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A Phorbol Ester and Phospholipid-Activated, Calcium-Unresponsive Protein Kinase in Mouse Epidermis: Characterization and Separation From Protein Kinase C

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The phosphorylation of an Mr 82,000 protein (p82) in the Triton X-100 extract of the particulate fraction of mouse epidermis is dependent on the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) or diacylglycerol and phospholipid and, contrary to protein kinase C (PKC)-catalyzed phosphorylation, cannot be activated by calcium plus phospholipid. The novel p82 kinase differs also from PKC in many other respects, such as substrate specificity, turnover rate, and sensitivity to inhibitors. The p82 kinase can be separated from PKC by chromatography on phenyl sepharose and does not react with a polyclonal PKC antiserum. Like PKC, the novel kinase phosphorylates its substrate on threonine and serine, but not on tyrosine. Similar to PKC, the epidermal p82-kinase system is down-modulated after TPA treatment of mouse skin, with a half-life of around 5 h. Down-modulation is also accomplished by the phorbol ester RPA, but not by the Ca^{2+} ionophore A23187, and it is inhibited by the immunosuppressive agent cyclosporin A. In addition to down-modulation, TPA treatment of the animals activates a phosphatase that dephosphorylates phosphorylated p82 in the extract of the particulate fraction.

Key words: protein phosphatase, protein kinase C antiserum, p82 kinase phosphorylation, down-modulation, TPA treatment

The molecular basis of mouse skin tumor promotion by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is still unknown. It is well established, however, that TPA in vitro, like the physiological activator diacylglycerol, lowers the

Abbreviations used: TPA, 12-O-tetradecanoylphorbol-13-acetate; RPA, 12-retinoylphorbol-13-acetate; PKC, protein kinase C; H9, N-(2-aminoethyl)-5-isoquinoline-sulfonamide; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; PS, phosphatidyl serine.

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calcium requirement of the Ca^{2+}/phospholipid-dependent protein kinase C (PKC), resulting in maximal activity of the enzyme at normal intracellular calcium concentration [1]. Thus TPA appears to be able to mimic the physiological activator of PKC. PKC-catalyzed phosphorylation of as yet unidentified proteins [1,2] is thought to play an important role in biological responses to TPA, including tumor promotion.

Recently, we discovered a phospholipid-dependent phosphorylation of an Mr 82,000 protein (p82) in the particulate fraction of mouse epidermis, which is also stimulated by TPA [3] but, contrary to PKC-catalyzed phosphorylation, cannot be activated by Ca^{2+} ions and phospholipid in the absence of TPA. Here we report on the further characterization of this novel TPA-dependent protein kinase and on its separation from PKC.

MATERIALS AND METHODS

Materials

12-O-tetradecanoylphorbol-13-acetate (TPA) and 12-retinoylphorbol-13-acetate (RPA) were kindly supplied by Prof. Dr. E. Hecker and Dr. G. Fürstenberger, respectively, German Cancer Research Center, Heidelberg, Federal Republic of Germany. Cyclosporin A (CsA) and the protein kinase inhibitor N-(2-aminoethyl)-5-isoquinolinesulfonamide (H-9) were generous gifts of Sandoz (Basel, Switzerland) and Dr. Hartenstein, Gödecke AG (Freiburg, Federal Republic of Germany), respectively. The inhibitor K252a was purchased from Kyowa Hakko Europe GmbH (Düsseldorf, Federal Republic of Germany). The Ca^{2+} ionophore A 23187 was obtained from Calbiochem GmbH (Frankfurt, Federal Republic of Germany). Phosphatidyl serine, the phosphoamino acids, cycloheximide, histone III-S, the calmodulin inhibitor N-(6-aminohexyl)-5-chloro-1-naphthalenesulfanamide (W7), anti-rabbit-IgG, peroxidase-antiperoxidase complex, and the V8 protease were purchased from Sigma (Munich, Federal Republic of Germany).

[γ-^{32}P]ATP (spec.act. 3,000 Ci/mmole) was obtained from NEN (Waltham, MA). PVDF (polyvinylidene difluoride) membranes were obtained from Millipore (Neu Isenburg, Federal Republic of Germany).

Animals

Female NMRI mice (age 7–8 weeks) were used in all experiments. The animals were kept under an artificial day–night rhythm and were fed a standard diet ad libitum.

Preparation of the Triton X-100 extract of epidermal particulate fraction, phosphorylation of proteins in the Triton X-100 extract, and polyacrylamide gel electrophoresis were performed as described previously [3]. Cytosol was the 100,000*g* supernatant of an epidermal homogenate and was obtained in the course of the preparation of the epidermal particulate fraction [see Ref. 3]. Kinase activity was measured with histone III-S as substrate as described previously [4].

