Volume 252, number 1,2, 5-11 FEB 07361 July 1989

Direct activation of a protein kinase activity from rat lacrimal gland by PMA in a phospholipid-free system

Philippe Mauduit, Driss Zoukhri and Bernard Rossignol

Laboratoire de Biochimie des Transports Cellulaires, Biochimie, CNRS UA 1116, Bat 432, Université de Paris Sud, 91405 Orsay Cedex, France

Received 13 May 1989

The analysis of the cytosolic fraction from rat exorbital lacrimal gland with DEAE-cellulose ion-exchange chromatography showed the presence of a peak of protein kinase activity which was dependent on the presence of phosphatidylserine and diolein as well as calcium. This activity showed the same properties as the previously reported protein kinase C (PKC). Moreover, we have shown for the first time that this kinase or a kinase that coeluted from the column with PKC could be activated by a phorbol ester, PMA, in a phospholipid-free system, i.e. in the absence of any cofactor of PKC. These findings emphasize the need for caution in the interpretation of experimental results obtained when using phorbol esters to probe for a role of PKC in many regulatory processes.

Exorbital lacrimal gland; Protein kinase C; Phorbol ester; Signal transduction; (Rat)

1. INTRODUCTION

Newly synthesized protein secretion from rat exorbital lacrimal glands is mainly controlled through the activation of cholinergic, muscarinic receptors [1,2]. Receptor activation leads to the generation of two intracellular messengers through the activation of a specific phospholipase C (PLC) activity. This PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) [3]. The role of IP_3 is to release calcium from a non-mitochondrial intracellular store which results in an increase of cytosolic free calcium concentration [4]. The role of DAG seems to favor the activation of a peculiar protein kinase, protein kinase C (PKC) [5]. Both the increased intracellular free calcium concentration, which could activate calcium-calmodulin-dependent protein kinase, and DAG generation, which activates PKC, act together to transduce extracellular signals into cellular responses (for review see [6]).

Since the discovery that tumor-promoters such as the phorbol esters were able to mimic the effect of DAG and to activate PKC [7], many studies showed that this kinase might be involved in the regulation of an increasing number of physiological processes, such as exocrine protein secretion [1,8,9].

In a recent paper, we showed that phorbol 12-myristate 13-acetate (PMA) could increase the release of newly synthesized proteins from rat exorbital lacrimal glands [1]. This result was recently confirmed by Dartt et al. [8] who showed a stimulatory effect of Phorbol 12-13 di-butyrate (PdBu) on peroxidase secretion by exorbital lacrimal gland acini. So, in the present study, we have partially purified protein kinase C from rat exorbital lacrimal gland and characterized the enzyme using lysine-rich histone as substrate. We also present evidences, for the first time, for the existence of a protein kinase activity that co-purify with protein kinase C and that is stimulated by PMA in the absence of Ca^{2+} and phospholipid.

Correspondence address: P. Mauduit, Biochimie, Bat 432. Université Paris Sud, 91405 Orsay Cedex, France

Published by Elsevier Science Publishers B.V. (Biomedical Division)

2. MATERIALS AND METHODS

2.1. *Materials*

Phospholipase C, phosphatidylserine, 1.2-diolein, phorbol esters, histone III-S, ATP, EDTA, EGTA and leupeptin were from Sigma, St. Louis, MO, USA. [γ-^{32}P] ATP (3000 Ci/mmol) was purchased from Amersham International Incorporation, England.

2.2. *Methods*

2.2.1. *Preparation of rat lacrimal gland homogenate and partial purification of soluble protein kinase C*

Male Sprague Dawley rats (5–6 weeks old) were killed by cervical dislocation, and exorbital lacrimal glands were removed and freed of connective tissue. The gland homogenate (usually glands from ten rats) was prepared by homogenization of 10 vol (w/v) of ice-cold 20 mM Tris-HCl (pH 7.5 at 4°C), 0.25 M sucrose, 10 mM EGTA, 5 mM EDTA, 50 mM β-mercaptoethanol, and 0.01% leupeptin. The homogenate was centrifuged at 700 × *g* for 5 min. The pellet was discarded and the supernatant centrifuged at 100 000 × *g* for 60 min or 450 000 *g* for 15 min. The high speed supernatant was referred as the cytosolic fraction.

