Modulation of the Substrate Specificity of Purified Human Protein Kinase C by its Activators

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The substrate specificity of purified human protein kinase C was modulated by 12-*O*-tetradecanoyl-4β-phorbol-13-acetate (TPA), dioleoylglycerol, arachidonic acid and lipid A when histone type III-S and myelin basic protein were used as phosphate acceptors. Each activator also showed a distinct pattern in the stimulation of phosphorylation of the kinase itself and of cytosolic placental proteins. The nature of the substrate and the presence of calcium and phospholipid determined the magnitude of the effect observed upon addition of all activators and also the dose dependency of kinase activation by TPA. The apparent K_m value for phosphorylation of histone type III-S by the kinase activated by phorbol ester alone and with calcium was 20–30 fold higher than that observed for the enzyme activated by calcium and phospholipid. These observations indicate that the nature and extent of cellular response induced by the activation of C-kinase(s) may be determined by the type of cellular stimulus.

The dose dependencies for cellular effects of phorbol esters and diacylglycerols are known to be different, and dependent on the system used. Addition of 1–10 nM 12-*O*-tetradecanoyl-4β-phorbol-13-acetate (TPA) to whole cells leads to modulation of differentiation[1-3] and stimulation of the metabolism of lipid and carbohydrate in various cell systems,[4-6] whereas the effects of diacylglycerols on whole cells have been observed for concentrations of 10–100 μM.[5,7] Similar concentrations of TPA and diacylglycerol, respectively, promote the phosphorylation *in vitro* of the transferrin receptor by protein kinase C.[8] However, stimulation of protein kinase C-dependent phosphorylation of histones *in vitro* is observed at 1000-fold lower concentrations of TPA (pM) and diacylglycerol (nM), respectively.[9] Anti-proliferative effects of TPA on different human tumor cell lines at a concentration of only 10 pM have been described.[1,2]

The different dose responses for the effects of TPA and diacylglycerols on protein kinase C, *in vivo* as well as *in vitro*, have prompted us to characterize the effects of known activators of the kinase. Our observations suggest that the nature of the substrate involved in a specific biological process, as well as the local availability of calcium and phospholipids, might determine the dose dependency of both exogenous and endogenous activators of protein kinase C.

Experimental

Materials. [γ-^{32}P]-ATP and [^{3}H]-4β-phorbol-12,13-dibutyrate ([^{3}H]-PDBU) (12.5 Ci mmol^{-1}) were obtained from New England Nuclear. Histone type III-S, bovine brain phosphatidylserine (PS), 1,3-dioleoylglycerol, TPA and 4β-phorbol-12,13-dibutyrate (PDBU) were purchased from Sigma. Arachidonic acid was obtained from Nu-Check Prep. Myelin basic protein and lipid A were purchased from Calbiochem. DEAE-Sepharose and molecular weight markers were obtained from Pharmacia, and hydroxylapatite (Bio-gel HTP) was purchased from Biorad.

Preparation of protein kinase C. Normal full-term placentae were obtained from local hospitals.

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Tissue (400–500 g) was mixed with 3 ml of 0.25 M sucrose, 20 mM Tris-Cl, 10 mM EDTA, 50 mM β-mercaptoethanol. 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.5) per g of tissue and treated in an ice-cold Waring blender for two 15 s periods at the highest setting. The resulting homogenate was centrifuged at $9000 \times g$ for 30 min. Polyethylene glycol 6000 (PEG) was added to the supernatant, and the material precipitating between 5 and 15 % (*w/v*) PEG was collected by centrifugation. The resulting pellet was dissolved in 20 mM Tris-Cl, 5 mM EDTA, 1 mM dithioerythritol, 0.25 mM PMSF (pH 7.5; Buffer A) and applied to a 750 ml DEAE-Sepharose column equilibrated in Buffer A. The column was re-equilibrated with 750 ml of Buffer A and the protein was eluted using a 2 l linear gradient of Buffer A to Buffer A containing 0.25 M NaCl. The active fractions were pooled, diluted with one vol. of Buffer A and applied to a 30 g hydroxylapatite column packed in Buffer A with 10 % glycerol (Buffer B). The column was developed directly with a 1.5 l linear gradient containing from 25 mM to 225 mM potassium phosphate in Buffer B. Fractions rich in protein kinase C were pooled, supplemented with 1/50 vol. of 0.1 M ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid, concentrated to 20–30 ml on an Amicon PM-10 ultrafiltration device and dialysed against two 1 l portions of Buffer B. The resulting enzyme preparation constitutes the partially purified protein kinase C. Highly purified enzyme was prepared using affinity chromatography on immobilised phosphatidylserine (PS), as described by Uchido[10] but with the modification that 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5) was used as buffer. When the binding of [^{3}H]-PDBU to the affinity-purified kinase was determined[11,12] under the standard conditions used in the kinase assay, K_a was found to be 8.0 nM. The kinase activity was 14.8±3.6 (N=4) pmol ^{32}P incorporated into histone per min and per pmol [^{3}H]-PDBU bound at 30–50 nM [^{3}H]-PDBU. A specific activity of 185±45 nmol ^{32}P incorporated into histone per min and per mg protein kinase C was calculated from this value, assuming 1:1 binding of phorbol ester to the M_r 80 000 kinase.

