Protein Kinase C Isoenzyme Expression in Normal Mouse Mammary Epithelial Cells Grown in Primary Culture (44037)

H. P. BIRKENFELD,* B. S. MCINTYRE,* K. P. BRISKI,† AND P. W. SYLVESTER*,[1]

Hormonal Carcinogenesis Laboratory, Pharmacology and Toxicology Program, College of Pharmacy, and Department of Veterinary Anatomy, Pharmacology and Physiology,† College of Veterinary Medicine, Washington State University, Pullman, Washington 99164-6510*

Abstract. **Mammary epithelial cells isolated from midpregnant BALB/c mice were grown within collagen gels and maintained in serum-free media containing 10 ng/ml epidermal growth factor (EGF) for an 8-day culture period. Western blot and scanning densitometric image analysis showed the presence of protein kinase C (PKC) α (82 kDa), δ (75 kDa), η (90 and 78 kDa), and ζ (82, 74, and 65 kDa), whereas PKCβ, γ, and θ were not detected in either the cytosolic or membrane fractions in these cells. Cytosolic and membrane levels of PKCα and 82 kDa PKCζ band progressively increased throughout the 8-day culture period. During this same time, cytosolic PKCδ levels decreased, while membrane levels of PKCδ showed no change. Cytosolic and membrane levels of PKCη and the 74- and 65-kDa PKCζ bands displayed some fluctuations but remained relatively constant during the 8-day culture period. Other studies showed that 24-hr treatment with 100 n*M* of phorbol 12-myristate 13-acetate (PMA), resulted in the downregulation of PKCα, δ, and η, and the 82-kDa PKCζ band. However, PMA treatment had no effect on cytosolic and membrane levels of the 74- and 65-kDa PKCζ bands. Since PKC activation is associated with hormone- and growth factor–dependent mammary epithelial cell proliferation, these findings suggest that increases and/or decreases in the relative levels of the different PKC isoenzymes in proliferating cells may indicate their possible role in mediating or regulating EGF-dependent mitogenesis.** [P.S.E.B.M. 1996, Vol 213]

Growth and development of normal mouse mammary epithelial cells is dependent on various hormones and growth factors, particularly epidermal growth factor (EGF) and/or EGF-like compounds (1). EGF activation of the EGF receptor (EGF-R) induces intrinsic tyrosine kinase activity which can autophosphorylate tyrosine residues on the C terminus of the receptor, as well as other intracellular substrates (2–4). Although the exact intracellular mechanism(s) involved in mediating EGF-induced mammary epithelial cell proliferation is not fully understood, activation of the EGF-R results in phospholipid-dependent protein kinase C (PKC) activation (5). Studies have demonstrated that treatments that enhance phospholipid-dependent PKC activation stimulate, whereas treatments that reduce phospholipid-dependent PKC activation inhibit, EGF-induced mitogenesis in normal mammary epithelial cells (5–8). These findings suggest that EGF-induced mammary epithelial cell proliferation is mediated, at least in part, through PKC-dependent mechanisms.

PKC consists of a large family of structurally homologous serine/threonine kinases, which display a wide range of tissue and cellular distribution, and have distinct sensitivities to lipid activation and regulation (9–13). The PKC family of isoenzymes has been divided into subfamilies based upon related characteristics. The first subfamily is referred to as the "conventional" PKC isoenzymes and includes PKCα, β, and

[1] To whom requests for reprints should be addressed at College of Pharmacy, Washington State University, Pullman, WA 99164-6510.

