Protein kinase C signaling is desensitized through a combination of dephosphorylation and proteolysis in intact cells. The process of dephosphorylation is analyzed here, as well as its relationship to degradation. It is established for protein kinase Cα that dephosphorylation occurs in a membrane compartment following activation and temporally preceding significant degradation. The phosphatase responsible for the dephosphorylation appears to be a heterotrimeric type 2A phosphatase, which is shown to be in part constitutively membrane associated. Consistent with a role for this activity, okadaic acid is shown to inhibit the phospholipid- and diacylglycerol-dependent dephosphorylation of protein kinase Cα that occurs in intact cells. Furthermore, phospholipid-induced down-regulation of protein kinase Cα is shown not to be dependent on the rate of dephosphorylation, indicating that these desensitizing pathways may operate in parallel.

Members of the protein kinase C (PKC) family constitute a class of diacylglycerol-dependent protein kinases (reviewed in Refs. 1–4). These ligand-dependent protein kinases display constitutive catalytic activity on partial proteolysis in vitro, indicating the presence of a fully functional kinase domain that does not require ligand-dependent modification for activity (see Ref. 5). Consistent with this view, point mutations in the autoinhibitory domain of PKCα render the protein constitutively active, i.e. ligand-independent (6). Thus PKC could be considered to behave like the Ca2+- and phosphatidylserine-dependent kinases, albeit with tethered regulatory and catalytic domains. Indeed, much of our understanding of PKC function in vivo reflects its transient, allosteric activation by its effectors, a paradigm much exploited for Ca2+-dependent protein kinases.

Although this view of a ligand-dependent protein kinase encompasses numerous observations of PKC behavior, it is well established that at least for PKCα and PKCBβ, phosphorylation controls intrinsic catalytic potential (see Ref. 7 and references therein). This was first demonstrated for the PKCα isotype, both in vivo (8) and in vitro (9). In the former case, the dephosphorylated, inactive form of PKCα accumulated following chronic activation by the phorbol ester TPA (8). It is clear that in other contexts, chronic activation by TPA also leads to the generation of dephosphorylated forms that migrate faster on SDS-polyacrylamide gel electrophoresis (PAGE) (for example, see Ref. 10); these forms usually accumulate in association with the cytoskeleton. In view of the loss of activity of PKCα on dephosphorylation, it is anticipated that in vivo this event serves an important role in desensitizing responses through the PKC pathway.

There is limited information available as to the specific components involved in the desensitization of PKC. In the case of down-regulation, it has been established that the loss of protein is a consequence of increased proteolysis (11). Furthermore, there is evidence that this is driven by some form of vesicle-dependent sorting process (12), but the molecular details remain elusive. Nothing is known about the dephosphorylation of PKC in intact cells or the relationship between this process and the coincident down-regulation that is observed. To understand the selective dephosphorylation of PKCα and its role, it is important to define the molecular mechanism(s) involved. This study describes the identification of a membrane-associated protein phosphatase 2A heterotrimer that acts on PKCα in vitro and in intact cells and further analyses the relationship between the proteolytic degradation and dephosphorylation of PKCα.

**EXPERIMENTAL PROCEDURES**

**Materials**—Okadaic acid and other biochemical reagents were obtained from Sigma unless stated otherwise. Other chemicals were of the highest grade available. Antisera to PKCα and to subunits of protein kinases 1 and 2A were as described previously (13–16). The horseradish peroxidase-conjugated donkey anti-rabbit IgG, ECL detection reagents, and [γ-32P]ATP were from Amersham Corp.

**Cell Culture and Transfection**—COS cells were grown in culture as described previously (17). Where indicated, subconfluent COS cells were transfected by electroporation as detailed elsewhere (18). Where analyzed for down-regulation of wild-type and mutant PKCs, cells were treated with TPA for the times indicated and harvested directly into sample buffer (19). Proteins were fractionated by SDS-PAGE on 10% acrylamide gels, and PKCα was detected by Western analysis.

**Protein Analysis**—Samples were subjected to SDS-PAGE (19). Separated proteins were transferred to Immobilon membranes (Millipore) and probed with PKC or protein phosphatase antisera as described previously (20). Detection of immunoreaction was by ECL. Protein concentration was measured by the method of Bradford (21).