Purification of Protein Kinase C (PKC) From Mouse Brain

Buffer A: 50 mM β-mercaptoethanol, 2 mM phenylmethylsulfonylfluoride, 25 μg/ml leupeptin, 20 mM Tris/HCl, pH 7.5; buffer B: 50 mM β-mercaptoethanol, 1 mM EGTA, 20 mM Tris-HCl, pH 7.5; buffer C: 50 mM β-mercaptoethanol, 0.1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5.

Mice were killed by cervical dislocation, brains were dissected, immediately frozen in liquid nitrogen, and stored at −70°C; 20 g of brain tissue were homogenized in 100 ml buffer A/1 mM $CaCl_2$. Under these conditions PKC is bound to the particulate fraction [5]. The homogenate was centrifuged at 100,000*g* for 30 min, and the pellet was washed once by rehomogenization in 100 ml buffer A/0.1 mM $CaCl_2$. After centrifugation as above, the pellet was rehomogenized once more in 50 ml buffer A/2 mM EDTA and 2 mM EGTA, incubated in ice for 30 min, and centrifuged as above.

The supernatant was mixed with one-half volume of buffer B/2 M NaCl and loaded onto a phenyl sepharose column (2.5 × 4 cm) equilibrated with buffer B/1 M NaCl. The column was washed with 50 ml buffer B/0.5 M NaCl, 25 ml buffer C/0.45 M NaCl, and with 100 ml buffer C/10% glycerol. PKC was then eluted by buffer B.

The pooled fractions from the phenyl sepharose column containing PKC activity were loaded onto a DEAE–cellulose column (0.6 × 2 cm) equilibrated with buffer B. The column was washed with 20 ml buffer B and eluted by a linear gradient of 0 to 0.3 M NaCl in buffer B (2 × 10 ml). The fractions with the highest PKC activity were pooled and found to be 95% homogeneous, as judged by SDS–polyacrylamide gel electrophoresis. This purification procedure takes about 8 h.

Phosphoamino acid analysis. The band containing phosphorylated p82 was cut out of the polyacrylamide gel, dried, and hydrolyzed in 6 N HCl for 2 h at 105°C. The sample was evaporated to dryness, dissolved in 10 μl water containing marker phosphoamino acids, and electrophoresed on Whatman 3MM paper in acetic acid/pyridine/water (52/5/943, pH 3.5).

Peptide mapping. After phosphorylation, samples (10 μl) were incubated with 10 μl of V8 protease dissolved in 10 mM Tris-HCl, pH 7.4 (12.5 μg/ml) for 1 min at 10°C. Controls were incubated without V8 protease. The reaction was stopped by the addition of 10% trichloroacetic acid; the precipitated proteins were dissolved in sample buffer and applied to SDS–polyacrylamide gel electrophoresis (9% acrylamide), as described previously [3].

DEAE–cellulose and phenyl sepharose chromatography. The Triton X-100 extract (28 ml) of the particulate fraction of mouse spleen (1,100 mg) was applied to a DEAE–cellulose column (1.6 × 2 cm) equilibrated with 50 mM Tris-HCl, 1 mM EDTA, pH 8 (TE). The column was washed with 6 ml TE and eluted with 6 ml 400 mM NaCl in TE. The eluate was applied to a phenyl sepharose column (0.9 × 3.5 cm) equilibrated with 300 mM NaCl in TE. The column was washed with 5 ml 300 mM NaCl in TE and eluted with 7 ml TE and then with 3 ml 0.2% Triton X-100 in TE.

Immunoblot. Extracts were subjected to SDS–polyacrylamide gel electrophoresis and proteins were transferred to a PVDF membrane according to Towbin et al. [6]. After blocking the membranes by overnight incubation with 10% fetal calf serum, 0.05% Triton X-100, and 0.05% sodium azide in TBS (Tris-buffered saline: 150 mM NaCl, 20 mM Tris-HCl, pH 7.4) at room temperature, they were incubated for 1 h at room temperature with a polyclonal PKC antiserum diluted 1:500 to 1:2,000 in incubation buffer (10% fetal calf serum and 0.05% sodium azide in TBS) with gentle agitation. The antiserum was raised in rabbits against a synthetic peptide with an amino acid sequence corresponding to part of the structure of rat brain PKC [Horn et al., in

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preparation]. The sequence of this peptide (D P N G L S D P Y V K L K) is conserved in the α, β, and γ PKC [7], but it is absent in the δ, ϵ and ζ PKC [8]. The membranes were washed three times for 5 min with 0.05% Triton X-100 in TBS and then incubated for 1 h with anti-rabbit IgG (2 μg/ml). After washing as above, the membranes were incubated with peroxidase/anti-peroxidase complex (diluted 1:1,000 in incubation buffer) for 1 h and washed as above, followed by an additional wash in 50 mM Tris-HCl (pH 7.6) buffer (peroxidase buffer). Immunoreactive bands were made visible by incubation in peroxidase buffer with 0.5 mg/ml 3,3-diaminobenzidine and 0.06% H_2O_2. The reaction was stopped with ethanol, and the membranes were air dried.