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γ. (13). Conventional PKC isoenzymes, which typify the classical definition of PKC by requiring calcium, phospholipid, and diacylglycerol for activation, catalyze the phosphorylation of histone H_1 and bind phorbol ester with high affinity (13). Tumor-promoting phorbol esters are potent activators of conventional PKC isoenzymes, substituting for diacylglycerol on the enzyme (9–13). However, chronic treatment with high concentrations of phorbol esters results in the downregulation of cytosolic and membrane levels of these PKC isoenzymes (9–13). Diacylglycerol binding to specific sites on conventional PKC isoenzymes induces the translocation of PKC from the cytosol to the cellular membrane, where the enzyme interacts with membrane phospholipids that are required for kinase activity (9–13). The second subfamily is called the "novel" PKC isoenzymes and includes PKCδ, ε, θ, and η (13). Novel PKC isoenzymes differ from conventional PKC isoenzymes in that their activation is calcium independent (13). The third subfamily is called the "atypical" PKC isoenzymes and includes PKCλ and ζ (13). Atypical PKC isoenzyme activation is calcium- and diacylglycerol-independent, and phorbol esters do not activate or downregulate these PKC isoenzymes (13). Recent studies have demonstrated that PKCα is the predominant conventional PKC isoenzyme present in murine mammary epithelial cells (5, 14). However, since different PKC isoenzymes display diversity in tissue levels and substrate specificity, these isoenzymes may also play a key role in mediating and/or regulating mammary epithelial cell mitogenesis. The following studies were conducted to determine which PKC isoenzymes are present in normal mouse mammary epithelial cells grown in primary culture and to characterize changes in their relative levels in proliferating cells throughout an 8-day culture period. Additional studies examined the effects of phorbol ester treatment on PKC isoenzyme downregulation in these cells.

Materials and Methods

Isolation and Culture of Mammary Epithelial Cells. Mammary epithelial cells were isolated from midpregnant BALB/c mice using the procedure described by Sylvester *et al.* (5). Briefly, mammary glands were removed, minced, and then incubated in a collagenase (250 IU/ml; Boehringer Mannheim Corp., Indianapolis, IN), followed by protease (0.5 mg/ml pronase E, Type XIV, from *Streptomyces griseus,* Sigma Chemical Co., St. Louis, MO) digestion media (5). The digested tissue suspension was then sequentially filtered through sterile 250- and 48-μm filter nylon membranes (Tetko Inc., Briarcliff Manor, NY). The retained mammary epithelial cell organoids were then rinsed off the 48-μm filter, pelleted by centrifugation, and then resuspended in fresh media. Recovered cells were plated within rat tail collagen gel and maintained on media containing 10% bovine calf serum for a 2-day incubation period (5). Mammary epithelial cell organoids were then recovered by collagenase digestion and filtration. The resulting mammary epithelial cell organoids, free of adipocyte and fibroblast contamination (5, 15), were then plated in 100 × 15–mm culture plates (1.0×10^7 cells/8 ml collagen gel/plate) and maintained on serum-free media consisting of DME/F12 containing 5 mg/ml bovine serum albumin (BSA), 10 μg/ml insulin, 100 U/ml soybean trypsin inhibitor, 10 μg/ml transferrin, 1 μg/ml *d*-α-tocopherol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10 ng/ml EGF. In other studies, cell were grown in similar media for 6 days and then treated for 24 hr with either 0 or 100 n*M* phorbol 12-myristate 13-acetate (PMA). PMA was first dissolved in 100% ethanol and then added to the media such that the final ethanol concentration in the media never exceeded 0.02% (v/v). Ethanol was added to all treatment media so that the final ethanol concentration was the same in all groups within a given experiment. All cells were fed fresh media every other day.

Electrophoresis and Western Blotting Analysis. Mammary epithelial cells in each treatment group were isolated from gels with collagenase, collected by centrifugation (300*g* for 10 min), washed, and then resuspended in buffer A, which consisted of 20 m*M* Tris-HCl buffer (pH 7.5) containing 2 m*M* EDTA, 0.5 m*M* EGTA, 2 m*M* PMSF, 25 μg leupeptin, and 0.33 *M* sucrose. Cells were then sonicated, and lysates were centrifuged (190,000*g* for 1 hr). The soluble cytosolic fraction was retained and the membrane pellet was resuspended in buffer B (buffer A minus 0.33 *M* sucrose) containing 1% nonidet P-40 to solubilize the membrane fraction. This detergent-treated suspension was shaken at 4°C for 30 min, and then centrifuged at 190,000*g* for 1 hr. Both the cytosolic and solubilized membrane fraction were purified by DEAE-cellulose (Whatman DE52 preswollen microgranular anion exchanger; Whatman Biosystems Ltd., Kent, England) chromatography as described previously (5). Protein concentration of each cytosolic and membrane fraction was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). All fractions were then diluted 1:3 with loading buffer (0.125 *M* Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 100 μ*M* sodium orthovanadate, pH 6.8), and boiled for 5 min. Equal amounts of cytosolic (25 μg) and membrane (25 or 50 μg) protein were fractionated in 7.5% polyacrylamide gels (16). Afterwards, each gel was equilibrated in transfer buffer (25 m*M* Tris, 192 m*M* glycine, and 20% methanol) and transblotted for 12–16 hr at 25 mV to 0.45 μm PVDF membranes (NEN Research Products, Dupont Co., Wilmington, DE) as described by Towbin (17). Membranes were then incu-

