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# A protein kinase C isozyme is translocated to cytoskeletal elements on activation

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**Protein kinase C (PKC)[1] isozymes comprise a family of related cytosolic kinases that translocate to the cell particulate fraction on stimulation. The activated enzyme is thought to be on the plasma membrane. However, phosphorylation of protein substrates occurs throughout the cell and is inconsistent with plasma membrane localization. Using an isozyme-specific monoclonal antibody we found that, on activation, this PKC isozyme translocates to myofibrils in cardiac myocytes and to microfilaments in fibroblasts. Translocation of this activated PKC isozyme to cytoskeletal elements may explain some of the effects of PKC on cell contractility and morphology. In addition, differences in the translocation site of individual isozymes—and, therefore, phosphorylation of different substrates localized at these sites—may explain the diverse biological effects of PKC.**

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[1] Abbreviations used in this paper: cAMP, cyclic adenosine monophosphate; DEAE, 2-diethylaminoethyl; DG, diacylglycerol; DMSO, dimethylsulfoxide; EGTA, ethylene glycol-bis(β-aminomethyl ether)-*N,N,N′,N′*-tetraacetic acid; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; mAb, monoclonal antibody; MEM, minimum essential medium; NE, norepinepherine; PBS, phosphate-buffered saline; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKM, catalytic fragment of PKC; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; Tris-HCl, tris(hy-droxymethyl)aminomethane.

## Introduction

Protein kinase C (PKC) isozymes are a family of calcium- and lipid-dependent protein kinases (Nishizuka, 1988). Binding of agonists to surface receptors coupled to phospholipase C generates diacylglycerol (DG), an activator of PKC (Nishizuka, 1984). Tumor-promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), also activate PKC by binding to the DG site on the regulatory domain of the enzyme (Nishizuka, 1984). PKC is involved in a variety of functions, including changes in cell morphology (Schliwa *et al.,* 1984; Keller *et al.,* 1985), secretion (Peppers and Holz, 1986), cell contractility (Scholz *et al.,* 1988), and regulation of other signal transduction systems (Sugden *et al.,* 1985). In addition to changes in cell physiology and morphology, cell growth (Huang and Ives, 1987) and gene expression (Greenberg and Ziff, 1984; Starksen *et al.,* 1986) are also altered on PKC activation. The protein substrates of PKC that may be responsible for these diverse effects are located throughout the cell, i.e., on the plasma membrane (Hunter *et al.,* 1984), in the cytosol (Ahmad *et al.,* 1984), on the cytoskeleton (Nishikawa *et al.,* 1983), and in the nucleus (Patskan and Baxter, 1985).

Cell fractionation experiments have demonstrated that activation of PKC results in translocation of PKC activity from the cytosol to the particulate fraction (Kraft and Anderson, 1983). Because PKC activity is dependent on lipids (i.e., DG and phosphatidylserine [PS]), translocation to the particulate fraction has been interpreted to reflect PKC binding to plasma membrane lipids (e.g., Bell, 1986). Furthermore, immunofluorescence localization experiments have shown that PKC appears on the plasma membrane (Shoji *et al.,* 1986). However, if the activated enzyme is restricted to the plasma membrane, it is not clear how activation of PKC results in phosphorylation of proteins throughout the cell. Because there are several PKC isozymes (Nishizuka, 1988), and more than one PKC isozyme per cell (e.g., certain glial cells and neurons in the CNS [Huang *et al.,* 1987; Mochly-Rosen *et al.,* 1987]), it is possible that not all of the ac-

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tivated PKC isozymes within a cell are restricted to the plasma membrane. Some may translocate to the intracellular sites of their protein substrates.

To address this question, we determined activation-dependent translocation of a PKC isozyme in rat cardiac myocytes, using an anti-PKC monoclonal antibody (mAb) CK 1.4, in an immunofluorescence study. CK 1.4 is an isozyme-specific mAb (Mochly-Rosen *et al.,* 1987); however, the identity of this isozyme has not yet been determined.[2] Primary cardiac myocytes are a useful cell system for study because PKC is suggested to be involved in a variety of functions, including modulating cell morphology (Claycomb and Moses, 1988) and contractility (Leatherman *et al.,* 1987; Yuan *et al.,* 1987; Allen *et al.,* 1988), as well as in the development of hypertrophy (Simpson, 1985; Starksen *et al.,* 1986; Henrich and Simpson, 1988). In addition, some of the PKC substrates in these cells are on the plasma membrane (Limas and Limas, 1985; Yuan and Sen, 1986) and others are on myofibrils (Katoh *et al.,* 1983; Nishikawa *et al.,* 1983; Mazzei and Kuo, 1984; Lim *et al.,* 1985; Ohta *et al.,* 1987), intracellular structures that are easily identified. The present study demonstrates activation-dependent association of a PKC isozyme with myofibrils. Because another PKC isozyme was found to translocate to the plasma membrane in the same cell type, these results suggest that, on activation, different PKC isozymes are translocated to different intracellular sites, probably to the vicinity of their respective substrates.

## Results

### *CK 1.4 antibody is specific for PKC*

We used a PKC isozyme-specific mAb, CK 1.4 (Mochly-Rosen *et al.,* 1987), to identify the intracellular sites to which this isozyme is translocated on activation. This mAb has been previously described (Mochly-Rosen and Koshland, 1988). It was raised against highly purified PKC isolated from the combined cytosolic and particulate fractions of rat brain and was selected for its ability to inhibit the activity of PKC (Mochly-Rosen and Koshland, 1988). CK 1.4 inhibits the activity of intact PKC, but does not inhibit the activity of PKM (the catalytic fragment of PKC [Mochly-Rosen and Koshland, 1987]) or other protein kinases, including cAMP-dependent protein kinase or calcium- and calmodulin-dependent protein kinase type II (Mochly-Rosen *et al.,* 1987). CK 1.4 appears to recognize one PKC isozyme (Mochly-Rosen *et al.,* 1987), but its identity has not been determined.[2]

Using partially purified PKC from primary cardiac myocytes cells in culture, we further demonstrated the specificity of CK 1.4 antibody for PKC by Western blot analysis (Figure 1A). The chromatographic profile on 2-diethylaminoethyl (DEAE) cellulose of the CK 1.4-, $\alpha$-, and $\beta$-PKC isozymes were similar; and fractions devoid of PKC kinase activity had no CK 1.4 immunoreactivity (data not shown). The $\gamma$ isozyme did not appear to be present in primary cardiac myocytes (Figure 1A). We found that CK 1.4 recognized a 70-kDa polypeptide in a PKC

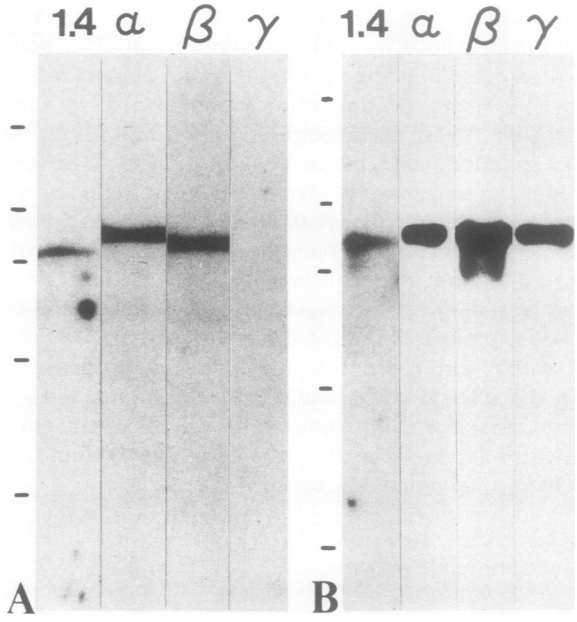


***Figure 1.* Western blot analysis with isozyme-specific monoclonal antibodies.** PKC from primary cardiac myocytes was partially purified by DEAE cellulose chromatography (see Methods). (A) The fraction containing PKC activity was analyzed for immunoreactivity using CK 1.4, anti-$\alpha$, anti-$\beta$, and anti-$\gamma$ PKC antibodies. (B) For comparison, PKC purified from rat brain soluble and particulate fractions was also analyzed by Western blot. Bars on the left indicate molecular mass standards of 200, 92, 69, 45, and 30 kDa from top to bottom.

