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Ca^{2+}- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain

(oxygen radicals/Ca^{2+}-dependent regulation/phorbol esters/protein phosphorylation/signal transduction)

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ABSTRACT **The susceptibility of purified protein kinase C to oxidative inactivation by H_2O_2 was found to be increased by Ca^{2+} either alone at a high (5 mM) concentration or at a low (≈50 μM) concentration along with phosphatidylserine and diacylglycerol and by tumor-promoting phorbol esters even in the absence of Ca^{2+}. This suggested that the membrane-bound and/or catalytically active form of protein kinase C is relatively more susceptible to oxidative inactivation. Although both the regulatory and catalytic domains of protein kinase C were susceptible to oxidative inactivation, a selective modification of the regulatory domain was obtained under mild oxidative conditions by protecting the catalytic site with ATP/Mg^{2+}. Under these conditions there was a loss of both phorbol ester binding and Ca^{2+}/phospholipid-stimulated kinase activity. However, this modified form of enzyme exhibited an increase in Ca^{2+}/phospholipid-independent kinase activity. This suggests that selective oxidative modification of the regulatory domain may negate the requirement for Ca^{2+} and lipids for activation. Treatment of intact C6 glioma or B16 melanoma cells with H_2O_2 resulted in a time- and temperature-dependent decrease in Ca^{2+}/phospholipid-dependent protein kinase C activity along with a concomitant transient increase in an oxidatively modified isoform of protein kinase C that exhibited activity in the absence of Ca^{2+} and phospholipids. Since protein kinase C can initially be activated by mild oxidative modification and subsequently inactivated by further oxidation, this dual activation–inactivation of protein kinase C in response to H_2O_2 suggests an effective on/off signal mechanism to influence cellular events.**

Protein kinase C (PKC) plays a crucial role in cellular signal transduction and also serves as an intracellular receptor for tumor-promoting phorbol esters (1). Besides the reversible activation of PKC by Ca^{2+}, phospholipids (PL), diacylglycerol, unsaturated fatty acids, and phorbol esters, this enzyme is also irreversibly activated by limited proteolysis involving the intracellular protease calpain (1–5). The calpain-mediated proteolysis of PKC also has been implicated in the down-regulation of this kinase in cells treated for prolonged periods of time with phorbol esters (6–8). Recently, we suggested that the oxidative inactivation of PKC, particularly the membrane-bound form, also may play a role in the down-regulation of this enzyme (9). Since phorbol esters induce not only the membrane association of PKC (10, 11) but also the generation of reactive oxygen species (12, 13), increased oxidative inactivation of PKC may occur in phorbol ester-treated cells (9). In turn, as demonstrated for other proteins (14), this inactive, oxidatively modified form of PKC may be more susceptible to proteolysis.

In this communication we identify factors that regulate the rate of H_2O_2-mediated oxidative inactivation of PKC. Further, it is shown that selective oxidative modification of the regulatory (phorbol ester/PL-binding) domain, under conditions where the catalytic site is protected, results in the generation of a modified form of PKC that exhibits kinase activity in the absence of Ca^{2+} and PL.

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MATERIALS AND METHODS

PKC and M-Kinase Preparations. PKC was purified from bovine brain by DEAE-cellulose chromatography followed by a modified Ca^{2+}-dependent hydrophobic chromatography. The detailed procedure will be published elsewhere. Enzyme having an activity of ≈600 units/mg of protein was obtained and employed in the studies presented. While partially purified preparations of PKC exhibit similar results, it is important to have PKC free from proteases, especially calpain. Apparently homogeneous preparations of PKC obtained by other published methods were, in our hands, contaminated to varying degrees with calpain I. This can be overcome by passing the PKC preparations through a small (1-ml) phenyl-Sepharose column equilibrated and eluted with 20 mM Tris·HCl, pH 7.5/0.1 M NaCl/1 mM EDTA. Under these conditions calpain I remained tightly bound to the phenyl-Sepharose while PKC came in the unbound protein fraction. Such preparations of PKC free from calpain retained full activity for at least 20 min when incubated at room temperature in the presence of Ca^{2+}. Because of the increased susceptibility of oxidatively modified proteins to proteolysis (14), it is important to include protease inhibitors such as leupeptin and pepstatin A in the PKC preparations during oxidative modification and during the isolation of oxidatively modified forms of PKC from cells. M-kinase was derived from native PKC by limited proteolysis using calpain II (15, 16).