bated for 2 hr at room temperature in TBST (25 m*M* Tris-HCl, pH 7.5, containing 150 m*M* NaCl, 0.05% [w/v] Tween-20) buffer supplemented with 0.3% nonfat milk. Membranes were then incubated for 2 hr with either mouse monoclonal antibodies specific against PKC isoenzymes α, β, δ, η, or θ (Transduction Labs, Lexington, KY, γ (Zymed Laboratories Inc., San Francisco, CA), or polyclonal antibodies specific against PKCη and ζ (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then rinsed four times with TBST buffer and then incubated with either [^{125}I]labeled (18) goat anti-mouse (specific against monoclonal antibodies) or goat anti-rabbit (specific against polyclonal antibodies) second antibody (dilution 1:5000) at room temperature for 1 hr. Blots were then rinsed four times with TBST buffer, and bands were then visualized on film (Kodak X-OMAT AR, Rochester, NY) by autoradiography at −70°C for 24 to 48 hr. Autoradiographic images were quantitated and molecular weights calculated using a Scanalytics Scanmaster 3$^+$densitometer (Billerica, MA) with RFLPscan software.

Results

Figure 1 shows autoradiographic visualization of PKC isoenzyme expression in normal mammary epithelial cells grown in primary culture and maintained on serum-free media. These cells displayed rapid and continued growth, resulting in a 3-fold increase in viable cell number by the end of the 8-day culture period, as determined by hemocytometer. Mammary epithelial organoids displayed a time-dependent increase in duct-like branching, characteristic of cell proliferation in this experimental model (1, 5, 15, 19). Western blot analysis showed that PKCα was the predominant conventional isoenzyme present in both the cytosolic and membrane fractions, and that the relative levels of PKCα progressively increased in these cells throughout the 8-day culture period (Fig. 1). Novel PKC isoenzymes, δ and η, were found to be present in less abundant concentrations in these cells (Fig 1). PKCδ was visualized as a single band with a molecular weight of approximately 75 kDa. The relative cytosolic levels of PKCδ decreased over time in culture, but membrane levels remained relatively constant (Fig. 1). PKCη was visualized on Western blots in two separate bands with approximate molecular weights of 90 and 78 kDa, respectively (Fig. 1). Relative cytosolic and membrane levels of PKCη showed little variation throughout the 8-day culture period (Fig. 1). The atypical isoenzyme, PKCζ, was also found in high concentrations in these cells, and was visualized on Western blots in three bands of approximate molecular weights of 82, 74, and 65 kDa, respectively. Relative cytosolic and membrane levels of the 74- and 65-kDa PKCζ bands fluctuated slightly, whereas cytosolic and membrane levels of the 82-kDa PKCζ band progressively increased during the 8-day culture period (Fig. 1). PKCβ, γ, and θ were not detected in either the cytosolic or membrane fractions of these cells at any time during the 8-day culture period (Fig. 1). Scanning densitometric quantification of the Western blot PKC isoenzyme bands visualized by autoradiography in Figure 1 is shown in Figure 2.

