Activated protein kinase C binds to intracellular receptors in rat hepatocytes

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The aim of this study was to identify in rat hepatocytes cellular polypeptides that bind protein kinase C (PKC) and may influence its activity and its compartmentation. At least seven proteins, with apparent M_r values between 12000 and 36000, that behave like Receptors for Activated C-Kinase (RACKs) were found in the Triton-X-100-insoluble fraction of these cells; i.e. PKC bound to these polypeptides when it was in its active form. RACKs seem to be PKC substrates. Studies using isotype-specific PKC antibodies suggested some selectivity of RACKs, i.e. RACKs in the $M_r \approx 28000$ –36000 region bound PKC- α and PKC- β in the presence of phosphatidylserine, diolein and Ca^{2+} ,

whereas those of $M_r \approx 12000$ –14000 bound all isoforms studied, and, in contrast with the other RACKs, they did this even in the absence of Ca^{2+} . Peptide I (KGDYEKILVALCGGN), which has a sequence suggested to be involved in the PKC–RACKs interaction [Mochly-Rosen, Khaner, Lopez and Smith (1991) *J. Biol. Chem.* **266**, 14866–14868], inhibited PKC activity. Preincubation of RACKs with antisera directed against peptide I prevented PKC binding to them. The data suggest that peptide I blocks PKC binding to RACKs by two mechanisms: inhibition of PKC activity and competition with a putative binding site.

INTRODUCTION

Protein kinase C (PKC) plays a key role in the signal-transduction mechanisms utilized by many growth factors, hormones and neurotransmitters [1,2]. This enzyme belongs to a family of kinases that are activated by diacylglycerol in the presence of acidic phospholipids such as phosphatidylserine; the activity of some members of the family is Ca^{2+} -dependent, whereas that of others is Ca^{2+} -independent [3]. PKC is also activated by tumour-promoting phorbol esters, which modulates many cellular functions [1,3]. Translocation of PKC between soluble and membrane-bound intracellular compartments appears to be involved in its activation and down-regulation [4–7]; however, the molecular mechanisms underlying such redistribution remain unclear. It is thought that increased intracellular Ca^{2+} may be initially involved in membrane association of PKC, as Ca^{2+} alone has been noted to modulate reversible PKC binding to membranes; then, the reversible association is converted into a stable binding when the enzyme interacts with phospholipid components of the membrane and binds diacylglycerol [8]. In addition, a role for membrane proteins in this process has also been suggested [9,10]. Several lines of evidence indicate that, upon activation, PKC interacts with cytoskeletal elements associated with particulate fractions of various cell types [10–13]. Recently, the existence of at least two intracellular proteins that fulfil the criteria of receptors for PKC (termed RACKs: Receptors for Activated C-Kinase) were detected in the particulate fraction from neonatal-rat heart [14]. A sequence that resembles the PKC binding site on these RACKs was identified ('peptide I'), suggesting that binding is mediated by direct protein–protein interaction [15].

Here we show that in rat hepatocytes at least seven proteins bind activated PKC. We also show some differences in the binding of PKC isoforms to these proteins, and that the sequence proposed to resemble the PKC-binding site (peptide I) itself inhibits PKC activation in a dose-dependent fashion.

EXPERIMENTAL

Materials

Peptide I (sequence KGDYEKILVALCGGN) [15] was purchased from Multiple Peptide Systems (San Diego, CA, U.S.A.). Murine monoclonal antibodies to PKC isoenzymes and polyclonal antibody against PKC were from Upstate Biotechnology Inc. or from Seikagaku Kogyo Co.; goat anti-mouse or anti-rabbit IgG–alkaline phosphatase conjugates were from Bio-Rad. Phorbol 12-myristate 13-acetate (PMA), histone H1–IIS, phosphatidylserine, Triton X-100 and 1,2-diolein were from Sigma. Calphostin-C was from Kamiya Biochemical Co., [γ - ^{32}P]ATP from ICN Radiochemicals and DEAE-cellulose (DE-52) from Whatman.

Isolation of rat hepatocytes

Female Wistar rats (200–250 g) fed *ad libitum* were used. Hepatocytes were isolated by the method of Berry and Friend [16].