For the purification of the PKC activity, the cytosolic fraction (about 10 ml) was loaded into a column (0.9 × 18 cm) packed with DE 52 resin equilibrated with buffer A [20 mM Tris-HCl (pH 7.5 at 4°C) 0.5 mM EGTA, 0.5 mM EDTA, 50 mM β-mercaptoethanol and 0.001% leupeptin). The column was washed with 50 ml of the above buffer. This was usually sufficient for non-interacting proteins to be eluted from the resin. Proteins were then eluted from the column by a continuous gradient of NaCl (0–300 mM) made in buffer A (100 ml total volume) at a flow rate of 1 ml/min. Fractions of 2 ml each were collected. Active fractions were pooled (generally 10 fractions), made 1.5 M NaCl and applied to a Phenyl-Sepharose CL-4B (Pharmacia) column (0.9 × 10 cm) at a flow rate of 0.5 ml/min as described by Kikkawa et al. [24]. Fractions of 2 ml each were collected. The collected fractions could be stored without any appreciable loss of activity for several weeks at −20°C after being made 37.5% glycerol. However, the amount of activity which was recovered from the two columns from different experiments showed some variability.

2.2.2. *Determination of PKC activity*

PKC activity was determined either on individual fractions from the DE 52, or the Phenyl-Sepharose chormatography columns or on pooled fractions (as indicated) containing the highest activity. The protein kinase activity was assayed in 200 μl of medium containing 20 mM Tris-HCl (pH 7.5 at 30°C), 5 mM $MgCl_2$, 0.5 mM EGTA, 20 μM ATP, 0.5 μCi [γ-^{32}P] ATP, 50 μg lysine-rich histone type III-S with or without 0.6 mM $CaCl_2$ in the presence or the absence of 25 μg PS + 10 μg diolein (DiC18) or phorbol esters where indicated. The solutions of phosphatidylserine or phosphatidylserine + diolein were prepared by mixing appropriate amounts of both compounds dissolved in chloroform, evaporating under a stream of nitrogen followed by a dispersion of the residue by sonication in 20 mM Tris-HCl (pH 7.5 at 30°C), 5 mM $MgCl_2$ and 0.5 mM EGTA. A fresh solution was prepared each time. Phorbol esters were prepared as stock solutions in DMSO at a concentration of 1 mg/ml. Control incubations with the highest concentrations of DMSO used were performed and we never found any effect of DMSO in these conditions. After a 5 min equilibration period at 30°C, the reaction was initiated by adding ATP and was carried out usually for 10 min in the same conditions. Incubations were stopped by the addition of 2 ml of ice-cold 10% trichloroacetic acid (TCA). Then, 0.5 mg BSA were added to each tube and the precipitate was centrifuged (10 min at 2500 × *g*), the pellet was washed once with 2 ml TCA, solubilized with NaOH and radioactivity was counted by liquid scintillation. PKC activity was defined as the amount of ^{32}P transferred from ATP to histone III-S in the presence of Ca^{2+} + PS + DiC18 compared with that in the presence of Ca^{2+} alone.

2.2.3. *Assay with PLC*

The active fractions from the DE 52 column were pooled and incubated with PLC in the presence of 5.5 mM total Ca^{2+} concentration (5 mM free calcium) that is necessary for the activity of PLC. The effects of PLC were tested on both the basal activity (in the presence or absence of calcium), the activity in the presence of Ca^{2+} + PS + DiC18 and the activity in the presence of PMA in a Ca^{2+} and phospholipid-free (PL-free) system.

After a 5 min equilibration period, the reactions were initiated by the addition of PLC and carried out for 10 min at 30°C in the same conditions. Then EGTA buffer was added to decrease the free calcium concentration of 0.1 mM or below 10 nM. The phosphotransferase actvity was initiated by the addition of a mixed solution of [γ-^{32}P]ATP (20 μM) + histone (50 μg) 5 min after the addition of EGTA and stopped 10 min later by the addition of 2 ml of ice-cold TCA 10%.