RESULTS

Incubation of a Triton X-100 extract prepared from the particulate fraction of mouse epidermis with phosphatidyl serine (PS), phorbol ester TPA, and (^{32}P)ATP resulted in the specific phosphorylation of an 82-kDa polypeptide. In contrast to PKC-catalyzed phosphorylation, p82 phosphorylation was unresponsive to Ca^{2+} ions, that is, Ca^{2+} could not replace TPA as a specific activator of the reaction (Fig. 1). Diacylglycerol at a 100-fold higher concentration than TPA exhibited a similar stimulatory effect as TPA (not shown).

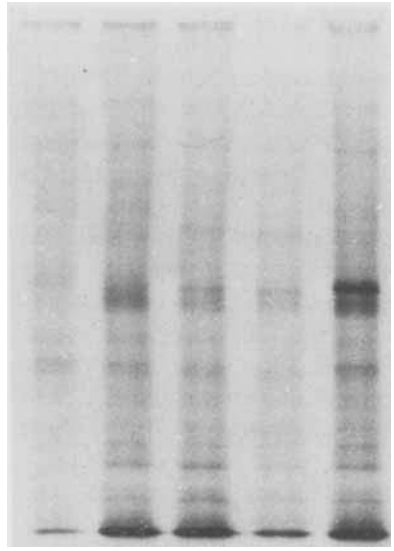

Fig. 1. Protein phosphorylation in a Triton X-100 extract of the particulate fraction of mouse epidermis: effect of Ca^{2+} and 12-O-tetradecanoylphorbol-13-acetate (TPA). The reaction mixture for protein phosphorylation contained 50 μg phosphatidyl serine (PS) and, where indicated, 4 $\times$ 10^{-6}M TPA. Phosphorylation was performed and evaluated as described previously [3].

As shown in Figure 2, purified PKC from mouse brain was unable to phosphorylate p82 when added to the extract of the epidermal particulate fraction. Several other proteins in this extract, however, were phosphorylated extensively by PKC. Histone has been used in most cell-free systems as a substrate for PKC. The difference in histone phosphorylation in the presence of Ca^{2+} alone versus Ca^{2+}/PS has been taken as a measure of PKC activity. Figure 3 shows that, under these conditions, PKC activity was undetectable in the Triton X-100 extract of the particulate fraction of untreated mouse epidermis, whereas TPA-dependent kinase activity was rather high. In contrast, epidermal cytosol exhibited distinct PKC activity but no significant TPA-dependent kinase activity.

To evaluate the effect of a PKC inhibitor, such as K252a, on p82-phosphorylation, a Triton X-100 extract from the particulate fraction of spleen, instead of epidermis, was used; in contrast with epidermal preparations the spleen fraction contains both PKC and p82 kinase [3]. K252a was reported to inhibit PKC and cyclic-nucleotide-dependent protein kinases with K_i values of 18–25 nM [9]. K252a suppressed the TPA/phospholipid-dependent phosphorylation of p82 only slightly, whereas the Ca^{2+}/phospholipid-dependent, that is, PKC-catalyzed, phosphorylation of several other proteins was inhibited effectively (Fig. 4). Topical application of 1 mg cycloheximide in 100 μl acetone to mouse skin resulted in a 50% reduction of p82 phosphorylation within 7 h (Fig. 5); 26 h after cycloheximide treatment, p82 phosphorylation was still inhibited by 40%. When added to the cell-free system, cycloheximide did not influence p82 phosphorylation (data not shown).

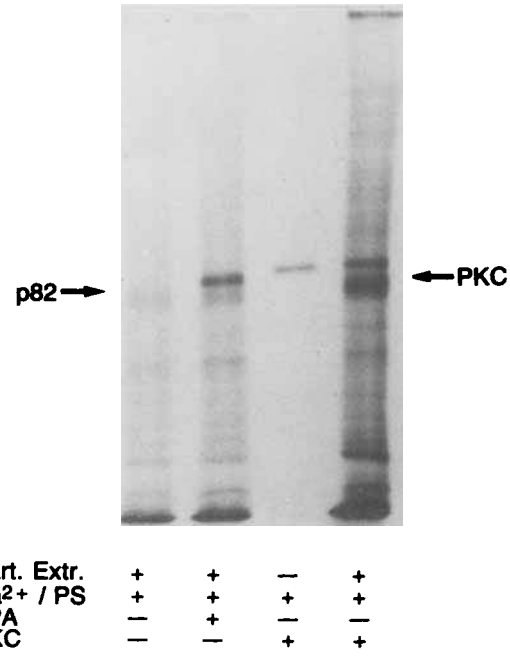

Fig. 2. Effect of purified protein kinase C (PKC) on protein phosphorylation in the Triton X-100 extract of the epidermal particulate fraction. Where indicated, 0.05–0.1 mU of PKC were added.

