α-Tocopherol specifically inactivates cellular protein kinase C α by changing its phosphorylation state

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The mechanism of protein kinase C (PKC) regulation by α-tocopherol has been investigated in smooth-muscle cells. Treatment of rat aortic A7r5 smooth-muscle cells with α-tocopherol resulted in a time- and dose-dependent inhibition of PKC. The inhibition was not related to a direct interaction of α-tocopherol with the enzyme nor with a diminution of its expression. Western analysis demonstrated the presence of PKCα, β, δ, ε, ζ and μ isoforms in these cells. Autophosphorylation and kinase activities of the different isoforms have shown that only PKCα was inhibited by α-tocopherol. The inhibitory effects were not mimicked by β-tocopherol, an analogue of α-tocopherol with similar antioxidant properties. The inhibition of PKCα by α-tocopherol has been found to be associated with its dephosphorylation. Moreover the finding of an activation of protein phosphatase type 2A *in vitro* by α-tocopherol suggests that this enzyme might be responsible for the observed dephosphorylation and subsequent deactivation of PKCα. It is therefore proposed that PKCα inhibition by α-tocopherol is linked to the activation of a protein phosphatase, which in turn dephosphorylates PKCα and inhibits its activity.

INTRODUCTION

Beneficial effects of α-tocopherol (vitamin E) in preventing the development of atherosclerosis [1–4] have stimulated new studies in understanding the mechanism of action of this vitamin. Recent research has focused the attention on the mode of α-tocopherol uptake [5,6], its selective retention in the body [7,8] and the finding of its cellular targets [9–11].

It has been observed that, at a cellular level, α-tocopherol negatively regulates the proliferation of a number of cell lines at physiological concentrations [9–12]. Moreover, α-tocopherol inhibits protein kinase C (PKC) activity [10,11,13–16]. Such an inhibition, which seems important in the control of cell growth, is not caused by the direct binding of α-tocopherol to the enzyme but presumably results from the prevention, at a cellular level, of its activation [10]. Cell proliferation and PKC inhibition are not caused by β-tocopherol, an analogue of α-tocopherol, provided with similar antioxidant properties; β-tocopherol is, however, able to reverse the inhibitory effects of α-tocopherol [9,10,13,17–19].

PKC belongs to a family of related Ser/Thr kinases with a central role in the transduction of signals elicited by external stimuli [20–22]. A post-translational phosphorylation has been shown to be necessary to produce a catalytically competent PKC [23–25]. In fact, signal transfer by PKC cannot occur until the enzyme is first phosphorylated by a still-unidentified PKC kinase [26,27]. It was found that mature PKCβ_{II} is phosphorylated at two distinct sites and lost its activity after dephosphorylation by protein phosphatase type 1 (PP_1) but not by protein phosphatase type 2A (PP_2A) [28].

The questions posed in this study have been the following: which PKC isoform(s) is (are) inhibited by α-tocopherol in smooth-muscle cells, what is the molecular basis of its inhibition, and what is the role of protein phosphatases in producing PKC inhibition? It was found that PKCα was inhibited specifically by α-tocopherol at a cellular level through a diminution of its phosphorylation state.

Purified PP_2A has been also shown to be activated by α-tocopherol and was found to be able to inhibit PKC *in vitro*. Taken together, the results are consistent with a model by which α-tocopherol-induced activation of PP_2A causes dephosphorylation and inactivation of PKCα.

The relationship between cell proliferation, PKC regulation and α-tocopherol is discussed.

MATERIALS AND METHODS

Materials

Rat A7r5 aortic vascular smooth-muscle cells were from the American Type Culture Collection. Purified PKCα, PP_1 and PP_2A were from UBI (New York, NY, U.S.A.). Tissue culture media and polyclonal antibodies against PKCα, δ and ε isoforms were purchased from Life Technologies (Grand Island, NY, U.S.A.). Fetal calf serum (FCS) was from PAA (Linz, Austria). Anti-rat PKCζ polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Polyclonal anti-PKCμ antibody, calyculin A, okadaic acid and PMA were from LC Laboratories (Woburn, MA, U.S.A.). [γ-^{32}P]ATP (3000 Ci/mmol), [^{32}P]P_i, monoclonal anti-PKCα antibody (clone MC5), the enhanced chemiluminescence (ECL) detection system and ECL Hyperfilm were from Amersham International (Little Chalfont, Bucks., U.K.). Anti-PKCα rabbit polyclonal antibody employed for the kinase reactions of the immunoprecipitated protein was from Oxford Biomedical Research (Oxford, MI, U.S.A.). Phosphorylase *b*, phosphorylase kinase, streptolysin-O, Histone III-SS and phorbol dibutyrate were from Sigma (St. Louis, MO, U.S.A.). C_2-ceramide was from BioMol (Hamburg, Germany). PMSF, leupeptin, pepstatin and aprotinin were from Boehringer (Mannheim, Germany). Protein A–Sepharose 4B was from Pharmacia Biotech. The peptide PLSRTLSVAAKK used as substrate for assaying PKC activity was synthesized by Dr. Servis (Epalinges, Switzerland). Myelin basic protein (4–14

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PKC, protein kinase C; PP_1, protein phosphatase type 1; PP_2A, protein phosphatase type $2A_1$; $MBP_{4–14}$, myelin basic protein peptide fragment 1–14.

