Isoenzymes of protein kinase C in rat mammary tissue: changes in properties and relative amounts during pregnancy and lactation

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Protein kinase isoenzymes belonging to the protein kinase C (PK-C) family present in rat mammary tissue have been resolved from one another by chromatography on hydroxyapatite, and characterized. PK-C α is the predominant isoenzyme and is present at a constant level of activity throughout mammary-gland development and differentiation. In contrast, marked changes in the relative abundance of other mammary PK-C isoenzymes accompany the transition from pregnancy to lactation. The sensitivity of mammary PK-C α to Ca^{2+} is greater in tissue from pregnant than from lactating rats. This isoenzyme

has other atypical properties consistent with its being more highly phosphorylated than PK-C α in rat brain and spleen. One of the protein kinase isoenzymes resolved from mammary tissue recognizes the peptide substrate used to assay AMP-activated kinase and may thus interfere in the determination of this activity. Another is fully active in the absence of Ca^{2+} and is more than 80% active in the absence of added lipid effectors. A 'housekeeping' role is proposed for PK-C α in mammary tissue, whereas the less abundant PK-C isoenzymes may be involved in mammary cell proliferation and differentiation.

INTRODUCTION

Intracellular signalling via protein kinase C (PK-C) has been implicated in many instances of the initiation of cell proliferation triggered by hormones, growth factors and mitogens (reviewed by Nishizuka, 1984, 1986, 1988; Woodgett et al., 1987; Asaoka et al., 1992). Furthermore, in several types of secretory cell, the Ca^{2+} -requiring and Ca^{2+} -independent processes of regulated and constitutive exocytosis (Burgess and Kelly, 1987) also involve PK-C (Rink et al., 1983; TerBush et al., 1988).

During the development of mammary tissue that occurs in pregnancy and into lactation, mammary secretory epithelial cells undergo a programme of cell proliferation, growth and differentiation. These changes are orchestrated by several hormones acting on mammary tissue (Neville and Daniel, 1987). In fully differentiated mammary cells, evidence for the involvement in casein secretion of both regulated and constitutive exocytotic pathways has recently been presented (Turner et al., 1992). Mammary tissue PK-C activity undergoes marked changes in the course of this developmental programme, being highest throughout early and mid-pregnancy, falling to much lower levels in late pregnancy and remaining low throughout lactation (Caulfield and Bolander, 1986; Lavandero et al., 1992). Broadly, therefore, there is a positive correlation between the proliferative phase of mammary development and PK-C activity, although existing knowledge of exocytotic mechanisms in other cell types indicates the probability of a role for PK-C in the secretory processes of the fully differentiated mammary epithelial cell. PK-C activity is the functional expression of a family of distinct isoenzymes (10 have been described to date; Asaoka et al., 1992) whose individual functions are not well defined, and whose relative contributions to total PK-C activity vary from tissue to tissue. The purpose of the work described here was to characterize and quantify the spectrum of PK-C isoenzymes in rat mammary tissue at different times during mammary development and differentiation to enable more precise correlations to be made between these two phases

of functional maturation of mammary cells and the activity of individual PK-C isoenzymes.

EXPERIMENTAL

Animals and cell lines

Female Wistar rats (A. Tuck and Sons) were used in their first pregnancy or lactation. They were initially mated on reaching a body weight of 180 g and thereafter maintained under conditions described previously (Clegg and Ottey, 1990). Spleen and mid-brain tissue was obtained from male rats (160 g body wt). COS-7 cells were grown in 75 mm² tissue-culture flasks to confluence at 37 °C in Dulbecco's modified Eagle's medium containing 10% foetal-calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin under 5% CO_2 in air. Attached layers were rinsed briefly with Hanks balanced salts medium without Ca^{2+} and Mg^{2+} (5 ml/flask at room temperature) before scraping into ice-cold homogenization buffer (1 ml/flask) and snap-freezing in liquid nitrogen.

