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Activation of hepatocyte protein kinase C by redox-cycling quinones

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The effects of quinone-generated active oxygen species on rat hepatocyte protein kinase C were investigated. The specific activity of cytosolic protein kinase C was increased 2–3-fold in hepatocytes incubated with the redox-cycling quinones, menadione, duroquinone or 2,3-dimethoxy-1,4-naphthoquinone, without alterations in particulate protein kinase C specific activity or Ca^{2+}- and lipid-independent kinase activities. Redox-cycling quinones did not stimulate translocation of protein kinase C; however, activated protein kinase C was redistributed from cytosol to the particulate fraction when quinone-treated hepatocytes were exposed to 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Quinone treatment did not alter cytosolic phorbol 12,13-dibutyrate (PDBu) binding capacity, and the cytosol of both control and quinone-treated hepatocytes exhibited a K_d for PDBu binding of 2 nM. Quinone-mediated activation of cytosolic protein kinase C was reversed by incubation with 10 mM-β-mercaptoethanol, dithiothreitol or GSH, at 4 °C for 24 h. Furthermore, protein kinase C specific activity in control cytosol incubated in air increased by over 100% within 3 h; this increase was reversed by thiol-reducing agents. Similarly, incubation of partially-purified rat brain protein kinase C in air, or with low concentrations of GSSG in the presence of GSH, resulted in a 2–2.5-fold increase in Ca^{2+}- and lipid-dependent kinase activity. In contrast with the effects of the redox-cycling quinones, when hepatocytes were treated with the thiol agents *N*-ethylmaleimide (NEM), *p*-benzoquinone (*p*BQ) or *p*-chloromercuribenzoic acid (*p*CMB), the cytosolic Ca^{2+}- and lipid-dependent kinase activity was significantly inhibited, but the particulate-associated protein kinase C activity was unaffected. The Ca^{2+}- and lipid-independent kinase activity of both the cytosolic and particulate fractions was significantly stimulated by NEM, but was unaffected by *p*BQ and *p*CMB. These results show that hepatocyte cytosolic protein kinase C is activated to a high-$V_{max.}$ form by quinone-generated active oxygen species, and this effect is due to a reduction-sensitive modification of the thiol/disulphide status of protein kinase C.

INTRODUCTION

The regulation of cellular growth, differentiation and function is critically dependent upon transduction of information from extracellular signals to various control elements within the cell. One of the major signal-transducing pathways involves the Ca^{2+}- and phospholipid-dependent, *sn*-1,2-diacylglycerol-stimulated protein kinase (protein kinase C) [1,2]. Protein kinase C activation appears to mediate a wide variety of cellular responses produced by both physiological and non-physiological stimuli; these stimuli include various growth factors, hormones, neurotransmitters and tumour promoters [2,3]. Physiological activation of protein kinase C depends on interaction of the enzyme with acidic phospholipid (in particular phosphatidylserine, presumably associated with intracellular membranes), Ca^{2+} and diacylglycerol [1,4]. Diacylglycerol is generated from inositol phospholipids by agonist-stimulated, receptor-mediated activation of phospholipase C. Phospholipase C-dependent breakdown of inositol phospholipid also produces the important second messenger, inositol 1,4,5-trisphosphate, which has been demonstrated to stimulate the release of Ca^{2+} from the endoplasmic reticulum (see [1,5] for reviews).

Recent studies by our laboratory and others have examined the effects of oxidative stress on various cellular functions. The generation of active oxygen species within, or in the vicinity of, virtually all aerobic cells occurs by a variety of physical, chemical, and biological mechanisms (for reviews, see [6–8]). Experimental systems commonly used to expose various types of cells and tissues to oxidative stress include enzymic generation of active oxygen species (e.g. using xanthine/xanthine oxidase [9]), direct exposure to peroxides or pro-oxidants [10] and intracellular generation of active oxygen species by treatment with redox-cycling chemicals such as quinones or bipyridilium compounds [11–13]. Notable consequences of cellular oxidative stress include marked alterations in the regulation of intermediary metabolism [10,14], disruption of intracellular thiol and Ca^{2+} homeostasis [15,16] and tumour promotion [17,18]. Certain biochemical effects associated with oxidant-induced tumour promotion, e.g. induction of transcription of the

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PMSF, phenylmethanesulphonyl fluoride; PS, L-α-phosphatidyl-L-serine; DAG, 1,2-dioleoyl-*sn*-glycerol; NEM, *N*-ethylmaleimide; PDBu, phorbol 12,13-dibutyrate; *p*CMB, *p*-chloromercuribenzoic acid; *p*BQ, *p*-benzoquinone; menadione, 2-methyl-1,4-naphthoquinone; duroquinone, 2,3,5,6-$(CH_3)_4$-benzoquinone; 2,3-$(MeO)_2$NQ, 2,3-dimethoxy-1,4-naphthoquinone; Me_2SO, dimethyl sulphoxide.

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proto-oncogenes c-*myc* and c-*fos* [9], are also associated with protein kinase C activation [19,20].

Protein kinase C is a cysteine-rich enzyme [21]. The exact functions of specific cysteine residues in protein kinase C are not known; however, it is possible that modification of the thiol redox status of one or more of these cysteine residues may alter protein kinase C function. Because several signal-transduction-dependent processes involving protein kinase C result in biochemical responses that are also produced by oxidative stress, in particular, responses relevant to tumour promotion, we examined the effects of oxidative stress on protein kinase C. In this study, we have used redox-cycling quinones to generate non-cytotoxic levels of intracellular active oxygen species. We found that protein kinase C was activated to a high-$V_{max.}$ form, and that activation of protein kinase C was due to reduction-sensitive modification of the thiol/disulphide status of the enzyme. Thus, modulation of protein kinase C activity by thiol oxidation may be an important factor in responses of cells to oxidative stress.