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fragment) was from Bachem (Bubendorf, Switzerland). RRR-α-tocopherol and RRR-β-tocopherol were from Henkel (LaGrange, IL, U.S.A.). Tocopherols were adsorbed on FCS before the addition to the cells, as described [5]. Protein concentration was determined with a Pierce kit in accordance with the manufacturer's instructions.

Cell culture

Rat A7r5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1.0 g/l glucose, 60 i.u./ml penicillin, 60 mg/ml streptomycin and supplemented with 10 % (v/v) FCS. Cells in a subconfluent state were made quiescent by incubation in DMEM containing 0.2 % (v/v) FCS for 48 h. Cells were then washed with PBS and treated as indicated in the figure legends. Cell viability, determined by the Trypan Blue exclusion method, was 90–95 % in all experiments. Cells were used between passages 7 and 15.

PKC activity in permeabilized cells

Quiescent A7r5 cells were subjected to different treatments as indicated in the figure legends. During the last hour of the preincubation period, cells were treated with 100 nM PMA to allow penetration of the compound into permeabilized cells. These amounts and the treatment time were found not to change the expression of PKC or to down-regulate it. Aliquots of cells were resuspended in a reaction buffer containing 5.2 mM $MgCl_2$, 94 mM KCl, 12.5 mM Hepes, pH 7.4, 12.5 mM EGTA and 8.2 mM $CaCl_2$, and assays were started by adding [γ-^{32}P]ATP (9 c.p.m./pmol, final concentration 250 μM), peptide substrate (final concentration 70 μM) and streptolysin-O (0.3 i.u.). Samples were incubated at 37 °C for 10 min, quenched and analysed as described previously [10,29]. The basal PKC activity in the absence of added PMA was constantly less than 20 % of that in its presence and coincided with the residual activity of samples treated with PMA plus calphostin C. This activity was considered non-specific and subtracted from the total.

Immunoprecipitation of PKC isoforms

After treatment, cells were harvested in 1 ml of lysis buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 % (v/v) Triton X-100/1 mM EGTA/2 mM EDTA/5 mg/l chymostatin/5 mg/l leupeptin/5 mg/l antipain/5 mg/l pepstatin/1 mg/l *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64)/1 mM PMSF]. Cell lysates were forced 15 times through a 25-gauge syringe and cleared by centrifugation at 15800 ***g*** for 10 min. Immunoprecipitation was performed on equal amounts of protein with the indicated anti-PKC antibody (3 μg) incubated for 1–3 h at 4 °C followed by adsorption on Protein A–Sepharose beads (10 mg) for 1 h at 4 °C. Precipitated samples were recovered by centrifugation and proteins were either resolved by SDS/PAGE or used in autophosphorylation and kinase reactions.

Western blot analysis

Immunocomplexes were dissolved in Laemmli's sample buffer and separated by electrophoresis on a 10 % (w/v) polyacrylamide gel followed by electrotransfer to PVDF membranes (DuPont–NEN Research Products). Membranes were incubated at room temperature with 1–4 μg/ml anti-(PKC isoforms). Proteins were detected with the enhanced chemiluminescence system (Amersham).

Autophosphorylation of PKC isoforms

Immunoprecipitated PKC isoforms bound to Protein A–Sepharose beads were washed three times with lysis buffer and once with the same buffer containing 0.4 M NaCl and without EDTA/EGTA. Samples were incubated in 40 ml of a mixture containing 5 μCi of [γ^{32}P]-ATP, 10 mM ATP, 400 μM $MgCl_2$, 5 mM $CaCl_2$, 400 μM phosphatidylserine, 100 nM phorbol 1,12-dibutyrate, 1 mM sodium orthovanadate and 20 mM Tris/HCl, pH 7.4, at 37 °C for 10 min. The reactions were terminated by the addition of 10 μl of boiling SDS sample buffer and subjected to electrophoresis on a 10 % (w/v) polyacrylamide gel. Gels were stained with the SYPRO protein gel stain kit (Molecular Probes) and blotted; the radioactivity in the membranes was detected by using a Bio-Rad GS-250 Molecular Imager. Alternatively, gels were dried down for autoradiography on Kodak X-Omat S films. Quantification was done by using a BioRad GS-700 imaging densitometer.