Resolution of PK-C isoenzymes

The following procedure was based on published methods (Kuo et al., 1980; Huang et al., 1986b). Mammary tissue was removed from anaesthetized animals (intraperitoneal pentobarbital; 60 mg/kg body wt.), rapidly frozen in liquid nitrogen and cryopulverized. Homogenates were prepared from powdered tissue (between 1.5 and 2.5 g was usually processed) in 6 vol. of ice-cold buffer (25 mM Tris/HCl, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 50 mM 2-mercaptoethanol, pH 7.5) containing 1 mM phenylmethanesulphonyl fluoride, 1 mM benzamidinium/HCl, 2 $\mu\text{g}/\text{ml}$ soya-bean trypsin inhibitor and 25 $\mu\text{g}/\text{ml}$ leupeptin, by using a Polytron homogenizer fitted with a PTA 10S probe operated at 18000 rev./min for 20 s. All further operations were at 0–4 °C. The homogenate was centrifuged at 6000 g for 10 min. The supernatant was retained after straining

through glass wool. Triton X-100 was added to a final concentration of 1% (v/v). After stirring for 60 min, the extract was centrifuged at 105 000 *g* for 1 h and the clear supernatant retained for further processing.

Other tissues, serving as positive controls for 'calibration' of the fractionation system, were treated in exactly the same manner. Snap-frozen suspensions of scraped COS-7 cells were allowed to thaw at room temperature and then immediately homogenized and treated in the manner described for solid tissues. Cells from approx. 15 flasks gave ultimate yields of PK-C activity comparable with those from 1 g of the solid tissues.

Triton-treated extracts were loaded on to DEAE-cellulose columns (19.5 mm diam. $\times$ 66 mm) equilibrated in 20 mM Tris/HCl/1 mM EDTA/1 mM EGTA/50 mM 2-mercaptoethanol, pH 7.5. Columns were then washed in the same buffer until the A_{280} of the eluate fell below 0.05. Adsorbed protein was then eluted with a 'step' of this buffer containing 300 mM NaCl. Fractions containing PK-C activity were pooled and dialysed into 5 mM potassium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM EGTA and 1 mM dithiothreitol, before loading on to a column (16 mm diam. $\times$ 140 mm) of hydroxyapatite equilibrated in the same buffer. Then 4 column vol. of the same buffer was used to wash through unbound protein before applying a linear gradient (130 ml) of 5–300 mM potassium phosphate buffer containing the same additions as above. Fractions (2.5 ml) were collected and assayed promptly for PK-C activity. For longer-term storage, fractions were pooled as appropriate and activity was stabilized by addition of 0.2 mg/ml BSA. After dialysis against 50% glycerol/10 mM Tris/HCl/0.5 mM EDTA/0.5 mM EGTA/25 mM 2-mercaptoethanol, pH 7.5, samples were stored at -20°C . Activity was stable for several weeks under these conditions.

Assay of PK-C activity

The method used was modified from that of House et al. (1987), by using as phosphate acceptor a synthetic peptide [Ala⁹,Ala¹⁰,Lys¹¹,Lys¹²]GS(1–12) modified from the sequence of residues 1–12 of glycogen synthase and referred to here as 'GS-peptide'. The complete assay mixture (60 μl ; pH 7.0) contained 13.3 mM Mops/KOH, 10.7 mM magnesium acetate, 66.7 μM [γ -³²P]ATP (approx. 10^5 c.p.m.), 6.7 mM dithiothreitol, 4.2 mM 2-mercaptoethanol, 0.5 mM CaCl₂, 83 μM EDTA, 83 μM EGTA, 50 $\mu\text{g}/\text{ml}$ phosphatidylserine, 3.3 mM Tris/HCl, 2 μg of BSA and 12 μg of GS peptide. Other additions or modifications were as noted in the text. Incubation was at 30°C . Phosphate incorporation into GS-peptide was measured by spotting samples of assay mixtures on to squares of P 81 paper and processing them as described by Roskowski (1983). Phosphorylation was linear with respect to time for at least 20 min and with kinase activity per assay up to 0.1 m-unit (where 1 unit of activity is that which catalyses the transfer to acceptor substrate of 1 μmol of phosphate/min).

