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CALCIUM AND PHOSPHOLIPID-DEPENDENT PROTEIN KINASE ACTIVITY IN MOUSE EPIDERMIS CYTOSOL. STIMULATION BY COMPLETE AND INCOMPLETE TUMOR PROMOTERS AND INHIBITION BY VARIOUS COMPOUNDS

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Calcium- and phospholipid-dependent protein kinase (Ca, PL-PK) activity is detectable in mouse epidermis cytosol. It can be stimulated in vitro by complete and incomplete tumor promoters (12-O-tetradecanoylphorbol-13-acetate (TPA) and 12-O-retinoylphorbol-13-acetate (RPA)), respectively. Effective inhibition of the enzyme activity is achieved with quercetin and phloretin, whereas the lipoxygenase and cyclooxygenase inhibitors nordihydroguaiaretic acid (NDGA) and esculetin show just weak or no inhibition. Quercetin inhibits the lipoxygenase and cyclooxygenase equally well as the Ca,PL-PK, whereas the strong Ca,PL-PK inhibitor phloretin is absolutely ineffective in inhibiting the lipoxygenase/cyclooxygenase. The application of these inhibitors in differentiating tumor promoter induced effects in vivo is proposed.

Phorbol ester binding sites are thought to play a key role in tumor promotion. Recently, data from various reports has been accumulated which strongly indicates that the Ca,PL-PK C binds the tumor promoting phorbol ester TPA with high affinity and specificity (1-4). Even though it is not known whether the Ca,PL-PK is the only high affinity binding site for TPA (5), it is at least one conceivable candidate as a key mediator of TPA-induced tumor promotion. Soluble phorbol ester binding and Ca,PL-PK activity have been demonstrated in many tissues (6-9). We now present data on Ca,PL-PK activity in mouse epidermis cytosol and report on the stimulation of this enzyme's activity by complete and incomplete tumor promoters, as well as on this kinases inhibition by quercetin and other compounds in vitro.

MATERIAL AND METHODS

Materials: TPA and 4-O-methyl-TPA were kindly supplied by Professor Dr. E. Hecker (German Cancer Research, Center). RPA was prepared as in (10). 20-^{3}H(N) TPA (spec.act. 20 Ci/mmole), γ-^{32}P ATP (spec.act. 8.3 Ci/mmole)

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and [^{14}C(U)]arachidonic acid (spec.act. 150-400 mCi/mmol), [^{3}H]-5-HETE (spec.ac.t 30-60 Ci/mmol) [^{3}H]-12-HETE (spec.act. 30-60 Ci/mmol) [^{3}H]-15-HETE (spec.act. 30-100 Ci/mmol), [^{3}H]PGE_2 (spec.act. 100-200 Ci/mmol) and [^{3}H]$PGF_{2\alpha}$ (150-180 Ci/mmol) were from New England Nuclear (Waltham, MA). [^{3}H]13,14-dihydro-15-keto-PGE_2 (spec.act. 80 Ci/mmol) was from Amersham/Buchler, Braunschweig, F.R.G.
Quercetin, phloretin, esculetin, were from Sigma, München, FRG.
NDGA was from Serva, Heidelberg, FRG. All organic solvents were of HPLC grade.

Animals: Female NMRI mice (age 7 to 8 weeks) were used in all experiments.

Preparation of mouse epidermis cytosol: back skin epidermis from 10 mice was scraped off at 4 °C, as described previously (11), and homogenized in 6 ml of a 20 mM Tris-HCl buffer (pH 7.4) containing 10 mM EDTA, 2 mM EGTA and 2 mM phenylmethanesulfonylfluoride. Homogenization was performed with 10 strokes (each 1 sec.) of a Polytron homogenizer set at half maximal speed (5th mark). The homogenate was centrifuged for 1 hr at 100,000 x g in a Ti-50 rotor (Beckman). The supernatant (cytosol) contained 1.9 mg protein/ml. The cytosol was diluted 1:2, and 10 µl aliquots were used for the protein kinase assay.

[^{3}H-TPA] binding assay and protein kinase assay: as described previously (6,7).

