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RAPID COMMUNICATIONS

PHOSPHOLIPASE C ACTIVITY IN NORMAL RAT MAMMARY TISSUES AND IN DMBA-INDUCED RAT MAMMARY TUMORS[1]

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Employing phosphatidylinositol as the substrate, phospholipase C (PLC) activity was measured in various cellular fractions derived from DMBA-induced rat mammary tumors and mammary tissues from 12-14 day pregnant rats. In the 2,000g pellet, 10,000g pellet, 100,000g pellet and 100,000g supernatant fractions, PLC activity was more than 5 fold higher in the fractions derived from the neoplastic tissues. This was true whether PLC activity was expressed on the basis of protein content or 5' nucleotidase activities.,

The actions of a variety of mitogens on cell division are associated with perturbations in the metabolism of the phosphoinositides (1-3). Evidence suggests that these mitogenic agents including certain oncogene products may express their proliferative actions via the stimulation of a phosphoinositide-specific phospholipase C (PLC). The products of the action of PLC on the phosphoinositides include diglycerides plus inositol phosphates; these substances have effects which may trigger cell division processes. In view of the apparently pivotal role of PLC in regulating mitogenesis, the activity of PLC was measured in various cell fractions derived from normal and neoplastic rat mammary tissues. PLC activities are compared based both on the protein content and 5' nucleotidase activities of the tissue fractions.

MATERIALS AND METHODS

Female Sprague-Dawley rats were purchased from Harlan Sprague-Dawley Inc. (Haslett, MI). The tissues used in these studies were mammary tumors from DMBA-injected rats or mammary glands from 2-4 month old mid-pregnant (12-14 days) rats. Mammary tumors were induced in female rats by a single intragastric infusion of 5 mg DMBA (contained in 1 ml vegetable oil) into 50-52 day-old animals. Growing tumors were obtained and used for experimentation 9-18 weeks after the infusion of DMBA. The tumors weighed 200-600 mg at the time of excision. L-3-phosphatidylinositol, 1-stearoyl-2-[1-^{14}C] arachidonyl (58 mCi/mMol), was purchased from Amersham Corp. (Arlington Heights, IL). Ascorbic acid, 5'-adenosine monophosphate and arachidonic acid were purchased from Sigma Chemical Co. (St. Louis, MO).

Mammary glands from mid-pregnant rats and DMBA-induced mammary tumors in rats were removed from animals killed by cervical dislocation. The tissues were then frozen at -80C until processed for the determination of enzyme activities. Processing of tissues was carried out in accord with the methods outlined by Huggins and Carraway (4). Briefly, the tissues were weighed and placed in ice cold buffer (0.25 M sucrose - 50 mM TRIS, pH 7.4). The tissues were minced with scissors, diluted to about 5 ml/gram of tissue,

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and homogenized with a polytron mixer (2 bursts of 30 sec each at a power setting of 5). The homogenates were then separated by centrifugation into a 2,000g pellet fraction, a 10,000g pellet fraction, a 100,000g pellet fraction, and a 100,000g supernatant fraction. After washing with the homogenization buffer, the pellets were resuspended in ice-cold 50 mM Tris, pH 7.4, and enzyme activities were determined after appropriate dilutions of each of the cell fractions.

5'-Nucleotidase activity was measured employing the method of Ames (5) for phosphate determinations. Aliquots of the tissue fractions were incubated for 15 min in a 1.6 ml volume containing 50 mM TRIS (pH 7.4), 6.25 mM 5'-AMP, 80 mM KCl and 5 mM $MgCl_2$. Inorganic phosphate generated during the incubation period was then determined.

Phospholipase C activity was determined via the rate of [^{14}C]-diglyceride release from L-3-phosphatidylinositol, 1-stearoyl-2-[1-^{14}C] arachidonyl ([^{14}C] PI). The enzyme reactions were carried out for 30 and 60 min at 37C in a total volume of 0.1 ml containing 50 mM TRIS (pH 7.4 at 37C), 5 mM $CaCl_2$, about 20,000 dpm of the [^{14}C] PI, and an appropriate amount of the tissue fractions containing the enzyme. Dilutions of tissue fractions were employed such that less than 10% of the substrate ([^{14}C] PI) was used during a 60 min incubation at 37C. After incubation, the lipids were extracted by the methods of Bligh and Dyer (6) with 2M KCl in 0.5 M phosphate buffer (pH 7.4) present in the aqueous phase. The lipids were then separated on 250 m silica gel G plates using a solvent system consisting of hexane: ethyl ether: acetic acid: methanol (90:20:3:2). Areas on the chromatograms corresponding to the diglycerides, arachidonic acid and phosphatidylinositol were removed from the plates and radioactivity determined by liquid scintillation techniques. The activity of phospholipase C was then expressed as the percentage of [^{14}C] in the diglyceride fraction of the total [^{14}C] in the extracts. Protein content of the tissue fractions was determined using the method of Lowry et al (7). In preliminary studies the validity of the PLC assay was determined by demonstrating that product formation was linearly related to the amount of enzyme added to the reaction mixtures; product formation was also linear with time for up to 60 min.