Activity of immunoprecipitated PKCα

Confluent A7r5 cells were treated as described in the figure legends, washed and lysed in a buffer containing 150 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1 % (v/v) Nonidet P40, 0.5 % deoxycholate, 0.1 % SDS, 10 mM NaF, protease inhibitor cocktail (Boehringer), 1 mM sodium orthovanadate and 1 mM PMSF. Extracts were prepared by passing the lysates 15 times through a 25-gauge needle and cleared by centrifugation at 15800 ***g*** for 10 min. Anti-PKCα antibody (3 μg) was added to the supernatants for 1 h at 4 °C and afterwards Protein A–Sepharose was added for a firther 1 h. The resulting immunocomplexes were collected by centrifugation, washed in lysis buffer and finally in kinase buffer [50 mM Tris/HCl (pH 7.4)/10 mM NaF/0.5 mM EDTA/0.5 mM EGTA/2 mM $MgCl_2$/1 μM PMSF/protease inhibitor cocktail]. Kinase reactions with the immunocomplexes were performed in a 40 μl final volume of an activation buffer containing 20 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM ATP, 2.5 μCi [γ-^{32}P]ATP (600 Ci/mmol), 0.4 mg/ml histone III-S, 1.2 mM $CaCl_2$, 40 mg/ml phosphatidylserine and 3.3 mM dioleylglycerol. Reactions were terminated by adding 20 μl of boiling SDS sample buffer and frozen until use. Samples were subjected to SDS/PAGE [10 % (w/v) gel] and blotted on a PVDF membrane for 1 h at 100 mA. Histone phosphorylation was detected by using a phosphorimager and the signals were quantified by densitometric scanning and normalized with respect to the amount of PKCα immunoprecipitated, which was detected by immunoblots with the MC5 anti-PKC antibody.

Labelling of cells *in vivo*

Quiescent A7r5 cells (6×10^5) were incubated in phosphate-free DMEM (Amimed) with 0.25 mCi/ml [^{32}P]P_i for 14 h at 37 °C. Cells were then stimulated with 10 % (v/v) dialysed FCS (Sigma) in the presence of the indicated agents and incubated for a further 7 h. During the last hour, cells received 100 nM PMA. Cells were then washed exhaustively with PBS, lysed in SDS buffer and subjected to immunoprecipitation for PKCα. PKCα was resolved by SDS/PAGE and its phosphorylation was analysed on a Bio-Rad Molecular Imager GS-250. Protein loading of the gels was controlled by staining with the SYPRO kit.

Protein phosphatase activity assay

Purified PP_2A (25 ng) resuspended in 10 μl of assay buffer [50 mM Tris/HCl (pH 7.6)/1 mM EDTA] was preincubated with a solution of α-tocopherol (50 μM) and/or β-tocopherol (50 μM)

for 10 min at 30 °C. Control reactions contained vehicle [0.1 % (v/v) ethanol] alone. Assays were started by addition to the mixtures of ^{32}P-phosphorylase *a* solution (8.5 mg of protein, 9×10^4 d.p.m.) and incubated for a further 10 min at 30 °C. Reactions were stopped by the addition of 120 μl of ice-cold 10 % (v/v) trichloroacetic acid and 150 mg of albumin in 20 μl of water. Samples were left on ice and centrifuged for 2 min at 12000 ***g***. The clear supernatant was counted in a liquid-scintillation counter.

PKCα/protein phosphatase assay

PKCα (16 nM) was incubated for 10 min at 30 °C with PP_2A or PP_1 at the indicated concentrations in 40 μl of activation buffer containing 10 mM Mops, pH 7.2, 0.5 mM dithiothreitol, 100 μM myelin basic protein peptide fragment 1–14 (MBP_{4-14}), 0.25 mM ATP, 20 mM $MgCl_2$, 5 μg of phosphatidylserine, 5 μg of diacylglycerides and 5 μCi of [^{32}P]ATP. The reaction was stopped with 20 μl of 25 % (w/v) trichloroacetic acid. Aliquots (50 μl) were spotted on 3 cm × 3 cm P81 Whatman filter, washed twice with 0.75 % (v/v) phosphoric acid and once with acetone. Radioactivity was counted in a liquid-scintillation analyser.

RESULTS

PKC isoforms in A7r5 smooth-muscle cells

The previous finding of PKC involvement in the inhibition by α-tocopherol of the proliferation of smooth-muscle cells [10] prompted the question of which isoform(s) is (are) affected by α-tocopherol. The presence of PKCα, δ, ε, ζ and μ in A7r5 cells was documented (Figure 1, lanes A). To determine the specificity of the reaction, the corresponding competitor peptides were used (Figure 1, lanes B). The PKCβ isoform was determined in a separate experiment as well and shown to be present in smooth-muscle cells. It was also found that long-term treatment with PMA down-regulated the PKCα, δ and ε isoforms (Figure 1, lanes C) as well as PKCβ and PKCμ (results not shown).

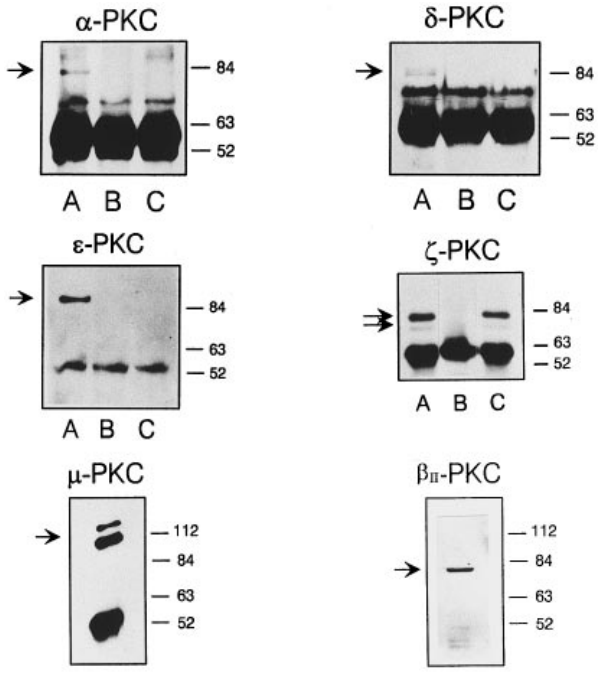

Figure 1 Characterization of PKC isoforms in A7r5 cells

Cells (10^6) at 90% confluence were harvested. PKC isoforms were immunoprecipitated with the corresponding antibodies and subjected to SDS/PAGE and immunoblotting as described in the Materials and methods section. Lanes A, control; lanes B, with competitor peptide; lanes C, cells treated for 24 h with 1 μM PMA. Molecular masses (in kDa) are indicated at the right of each panel.