Kinase assay. Protein kinase C activity was determined as described previously,[12,13] using 2 min pre-incubation and 3 min incubation at 30 °C, unless otherwise stated. The standard incubation mixture contained 50 μg of substrate in a final volume of 250 μl and, where indicated, also 0.4 mM (calculated) free Ca^{2+} and 10 μg PS per tube or indicated activator dissolved in ethanol. The presence of ethanol at the concentrations used (2–4 %) had no observable effect on the kinase activity. The assay was linear with respect to time for all substrates. Where indicated, incubation was stopped by the addition of solubilization buffer[14] and rapid chilling in an ice-bath, followed by analysis of the phosphorylated proteins by gel electrophoresis in the presence of sodium dodecylsulfate (SDS)[14] and preparation of autoradiograms from the dried gels. Phosphorylase, bovine albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lactalbumin (M_r 94 000, 67 000, 43 000, 30 000, 20 100 and 14 400, respectively) were used as molecular weight markers.

Kinetic analysis. Highly purified protein kinase C was incubated with histone III–S at 5–7 different concentrations centered around the apparent K_m (0.08–12.4 μM), using 1 min pre-incubation and 2 min incubation time. The data were analysed by adjusting K_m and V_{max} in the Michaelis-Menten equation to minimize the relative error in the reaction velocity, $[v(\text{obs.})-v(\text{calc.})]/v(\text{obs.})$, by the least-squares method, using an iterative program written in PASCAL (available upon request from the authors). The apparent V_{max} values in the presence of TPA, calcium and TPA, or calcium, PS and TPA were 67.4±27.8, 129.1±34.4 and 113.4±16.5 % of the value in the presence of calcium and PS (N=5). The major protein component in the histone type III-S preparation was the dominant phosphoprotein product under these conditions. The apparent molecular weight of this protein, 31 000, was used in the calculation of molar concentrations of substrate.

Results

Phosphorylation of cytosolic placenta proteins in the presence of activators of protein kinase C. Incubation of a partially purified preparation of protein kinase C with labelled ATP in the presence of different combinations of activators resulted in the incorporation of [^{32}P]-phosphate into several different proteins (Fig. 1). The extent of incorporation of phosphate into these protein

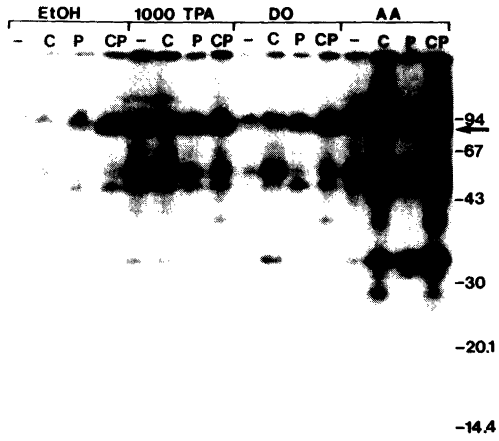

Fig. 1. Phosphorylation of the protein kinase C preparation. Partially purified protein kinase C (16 μg protein per tube) was incubated under standard assay conditions with solvent (EtOH), 1000 nM TPA (1000 TPA), 100 μM dioleoylglycerol (DO), 100 μM arachidonic acid (AA) or 20 μg of lipid A per ml (LA) in the presence of calcium (C), phosphatidylserine (P) or both (CP), as indicated. No exogenous substrate was added. The incubated samples were analysed by SDS gel electrophoresis[14] in a 12 % polyacrylamide slab gel. The position of protein kinase C is marked with an arrow.

bands was affected to different extents by the tested activators. The phosphorylation of a M_r 80 000 protein, corresponding to protein kinase C, as judged by its co-elution with the kinase activity upon chromatography using DEAE-Sepharose, hydroxylapatite and Sephacryl S-200, was particularly enhanced by PS, TPA or arachidonic acid in the presence of calcium.