Oxidative Modification of PKC. 2-Mercaptoethanol present in the PKC preparation was removed by dialysis versus buffer A (10 mM Tris·HCl, pH 7.4/0.1 mM EGTA/0.15 μM pepstatin A). To PKC (0.5–1 unit) were added 25 μM $FeCl_2$, 1 mM EGTA or ≈0.05–5 mM $CaCl_2$, 25 μM leupeptin, and the indicated amounts of other ligands [phosphatidylserine (PS) from bovine brain, diolein, phorbol 12-myristate 13-acetate (PMA), ATP, Mg^{2+}] in a total volume of 0.25 ml. These samples were brought to room temperature (20°C) in a water bath. Oxidative modification was initiated by adding the indicated concentration of H_2O_2 and the samples were further incubated for 1–20 min. To these samples, 12 μl of 0.2 M dithiothreitol or 0.5 M 2-mercaptoethanol was added at the indicated times to prevent further oxidative modification. The samples were placed on ice; both PKC activity (in the presence and absence of Ca^{2+}/PL) and [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding were determined immediately.

Abbreviations: PKC, protein kinase C; M-kinase, Ca^{2+}/PL-independent form, proteolytically derived from PKC; PL, phospholipid(s); PS, phosphatidylserine; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate.

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Isolation of Oxidatively Modified PKC from Cells Treated with H_2O_2. Cells [C6 glioma, B16 melanoma, or normal rat kidney (NRK)] were grown in 150-cm^2 flasks in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. When the cells reached confluency, the medium was changed and the cultures were left overnight in the incubator. The cells then were treated with various concentrations of H_2O_2 for the indicated periods of time. The treated cells were Dounce homogenized in 7 ml of buffer B containing detergent [20 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM 2-mercaptoethanol/0.5 mM phenylmethanesulfonyl fluoride/25 μM leupeptin/0.15 μM pepstatin A/1% (vol/vol) Nonidet P-40]. The homogenates were centrifuged at 13,000 × g for 15 min, and the supernatant was used to isolate PKC as described in the legend to Fig. 3.

Other Procedures. PKC activity was measured using histone (Sigma type III-S) as the substrate (10, 11). Because the basal activity observed in the presence of EGTA was altered by oxidative modification, the total activity observed in the presence of Ca^{2+}/PL was expressed without subtracting basal activity, unless stated otherwise. Both forms of protein kinase activities were expressed in units, where 1 unit of enzyme transfers 1 nmol of phosphate to histone H1 per min at 30°C. Specific phorbol ester binding was determined with [^{3}H]PDBu as the ligand (2).

RESULTS AND DISCUSSION

Previously we showed that the exposure of PKC to low concentrations of oxidizing agents such as N-chlorosuccinimide and H_2O_2 resulted in the loss of both kinase activity and phorbol ester binding (9). In the present studies, we attempted to selectively block the oxidative modification of one of the domains in an attempt to obtain a protein exhibiting either kinase activity or phorbol ester binding. Ca^{2+} can protect other proteins such as calmodulin and troponin C from oxidative inactivation (17). Substrate ligands also have been shown to protect some enzymes from oxidative inactivation (18). Thus, two types of ligands that bind to or influence PKC were examined for their ability to selectively modulate the oxidative modification of either domain. One type were those ligands that bind to the regulatory domain (Ca^{2+}, PL, and phorbol esters) and the other type were those that bind to the catalytic domain (ATP, P_i, and Mg^{2+}).