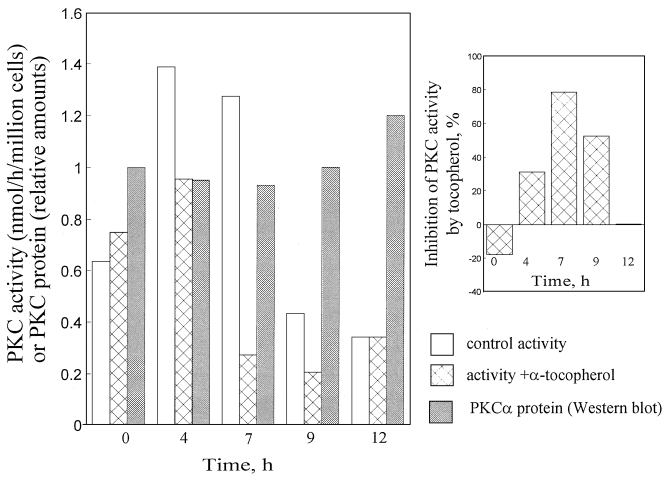

Figure 2 Inhibition of PKC activity by α-tocopherol is a function of the cell cycle

Quiescent A7r5 cells were stimulated for different durations with FCS in the absence or presence of 50 μM α-tocopherol. At the indicated time points, PMA-stimulated PKC activity in permeabilized cells was measured. PKCα levels were analysed by Western analysis and the signals were scanned by densitometry. Results are expressed as arbitrary units of absorbance. Inset: percentages of PKC inhibition by α-tocopherol at different incubation durations. Results are representative of four independent experiments. The percentage values in the inset were calculated from the results shown in the main graph.

α-Tocopherol inhibits the activity, but not the expression, of PKC in smooth-muscle cells

Recombinant PKCα was not inhibited when incubated *in vitro* with α-tocopherol (results not shown). Consequently the possibility was studied that the inhibition at a cellular level was due to a decrease in protein synthesis or to a post-translational modification of the enzyme.

During the transition between the G_0 and G_1 phases of the cell cycle an α-tocopherol-sensitive increase in PKC activity was observed (Figure 2) that was not paralleled by changes in the mRNA levels of the PKCα, β, δ, ε and ζ isoforms (B. Chatelain, D. Boscoboinik and A. Azzi, unpublished results). Similarly, no changes in the protein levels of the major isoform (PKCα) expressed during the transition were observed in the presence or absence of α-tocopherol (Figure 2).

Maximal PKC inhibition by α-tocopherol was found 6–7 h after the entry of cells into the G_1 phase (Figure 2, inset) and this inhibition was observed only when α-tocopherol was added at the time of restimulation [10].

Effect of α-tocopherol on the autophosphorylation of PKC isoforms

Nanomolar concentrations of Gö 6976 {12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo-[3,4-*c*]carbazole} have been shown to inhibit PKCα and PKCβ, whereas even micromolar concentrations have no effect on