Phosphorylation of highly purified protein kinase C in the presence of its activators. Chromatography of the partially purified protein kinase C on immobilized PS resulted in a preparation exhibiting a major band in SDS gel electrophoresis corresponding to the molecular weight of protein kinase C (Fig. 2). Gel-electrophoretic analysis of samples of highly purified protein kinase C incubated with labelled ATP in the presence of different combinations of activators but in the absence of exogenous substrate demonstrated a calcium- and PS-dependent incorporation of radioactive phosphate into the kinase (Fig. 3A). This autophosphorylation was markedly stimulated by the addition of TPA in the absence of any other addition, as well as in the presence of PS alone. Diacylglycerol enhanced the autophosphorylation in the absence of calcium and PS. Arachidonic acid decreased this kinase activity, except in the presence of PS, whereas lipid A inhibited the reaction in the presence of PS and stimulated the autophosphorylation in the presence of calcium.

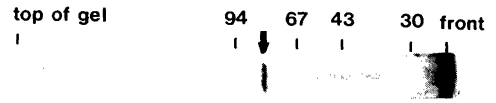

Fig. 2. Purity of the human placental protein kinase C. Highly purified protein kinase C was subjected to SDS gel electrophoresis in a 6.5 % polyacrylamide slab gel.[14] The figure shows the silver-stained gel[22] with the position of protein kinase C indicated by an arrow.

Phosphorylation of histone type III-S and myelin basic protein by protein kinase C. When incubated samples containing highly purified protein kinase C and histone type III-S or myelin basic protein were analysed by SDS gel electrophoresis, a substrate dependency for the effects of TPA, arachidonic acid and lipid A was observed (Figs. 3B and 3C). TPA stimulated the phosphorylation of myelin basic protein in the absence of added calcium. Arachidonic acid activated protein kinase C in the presence of calcium when using myelin basic protein as substrate. Lipid A was the activator which most markedly stimulated the phosphorylation of histone type III-S in the presence of calcium.

Analysis of the substrate dependency of histone phosphorylation by the kinase demonstrated that the apparent K_m value was markedly affected by the choice of combination of activators. Upon stimulation of the enzyme by 1 μM TPA alone, and in combination with calcium, apparent K_m values of 6.34±3.57 and 5.25±1.77 μM were found, respectively. Activation by PS in the presence of calcium resulted in a 20–30-fold lower apparent K_m value, 0.237±0.123 μM, and a similar value (0.267±0.155 μM) was observed in the additional presence of TPA.

Stimulation of histone phosphorylation by TPA was dose-dependent (Fig. 4A). Addition of 0.8 mM calcium increased the sensitivity of the kinase to TPA-induced stimulation with a half-

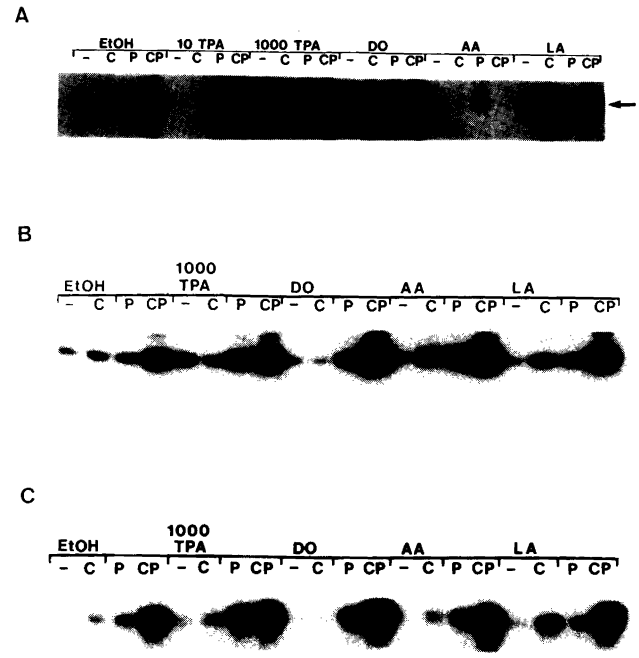

Fig. 3. Activation of highly purified protein kinase C. The enzyme was incubated with the indicated additions (see Fig. 1) and also with 10 nM TPA (10 TPA). The incubated samples were treated and analyzed as described for Fig. 1. (A) Autophosphorylation of highly purified protein kinase C upon 10 min incubation under standard conditions without any added substrate. Only the part of the gel corresponding to protein kinase C is shown. Phosphorylation of (B) myelin basic protein and (C) histone type III-S upon incubation of the kinase under standard conditions with 10 μg substrate per tube. Only the parts of the autoradiograms corresponding to the major phosphoprotein band, M_r 18 000 in B and 31 000 in C, are shown.

maximal effect at around 10 nM at a calculated free calcium concentration of 400 μM. The presence of calcium apparently increased the sensitivity to TPA by two orders of magnitude. The stimulatory effect of TPA on protein kinase C was also observed using myelin basic protein as phosphate acceptor; however, the effect of the phorbol ester was less pronounced and the sensitivity to TPA decreased by two orders of magnitude relative to that observed using histone type III-S (Fig. 4B).