[2] In a preliminary study, PKC cDNAs for the $\alpha$-, $\beta$-, and $\gamma$-isozymes have been transiently expressed in RAT-1 cells. No change in CK 1.4 binding was found in any of the transfected cells, suggesting that CK 1.4 does not recognize the $\alpha$-, $\beta$-, or $\gamma$-isozymes of PKC. In contrast, increased binding of CK 1.3 was observed in cells expressing only the $\beta$-PKC cDNA (Mochly-Rosen, D., and Rosenthal, A., unpublished results). These observations suggest that CK 1.3 may be specific for the $\beta$-isozyme and that CK 1.4 may be specific for one of the other PKC isozymes that have been recently identified (Ono *et al.,* 1988).

preparation purified from cardiac myocytes (Figure 1A). In PKC prepared from brain particulate and soluble fractions (Mochly-Rosen and Koshland, 1987), a 78-kDa band was the major immunoreactive band recognized by CK 1.4 (Figure 1B). Additional CK 1.4 immunoreactive polypeptides of higher molecular mass were occasionally observed in PKC preparations from brain or whole heart (data not shown). The different polypeptides recognized by CK 1.4 may be generated by posttranslational modification. Similar multiple PKC immunoreactive polypeptides were also found with the anti-$\beta$ isozyme mAb (Figure 1B). Variation in electrophoretic mobility of a PKC isozyme in different organs has been reported previously (e.g., Kosaka *et al.*, 1988).

When cardiac myocytes were incubated with CK 1.4 antibody, diffuse cytosolic staining was observed (Figure 2A). No immunostaining was observed with control hybridoma media (Figure 2B). To further demonstrate the specificity of CK 1.4 for PKC in these cells, the antibody was preadsorbed with different proteins immobilized on nitrocellulose before immunostaining of the cultured cardiac cells. Preadsorption with PKC (a mixture of PKC isozymes) abolished CK 1.4 staining (Figure 2D), whereas preadsorption with cAMP-dependent protein kinase (Figure 2C) or with normal goat serum (Figure 2A) did not. CK 1.4 stained 94 ± 3% and 82 ± 7% of the cells after preadsorption with 0.5 U of cAMP-dependent protein kinase (bovine brain) or with normal goat serum, respectively (mean ± range, n = 2). In contrast, CK 1.4 stained only 2.6 ± 4% of the cells after preadsorption with 0.05 U of PKC (1 U = 1 nmol · $min^{-1}$ · $mg^{-1}$ protein; Mochly-Rosen *et al.*, 1987). Taken together, these data strongly suggest that CK 1.4 specifically recognizes a PKC isozyme in rat cardiac myocytes.

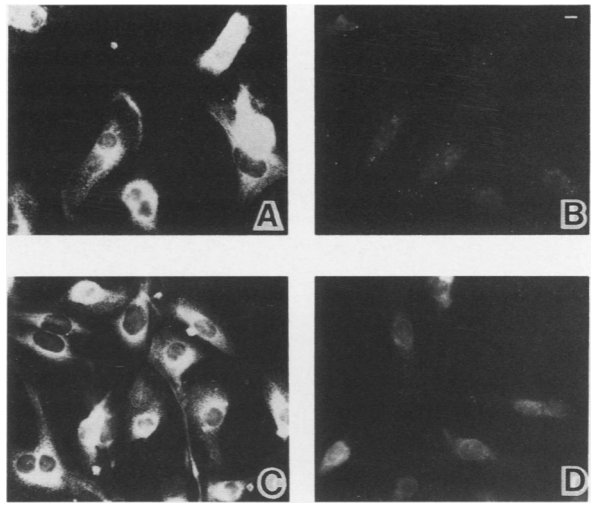


***Figure 2.*** **CK 1.4 immunoreactivity is specific for PKC.** CK 1.4 hybridoma medium was adsorbed in solid phase on (A) normal goat serum, (C) cAMP-dependent protein kinase, or (D) PKC before immunostaining of the cultured cardiac cells. (B) Background staining with control hybridoma medium. Antibody binding was detected with fluorescein isothiocyanate (FITC)-conjugated second antibody. CK 1.4 immunoreactivity is adsorbed by PKC (D), but not by cAMP-dependent protein kinase (C) or by normal goat serum (A). Bar equals 10 $\mu$m.

### *On activation, CK 1.4 isozyme is translocated to myofibrils in cardiac myocytes*

PKC was activated in the cardiac myocytes with 2 $\mu$M norepinephrine (NE), and the intracellular localization of PKC before and after activation was determined by immunofluoresence. Immunostaining of control cardiac myocytes with CK 1.4 was diffusely cytosolic and perinuclear (Figure 3A). After a 1-min incubation with NE, PKC immunoreactivity was localized on myofibrils (Figure 3, C and E).

To confirm the myofibrillar localization of CK 1.4 isozyme, the fixed cells were incubated with rhodamine-phalloidin, a toxin that binds polymerized actin (Schliwa *et al.*, 1984). The phalloidin-stained cells showed the same cross-striated pattern as CK 1.4 immunostaining (Figure 3, D and F). CK 1.4 staining of myofibrils after adrenergic stimulation (Figure 3C) reflected binding to preexisting myofibrils, rather than reorganization of these structures during the brief NE stimulation, because phalloidin staining of control cells also showed a cross-striated pattern (Figure 3B). At higher magnification (Figure 3, E and F) and with double-exposure micrography (data not shown), the striated staining patterns observed with phalloidin and CK 1.4 appeared to be on adjacent bands, rather than superimposed. In addition, cross-striated staining of myofibrils on activation was also observed in cells treated with 0.05% Triton before cell fixation (data not shown). This treatment is thought to remove all cellular components except for the cytoskeletal elements (Ben-Ze'ev *et al.*, 1979). These data further support CK 1.4 association with the cytoskeleton. Anti-myosin antibodies also showed the same cross-striated pattern (data not shown) and after 1 h incubation with cytochalasin B, which disrupts myofibril structure, the cross-striated pattern of staining with CK 1.4 was lost (data not shown). All these data sup-