**Cell Extraction and Fractionation**—For the partial purification of the membrane-associated protein phosphatase, COS cells were harvested and snap frozen in aliquots of ~106 cells. Extracts from frozen COS cells were prepared in 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 5 mM EGTA, 10 mM benzamidine, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 100 μg/ml aprotinin, and 0.3% β-mercaptoethanol; 2 ml of extraction buffer was used per 106 cells. The extract was homogenized using a Dounce homogenizer, and the crude membrane fraction was prepared by centrifugation at 14,000 rpm in a bench-top

---

*This work was supported in part by a fellowship from the European Commission (to F. P.) and as part of a European Commission Biomed Program (to P. J. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Institut de Recherches Servier, 125 Chemin de Ronde, 78290 Croissy-sur-Seine, France.

§ To whom correspondence should be addressed.

¶ The abbreviations used are: PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; PAGE, polyacrylamide gel electrophoresis; OA, okadaic acid.
The Dephosphorylation of PKCα by Protein Phosphatase 2A

FIG. 1. Dephosphorylation of PKCα at the membrane. COS cells transfected with PKCα were either treated or not with TPA (500 nM) for 60 min as indicated. Cells were then extracted and fractionated into cytosol (C) and Triton X-100-soluble (TS) and Triton X-100-insoluble (TI) fractions as described under “Experimental Procedures.” Equivalent samples from each fraction were separated by SDS-PAGE, and PKCα was detected by Western analysis. Arrows, upper and lower (dephosphorylated) PKCα species. The markers are 97 kDa (upper bar) and 67 kDa (lower bar).

FIG. 2. A membrane-associated PKCα-phosphatase. Membranes from ~10^7 COS cells were assayed for the presence of phosphatase activity using recombinant PKCα (see “Experimental Procedures”). Dephosphorylation was detected by altered migration on SDS-PAGE, as indicated by the arrows. The detection was by Western analysis. Arrows, upper and lower (dephosphorylated) PKCα species. The markers are 97 kDa (upper bar) and 67 kDa (lower bar).

FIG. 3. TPA independence of PKCα-phosphatase membrane association. Cells were either pretreated or not with TPA (500 nM) for 60 min as indicated. Membranes were isolated, and PKCα-phosphatase activity was assayed in the presence or absence of microcystin as shown. Dephosphorylation was detected by altered migration on SDS-PAGE; arrows, phospho-PKCα and dephospho-PKCα species. The markers shown are 97 and 67 kDa (upper and lower bars, respectively).

FIG. 4. Membrane-associated PKCα-phosphatase is a protein phosphatase 2A heterotrimeric complex. The membrane-associated phosphatase activity was extracted in Triton X-100 and fractionated on a Mono Q anion exchange column. A, the PKCα-phosphatase activity detected by altered migration of dephosphorylated PKCα peaked in fractions 11 and 12. B, the fractions through the peak of phosphatase activity (9–15) were analyzed for protein phosphatase 1 and 2A catalytic subunits. Whole cell extracts (extract) were reactive with both protein phosphatase 1c (PP1c) and protein phosphatase 2Ac (PP2Ac) antisera, demonstrating the presence of these 37-kDa subunits. Antiserum to protein phosphatase 1c detected no antigen in the fractionated membrane samples (not shown), whereas the 2Ac serum identified the 37-kDa subunit coincident with the PKCα-phosphatase activity. C, the same Mono Q fractions as above were analyzed for the p55α and p65α subunits by Western blotting. Arrows, reactive antigens migrating at 55 and 65 kDa.

Results

Preliminary data indicated that dephosphorylation of COS cell PKCα could be detected within 60 min of TPA treatment (see below). To assess the compartment in which PKCα is dephosphorylated, COS cells were stimulated with TPA and fractionated. Cytosol, the Triton X-100-soluble membrane fraction, and the Triton X-100-insoluble fraction were analyzed. As shown in Fig. 1, untreated cells showed an exclusively cytosolic localization of PKCα. However, after 60 min of TPA treatment, the PKCα antigen was no longer present in the cytosolic fraction but was found in the Triton X-100-soluble membrane fraction. The antigen migrated as a doublet characteristic of phospho- and dephospho-PKCα species. No dephosphorylation was observed following much shorter treatments with TPA (15 min; not shown) even though PKCα becomes fully membrane asso-
The Dephosphorylation of PKCα by Protein Phosphatase 2A

Fig. 5. Protein phosphatase 2A does not translocate in response to TPA. COS cells were treated (60 min) or not with TPA (500 nM) as indicated. Cytosol (C) and membrane (M) extracts were prepared as described under “Experimental Procedures.” Samples were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane for Western analysis. Strips of membrane covering the 57- , 65- , and 55-kDa ranges were excised and blotted with sera to protein phosphatase 2A, (C; p65α and p55α subunits are as indicated.