Partial purification of PKC

After washing, the cells were homogenized in ice-cold buffer (20 mM Tris/HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 0.5% Triton X-100, 50 mM 2-mercaptoethanol and 0.1 mg/ml trypsin inhibitor), incubated at 4 °C for 30 min and centrifuged [17]. Portions of the supernatant were applied to DEAE-cellulose columns that had been previously equilibrated with 'column buffer' (20 mM Tris/HCl, 50 mM 2-mercaptoethanol, pH 7.5) at 4 °C. After washing with column buffer, the PKC activity was eluted batchwise, first with column buffer containing 0.08 M NaCl, 2 mM EDTA and 0.1 mg/ml trypsin inhibitor and then with column buffer containing 0.25 M NaCl, 2 mM EDTA and 0.1 mg/ml trypsin inhibitor. By using this chromatographic system, two peaks of PKC were eluted, the first at 0.08 M NaCl

(PKC 1) and the second at 0.25 M NaCl (PKC 2) [17]. PKC 1 or PKC 2 fractions were concentrated with an Amicon concentrating system (YM-30 membrane) and used for overlay assays.

Overlay assay

PKC binding was determined by the method of Wolf and Sahyoun [10] as modified by Mochly-Rosen et al. [14]. Briefly, polypeptides from the Triton X-100 homogenates from rat hepatocytes (soluble and insoluble fractions) were separated by SDS/PAGE (10% gels) and transferred to nitrocellulose membranes, which were then blocked with 3% (w/v) BSA. Partially purified and concentrated PKC 1 or PKC 2 (100–200 units; one unit is defined as 1 pmol of ^{32}P transferred to histone/min per mg of protein; final protein concn. 0.5 mg/ml) fractions were incubated for 1 h at 30 °C with the nitrocellulose strips in blotting buffer (50 mM Tris/HCl, pH 7.5, 1% polyethylene glycol and 0.2 M NaCl) in the presence of phosphatidylserine (20 $\mu\text{g}/\text{ml}$), 1,2-diolein (0.8 $\mu\text{g}/\text{ml}$) and CaCl_2 (1 mM) or in their absence plus EGTA (0.5 mM). The PKC/blotting-buffer mixture was removed and the membranes were washed three times with blotting buffer. Bound PKC was detected either (a) by using anti-PKC antibodies and second goat anti-mouse or anti-rabbit IgG antibodies conjugated to alkaline phosphatase [14], or (b) by PKC-dependent phosphorylation, i.e., nitrocellulose strips were incubated at 30 °C with 20 mM Tris/HCl (pH 7.5) containing 10 μM [$\gamma\text{-}^{32}\text{P}$]ATP (800 c.p.m./pmol) and 20 mM $\text{Mg}(\text{NO}_3)_2$ in the absence or presence of PKC activators; after 20 min, the reaction mixture was removed, and the nitrocellulose was washed, dried and exposed for autoradiography.

Phosphorylation studies

For these, 100 μg of sample protein (detergent-insoluble extracts) was phosphorylated with partially purified PKC in phosphorylation assay buffer (250 μl) containing 20 mM Tris/HCl (pH 7.5), 5 mM $\text{Mg}(\text{NO}_3)_2$, 10 mM 2-mercaptoethanol, 10 μM [$\gamma\text{-}^{32}\text{P}$]ATP (800 c.p.m./pmol), 5 μg of phosphatidylserine, 0.2 μg of 1,2-diolein and 0.5 mM CaCl_2 . The reaction was terminated after 30 min at 30 °C by addition of 25% trichloroacetic acid (1 ml) plus 100 μg of BSA. After centrifugation, protein precipitates were solubilized, diluted with electrophoresis sample buffer and analysed by SDS/PAGE (10% gels). Gels were dried and exposed to X-ray film for 2–4 days.

Effect of peptide I on PKC activation

PKC isoforms from rat hepatocytes were partially purified by DEAE-cellulose column chromatography and assayed *in vitro* by measuring the incorporation of ^{32}P from [$\gamma\text{-}^{32}\text{P}$]ATP into H1-HIIS histone in the presence of 10 μM CaCl_2 , 20 $\mu\text{g}/\text{ml}$ phosphatidylserine, 0.8 $\mu\text{g}/\text{ml}$ 1,2-diolein, 10 μM PMA [17] and the indicated concentrations of peptide I. PKC activity is expressed as pmol/mg wet wt. of hepatocytes in 4 min.