Factors That Increase the Oxidative Inactivation of PKC by H_2O_2. *Limited proteolysis to produce M-kinase.* We compared the relative rates of oxidative inactivation of native PKC (in the presence of EGTA) and the so-called M-kinase form derived from PKC by calpain-mediated proteolysis. As shown in Fig. 1, M-kinase was more rapidly inactivated by H_2O_2 than intact PKC in the presence of EGTA. That the M-kinase form is catalytically active without further requirement for other regulators, whereas holo PKC requires other regulators to express catalytic activity, suggested that the oxidation-susceptible amino acid residues at the catalytic site of intact, nonactivated PKC are not readily accessible to oxidative modification. Hence, we tested various regulators to determine how they might influence the susceptibility of intact PKC to oxidation.

Regulation by Ca^{2+}. First, the rate of oxidative inactivation of PKC was compared in the presence and absence of Ca^{2+}. The control preparation of PKC, which was exposed to Ca^{2+} but not to H_2O_2, lost only 5–10% of its activity and this basal loss of activity was taken into account. PKC lost about 52% of its activity within 3 min of exposure to 4 mM H_2O_2 when in the presence of 5 mM Ca^{2+}, whereas it lost only 7% of its activity in the presence of EGTA (Fig. 1). Further, the rate of inactivation of PKC in the presence of Ca^{2+} was more rapid than even that noted with M-kinase. In this regard, PKC differs from calmodulin, which is relatively more stable to oxidative inactivation in the presence of Ca^{2+} than in the absence of Ca^{2+} (17). The optimal amount of Ca^{2+} needed to increase the sensitivity of PKC to oxidative inactivation was high (2–5 mM). Since PL, and diacylglycerol, reduces the concentration of Ca^{2+} required for the activation of PKC, the effect of these ligands was determined.

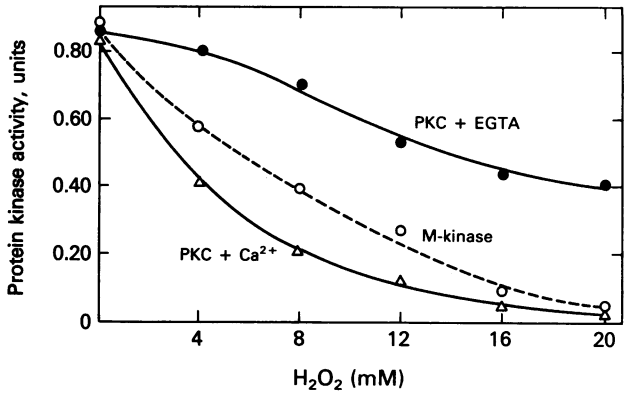

FIG. 1. Relative susceptibility of purified bovine brain PKC and M-kinase to oxidative inactivation in the presence or absence of Ca^{2+}. M-kinase or holo PKC, in the presence of either 1 mM EGTA or 5 mM Ca^{2+}, was exposed to various concentrations of H_2O_2 for 3 min at 20°C. Protein kinase activity remaining in the treated samples was measured in the presence of Ca^{2+} and PL and expressed without subtraction of basal (Ca^{2+}/PL-independent) activity. ●, PKC with 1 mM EGTA; ○, M-kinase with 1 mM EGTA; △, PKC with 5 mM $CaCl_2$.

Regulation by PL. PS (20 μg/ml) and diacylglycerol (0.8 μg/ml), either alone or in combination, had no effect on the rate of H_2O_2-induced inactivation of PKC in the absence of Ca^{2+} or the presence of a high (5 mM) concentration of Ca^{2+}. However, these regulators did increase the rate of oxidative inactivation observed in the presence of a low (≈50 μM) concentration of Ca^{2+} (Table 1). This suggests that these lipid regulators, by increasing the affinity of the PKC for Ca^{2+}, can enhance the rate of oxidative inactivation of PKC at a physiologically low (micromolar) concentration of Ca^{2+}. Prior incubation with PS, diacylglycerol, and 50 μM Ca^{2+} followed by incubation with 1 mM EGTA also increased the sensitivity of PKC to oxidative inactivation (data not shown).