Other methods

SDS/PAGE was performed as described by Laemmli and Favre (1973), and electrotransfer of proteins to nitrocellulose and Western blotting with antisera were done as described by Towbin et al. (1979). Autophosphorylation reactions with resolved PK-C fractions were carried out by using a modification of the assay incubation described above. Specific radioactivity was increased 10-fold, GS-peptide was omitted and concentrations of EGTA

and CaCl₂ were altered to give a free Ca²⁺ concentration of 5.6 μM . The peptide inhibitor of protein kinase A (PK-A) was included at 1 $\mu\text{g}/\text{ml}$. After incubation at 30°C for 30 min, reaction mixtures were disaggregated for SDS/PAGE and electrophoresed on 8%-acrylamide gels. Phosphoproteins were located by autoradiography after fixing, Coomassie Blue staining and destaining the gels.

Sources of consumables and reagents

Radioisotopes were from Amersham or ICN; P 81 paper was from Whatman; nitrocellulose membrane (Hybond-C Extra) and ECL reagents for signal development on Western blots were from Amersham. X-ray film was Agfa 'Curix'. PK-C isoenzyme-selective antisera were raised in rabbits against synthetic peptides corresponding to regions of sequence unique to each isoenzyme; these and their respective peptide antigens were generously given by Dr. Peter Parker (ICRF, London). Dr. Parker also provided the γ -isoenzyme-selective mouse monoclonal antibody 36G9. The α - and β -selective anti-PK-C mouse monoclonal antibody MC5 was from Amersham International. Other antisera, peroxidase/anti-peroxidase (PAP) complexes and non-immune sera were from the Scottish Antibody Production Unit. The following synthetic peptides were prepared with an Applied Biosystems 431A solid-phase synthesizer: GS-peptide and PK-C pseudo-substrate inhibitor peptide (House et al., 1987; House and Kemp, 1987); Kemptide (Kemp et al., 1976); Walsh inhibitor peptide (Cheng et al., 1986); 'Farago' PK-C substrate peptide (Farago et al., 1989); SAMS peptide substrate for AMP-activated protein kinase (Davies et al., 1989). Lysine-rich histone (type III) was from Sigma. Other reagents were from the sources previously described.

RESULTS

PK-C activity of mammary tissue

PK-C activity was low in mammary tissue compared with that in brain and spleen. As has been found previously in mice (Caulfield and Bolander, 1986) and rats (Lavandero et al., 1992), more activity was measurable in extracts from mammary tissue of early- and mid-pregnant rats (18.3 ± 0.48 m-units/g wet wt. for 8-day-pregnant; mean $\pm$ S.E.M. for 7 determinations) than from lactating animals (8.4 ± 0.51 m-units/g wet wt. for 10-day-lactating; mean $\pm$ S.E.M. for 5 determinations). During late pregnancy (20 days), activity fell to that maintained throughout lactation.

For these measurements of total tissue activity given above, the mammary extracts were eluates from DEAE-cellulose, prepared as described in the Experimental section for the resolution of PK-C isoenzymes. Kinase activity was measured with GS peptide as a phosphate acceptor; PK-C activity was arbitrarily defined as that fraction of the increment in activity provoked by the addition of Ca²⁺ plus phosphatidylserine (final concns. 33 μM free cation and 50 $\mu\text{g}/\text{ml}$ respectively) which was inhibitable by the PK-C pseudosubstrate inhibitor peptide (final concn. 420 $\mu\text{g}/\text{ml}$). Measurements were also routinely performed of the increment above basal activity caused by the separate addition of Ca²⁺ and of phosphatidylserine; furthermore, both pseudo-substrate-sensitive and pseudosubstrate-insensitive components of all these activity parameters were measured. If histone (final concn. 500 $\mu\text{g}/\text{ml}$) was substituted for GS peptide, the resulting estimates of PK-C activity (by using otherwise the same protocol) were 6.7 and 1.4 m-units/g wet wt. of tissue (means of 3 determinations) from 8-day-pregnant and 10-day-lactating rats respectively. The pseudosubstrate-inhibitor-insensitive com-