Production of antibodies against peptide I

Peptide I was conjugated to BSA, and New Zealand rabbits were immunized by using the protocol described by Goldsmith et al. [18]. After the initial inoculation, animals were boosted five more times at 4-week intervals before antisera were obtained.

RESULTS

Previously we reported that in rat hepatocytes two main groups of PKC forms are detected by DEAE-cellulose chromatography: PKC 1 and PKC 2 [17]. Hydroxyapatite column chromatography further resolved PKC 1 into three distinct peaks, 1a, 1b, 1c, and PKC 2 into at least four peaks of activity, 2a, 2b, 2c and 2d [17]. Western-blot analysis showed that both PKC 1 and 2 fractions were recognized by polyclonal antibody against all PKC forms (results not shown), whereas monoclonal anti-PKC-isoenzyme-specific antibodies identified peak 1a as PKC- β and peak 1b as PKC- α [17]. The other peaks of activity are as yet unidentified, but there is evidence that they may be composed of novel Ca^{2+} -independent PKC forms [19].

Figure 1 Identification of hepatocyte RACKs

Triton-X-100-soluble (lanes c and d) or -insoluble (lanes a and b) fractions from rat hepatocyte homogenates were subjected to SDS/PAGE (10% gels) and blotted on to nitrocellulose. PKC binding was determined by incubating the blots with PKC in the presence (right panels) or absence (left panels) of phosphatidylserine, 1,2-diolein and Ca^{2+} . (a) Immunological detection: polypeptides that bound PKC were identified by using monoclonal antibodies to the PKC- α isoenzyme and alkaline phosphatase-conjugated second antibody. (b) Detection by PKC-dependent phosphorylation: PKC binding to proteins were identified by incubating PKC in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP and 20 mM MgCl_2 , followed by autoradiography. The amount of protein loaded was 50 μg for lanes a and c, and 100 μg for lanes b and d, in each case. The results shown on both panels were obtained from the same cell preparation and by running the assays in parallel, and are representative of three independent experiments.

Figure 2 Phosphorylation of proteins by PKC (a) and effect of MgATP on PKC binding to RACKs (b)

(a) Samples (100 μ g of protein) from Triton-insoluble rat hepatocyte extracts were phosphorylated with (lane 2) or without (lane 1) PKC in the presence of 10 μ M [γ - 32 P]ATP (800 c.p.m./pmol), phosphatidylserine, dioleoin and CaCl_2 as described in the text. The reaction was terminated after 30 min at 30 $^\circ\text{C}$ by addition of 25% trichloroacetic acid plus 100 μ g of BSA. Protein precipitates were solubilized, diluted with electrophoresis sample buffer and subjected to SDS/PAGE (10% gels). The gel was dried and exposed for autoradiography. (b) Protein (100 μ g) from the Triton-insoluble fraction of hepatocyte homogenates was subjected to SDS/PAGE (10% gel), blotted on to nitrocellulose and incubated with PKC in the absence (lane 1) or presence (lane 2) of 5 mM $\text{Mg}(\text{NO}_3)_2$, 10 μ M ATP and PKC activators as described, by using the overlay assay. PKC bound was detected with monoclonal antibody against PKC- α and developed with alkaline-phosphatase-conjugate second antibody. Results shown are representative of three independent experiments.

Figure 3 Effect of lipids and Ca^{2+} on PKC binding to RACKs

Protein (100 μ g) from the Triton-insoluble fraction of hepatocyte homogenates was subjected to SDS/PAGE (10% gels), blotted and incubated with PKC 1 in the absence or presence of the agents indicated, by using the overlay assay. The positions of M_r standards are indicated. The results are representative of four independent experiments. Abbreviation: PL, phosphatidylserine plus dioleoin.

