State-specific Monoclonal Antibodies Identify an Intermediate State in Epsilon Protein Kinase C Activation*

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Evaluation of the activation state of protein kinase C (PKC) isozymes relies on analysis of subcellular translocation. A monoclonal antibody, 14E6, specific for the activated conformation of ePKC, was raised using the first variable (V1) domain of PKC as the immunogen. 14E6 binding is specific for ePKC and is greatly increased in the presence of PKC activators. Immunofluorescence staining by 14E6 of neonatal rat primary cardiac myocytes and the NG108-15 neuroblastoma glioma cell line, NG108-15/D2, increases rapidly following cell activation and is localized to new subcellular sites. However, staining of translocated ePKC with 14E6 is transient, and the epitope disappears 30 min after activation of NG-108/15 cells by a D2 receptor agonist. In contrast, subcellular localization associated with activation, as determined by commercially available polyclonal antibodies, persists for at least 30 min. In vitro, eRACK, the receptor for activated PKC, inhibits 14E6 binding to ePKC, suggesting that the 14E6 epitope is lost or hidden when active ePKC binds to its RACK. Therefore, the 14E6 antibody appears to identify a transient state of activated but non-anchored ePKC. Moreover, binding of 14E6 to ePKC only after activation suggests that lipid-dependent conformational changes associated with ePKC activation precede binding of the activated isozyme to its specific RACK, eRACK. Further, monoclonal antibody 14E6 should be a powerful tool to study the pathways that control rapid translocation of ePKC from cytosolic to membrane localization on activation.

Several isozymes of protein kinase C (PKC), 1 lipid-dependent protein kinases, are present within a single cell, each mediating unique intracellular functions. Studies using conventional or confocal microscopy reveal a complex and specific localization of PKC isozymes in their inactive as well as their active state (1–4). Most isozymes are localized to unique sites prior to cell stimulation, and translocate upon activation to new distinct intracellular sites. PKC isozyme localization is determined by binding to specific anchoring molecules termed RACKs (receptors for activated C-kinase) (5). Two RACKs have been identified and characterized to date: the βIPPKC-specific RACK (RACK1) (6) and the ePKC-specific RACK (eRACK), also known as β′ COP (7).

Many aspects of PKC activation are not fully understood. Are conformational changes associated with PKC activation? Does lipid binding precede RACK binding? In order to answer these and other questions, it is necessary to develop new “state-specific” reagents to distinguish between active and inactive individual PKC isozymes.

ePKC is an isozyme that regulates many cellular functions (8–14). In cardiac myocytes, ePKC mediates cardioprotection from an ischemic episode (9, 11, 14–16), cardiac hypertrophy (17, 18), regulation of L-type calcium channel (19, 20), and regulation of contraction rate (10, 21). The V1 domain of ePKC is involved in the binding of activated ePKC to its RACK in these cells (7, 10, 22). Because binding to RACKs occurs only after activation of ePKC (7, 23, 24), we expected that a conformational change in this domain must occur upon activation. We therefore predicted that some antigenic determinants on the V1 domain should be exposed only following ePKC activation and that some of the antibodies raised against V1 might recognize the active state of ePKC and be isozyme-selective.

We report here the production and characterization of an isoyme-selective monoclonal antibody (mAb) to ePKC that is specific for the activated form of this isozyme. Activation is required to expose the epitope for this antibody, but 14E6 no longer binds to the activated enzyme when the latter is bound to eRACK. Together, these studies suggest the existence of a previously unidentified state in PKC activation: a transient, activated, but non-anchored state. Immunostaining with 14E6 should help in identifying cells in tissues where ePKC has been activated by a physiological trigger. In addition, 14E6 will be a useful marker to follow the pathway of ePKC translocation and help elucidate the mechanism involved in this pathway.

EXPERIMENTAL PROCEDURES

Materials—Recombinant δ, ε, and γ PKCs produced in S99 cells were obtained from PanVera (Madison, WI). Phorbol myristate acetate (PMA) was from Alexis (San Diego, CA), and phosphatidyserine (PS) and dioleoylglycerol (DG) were purchased from Avanti (Alabaster, AL). PKC was partially purified from rat brain by DEAE-cellulose chroma-
tography, ammonium sulfate precipitation, and gel filtration, as described previously (25). A rabbit IgG raised to the C terminus of ePKC (sc-214, Santa Cruz Biotechnology, Santa Cruz, CA) was used in some of the Western blots and immunoprecipitation experiments. Rabbit antisem to the C terminus of ePKC (AS-2453S, Research & Diagnostics Antibodies, Berkeley, CA) was used for immunofluorescence in cardiac myocytes. A monoclonal antibody to ePKC (SC-1681, Santa Cruz Biotechnology) was used for immunofluorescence in NIH/81-15 cells.