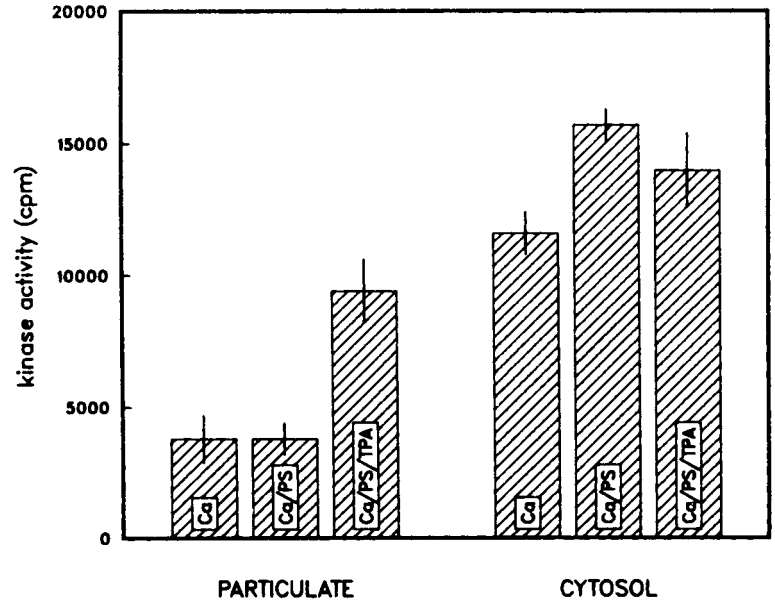

Fig. 3. Phosphorylation of histone III-S in the Triton X-100 extract of the particulate fraction and in the cytosol of mouse epidermis: effect of Ca^{2+} (0.5 mM), phosphatidyl serine (PS) (50 μg), and 12-O-tetradecanoylphorbol-13-acetate (TPA) (4×10^{-6}M).

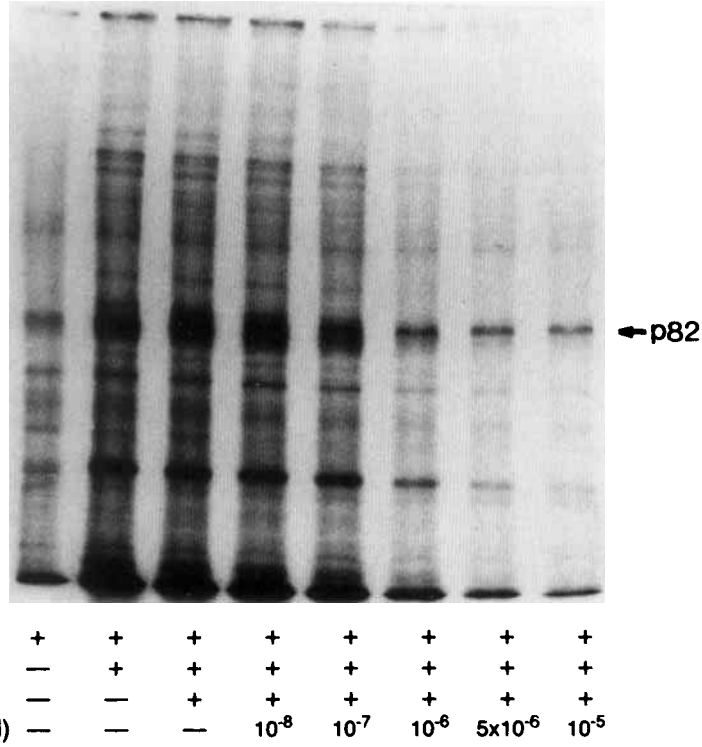

Fig. 4. Protein phosphorylation in a Triton X-100 extract of the particulate fraction of mouse spleen: effect of protein kinase C (PKC) inhibitor K252a. The cell-free system from spleen was prepared as was that from epidermis [3]. The final concentrations of Ca^{2+}, phosphatidyl serine (PS), and 12-O-tetradecanoylphorbol-13-acetate (TPA) were as in Figure 3, and that of K252a (M) was as indicated.

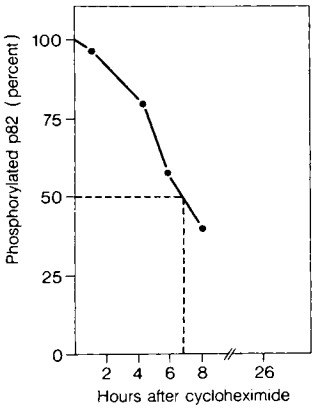

Fig. 5. Phosphorylation of p82 in the Triton X-100 extract of the epidermal particulate fraction after topical application of cycloheximide to mouse skin. Various times after application of 100 μl of cycloheximide (10 mg/ml acetone) to the back skin of mice, protein phosphorylation in the Triton X-100 extract of the epidermal particulate fraction was performed in the presence of 12-O-tetradecanoylphorbol-13-acetate (TPA) as described previously [3]. The intensity of the p82 band was determined by scanning the autoradiograms and is given in percent of the control (untreated).