Figure 4 Lack of specificity of RACKs with respect to PKC- α and - β isoforms

Protein (100 μ g) from detergent-insoluble (lanes a) or soluble (lanes b) fractions from rat hepatocyte homogenates were subjected to SDS/PAGE (10% gels), blotted on to nitrocellulose and incubated with PKC 1 in the presence of lipids and Ca^{2+} . Polypeptides that bound PKC were detected by using isoenzyme-specific monoclonal antibodies against PKC- α , PKC- β or both, as indicated in the Figure, and developed with alkaline-phosphatase-conjugated second antibody. Results shown are representative of six independent experiments.

In order to search for cellular polypeptides which may bind PKC in these cells, soluble and insoluble fractions of Triton X-100 homogenates were separated by SDS/PAGE, transferred to nitrocellulose and overlaid with the PKC I group of isoenzymes. As shown in Figure 1(a) (right panel, lanes a and b), several (6–7) immunoreactive bands were detected in the Triton-insoluble fraction of rat hepatocyte homogenates by using an anti-PKC- α antibody. No immunoreactive bands were detected in the Triton-soluble fraction. The PKC receptors were observed in two zones, one in the $M_r \approx 28000$ – 36000 region and another in the $M_r \approx 12000$ – 14000 region (Figure 1). The most abundant bands were those with apparent M_r values of ≈ 14000 and ≈ 31000 . An identical pattern was observed when the PKC-dependent phosphorylation reaction was employed to make visible the receptor proteins in experiments running in parallel (Figure 1b). It is clear that binding of PKC to the polypeptides occurred only when the enzyme was in its activated form, since no bands were detected in the absence of phospholipid and Ca^{2+} (Figures 1a and 1b, left panels). These data indicated that in hepatocytes at least six proteins behave like RACKs, i.e. receptors for activated C-kinase.

On the other hand, the phosphorylation reaction employed to detect bound PKC suggested that RACKs may be substrates for PKC. In order to examine this possibility, detergent-insoluble proteins from rat hepatocyte extracts were incubated *in vitro* with partially purified PKC and [γ - 32 P]ATP as described in the Experimental section. Analysis of proteins by SDS/PAGE (10% gels) followed by autoradiography (Figure 2a, lane 2) showed that several Triton-insoluble proteins are PKC substrates, including bands whose M_r corresponded to those of the RACKs previously detected by the PKC-phosphorylation detection method. We next examined the effect of MgATP on the binding of PKC to electroblotted proteins. As shown in Figure 2b (lane 2), inclusion of MgATP in the overlay assay mixture diminished

Figure 5 Specificity of RACKs with respect to PKC 2 isoforms

Polypeptides (100 μ g) from Triton-insoluble fraction from cell extracts were subjected to SDS/PAGE (10% gels), blotted and incubated with PKC 1 (lane 1) or PKC 2 (lane 2) in the presence of Ca^{2+} and lipids. Proteins that bound PKC were detected with monoclonal anti-PKC- α antibodies for lane 1 or with polyclonal anti-PKC (all forms) antibody for lane 2. Both blots were developed with alkaline-phosphatase-conjugated second antibody. Results shown are representative of four independent experiments.

PKC binding to RACKs, suggesting that phosphorylation may modulate the interaction between these proteins.

To understand better the factors that might regulate the binding of PKC to RACKs, PKC 1 was incubated with polypeptides from the Triton-X-100-insoluble fraction in the absence or presence of lipids (phosphatidylserine plus 1,2-diolein), Ca^{2+} or EGTA, by using the overlay assay. Results in Figure 3 showed that the binding of PKC to the $M_r \approx 28000$ – 36000 proteins appeared to be Ca^{2+} -dependent and that phosphatidylserine plus diolein was essential for the binding of PKC to all RACKs. Neither Ca^{2+} alone nor EGTA induced PKC binding to these proteins. The most effective binding of PKC to RACKs was obtained in the presence of both lipids and Ca^{2+} .