Preparation of Recombinant Proteins—The V1 regions of rat 8 and ePKC (amino acids 2–144 and 2–145, respectively) were expressed as fusion proteins with MBP (maltose-binding protein; pMAL-c2 vector, New England Biolabs) in Escherichia coli. Vectors were also constructed expressing the C-terminal half of eRACK as an MBP fusion protein (MBP-eRACK-C, amino acids 425–905), MBP-LaZ, and MBP-MifIPK-C-V5 (amino acids 622–935). MBP fusion proteins were purified by affinity chromatography on amylose resin according to the New England Biolabs protocol. Where indicated, cleavage of the fusion protein and removal of MBP were carried out with 5 μg/ml Factor Xa (New England Biolabs) for 2 h at 4 °C.

Monoclonal Antibody Production—Purified ePKC-V1 was incubated with 120 μg/ml PS, 4 μg/ml DG, and eRACK-C for 15 min at room temperature (PS and DG in ethanol were dried under nitrogen, 20 mM Tris pH 7.5, 5 mM EDTA, 12 mM NaCl, and DG to PBS plus PS). The mixture was sonicated on ice, with a Branson Sonifier at 20% output at setting 2.5, using 3 cycles of 1 min each, with 30 s cooling periods on ice between cycles. Eight-week-old BALB/c mice (Harlan) were injected subcutaneously three times at 3–4-week intervals with 10 μg of ePKC-V1 and 10 μg of eRACK-C with PS and DG, emulsified with Freund’s adjuvant. Four days after the final boost, splenocytes (108) were fused with 2 × 104 murine myeloma Fox-NY cells (from Thomas A. Stamey) according to Kohler and Milstein (26). Cells were grown in HAT-containing medium as described (25), and positive cultures were subcloned three times. Antibodies were obtained either as ascites in BALB/c mice or as supernatant in serum-free medium in roller bottles, and purified by affinity chromatography with 5 μg/ml of ePKC-V1 and 5 μg/ml of eRACK-C with PS and DG. They were subsequently affinity purified by gel filtration chromatography on Sephacryl S-300. SDS-PAGE was performed with 12% gel. Western blotting was carried out with rabbit polyclonal IgG (sc-214, Santa Cruz Biotechnology, Inc.) or mouse polyclonal IgG (905), MBP-LacZ, and MBP-MifIPK-C-V5 (amino acids 622–935). MBP fusion proteins were purified by affinity chromatography on amylose resin according to the New England Biolabs protocol. Where indicated, cleavage of the fusion protein and removal of MBP were carried out with 5 μg/ml Factor Xa (New England Biolabs) for 2 h at 4 °C.

Preparation of Cell Lysates—For immunoprecipitation, the heart was homogenized in 1 ml of 50 mM sodium phosphate/25 mM citric acid, pH 5.5 with 2 μM N-ethylmaleimide, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 50 μg/ml of 6E4 was incubated with 30 μl of protein G-agarose (Invitrogen, Life Technologies, Inc.). Following the indicated times and concentrations, cells were incubated with fluorescein labeled-goat anti-mouse IgM, anti-mouse IgG, or anti-rabbit IgG (Organon Tecnika Corp., Bohemia, NY) for 2 h at room temperature, washed with PBS三次 followed by 1 ml of goat anti-mouse fluorescent antibody (goat anti-mouse IgG, Jackson Laboratories, West Grove, PA; diluted 1:5000 in PBS containing 1% BSA) was added and incubated for 1 h, followed by three washes with PBS/Tween. 100 μg of O-phtalaldehydime-1,6-diamine and 0.03% H2O2 in 100 mM citrate buffer, pH 6.0, was added to assay HRP activity; after 15 min, 50 μl of 2 n H2O2 was added to stop the reaction and absorbance at 490 nm determined. To determine whether binding of eRACK to ePKC affects the subsequent binding of antibodies, 5 μg/ml of MBP-eRACK-C was added to immobilized MBP-ePKC-V1 for 1 h at room temperature, followed by three washes with PBS/Tween, prior to antibody addition.