Statistical comparisons were made in these studies with Student's t-test.

RESULTS

Table 1 shows the 5' nucleotidase activities in several tissue fractions derived from normal and neoplastic rat mammary tissues. The higher specific activity of 5' nucleotidase in the 100,000g pellet fractions indicates the prevelance of the plasma membranes in that fraction. The specific activity of 5' nucleotidase in the 100,000g pellet and supernatant fraction were not different in the normal and neoplastic tissues. In the 2,000g and 10,000g fractions, however, 5' nucleotidase activity was higher in the fractions derived from the tumor tissues, thus indicating a relatively poor

Table 1. 5' Nucleotidase activity in normal and neoplastic rat mammary tissues

Tissue fraction	5' Nucleotidase activity (nMol/hr./mg protein) Normal Tissue	Neoplastic Tissue
2,000g Pellet	8.15 ± 0.59*	15.6 ± 1.7
10,000g Pellet	6.83 ± 0.80	29.7 ± 4.8
100,000g Pellet	62.1 ± 12.7	59.5 ± 8.1
100,000g Supernatant	12.5 ± 1.5	19.1 ± 4.4

*Numbers represent the mean ± SE of tissues derived from 6 animals.

Table 2. Phospholipase C activity in normal and neoplastic mammary tissues

Tissue Fraction	Phospholipase C Activity (% [^{14}C] PI converted to [^{14}C] DG/30 min/mg protein)		Fold higher in neoplastic tissues based on	
	Normal Tissues	Neoplastic Tissues	Protein Content	5' Nucleotidase Activity
2,000g Pellet	0.034 ± 0.003*	0.370 ± 0.054	10.9	5.70
10,000g Pellet	0.094 ± 0.010	2.29 ± 0.20	67.3	15.4
100,000g Pellet	0.369 ± 0.049	2.42 ± 0.49	6.56	6.8
100,000g Supernatant	0.520 ± 0.060	8.89 ± 1.22	17.1	11.2

*Numbers represent the mean ± SE of tissues derived from 6 animals.

dissociation of the plasma membranes from the homogenates of neoplastic tissues.

Table 2 shows the PLC activities in the extracts of normal and neoplastic mammary tissues. PLC activities in all the tissue fractions derived from the tumor tissues were between 5 and 67 fold higher than the corresponding tissue fractions derived from normal mammary tissues. This was the case whether the comparisons were made on the basis of protein content or 5' nucleotidase activities. Under conditions employed for the results shown in Table 3, no free [^{14}C] arachidonic acid was found in the enzyme reaction mixtures, thus indicating a low activity of phospholipase A_2 and/or diglyceride lipase in the tissue extracts.

DISCUSSION

These studies clearly show that phosphatidylinositol phosphodiesterase (PLC) activity is elevated several-fold in all tissue fractions derived from growing mammary tumors compared to mammary tissues derived from pregnant rats. The tissues from pregnant rats were chosen as control, normal tissue for these studies because they are in a highly differentiated state and contain a high percentage of epithelial cells (8). Although phospholipase A_2 (PLA_2) activity, employing phosphatidylcholine as a substrate, was shown in earlier studies to be elevated several-fold in DMBA-induced rat mammary tumors compared to normal mammary tissues, no PLA_2 activity was detectable under conditions used in the present studies when PI was employed as the substrate. This was the case with all tissue fractions derived from normal and neoplastic mammary tissues. It is possible that substrate specificity for the phospholipase enzymes may explain these observations.

The fact that PLC activity with PI as the substrate is many-fold higher in the mammary tumor tissues may have a direct bearing on the neoplastic properties of these tissues. One of the most rapid effects of mitogenic agents, including certain oncogene products, on cultured cells is the stimulation of the turnover of phosphoinositides (1-3,9). The stimulation of PLC appears to be the initial event affected by the mitogens. When calcium ions are available at mM concentrations, PLC will catalyze the breakdown of PI as well as the 4-phospho- and 4,5 diphospho-derivitives of PI (3). The products of PLC's action on phosphoinositides are therefore diglycerides, inositol phosphate, inositol diphosphate and inositol triphosphate (IP_3). IP_3 has been reported to increase free calcium ion concentrations in cells, and it may be responsible for the elevated calcium ion concentrations observed in dividing cells (1-3,9-12). An elevated PLC activity may also explain the lower total calcium content of neoplastic cells (13), since the IP_3 is thought to primarily increase intracellular calcium ion concentrations by releasing calcium from intracellular organelles; intracellular stores of calcium could thus be depleted. The

diglycerides produced in response to PLC actions on phospholipids are known to activate protein kinase C. This enzyme is also activated by the tumor-promoting phorbol esters and this is the only known specific site of action of these tumor promoters. Although it is not known specifically how protein kinase C and calcium ions function in the regulation of cell division, many observations suggest that they are involved and PLC is thought to have a profound effect on regulating them through the products generated via phospholipid degradation. If the elevation of PLC activity is found to be a universal phenomenon in tumor tissues, it may represent a primary difference between normal and neoplastic tissues.

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