Fig. 6. Okadaic acid inhibits TPA-induced dephosphorylation of PKCα in COS cells. A, COS cells were transfected with PKCα and then treated with TPA (500 nM) for 60 min in the presence of increasing concentrations of OA as indicated. PKCα dephosphorylation was observed as a shift in migration on SDS-PAGE, as indicated by the arrows; lower dotted arrow, dephosphorylated form. B, COS cells as in A, were treated with TPA for 60 or 90 min as indicated, in the presence or absence of 500 nM OA. Although okadaic acid inhibited the accumulation of the faster migrating dephosphorylated PKCα species (dotted arrow) at 60 min, this was incomplete, as evidenced by further dephosphorylation even in the presence of okadaic acid at 90 min.

Fig. 7. Wild-type and T638A PKCα down-regulate at equivalent rates. COS cells were transfected with either wild-type (Wt) PKCα or phosphorylation site mutant T638A (638A) PKCα. Cells were then treated with TPA (500 nM) for increasing times and extracted directly for SDS-PAGE. Samples were run on 10% polyacrylamide gels at a 30:0.6 acrylamide/methylene bisacrylamide ratio (not high resolution). Under these conditions, shifts in migration are less apparent; however the less diffuse migration permits more accurate quantitation (see text).

trimeric form of protein phosphatase 2A.

Having established the identity of the membrane-associated PKCα-phosphatase, confirmation of the TPA-independent localization (see Fig. 3) was obtained. Control and TPA-stimulated cells were fractionated, and protein phosphatase 2A subunits were localized. It is evident (Fig. 5) that the heterotrimeric complex is in part constitutively membrane-associated; this distribution is entirely insensitive to TPA treatment. The lack of TPA response on the localization of the phosphatase is consistent with the effect of TPA being PKCα (i.e. substrate)-directed.

To assess the role of protein phosphatase 2A in intact cells, PKCα-transfected COS cells were treated with TPA, and the effect of okadaic acid (OA) was determined. As illustrated in Fig. 6, okadaic acid inhibited TPA-induced dephosphorylation with an almost complete effect at 500 nM. The slow time-dependent increase in PKCα dephosphorylation even in the presence of OA (Fig. 5B) suggests either incomplete inhibition by OA or the action of an additional inhibitor-insensitive activity.

It would appear from Fig. 6A that OA may protect PKCα from TPA-induced degradation. However, the OA effect on antigen loss was variable, and furthermore, any response to OA would not distinguish between an effect on the proteolytic “machinery” and an effect via inhibition of PKCα dephosphorylation. To resolve the relationship between TPA-induced PKCα dephosphorylation and degradation, use was made of the T638A PKCα phosphorylation site mutant, which is rapidly dephosphorylated in vivo on TPA stimulation (23). It is evident from Fig. 7 that the wild-type PKCα and the T638A PKCα down-regulate at similar rates. In fact, wild-type PKCα is lost more rapidly than the T638A mutant, with 20% (wild-type) and 34% (T638A) of antigen remaining after 3 h of TPA treatment.

DISCUSSION

It is demonstrated here that the TPA-dependent dephosphorylation of PKCα at the membrane is okadaic acid-sensitive in vivo, correlating with the presence of a membrane-associated protein phosphatase 2A heterotrimeric complex. TPA does not appear to influence the membrane-associated heterotrimeric protein phosphatase 2A activity or its subcellular distribution; the effect on PKCα dephosphorylation correlates with TPA-induced PKC activation and the associated conformational change (7). It is further shown that although TPA-induced PKCα dephosphorylation is inhibited in vivo by okadaic acid, dephosphorylation is not rate-limiting with respect to down-regulation; these two desensitizing pathways appear to operate independently. Thus, although it is possible that other okadaic acid-sensitive phosphatases may act on PKCα in vivo, the simplest interpretation is that the membrane-associated, heterotrimeric 2A complex is responsible for this desensitizing process.