Cyclooxygenase and lipoxygenase assay: Neonatal mouse epidermis was homogenized in 150 mM PBS/1 mM Ca^{2+} buffer, pH 7.4, with a Polytron-PT-1-homogenizer (setting 6; three times for 5 to 10 sec.) at 4°C. The homogenate was filtered through gauze. [^{14}C(U)]arachidonic acid (final concentration 0.5 µM) was added to the reaction tubes containing 500 µl of homogenate (0.9 mg protein) and 10 µl of acetone or inhibitors dissolved in 10 µl of acetone. After incubation at 37°C for 15 minutes 50 µl of 1 N HCL and 2 nCi [^{3}H]13,14-dihydro-15-keto-PGE_2 as external standard were added and the mixture was extracted twice with 4 ml ethylacetate each. The combined ethylacetate phase was evaporated. The residue was redissolved in 200 µl of diethylether and chromatographed on a silica cartridge (Sep Pak, Water Associates) which was eluted subsequently with 30 ml of diethylether/hexane (20/80 and 40/60; by vol.) and ethyl acetate/methanol each (90/10; by vol.). The ethyl/acetate/methanol fraction was collected. After evaporation the residue was dissolved in 100 µl of methanol and aliquots of 20 µl were subjected to HPLC on a Waters HPLC instrument equipped with a 6000 A pumping system, a U6K injector and a reverse phase column (5 µ spherical C_{18} resolve column; 3.9 x 150 mm; Waters Associates). Stepwise elution of prostaglandins and hydroxylated arachidonic acid derivatives was achieved according to ref. 12,13 using the solvent systems acetonitrile/0.017 M H_3PO_4 (33/67 by vol.), acetonitrile/0.017 M H_3PO_4 (50/50 by vol.) and acetonitrile at a flow rate of 1.5 ml/min. Fractions were collected every 30 sec and the radioactivity was measured by liquid scintillation counting. This chromatography separates all prostaglandins, but separates the HETES as a single peak as shown by chromatography with authentic samples. Cyclooxygenase activity was determined by measuring the amount of PGE_2, the predominant cyclooxygenase product in epidermis, and lipoxygenase activity by measuring the amount of HETES, with 12-HETE being the predominant epidermal lipoxygenase metabolite. Quantitation of PGE_2 and HETES was achieved by comparison with the external standard added. Duplicate determinations of two independent assays were performed.

Determination of protein: was carried out according to Lowry (14).

RESULTS

The cytosol from mouse epidermis was found to contain TPA-binding activity (3.5 pmoles/mg cytosol protein), as well as calcium- and phospholipid-(phosphatidylserin (PS)) dependent protein kinase activity (Table 1). TPA, as well as RPA, were able to reduce the requirement for calcium and to stimulate Ca,PL-PK activity, whereas 4-O-methyl-TPA (MTPA) was ineffective in stimulation (Table 1). Kinase activity increased with the concentration of TPA added to the cytosol and was optimal at 10^{-7}M TPA (Fig. 1). 10^{-4}M quercetin and 10^{-3}M NDGA were able to completely inhibit the calcium- as well as the TPA-stimulated kinase activity (Table 1). In addition, NDGA also reduced to some extent the calcium- and PS-independent kinase activity. For a valid differentiation of Ca,PL-PK and lipoxygenase activity the concentration dependency of the various suppressors of these enzymes were determined (Table 2). A 50% inhibition of Ca,PL-PK, lipoxygenase and cyclooxygenase activities were obtained with 10^{-5}M to 10^{-6}M quercetin. Phloretin was almost equally effective as quercetin in inhibiting Ca,PL-PK but did not show any suppression of lipoxygenase and cyclooxygenase at concentrations up to 10^{-4}M. Esculetin was found to be a strong lipoxygenase and cyclooxygenase inhibitor, whereas no inhibition of Ca,PL-PK was detectable even at a concentration of 10^{-3}M. The well-known lipoxygenase inhibitor NDGA (15) had to be applied at a concentration of 5×10^{-4}M in order to obtain 50% inhibition of Ca,PL-PK.

Table 1: Stimulation of protein kinase activity (cpm x 10^{-3}/mg protein) in epidermis cytosol by 10^{-3}M Ca^{2+}, 60 µg PS/ml, 10^{-7}M TPA, 10^{-7}M RPA, 10^{-7}M MTPA as well as inhibition by 10^{-4}M quercetin (Qu) or 10^{-3}M NDGA. Assays without Ca^{2+} contained 1 mM EGTA. In order to obtain Ca,PL-PK activity the value measured in the absence of Ca^{2+} has to be subtracted.
n.d.: not determined.