ELISA Analysis for ePKC Activation—Activation of ePKC was carried out by incubation of 1–5 μg of PanVera S19 ePKC in a final volume containing 30 mM MOPS, pH 7.5, 5 mM EDTA, 125 μM of a- to gPKC, 0.25% Triton X-100, and 10 nM of ATP gamma S. The reaction was started with 0.2% NaN3 and rocked for 2 h at 4 °C. The beads were blocked with 3% BSA in PBS, incubated with proteins G- and IgM-agarose (Sigma) overnight at 4 °C. Antibody-bound beads were then washed twice with PBS and blocked with 3% BSA for 2 h at 4 °C. Triton-soluble material was diluted 5 times in homogenization buffer to dilute the Triton X-100 to 0.2%, then precleared with protein G- or IgM-agarose beads for 30 min at 4 °C, incubated with antibody-bound beads overnight at 4 °C, and subsequently washed four times with PBS containing 0.1% Triton X-100. Bound material was eluted with SDS sample buffer, run on an 8% SDS-PAGE, transferred and probed for ePKC (anti-V5 Santa Cruz) and eRACK (monoclonal antibody 4B12).

Immunoprecipitation of Rat Brain PKC—For immunoprecipitation, 5 μg of each monoclonal antibody was added to 50 μl of goat anti-mouse IgM (Jackson Laboratories) conjugated to Sepharose beads using cyanogen-bromide-activated Sepharose 4B (Amersham Biosciences), or to 50 μl of protein G-Agarose beads (Invitrogen, Life Technologies, Inc.), and rocked for 2 h at 4 °C. The beads were washed three times with PBS/Tween, washed with PBS/2% Triton X-100, and washed again. Plates were incubated with anti-mouse IgM-horseradish peroxidase conjugate (Pierce Endogen 31440) for 1 h, washed, and developed with 3,3’,5’-tetramethylbenzidine (Sigma T3405; 1 mg per 10 ml of 50 mM sodium phosphate/25 mM citric acid, pH 5.5 with 2 μM of 30% hydrogen peroxide). The reaction was stopped with 50 μl of 2 M H2SO4, and the absorbance was measured at 490 nm.

Cardiac Myocyte Preparation—Primary cardiac myocytes were prepared from the hearts of 1-day-old Sprague-Dawley rats by gentle trypsinization as described (27). Non-myocytes were removed by pre-plating for 30 min. Myocytes were plated at 800 cell/mm2 in M-199 medium (Invitrogen, Life Technologies, Inc.) with 0.1 mM bromodeoxyuridine and 0.5% FeCl3. Myocytes were incubated at 37 °C in 1% CO2. After 24 h, the culture medium was changed to the above medium containing 80 μM ascorbic acid, and cells were cultured an additional 72 h. Myocytes were then incubated in a defined medium (M-199 with penicillin and streptomycin, 2 μg/ml vitamin B12, and 10 μg/ml insulin and transferrin) for 24 h, after which experiments were initiated.

NG108-15 Cell Culture—NG108-15 cells were cultured as described (28), and maintained in a serum-free defined medium for 6 days prior to immunofluorescence or immunoprecipitation experiments. Experiments with the dopamine receptor agonist NPA were carried out with NG108-15 cells stably transfected with the dopamine D2 receptor, NG108-15/D2 (29).

Immunofluorescence—Cardiac myocytes were plated on glass cover slips or in chamber slides coated with 1.2 μg/ml laminin from mouse sarcoma cells (Invitrogen, Life Technologies, Inc.). Following the indicated treatments, cells were fixed for 5 min on ice in methanol/aceton (1:1, −20 °C). Slides were washed three times with cold PBS, blocked with PBS plus 0.1% Triton X-100 (PBS/Triton) containing 1% normal goat serum, and incubated with primary antibodies overnight at 4 °C in PBS/Triton containing 1% normal goat serum. After three washes with PBS/Triton, cells were incubated with fluorescein labeled-goat anti-mouse IgM, anti-mouse IgG, or anti-rabbit IgG (Organon Teknika Corp., Bohemia, NY) for 2 h at room temperature, washed three times with PBS/Triton, and mounted in Vecta Shield (Vector Labs, Burlingame, CA). Fluorescence microscopy was carried out with a ×40 water immersion objective or by confocal microscopy. NG108-15/D2 cells were fixed with methanol and confocal microscopy carried out as described (28). These fixation conditions were found optimal to conserve antibody epitopes and cell structure.