Figure 1 PK-C isoenzymes of rat mammary tissue: comparison with other rat tissues

Extracts were prepared from rat tissues and COS-7 cells, and fractionated by hydroxyapatite chromatography as described in the Experimental section. PK-C activity was measured with GS peptide as substrate. The separations shown all started from 2 g of tissue or from COS-7 cells harvested from 17 flasks. (a) Spleen; (b) mammary tissue (mid-lactating rat); (c) COS-7 cells; (d) brain. The separation using COS-7 cells has not been repeated; each of the rat tissue separations shown has been performed at least twice with similar results.

ponent of histone kinase activity rose sharply, from 1.2 m-units/g of tissue during pregnancy to 11.6 m-units/g during mid-lactation; this activity was also insensitive to Ca^{2+} (at free concentrations between < 10 pM and $33 \mu\text{M}$), phosphatidylserine and the peptide inhibitor of PK-A. The timing of its increase reflects that of casein secretion, and it may be a measure of the Golgi-vesicle kinase activity which is responsible for the phosphorylation of caseins before their secretion in milk, although the latter is inactive against rat caseins when the concentration of free Ca^{2+} is below 0.1 mM (West and Clegg, 1983). In contrast, the extent of increase in the pseudosubstrate-inhibitor-insensitive component of GS kinase activity between pregnancy and lactation was much less marked. It rose from 2.8 m-units/g at day 8 of pregnancy to 7.2 m-units/g at day 10 of lactation. Like the inhibitor-resistant component of histone kinase activity, it too was Ca^{2+} - and phospholipid-insensitive and not significantly inhibited by the peptide inhibitor of PK-A. These results illustrate the much greater sensitivity afforded to the assay of mammary tissue PK-C (especially tissue from lactating animals) by the use of GS peptide rather than histone.

Resolution of PK-C isoenzymes from mammary tissue

Separation of PK-C activity into its major isoforms can be achieved by hydroxyapatite chromatography. Figure 1 shows a typical profile for mammary tissue (from lactating animals) compared with those obtained for control tissues and cells having known composition with respect to the Ca^{2+} -dependent isoenzymes: brain (all isoenzymes present); spleen (β -isoenzyme predominant); COS-7 cells (α -isoenzyme predominant). Minor but significant differences in the conditions of PK-C assay, compared with those described in the previous section, were employed when screening fractions from hydroxyapatite. The

activity profiles illustrated in Figure 1 (and also in Figure 3) were obtained by measuring kinase activity with GS peptide as phosphate acceptor under the conditions of Ca^{2+} - and phosphatidylserine-sufficiency given in the Experimental section but with the peptide inhibitor of PK-A also present at a final concentration of $1 \mu\text{g/ml}$. Thus, at this stage in the study, conditions of assay rigorously selective for PK-C had not been used when measuring GS kinase activity in these samples. However, the results described in the previous section support the provisional identification of the measured activity peaks as PK-Cs, and this is further sustained by the effector-sensitivity data presented below. Four major activity peaks, one with a small shoulder, were resolved from the mammary tissue extract; these are identified, in order of elution, as I–V in Figure 1. On the basis of elution position from the hydroxyapatite column, correspondence of selected mammary peaks to authentic isoenzymes is as follows: I to γ (although γ may not occur outside the central nervous system), III to β_1 , IV to β_{11} , V to α . The response of activity peaks I, II, III and V to PK-C effectors was tested; the results (Figure 2) confirm this correspondence for I, III and V, but insufficient quantities of IV were available for full analysis. Furthermore, the effective inhibition of kinase activity by the PK-C pseudosubstrate inhibitor supports the contention that all five mammary protein kinases are members of the PK-C family.