3. RESULTS AND DISCUSSION

3.1. *Protein kinase C characterization*

In a first attempt, we tried to show the presence of protein kinase C directly in the cytosol of the rat exorbital lacrimal gland cells. However, we failed to detect any increase in the incorporation of ^{32}P from [γ-^{32}P]ATP into histone III-S in the presence of all the PKC cofactors (calcium, phosphatidylserine, and diolein) when compared with that in the presence of calcium alone. Moreover, calcium alone (0.1 mM free concentration) was able to increase histone phosphorylation by itself (data not shown) probably because of the presence of calcium/calmodulin-dependent protein kinase activities in this cytosolic fraction [10,11]. The lack of effect of the PKC cofactors on crude preparation has been described in other tissues [12,13] and could result from: (i) the absence of PKC in this tissue; (ii) the presence of a high level of other protein kinase activities which could mask the PKC activity through an increase in the basal rate of ^{32}P incorporation; (iii) the presence of an endogenous inhibitor of PKC.

We then performed a partial purification of the cytosolic fraction by anion-exchange chromatography on a DE 52 column in order to separate PKC activity from either an inhibitor or other protein kinases.

Fig.1A shows the effect of 0.1 mM free calcium on the histone kinase activity contained in the gradient fractions. As evidenced, no calcium-dependent activity could be detected in any of the fractions tested. This result suggests that either the calcium-dependent protein kinase activity measured in the crude cytosolic fraction was not retained or not eluted from the column or that an endogenous calcium-dependent activator such as calmodulin for example was lost or separated from the kinase. One could notice that a calcium-independent histone kinase activity eluted from the column at a salt concentration of 220 to 240 mM (fraction 30 through 48). The relative amount of activity in these fractions increased when the tissue was homogenized in a buffer containing only 2 mM EGTA and 2 mM EDTA instead of 10 mM EGTA and 5 mM EDTA and upon storage without glycerol (data not shown). This activity may correspond to the enzyme protein kinase M (PKM) which was first described by Takai et al. [14] as the constitutively activated form of PKC through proteolysis by a calcium-dependent protease [15].

As shown in fig.1B, an activity which was greatly increased by the simultaneous presence of calcium, phosphatidylserine and diolein eluted from the column at a salt concentration of NaCl ranging from 80 to 100 mM. In this experiment, the maximal phosphotransferase activity (fractions 17–18) corresponded to a 33-fold increase in the basal rate of ^{32}P incorporation into histone obtained in the presence of 0.1 mM free calcium alone (see fig.1A for comparison).

To characterize further the histone kinase activity which was stimulated in the presence of calcium and phosphatidylserine, fractions containing the highest activity were pooled (fractions 15 through 19 of fig.1 for example). This protein kinase activity was then tested for its dependence on substrates (ATP and histone III-S) and magnesium. The apparent K_m for ATP and histone were found to be 8.7 μM and 2.4 μM, respectively. These values were in agreement with those obtained with more purified preparations of PKC [16] and would further indicate that the kinase activity measured in

