Volume 225, number 1,2, 233-237 FEB 05386 December 1987

Susceptibility of protein kinase C to oxidative inactivation: loss of both phosphotransferase activity and phorbol diester binding

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Received 15 October 1987

Exposure of protein kinase C to low concentrations of either *N*-chlorosuccinimide or H_2O_2 resulted in rapid and parallel loss of phosphotransferase activity and phorbol ester binding. This oxidative inactivation of protein kinase C also occurred in intact cells exposed to a low concentration of H_2O_2. With H_2O_2 treatment the rate of inactivation of protein kinase C in the cytosol of MCF-7 cells was rather slower than that which occurred in the cytosol of PYS cells. However, in both cell types, the oxidative inactivation of membrane-associated protein kinase C occurred rapidly in comparison to the enzyme in the cytosol. Prior treatment of cells with phorbol ester to induce membrane association (stabilization) of protein kinase C, followed by exposure to H_2O_2, resulted in increased inactivation of protein kinase C, suggesting that membrane association of protein kinase C increases its susceptibility to oxidative inactivation

Protein kinase C; Oxidative inactivation; Oxygen radical; Phorbol ester; Membrane binding

1. INTRODUCTION

Ca^{2+}- and phospholipid-dependent protein kinase C plays a crucial role in the signal transduction of a variety of extracellular stimuli and also serves as an intracellular receptor for tumor promoter phorbol esters [1,2]. Tumor promoter treatment of cells has been shown to generate reactive oxygen species which appear to play a major role in the process of tumor promotion [3]. Oxidative inactivation has been reported for a number of proteins including Ca^{2+}-regulated calmodulin and troponin C [4,5]. Loss of both protein kinase C activity and phorbol ester binding in cells treated for prolonged periods of time with phorbol esters has been reported [6–8]. While proteolytic degradation of the activated enzyme does occur in several cell types [6–8], other mechanisms might also be involved in this inactivation process. Here, we show that protein kinase C both in the isolated form and in the intact cells is highly susceptible to oxidative inactivation upon exposure to hydroxyl radicals ($OH^{\cdot}$). This inactivation results in the loss of both phosphotransferase activity and phorbol ester binding.

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Abbreviations: PDBu, phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PYS, parietal yolk sac

2. MATERIALS AND METHODS

N-Chlorosuccinimide and H_2O_2 (30%, w/w) were obtained from Sigma. The concentration of H_2O_2 was standardized by using the spectrophotometric method of Luck [9]. Protein kinase

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00145793/87/$3.50

C was partially purified from bovine brain as described [10] and enzyme having an activity of approx. 400 U/mg protein was used. The protein kinase C preparation was dialyzed to remove 2-mercaptoethanol present in the buffer prior to oxidative modification. Laboratory water (reverse osmosis) was further passed through a deionizer and an activated-charcoal unit to remove traces of *N*-chloramines present. Protein was estimated by the procedure of Lowry et al. [11] using bovine serum albumin as the standard.

PYS and MCF-7 cells were grown in DMEM medium supplemented with 10% fetal calf serum in 150-cm^2 flasks. Cytosolic and membrane fractions were prepared from confluent cells and protein kinase C was isolated from these fractions by DEAE-cellulose chromatography as in [10]. Protein kinase C activity was assayed using histone H1 as substrate [10,12] and phorbol ester binding was measured using [^{3}H]PDBu as ligand [13]. Only the stimulated activity observed in the presence of Ca^{2+} and phospholipid was expressed as units of protein kinase C activity (1 nmol phosphate transferred to histone H1/min at 30°C). Calmodulin, calpain and protein kinase A were measured as described [14–16].

3. RESULTS AND DISCUSSION

N-Chlorosuccinimide is a mild oxidizing agent which modifies oxidation-susceptible surface amino acid residues in proteins [17]. When purified protein kinase C was treated with a low concentration (10 μM) of *N*-chlorosuccinimide for 5 min, about 40% of protein kinase C activity was lost. Exposure of the enzyme to increasing *N*-chlorosuccinimide concentrations up to 100 μM resulted in a progressive loss in phosphotransferase activity (fig.1A). At 100 μM, *N*-chlorosuccinimide caused about 50% loss of enzyme activity within 30 s, and all activity was lost after 5 min of treatment (fig.1B). As shown in fig.1, this oxidation treatment also resulted in a parallel loss of [^{3}H]PDBu binding. This suggests either that catalytic and phorbol ester-binding sites have the same degree of susceptibility to oxidation or that these two sites are influenced by a common oxidation-susceptible domain in the enzyme. Protein kinase C was inactivated when stored (at 4°C) in the absence of reducing agents in buffers