MATERIALS AND METHODS

Materials

Collagenase (grade II), glutathione reductase (from yeast), phenylmethanesulphonyl fluoride (PMSF), ATP, dithiothreitol and leupeptin were purchased from Boehringer, Mannheim, Germany. Histone type III-S, L-α-phosphatidyl-L-serine (PS), 1,2-dioleoyl-*sn*-glycerol (DAG), β-mercaptoethanol, GSH, GSSG, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), *N*-ethylmaleimide (NEM), phorbol 12,13-dibutyrate (PDBu) and *p*-chloromercuribenzoic acid (*p*CMB) were obtained from Sigma Chemical Co. (St. Louis, MO. U.S.A.). DEAE-cellulose (DE-52) was from Whatman International (Maidstone, Kent, U.K.). [γ-^{32}P]ATP (5000 Ci/mmol) and [U-^{3}H]PDBu (18.9 Ci/mmol) were obtained from New England Nuclear. 2-Methyl-1,4-naphthoquinone (menadione) and 2,3,5,6-$(CH_3)_4$-benzoquinone (duroquinone) were obtained from Chemical Dynamics (South Plainfield, NJ, U.S.A.) and Aldrich Chemical Co., respectively. 2,3-Dimethoxy-1,4-napthoquinone [2,3-$(MeO)_2$NQ] was a gift from Dr. G. M. Cohen (School of Pharmacy, University of London, U.K.) *p*-Benzoquinone (*p*-BQ) (Merck, Darmstadt, Germany) was purified by sublimation prior to use. All other chemicals were of at least analytical grade.

Hepatocyte isolation and incubation

Male Wistar rats (ALAB AB, Sollentuna, Sweden; 200–250 g, fed *ad libitum*) were used in all experiments. Hepatocytes were isolated between 10:00 and 11:00 am by collagenase perfusion of the liver as previously described [22]. More than 90% of the cells excluded Trypan Blue. Hepatocytes were preincubated for 30–60 min, at a concentration of 10^6 cells/ml, in modified Krebs–Henseleit buffer (pH 7.4) (described in [22]), supplemented with 12.5 mM-Hepes in rotating, round-bottom flasks at 37 °C under an atmosphere of O_2/CO_2 (19:1) before addition of the test compounds. All test compounds were dissolved in Me_2SO at concentrations such that the final concentration of Me_2SO in the hepatocyte incubations did not exceed 0.25% (v/v).

Preparation of protein kinase C fractions from isolated hepatocytes

Hepatocyte fractionation methods were adapted from [23]. Aliquots of the hepatocyte incubations (10^7 cells) were collected, centrifuged for 5 s at 500 *g* and the cell pellets were resuspended in 2 ml of ice-cold buffer A (20 mM-Tris/HCl/250 mM-sucrose/10 mM-EGTA/2 mM-EDTA, pH 7.5 at 4 °C) supplemented with 0.1 mM-PMSF and 100 μg of leupeptin/ml; PMSF and leupeptin were added to the buffer immediately prior to use. The cell suspensions were then sonicated on ice using a Soniprep 150 probe sonicator (4 × 10 s bursts; 10 μm amplitude). The resulting homogenates were centrifuged at 100000 *g* for 60 min and the supernatants were collected for determining 'cytosolic' protein kinase C activity. The pellets were rinsed with cold buffer A and then resuspended by hand in buffer A containing 0.2% (v/v) Triton X-100, using a glass–glass homogenizer. The resuspended pellets were incubated for 45 min at 4 °C, then centrifuged at 100000 *g* for 30 min. The supernatant was collected for determining 'particulate-associated' protein kinase C activity. Protein content of the protein kinase C fractions was determined as in [24], using crystallized and lyophilized bovine serum albumin as the standard. All protein kinase C samples were kept frozen at −70 °C until use.

Partial purification of protein kinase C

Protein kinase C from hepatocyte cytosol or rat brain cytosol [supernatant from a 60 min, 100000 *g* centrifugation of a 25% (w/v) whole brain homogenate in buffer A containing 0.1 mM-PMSF, 100 μg of leupeptin/ml and 6 mM-β-mercaptoethanol] was partially purified by DE-52 column chromatography as reported in [25], except the cyclic AMP was omitted from the first column elution step. Typically, protein kinase C specific activity was increased 10–50-fold by the column purification procedure.

Determination of protein kinase C activity

Methods for determining cytosolic and particulate-associated hepatocyte protein kinase C activity were adapted from [26]. Ca^{2+}-activated, phospholipid-dependent kinase activity was determined at 30 °C in assays containing (in a final volume of 150 μl) 10 μM-[γ-^{32}P]ATP (0.2–0.3 μCi), 200 μg of histone III-S/ml, 20 μg of PS/ml, 1 μg of DAG/ml, 5 mM-$CaCl_2$, 20 μM-Tris/HCl, pH 7.5 at 30 °C, and up to 20 μg of protein (in 40 μl of buffer A) from hepatocyte fractions, or 20–50 ng of partially purified brain protein kinase C, which was added last to initiate the assay. Ca^{2+}- and lipid-independent kinase activity was assayed by replacing Ca^{2+} and the lipids (PS and DAG) in the assay mixture with 0.5 mM-EGTA and 20 mM-Tris/HCl buffer (pH 7.5) respectively. Concentrations of histone, ATP and cofactors were not rate-limiting, so that the protein kinase C assay was run under apparent $V_{max.}$ conditions. Autophosphorylation was quantified by omitting histone from the assays, and filter blanks were obtained by replacing the protein kinase C sample with an equal amount of bovine serum albumin. Assay reactions containing partially purified brain protein kinase C or hepatocyte protein kinase C were stopped after 5 or 15 min respectively, by addition of 0.5 ml of 50% (w/v) trichloroacetic acid at 4 °C, and samples were stored at 4 °C for 1 h to allow for protein