Regulation by phorbol esters. PMA, when added along with PS, was able to increase the rate of oxidative inactivation of PKC even in the presence of EGTA (Table 1). This increase

Table 1. Effect of various PKC regulators on the susceptibility of PKC to oxidative inactivation

Treatment and regulator(s)	PKC activity with Ca^{2+}/PL, unit*	Decrease, %
Control (not exposed to H_2O_2)	0.79	—
Exposed to H_2O_2		
In the absence of Ca^{2+}		
EGTA (1 mM) alone	0.57	28
EGTA, PS, and diolein	0.55	30
EGTA, PS, and PMA	0.36	54
In the presence of Ca^{2+}		
High Ca^{2+} (5 mM) alone	0.17	78
Low Ca^{2+} (≈50 μM) alone	0.51	35
Low Ca^{2+}, PS, and diolein	0.14	82
Low Ca^{2+}, PS, and PMA	0.15	81

Preparations of purified PKC in the presence or absence of Ca^{2+} along with PS (20 μg/ml), diolein (0.8 μg/ml), and PMA (0.1 μM) as indicated were exposed to 10 mM H_2O_2 for 3 min at 20°C. PKC activity remaining in each treated sample was determined in the presence of Ca^{2+}/PL. Suitable controls were set and loss of any activity without exposure to H_2O_2 was corrected.

*Basal activity was not subtracted.

was even more pronounced in the presence of a low (≈50 μM) concentration of Ca^{2+}. Thus PMA, in a manner similar to that noted with diacylglycerol, appears to reduce the Ca^{2+} requirement necessary to promote oxidative inactivation of PKC. However, the effect of PMA differs from that of diacylglycerol, in that PMA is able to enhance the rate of oxidative inactivation of PKC even in the absence of Ca^{2+}. Not only was the oxidatively inactivated form of PKC found to have lost [^{3}H]PDBu binding, but in addition phorbol ester initially bound to the native, unmodified enzyme was found to be dissociated from PKC upon oxidative modification of the enzyme (data not shown). The presence of a protein substrate, histone H1 (0.1 mg/ml), did not alter the rate of oxidative inactivation of PKC either in the presence or in the absence of Ca^{2+}.

Isolated PKC did not exhibit any kinase activity in the presence of 5 mM Ca^{2+} alone. Yet, this high concentration of Ca^{2+} did increase the rate of oxidative inactivation of isolated PKC, suggesting that the catalytically active (histone-phosphorylating) form of PKC is not absolutely required to exhibit enhanced susceptibility to inactivation. In intact cells, however, the lipid-bound form of PKC binds Ca^{2+} at physiologically low (micromolar) concentration. Hence the membrane-bound and/or catalytically active form of PKC appears to be more susceptible to oxidative inactivation.

Selective Modification of Regulatory Domain: Oxidative Activation of PKC. Previously, we reported that the strong oxidizing agent *N*-chlorosuccinimide caused the simultaneous loss of both [^{3}H]PDBu binding and Ca^{2+}/PL-stimulated kinase activity (9). In the present studies, however, when PKC was treated for a short time (2 min) with a low concentration (4 mM) of H_2O_2 in the presence of 5 mM Ca^{2+}, there was a proportionate loss of Ca^{2+}/PL-stimulated activity and [^{3}H]PDBu binding, accompanied by a 5-fold increase in basal (Ca^{2+}/PL-independent) activity (Fig. 2*A*). This increase in Ca^{2+}/PL-independent kinase activity was only 25% of the initial Ca^{2+}/PL-stimulated activity noted with the unmodified control enzyme, and the enzyme exhibited a progressive decrease in activity with exposure to H_2O_2 for longer periods of time. Assuming that the initial oxidative modification of PKC in the presence of Ca^{2+} opened the catalytic site to make it more susceptible to oxidative inactivation, we attempted to selectively protect the catalytic site.