Preparation of Cell Lysates—For immunoprecipitation, the heart was homogenized in 1 ml of 50 mM sodium phosphate/25 mM citric acid, pH 5.5, 2 mM EDTA, 2 mM NaCl, 2 μg/ml bovine serum albumin, 50 mM methylsulfonyl fluoride (17 μg/ml), SBDT (20 μg/ml), leupeptin (25 μg/ml), and aprotinin (25 μg/ml). Cells were then fractionated into soluble and particulate fractions; cells resuspended in homogenization buffer were ultracentrifuged at 100,000 × g for 30 min. The pellet was resuspended in one-fifth of the initial volume in DE buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM NaCl, 2 μg/ml bovine serum albumin, 50 mM methylsulfonyl fluoride (17 μg/ml), SBDT (20 μg/ml), leupeptin (25 μg/ml), and aprotinin (25 μg/ml). The entire immunoprecipitate was analyzed by SDS-PAGE using 8% polyacrylamide. Western blotting was carried out with rabbit polyclonal IgG.
whether the 14E6 epitope overlaps with the site. We used recombinant MBP-fusion proteins containing the V1 domain, MBP-PKC-V1 to allow complex formation. Binding of MBP-eRACK-C to MBP-PKC-V1 is maximal under the conditions used here, as quantitated with antibodies to eRACK-C (not shown). 14E6 (A) or 6E10 (B) was then added, and the amount of each antibody bound was quantitated by ELISA as described under “Experimental Procedures.”

RESULTS

Our purpose was to generate mAbs that distinguish between active and inactive ePKC. The V1 domain of this isozone was chosen as the immunogen because it contains a RACK binding site (7, 10) that is exposed only after ePKC activation. We therefore expected that certain epitopes in this domain may be exposed only following activation of ePKC and therefore should be recognized by antibodies that would only recognize activated ePKC. Mice were injected with recombinant protein comprising the V1 region of rat ePKC (amino acids 2–145). Hybridoma culture supernatants were screened by ELISA for binding to MBP-ePKC-V1.

Identification of Antibodies to the V1 Domain of ePKC That Are Isozyme-specific—An IgM antibody, 14E6, was shown by ELISA to be specific for ePKC. 14E6 bound to MBP-ePKC-V1 and to full-length recombinant ePKC expressed in Sf9 cells (Sf9-ePKC), but not to Sf9-6 PKC, Sf9-γ PKC, MBP-βPKC-V1, MBP-βIIPKC-V5, or to the eRACK fragment that binds the ePKC-V1 domain (MBP-eRACK-C) (Fig. 1). In Western blot analyses, 14E6 did not recognize full-length Sf9-ePKC or purified rat brain ePKC, but did recognize MBP-e V1 (data not shown). 6E10, a second IgM antibody raised against MBP-ePKC-V1, has a specificity similar to 14E6 in ELISA and Western blot analyses (Figs. 1 and 4, and data not shown).

14E6 Competes with eRACK for Binding to ePKC-V1—Because the 14E6 binding site on ePKC is within the V1 domain (Fig. 1), the same domain that contributes significantly to the binding of ePKC to full-length eRACK (7), we next determined whether the 14E6 epitope overlaps with the eRACK binding site. We used recombinant MBFusion proteins containing either the ε V1 domain, MBP-ePKC-V1 (MBP-εPKC-2–145) or the C-terminal half of eRACK, MBP-eRACK-C (β′-COP 425–905), which contains the ePKC binding site. Both 14E6 and the control anti-ePKC-V1 mAb, 6E10, bound to MBP-ePKC-V1 (Fig. 2, A and B, solid bar and Fig. 1), and did not bind to MBP-eRACK-C or to MBP (Fig. 2, A and B, striped bars). However, when ePKC-V1 and eRACK-C were preincubated before addition of 14E6, the binding of 14E6 to this complex (Fig. 2A, open bar) was 70% lower than to ePKC-V1 alone (Fig. 2A, filled bar), suggesting that binding of eRACK to ePKC prevents the binding of 14E6 to the enzyme. In contrast, binding of 6E10 to ePKC-V1 was not significantly decreased by concurrent eRACK binding (Fig. 2B, open bar), making it unlikely that the inhibition of 14E6 binding to ePKC by eRACK was due to a nonspecific steric effect. Our data indicate that the epitope for 14E6 overlaps with or is close to an eRACK binding site on ePKC-V1 and predict that 14E6 will not bind to complexes of active ePKC with eRACK in vivo.