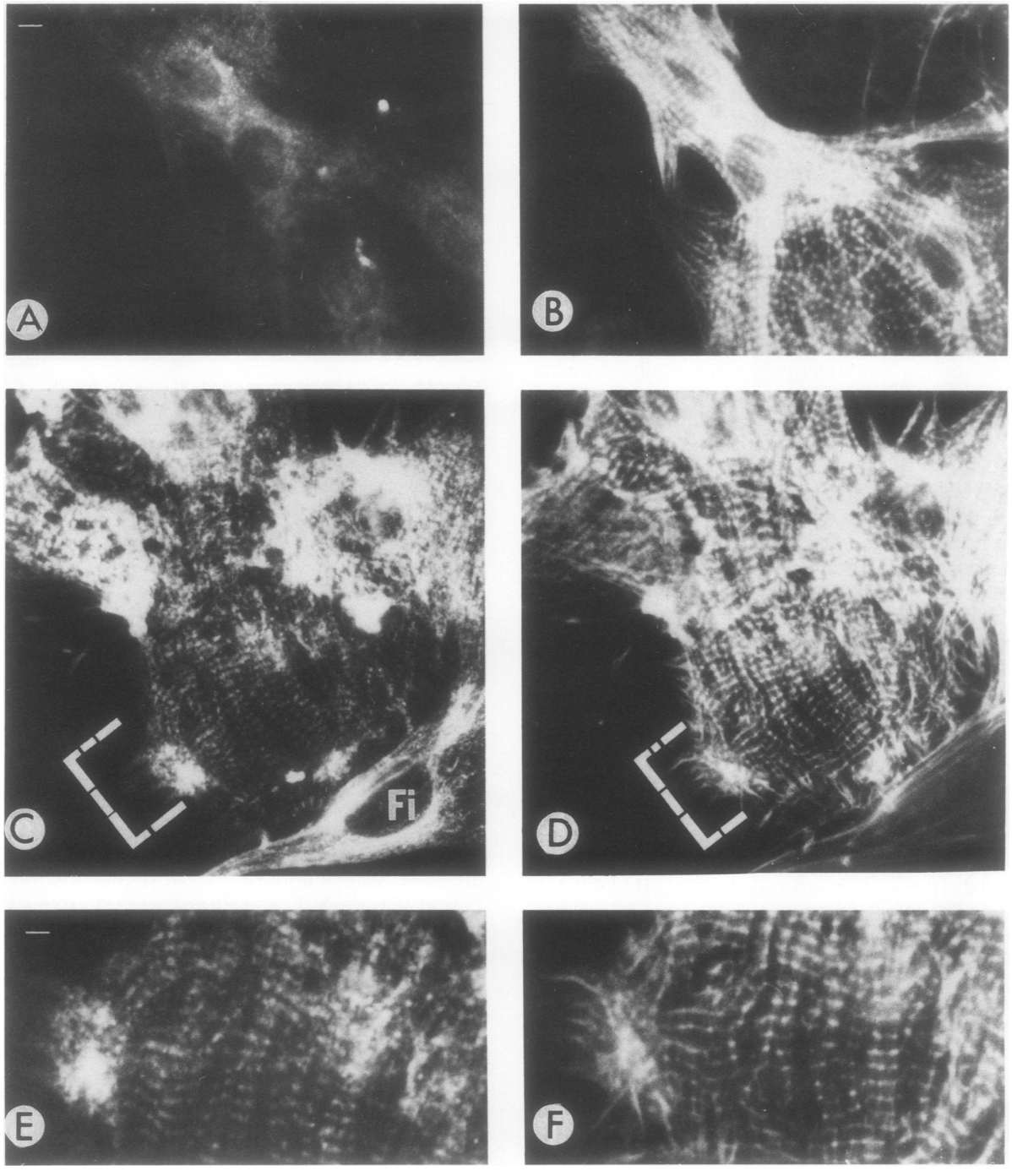


***Figure 3.*** **Translocation of CK 1.4 isozyme to myofibrils in cultured cardiac myocytes by treatments with NE.** Shown are pairs of identical microscopic fields with double-label fluorescence staining. Left (A, C, and E): PKC immunoreactivity was visualized with antibody CK 1.4 and FITC-conjugated second antibody. Right (B, D, and F): myofibrils were stained with rhodamine-conjugated phalloidin. Cells were treated with vehicle (A and B) or with 2 $\mu$M NE (C–F) for 1 min. PKC immunoreactivity is diffusely cytosolic and perinuclear in controls (A), but is present in a myofibrillar pattern after incubation with NE (C and E). Myofibrils are present in all myocytes regardless of treatment (B, D, and F). "Fi" in panel C identifies a cardiac fibroblast in which CK 1.4 isozyme was not activated (the immunostaining is cytosolic and perinuclear). Bar equals 5 $\mu$m, except for E and F, which are twofold magnifications of the bracketed area in C and D, respectively.

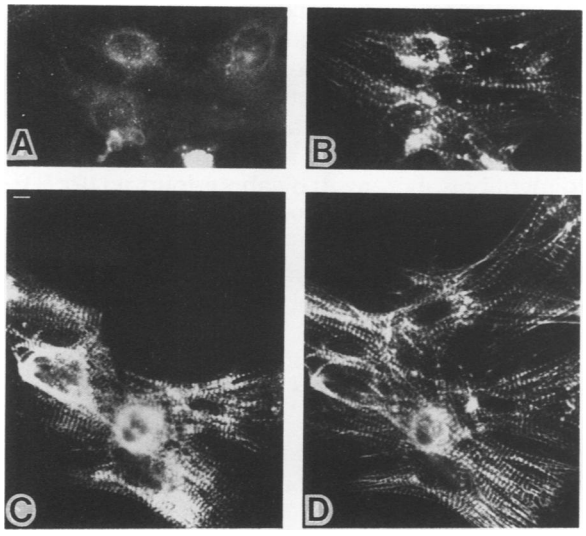


***Figure 4.*** **Translocation of the CK 1.4 isozyme to myofibrils in cultured cardiac myocytes after incubation with PMA.** Shown are pairs of identical microscopic fields with double-label fluorescence staining. Left (A and C): PKC immunoreactivity was visualized with antibody CK 1.4 and FITC-conjugated second antibody. Right (B and D): myofibrils were stained with rhodamine-conjugated phalloidin. Cells were treated with vehicle (A and B), or with 100 nM PMA (C and D) for 5 min. PKC immunoreactivity is diffusely cytosolic in controls (A), but is present in a myofibrillar pattern after PMA treatment (C). Note that a cross-striated staining with CK 1.4 is not observed in all the myocytes (C vs. D). Bar equals 5 $\mu$m.

port our conclusion that CK 1.4 isozyme is localized on myofibrils.

When cardiac myocytes were incubated with 100 nM PMA and immunostained with CK 1.4, the same cross-striated pattern was observed as with NE activation (Figure 4C). The vehicle, dimethylsulfoxide (DMSO), at 0.01% had no effect on CK 1.4 staining (Figure 4A). Although treatment with PMA disrupts myofibrils in cultured skeletal muscle cells (Croop *et al.,* 1980), no change in myofibrillar organization was seen in the cardiac myocytes after the brief PMA incubation used in this study.

The intensity of immunostaining of the CK 1.4 isozyme increased on PKC activation (Figures 3, A and C, and 4, A and C). This increase may reflect an activation-induced conformational change in the CK 1.4 isozyme resulting in an increased affinity for the CK 1.4 antibody. Alternatively, the change in intensity may be only apparent, reflecting the concentration of the isozyme from diffused cytosolic into a small area.

In many cell systems, stimulation leading to activation of PKC appears to produce an immediate and transient response (i.e., Henrich and Simpson, 1988; Messing *et al.,* 1989). Similarly, an immediate and transient translocation of CK 1.4 immunoreactivity in response to PKC activation should be observed. Indeed, the number of myocytes in which myofibrils were stained with CK 1.4 increased after a 15-s incubation with NE (the shortest time point studied), reached a maximum after 1 min, and returned to control levels by 1 h (Figure 5A).