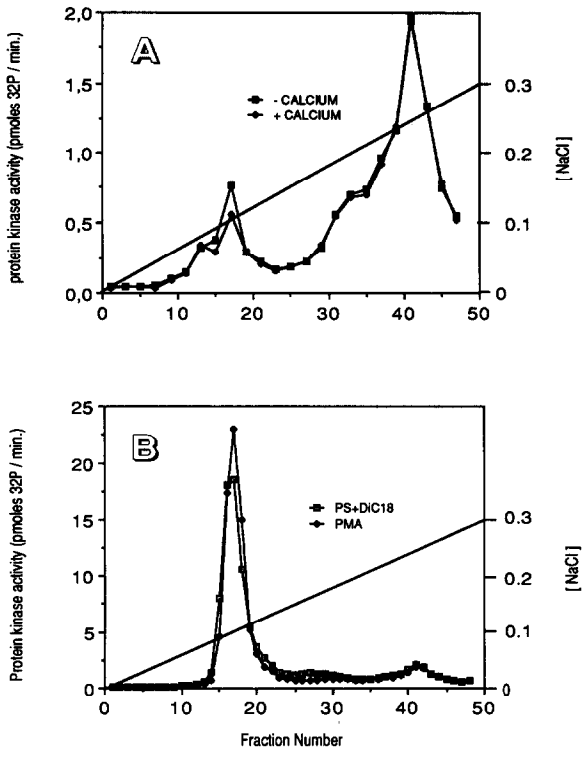

Fig.1. DEAE-cellulose chromatography of cytosolic protein kinase C. Proteins from the cytosolic fraction of rat exorbital lacrimal glands were chromatographed on a DE 52 column as described in section 2. The column was developed with a linear salt gradient of 0–0.3 M NaCl in Tris-HCl buffer (straight line). The amount of phosphotransferase in each fraction was measured on a aliquot of 25 μl. (A) Protein kinase activity was determined in the absence (■) or presence (◇) of 0.1 mM free calcium. (B) Protein kinase activity measured in the presence of 0.1 mM free calcium + 25 μg/ml PS + μg/ml DiC18 (⧈) or in the absence of calcium + 0.1 μM PMA (◆).

the presence of Ca^{2+}, PS and DiC18 might be PKC.

This calcium-phospholipid dependent protein kinase activity has an absolute requirement for magnesium. No activity could be detected below a threshold concentration of 0.3 mM Mg^{2+}. The maximal activity was observed at concentrations of 5–10 mM, while higher concentrations were inhibitory with a nearly complete inhibition at a Mg^{2+} concentration of 100 mM (data not shown). Similar results were also obtained in other PKC preparations [17].

3.2. *Effect of phorbol esters*

Since the discovery that phorbol ester such as

PMA and PdBu could directly activate protein kinase C [7], it has been proposed that this effect was archieved by mimicking the effect of diacylglycerol [18]. The activation process involves the formation of a quaternary complex between kinase C, calcium, phospholipid and phorbol ester. In some studies, it has been shown that calcium is not absolutely necessary for the action of PMA, whilst the effect of phorbol esters is dependent upon the presence of phospholipids [7,19]. However, in the course of our study, as a control, we tested the effect of PMA on the kinase activity of the fractions eluting from the DE 52 column in the absence of both calcium and phosphatidylserine. As can be seen from the elution profile of protein kinase C activity (fig.1B), we found that 0.1 μM PMA alone was able to increase the phosphotransferase activity towards histone greatly. This activity was superimposable on the PKC activity peak. The stimulatory effect of PMA in the calcium-phospholipid-free reaction was dose-dependent and with a half-maximal stimulation obtained at a PMA concentration in the 10 nM range (see figs 2, 3 and 4).

To our knowledge, this is the first report of a stimulatory effect of PMA on a protein kinase activity in the absence of phospholipid and calcium. It has been previously shown that, as PMA, another tumor-promoting phorbol ester, PdBu, could increase PKC activity in the presence of phosphatidylserine whereas a non-tumor-promoting phorbol ester, 4α-phorbol 12,13-dideconoate (4α-Pdd) could not. So, to test the nature and the specificity of the effect of PMA in the calcium-phospholipid-free assay, we analyzed the effect of increasing concentrations of PdBu and 4α-Pdd in these conditions. The results are reported in fig.2. They were quite different from those expected from previous assumptions, since PdBu and 4α-Pdd had no significant effects, a slight increase in kinase activity could only be observed at 1 μM PdBu. At least two reasons could explain the lack of effect of PdBu compared to PMA. Indeed, it has been shown that the presence of PS was absolutely necessary for the binding of radioactive PdBu to protein kinase C. So, the first possibility was that PdBu had no effect in the absence of PS because it dit not bind to PKC. The second possibility was that PdBu could bind to PKC but in contrast with PMA could not fully activate the enzyme. If the second possibility were true, PdBu might act as a competitive inhibitor of PMA if added to the medium simultaneously.