In Vitro Activation of ePKC with PS and DG Increases Binding by 14E6—PKC is activated in vivo through binding of lipid activators such as PS and DG. To determine whether 14E6 or 6E10 could distinguish between the active and inactive forms of ePKC, recombinant ePKC was activated by addition of PS and DG, and 14E6 or 6E10 binding was assessed by ELISA. Antibody binding to ePKC is expressed as the ratio of binding in the presence of lipid activators to binding in the absence of lipid activators (Fig. 3). Binding of 14E6 was increased almost 3-fold by inclusion of lipid activators to 10 ng of ePKC (Fig. 3). The concentration of enzyme appeared to be critical for optimal sensitivity in this assay. When 25 or 50 ng of ePKC was used, binding of 14E6 was already high (data not shown) and was minimally affected by lipid activation of the enzyme (Fig. 3). This explains the binding of 14E6 to ePKC in the absence of activators shown in Fig. 1, since the standard ELISA protocol used over 300 ng of protein per well. The increased binding to ePKC in the presence of lipid activators when limiting amounts of ePKC are used suggests that the 14E6 epitope on the enzyme becomes exposed upon activation of ePKC. We also found that the binding of mAb 6E10 to activated ePKC is slightly but significantly lower than binding to inactive ePKC (Fig. 3).
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**Fig. 3.** Specificity of anti-\(\varepsilon\)PKC antibodies for activated ePKC. The binding of 14E6 and 6E10 to ePKC in the presence or absence of PS and DG was determined by ELISA using variable amounts of full-length enzyme expressed in S9 cells. The ratio of protein to lipid was kept constant. Data are shown as the ratio of binding in the presence of activators to that in the absence of activators. (See “Experimental Procedures”; results are mean ± S.E. of three independent experiments each carried in triplicates: *\(, p < 0.05\).)

Taken together, our data suggest that mAb 14E6, but not 6E10, specifically recognizes the active form of PKC in an ELISA assay.

We next determined whether 14E6 recognizes the active form of ePKC in solution using an immunoprecipitation assay. When a partially purified preparation of PKC from rat brain was incubated with 14E6, little ePKC was immunoprecipitated (Fig. 4A). However, incubation of brain PKC with the activating lipids PS and DG for 3 or 6 min at room temperature resulted in significant immunoprecipitation of ePKC by 14E6 (Fig. 4A). These activators increase the catalytic activity of calcium-independent PKC in the brain PKC preparation by over 10-fold as determined by phosphorylation of myelin basic protein (data not shown). In contrast, 6E10 immunoprecipitated more ePKC in the absence of PKC activators (Fig. 4B) and commercial antiserum to the C terminus of ePKC-V5 immunoprecipitated equal amounts of enzyme regardless of the presence of PKC activators (data not shown). The level of δPKC in the 14E6 immunoprecipitate was very low, indicating the specificity of 14E6 for ePKC (Fig. 4A). In addition, SDS electrophoresis of the supernatants of the immunoprecipitates demonstrated that degradation of ePKC did not occur during the experiment (Fig. 4A, upper blot), indicating that the differences in immunoprecipitation observed in Fig. 4 reflect differences in epitope exposure in the various samples. Therefore, 14E6 is a unique antibody; it specifically recognizes an antigenic determinant on ePKC that becomes exposed following activation by PS and DG.

**The 14E6 Epitope Is Exposed Following Stimulation of NG108-15/D2 Cells—**To determine whether 14E6 identifies activated ePKC in cells, we used a rat/mouse neuroblastoma X glioma cell line stably expressing the dopamine D2 receptor, NG108-15/D2. These cells respond to the dopaminergic agonist NPA with a robust translocation of ePKC (29). We compared staining with 14E6 and with commercial ePKC monoclonal antibodies (raised against the last 15 amino acids in the V5 domain of ePKC) as a function of time following cellular activation. Immunofluorescence confocal microscopy with the commercial anti-ePKC mAbs revealed an NPA-induced translocation from the nucleus and a narrow perinuclear region to a broader perinuclear/Golgi distribution as well as to cytosolic sites (Fig. 5A). In contrast, very little staining with 14E6 was observed in resting cells (Fig. 5B, left panel), but was induced by NPA treatment, yielding staining in broad perinuclear and Golgi regions (Fig. 5B); there was little cytosolic staining, however.