Following chronic activation of PKC by exposure to the
poorly metabolized activator TPA, TPA-responsive PKC iso-
types are frequently found to become down-regulated through
increased proteolysis (e.g. Ref. 11). Associated with this down-
regulation is a shift in mobility on SDS-PAGE of the remaining
antigen and an accumulation in the Triton X-100-insoluble
cytoskeleton fraction (8). The down-regulation of PKCa and cytoskeleton association is shown here to be preceded by a dephosphorylation of the mem-
brane-associated, activated PKC. PKCa is phosphorylated on
at least three sites in vivo (24), and the extent of the shift in
mobility is consistent with the complete dephosphorylation of
the protein. The time frame of these events is minutes or hours,
not seconds; hence this would seem to be part of an adaptive
process responding to chronic activation. It should be noted
that prolonged activation and down-regulation is not unique to
TPA stimulation but is observed also on exposure to physiological
agonists (e.g. Refs. 25 and 26).

The inhibition of TPA-induced PKCs dephosphorylation by OA gave a variable effect on the subsequent loss of polypeptide,
normally associated with chronic TPA exposure. To resolve any
possible causal relationship between dephosphorylation and degradation of PKCa, a mutant of PKC (T638A), which is
hypersensitive to dephosphorylation (23), was used. In this
context, when the rate of dephosphorylation was very greatly
increased, no difference was observed with respect to PKCa
polypeptide loss. Thus the rate of dephosphorylation does not
determine the rate of down-regulation.

As yet there is no evidence on the specific nature of the membrane
compartment in which PKCa is dephosphorylated. Although there is no cause-and-effect relationship between dephosphorylation and down-regulation, it is evident that these
events occur within the same time frame. We have shown previously that the process of down-regulation appears to be a
vesicle traffic-directed event (12). It is thus quite likely that the
membrane-associated heterotrimeric protein phosphatase 2A
complex is also present in the same vesicle compartment and is
not (solely) resident at the plasma membrane.

Although the heterotrimeric protein phosphatase 2A complex
was first identified as a soluble activity, a membrane-
associated form(s) of protein phosphatase 2A has been identified
in T cell membranes (27). In COS cells, approximately 10%
of the protein phosphatase 2A catalytic subunit is membrane-
associated (see Fig. 5). Whether there is a specific membrane-
targeting protein or whether there are modifications to one or
more subunits rendering the protein membrane-bound remains to
determine.

During the course of these studies, a similar particulate
protein phosphatase 2A complex was identified as being re-
sponsible for the inactivation of c-Raf (28) and for the dephos-
phorylation of the β-adrenergic receptor (29). It would appear
that this membrane-associated activity plays a key role in the
desensitization and reversal of a number of distinct signaling
events. In the case of PKCα the trigger for dephosphorylation is
the TPA-induced membrane association and change in confor-
mation. No change in the localization of the protein phospha-
tase 2A complex or in its extractable membrane-associated activity was observed following TPA treatment of cells.

The existence of a mechanism for the dephosphorylation (and inactivation) of PKCα suggests that either sustained activation has detrimental effects on the cell and hence must be desensi-
tized or that the cytoskeleton-associated, dephosphorylated
protein that accumulates has some unique function in vivo. Although this distinction remains to be resolved, the simple
view would be that this is part of a desensitization process.

As a consequence of being shown to activate protein phosphatase
2A (30), it was possible that increased dephosphorylation of
PKC may play a role in the antagonism between ceramide (or perhaps a metabolite) and PKC function. However, in COS
cells treated acutely (15 min) with either short chain ceramides or sphingomyelinase, no difference in the rate of TPA-induced
dephosphorylation of PKCa was observed (data not shown).

This contrasts with recent data for T cell lines, in which on
prolonged exposure in the absence of TPA, ceramide has been
shown to induce PKCa dephosphorylation (31). Whether this
distinction reflects cell specificity or subcellular localization
remains to be determined.

In conclusion, PKCa is shown to be dephosphorylated by a
membrane-associated heterotrimeric protein phosphatase 2A
complex. This process is triggered by the membrane-associated
activation of PKCa itself. Whether physiological modifiers of
protein phosphatase 2A activity control the extent of activa-
tion of PKCa remains to be determined. However, irrespective of
the acute control of the dephosphorylation itself, it would appear
that this step temporally precedes the enhanced degrada-
tion observed in response to agonists and thus plays a primary
role in desensitizing this pathway.