precipitation. Samples were then collected on 13 mm Sartorius nitrocellulose filters (pore size 0.45 μm) by vacuum filtration. The filters were rinsed four times with 0.75 ml of 10 % (w/v) trichloroacetic acid, and the radioactivity of the acid-precipitated material trapped on the filters was determined using a Beckman LS 1801 liquid scintillation counter (Instagel scintillation cocktail, Packard). Protein kinase C activity was defined as histone kinase activity in the presence of Ca^{2+}, PS and DAG, minus the histone kinase activity in the absence of these activators.

[^{3}H]PDBu binding to protein kinase C

Binding of [^{3}H]PDBu to hepatocyte protein kinase C was measured using methods derived from the polyethylenimine filter receptor binding procedure reported in [27]. Standard incubations (final volume of 200 μl) were run for 20 min at 30 °C and contained 20 mM-Tris/HCl, pH 7.2 at 30 °C, 100 mM-KCl, 5 mM-$CaCl_2$, 4 mg of bovine serum albumin/ml, 100 μg of PS/ml, 1.25 % (v/v) Me_2SO, and 0–5 nM-[^{3}H]PDBu. Non-specific [^{3}H]PDBu binding was measured by including an excess of unlabelled PDBu (15 μM final concentration) in parallel incubations. The binding assays were stopped by addition of 3 ml of ice-cold 0.5 % (v/v) Me_2SO, followed by rapid vacuum filtration of the samples on to Whatman GF/B filters presoaked for at least 2 h in a 0.5 % (v/v) solution of polyethylenimine. The filters were rinsed four times with 3 ml of 0.5 % Me_2SO, air dried and the radioactivity trapped on the filters was quantified by liquid scintillation counting.

Chemical reduction and oxidation of protein kinase C

Reducing agents (GSH, β-mercaptoethanol or dithiothreitol) were dissolved in buffer B (20 mM-Tris/HCl, 10 mM-EGTA, 2 mM-EDTA, pH 7.5 at 4 °C), which had been bubbled with argon gas. Aliquots of cytosol from control or quinone-treated hepatocytes were rapidly mixed with equal volumes of the thiol-reducing solutions in vials fitted with rubber septa; the final concentration of reducing agent in each reaction mixture was 10 mM. The reaction mixtures were gassed with argon for 5 min at 4 °C and were then incubated at 4 °C. After 24 h, the reaction mixtures were diluted with buffer B, and aliquots were immediately assayed for protein kinase C activity as described above.

Oxidation of partially-purified rat brain protein kinase C was carried out in a glutathione redox buffer consisting of buffer A supplemented with 1 mM-β-mercaptoethanol and 4 mM-GSH, plus increasing concentrations of GSSG. Incubations were run at 22 °C, exposed to air, using a final protein concentration of 1 μg/ml; after 30 min, aliquots were assayed for protein kinase C activity. Commercial GSH preparations typically contain 1–2 % GSSG (for example, a freshly prepared 4 mM-GSH solution contains approx. 30 μM-GSSG, as determined by h.p.l.c. [28]). To obtain GSH solutions containing very low concentrations of GSSG, stock solutions of GSH (16 mM) in buffer A containing 1 mM-β-mercaptoethanol were preincubated for 2–5 min at 22 °C with glutathione reductase (10 units/ml) and 2 mM-NADPH before dilution and incubation with brain protein kinase C, as described above. The presence of glutathione reductase and NADPH did not affect protein kinase C activity.

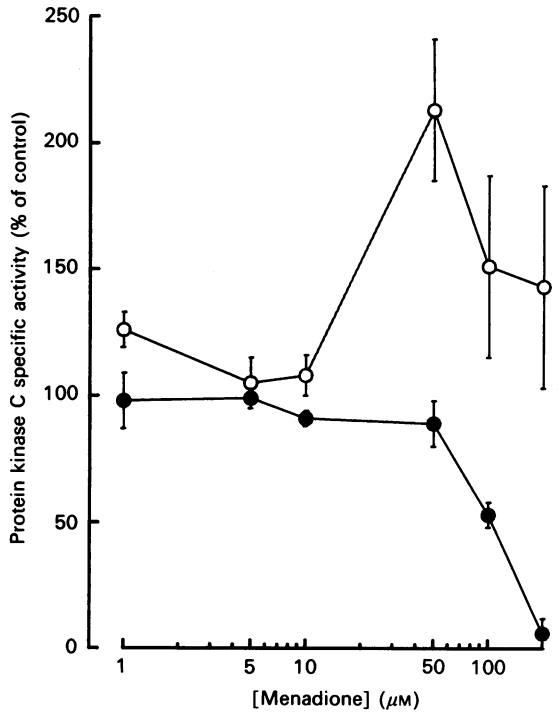

Fig. 1. Dose-response for activation of protein kinase C by menadione

Hepatocytes were incubated with increasing concentrations of menadione for 30 min before isolation of the cytosolic (○) and particulate-associated (●) protein kinase C fractions. The highest concentration of menadione tested was 200 μM. Protein kinase C specific activity was measured as described in the Materials and methods section. Each point represents the mean ± S.E.M. of three to five independent experiments; control cytosolic and membrane-bound protein kinase C specific activities were 14.7 ± 4.6 and 32.9 ± 3.9 pmol of [^{32}P]P_i incorporated into histone/min per mg of protein respectively.