Activity peaks resolved from brain, spleen and COS-7 cells were used as sources of authentic isoenzymes in subsequent experiments. Each was confirmed by Western blotting with isoenzyme-specific antisera to be homogeneous with respect to isoenzyme content (results not shown), although all were heterogeneous with respect to protein content, with greatest heterogeneity being apparent in the fractions eluted earliest from hydroxyapatite.

Figure 2 Effector-responses of mammary PK-C isoenzymes

Each panel displays the responses of an individual isoenzyme, identified by Roman numerals as in Figure 1. Conditions of assay (see the Experimental section for full details) are summarized beneath each histogram bar. Phosphatidylserine (PtdSer) was either present at 50 μ g/ml or absent. The condition designated ' μ M Ca²⁺' resulted when neither Ca²⁺ nor extra EGTA was added. 'mM Ca²⁺' was achieved by adding CaCl₂ to a final concentration of 0.5 mM, and zero Ca²⁺ by adding 2 mM EGTA. All assays contained the peptide inhibitor of PK-A (15 μ g/ml); where indicated, the pseudosubstrate (P-S) peptide inhibitor of PK-C was also present at 420 μ g/ml. The results shown are from a single experiment, representative of at least four that have been done with each isoenzyme. The activities (μ -units/assay) represented by the 100% values were: I, 1.5; II, 1.4; III, 2.8; V, 7.6.

Figure 3 PK-C isoenzymes of mammary tissue from pregnant rat

Conditions were exactly as in Figure 1; 2 g of tissue from an 8-day-pregnant rat was processed. A representative separation is shown from three that have been performed.

The relative abundance of the resolved isoenzymes in mammary tissue from pregnant rats was markedly different from that in lactating animals (Figure 3). The absolute amount of isoenzyme V was similar between the two physiological states, but amounts of isoenzymes I, II and III were much greater in tissue from pregnant animals, with isoenzyme II being most markedly affected.

Different PK-C isoenzymes show different preferences among

the many phosphate-acceptor substrates which have been used for their assay. Exploiting these properties with the intention of confirming the identification of individual mammary isoenzymes with authentic PK-C isoenzymes from rat brain, each of the activity peaks resolved from mammary tissue extract was tested with an alternative peptide substrate described by Farago et al. (1989) (related to the sequence of a known PK-C site on histone H1), and also with Kemptide (in the absence of the PK-A inhibitor peptide) and the SAMS peptide (Figure 4). None of the brain isoenzymes phosphorylated Kemptide significantly (indicating a lack of contamination with PK-A); surprisingly, the γ isoenzyme was active with the SAMS peptide, which has been reported to be a selective substrate for AMP-activated protein kinase. Other experiments (results not shown) demonstrated that the SAMS peptide kinase activity of PK-C γ was not attributable to AMP-activated kinase, because (a) it was not stimulated by AMP, (b) it was not inhibited by 8-bromo-AMP, and (c) it was not dependent on inhibition of protein phosphatases for continued kinase activity. The γ isoenzyme was inactive against the Farago peptide substrate, whereas both the β and α isoenzymes phosphorylated this peptide at around 40% of the rate achieved with GS-peptide. These substrate preferences of the γ , β and α isoenzymes from brain were exactly reflected in those of activity peaks I, III and V, respectively, separated from mammary tissue (Figure 4). Mammary peak II had no corresponding homologue in brain; it was insensitive to Ca²⁺, and the extent of its stimulation by phosphatidylserine was small. It was unable to catalyse phosphorylation of either the Farago peptide or the SAMS peptide (Figures 2 and 4).