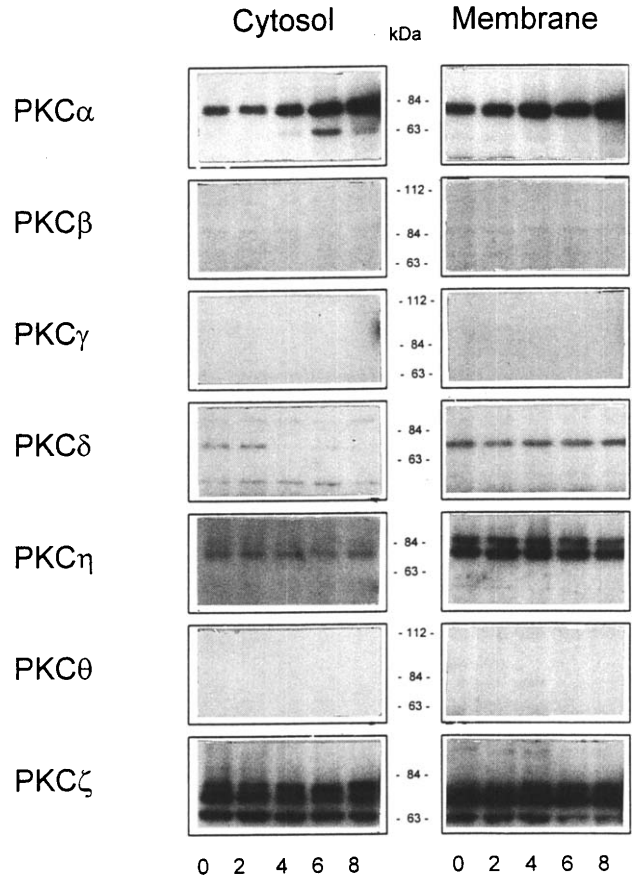

Figure 1. Autoradiographic visualization of Western blot analysis of PKC isoenzyme expression on Day 0, 2, 4, 6, and 8 in culture in cytosolic (left column) and membrane (right column) fractions of isolated mammary epithelial cells. Each lane contains 25 μg of cytosolic protein (left column) or 50 μg of membrane protein (right column).

The effects of phorbol ester treatment on the relative levels of the various PKC isoenzymes in normal mammary epithelial cells are shown in Figure 3. After a 24-hr exposure to 100 n*M* PMA, cytosolic and membrane levels of PKCα, δ, η, and the 82-kDa PKCζ band displayed a downregulation in these cells, compared with untreated controls (Fig. 3). In contrast, PMA treatment had no effect on cytosolic and membrane levels of the 74- and 65-kDa PKCζ bands (Fig. 3). Scanning densitometric quantification of the Western blot PKC isoenzyme bands visualized by autoradiography in Figure 3 is shown in Figure 4.

Verification that the bands identified by Western

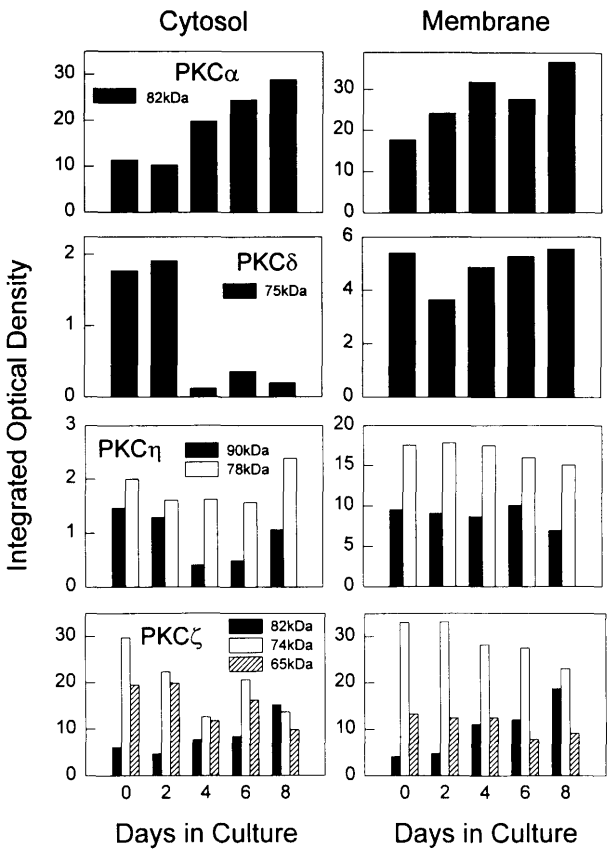

Figure 2. Scanning densitometric analysis of Figure 1 autoradiographs visualizing PKC isoenzyme expression on Day 0, 2, 4, 6, and 8 in cytosolic (left column) and membrane (right column) fractions of isolated mammary epithelial cells. Vertical bars represent the integrated optical density of bands visualized in each lane for individual PKC isoenzymes.