For the reasons mentioned above, spleen was used, instead of epidermis, in an attempt to separate the p82 kinase from PKC. The Triton X-100 extract of the spleen particulate fraction was applied to DEAE–cellulose, and both kinase activities were eluted with 0.4 M NaCl. After application of this salt-containing kinase preparation to phenyl sepharose, PS/Ca^{2+}-dependent PKC activity could be eluted with a Tris/EDTA buffer, whereas PS/TPA-dependent p82-kinase activity required Triton X-100 for elution (Fig. 6). The immunoblot, which used a polyclonal PKC antiserum raised against a PKC peptide and reacts with α, β, and most likely also with γ PKC, (Horn et al., in preparation), proved that PKC was present predominantly in the Tris/EDTA fraction and showed that p82 kinase did not react with this antiserum (Fig. 6).

Several attempts to separate p82 from its kinase had failed. Since this indicated that p82 itself might be the kinase and that phosphorylation of p82 might be autophosphorylation, it was important to show by proteolytic fragmentation that p82 and PKC were different proteins. Limited digestion of both phosphorylated p82 and PKC by V8 protease revealed a significant difference in the fragmentation, as shown by Figure 7. PKC was partially digested to a fragment with a molecular weight of ~80,000 daltons, whereas p82 gave rise to a smaller fragment with a molecular weight of ~65,000 daltons. Upon digestion of an extract lacking phosphorylated p82, the 65,000 dalton-fragment was not observed (data not shown). This proved that this fragment indeed originated from p82.

To determine the phosphoamino acid(s) of phosphorylated p82 (pp82), the respective band on the polyacrylamide gel was cut out, the protein was hydrolyzed by acid treatment, and the hydrolysate was applied to high-voltage paper chromatography. Phosphothreonine and phosphoserine, but no phosphotyrosine, were observed, as shown in Figure 8. After topical application of 20-nmole TPA to mouse skin, the p82 phosphorylation system (kinase and/or substrate) disappeared from the particulate fraction of epidermis (Fig. 9 and Table I). No effect was seen up to 2 h after TPA