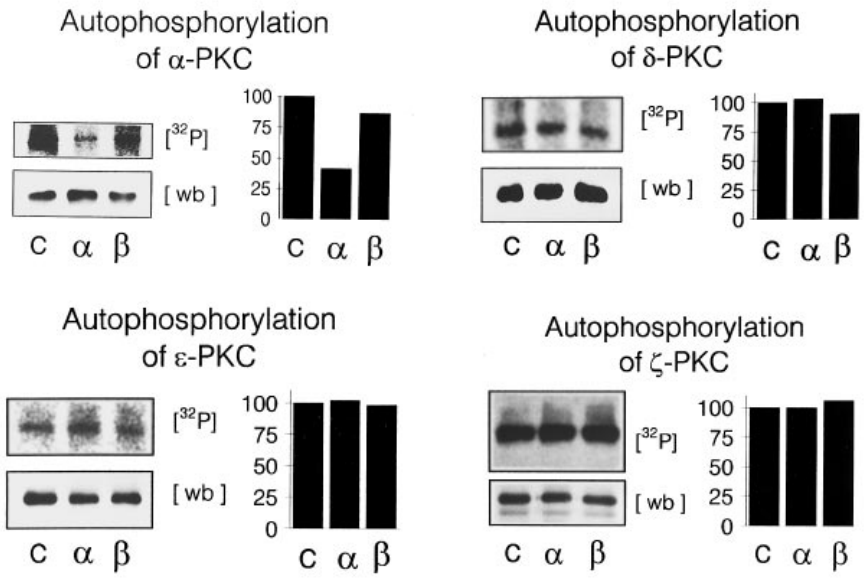

Figure 3 Autophosphorylation activity of different PKC isoforms

Quiescent cells were restimulated for 7 h with FCS in the absence (lanes C) or presence of either 50 μM α-tocopherol (lanes α) or β-tocopherol (lanes β). PMA (100 nM) was added for the last 1 h of the preincubation period. Then cell extracts were prepared and PKC isoforms were immunoprecipitated with the indicated antibodies. Autophosphorylation reaction of the individual isoforms was performed as described in the Materials and methods section. Samples were subjected to electrophoresis and blotted; the calculated ratio between incorporated radioactivity (^{32}P) and the protein levels (Western blot, wb) is represented in the bar graph for each condition. Data are representative of three independent experiments.

the activity of PKCδ, ϵ or ζ [30]. In the presence of this inhibitor a residual PKC activity of approx. 30% was present that was insensitive to α-tocopherol (results not shown). This experiment suggested that α-tocopherol was specifically acting on PKCα and/or PKCβ.

To substantiate this finding, immunoprecipitation of the different PKC isoforms and a determination of the kinase and autophosphorylating activities were performed.

PKC autophosphorylation in immunoprecipitates has been found to be correlated with its enzymic activity and has been taken as a reliable indication of PKC activity [23].

Cells were incubated in the absence or the presence of α-tocopherol or β-tocopherol for 7 h during the G_1 phase. Then extracts were prepared and immunoprecipitation of the individual PKC isoforms was performed. Autophosphorylation activity and protein amounts were determined for each isoform. In Figure 3 the effects on PKCα, δ, ϵ and ζ are shown. The bar graphs correspond to the PKC activity and values are normalized with respect to the protein content. As can be seen, only PKCα from α-tocopherol-treated cells was less active relative to its control. The activity of all the other PKC isoforms tested was not affected by the treatment of cells with α-tocopherol or β-tocopherol. The immunoprecipitated PKCβ isoform was inactive, possibly owing to the specific precipitating antibody employed.

α-Tocopherol selectively inhibits the activity and the phosphorylation state of PKCα

To establish whether the incubation of cells with α-tocopherol resulted in a change in the phosphorylation state of PKCα a labelling reaction was performed *in vivo*.

Cells were labelled overnight with [^{32}P]P_i and, after stimulation with FCS for 7 h in the presence or absence of α-tocopherol, PKCα was immunoprecipitated and blotted; the [^{32}P]P_i incorporation was then measured. Figure 4 shows (from top to bottom) the autoradiogram of the gel, the immunoblot and a bar graph presentation of the relative radioactivity intensities, integrated and normalized relative to the protein levels of each sample. Relative to the control (Figure 4, lane A), the PMA-treated cells (lane B) showed a significant increase in P_i incorporation into PKCα. Cells pretreated with α-tocopherol (lane C) showed a large inhibition of PKCα phosphorylation, whereas cells preincubated with β-tocopherol showed much less inhibition (lane D). The inhibitory effect of α-tocopherol was reversed by two potent protein phosphatase inhibitors, 2 nM okadaic acid

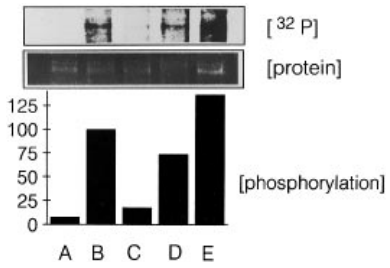

Figure 4 Effect of α-tocopherol and β-tocopherol on the phosphorylation state of PKCα

Quiescent A7r5 cells were incubated in phosphate-free DMEM medium (ICN) for 48 h. They received 0.25 mCi/ml [^{32}P]P_i for the last 14 h. Cells were restimulated for 7 h with FCS in the absence (lane B) or presence of either 50 μM α-tocopherol (lane C) or β-tocopherol (lane D). PMA (100 nM) was added for the last 1 h to all samples except that in lane A. Calyculin A (2 nM) was added to cells for 1 h where indicated (lane E). Cell extracts were prepared and immunoprecipitated with anti-PKCα. Proteins were resolved by SDS/PAGE and radioactivity (^{32}P) and protein levels were quantified as described in the Materials and methods section. The bar graph represents the ratio between radioactive counts incorporated into PKCα and the protein levels. Results are representative of three independent experiments.

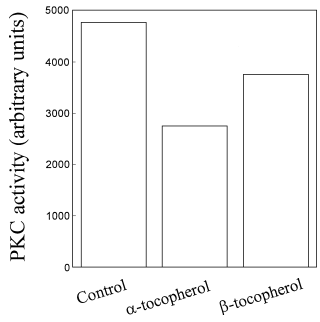

Figure 5 Determination of PKCα activity after its immunoprecipitation from cells treated with α-tocopherol or β-tocopherol

Cells were stimulated for 7 h with FCS in the absence (control) or presence of either 50 μM α-tocopherol or 50 μM β-tocopherol as indicated. During the last 1 h of preincubation they received 100 nM PMA. Then extracts were prepared, PKCα was immunoprecipitated and a kinase reaction with histone III-SS was performed as described in the Materials and methods section. Proteins were resolved by electrophoresis and radioactive bands were quantified with Bio-Rad Molecular-Analyst software. Protein levels were estimated by staining the gel with the SYPRO kit or by immunoblotting with the MC5 monoclonal antibody. The ratio between radioactivity incorporated into the substrate and the amount of PKCα precipitated was expressed in arbitrary units of the densitometric scanning of the bands. Results are representative of three independent experiments.