With the commercial anti-ePKC mAb, translocation was not obvious until 5 min after exposure to NPA, and persisted at 30 min. In contrast, 14E6 staining of NG108-15/D2 cells increased within 1 min after addition of NPA, was maximal by 10 min, and was low again by 30 min. The level of exposure of the 14E6 epitope in these cells is very low, as the signal from 14E6 (Fig. 5A) was amplified 10 times compared with the signal from the commercial antibody (Fig. 5A). Notably, 14E6 staining is not observed in the nucleus either before or after NPA is added, in contrast to staining with the commercial anti-ePKC antibody. These results suggest that 14E6 does not recognize inactive ePKC in the nucleus of NG108-15/D2 cells.

Translocation of ePKC, as determined by the commercial antibodies, persisted at 30 min (Fig. 5A), when activated enzyme was no longer detected by 14E6 (Fig. 5B), suggesting that exposure of the epitope for 14E6 is more transient than is localization to sites usually associated with activated enzyme. Further, 30 min after treatment of cells, the commercial anti-ePKC antibody (Fig. 5A), but not 14E6 (Fig. 5B), stained the cell cytosol peripherally.

We also carried out immunoprecipitation experiments on the solubilized particulate fraction of lysates from NG108-15/D2 cells that were incubated with NPA over the same time course as the immunofluorescence studies. Western blots of immunoprecipitation studies with monoclonal anti-ePKC indicate that the total amount of ePKC in the particulate fraction increases with time of incubation with NPA, as expected (Fig. 5C). In contrast, 14E6 only immunoprecipitates a protein recognized by antibodies to ePKC at the 1 min time point (Fig. 5D), supporting the transient nature of the 14E6 epitope indicated in the immunofluorescence studies (Fig. 5B). The exact time of appearance of the 14E6 epitope in the immunofluorescence and immunoprecipitation studies cannot be compared since the cells are fixed immediately for the former and must be lysed and fractionated after the indicated time of incubation with NPA before immunoprecipitation can be carried out. Nevertheless, the immunoprecipitation studies support the conclusion of the immunofluorescence studies that the 14E6 epitope only occurs transiently after activation of the D2 receptor.
Both the immunofluorescence and immunoprecipitation data indicate that the anti-\(\alpha\)/H9280 PKC antibodies recognize activated \(\alpha\)/H9280 PKC at 30 min. However, there is no 14E6 staining at this time. One possible interpretation of this data is that \(\alpha\)/H9280 PKC is active but bound to \(\alpha\)/H9280 RACK and therefore cannot bind to 14E6 (Fig. 2). Therefore we incubated the blots from the immunoprecipitation experiments with antibodies to \(\alpha\)/H9280 RACK. We found that \(\alpha\)/H9280 RACK is co-immunoprecipitated by the anti-\(\alpha\)/H9280 PKC antibody (Fig. 5C), but not by 14E6 (Fig. 5D). Taken together, our data suggest that the 14E6 epitope becomes inaccessible when \(\alpha\)/H9280 PKC is bound to \(\alpha\)/H9280 RACK in cells. To further investigate this possibility, we used neonatal cardiac myocytes, where the localization of activated \(\alpha\)/H9280 PKC is very characteristic and more easily discernable (3, 4).