Enzymes that possess binding sites for ligands with a phosphate group have been shown to have vicinal thiols at the binding site, and these sites have been shown to be susceptible to oxidative modification (18). These oxidation-susceptible sites can be protected by appropriate phosphate-containing ligands or by P_i and other tetrahedral anions (18). Hence we examined the ability of ATP and P_i to protect the catalytic site from oxidative inactivation. The addition of either ATP (0.1 mM) or Mg^{2+} (8 mM) alone had no protective effect. However, when ATP was added along with Mg^{2+} during oxidative modification, the basal (Ca^{2+}/PL-independent) activity was increased to a greater extent (40–50% of the Ca^{2+}/PL-stimulated activity) and remained relatively stable for about 10 min. This increase in Ca^{2+}/PL-independent kinase activity was accompanied by the loss of both [^{3}H]PDBu binding and Ca^{2+}/PL-stimulated kinase activity. While the presence of ATP-Mg^{2+} did protect the catalytic site from initial oxidative inactivation by H_2O_2, prolonged exposure to H_2O_2 nonetheless resulted in the loss of kinase activity.

Since PKC is oxidatively inactivated rapidly in the presence of Ca^{2+}, studies were carried out to control the rate of oxidative modification of the regulatory domain of PKC in the absence of Ca^{2+} (0.1 mM EGTA) by using a higher (10 mM) concentration of H_2O_2. When compared to the extent of oxidative activation noted in the presence of Ca^{2+}, the increase in basal, Ca^{2+}/PL-independent activity observed in the absence of Ca^{2+} was greater (50–70% of Ca^{2+}/PL-stimulated activity of unmodified enzyme) and declined at a

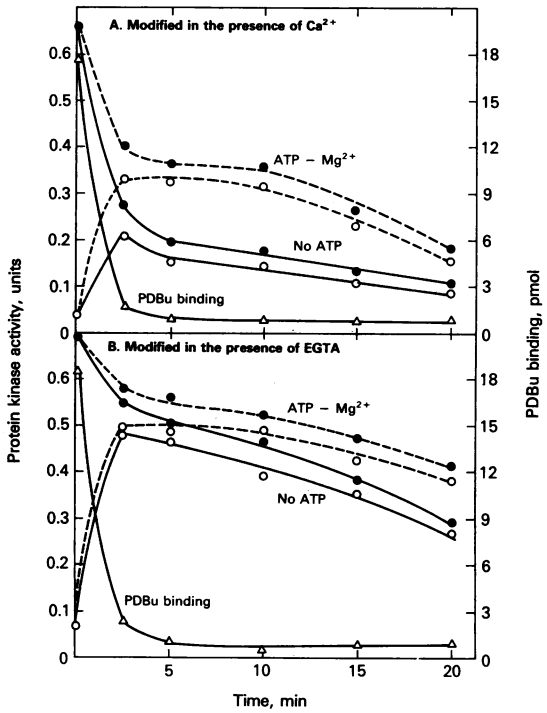

FIG. 2. Time course of the oxidative modification of purified bovine brain PKC in the presence or absence of Ca^{2+} with or without ATP/Mg^{2+}. PKC was incubated with 4 mM H_2O_2 in the presence of 5 mM $CaCl_2$ (*A*) or with 10 mM H_2O_2 in the presence of 1 mM EGTA (*B*). PKC samples (0.6 unit) were treated with H_2O_2 at 20°C in the presence (broken lines) or absence (solid lines) of 0.1 mM ATP and 8 mM Mg^{2+} for the times indicated. Then the remaining PKC activity was determined in the presence of Ca^{2+}/PL (●) or in the presence of 1 mM EGTA (○). [^{3}H]PDBu binding (△) was determined in samples treated with H_2O_2 in the absence of ATP/Mg^{2+}. The observed PKC activity was corrected for the dilution of specific radioactivity of the [γ-^{32}P]ATP substrate by the unlabeled ATP introduced into the protein kinase assay with the H_2O_2-treated PKC samples.

relatively slower rate with continued exposure to H_2O_2 (Fig. 2*B*). The protection offered by ATP plus Mg^{2+} was not very significant. These results suggest that limited oxidative modification of the regulatory domain may alter the conformation of PKC to an active form similar, but not identical, to that obtained with the binding of Ca^{2+}/PL activators to the regulatory domain. This mechanism might enable the conversion of PKC to a catalytically active form without a requirement for Ca^{2+}/PL.