The specificity of RACKs to bind PKC isoforms was next explored, by again using the overlay assay. It is important to emphasize that PKC 1 contains PKC- α , PKC- β and another uncharacterized form, whereas PKC 2 is composed of four yet unidentified peaks of PKC activity [17]. We made use of a polyclonal antibody (not subtype-specific) to detect PKC 2 isoforms and isoenzyme-specific monoclonal antibodies to detect PKC 1 isoforms. Figure 4 shows the results obtained with the PKC 1 group. Both PKC- α and PKC- β isoenzymes bound to the previously identified polypeptides of the Triton-X-100-insoluble fraction of cell extracts (lanes a). In contrast, the PKC 2 isoforms (Figure 5, lane 2) bind mainly to the polypeptides of M_r 12000–14000 even in the presence of both Ca^{2+} and phospholipid. However, such selective binding properties of

Figure 6 Effect of peptide I and Calphostin-C on PKC binding to RACKs (a), in comparison with the effect of peptide I on PMA-stimulated PKC activity (b)

(a) SDS/PAGE (10% gel) blots of Triton-insoluble fraction (100 μ g each lane) were overlaid with PKC in the presence of its activators and in the absence or presence of peptide I (170 μ M) or Calphostin-C (15 μ M). Peptides that bound PKC were detected with anti-PKC- α monoclonal antibody and with alkaline-phosphatase-conjugated second antibody. Abbreviation: PL, phosphatidylserine plus diolein. (b) PKC was partially purified by DEAE-cellulose chromatography and assayed *in vitro* in the presence of 10 μ M CaCl_2 , 20 μ g/ml phosphatidylserine, 0.8 μ g/ml 1,2-diolein, 10 μ M PMA and the indicated concentrations of peptide I. Controls were assayed under the same conditions, except that no phorbol ester was added (■); ○, with 1 μ M PMA; ■, activity in the absence of PMA. Values plotted are means $\pm$ S.E.M. of four experiments using different cell preparations.

PKC 2 can only be evident if PKC 1 and PKC 2 are overlaid under identical conditions, i.e. the same amount of RACKs exposed to the same amount of PKC for 1 h. Selectivity is lost when double the amount of RACKs is overexposed (2 h) with an excess (3-fold usual amount; 1.5 mg/ml final concn.) of PKC 2 (results not shown). These results indicate that, although PKC 2 isoforms can bind all RACKs, they have lower affinity for them in comparison with PKC 1, particularly to M_r 28000–36000 proteins. Taken together, the data suggest that RACKs may not

Figure 7 Immunological studies with polyclonal antisera against peptide I

(a) Identification of proteins that bind antisera against peptide I. Triton X-100-soluble (lanes B) or -insoluble (lanes A) fractions from rat hepatocyte homogenates were separated by SDS/PAGE (10% gels) and transferred to nitrocellulose membranes. Immunoblot analysis was done with pre-immune (lanes 1A and 1B) or immune (lanes 2A and 2B) sera and developed with anti-(rabbit IgG)-alkaline-phosphatase-conjugated second antibody. (b) Effect of antisera against peptide I on PKC binding to RACKs. Blots of detergent-insoluble fractions from rat hepatocytes (100 μ g each lane) were preincubated for 2 h with pre-immune serum (lane 1) or with immune serum (lane 2). Blots were then washed to remove unbound material, and PKC binding to RACKs was determined in the presence of its activators by using the overlay-assay method described. Results shown are representative of four independent experiments.

be absolutely isoenzyme-specific, but may have some binding selectivity for groups of PKC isoforms.

The sequence KGDYEKILVALCGGN (peptide I) is similar to that of a potent PKC inhibitor [20,21]. We consider the possibility that peptide I could inhibit PKC activity and that by doing so it may block PKC binding to RACKs. Using the overlay assay, we incubated the PKC 1 in the presence of its activators and in the absence or presence of the PKC inhibitor Calphostin-C (15 μ M) or of peptide I [at a concentration (170 μ M) corresponding to 100-fold the K_i reported for the PKC inhibitor from which it derives [20]]. The results in Figure 6(a) indicated that both peptide I and Calphostin-C inhibited PKC binding to RACKs. We also examined the effect of peptide I on PMA-stimulated PKC activity *in vitro*. Figure 6(b) shows that the effect of the active phorbol ester was dose-dependently blocked by peptide I, with an IC_{50} of 17 μ M.