RESULTS

Effects of menadione on protein kinase C activity

We have previously reported that incubation of isolated hepatocytes with redox-cycling quinones such as menadione and duroquinone is a useful model for studying the effects of active oxygen species on cellular systems [11,29]. When isolated hepatocytes were exposed to between 5 and 50 μM-menadione for 30 min, there was a concentration-dependent increase in the specific activity of cytosolic protein kinase C, but no significant change in the particulate-associated protein kinase C activity (Fig. 1). At concentrations above 50 μM-menadione, both cytosolic and particulate-associated protein kinase C activities decreased. Inhibition of protein kinase C by the higher concentrations of menadione (100 and 200 μM) was most probably due to some arylation of the kinase by menadione [30]. For all menadione concentrations tested, the cytosolic and particulate Ca^{2+}- and phospholipid-independent kinase activities did not change (results not shown). No loss of hepatocyte viability (as assessed by Trypan Blue exclusion) was observed at any of the menadione concentrations tested. At menadione concentrations below 100 μM, the hepatocytes showed no detectable morphological changes; thus, the increase in

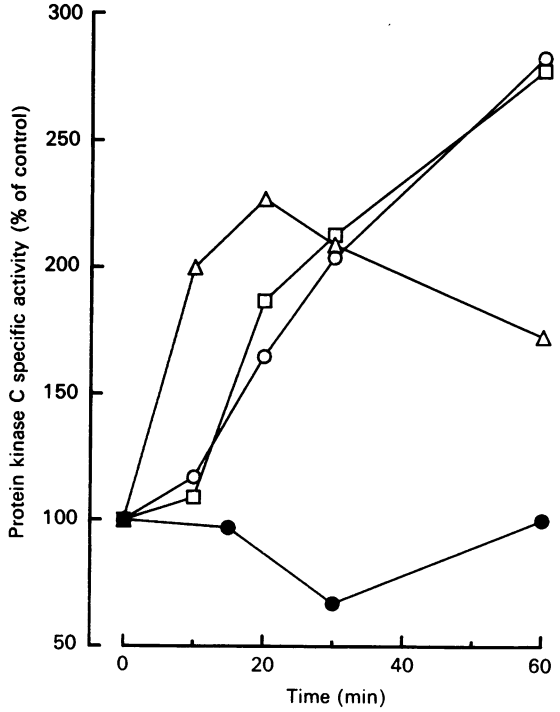

Fig. 2. Time-dependent activation of cytosolic protein kinase C by redox-cycling quinones

Hepatocytes were incubated with menadione (50 μM), duroquinone (250 μM) or 2,3-$(MeO)_2$NQ (250 μM) for the indicated time periods. The cytosolic protein kinase C fractions were then isolated and assayed as described in the Materials and methods section. Each point represents the mean of two or three separate experiments. Symbols are: ●, control; △, menadione; ○, duroquinone; □, 2,3-$(MeO)_2$NQ.

specific activity of cytosolic protein kinase C induced by menadione occurred without any observable cytotoxicity. However, at higher menadione concentrations (100 and 200 μM) there was significant plasma-membrane blebbing.

Time-dependent activation of cytosolic protein kinase C by menadione, duroquinone, and 2,3-$(MeO)_2$NQ

Incubation of hepatocytes with menadione (50 μM), duroquinone (250 μM) or 2,3-$(MeO)_2$NQ (250 μM) induced a time-dependent increase in protein kinase C specific activity (Fig. 2). Menadione induced a rapid increase in cytosolic protein kinase C activity (225 % of control at 20 min), that was followed by a decrease to 175 % of control by 60 min. In contrast with menadione, the pure redox-cycling quinones, duroquinone [29] and 2,3-$(MeO)_2$NQ [31], induced a steady increase in cytosolic protein kinase C specific activity up to 60 min. As observed in the menadione concentration–response experiment, cytosolic Ca^{2+}- and lipid-independent kinase activity remained constant over the entire incubation period (for all three quinones), and no change in particulate-associated protein kinase C specific activity was observed. Ca^{2+}- and lipid-dependent autophosphorylation of cytosol from control and quinone-treated hepatocytes was not detectable, demonstrating that the quinone-mediated increase in protein kinase C specific activity was due only to an increase in the rate of histone phosphorylation.

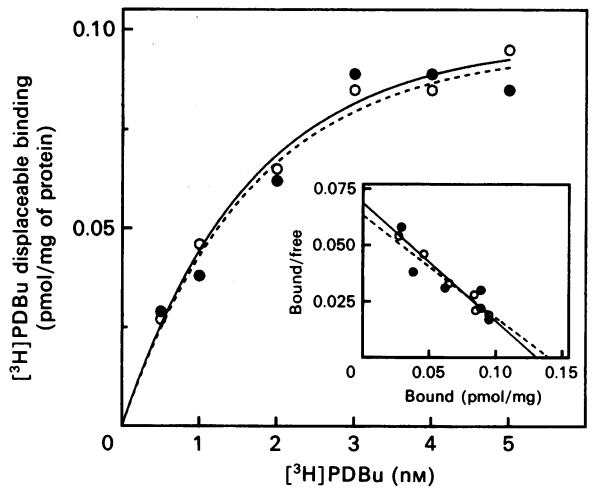

Fig. 3. [^{3}H]PDBu binding to hepatocyte cytosolic protein kinase C

The cytosolic protein kinase C fraction was isolated from control hepatocytes (○; broken line) or hepatocytes treated with menadione (50 μM; 30 min) (●; solid line), and displaceable [^{3}H]PDBu binding to the cytosolic fractions was measured as described in the Materials and methods section. One experiment representative of three is shown. Inset: Scatchard plot analysis of the [^{3}H]PDBu binding data. Similar results were obtained when [^{3}H]PDBu binding to control cytosol was compared with the binding to cytosol isolated from cells treated with duroquinone (250 μM; 30 min).