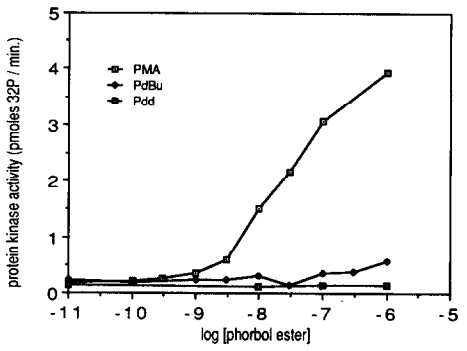

Fig.2. Effect of phorbol esters on protein kinase activity. The fractions containing the highest calcium-phospholipid dependent histone kinase activity were pooled and tested for activity as described in section 2. Kinase activity was assayed with increasing concentrations of PMA (▣), PdBu (◆) and 4α-phorbol (□) in the absence of calcium and PS. Each point is the mean of triplicates from a typical experiment.

In order to discriminate between these hypothesis, we performed the following experiments. First, we tested the effect of phosphatidylserine on the dose-response curve to PdBu (fig.3). As can be observed, in the presence of PS, PdBu became active for concentrations which were ineffective in the absence of PS. This result favoured our first hypothesis. Additionally, we observed that; if the presence of PS is not absolutely necessary in order to observe an effect of PMA, its presence increased the apparent affinity of PMA for the kinase.

In order to test the second hypothesis, we measured the effect of a high concentration of PdBu (1 μM) which has only a small stimulatory effect by itself, on the dose-response curve to PMA. The results reported in fig.4 show that PdBu did not exert any inhibitory effect on the phosphotransferase activity induced by PMA. The effect of PdBu is rather additive with that of PMA at low concentrations where the inhibitory effect might have been maximal in the case of the second hypothesis.

Up to now, we have no definitive explanation for these results, but several hypotheses can be proposed. Firstly, our fractions could contain amounts of phospholipids (PS for example), sufficient to permit the activation of PKC by PMA in our assay conditions. The second hypothesis is that we are in

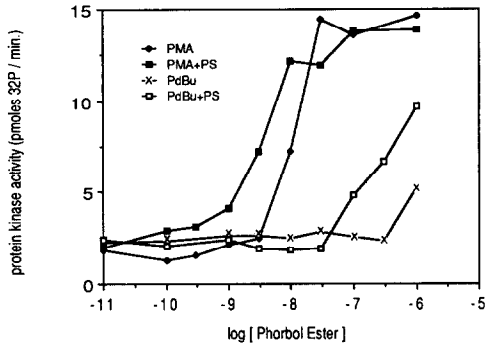

Fig.3. Effect of phosphatidylserine on the dose response curve to PMA and PdBu. Kinase activity was assayed as described in section 2 in the presence of increasing concentrations of either PMA (◆, ■) or PdBu (×, □) in the absence (◆, ×) or presence (■, □) of 25 μg/ml PS. Each point is the mean of triplicates from a typical experiment.

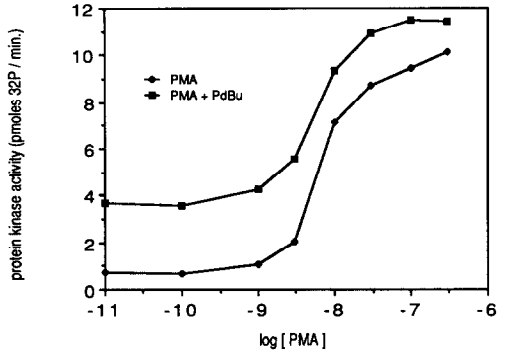

Fig.4. Effect of PdBu on the dose-response curve to PMA. Kinase activity was assayed as decribed in section 2 in the presence of increasing concentrations of PMA in the absence (◆) or presence (■) of 1 μM PdBu.

the presence of two different kinases which co-elute upon DEAE-cellulose ion-exchange chromatography. One is PKC, which is activated by both PMA and PdBu only in the presence of PS, and the other one is a PMA-activated and PL-independent protein kinase, which could not be activated by PdBu in the PL-free system.