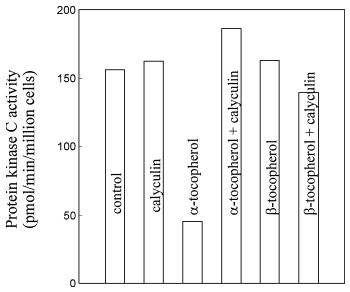

Figure 6 Lack of PKC inhibition by α-tocopherol in the presence of calyculin A

Cells were made quiescent by serum deprivation for 48 h and restimulated for 7 h with 10% (v/v) FCS in the presence or absence of 50 μM α-tocopherol or 50 μM β-tocopherol, as indicated. PMA (100 nM) and, when indicated, calyculin A (2 nM) were added 1 h before the activity measurement. PMA-stimulated PKC activity was determined as described in the Materials and methods section by using the phosphatase-insensitive peptide $MBP_{4–14}$ as substrate. Results are representative of four independent experiments.

(results not shown) or 2 nM calyculin A (lane E). Figure 5 shows the PKCα activity measured after immunoprecipitation of the enzyme from cells preincubated with α-tocopherol or β-tocopherol at the late G_1 phase of the cell cycle. As can be seen, α-tocopherol inhibited PKCα activity more strongly than did β-tocopherol.

With PKCβ, no significant PKC activity changes were observed if the cells were preincubated either with α-tocopherol or β-tocopherol (results not shown).

PP_2A is activated by α-tocopherol and can dephosphorylate PKCα

The role of PP_2A in the deactivation of PKCα was postulated on the basis of the experiment reported in Figure 6. This experiment shows that, in the presence of 2 nM calyculin A (a concentration that produces specific PP_2A inhibition), the activity of PKC was not inhibited by α-tocopherol (compare with [10]). To establish whether a direct effect of PP_2A on PKC could take place, the two

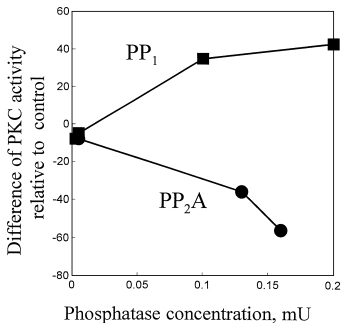

Figure 7 Inhibition of PKCα activity by PP_2A, and activation by PP_1

Purified PKCα (16 nM) was incubated for 10 min at 30 °C with PP_2A or PP_1 in 40 μl of activation buffer as described in the Materials and methods section. PKCα activity was determined by using a peptide substrate as described previously. Results are representative of three independent experiments. The control value of PKC activity was 0.6 μmol of P_i/min per mg of kinase. Abbreviation: mU, m-units.

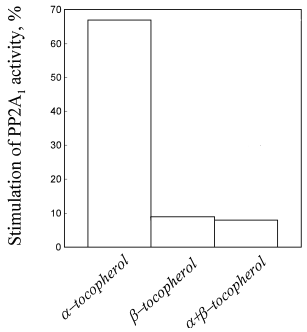

Figure 8 Effect of α-tocopherol and β-tocopherol on PP_2A activity

Phosphatase activity with pure PP_2A was assayed in the presence of different α-tocopherol or β-tocopherol concentrations as indicated. Stimulation of PP_2A_1 activity was calculated with respect to control samples. The background in the absence of the enzyme represented less than 3.5% of the initial total radioactive counts. Results are representative of three separate experiments. The control value of PP_2A activity was 106 m-units/mg of phosphatase.

enzymes were incubated together and the activity of PKCα was measured. It was observed that PP_2A produced a deactivation of PKCα (Figure 7). The inhibition of approx. 50% in PKCα activity obtained at a PP_2A-to-PKC molecular ratio of 1:16 indicates a catalytic role of PP_2A on PKC inactivation. The effect of another protein phosphatase, namely PP_1, was also tested. The same concentrations of PP_1 (approx. 0.2 m-unit per assay) resulted in an activation of PKCα, suggesting the existence of an inhibitory phosphorylation site on the enzyme (Figure 7). This phenomenon is being investigated further.