	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$ +TPA	$-Ca^{2+}$ +RPA	$-Ca^{2+}$ +MTPA	$+Ca^{2+}$ +Qu	$-Ca^{2+}$ +TPA +Qu	$+Ca^{2+}$ +NGDA	$-Ca^{2+}$ +TPA +NDGA
-PS	172	303	281	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
+PS	163	436	339	341	170	174	150	109	119

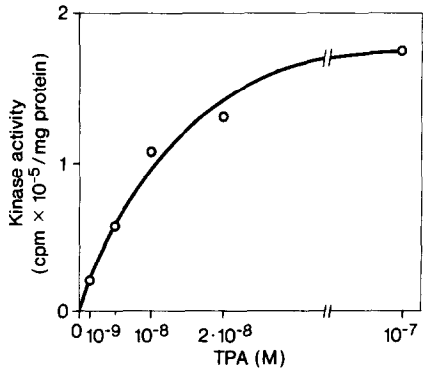

Fig. 1: Concentration dependence of the stimulation of Ca,PL-PK activity by TPA.
Assays were performed with 60 µg PS/ml without calcium (mM EGTA). A blank without TPA was subtracted from each value.

DISCUSSION

Mouse epidermis is the classical target tissue for tumor promoting phorbol esters and is probably the best established system of multistage carcinogenesis (16). Recently, Ashendel et al. (9) have shown that mouse epidermis contains TPA binding sites and Ca,PL-PK activity. This is of interest, since Ca,PL-PK C has been found to exhibit TPA receptor properties (4). We were able now to confirm the existence of TPA binding sites and of protein kinase activity, dependent both on calcium and PS in mouse epidermis. Furthermore, we could show that complete as well as incomplete tumor promoters (TPA and RPA, respectively), but not the non-promoting phorbol

Table 2: Inhibition of epidermal Ca,PL-PK, lipoxygenase and cyclooxygenase by various compounds

Enzyme	50% Inhibition			
	Quercetin	Phloretin	NDGA	Esculetin
Ca,PL-PK	10^{-5}M	$2x10^{-5}$M	$5x10^{-4}$M	$>10^{-3}$M
Lipoxygenase	$5x10^{-6}$M	$>10^{-4}$M	n.d	$5x10^{-5}$M
Cyclooxygenase	$3.5x10^{-5}$M	$>10^{-4}$M	n.d.	$7x10^{-5}$M

Ca,PL-PK, lipoxygenase and cyclooxygenase activity were determined as described in Methods. The basal activities of epidermal cyclooxygenase and lipoxygenase were 22 pmol PGE_2/mg protein/15 min and 30 pmol HETES/mg protein/15 min, respectively. for the maximal activity of Ca,PL-PK in the presence of calcium and PS see Table 1.

ester 4-O-methyl TPA were able to stimulate Ca,PL-PK activity. We obtained similar results recently with Ca,PL-PK from chick brain and oviduct (7). Since incomplete (second stage) tumor promoters increased Ca,PL-PK activity, we suspected that the activation of this kinase might be related to the second stage of tumor promotion. In addition, we cannot exclude, that the kinase is necessary, although probably not sufficient, for the first stage of tumor promotion.

The flavonoid quercetin is known to suppress tumor promotion (17). Recently, we found quercetin to be a potent inhibitor of Ca,PL-PK activity in mouse brain cytosol (18). Using a partially purified Ca,PL-PK from chick oviduct, we were able to prove that the inhibition by quercetin is reversible and due to the competition of quercetin with ATP (M.G., F.H. and F.M., in preparation) . We can now show that the activity of this enzyme in mouse epidermis cytosol is equally well inhibited by quercetin. Quercetin, however, is known also to inhibit lipoxygenase activity (17, compare also Table 2). Thus, TPA effects, which are inhibited by quercetin, cannot be unequivocally associated with Ca,PL-PK activity. Therefore, we looked for other inhibitors with more specific properties which could be used in addition to quercetin. Phloretin, which is known to interfere with carrier mediated transport processes (19-22), suppresses Ca,PL-PK but not lipoxygenase and cyclooxygenase activity. On the other hand, esculetin, a strong inhibitor of lipoxygenase and cyclooxygenase (Table 2 and ref. 23) does not inhibit Ca,PL-PK at all. NDGA, another strong lipoxygenase and tumor promotion inhibitor (15), is a very weak inhibitor of the Ca,PL-PK. Taken together, with respect to Ca,PL-PK and lipoxygenase/cyclooxygenase, there are only two specific inhibitors (phloretin and esculetin, respectively) of these enzymes. Quercetin cannot differentiate these enzymes, whereas NDGA appears to be specific for lipoxygenase/cyclooxygenase at low concentrations only. Thus, these inhibitors applied <u>in vivo</u>, might be valuable for the association of TPA effects either with lipoxygenase and cyclooxygenase- or with Ca,PL-PK activity. For example, we found recently that TPA