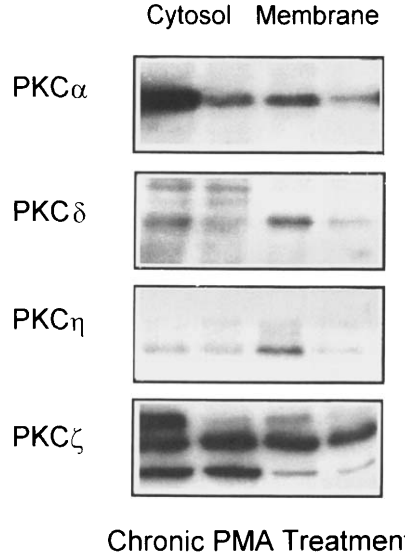

Figure 3. Autoradiographic visualization of Western blots analysis of PKC isoenzyme expression in the cytosolic and membrane fractions of isolated mammary epithelial cells cultured for 6 days and then treated for 24 hr with 0 (−) or 100 n*M* (+) PMA. Each lane contained 25 μg of cytosolic or membrane protein.

blot analysis were specific for PKCα, β, γ, η, and ζ, was determined by competition studies with the specific peptides as described previously (5). In general, bands visualized by the different PKC isoform antibodies were not visualized in the presence of excess competing peptide (data not shown). An exception to this observation was the finding that the 82-kDa PKCζ band was still visualized in Western blots incubated in the presence of excess competing peptide (Fig. 5). Specific peptides for PKCδ and θ were not available for similar competition studies. However, specificity for these antibodies was confirmed in Western blots using nonspecific mouse IgG2A antibody (isotype antibody controls). Isotype antibody Western blots displayed a complete absence of band visualization for each of these EGF isoenzymes (data not shown). These findings confirm previous reports from our laboratory (5) and demonstrate that the bands visualized in our Western blots are specific for each PKC isoenzyme.

Discussion

These studies demonstrate that at least four different PKC isoenzymes are present in normal mouse mammary epithelial cells grown in primary culture and maintained on serum-free media. PKCα was present in the highest concentrations in these cells, and the relative levels of this conventional PKC isoenzyme progressively increased throughout the 8-day culture period. Experimental findings also confirmed previous studies which showed that conventional isoenzymes PKCβ and γ are not present in these cells (5). PKCζ was also found to be present in high concentrations in normal mammary epithelial cells. However, relative levels of the 74- and 65-kDa PKCζ bands showed little change throughout the 8-day culture period, while cytosolic and membrane levels of the 82-kDa PKCζ band increased over time in culture. PKCδ and η were found to be present in less abundant concentrations in these cells, but the relative levels of these novel PKC isoenzymes were found to decrease over time in culture.

Previous reports have demonstrated that PKCα, δ, and ζ are present in normal and neoplastic mammary epithelial cells of other species (14, 20, 21). Additional studies using Western blot analysis of PKC isoenzymes in brain tissue showed that PKCα and δ are visualized in a single band whereas PKCη and ζ are visualized as a series of two to three bands (22). The multiple forms that exist for each of these PKC isoenzymes are believed to represent different phosphorylation states of the enzyme, which affects band migration during electrophoretic separation (22). However, experimental evidence in this present study suggests that the 82-kDa band visualized in Western blots for PKCζ may not represent an alternative phosphorylation state of this atypical PKC isoenzyme. Rather, this 82-kDa band may represent another protein which