**Activation of Cardiac Myocytes with Phorbol Ester Results in Induction of the Epitope for 14E6**—Our previous studies in resting cardiac myocytes using commercially available anti-\(\alpha\)/PKC antibodies raised against the V5 region of \(\alpha\)/PKC localized \(\alpha\)/PKC to the nucleus, with only some cells showing \(\alpha\)/PKC at the perinucleus and in cross-striated structures in the cell body (3, 4). After activation, \(\alpha\)/PKC is localized to cross-striated structures and the perinucleus in most cells. This suggests that inactive \(\alpha\)/PKC is found in the nucleus and activated \(\alpha\)/PKC in the perinucleus and cross-striated structures (4). The localization of \(\alpha\)/PKC in resting and activated cardiac myocytes was compared using commercial polyclonal anti-\(\alpha\)/PKC antiserum and the \(\alpha\)/PKC-V1 antibody, 14E6. In agreement with our published observations, when cells were stained with the commercial anti-\(\alpha\)/PKC antibodies, the ratio of perinuclear and cell body staining to nuclear staining increased upon activation of the cells with PMA (Fig. 6A, *top panels*). In contrast, staining with 14E6 was always non-nuclear, consisting of perinuclear, punctate, and cross-striated patterns in the cell body (Fig. 6A, *middle panels*). In addition, there was an increase in the intensity of 14E6 immunofluorescence staining in PMA-treated cells compared with that in unstimulated cells (*left versus right middle panels*, Fig. 6A). 14E6 staining was not seen when the...
antibody was pre-incubated with 1 mg/ml MBP-ePKC-V1 (Fig. 6A, lower panels), indicating again that 14E6 is specific for the V1 domain of ePKC. Both the extranuclear localization of the 14E6 epitope and the increase in staining following activation of PKC suggest that 14E6 is specific for activated ePKC. Moreover, as was observed in NG108-15/D2 cells stimulated with a dopaminergic agonist, translocation of ePKC observed with 14E6 could be detected in myocytes before that seen with the polyclonal antibody and it was more transient (not shown). Taken together, these data suggest that 14E6 is selective for activated ePKC, recognizing a transient form of the activated enzyme.

We recently identified a peptide activator of ePKC. This peptide, ψεRACK, is thought to disrupt intramolecular interaction within PKC and thus expose both the RACK binding site and the catalytic site, rendering the enzyme active (9, 30). Addition of this peptide to cardiac myocytes causes selective ePKC translocation and function (9). In addition, it increases the function of ePKC in the presence of suboptimal levels of PMA. We predicted that if 14E6 recognizes activated ePKC, we should see an increase in staining when cells are treated with ψεRACK. As seen in Fig. 6B, cardiac myocytes treated for 1 min with 1 nM PMA had immunostaining levels with 14E6 that were not different from those in control-treated cells (compare with Fig. 6A, middle left panel). However, in the presence of ψεRACK and 1 nM PMA, immunostaining with 14E6 was similar to that seen with fully activated ePKC (compare Fig. 6A, middle right panel and 6B, right panel.) These data support our hypothesis that 14E6 specifically recognizes the active state of ePKC.

14E6 Recognizes Active but Not RACK-associated ePKC in Cells—The ELISA data shown in Fig. 2 and the co-immunoprecipitation experiments shown in Fig. 5D support our hypothesis that following cellular activation, binding of ePKC to eRACK in cells prevents binding of 14E6 to ePKC, thus leading to the transient appearance of the 14E6 epitope observed in NG108–15/D2 cells (Fig. 5). To determine whether eRACK binding to ePKC precludes 14E6 binding to ePKC in cardiac myocytes, we used confocal microscopy to assess co-localization of ePKC and eRACK in cardiac myocytes. Because both 14E6 and the only anti-eRACK antibodies available are mouse IgM mAbs, we first examined simultaneous staining of ePKC with a rabbit polyclonal antibody (Fig. 7A) and anti-eRACK mAbs (Fig. 7B). In resting cells, there is very little overlap between eRACK and ePKC as stained with the polyclonal ePKC antibodies (data not shown; see also Refs. 5, 7, 10). However, after activation most of the ePKC co-localizes with eRACK (Fig. 7C, yellow). After even brief and mild activation, only small areas of unique staining for polyclonal anti-ePKC (red staining) remain. This implies that most of the ePKC stained by the polyclonal antibody is also bound to eRACK. If binding of activated ePKC to eRACK precludes 14E6 binding, then the 14E6 epitope should not co-localize with polyclonal anti-ePKC staining in activated cells. Therefore, we next examined simultaneous staining of ePKC using the polyclonal anti-ePKC (Fig. 8, left, red) and 14E6 (Fig. 8, right, green). After activation, although both antibodies indicated translocation of ePKC to cross-striated structures (see also Fig. 5), we observed very few areas where the cross-striated staining by the two antibodies merged (Fig. 8, merged). In most areas, there was alternate green-red staining. These data are consistent with the existence of at least two populations of activated ePKC in the cross striations of cardiac myocytes. One is ePKC that is stained by the polyclonal anti-ePKC antibody, which is co-localized with eRACK and that makes up the overwhelming majority of ePKC. The second, is a transient and small population of ePKC that is stained by 14E6. We propose therefore that the commercial polyclonal antibodies stain the cross-striated structures by binding to activated ePKC bound to its RACK; 14E6 recognizes a transient, activated, but non-anchored state of ePKC that has not yet reached the site of eRACK.