Although we conducted these oxidative modification experiments under protective conditions, we were unable to achieve an oxidatively activated (Ca^{2+}/PL-independent) form of PKC that exhibited any more than 50–70% of the initial Ca^{2+}/PL-stimulated activity expressed by the unmodified control PKC. With PKC isolated from rat brain we were able to achieve oxidative activation to an extent equal to only 40–50% that noted with Ca^{2+}/PL.‡ Since oxidative modifi-

‡Some stored preparations of bovine brain PKC did show an increase in Ca^{2+}/PL-independent activity with oxidative modification, to a level 80–90% of that exhibited by unmodified PKC activated with Ca^{2+}/PL. However, freshly purified bovine PKC exhibited only 35–50% of this activation by oxidative modification. Fresh preparations of purified bovine PKC showed 10–12 pmol of PDBu binding per unit of enzyme. However, after storage for 2 or 3 weeks at 4°C, PKC exhibited 20–25 pmol of PDBu binding per unit of enzyme, apparently due to a decrease in the kinase activity with no change in total PDBu binding. However, this change in bovine brain kinase activity/PDBu binding with prolonged storage was not observed during storage of PKC isolated from rat brain. This difference might be responsible for the lower extent of oxidative activation observed with the rat brain PKC.

cation results in initial activation followed by inactivation, it is difficult to control the extent of oxidation to obtain a fully activated state. However, other considerations cannot be excluded. Conceivably, the oxidatively activated form may be inherently less active than the unmodified PKC stimulated with Ca^{2+}/PL. Alternatively, differences in the rates at which the various isoenzymes of PKC undergo oxidative activation may influence the extent of stimulation noted. Certainly further studies are required to determine the relative susceptibility of the isolated isoenzymes of PKC to oxidative activation or inactivation. The extent of oxidative activation of PKC achieved was comparable to the proteolytic activation of PKC obtained using trypsin.

Some proteins, such as hemoglobin, have been shown to undergo fragmentation upon exposure to H_2O_2 (19). However, the H_2O_2-exposed PKC showed no decrease in molecular weight as judged by SDS/polyacrylamide gel electrophoresis, indicating that the observed increase in Ca^{2+}/PL-independent activity of PKC was most likely not due to limited fragmentation of the protein induced by H_2O_2.

Isolation of Oxidatively Activated PKC from H_2O_2-Treated Cells. A mixture of oxidatively modified and native PKC along with bovine serum albumin (1 mg/ml) was subjected to DEAE-cellulose chromatography. The majority of the unmodified PKC was eluted with 0.1 M NaCl, whereas the oxidatively modified PKC was eluted with 0.25 M NaCl. When detergent-solubilized extract of control C6 glioma cells was subjected to DEAE-cellulose chromatography, two peaks of PKC activity were found; peak I was eluted with 0.1 M NaCl, and peak II was eluted with 0.25 M NaCl (Fig. 3*A*). In peak I, both Ca^{2+} and PL stimulated the kinase activity by 11-fold over basal. However, peak II was found to contain high basal (Ca^{2+}/PL-independent) activity, and this was further stimulated only 2-fold by Ca^{2+}/PL. Although the present study was not aimed at a detailed characterization of total protein kinases present in this peak II, the preliminary results suggest that peak II contains endogenous oxidatively modified PKC that may exhibit Ca^{2+}/PL-independent kinase activity. However, we cannot exclude the possibility of phosphorylation of substrate (type III-S histone) by other nonspecific protein kinases. The Ca^{2+}/PL-stimulated activity in peak II might be attributable to a modified proform of PKC that otherwise would be eluted in peak I. Thus, the PKC activity found in peak II should not be treated as a different isoenzyme of PKC but rather it appears to represent a modified form (isoform) of PKC.[§] All the three known isoenzymes (20, 21) were present in the peak I fraction. The kinase activity in both isolated peaks I and II was lost at a similar rate when exposed to increasing concentrations (1–20 mM) of H_2O_2 for 1–15 min (data not shown).