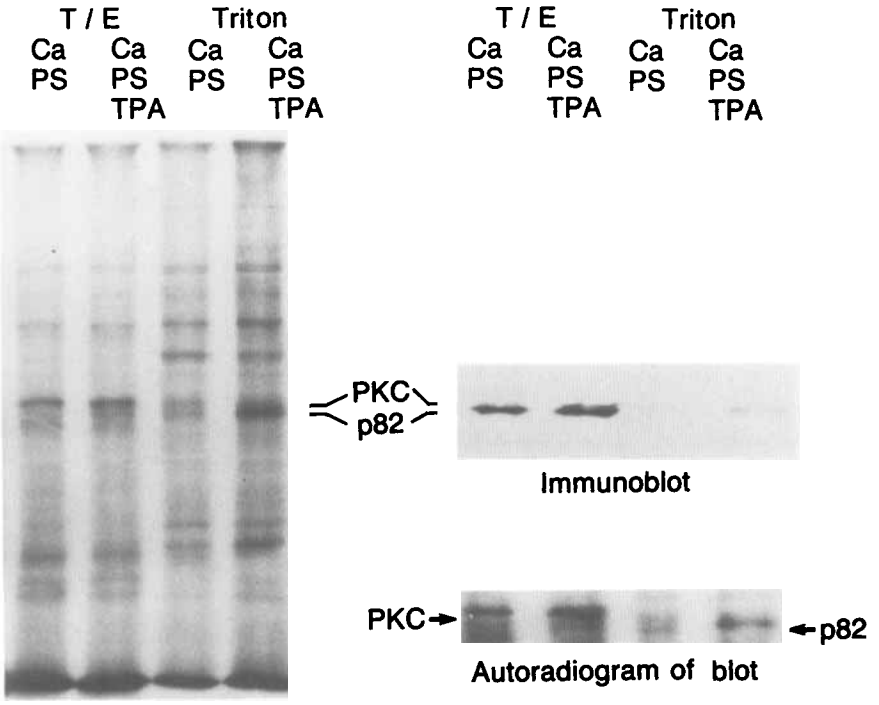

Fig. 6. Polyacrylamide gel of phenyl sepharose fractions after phosphorylation of proteins and immunoblotting of the proteins using a protein kinase C (PKC) antiserum. Chromatography of the Triton X-100 extract of the particulate fraction of mouse spleen on DEAE–cellulose and then on phenyl sepharose was performed as described in Methods. The Tris/EDTA (T/E) and the Triton X-100 (Triton) eluates of the phenyl sepharose were phosphorylated with ^{32}P-ATP in the presence of Ca/PS and Ca/PS/TPA, as described previously [3]. The proteins were separated on an SDS–polyacrylamide gel (autoradiogram of the gel: **left** side) and transferred to a polyvinylidene difluoride membrane (autoradiogram of the blot: **right** side). The membrane was incubated with a PKC antiserum and bound immunoglobulin was detected as described in Materials and Methods. Only the relevant section of the immunoblot (right side) containing the stained immunocomplex is shown. To allow for a distinctly visible immunoreaction to lanes **1, 2,** and **4** (from left to right), the respective unlabelled sample at a 40-fold concentration (concentrated by trichloroacetic acid [TCA] precipitation) was added. Lane **3** contained just the labeled sample. Ca/PS, calcium/phosphatidyl serine; TPA, 12-O-tetradecanoylphorbol-13-acetate.

treatment, while phosphorylation of p82 was reduced by 30% after 4 h. Ninety percent inhibition was reached 24 h after TPA application. After 48 h, p82 phosphorylation began to reappear (43% of the control). This "down-modulation" of the p82 phosphorylation system was also observed after treatment of the animals with the phorbol ester RPA but not with the calcium ionophore A 23187; it could be inhibited by cyclosporin A (CsA, Fig. 9).

We observed a distinct and specific reduction of p82 phosphorylation in the Triton X-100 extract from untreated animals when adding the extract from TPA-treated animals, which lacked p82 phosphorylation system because of down-modulation (Fig. 10). This inhibitory potency could be destroyed by heating the extract to 60°C for 5 min before mixing it with the extract from untreated animals or by adding molybdate,

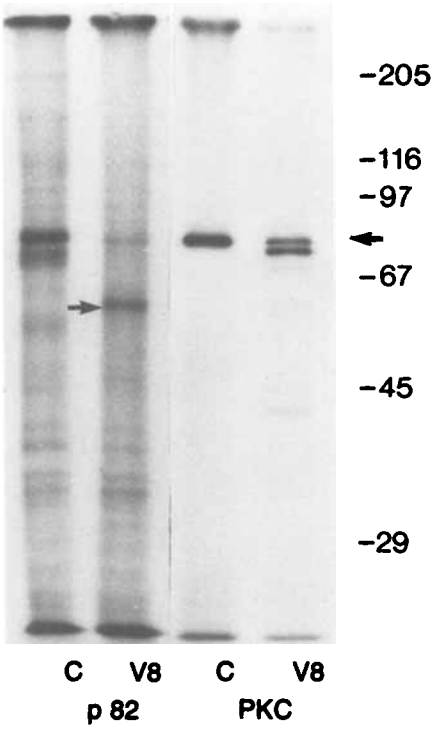

Fig. 7. Effect of limited digestion with the V8 protease on phosphorylated p82 and autophosphorylated protein kinase C (PKC). The Triton X-100 extract of the particulate fraction of mouse epidermis containing p82 as well as purified PKC from mouse brain were phosphorylated with ^{32}P-ATP in the presence of Ca^{2+}/PS/TPA [3]. The phosphorylated samples were incubated with V8 protease as described in Materials and Methods and applied to a 9% polyacrylamide gel. C is the control (without V8). The following molecular weight markers were used: myosin (205 kD), β-galactosidase (Escherichia coli, 116 kD), phosphorylase B (97 kD), bovine serum albumin (67 kD), ovalbumin (45 kD), and carbonanhydrase (29 kD). Ca^{2+}/PS/TPA, calcium/phosphatidyl serine/12-O-tetradecanoylphorbol-13-acetate.

an inhibitor of phosphoprotein–phosphatase, to the mixture, before starting the phosphorylation reaction (Fig. 10). Molybdate could not restore, however, the "down-modulated" p82 phosphorylation in an extract from TPA-treated (19 h) animals (Fig. 10). In the presence of molybdate, the Ca^{2+}/PS-dependent phosphorylation of at least two proteins (indicated by the arrows in Fig. 10) became visible in this cell-free system; this phosphorylation was not observed in the absence of molybdate.

DISCUSSION

In a search for PKC substrates in mouse epidermis, we recently discovered the in vitro phosphorylation of an Mr 82,000 protein (p82) in the Triton X-100 extract of the particulate fraction of mouse epidermis. The phosphorylation of p82 was found to be dependent on the phorbol ester TPA (or diacylglycerol) and phospholipid [3]. Here we show that p82 phosphorylation differs profoundly from orthodox PKC-catalyzed