NE activates $\alpha_1$- and $\beta$-adrenergic receptors in cardiac myocytes (Simpson, 1985). However, only the $\alpha_1$ receptors are coupled to phosphatidylinositol bisphosphate hydrolysis and PKC activation (Brown *et al.,* 1985; Abdel-Latif,

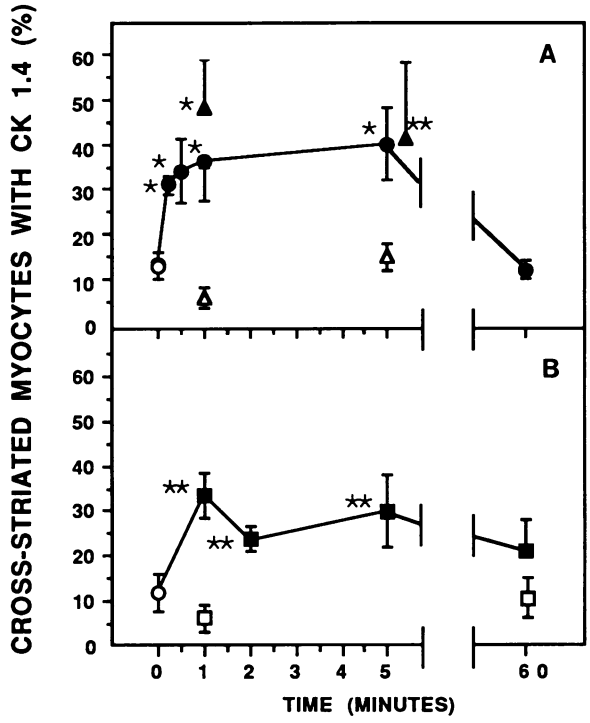


***Figure 5.*** **Activation of PKC increases the number of cardiac myocytes with CK 1.4 isozyme on myofibrils.** (A) Cells were treated for the indicated times with NE. Treatment with NE (2 $\mu$M) was carried out in the absence (●) or presence of 2 $\mu$M propranolol (▲), a $\beta$-adrenergic antagonist, or 2 $\mu$M terazosin (△), an $\alpha_1$-adrenergic antagonist. (B) Cells were also treated with 100 nM PMA (■), 4-$\alpha$PMA (□) or vehicles (○). The percentage of cardiac myocytes having CK 1.4 staining in a striated pattern was determined as described in Methods. A mean of 150 myocytes in randomly selected microscopic fields were counted in each experiment with a total of 90–1069 (mean = 479) myocytes counted per data point. Values are the mean ± SE for three to six experiments, each carried out on a different culture preparation. The values for the vehicles (ascorbic acid and dimethylsulfoxide) were the same as no addition, and all three groups are averaged in the figure. *$p < 0.01$ vs. control.; **$p < 0.05$ vs. control.

1986), and activation of $\alpha_1$- but not $\beta$-receptors resulted in translocation of PKC enzymatic activity from the cytosolic to the particulate fraction (Henrich and Simpson, 1988 and data not shown). Similarly, we found that only stimulation of the $\alpha_1$ receptors resulted in CK 1.4 immunostaining of myofibrils. Terazosin, an $\alpha_1$-adrenergic antagonist, inhibited the NE-induced CK 1.4 staining of myofibrils, whereas propranolol, a $\beta$-adrenergic antagonist, had no effect (Figure 5A).

We also observed an increase in the number of myocytes stained in a cross-striated pattern with CK 1.4 after 1–5 min treatment with PMA (Figure 5B). The PMA effect on the localization of the CK 1.4 isozyme also appeared to be transient; after a 2-h incubation with PMA, the amount of myofibril-associated CK 1.4 isozyme was not significantly different from control levels (analyzed by Student's *t* test; Figure 5B). The biologically inactive phorbol ester, 4$\alpha$-PMA, did not cause redistribution of CK 1.4 isozyme (Figure 5B).

After stimulation with NE, ~50% of the myocytes exhibited a striated pattern with CK 1.4 (Figure 5A). The absence of cross-striated staining in some of the cardiac myocytes may be due to differences between myocytes in the amount of the CK 1.4 isozyme present or the time course of the translocation of the CK 1.4 isozyme. Alternatively, the myocytes may differ in the expression of the myofibrillar element(s) to which the CK 1.4 isozyme is bound. Such heterogeneity in the expression of myosin isoforms has been found in cardiac myocytes (Samuel *et al.*, 1983).

### *Exogenously added PKC binds myofibrils*

Because the time course and agonist dependence of the translocation of CK 1.4 immunoreactivity correlated with the translocation of the PKC activity (Figure 5; see also Henrich and Simpson, 1988; Messing *et al.*, 1989), it seems very unlikely that CK 1.4 antibody bound a non-PKC protein that shares an antigenic determinant with PKC. To further support this conclusion, we determined whether exogenously added PKC can bind directly to myofibrils in control cells. To this end, control cardiac myocytes were fixed and permeabilized and various proteins added. The unbound material was removed by extensive washing and the intracellular localization of the bound protein was determined with CK 1.4 antibody.

When permeabilized fixed cardiac myocytes were incubated with two rat brain PKC preparations (containing a mixture of PKC isozymes; see Methods), a cross-striated pattern was obtained, indistinguishable from that observed after NE or PMA activation. No other cellular structures were "decorated" (data not shown). The percentage of cells showing CK 1.4 binding to myofibrils increased by about fourfold (PKC1, PKC2; Figure 6A), to a value similar to that observed on activation of PKC in intact cells (see Figure 6A vs. 5A). This decoration of myofibrils with CK 1.4 did not occur with cAMP-dependent

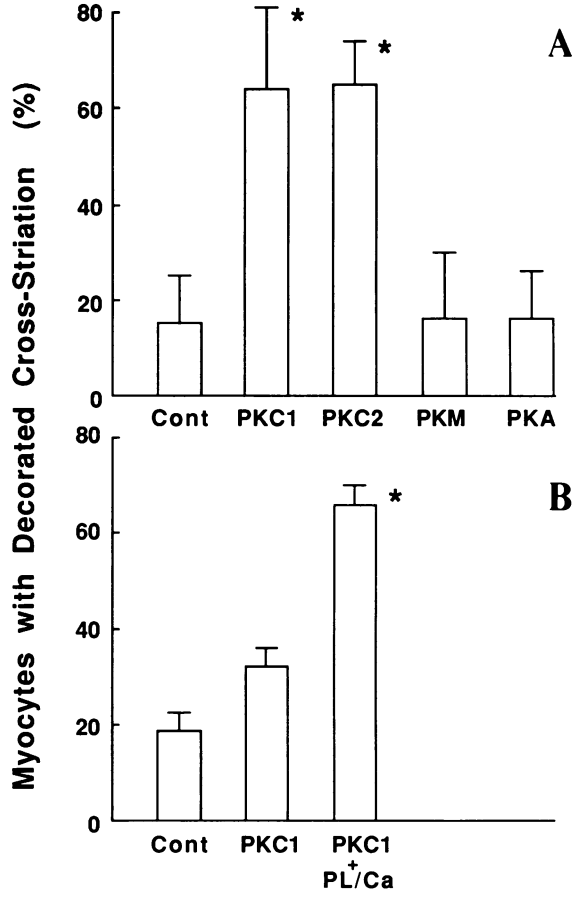


***Figure 6.*** **Exogenously added PKC binds to myofibrils.** Binding of different proteins to myofibrils of control, fixed, and permeabilized cardiac myocytes was visualized with CK 1.4 antibody. The percentage of cells showing cross-striated staining with CK 1.4 was determined as in Figure 5. (A) PKC-enriched preparations (0.068 and 0.05 U) decorate myofibrils of nonstimulated myocytes. PKM, PKA, or nonrelated protein mixtures do not alter the percentage of myocytes with cross-striated staining as determined by CK 1.4 antibody binding. Results are mean ± SE determined by counting three to seven randomly selected microscopic fields (34–79 cells) in two separate experiments. (B) Decoration of myofibrils by lower amounts of PKC (0.01 U) is dependent on PS, DG, and calcium. Results are mean ± SE determined by counting 10 randomly selected microscopic fields (104–124 cells). *$p < 0.001$ vs. control.