Several chemicals are known to interact with and directly activate protein kinase C (e.g. tumour-promoting phorbol esters [2], chloroform [32] and sulphatide [33]). When menadione, duroquinone or 2,3-$(MeO)_2$NQ were added directly to control cytosolic protein kinase C preparations (at 50, 250, and 250 μM respectively), they did not alter protein kinase C activity (results not shown).

[^{3}H]PDBu-displaceable binding and PS- and Ca^{2+}-dependence of cytosolic protein kinase C isolated from control and quinone-treated hepatocytes

Cytosol from control and duroquinone- or menadione-treated hepatocytes exhibited measurable [^{3}H]PDBu binding (Fig. 3). [^{3}H]PDBu binding was saturable and displaceable by an excess of unlabelled PDBu; there was no detectable difference in [^{3}H]PDBu binding between the control and activated protein kinase C fractions. Scatchard analysis yielded similar K_d values of 2.3 and 2.1 nM for control and activated protein kinase C respectively, and the quinone treatment did not alter [^{3}H]PDBu binding capacity (Fig. 3, inset). In the absence of added PS, cytosol from both control and duroquinone-treated hepatocytes exhibited significant Ca^{2+}-stimulated kinase activity (50–80 % of maximal activity), indicating that the crude fractions contained considerable concentrations of acidic phospholipids. After partial purification (without β-mercaptoethanol) of the control and quinone-treated cytosolic protein kinase C preparations, no histone phosphorylation activity was detected in the absence of added PS. There was no difference in the PS-concentration-dependence for activation of protein kinase C between the two partially-

Table 1. TPA-stimulated translocation of activated cytosolic protein kinase C isolated from hepatocytes treated with the redox-cycling quinone, duroquinone

Isolated hepatocytes (10^7 cells in 10 ml) were incubated as described in the Materials and methods section with 0.25% (v/v) Me_2SO for 45 min (control), 50 nM-TPA for 15 min (TPA), 250 μM-duroquinone for 30 min (DQ), TPA (50 nM, 15 min) followed by duroquinone (250 μM, 30 min) (TPA → DQ), or duroquinone (250 μM, 30 min) followed by TPA (50 nM, 15 min) (DQ → TPA). Cytosolic and particulate-associated protein was then isolated and assayed for protein kinase C activity. Values represent means ± S.E.M. of experiments run on three separate hepatocyte preparations.

Treatment	Protein fraction	Protein kinase C specific activity*	Protein kinase C activity (mU/10^7 cells)†		Net change in protein kinase C‡	
Control	Cytosolic	12.2 ± 2.1	50		–	
	Particulate	14.0 ± 2.1	33		–	
			Total	83	–	
TPA	Cytosolic	9.3 ± 1.0	38		−12	
	Particulate	19.6 ± 3.5	46		+13	
			Total	84	Total	+ 1
DQ	Cytosolic	21.3 ± 3.9	88		+38	
	Particulate	10.8 ± 3.0	25		− 8	
			Total	113	Total	+30
TPA → DQ	Cytosolic	8.6 ± 1.8	36		−14	
	Particulate	16.2 ± 0.7	38		+ 5	
			Total	74	Total	− 9
DQ → TPA	Cytosolic	10.9 ± 1.8	45		− 5	
	Particulate	29.1 ± 2.9	68		+35	
			Total	113	Total	+30

* pmol of $[^{32}P]P_i$ incorporated into histone type III-S/min per mg of cytosolic or particulate protein.

† One unit (U) of activity catalyses the incorporation of 1 nmol of $[^{32}P]P_i$ from [γ-^{32}P]ATP into histone type III-S per min at 30 °C. Units of protein kinase C activity were derived from specific activities by correcting for the protein content of the cytosolic and particulate-associated protein fractions (4.13 ± 0.26 and 2.32 ± 0.13 mg per 10^7 cells respectively; means ± S.E.M., $n = 15$).

‡ Increase (+) or decrease (−) in protein kinase C (units), relative to the activity of the corresponding control protein fraction.

purified preparations, and no difference in the Ca^{2+} requirements for protein kinase C activity in cytosol prepared from control or duroquinone-treated hepatocytes (results not shown).

Translocation of activated protein kinase C

This series of experiments was designed to determine if activation of protein kinase C by active oxygen species disrupted the ability of TPA to induce translocation of protein kinase C from the cytosol to the particulate fraction of hepatocytes. In control hepatocytes, 60% of the total protein kinase C activity was recovered in the cytosolic fraction and 40% was associated with the particulate fraction (Table 1). Treatment of control cells with 50 nM-TPA for 15 min caused a redistribution of protein kinase C activity from the cytosol to the particulate fraction, without altering the total protein kinase C activity of the hepatocytes. When hepatocytes were pretreated with duroquinone for 30 min, total hepatocyte protein kinase C activity increased due to a rise in cytosolic protein kinase C specific activity. Under these conditions, there was no significant change in the particulate-associated activity. Subsequent exposure of quinone-treated cells to TPA (for 15 min) caused nearly quantitative translocation of the activated cytosolic protein kinase C to the particulate fraction (Table 1). Treatment of cells with TPA for 15 min prior to administration of duroquinone stimulated protein kinase C translocation and prevented the quinone-induced increase in total hepatocyte protein kinase C activity.

