to the perinuclear structures in CHO cells and to neurites in NG108 cells. We suggest that RACK1 can localize.
activated βIIPKC to different sites because it is a mobile rather than a fixed protein. This is consistent with our finding that RACK1 is not associated to a specific organelle and with other reports that RACK1 is localized at different sites (10, 27). Indeed, sequence analysis reveals that RACK1 does not contain consensus sequence motifs that could anchor it to a particular subcellular site. Thus, the mobility of RACK1 enables it to shuttle βIIPKC to different sites in different cells. These observations also suggest that RACK1 movement may be affected by other signaling cascades. Indeed, we have found that treatment with ethanol induces RACK1 to move to the nucleus, whereas βIIPKC localization is unchanged in three different cell lines (NG108-15, CHO, and C6), as well as in certain brain areas of mice. Furthermore, we found that forskolin (an activator of adenylate cyclase) also induces the nuclear movement of RACK1 but not βIIPKC. Taken together, our studies indicate that different stimuli induce the recruitment of RACK1 to different sites. These results also suggest that the intracellular localization of RACK1 does not depend exclusively upon PKC activation, whereas the movement of βIIPKC is directed by RACK1.

RACK1 may represent a new class of mobile targeting proteins. It is conceivable that other anchoring, scaffolding, or adaptor proteins may also shuttle signaling proteins between intracellular sites. One possible candidate is the adaptor protein 14-3-3 protein, which has recently been shown to bind both inactive Raf in the cytosol and active Raf at the plasma membrane (28). 14-3-3 protein could be a Raf shuttling protein that is responsible for movement of Raf from the cytosol to the plasma membrane. Other candidates are members of the AKAP family of proteins that are redistributed in response to stimuli (29, 30).

What mediates the localization of RACK1? It is possible that RACK1 localization is determined by interaction with organelle specific proteins. One intriguing possibility is that RACK1 targets membranes of organelles via binding to the pleckstrin homology (PH) domains that bind both phospholipids and proteins. Indeed, WD40-containing proteins have been found to interact with PH domain-containing proteins (31). The WD40 motif of Gβγ binds to the PH domain of β-adrenergic receptor kinase (31, 32), and RACK1 itself was found to bind PH domains in vitro. Another possibility is that RACK1 is associated with a PKC substrate after movement. Some of the PH-containing proteins, such as pleckstrin, are PKC substrates, and RACK1 associates with the cytoplasmic domain of β-integrins only in the presence of PMA (10). Based on the translocating properties of RACK1, it is possible that activation of PKC causes movement of RACK1 together with βIIPKC to the plasma membrane, where RACK1 binds to the cytoskeletal tail of β-integrin, allowing PKC to phosphorylate either β-integrin or neighboring proteins.

In summary, our data show that RACK1 localization is regulated by PKC activation and suggest that RACK1 is a PKC shuttling protein. The shuttling properties of RACK1 and other members of its class may add another dimension to our understanding of how PKC isoforms are localized to different sites after activation and movement.

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