induces the appearance of alkaline phosphatase activity in mouse epidermis. Since this effect could be inhibited by quercetin but not by NDGA or esculetin, we concluded that it might be mediated by Ca,PL-PK (24). This idea gained support by our recent finding that the induction of phosphatase activity by TPA is suppressed also by phloretin (unpublished results).

REFERENCES

1. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257. 7847-7851.
2. Niedel, J.E., Kuhn, L. J., and Vandenbark, G.R. (1983). Proc. Natl. Acad. Sci. USA 80, 36-40.
3. Ashendel, C. L., Staller, J.M., and Boutwell, R.K. (1983). Cancer Res. 43, 4333-4337.
4. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 11442-11445.
5. Dunn, J.A., and Blumberg, P.M. (1983) Cancer Res. 43, 4632-4637.
6. Gschwendt, M., and Kittstein, W. (1983) Cancer Letters 19, 159-164.
7. Gschwendt, M., Horn, F., Kittstein, W., Fürstenberger G., and Marks, F. (1983) FEBS Letters 162, 147-150.
8. Leach, K.L., James, M.L., and Blumberg, P.M. (1983) Proc. Natl. Acad. Sci. USA 80, 4208-4212.
9. Ashendel, C.L., staller, J.M., and Boutwell, R.K. (1983) Biochem. Biophys. Res. Commun. 111, 340-345.
10. Fürstenberger, G., Berry, D.L., Sorg, B., and Marks, F. (1981) Proc. Natl. Acad. Sci. USA 78, 7722-7726.
11. Krieg, L., Kühlmann, I. and Marks, F. (1974) Cancer Res. 34, 3135-3146.
12. Terragno, A., Rydrik, R. and Terragno, N.A. (1981) Prostaglandins 21, 101-112.
13. Eling, Th., Tainer, B., Ally, A. and Warnock, R. (1982) Methods in Enzymol. 86, 511-517.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, J.R. (1951) J. Biol. Chem. 193, 265-275.
15. Nakadate, T., Yamamoto, S., Iseki, H., Somoda, S., Takemura, S., Ura, A., Hosoda, Y., and Kato, R. (1982) Gann 73, 841-843.
16. Hecker, E., Fusenig, N.E., Kunz, W., Marks, F., and Thielmann, H.W. (eds.) (1982) Carcinogenesis, Vol. 7, Raven Press, New York.
17. Kato, R., Nakadate, T., Yamamoto, S., and Sugimura, T. (1983) Carcinogenesis 4, 1301-1305.
18. Gschwendt, M., Horn, F., Kittstein, W., and Marks, F. (1983) Biochem. Biophys. Res. Commun. 117, 444-447.
19. Le Ferre, P.G., and Marshall, J.K. (1959) J. Biol. Chem. 234, 3022-3026.
20. Owen, J.D., and Solomon, A.K. (1972) Biochim. Biophys. Acta 290, 414-418.
21. Macey, R.I., and Farmer, E.L. (1970) Biochim. Biophys. Acta 211, 104-106.
22. Kaplan, M.A., Hays, L., and Hays, R.M. (1974) Am. J. Physiol. 226, 1327-1332.
23. Neichi, T., Koshihara, Y., and Murota, S.-I. (1983) Biochim. Biophys. Acta 753, 130-132.
24. Gschwendt, M., Kittstein, W., and Marks, F. (1984) Cancer Letters 22, 219-225.