Effect of thiol-reducing agents on protein kinase C

Incubation of control protein kinase C and activated protein kinase C from quinone-treated hepatocytes with thiol-reducing agents decreased the protein kinase C activities of the two preparations to essentially the same final specific activity, which was around 70% of the specific activity of control cytosolic protein kinase C incubated in the absence of reducing agents (Fig. 4). The decrease in control protein kinase C activity in response to treatment with reducing agents suggested that the enzyme was susceptible to oxidative activation if manipulated in the absence of reductants. We further investigated this phenomenon and found that when cytosolic protein kinase C fractions from untreated hepatocytes were incubated in air at 30 °C in 20 mM-Tris/HCl, pH 7.5, the specific activity of protein kinase C had increased to 220% of the zero-time level by 3 h (Fig. 5). The elevated specific activity of control protein kinase C incubated in air was rapidly decreased to its original activity following the addition of 5 mM-β-mercaptoethanol. To examine whether the observed increase in kinase activity could be attributed to a modification of the thiol/disulphide status of the protein kinase C enzyme molecule, we studied the effects of glutathione redox buffers on protein kinase C activity. On incubation of partially-purified rat brain protein kinase C with GSH essentially free of GSSG (i.e. GSH/GSSG ratio > 1000), a condition which was achieved by preincubating GSH with glutathione reductase and NADPH [34], protein kinase C activity

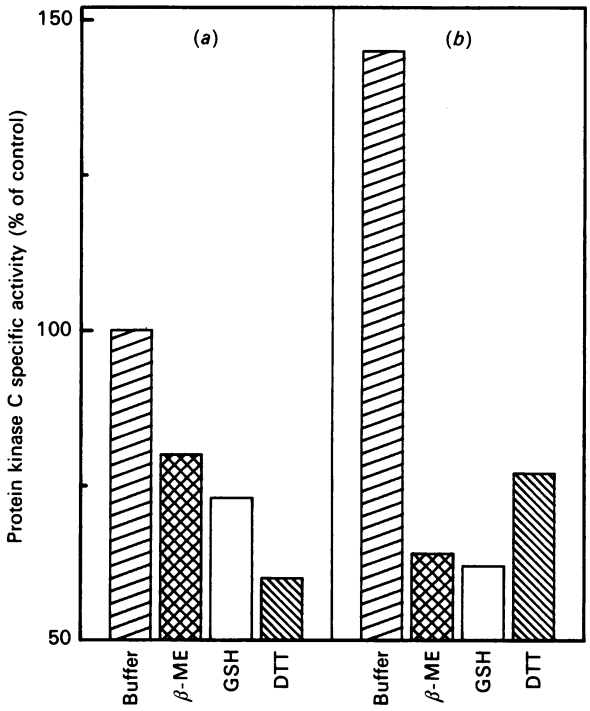

Fig. 4. Effect of thiol-reducing agents on cytosolic protein kinase C activity

Protein kinase C fractions from control (*a*) and duroquinone-treated hepatocytes (250 μM; 30 min incubation) (*b*) were incubated under argon for 24 h at 4 °C, in the presence of buffer B or buffer B plus β-mercaptoethanol (β-ME; 10 mM), GSH (10 mM) or dithiothreitol (DTT) (10 mM) prior to assay of protein kinase C activity. Details are given in the Materials and methods section. The specific activity of the buffer-treated control fraction was arbitrarily set to 100%. One experiment representative of four is shown.

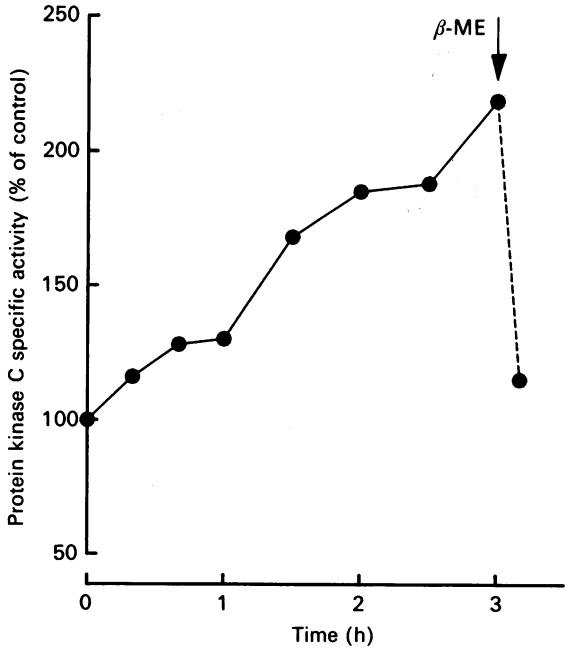

Fig. 5. Time-dependent activation of cytosolic protein kinase C by air

The cytosolic protein kinase C fraction was isolated from untreated hepatocytes as described in the Materials and methods section. After dilution to a final protein concentration of 0.4–0.5 mg/ml with 20 mM-Tris/HCl, pH 7.5, the samples (total volume = 0.75 ml) were incubated in plastic tubes at 30 °C and aliquots were removed at the indicated time points and immediately assayed for protein kinase C activity. At 3 h, β-mercaptoethanol (β-ME; 5 mM) was added and protein kinase C activity measured after 5 min. One experiment representative of three is shown, and each point represents the mean of duplicate incubations.

remained unaltered. However, in the presence of GSSG, a 2.5-fold increase in Ca^{2+}- and lipid-dependent kinase activity was observed (Table 2); a near-maximum increase in activity was observed with GSH/GSSG ratios as high as 130. Similarly, brain protein kinase C exposed to air for 30 min at 22 °C, in the absence of reducing agents, exhibited a 2-fold higher activity than control enzyme incubated in the presence of the reducing agents β-mercaptoethanol or GSH plus glutathione reductase and NADPH (Table 2).