protein kinase (PKA; Figure 6A). In addition, limited trypsin digestion of PKC, which generates a catalytic fragment of PKC that is independent of phospholipid and calcium (Takai *et al.,* 1977; Mochly-Rosen and Koshland, 1987), did not decorate myofibrils (PKM; Figure 6A).

In intact myocytes, binding to myofibrils depended on the activation of PKC (Figures 3–5), whereas in fixed cells, binding of exogenous nonactivated enzyme occurred (Figure 6A). If the in vitro decoration mimics the activation-induced translocation in intact cells, binding of PKC should be dependent on the presence of PKC activators. It is probable that activation of PKC increases the affinity of the enzyme for myofibrils. This could explain the observation that nonactivated enzyme could also bind myofibrils providing that an excess of exogenous enzyme is applied. If this were the case, PKC activators should increase binding to myofibrils in the presence of limiting amounts of PKC. When decoration was carried out with 0.01 U of PKC, only 33 ± 4% of the myocytes showed cross-striation (Figure 6B) in comparison with 64 ± 14% obtained with 0.05–0.068 U of PKC (Figure 6A). When PS, DG, and calcium were co-incubated with the lower amount of PKC, 66 ± 4% of the myocytes were decorated with cross-striation, indicating that binding to myofibrils was dependent on PKC activators. These data strongly suggest that the protein translocated to the myofibrils on activation is a PKC isozyme.

### *On activation, another PKC isozyme is translocated to the plasma membrane of cardiac myocytes*

CK 1.4 did not stain the plasma membrane either in control cardiac myocytes or after treatment with NE or PMA. However, translocation of PKC immunoreactivity to the surface membrane could be detected in these cells. CK 1.3 is a monoclonal antibody that appears to be specific for a PKC isozyme other than the one to which CK 1.4 binds (Mochly-Rosen *et al.,* 1987). Preliminary results suggest that CK 1.3 is specific for the β-isozyme.[2] In nonstimulated cells, CK 1.3 immunostaining appeared to be cytosolic (Figure 7A). After a 5-min treatment with PMA, CK 1.3 immunoreactivity was translocated to the plasma membrane (Figure 7B, arrows). An increase in perinuclear staining by CK 1.3 after PMA treatment was also observed (Figure 7B). Membrane staining with CK 1.3 after PMA stimulation was not evenly distributed (Figure 7B, arrows). This uneven staining may reflect differences in the amount of CK 1.3 isozyme at different parts of the plasma membrane.[3]

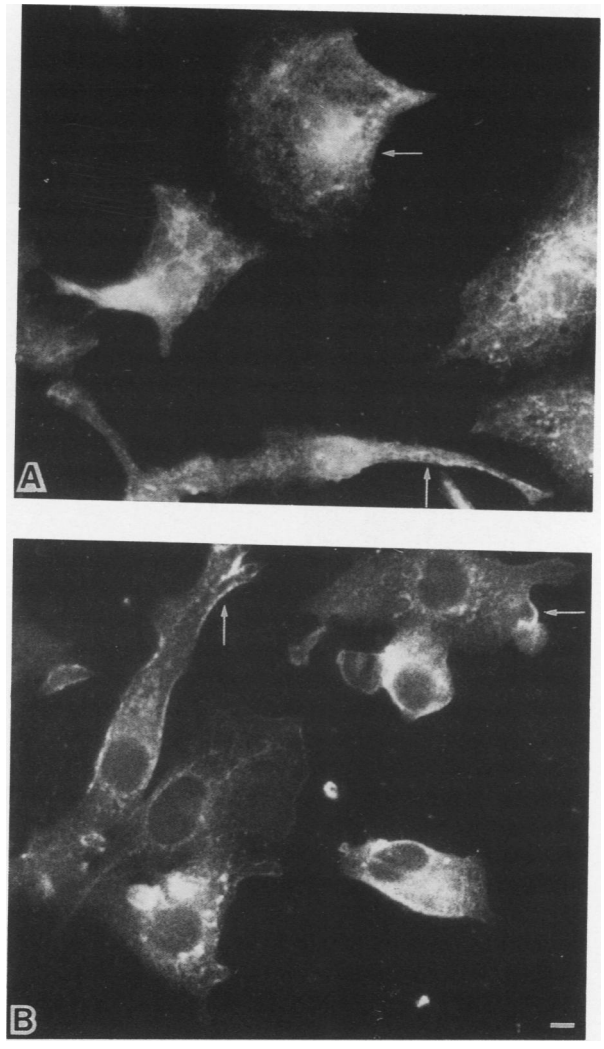


***Figure 7.*** **Translocation of CK 1.3 isozyme to the plasma membrane in cultured cardiac myocytes after treatment with PMA.** Cells were treated with (B) 100 nM PMA or (A) vehicle for 5 min as in Figure 4 and stained with mAb CK 1.3. (A) In control cells, CK 1.3 staining is diffusely cytosolic and obscures the nuclei. Faint fibrillar staining is observed in some cells. Membrane staining is not observed (arrows). (B) After activation of PKC with PMA, CK 1.3 immunoreactivity is localized to the plasma membrane (arrows). Perinuclear staining appears in some of the cells. Bar equals 10 μm.

[3] As mentioned earlier, under certain conditions, microfilaments and myofibrils can collapse and accumulate close to the plasma membrane. Staining of these collapsed structures with phalloidin, for example, may appear as membranal staining. However, under the conditions described in our study, these cytoskeletal elements remained intact and no

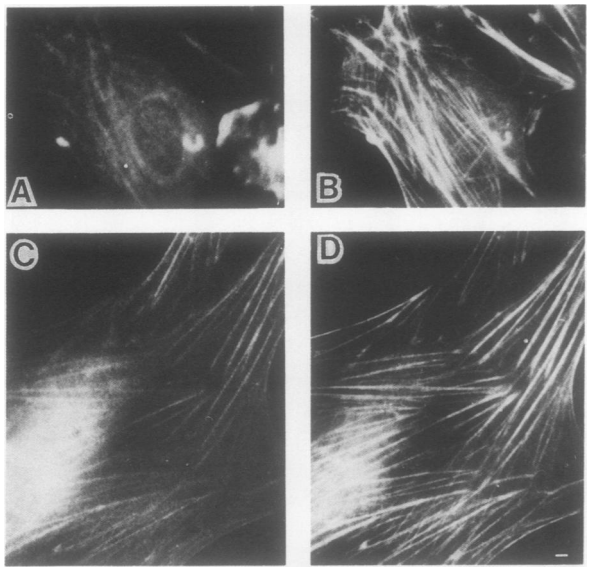


***Figure 8.*** **Translocation of CK 1.4 isozyme to microfilaments in cultured cardiac fibroblasts after treatment with PMA.** Shown are pairs of identical microscopic fields with CK 1.4 staining (left) or rhodamine-phalloidin staining (right). Cardiac fibroblasts were treated with 100 nM PMA (C and D) or vehicle (A and B) as in Figure 4. (B and D) Microfilaments are seen by phalloidin staining in both conditions. (A) Faint diffuse fibrillar and perinuclear staining with CK 1.4 is seen in control cells. (C) CK 1.4 staining of microfilaments appears after PMA treatment. Bar equals 5 $\mu$m.