We further studied the possibility that peptide I can be part of the PKC-binding site on RACKs by producing antibodies against this sequence and examining their ability to block PKC binding to them. Selective recognition of RACKs by antisera against peptide I was first examined by Western-blot analysis. Figure 7(a) shows that the immune sera (panel a2) bound to proteins from detergent-insoluble proteins (panel a2, lane A) corresponding in M_r to RACKs. Preincubation of blotted RACKs with antisera to peptide I blocked almost completely subsequent PKC binding to all RACKs, as shown in Figure 7(b) (lane 2). Pre-immune sera were without effect (Figure 7b, lane 1).

DISCUSSION

Translocation of PKC from cytosol to membranes has become a widely recognized index of PKC activation. Important questions remain concerning the biological significance and the molecular

mechanism(s) involved in the membrane-enzyme association. In this study, we provide evidence that in rat hepatocytes PKC can bind to intracellular proteins (RACKs) upon activation. These polypeptides were localized only in the detergent-insoluble material of the cells, supporting the notion suggested by some authors [10–14] that cytoskeletal elements may participate in the PKC activation process. The same RACKs were detected using two different methods, indicating that they are not artifacts of a detection technique. Some authors [10,14,22,23] have used an overlay-assay approach to identify PKC-binding proteins, some of which have been found to be PKC substrates. Here we found that in rat hepatocytes several proteins that behave like RACKs are also PKC substrates. Interestingly, MgATP was able to produce a partial blockade of PKC binding to these RACKs (see Figure 2b), suggesting that interaction between RACKs and PKC may be modulated by phosphorylation. Further experiments need to be performed to address this possibility.

The fact that PKC binds RACKs in its activated state suggests that only in the active conformation does PKC expose the region that interacts properly with its receptors. On the other hand, RACKs do not seem to trigger activation of PKC, and the physiological roles they play in whole cells remain to be clarified.

It is noteworthy that the PKC-binding domain of RACKs survives SDS/PAGE, electroblotting and repetitive washes, suggesting that activated PKC recognizes a specific sequence rather than a tertiary structure on RACKs and further indicating that binding of PKC to them is not easily reversible. It is also remarkable that the apparent M_r values of two of the RACKs found in rat hepatocytes are very similar to those found in neonatal-rat heart (30000–33000) [18].

In the present study we compared the binding abilities of two main groups of PKC isoforms. As mentioned previously, PKC 1 is composed of PKC- α , PKC- β and another as yet unidentified form [17]. PKC 2 is not yet well characterized, but several lines of evidence indicate that it may contain some of the unconventional Ca^{2+} -independent subfamily members of PKC [17,19,24,25]. A salient finding was obtained here with respect to the specificity of RACKs and the factors needed for PKC binding to them. We found that lipids (phosphatidylserine plus dioleoin) are essential for binding to all RACKs, but Ca^{2+} is not. The Ca^{2+} -dependence or -independence exhibited by the $M_r \approx 28000$ –36000 or the $M_r \approx 12000$ –14000 RACKs, respectively, may reflect the way PKC isoforms are regulated. Whereas $M_r \approx 28000$ –36000 RACKs showed Ca^{2+} -dependence and bound PKC 1 (PKC- α and PKC- β), under similar overlay-assay conditions they did not bind PKC 2, which presumably contains Ca^{2+} -independent PKC isoforms. This is also consistent with the results obtained regarding the binding capacities of the RACKs of $M_r \approx 12000$ –14000, which bound PKC in the absence of Ca^{2+} and were the main forms which bound PKC 2 isoenzymes. An attractive possibility, therefore, is that RACKs may be subfamily-specific instead of isoenzyme-specific for PKC.

The 'peptide I' sequence has been proposed to resemble the PKC-binding site on RACKs on the basis that it can bind PKC and blocks PKC binding to RACKs [15]. Our data using the antiserum against peptide I were consistent with this suggestion; we also obtained evidence that peptide I blocked PKC binding to RACKs indirectly by inhibiting PKC activation.

It should be kept in mind that the property of PKC to associate with membranes containing acidic phospholipids in a Ca^{2+} -dependent manner is shared by other diverse groups of proteins [26–30]. It remains to be explored whether proteins that can be translocated possess sequences similar to PKC that enable them to interact with proteins containing peptide I-like sequences. Further investigations are required to understand the

molecular basis of translocation and the role(s) it plays in the activation and/or regulation of PKC actions.

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