Acknowledgments—We are indebted to Dr. M. Bollen (Katholieke
Universiteit, Leuven, Belgium) for providing the protein phosphatase 1
antiserum. We gratefully acknowledge Louise Mansi for preparation of
the text.

REFERENCES

1. Nishizuka, Y. (1992) Science 258, 607–614
2. Hug, H., and Sarre, T. F. (1993) Biochem. J. 291, 329–343
3. Dekker, L. V., and Parker, P. J. (1994) Trends Biochem. Sci. 19, 73–77
4. Nishizuka, Y. (1995) FASEB J. 9, 484–496
5. Kishimoto, A., Kajikawa, N., Shiota, M., and Nishizuka, Y. (1983) J. Biol.
Chem. 258, 1156–1164
6. Pears, C. J., Kour, G., House, C., Kemp, B. E., and Parker, P. J. (1990) Eur.
J. Biochem. 194, 89–94
7. Newton, A. C. (1995) J. Biol. Chem. 270, 28495–28498
8. Bornancin, F., Filippuzi, L., Wartmann, M., Eppenberger, U., and Fabbro, D.
(1989) J. Biol. Chem. 264, 13098–13099
9. Pears, C., Stabel, S., Cazaubon, S., and Parker, P. J. (1992) Biochem. J. 283,
515–518
10. Junco, M., Webster, C., Crawford, C., Bosca, L., and Parker, P. J. (1994) Eur.
J. Biochem. 223, 259–263
11. Young, S., Parker, P. J., Ulrich, A., and Stabel, S. (1987) Biochem. J. 244,
759–779
12. Goode, N. T., Hajibagheri, M. A. N., and Parker, P. J. (1995) J. Biol. Chem.
270, 2669–2673
13. Makita, R. M., and Parker, P. J. (1989) Eur. J. Biochem. 182, 129–137
14. Wera, S., Fernandez, A., Lamb, N. J., Turwosp, P., Hemmings, M. M., Mayer,
J. R., and Hemmings, B. A. (1995) J. Biol. Chem. 270, 21374–21381
15. Turowski, P., Fernandes, A., Favre, B., Lamb, N. J., and Hemmings, B. A.
(1995) J. Cell Biol. 129, 397–410
16. Favre, B., Zolnierowicz, S., Turwosp, P., and Hemmings, B. A. (1994) J. Biol.
Chem. 269, 16311–16317
17. Peretz, C., and Parker, P. J. (1991) FEBS Lett. 284, 120–122
18. Palmer, R. H., and Parker, P. J. (1995) Biochem. J. 309, 315–320
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Olivier, A. R., and Parker, P. J. (1992) J. Cell Physiol. 152, 240–244
21. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
22. Stabel, S., Schaap, D., and Parker, P. J. (1991) in Methods in Enzymology (Hunter
and Sehon, B. M., eds) pp. 670–673, Academic Press, Inc., Orlando, FL
23. Bornancin, F., and Parker, P. J. (1996) Curr. Biol., 6, 1114–1123
24. Kerenan, L. M., Dutill, E. M., and Newton, A. C. (1995) Curr. Biol. 5,
1394–1403
25. Riley, S. C., Parker, P. J., Fabbro, D., and Jakin, S. (1991) J. Biol. Chem. 266,
23761–23768
26. Olivier, A. R., and Parker, P. J. (1994) J. Biol. Chem. 269, 27578–2763
27. Alexander, D. R., Brown, M. H., Tutt, A. L., Crompton, M. J., and Shivnan, E.
(1992) Biochem. J. 286, 69–77
28. Dent, P., Selinus, T., Morrison, D. K., and Sturgill, T. W. (1995) J. Biol.
Chem. 269, 1657–1657
29. Pitcher, J. A., Payne, E. S., Cenuto, C., Depaoliroach, A. A., and Lefkowitz, R.
(1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8343–8347
30. Dekker, L. V., Kobayashi, C., Munly, M. C., and Hannu, Y. A. (1993) J.
Biol. Chem. 268, 15523–15530
31. Lee, J. M., Hannu, Y. A., and Obeid, L. M. (1998) J. Biol. Chem. 271,
13169–13174

P. J. Parker, unpublished observations.

F. Bornancin and P. J. Parker, unpublished observations.