### *On activation, the CK 1.4 isozyme is translocated to microfilaments in fibroblasts*

Translocation of the CK 1.4 isozyme to cytoskeletal elements on activation was not unique to cardiac myocytes. Fibroblasts comprise 5%–10% of the cells in the primary culture system used in this study (Simpson, 1985). Microfilaments, actin- and myosin-containing cytoskeletal elements, were identified in the cardiac fibroblasts by staining with phalloidin (Figure 8, B and D) and by their disruption after 1 h treatment with cytochalasin B (not shown). In control fibroblasts (treated with 0.01% DMSO for 5 min), these cytoskeletal elements were very lightly stained by CK 1.4 (Figure 8A). A 5-min incubation with 100 nM PMA increased CK 1.4 immunostaining of microfilaments (Figure 8C). Although treatment of a variety of cells with phorbol esters has resulted in reorganization of microfilaments (Dugina *et al.*, 1987; Schliwa *et al.*, 1984), no change in actin filament structure in fibroblasts was observed during the 5-min treatment of the cardiac culture with PMA or with the vehicle (Figure 8, B vs. D).

The ACAS 470 fluorescence imaging cytometer was used to quantitate the increase in CK 1.4 immunostaining of the microfilaments after PMA treatment. A digitized image of the immunofluorescence staining of elongated elements, probably microfilaments, was clearly observed after a 5-min PMA treatment and not after control treatment (Figure 9, B vs. A). In addition, fluorescence in the area of the nucleus

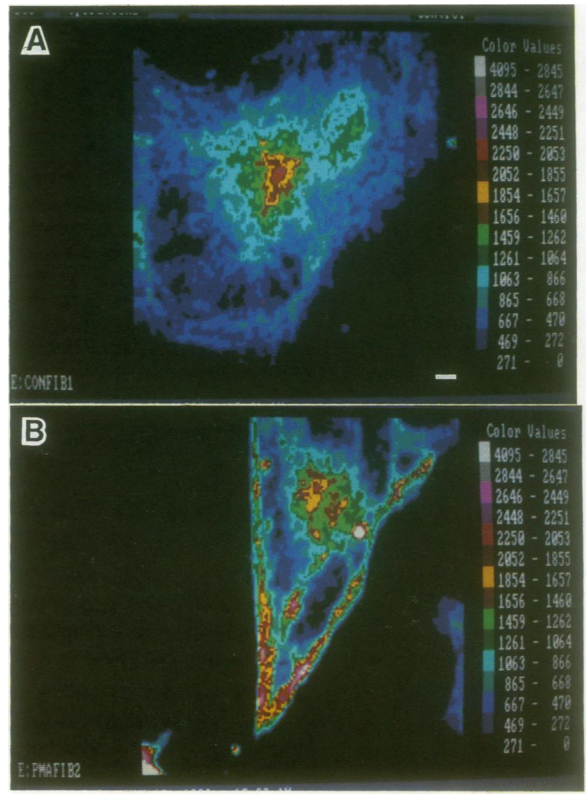


***Figure 9.*** **Digitized image of CK 1.4 isozyme localization in control and PMA-treated cardiac fibroblasts.** Shown are digitized images of the intensity of the fluorescence emission from (A) a control fibroblast and (B) a PMA-treated fibroblast obtained on an ACAS 470 argon laser fluorescence imaging cytometer. The fluorescence intensity maps are color coded and the scale (in arbitrary units) is given on the right of each panel. The nuclear area (green spheroid in the middle of the cell in both A and B) has higher fluorescence emission due to greater cell thickness in this region. (B) Fibrillar staining is found in the PMA-treated cell and is defined by the brightly colored red/yellow ridges. Because the depth of focus of the laser beam is ~8–10 $\mu$m (greater then the cell thickness), the image represents integration of the fluorescence signal emitted at any point of this region of the *Z* axis. Therefore, only cells with aligned microfilaments at the *Z* axis could be chosen for this analysis. An example of such image analysis is shown in the figure, and combined data from several determinations are given in the text. Bar equals 10 $\mu$m.

membranal staining with phalloidin was observed (Figure 4, B and D, and data not shown). Therefore, it is unlikely that the membranal staining with CK 1.3 on activation reflects CK 1.3 association with the structures with which CK 1.4 associates.

was also found in both control and PMA-stimulated cells, most likely reflecting the higher cell thickness in this part of the cell (Figure 9, B vs. A). ACAS 470 quantitation analysis demonstrated similar intensity of this fluorescence at the nuclear area before and after PMA stimulation (1368 vs. 1412 of arbitrary units of fluorescence intensity per pixel in control and PMA-treated cultures, respectively; Figure 9, A and B). In contrast, the ratio of fibrillar staining to nuclear staining within each cell increased by about threefold, from 0.47 ± 0.02 (mean ± SE; n = 6) in control cells to 1.47 ± 0.06 (mean ± SE; n = 9) in PMA-treated cells.

## Discussion

### *A PKC isozyme is translocated to cytoskeletal elements on activation*

The central finding of this study is that activation of PKC resulted in translocation, from the cytosol to the cytoskeleton, of a PKC isozyme recognized by CK 1.4 mAb. In cardiac myocytes the CK 1.4 isozyme was translocated to myofibrils, whereas in cardiac fibroblasts this isozyme was translocated to microfilaments. In addition, exogenously added PKC bound to these cytoskeletal elements in control cells in a phospholipid- and calcium-dependent manner, indicating the presence of a PKC binding site(s) on these structures. Because another PKC isozyme, recognized by CK 1.3, translocated to the plasma membrane and the perinuclear area in the same cell type, these data also suggest that the various PKC isozymes translocate to distinct intracellular sites on activation. Therefore, PKC isozymes are likely to phosphorylate different protein substrates present at these sites and, consequently, to regulate different cellular responses.

Our observation of activation-dependent translocation of a PKC isozyme to the plasma membrane is in accordance with published data (see below). However, activation-dependent translocation of PKC to cytoskeletal elements has not been previously demonstrated. The following data support the conclusion that the translocation of CK 1.4 immunoreactivity to cytoskeletal elements on activation reflects translocation of a PKC isozyme and not translocation of a nonrelevant protein with CK 1.4 cross-reactivity. 1) The translocation of CK 1.4 immunoreactivity from the cytosol to the cytoskeleton had similar agonist dependence and time course as does the translocation of PKC enzymatic activity from the soluble fraction to the particulate fraction (Henrich and Simpson, 1988). 2) CK 1.4 staining of cardiac myocytes was abolished after preadsorption with PKC, but not after preadsorption with other proteins. 3) Exogenously added PKC can bind myofibrils as demonstrated in the decoration experiment; when we used limited amounts of added PKC, binding increased in the presence of PKC activators. If this hypothetical nonrelevant PKC cross-reactive protein is present in the PKC preparation used in the decoration experiments, it must have similar chromatographic properties to the CK 1.4 isozyme on ion exchange (DEAE) phenyl-Sepharose and sizing column ($AcA_{34}$) chromatography. It also must have similar properties with regard to dependence on PS and DG. In addition, no decoration was found after a very limited trypsin digestion, which generates the catalytic fragment of PKC, PKM. We have previously demonstrated that CK 1.4 inhibition of PKC activity is lost if PKC was partially proteolysed by trypsin (Mochly-Rosen and Koshland, 1987). If CK 1.4 binds to a non-PKC protein, the antigenic determinant for CK 1.4 antibody on this protein would have to be as sensitive to limited proteolysis as is the determinant on PKC; and 4) Recently, we have identified another anti-PKC mAb, CK 1.13, that also binds myofibrils of cardiac myocytes in a cross-striated pattern (unpublished observation). Taken together, these data strongly suggest that the translocation of CK 1.4 immunoreactivity from the cytosol to the cytoskeleton indeed reflects activation-induced translocation of a PKC isozyme.