**DISCUSSION**

Monoclonal antibody 14E6, raised against the V1 domain of ePKC, appears to recognize an epitope exposed only after activation of the enzyme. First, binding of 14E6 to ePKC in ELISA or immunoprecipitation experiments is increased in the presence of lipid PKC activators when using limiting amounts of the enzyme (Figs. 3 and 4). Second, immunofluorescence localization of ePKC indicates that the 14E6 epitope is induced upon activation of cardiac myocytes by PMA, by the selective ePKC activator peptide, ψεRACK (Fig. 6), or by stimulation with norepinephrine (data not shown); in NG108–15/D2 cells, staining with 14E6 is only observed after activation of the D2 receptor. Third, in cardiac myocytes (Figs. 6 and 8) and in NG108–15 cells (Fig. 5), 14E6 does not stain subcellular compartments where inactivate ePKC is localized. In cardiac myocytes, for example, activation with PMA leads to an increased...
Fig. 9. A model of ePKC activation. Shown are three stages of PKC activation: cytosolic inactive PKC (State I, red) anchors to membranes on elevation of DG (State II, yellow). This transient state (II) is selectively detected by 14E6 and is induced by PKC binding to DG in the presence of lipids (bold in lipid scheme). The activated enzyme then binds to eRACK, but is not detected by 14E6 (green, state III). This last state represents the active stable form of PKC. On activation (step 1), PKC translocates from the cytosol to the membrane. This activation state (detected by 14E6) is transient and then this lipid-bound PKC binds to its RACK (step 2). Finally, by an as yet unknown mechanism, PKC detaches from its RACK and returns to the inactive state in the cytosol (step 3).

ratio of extranuclear to nuclear ePKC (Fig. 6), supporting our earlier observation (4) that nuclei contain a pool of inactive ePKC that translocates to extranuclear structures upon activation. 14E6 staining of PMA-treated cardiac myocytes is exclusively non-nuclear, localized to sites near those where activated ePKC is found (4), including a perinuclear ring, a punctate pattern in the cell body, and in cross-striated structures. Importantly, 14E6 only stains the activated but non-anchored enzyme, since staining by 14E6 of activated ePKC does not overlap with staining of eRACK where activated ePKC is anchored (Fig. 8).

The V1 domain of ePKC was used as the immunogen for raising activation-specific antibodies because it contains an activation-specific-binding site for eRACK (7, 22). The activation-specific epitope for 14E6 on ePKC appears to be within or near the binding site for eRACK (Fig. 2), suggesting that 14E6 only recognizes activated but non-anchored ePKC. Indeed, the data in Figs. 7 and 8 show that 14E6 stains cross-striations distinct from those stained by both an antibody to eRACK and a polyclonal anti-ePKC antibody, supporting this conclusion. Activation by DG, eRACK, or PMA causes ePKC to unfold into an active conformation (30) that is recognized by 14E6. We propose that this conformational change unmasksthe eRACK binding site in the V1 domain, leading to subsequent binding of the enzyme to eRACK, which in turn prevents the binding of 14E6.

Our data suggest a model of activation of ePKC where the enzyme has at least three states of activation: State I, inactive ePKC; State II, a lipid-activated transient state of ePKC to which 14E6 can bind; and State III, active, RACK-associated ePKC to which 14E6 cannot bind (Fig. 9). This model agrees with the time course of immunofluorescence and immunoprecipitation studies in NG108-15/D2 cells (Fig. 5). In the immunofluorescence experiments, the 14E6 epitope appeared within 1 min following treatment with NPA, before translocation of the majority of the ePKC from distinct perinuclear localization to a broader perinuclear/Golgi and cytosolic localization (Fig. 5, A and B; step 1, Fig. 9). Thirty minutes after treatment with NPA, the 14E6 epitope disappeared (Fig. 5B, step II, Fig. 9), whereas commercial anti-ePKC antibodies showed staining at sites where ePKC is bound to eRACK.

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