Alteration of protein kinase C activity by thiol alkylating and arylating agents

In order to compare the effects of active oxygen species on protein kinase C with those of thiol arylating and alkylating agents, hepatocytes were incubated with NEM, *p*BQ or *p*CMB for 10 min before isolating protein kinase C. All three agents significantly decreased cytosolic protein kinase activity measured in the presence of Ca^{2+} and lipid (Table 3), whereas the particulate kinase activity measured in the presence of Ca^{2+} and lipid was not significantly altered by *p*BQ and *p*CMB, and was somewhat increased by NEM. In both the cytosolic and particulate-associated protein kinase C fractions, NEM markedly stimulated Ca^{2+}- and lipid-independent kinase

Table 2. Effect of GSSG and exposure to air on brain protein kinase C activity

Partially-purified rat brain protein kinase C was incubated at 22 °C for 30 min in buffer A supplemented with β-mercaptoethanol (1 mM) and containing GSH (4 mM) and various concentrations of GSSG, as described in the Materials and methods section. Oxidation of protein kinase C by air was achieved by incubating protein kinase C as described above in buffer A without β-mercaptoethanol. The protein concentration was 1 μg of protein/ml. Data are expressed as percentages of the activity measured in the absence of GSSG (100% = 68.3±9.5 nmol/min per mg of protein)

Treatment	Protein kinase C (% activity)
GSH + 0 μM-GSSG	100
GSH + 30 μM-GSSG	242
GSH + 280 μM-GSSG	242
GSH + 530 μM-GSSG	252
Air (no reducing agent)	215

activity; more than 90% of the kinase activity measured in the presence of Ca^{2+} and lipid was in fact attributable to the NEM-stimulated, Ca^{2+}- and lipid-independent

Table 3. Effect of thiol alkylating and arylating agents on hepatocyte protein kinase C activity

Hepatocytes were incubated for 10 min in the absence or presence of NEM (100 μM), *p*BQ (100 μM) or *p*CMB (100 μM) before isolation of cytosolic and particulate-associated protein kinase C fractions. Histone phosphorylation activity was assayed as described in the Materials and methods section. Data are expressed as means ± S.E.M. of three separate experiments. * $P < 0.05$; ** $P < 0.005$.

		Protein kinase C activity (pmol of $[^{32}P]P_i$/min per mg of protein)			
	Fraction...	Cytosolic		Particulate	
Treatment	Conditions...	Ca^{2+}, PS	EGTA	Ca^{2+}, PS	EGTA
Control		9.1±0.9	2.1±0.5	10.1±1.7	4.3±1.0
NEM		4.9±0.9*	5.0±0.1**	15.1±3.1	13.6±2.4*
*p*BQ		4.3±1.6*	2.7±0.8	9.1±2.1	5.5±0.8
*p*CMB		2.5±1.0**	2.3±1.4	6.8±1.7	6.5±1.5

kinase activity. Similar effects were produced by *p*BQ and *p*CMB, though to a lesser extent than with NEM.

DISCUSSION

In the present investigation, we have studied the effects of active oxygen species on protein kinase C in hepatocytes. Active oxygen species were generated using the redox-cycling quinones menadione [35], duroquinone [29] and 2,3-$(MeO)_2$NQ [31]. These quinones are substrates for various one- and/or two-electron quinone reductases in hepatocytes; the one-electron reductases catalyse the production of a semiquinone free radical which, when re-oxidized by molecular oxygen, yields the parent quinone and superoxide anion radical ($O_2^{-\cdot}$): the so-called 'redox cycling' process [11,12]. Further metabolism of $O_2^{-\cdot}$ generates a variety of active oxygen species, such as hydrogen peroxide and hydroxyl radicals, which oxidize cellular thiols.

Several lines of evidence suggest that the observed increase in cytosolic protein kinase C specific activity is due to an increase in the $V_{max.}$ of protein kinase C resulting from quinone-mediated oxidative modification(s) of the enzyme, rather than to an increase in enzyme concentration. (i) Treatment of hepatocytes with redox-cycling quinones caused a marked increase in cytosolic protein kinase C specific activity without significantly altering protein kinase C activity associated with the particulate fraction. (ii) Despite the difference in cytosolic protein kinase C specific activities between control and quinone-treated hepatocytes, there was no difference in PDBu receptor binding. This suggests that the number of protein kinase C enzyme molecules was not altered by quinone treatment. (iii) Under the proper conditions, the elevated specific activity of cytosolic protein kinase C isolated from quinone-treated hepatocytes was readily decreased to control levels by treating the cytosolic fraction with thiol reducing agents. (iv) Exposure of hepatocyte cytosolic protein kinase C or partially purified brain protein kinase C to air at 22 or 30 °C respectively, in the absence of added reducing agents, resulted in a 2–2.2-fold increase in specific activity. The observation that the crude cytosolic protein kinase C fraction responded to oxidation in the same way as did the partially-purified brain enzyme suggests that the observed increase in $V_{max.}$ was unlikely to be due to the effects of active oxygen species on some extrinsic activator(s) or inhibitor(s) present in the hepatocyte cytosol.