Previous biochemical studies provide indirect support for the immunolocalization observed in our study. After sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the Triton-insoluble fraction of erythrocyte and brain membranes, two polypeptides were found to bind PKC in vitro (Wolf and Sahyoun, 1986). These two polypeptides have the same molecular weight as two known cytoskeletal proteins (Wolf and Sahyoun, 1986). In addition, it was suggested that PKC activity present in a particulate fraction purified from lymphocytes (Chen *et al.*, 1987; Zalewski *et al.*, 1988), from $GH_4C_1$ cells (Kiley and Jaken, 1990), and from Y-1 adrenal cells (Papadopoulos and Hall, 1989) was due to interaction of PKC with cytoskeletal proteins in this fraction. This interpretation was based on the finding that activation of PKC results in translocation of PKC activity to the detergent insoluble fraction, a fraction enriched in several cytoskeletal elements (Ben-Ze'ev *et al.*, 1979). However, no direct evidence was provided to support this interpretation; the data in lympho-

cytes could reflect incomplete extraction of activated PKC from the membrane lipids that contaminate the detergent-insoluble fraction, rather than direct association of PKC with cytoskeletal elements. Using anti-PKC polyclonal antibodies, Liu *et al.* (1989) recently reported localization of PKC immunoreactivity in the striated region of rat heart. However, no data is provided on the effect of activation of PKC on the localization of PKC immunoreactivity (Liu *et al.,* 1989), and therefore comparison with our study cannot be carried out. Finally, Jaken *et al.* (1989) reported association of PKC with focal contacts in rat embryo fibroblasts. Although the organization of this cytoskeletal structure was altered on phorbol ester exposure, the amount of PKC associated with the cytoskeleton remained the same as in nontreated cells (Jaken *et al.,* 1989). The data presented here provide the first direct evidence that, in intact cells, a PKC isozyme transiently associates with myofibrils and microfilaments on PKC activation.

There are several possible explanations for the failure to observe activation-dependent translocation of PKC to cytoskeletal elements in previous studies. 1) The PKC isozyme identified by CK 1.4 may not be present in the cells studied. Not all cells express all PKC isozymes (Mochly-Rosen *et al.,* 1987; Shearman *et al.,* 1987). 2) Because cytoskeletal elements in the different cell types previously studied are not organized in the same structures as in the cells presented here, they might not bind activated PKC; and 3) The antibodies employed in the other studies may be specific to different isozymes that do not cross-react with the CK 1.4 isozyme.

### *Potential mechanisms for association of activated PKC with the cytoskeleton*

Two potential mechanisms could explain the association of activated PKC with cytoskeletal elements. PKC may be activated at the plasma membrane and subsequently translocated to a binding element or elements in the cytoskeleton. Alternatively, PKC may be activated at the cytoskeleton.

The first mechanism is most consistent with current concepts of PKC activation. PKC requires PS and DG for activation (Bell, 1986). PS is a plasma membrane lipid and DG is generated at the plasma membrane after activation of surface receptors, such as the $\alpha_1$-adrenergic receptor. If PKC were activated at the plasma membrane, it would have to detach from the membrane and then translocate to a protein or proteins in the cytoskeleton. Postactivation detachment of PKC from the plasma membrane in vitro has been previously described (Wolf *et al.,* 1985).

Activation of PKC at the cytoskeleton is a novel idea, but there is some evidence in support of such a mechanism. Data from several laboratories indicate that the lipids required for binding and activation of PKC are either present on or can bind to cytoskeletal elements. First, PS binds to two cytoskeletal proteins, which also bind PKC in the presence of this lipid (Wolf and Sahyoun, 1986). Second, DG is present in purified preparation of $\alpha$-actinin, a protein associated with myofibrils and microfilaments. Moreover, the amount of DG in the $\alpha$-actinin preparation increases 30-fold under conditions that activate PKC in vivo (Burn, 1988; Burn *et al.,* 1985, 1988; Shearman *et al.,* 1987). Third, phosphatidylinositol bisphosphate ($PIP_2$), a source of DG, binds two cytoskeletal proteins, gelsolin (Janmey and Stossel, 1987; Shearman *et al.,* 1987; Yin *et al.,* 1988) and profilin (Lassing and Lindberg, 1985). These proteins are involved in regulation of actin polymerization and actin filament formation, and their function is modulated by $PIP_2$ hydrolysis (Lassing and Lindberg, 1985; Janmey and Stossel, 1987; Shearman *et al.,* 1987; Yin *et al.,* 1988). Therefore, it appears that all the lipids (or their precursor) required for binding and activation of PKC are associated with cytoskeletal elements, suggesting that PKC could be activated on these structures.

### *Different PKC isozymes are translocated to different intracellular locations*

Anti-PKC antibodies have been used previously to demonstrate activation-induced translocation of PKC. Translocation of PKC to the plasma membrane (Shoji *et al.,* 1986) to the perinuclear area (Halsey *et al.,* 1987; Leach *et al.,* 1989) and into nuclei (Cambier *et al.,* 1987) has been reported. Similar to our result with CK 1.3 in cardiac cells (Figure 7), the $\beta$-isozyme of PKC was found to translocate to the plasma membrane of human megakaryoblastic leukemic cells on activation (Ito *et al.,* 1988). It is not yet clear whether phorbol ester–induced translocation of the CK 1.3 isozyme and CK 1.4 isozyme to different intracellular sites occurs within the same cell. It is possible that different cardiac myocytes express only one PKC isozyme. Nevertheless, translocation of PKC isozymes to distinct intracellular sites may explain the variety of cellular responses and protein substrates phosphorylated by PKC.

### Summary and implications

In intact cardiac myocytes and fibroblasts, activation of PKC resulted in translocation of a PKC isozyme to myofibrils and microfilaments, respectively. Association of a PKC isozyme with cytoskeletal elements may explain some of the effects of PKC on cell contractility and morphology. Furthermore, it is possible that the various PKC isozymes are translocated to different intracellular sites and thus each may be involved in different cellular functions.

## Methods

### Monoclonal antibodies

The anti-PKC mAbs CK 1.4 and CK 1.3 have been previously described (Mochly-Rosen and Koshland, 1988). Both mAbs appear to be isozyme-specific (Mochly-Rosen *et al.*, 1987); however, the identity of these isozymes has not yet been determined. The anti-myosin mAb, MF20, which is specific for myosin of striated muscle (Bader *et al.*, 1982), was purchased from the Developmental Studies Hybridoma Bank. Anti-PKC isozyme-specific mAbs anti-$\alpha$ (MC-3a), anti-$\beta$ (MC-2a), and anti-$\gamma$ (MC-1a) were obtained from Seikagaku Kogyo (Tokyo, Japan).