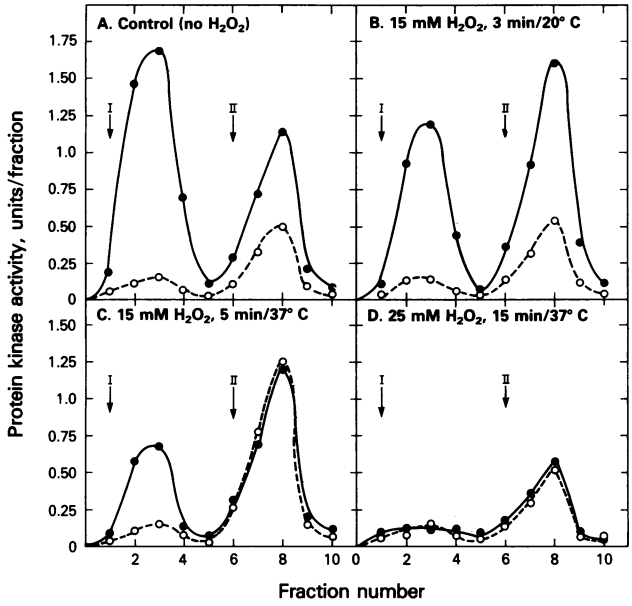

FIG. 3. Isolation of the native and oxidatively activated forms of PKC from H_2O_2-treated C6 glioma cells. (*A*) Control cells not treated with H_2O_2. (*B*) Cells treated with 15 mM H_2O_2 for 3 min at 20°C. (*C*) Cells treated with 15 mM H_2O_2 for 5 min at 37°C. (*D*) Cells treated with 25 mM H_2O_2 for 15 min at 37°C. Detergent-solubilized cell extract (5 ml; ≈18 mg of protein) was applied to a 1-ml DEAE-cellulose (DE52, Whatman) column (0.8 × 2 cm) previously equilibrated with buffer C (20 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM 2-mercaptoethanol/20 μM leupeptin/0.15 μM pepstatin A). After the column was washed with 5 ml of buffer C, the bound native form of PKC was eluted with 2.5 ml of 0.1 M NaCl in buffer C (peak I). The oxidatively modified and activated PKC then was eluted with 2.5 ml of 0.25 M NaCl in buffer C (peak II). Fractions of 0.5 ml were collected and the PKC activity present in these fractions was measured in the presence of Ca^{2+}/PL (●) or 1 mM EGTA (○).

Treatment of C6 glioma cells with 15 mM H_2O_2 for 3 min at 20°C resulted in an ≈40% decrease in the Ca^{2+}/PL-stimulated kinase activity found in peak I, along with a concomitant increase in Ca^{2+}/PL-dependent activity in peak II (Fig. 3*B*). About 50% of the activity lost from peak I was recovered in peak II in the Ca^{2+}/PL-dependent form. In cells treated with 15 mM H_2O_2 for 5 min at a higher temperature (37°C), peak I activity decreased 65%, while Ca^{2+}/PL-stimulated activity in peak II disappeared completely. However, the Ca^{2+}/PL-independent activity in peak II increased by 220% compared to untreated control (Fig. 3*C*). This suggests that the Ca^{2+}/PL-stimulated activity in peak II represents an intermediate form in the conversion of peak I kinase to Ca^{2+}/PL-independent peak II kinase. Treatment of the cells for 10 min with 15 mM H_2O_2 resulted in the complete disappearance of peak I kinase activity, and no increase was observed in peak II activity (data not shown). Cells treated with 25 mM H_2O_2 for 15 min exhibited not only a complete loss of peak I kinase but also a 50% decrease in peak II kinase activity (Fig. 3*D*).