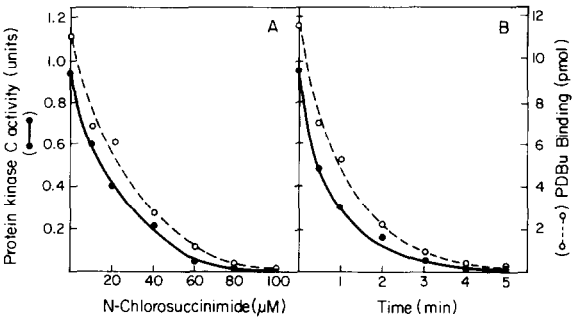

Fig.1. Oxidative inactivation of protein kinase C by *N*-chlorosuccinimide. Purified protein kinase C (approx. 1 U) was treated with the indicated concentrations of *N*-chlorosuccinimide for 5 min (A) or 100 μM *N*-chlorosuccinimide for the indicated time periods (B) and dithiothreitol was then added to a final concentration of 10 mM to prevent further inactivation. Aliquots of the treated samples were assayed for both protein kinase C phosphotransferase activity (●—●) and [^{3}H]PDBu binding (○---○).

prepared with regular laboratory-supplied (reverse osmosis) water, apparently due to traces of *N*-chloramines. No loss in activity was noted if the water used was processed to remove *N*-chloramines as described in section 2.

The oxidatively inactivated protein kinase C exhibited neither phosphotransferase activity nor phorbol ester binding even when the concentrations of components (histone H1, ATP, phospholipids, and PDBu) used in the assay reaction mixtures were increased by 10-times the standard amount. This suggests that the observed losses in activity and PDBu binding after oxidative modification were not due to decreased affinity for these ligands. Even without modification at the catalytic site, alterations of the lipid/phorbol-binding domains alone could result in a decrease in Ca^{2+}- and phospholipid-stimulated activity. Hence, we subjected modified protein kinase C to limited proteolysis using trypsin or calpain II to release active catalytic fragment free of the regulatory portion of the molecule. However, no increase in Ca^{2+}/phospholipid-independent activity was found. Even the M-kinase (Ca^{2+}/phospholipid-independent form) derived from native, unmodified protein kinase C by limited proteolysis [12], was inactivated with exposure to low concen-

trations of *N*-chlorosuccinimide suggesting that both the catalytic and phorbol ester-binding domains of protein kinase C are susceptible to oxidative modification.

Protein kinase C was also inactivated with exposure to a low concentration (5 mM) of H_2O_2 for only a few minutes. This inactivation was prevented by the addition of scavengers of $OH^{\cdot}$ radicals, mannitol (100 mM) and *N*-acetylcysteine (100 mM), suggesting that the $OH^{\cdot}$ radicals formed from H_2O_2 were involved. This oxidative inactivation of protein kinase C by $OH^{\cdot}$ radicals may be of regulatory significance since $OH^{\cdot}$ and other reactive oxygen species are formed in cells under certain physiological and pathological conditions. Several agents such as chemotactic factors (*N*-formyl-methionyl-leucyl-phenylalanine), tumor promoters and anticancer drugs (adriamycin) are known to generate various reactive oxygen species including $OH^{\cdot}$ radicals [17].

While a number of enzymes are inactivated in vitro by reactive oxygen species, only a few enzymes are inactivated by these radicals in intact cells as there are several scavenger mechanisms operative to protect the cell from oxygen toxicity [18]. Further, the binding of endogenous ligands can mask oxidation-susceptible sites thereby protecting some proteins from inactivation. Thus, we tested whether oxidative inactivation of protein kinase C can be observed in intact cells exposed to a low (5 mM) concentration of H_2O_2. As shown in fig.2A, protein kinase C activity decreased in both membrane and cytosol within a few minutes following exposure of intact PYS cells to H_2O_2 and no activity was detectable after 30 min of treatment. The inactivation of membrane-bound protein kinase C was found to be more rapid under these conditions. With MCF-7 cells, about 75% of membrane-associated protein kinase C activity was lost within 30 min of exposure to 5 mM H_2O_2 (fig.2B). In contrast, there was little change in cytosolic protein kinase C activity with treatment of intact MCF-7 cells with 5 mM H_2O_2 for time periods up to 30 min. However, by raising the concentration of H_2O_2 used to treat MCF-7 cells to 15 mM, cytosolic protein kinase C also was inactivated, although at a slower rate than that noted for the loss of membrane-associated activity. Under all these conditions described the loss of protein kinase C activity correlated with a corresponding decrease in [^{3}H]PDBu binding (table 1).

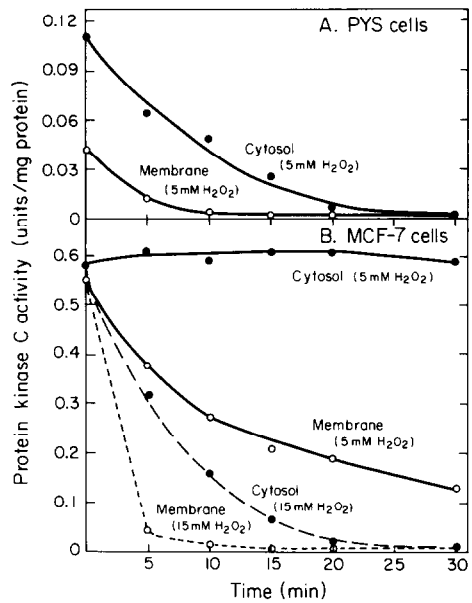

Fig.2. Oxidative inactivation of protein kinase C in intact cells treated with H_2O_2. PYS (A) and MCF-7 (B) cells ($4–6 \times 10^7$) were treated with either 5 or 15 mM H_2O_2 as noted at 37°C for the indicated time intervals. Protein kinase C activity present in the cytosolic and detergent-solubilized membrane fractions was determined.