The inhibition by α-tocopherol of PKCα at a cellular level might thus be related to a possible activation of PP_2A by α-tocopherol. This hypothesis was investigated in the experiment shown in Figure 8. Purified PP_2A was incubated with ^{32}P-phosphorylase *a* as a substrate, and its activation by α-tocopherol and/or β-tocopherol was analysed: α-tocopherol produced an almost 2-fold activation, β-tocopherol was slightly inhibitory and the mixture of both tocopherols was without significant effect. In Figure 9 the concentration dependence of α-tocopherol activation on PP_2A is shown. It therefore seems that one of the cellular targets of α-tocopherol might be PP_2A. PP_1 has been also

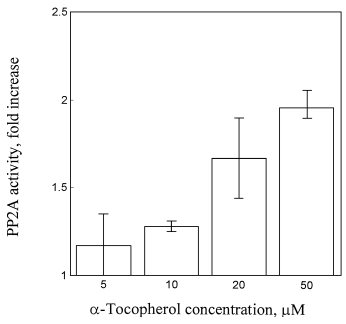

Figure 9 Effect of different α-tocopherol concentrations on PP_2A activity

Phosphatase activity with pure PP_2A was assayed in the presence of different α-tocopherol concentrations as indicated. Stimulation of PP_2A activity was calculated with respect to control samples. The background in the absence of the enzyme represented less than 3.5% of the initial total counts. The bars represent the means ± S.D. for four experiments. The control value of PP_2A activity was 106 m-units/mg of phosphatase.

tested (results not shown) and found to be insensitive to α-tocopherol treatment under the conditions employed for PP_2A.

DISCUSSION

Inhibition of PKC activity by α-tocopherol has been previously shown to be indirect and to depend on the cell type and the time, within the cell cycle, of tocopherol addition. Moreover it has been found not to be the consequence of a decreased protein expression [10,17,31,32].

One of the goals of this study was directed towards the analysis of which PKC isoform(s) is (are) affected by the treatment of cells with α-tocopherol. The isoforms expressed in A7r5 smooth-muscle cells are PKCα, β, δ, ε, ζ and μ. Experiments with inhibitors for PKCα and β had suggested that PKCα was the target of α-tocopherol. More direct evidence for this conclusion has been obtained by immunoprecipitating the different isoforms of PKC from smooth-muscle cells treated or not with α-tocopherol and by measuring their activity. Treatment of smooth-muscle cells with α-tocopherol results in a specific inhibition of PKCα autophosphorylation activity, all the other tested isoforms being unaffected. These experiments have also confirmed that PKCα is affected by α-tocopherol and to a much smaller extent by β-tocopherol.

The second goal of this study was to understand the molecular basis of the effect of α-tocopherol on PKCα. The results obtained by incubating smooth-muscle cells with [^{32}P]P_i have shown that the phosphorylation of PKCα is strongly diminished by the incubation of the cells with α-tocopherol. It is known that PKC undergoes a permissive phosphorylation and an auto-phosphorylation allowing full activatability of the enzyme. Thus a lack of phosphorylation of PKC might be the basis for the effect of α-tocopherol.

The third goal of this study was to investigate the possible cause of the observed changes on the phosphorylation state of PKC. The following effect of α-tocopherol has been considered: in its presence and during the cell cycle transition from G_0 to G_1, an inhibition of PKCα phosphorylation or an activation of its dephosphorylation occurs. This post-translational change results in a less active enzyme. As to the two alternatives, inhibition of PKC phosphorylation or activation of its dephosphorylation, results from this study (Figure 6) and from previous studies [10] indicate that in the presence of calyculin or okadaic acid (two phosphatase inhibitors) the effect of α-tocopherol disappears. This suggests that the inhibition of PKC phosphorylation might occur by activation of a phosphatase.

It has been shown that ceramide in Molt-4 cells caused the inactivation of PKCα. Okadaic acid, a potent phosphatase inhibitor, blocked the effects of ceramide on PKCα, suggesting that the effects of ceramide might be mediated by a protein phosphatase [33].

Our results show that inhibition of PKCα activity can be observed *in vitro* by incubating the enzyme with PP_2A. The inhibition is caused not by the formation of a complex between the two proteins but rather by a catalytic event. In fact, PP_2A was capable of producing 50% inactivation of PKCα even when the latter was present in a 16-fold excess. PP_1 was not able to inhibit PKC activity under the same experimental conditions. In fact, it was instead activating the enzyme, suggesting the possible existence of an inhibitory phosphorylation site on PKCα. It has been also observed that PP_2A *in vitro* can be activated by α-tocopherol. This event is not produced by β-tocopherol, which instead protects against the activation by α-tocopherol. It should be noticed that a similar situation was observed when PKC activity or cell proliferation was measured. PP_1 was found to be unaffected by α-tocopherol. Whether or not the only target of α-tocopherol is PP_2A is the object of current studies. At a cellular level, Parker's group has shown that both PP_1 and PP_2A are capable of dephosphorylating and inhibiting PKC [34], which supports our conclusions of a role for PP_2A in the inactivation of PKCα. The novelty in this report also resides in the activatory effect of α-tocopherol on PP_2A, which would result in an inhibition of PKCα activity.

In conclusion, the results presented here show that α-tocopherol selectively inhibits PKCα activity in A7r5 smooth-muscle cells. The effect of α-tocopherol is specific and is not shared by its β-tocopherol analogue. The inhibition of PKC seems to be linked to an α-tocopherol-induced decrease of its permissive phosphorylation. This event seems to be linked to the activation by α-tocopherol of a PP_2A phosphatase. A stimulation of expression of PP_2A by α-tocopherol is also currently under investigation.