### Cell culture

Cardiac myocytes were dissociated from the hearts of day-old Sprague-Dawley rats and were maintained at 150–200 cells/mm$^2$ on laminin-coated Lab-Tek 8-chamber slides or on 100-mm culture dishes in minimum essential medium (MEM) with 5% calf serum. In some of the experiments, cells were grown in calf serum–supplemented media for 4 d (from day 1 to 5) and in defined media (Simpson and Savion, 1982) for the last 48 h. Treatment with 0.1 mM bromodeoxyuridine through culture day 3 kept fibroblasts to 10% or less of total cells (Simpson and Savion, 1982; Simpson, 1985). On culture day 6, fresh *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered MEM (pH 7.3) without serum was added, and experiments were performed.

### Western blot analysis

PKC was partially purified from cultured cardiac myocytes. Cells from four, 100-mm plates were homogenized in 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl pH 7.5, 2 mM EDTA, 10 mM ethylene glycol-bis($\beta$-aminomethyl ether)-*N,N,N',N'*,-tetraacetic acid (EGTA), 12 mM $\beta$-mercaptoethanol, 1% Triton X-100, $10^{-5}$ M phenylmethylsulfonylfluoride, and 20 $\mu$g/ml each of leupeptin, aprotonin, and soybean trypsin inhibitor. Insoluble material was removed by centrifugation at 100 000 $\times$ *g* for 30 min. The extract was then chromatographed on DEAE cellulose as described (Mochly-Rosen and Koshland, 1987), and fractions containing PKC activity were pooled. PKC immunoreactivity was determined in the eluent fractions using CK 1.4, anti-$\alpha$, anti-$\beta$, and anti-$\gamma$ PKC-specific mcAbs at 1/300 dilution followed by rabbit anti-mouse antibodies (Cappel) and $^{125}$I-protein A (5 $\mu$Ci/30 ml). For comparison, equal amounts of PKC purified from rat brain were also analyzed.

### Immunocytochemistry

On culture day 6, cells were treated with 2 $\mu$M NE, 100 nM PMA, or their vehicles (final concentrations 100 $\mu$M ascorbic acid or 0.01% DMSO for NE and PMA, respectively) in serum-free HEPES-buffered MEM (pH 7.3), at 37°C. After the times indicated in the figure legends, medium was removed, and the cells were fixed for 30 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline (PBS). Fixed cells were permeabilized and nonspecific binding blocked by preincubation with PBS, 0.1% Triton X-100, and 1% normal goat serum (Mochly-Rosen *et al.*, 1987). The cells were double-stained to visualize PKC and myofibrils in myocytes, or PKC and microfilaments in fibroblasts. To visualize PKC, cells were incubated with CK 1.4 or CK 1.3 (<1 $\mu$g of immunoglobulins) in PBS containing 0.3% Triton X-100, followed by fluorescein isothiocyanate (FITC)-conjugated second antibody. To visualize myofibrils and microfilaments, cells were stained with rhodamine-conjugated phalloidin (Schliwa *et al.*, 1984) in PBS containing 0.3% Triton X-100. Controls included hybridoma medium or normal goat serum followed by FITC-labeled second antibody. Slides were mounted as described (Mochly-Rosen *et al.*, 1987) and viewed with a Zeiss (Thornwood, NY) IM35 microscope with a 40$\times$ water immersion objective. Images were recorded on Kodak technical pan film; exposure times were 45 and 30 s for all the fluorescein and rhodamine photomicrographs, respectively.

Adsorption of CK 1.4 was carried out in solid phase. Rat brain PKC (0.05 U), bovine heart PKA (Sigma [St. Louis, MO] 0.5 U) or 3% normal goat serum were spotted on 0.5 cm$^2$ nitrocellulose paper. After 5 min at room temperature, the papers were washed and blocked with 3% normal goat serum in PBS for 1 h. The papers were then incubated with CK 1.4 (~2 $\mu$g) for 1 h at room temperature in a humidified box, and the unadsorbed material was used for immunostaining.

### PKC and PKM preparation

Rat brain PKC was partially purified from the soluble and the particulate fractions by the use of DEAE and $AcA_{34}$ columns (PKC1) and on DEAE, phenyl-Sepharose and $AcA_{34}$ columns (PKC2) as described elsewhere (Mochly-Rosen and Koshland, 1987). PKM was generated by a 3-min trypsin digestion of the partially purified PKC (Mochly-Rosen and Koshland, 1987). Under these conditions, all the PKC is converted to PKM (the phospholipid and calcium independent catalytic fragment), but most of the other proteins remain intact.

### Quantitation of immunocytochemical data

Myocytes were identified by their cross-striated staining pattern with rhodamine-phalloidin. Over 90% of myocytes identified by morphological criteria (Simpson and Savion, 1982) were also observed to have cross-striated staining with rhodamine-phalloidin. The percentage of myocytes showing a cross-striated pattern with CK 1.4-FITC staining was determined.

### Decoration of myofibrils by exogenously added PKC

Control cardiac myocytes were fixed, blocked, and permeabilized with Triton X-100 as described for immunocytochemistry. Cells were then incubated with 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 12 mM $\beta$-mercaptoethanol, 5 $\mu$g/ml soybean trypsin inhibitor, 5 $\mu$g/ml aprotinin, 5 $\mu$g/ml leupeptin, and $10^{-5}$ M phenylmethylsulfonylfluoride containing one of the following: 1% normal goat serum (control); 0.068 U (PKC1) or 0.05 U (PKC2) of a mixture of PKC isozymes; trypsin-digested PKC from PKC1 (PKM); 0.5 U of PKA; or 0.01 U of PKC 1 in the presence or absence

of 20 μg/ml PS, 0.8 μg/ml DG, and 2 mM calcium. After three washes with PBS-Triton, bound PKC immunoreactivity was visualized with CK 1.4 antibody as described above. The percentage of cardiac myocytes showing cross-striated staining with CK 1.4 was then determined.

### *Quantitation of immunofluorescence on microfilaments by fluorescence imaging*

Slides stained as above were also analyzed for fluorescence intensity using the ACAS 470 argon laser fluorescence imaging cytometer (Meridian Instruments, Okemos, MI). This is an automated digital imaging system with which the subcellular distribution and the quantity of fluorescence intensity in a single cell can be determined. The instrument includes both an Olympus inverted phase contrast microscope equipped with a 40× objective with a high-speed, computer-controlled, two-dimensional stage and an IBM PC/AT microcomputer for data analysis. The image is obtained by focusing the laser beam and successively illuminating spots 0.5 μm in diameter (a pixel dimension) for 8 μs as the stage moves at high speed in the X/Y plane. Each pixel is given a coordinate, and this digitized information is stored for reconstruction of the entire image. FITC is exited with the argon laser beam at 488 nm, and fluorescence emission measured above 530 nm using a 530/30 nm longpass filter. The intensity of each single point emission is recorded and later displayed as a color-coded image. Fluorescence intensity is given in arbitrary units per pixel. Quantitation of fluorescence in a specific subcellular region is determined after electronically defining the desired area by drawing a polygon around it. The fluorescence intensity is divided by the number of pixels in the polygon and is given in arbitrary units.

### *Statistics*

Data were analyzed by Student's *t* test.

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