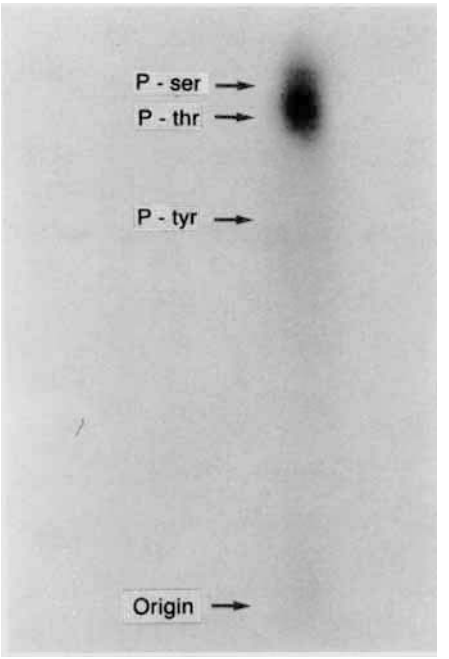

Fig. 8. Analysis of phosphoamino acids derived from phosphorylated p82. For the details of the procedure see Materials and Methods. The marker phosphoamino acids, phosphotyrosine, phosphothreonine, and phosphoserine, were applied to the chromatography paper together with the ^{32}P-labeled sample. Markers were visualized with ninhydrin spray and ^{32}P-labeled phosphoamino acids by autoradiography.

phosphorylation. This conclusion is based on the following properties of the p82 phosphorylation system:

1. Phorsphorylation of p82 is completely unresponsive to Ca^{2+} ions.
2. Purified PKC from mouse brain is unable to phosphorylate p82.
3. Epidermal p82-kinase activity is restricted to the particulate fraction, whereas PKC is predominantly located in the cytosol.
4. Phosphorylation of p82 is relatively insensitive to the PKC inhibitor K252a.
5. Contrary to PKC with a half-life of more than 24 h [10], the p82-kinase system has a rather high turnover rate with a half-life of only 7 h.
6. p82-kinase activity can be separated from PKC activity, e.g., by chromatography on phenyl sepharose.
7. A polyclonal PKC antiserum, which was raised against a conserved PKC peptide present in α, β, and γ but not in δ, ϵ and ζ PKC, does not react with p82 kinase.
8. As indicated by the failure to separate p82 from its kinase, p82 might itself be the novel kinase. Proteolytic fragmentation proves that p82 and PKC are different proteins.

Aside from the differences stated above, the behavior of the TPA-dependent p82 kinase is very similar to that of the PKC, indicating that both enzymes are related.

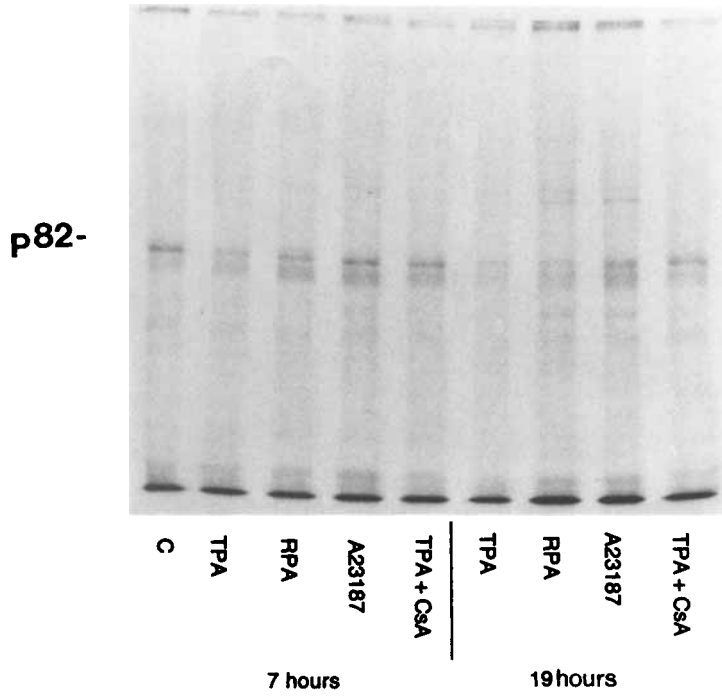

Fig. 9. Phosphorylation of proteins in the extract of the epidermal particulate fraction 7 and 19 h after topical application of various compounds to mouse skin in vivo: 20 nmole TPA, 20 nmole RPA, 200 nmole A 23187, 20 nmole TPA + 2 mg CsA (cyclosporin A). TPA, 12-O-tetradecanoylphorbol-13-acetate; RPA, 12-retinoyl phorbol-13-acetate.

TABLE I. Phosphorylation of p82 in the Triton X-100 Extract of the Epidermal Particulate Fraction Various Times After Topical Application of 20 nmole TPA to Mouse Skin

Time after TPA (h)	Phosphorylation of p82 (%)
0	100
0.5	102
2	101
4	68
7	26
24	9
48	43

The intensity of the p82 band was measured by densitometric scanning of the autoradiograms. The values are given in percent of the control (untreated).