Similar results were obtained with H_2O_2 treatment of other cell types (B16 melanoma and NRK cells). However, to reproduce the results reported, it was necessary to treat the other cell types with H_2O_2 for various times (2–20 min) rather than for one particular time, to determine the required conditions to obtain selective oxidative modification of PKC. The intracellular rate of oxidative modification of PKC varied depending on the cell type and culture conditions employed. The inactivation of PKC in peak I without significant recovery of the oxidatively activated form in peak II was found in cells grown in iron-supplemented calf serum (HyClone). Changing the culture medium at confluency the day prior to H_2O_2 treatment improved the recovery of oxidatively modified PKC in peak II. In all three cell types examined, when conditions were such that H_2O_2 treatment caused the complete disappearance of peak I PKC, no increase in peak II activity was observed. Under conditions where H_2O_2 treatment resulted in a 30–70% decrease in peak I activity, an increase in peak II activity consistently was found. Although the PKC forms peaks I and II are equally susceptible to oxidative inactivation after DEAE-cellulose isolation, the

[§]Western blot analysis of immunoreactive protein in peak II detected with specific PKC antisera revealed several polypeptide bands including a major M_r 80,000 band corresponding to PKC and a minor M_r 55,000 band. A low-affinity [^{3}H]PDBu binding also was observed in peak II. Treatment of the cell extracts with a high (50 mM) concentration of 2-mercaptoethanol for 6 hr at 4°C resulted in a decrease in the Ca^{2+}/PL-stimulated activity found in peak II, accompanied by an increase in Ca^{2+}/PL-dependent activity in peak I. Preliminary results suggest that a Ca^{2+}/PL-stimulated isoform of PKC in peak II might have been formed by formation of inter- or intramolecular disulfide bridge(s).

observed rapid decrease in peak I PKC activity along with the observed increase or slow rate of decrease of peak II kinase activity in intact cells exposed to H_2O_2 suggests that conversion of peak I kinase to peak II kinase compensates for the loss of peak II kinase activity.¶

Physiological Susceptibility of PKC to Oxidative Modification. Oxidative modification of PKC by exogenously added H_2O_2 in intact cells may require higher amounts of H_2O_2 because of the presence of other oxidation-susceptible components in culture medium (amino acids and serum proteins) and because of the presence of intracellular scavenging enzymes such as catalase, glutathione peroxidase, etc. Although C6 glioma and B16 melanoma cells require up to 15 mM H_2O_2 to induce oxidative modification of PKC, cell types such as parietal yolk sac cells, which have high cellular lipid concentrations, require only 3–5 mM H_2O_2 to induce oxidative modification of PKC. However, in view of the low PKC activity in the latter cell type, we preferred to use glioma or melanoma cell lines in this study.

H_2O_2, or reactive oxygen species generated *in situ*, may bring oxidative modification of PKC at much lower concentrations either directly or indirectly by initiating such events as lipid peroxidation (22). PKC binds to plasma membrane in response to certain stimuli (10, 11). Since PKC has binding sites for PL, diolein, or other compounds containing unsaturated fatty acyl groups, and since peroxidation of these highly susceptible protein-bound unsaturated fatty acyl chains results in the formation of hydroperoxides, rapid oxidative modification of amino acid residues that are in close proximity to these lipid binding sites may occur. This could lead to selective oxidative modification of the regulatory domain and activation of PKC. In pathological conditions accompanied by a net increase in the generation of high amounts of oxygen radicals (12, 13) along with an increase in intracellular Ca^{2+}, an increase in oxidative inactivation of PKC may occur.

Significance of Oxidative Activation of PKC in Signal Transduction. H_2O_2 and oxygen radicals have been implicated as second messengers in regulating cell division, chemotaxis, and the action of insulin and other hormones (23–27). In cells treated with oxidants, an increase in serine and tyrosine phosphorylation of some endogenous proteins, including insulin receptor, has been reported (25–27). Although oxygen radicals can inactivate some enzymes and can have toxic effects, they also are capable of activating certain enzymes such as guanylate cyclase (28). Oxidative modification of enzymes studied to date has been shown to result in either activation or inactivation, but not both. PKC, on the other hand, appears to undergo bimodal regulation in response to oxidative modification: initial activation followed by inactivation with further oxidation. This dual effect may provide an important on/off signaling mechanism and supports the suggestion that H_2O_2 and oxygen radicals generated by biological oxidation mechanisms might play a key role in cellular regulation.

¶If the catalytic activity of oxidatively activated PKC is lower than that of initial Ca^{2+}/PL-stimulated, unmodified PKC, this might account for the apparent lower recoveries of activity in peak II.

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