Discussion

The results of the present investigation indicate that known activators of protein kinase C may affect not only the kinase activity, but also the apparent substrate specificity of the enzyme. The intracellular activation of protein kinase C by a specific compound could thus induce the phosphorylation of a limited subset of the several potential kinase substrates. This principle may be

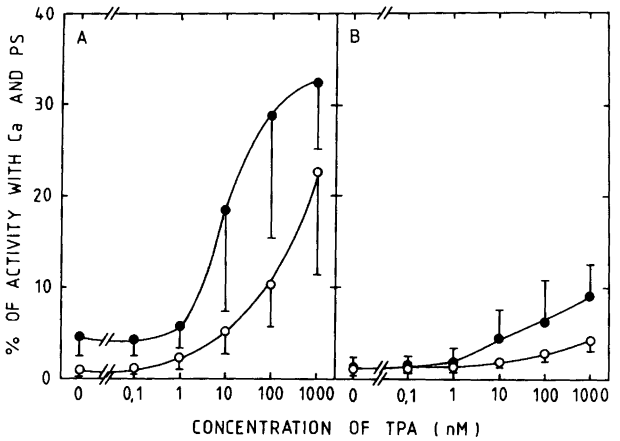

Fig. 4. Effect of TPA on the phosphorylation of (A) histone type III-S and (B) myelin basic protein. Highly purified protein kinase C was incubated under standard conditions without any addition of calcium or PS, ○—○, and with addition of calcium only, ●—●, in the presence of TPA added to give the concentrations indicated. The kinase activity is calculated as % of that observed in the presence of calcium and PS, and the result is presented as mean ± S.E. for three independent experiments.

reflected in the recent observation of differences in the actions of TPA and diacylglycerol on granulosa cell maturation.[15]

Similarly, addition of diacylglycerol to HL-60 cells was found to result in the phosphorylation of ten proteins, whereas the formation of four additional phosphoproteins was induces by TPA.[16] Furthermore, bryostatin, a substance that activates protein kinase C *in vitro* and induced translocation *in vivo,* has no effect on differentiation and has an antagonistic effect on TPA-induced differentiation in HL-60 cells.[17]

The calcium-dependent complex formation of the kinase with phospholipids and membrane preparations *in vitro* is known to be facilitated by the addition of phorbol esters or diacylglycerols and to be associated with the enzymatic activation.[9,14,18,19] Our findings indicate that the substrate specificity of the kinase may be altered by the association with the phospholipids. Phorbol esters, diacylglycerols and calcium may thus affect the rate of phosphorylation of a given substrate, both by direct effects on the enzyme and by modulating the binding to phospholipids and cellular membranes.

The sensitivity of activation of the kinase by TPA was found to vary by two orders of magnitude, depending on the presence of calcium and on the phosphate acceptor used. This might explain the different dose dependencies for activation of protein kinase C *in vitro*.[8,9] The presence of TPA inhibits proliferation of K-562 and U-937 cells at 10^{-10} M, whereas 10^{-8} M is required to modulate differentiation and to induce translocation of the kinase.[1] The present observations indicate that protein kinase C may indeed participate in both low-dose and high-dose responses to phorbol esters.

Cloning of protein kinase C cDNA has demonstrated the presence of at least three different forms of protein kinase C-related genes in bovine, human and rat tissues.[20,21] Based on differences in the deduced amino acid sequences of a putative calcium-binding site, it has been postulated that the multiple forms of the kinase may express different calcium dependencies.[21] The degree of membrane association of protein kinase C after expression of cDNA clones in transfected COS cells appears dependent on the particular type of kinase coded for.[20] Thus, the types of protein kinase C present might influence the activator requirements, and probably also the apparent substrate specificity, of protein kinase C-dependent reactions *in vivo* and *in vitro*. The role of multiple forms of protein kinase C in the observations described here remains to be established.

In the traditional model for intracellular receptors of, e.g., steroids, the concentrations of ligand and receptor and the affinity of the ligand for the receptor determine the magnitude of the response. Based on the data presented here, it may be speculated that recognition of protein substrates by protein kinase C is specified through the sensing of several different intracellular agents. The availability of critical phosphate-accepting proteins, governed by such factors as state of differentiation and events related to the cell cycle, may also contribute to the generation of a unique response for each combination of stimulus and cell.

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