Several recent reports prove the existence of a PKC family [7,8; for review see Ref. 11]. While this paper was reviewed, Ohno et al. [12] reported on a novel protein kinase, the so-called nPKC, which is distantly related to the PKC family. nPKC is regulated by phospholipid, diacylglycerol, and phorbol ester but is independent of Ca^{2+}. It is conceivable that the p82 kinase is a member of a novel nPKC family, that is, of a Ca^{2+}-independent PKC family. Like PKC [13], the novel p82 kinase can be inhibited by the inhibitor H9 but not by the calmodulin inhibitor W7 (data not shown). And like PKC [14], the TPA-dependent kinase phosphorylates its substrate

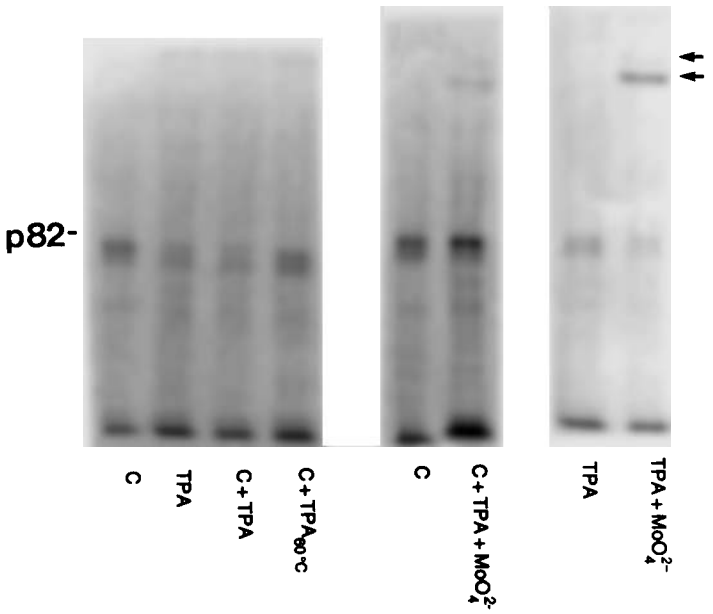

Fig. 10. Stimulation of phosphatase activity in the Triton X-100 extract of the epidermal particulate fraction by treatment of the animals with 12-O-tetradecanoylphorbol-13-acetate (TPA) (19 h). Equal amounts of Triton X-100 extracts from treated (TPA) and untreated (C) animals were mixed. An aliquot of the mixture or of each extract alone was taken for the phosphorylation that was performed in the presence or absence of 2.5 mM molybdate (MoO_4^{2-}), as described previously [3]. In one experiment the Triton X-100 extract from TPA-treated animals was heated to 60°C for 5 min (TPA 60°C) before mixing it with the extract from untreated animals.

at threonine and serine. Finally, application of TPA to the back skin of mice induces a down-modulation of the p82-kinase system in the particulate fraction of epidermis. A similar observation has been reported for various PKC-catalyzed phosphorylation systems (for a review see Ref. 11).

The half-life of the p82-kinase system after TPA treatment of the animals is around 5 h. The respective half-life of PKC varies depending on the cell line used [11]. In contrast to the p82 kinase, which is already located in the particulate fraction, a large portion of PKC is translocated from the cytosol to the particulate fraction as a consequence of TPA-treatment and then down-modulated [15]. The non-convertogenic tumor promoter RPA [16] is also able to induce down-modulation of the p82-kinase system, albeit at a later time after application to mouse skin than TPA. The Ca^{2+}-ionophore A 23187 that mimics several TPA effects in mouse skin but lacks tumor promoting activity [17] is ineffective in causing down-modulation of the epidermal p82-kinase system. The immunosuppressive agent cyclosporin A, an inhibitor of various TPA effects in mouse skin, including tumor promotion [18,19], suppresses the TPA-induced down-modulation of the p82-kinase system.

Experiments with mixed Triton X-100 extracts of the epidermal particulate fraction from untreated (control) and TPA-treated animals showed that TPA causes the induction or activation of a factor that reduces the amount of the phosphorylated form of p82 (pp82) in the control preparation. Since this effect is prevented by molyb-

date, it is most likely due to a protein phosphatase that dephosphorylates pp82. This "pp82-phosphatase" is heat sensitive. It is active predominantly in the particulate fraction but only after TPA treatment of the animals. This finding led us to suspect that the down-modulation of pp82 was, in reality, a dephosphorylation of pp82 by a TPA-activated phosphatase. Since, however, molybdate is unable to restore p82-phosphorylation in "down-modulated" skin extracts, it is likely that TPA-induced down-modulation of the p82-kinase system and TPA-induced activation of a pp82 phosphatase are independent processes, both of which result in a decrease in pp82. It is conceivable that these processes are part of a feedback mechanism that regulates the amount of pp82. This may indicate an important, but as yet unknown, physiological role of the p82-kinase system and of pp82 in mouse epidermis.

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