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REFERENCES

1 Stephens, N. G., Parsons, A., Schofield, P. M., Kelly, F., Cheeseman, K., Mitchinson, M. J. and Brown, M. J. (1996) Lancet **347**, 781–786
2 Rimm, E. B., Stampfer, M. J., Ascherio, A., Giovannucci, E., Colditz, G. A. and Willett, W. C. (1993) New Engl. J. Med. **328**, 1450–1456
3 Stampfer, M. J., Hennekens, C. H., Manson, J. E., Colditz, G. A., Rosner, B. and Willett, W. C. (1993) New Engl. J. Med. **328**, 1444–1449
4 Gey, K. F., Puska, P., Jordan, P. and Moser, U.K. (1991) Am. J. Clin. Nutr. **53**, 326S–334S
5 Chan, A. C. and Tran, K. (1990) Lipids **25**, 17–21
6 Dutta Roy, A. K., Leishman, D. J., Gordon, M. J., Campbell, F. M. and Duthie, G. G. (1993) Biochem. Biophys. Res. Commun. **196**, 1108–1112
7 Traber, M. G., Sokol, R. J., Kohlschutter, A., Yokota, T., Muller, D. P., Dufour, R. and Kayden, H. J. (1993) J. Lipid Res. **34**, 201–210
8 Traber, M. G. (1994) Free Radical Biol. Med. **16**, 229–239
9 Azzi, A., Boscoboinik, D., Marilley, D., Özer, N. K., Stäuble, B. and Tasinato, A. (1995) Am. J. Clin. Nutr. **62**, 1337S–1346S
10 Tasinato, A., Boscoboinik, D., Bartoli, G. M., Maroni, P. and Azzi, A. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 12190–12194
11 Boscoboinik, D., Szewczyk, A., Hensey, C. and Azzi, A. (1991) J. Biol. Chem. **266**, 6188–6194
12 Traber, M. G. and Packer, L. (1995) Am. J. Clin. Nutr. **62**, 1501S–1509S

13 Azzi, A., Boscoboinik, D., Chatelain, E., Özer, N. K. and Stäuble, B. (1993) Mol. Aspects Med. **14**, 265–271
14 Chakraborti, S. and Michael, J. R. (1993) Mol. Cell Biochem. **122**, 9–15
15 Kanno, T., Utsumi, T., Kobuchi, H., Takehara, Y., Akiyama, J., Yoshioka, T., Horton, A. A. and Utsumi, K. (1995) Free Radical Res. **22**, 431–440
16 Kunisaki, M., Bursell, S. E., Umeda, F., Nawata, H. and King, G. L. (1994) Diabetes **43**, 1372–1377
17 Boscoboinik, D., Chatelain, E., Bartoli, G. M. and Azzi, A. (1992) in Free Radicals and Aging (Emerit, I. and Chance, B., eds.), pp. 164–177, Birkhäuser Verlag, Basel
18 Boscoboinik, D., Özer, N. K., Moser, U. and Azzi, A. (1995) Arch. Biochem. Biophys. **318**, 241–246
19 Özer, N. K., Boscoboinik, D. and Azzi, A. (1995) Biochem. Mol. Biol. Int. **35**, 117–124
20 Azzi, A., Boscoboinik, D. and Hensey, C. (1992) Eur. J. Biochem. **208**, 547–557
21 Nishizuka, Y. (1984) Nature (London) **308**, 693–698
22 Dekker, L. V. and Parker, P. J. (1994) Trends Biochem. Sci. **19**, 73–77
23 Newton, A. C. (1995) J. Biol. Chem. **270**, 28495–28498
24 Cazaubon, S., Bornancin, F. and Parker, P. J. (1994) Biochem. J. **301**, 443–448
25 Pears, C., Stabel, S., Cazaubon, S. and Parker, P. J. (1992) Biochem. J. **283**, 515–518
26 Orr, J. W. and Newton, A. C. (1994) J. Biol. Chem. **269**, 8383–8387
27 Borner, C., Filipuzzi, I., Wartmann, M., Eppenberger, U. and Fabbro, D. (1989) J. Biol. Chem. **264**, 13902–13909
28 Dutil, E. M., Keranen, L. M., DePaoli-Roach, A. A. and Newton, A. C. (1994) J. Biol. Chem. **269**, 29359–29362
29 Alexander, D. R., Graves, J. D., Lucas, S. C., Cantrell, D. A. and Crumpton, M. J. (1990) Biochem. J. **268**, 303–308
30 Martiny Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D. and Schachtele, C. (1993) J. Biol. Chem. **268**, 9194–9197
31 Chatelain, E., Boscoboinik, D. O., Bartoli, G. M., Kagan, V. E., Gey, F. K., Packer, L. and Azzi, A. (1993) Biochim. Biophys. Acta **1176**, 83–89
32 Boscoboinik, D. O., Chatelain, E., Bartoli, G. M., Stäuble, B. and Azzi, A. (1994) Biochim. Biophys. Acta **1224**, 418–426
33 Lee, J. Y., Hannun, Y. A. and Obeid, L. M. (1996) J. Biol. Chem. **271**, 13169–13174
34 Hansra, G., Bornancin, F., Whelan, R., Hemmings, B. A. and Parker, P. J. (1996) J. Biol. Chem. **271**, 32785–32788

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