Our results indicate that protein kinase C is very sensitive to oxidation and, further, that the mechanism by which the $V_{max.}$ of protein kinase C is increased by exposure to active oxygen species appears to involve alteration(s) of the thiol/disulphide status of the enzyme. Redox cycling of menadione, duroquinone and 2,3-$(MeO)_2$NQ in hepatocytes has been shown to cause GSH depletion accompanied by GSSG formation [29–31], and formation of protein mixed-disulphides has been reported to occur in hepatocytes treated with menadione [15,30]. The role of thiol/disulphide exchange in the regulation of enzyme activity has been the subject of many recent investigations [10,14,34]. Several studies suggest that perturbation of tissue thiol/disulphide balance during oxidative stress leads to changes in the activities of key metabolic enzymes, as well as changes in intermediary metabolism [14]. Whereas the $V_{max.}$ of the majority of redox-sensitive enzymes studied is either unaffected or decreased by interaction with low-molecular-mass disulphides (e.g. GSSG), a few enzymes, such as human polymorphonuclear leukocyte collagenase [36] and fructose-1,6-bisphosphatase [37], are stimulated by disulphide effectors.

Two protein kinases have been shown to be activated through a mechanism involving thiol/disulphide exchange. A cyclic AMP-independent protein kinase that phosphorylates the α-subunit of eukaryotic initiation factor 2 has been shown to be activated by low concentrations of GSSG [38]. Recently, the formation of active oxygen species from redox-cycling naphthoquinones was shown to lead to the stimulation of a tyrosine-specific protein kinase located in the rat liver plasma membrane fraction [39]. This tyrosine kinase appears to be the insulin receptor protein kinase [39–41]. Analysis of the primary structure of the various isoforms of protein kinase C has indicated a substantial sequence similarity of the catalytic (kinase) domain with other serine, threonine and tyrosine protein kinases [21]. Thus, a common structural feature may predispose different protein kinases to activation through alteration(s) of their thiol:disulphide status. One could speculate that, under our experimental conditions, quinone-generated active oxygen species alter the thiol/disulphide balance within the protein kinase domain of protein kinase C (via

stimulation of protein-S-S-protein or protein-S-SG formation), leading to the observed increase in $V_{max.}$ The possibility that protein kinase C isoenzymes may be differentially susceptible to oxidative activation remains to be explored.

Our data show that intracellular generation of active oxygen species causes an increase in the $V_{max.}$ of protein kinase C. However, exposure of partially-purified brain protein kinase C or MCF-7 cells to H_2O_2 or *N*-chlorosuccinimide has been reported to inactivate protein kinase C [42]. Similar results were obtained in our laboratory using partially-purified hepatocyte protein kinase C incubated in the presence of glucose/glucose oxidase and/or xanthine/xanthine oxidase (S. K. Duddy, G. E. N. Kass & S. Orrenius, unpublished work). Thus, it appears that a critical thiol/disulphide balance is required for activation of protein kinase C to the high $V_{max.}$ form, and that excessive oxidation of protein kinase C leads to inactivation of the enzyme.

Activation of protein kinase C by active oxygen species occurred without translocation; however, when quinone-treated hepatocytes were subsequently exposed to TPA, the high-$V_{max.}$ form of protein kinase C was translocated from the cytosol into the particulate fraction (Table 1). Although tumour-promoting phorbol esters induce protein kinase C translocation in virtually every cell system so far examined [2], and translocation of protein kinase C is often assumed to be a necessary step in signal transduction, accumulating evidence suggests that translocation of the kinase may not always be required for the expression of protein kinase C activity [43,44]. Whether protein kinase C which has been activated by active oxygen species, as measured in the histone phosphorylation assay *in vitro*, is also stimulated within the intact cell (i.e. actively phosphorylating endogenous substrates) remains to be determined. Several lines of evidence suggest that this may indeed be the case. Submicromolar concentrations of H_2O_2 have been shown to potentiate the aggregation of platelets induced by stimuli such as thrombin, ADP and TPA [45]. Similarly, ornithine decarboxylase induction and skin tumour promotion by TPA is enhanced by adriamycin, a redox-cycling anthracycline antibiotic [46]. More recently, it has been found that 30–100 μM-H_2O_2 has the same effect as TPA in U-937 cells, stimulating adhesion of the cells to plastic and phosphorylation of two (or more) proteins (corresponding to 28 kDa and 43 kDa) [47].

Although many studies of tumour promoter action point to protein kinase C as being a major effector of tumour promotion, the mechanism(s) by which pro-oxidant tumour promoters act is still unclear. Cerutti and coworkers have recently demonstrated that active oxygen species produce DNA damage, with a concomitant stimulation of poly(ADP)-ribosylation [9]. They have suggested that non-cytotoxic concentrations of active oxygen species would produce only moderate levels of DNA damage and poly(ADP)-ribosylation, which could alter chromatin structure and function and lead to transcription of growth-related genes such as the proto-oncogenes c-*fos* and c-*myc* [9]. However, induction of c-*fos* and c-*myc* is also regulated by signal transduction via protein kinase C [19,20]. Thus, our data suggest an additional pathway by which active oxygen species may function as tumour promoters, i.e. oxidative modification of protein kinase C to a high $V_{max.}$ form which may overexpress responses to various stimuli.

In summary, we have shown that exposure of hepatocytes to active oxygen species, generated intracellularly by redox-cycling quinones, leads to an increase in the $V_{max.}$ of protein kinase C. This effect was a consequence of reduction-sensitive modification(s) of the enzyme's thiol/disulphide status.

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