To test for this hypotheses, we performed the following experiments. Firstly, we introduced a second step of purification upon a Phenyl-Sepharose column in order to remove contingent endogenous phospholipids or the contaminant kinases. As evidenced in fig.5, the peak of the PMA-activated and PL-independent kinase activity (fig.5A) is still superimposed with that of the PKC activity (fig.5B). Secondly, we incubated the DE 52 column fractions with PLC in order to hydrolyse the endogenous phospholipids that might coelute with PKC. As reported on fig.6, incubations with PLC did not inhibit the kinase activity in response to PMA in the PL-free system. As a control, we tested the effects of PLC on the PKC activity in the presence of calcium + PS + DiC18. In this case, we observed an inhibition when compared with pre-incubations with boiled PLC. A complete inhibition could not be observed probably because (i) PS is present in the incubation medium at a high concentration (25 μg/ml), (ii) DiC18, which increases the activity of PKC in the presence of subsaturating concentrations of PS, is also present in the assay medium and (iii) DG,

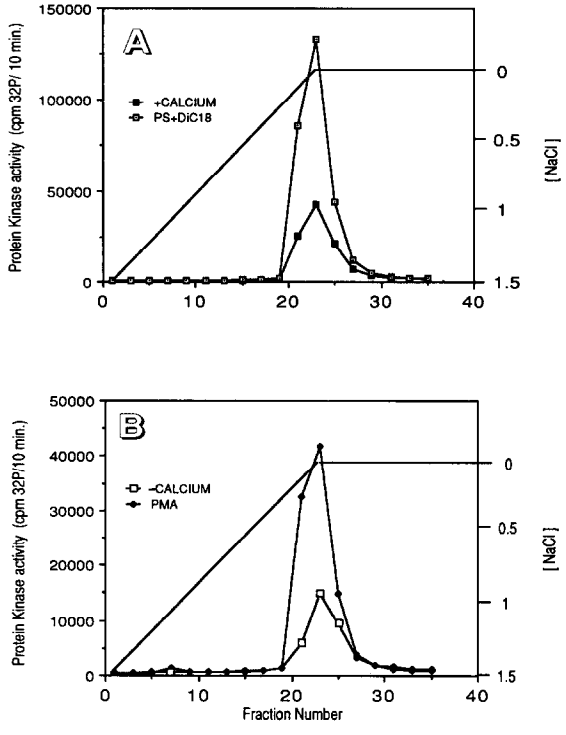

Fig.5. Phenyl-Sepharose chromatography of the active fractions from the DEAE-cellulose chromatograhic step. The experimental protocol was as described in section 2. The amount of phosphotransferase activity in the fractions was measured on an aliquot of 25 μl for 10 min. (A) Protein kinase activity was determined in the presence of 0.1 mM free calcium in the absence (■) or presence (⧈) of PS (25 μg/ml) + DiC18 (10 μg/ml). (B) Protein kinase activity measured in the absence of calcium in the absence (□) or presence (◆) of 0.1 μM PMA.

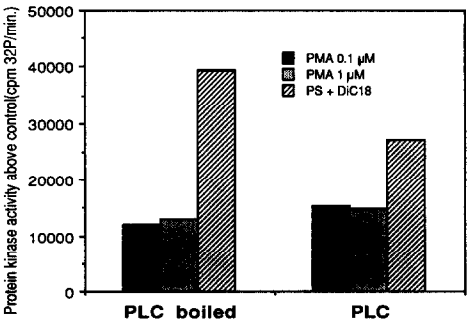

Fig.6. Effect of PLC pretreatment on the protein kinase activity. Active fractions from the DEAE-cellulose chromatographic step were pooled and treated in the presence of native (PLC) or denaturated (PLC boiled) PLC as described in section 2. Phosphotransferase activity was tested either in the presence of PMA (0.1 or 1 μM) in the absence of calcium or in the presence of PS + DiC18 in the presence of 0.1 mM free calcium. The amount of protein kinase activity in the control incubations, i-e, in the absence of calcium for PMA and in the presence of 0.1 mM free calcium for PS + DiC18 has been substracted from the results.

which is formed from PS as the result of PLC activity, would also potentiate the effect of PS on PKC. On the contrary, if contaminant phospholipids were present, they would be expected to be broken down and the response to PMA would have been inhibited.