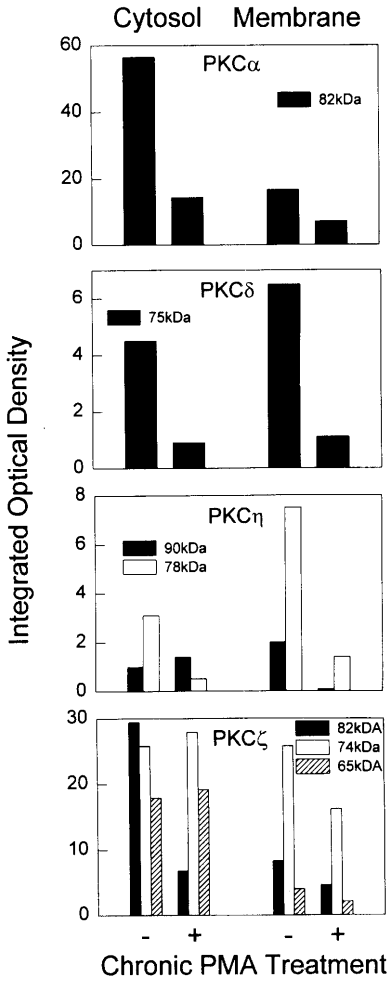

Figure 4. Scanning densitometric analysis of Figure 3 autoradiographs visualizing PKC isoenzyme expression in the cytosolic and membrane fractions of isolated mammary epithelial cells cultured for 6 days and then treated for 24 hr with 0 or 100 n*M* PMA. Vertical bars represent integrated optical density of bands visualized in each lane for individual PKC isoenzymes.

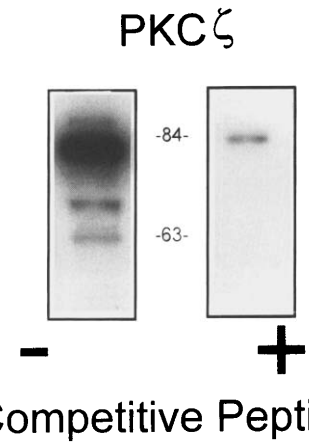

Figure 5. Competitive inhibition of PKCζ band visualization on Western blot autoradiographs by PKCζ-specific peptide. Transblotted membranes were incubated with antibodies specific for PKCζ in the absence (−) or presence (+) of a competitive PKCζ-specific peptide. Each lane contained 25 μg of cytosolic protein.

nonspecifically binds to the PKCζ antibody. This suggestion is supported by the finding that only the 82- and not the 74- or 65-kDa PKCζ band was still visualized on Western blots incubated in the presence of excess PKCζ-specific competing peptide. In addition, PKCζ has previously been shown to be insensitive to phorbol ester activation and/or downregulation (21, 23, 24), yet PMA treatment induced a downregulation of only the 82- and not the 74- or 65-kDa bands visualized on PKCζ Western blots. The findings also suggest that the PKCζ antibody used in Western blot analysis may have some cross-reactivity with PKCα, since both PKCα and ζ are visualized as a 82-kDa band in their respective Western blots, the intensity of both these 82kDa bands progressively increase during the culture period, and both bands are downregulated by phorbol ester treatment.

Various hormones and growth factors are involved in mammary epithelial cell growth and differentiation (1). In the nonpregnant adult female, the mammary gland is not functionally developed and consists of relatively simple ducts radiating from the nipple that contain few epithelial cells (1). During pregnancy, there is rapid mammary epithelial cell proliferation which leads to extensive ductal branching and alveoli formation (1). Previous studies indicate that EGF appears to be the major mitogenic stimulus in mammary epithelial cells isolated from midpregnant mice (25), and EGF stimulates phospholipid-dependent PKC activation in these cells (5). Furthermore, treatments that enhance PKC activation have been shown to stimulate, whereas treatments which reduce PKC activation inhibit, EGF-induced mammary epithelial cell proliferation (5–8). These findings strongly suggest that EGF-induced proliferation in normal mouse mammary epithelial cells is mediated by PKC-dependent mitogenic pathways. Although the specific roles for each PKC isoenzyme in regulating mammary epithelial cell proliferation have not yet been elucidated, differential changes in the relative levels of PKC isoenzymes have been observed in rodent mammary tissue during pregnancy and lactation, suggesting that individual PKC isoenzyme may selectively elicit specific cellular responses at different times during mammary epithelial cell proliferation and differentiation (13, 14). In addition, overexpression of specific PKC isoenzymes in various tumor cells lines has been correlated with increased or decreased cell proliferation (26–29).

In summary, the present investigation characterizes the expression of various PKC isoenzymes in normal mouse mammary epithelial cells grown in primary culture. The relative levels of individual PKC isoenzymes increased, decreased, or showed no change during EGF-induced cell proliferation. These findings suggest that various PKC isoenzymes in mammary epithelial cells may play a differential role in regulating

and/or mediating EGF-dependent mitogenic signaling in these cells. Further studies are required to determine the exact role of each PKC isoenzymes during EGF-induce mammary epithelial cells mitogenesis.

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