The relatively rapid inactivation of protein kinase C in the membrane fraction might be due to enhanced lipid peroxidation by $OH^{\cdot}$ radicals. Lipid peroxidation (chain reaction) in turn can generate other reactive oxygen species [19]. Alternatively, the membrane-bound protein kinase C may be in a form with increased susceptibility to oxidation. Most probably, the cytosolic protein kinase C in MCF-7 cells might be protected by catalase, glutathione peroxidase and other scavenger mechanisms which may be less operative in PYS cells. Although calmodulin is oxidatively inactivated in the isolated form [4,5], no inactivation of this protein occurred in H_2O_2-treated intact cells under these conditions (unpublished). Similarly, other related enzymes like Ca^{2+}-activated protease (calpain) and cAMP-stimulated protein kinase A were also not inactivated under these conditions suggesting that high susceptibility to oxidative inactivation may be limited to few en-

Table 1

Loss of [^{3}H]PDBu binding in PYS and MCF-7 cells treated with H_2O_2

Cell type	Treatment	[^{3}H]PDBu binding (pmol)	
		Cytosol	Membrane
PYS cells	control	0.8	0.5
	5 mM H_2O_2, 10 min	0.3	0.1
	5 mM H_2O_2, 30 min	0	0
MCF-7 cells	control	5.3	3.1
	5 mM H_2O_2, 10 min	5.1	1.4
	5 mM H_2O_2, 30 min	5.4	0.8
	15 mM H_2O_2, 10 min	3.2	0.1
	15 mM H_2O_2, 30 min	0.2	0

Both PYS cells (6×10^7) and MCF-7 cells (4×10^7) were treated with low (5 mM) and high (15 mM) H_2O_2 concentrations for the indicated periods of time. Protein kinase C present in the cytosolic and membrane fractions was isolated by DEAE-cellulose chromatography as described in section 2 and [^{3}H]PDBu binding was then determined

zymes such as protein kinase C in intact cells.

Since phorbol esters induce the redistribution of protein kinase C from cytosol to plasma membrane [10,20,21], we investigated whether this shift in protein kinase C from cytosol to membrane leads to increased inactivation of protein kinase C upon exposure to low concentrations (5 mM) of H_2O_2 (table 2). As previously noted in fig.2B, exposure of control (not treated with TPA) MCF-7 cells to 5 mM H_2O_2 caused only a slight change in cytosolic protein kinase C activity, such that total (membrane plus cytosolic) activity was decreased by only 25% under these conditions (table 2). However, with MCF-7 cells pretreated with TPA for 1 h to enhance protein kinase C association with membranes, exposure to 5 mM H_2O_2 resulted in a much greater (60%) loss in total protein kinase C activity, again accounted for by inactivation of the enzyme associated with the membrane fraction. This further indicates that membrane binding of protein kinase C increases its susceptibility to oxidative inactivation.

The disappearance of protein kinase C (both activity and immunoreactive protein) in cells treated for prolonged (24 h) periods with TPA has been reported [6–8]. It has been postulated that calpain-mediated proteolysis of membrane-bound enzyme may be responsible for this desensitization [6–8]. In addition to promoting the association of protein kinase C with membranes [10,20,21], TPA can also promote the formation of reactive oxygen radicals [3]. TPA treatment has also been shown to decrease the activity of scavenger enzymes like superoxide dismutase and catalase [22]. In view of the present results which demonstrate increased susceptibility of membrane-bound protein kinase C to oxidative inactivation, it is conceivable that increased oxidative inactivation of protein kinase

Table 2

Susceptibility of TPA-induced membrane-associated protein kinase C to oxidative inactivation

Treatment	Protein kinase C activity (units)			[^{3}H]PDBu binding (pmol)		
	Cytosol	Membrane	Total	Cytosol	Membrane	Total
Control	4.1	1.5	5.6	4.9	3.3	8.2
TPA (1 h)	1.5	3.7	5.2	1.8	6.9	8.7
5 mM H_2O_2	3.9	0.3	4.2	5.1	0.9	6.0
TPA followed by 5 mM H_2O_2	1.7	0.6	2.3	1.6	1.5	3.1

Confluent MCF-7 cells (4×10^7) were treated with 100 nM TPA for 1 h where indicated and then both TPA-treated and control cells were exposed to 5 mM H_2O_2 for 30 min

C may occur in TPA-treated cells. This may occur in addition to, or along with, proteolytic inactivation. In fact, oxidatively modified proteins have been reported to show enhanced susceptibility to proteolysis [23].

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