These results taken together rule out the possibility of a contamination of our enzyme preparation by endogenous PL. If the introduction of a second step of purification upon Phenyl-Sepharose column could not entirely rule out the possibility of a contamination by some other kinases, it render it however unlikely. Furthermore, considering the fact that the isoforms of PKC described in the literature show different enzymatic properties [19,20], we can hypothesize that in our preparation of rat lacrimal gland PKC, PMA, but not PdBu, would activate one peculiar isoform of PKC in the PL-free reaction. Such a hypothesis requires further studies and especially a better purification of the lacrimal gland PKC up to the characterization of the isoforms to determine if one of them is specifically involved in the observed phenomenon. Moreover, our results would further indicate that an activation mechanism of the soluble form of PKC might exist beside the well-documented one which involves an interaction of PKC with the membrane phospholipids in the presence of calcium and diacylglycerol. This last hypothesis is certainly speculative and rather non-classical but could explain some of the discrepancies observed between the effects of DAG and PMA at a more physiological level [21,22]. Our data also emphasize the need for caution in the analysis of experimental results obtained with phorbol esters to probe the involvement of PKC in the regulation of exocrine protein secretion since, as reported by Nishizuka [23], PKC may not be the only one target of tumor promoters.

Acknowledgements: Thanks are due to J. Dujancourt for her skilful technical assistance. This work was supported by the Institut National de la Santé et de la Recherche Medicale (887 015).

REFERENCES

[1] Mauduit, P., Herman, G. and Rossignol, B. (1987) Am. J. Physiol. 253, C514–C524.
[2] Herman, G., Busson, S., Ovtracht, L., Maurs, C. and Rossignol, B. (1978) Biol. Cell. 31, 255–264.
[3] Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473–482.
[4] Berridge, M.J. (1984) Biochem. J. 220, 345–360.
[5] Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979) Biochem. Biophys. Res. Commun. 91, 1218–1224.
[6] Berridge, M.J. (1987) Annu. Rev. Biochem, 56, 159–193.
[7] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[8] Dartt, D.A., Ronco, L.V., Murphy, S.A. and Unser, M.F. (1988) Invest. Ophthalmol Vis. Sci. 29, 1726–1731.
[9] Putney, J.W., McKinney, J.S., Aub, D.L. and Leslie, B.A. (1984) Mol. Pharmacol. 26, 261–266.
[10] Dartt, D.A., Guerina, V.J., Donowitz, M., Taylor, M. and Sharp, W.G. (1982) Biochem, J. 202, 799–802.
[11] Mauduit, P., Herman, G. and Rossignol, B. (1983) FEBS Lett. 152, 207–211.
[12] Skoglund, G., Hansson, A. and Ingelman-Sundberg, M. (1985) Acta Chem. Scand. 39, 219–226.
[13] Velasco, G., Iglesias, C.F., Dominguez, P., Barros, F., Gascon, S. and Lazo, P.S. (1986) Biochem. Biophys. Res. Commun. 139, 875–882.
[14] Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603–7609.
[15] Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610–7616.
[16] Wise, B.C., Glass, D.B., Jen Chou, C.-H., Raynor, R.L., Katoh, N., Schatzman, R.C., Turner, R.S., Kibler, R.F. and Kuo, J.F. (1982) J. Biol. Chem. 257–8489–8495.
[17] Wise, B.C., Raynor, R.L. and Kuo, J.F. (1982) J. Biol. Chem. 257, 8481–8488.

[18] Sharkey, N.A., Leach, K.L. and Blumberg, P.M. (1984) Proc. Natl. Acad. Sci. USA 81, 607–610.
[19] Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U. and Nishizuka, Y. (1988) J. Biochem. 103, 759–765.
[20] Nishizuka, Y. (1988) Nature 334, 661–665.
[21] Kreutter, D., Caldwell, A.B. and Morin, M.J. (1985) J. Biol. Chem. 260, 5979–5983.
[22] Kiss, Z. and Luo, Y. (1986) FEBS Lett. 198, 203–207.
[23] Nishizuka, Y. (1986) Science 233, 305–312.
[24] Kikkawa, U., Minakuchi, R., Takai, Y. and Nishizuka, Y. (1983) Methods Enzymol. 99, 288–298.