Figure 4 Relative rates of phosphorylation of different peptide substrates by mammary PK-C isoenzymes and by authentic PK-C α , β and γ

Phosphorylation of the peptide substrates indicated (all at 200 $\mu\text{g}/\text{ml}$) was assayed by using partially purified PK-C isoenzyme preparations. (a) PK-C isoenzymes, I, II, III and V from mid-lactating rat mammary tissue as shown. (b) PK-C isoenzymes α , β and γ from rat brain as shown. The activities ($\mu\text{-units}/\text{assay}$) represented by the 100% values were: I, 1.4; II, 2.2; III, 3.4; V, 7.7; α , 16; β , 11; γ , 8.

Sensitivity of PK-C isoenzymes to Ca^{2+}

The effect of diacylglycerol on PK-C is to diminish the EC_{50} for Ca^{2+} (Kishimoto et al., 1980). Because responses of PK-C to effectors are well known to vary as a function of the identity of phosphate acceptor during assay, the ability of diolein to modify Ca^{2+} -sensitivity was re-examined in the present study by using authentic isoenzyme preparations and the assay conditions described in the Experimental section. It was found that the Ca^{2+} -sensitizing effect of diolein was expressed only when the β isoenzyme was the source of PK-C activity (Figure 5); the Ca^{2+} -sensitivity of γ and α isoenzymes was unaffected (results not shown); a similar overall pattern was found for brain isoenzymes by Sekiguchi et al. (1988). Also shown in Figure 5 is the Ca^{2+} -sensitivity of mammary isoenzyme III and the effects on it of diolein. Diolein had no effect on the activity of the Ca^{2+} -insensitive mammary isoenzyme II, whether measured in the absence or presence of Ca^{2+} ; it also had no effect on the Ca^{2+} -sensitivity of isoenzymes I and V. These data for mammary PK-C isoenzymes were obtained by using material from 8-day-pregnant rats. In the absence of diolein, the Ca^{2+} -sensitivity (EC_{50}) of mammary isoenzymes I and III from such rats was 1.2 μM and 1.1 μM respectively (means of 3 determinations). These values did not change as pregnancy proceeded, or during lactation. In contrast, isoenzyme V from lactating rats was markedly less sensitive to Ca^{2+} than the same isoenzyme from pregnant animals (EC_{50} 2.1 μM and 0.88 μM respectively) as shown in Figure 6.

Figure 5 Diolein causes sensitization of mammary PK-C III to Ca^{2+}

PK-C III from mid-pregnant rat mammary tissue (a) and PK-C β from rat spleen (b) were assayed for GS kinase activity at a range of free concentrations of Ca^{2+} (achieved by the use of Ca^{2+} -EGTA buffers), in the presence ($\blacktriangledown$, $\bullet$) or absence (∇ , $\circ$) of diolein (10 $\mu\text{g}/\text{ml}$). Results shown are representative of those obtained in four replicates of this experiment using different isoenzyme preparations.

Figure 6 Ca^{2+} -sensitivity of mammary PK-C V varies with extent of mammary gland development

PK-C V preparations from mammary tissue of rats in mid-lactation ($\blacktriangle$) and mid-pregnancy ($\bullet$) were assayed for GS kinase activity at a range of free concentrations of Ca^{2+} , in the presence of phosphatidylserine (50 $\mu\text{g}/\text{ml}$). The 100% values were 8.8 $\mu\text{-units}$ ($\blacktriangle$) and 6.7 $\mu\text{-units}$ ($\bullet$) per assay. Replication was as in Figure 5.

Autophosphorylation of mammary PK-C isoenzymes

Isoenzymes I, II, III and V from mammary tissue at various stages of development were incubated under autophosphorylating conditions. Additional experiments were performed in parallel, in which authentic PK-C isoenzymes were auto-

Figure 7 Autophosphorylation of mammary PK-C isoenzymes

PK-C preparations from mammary tissue of mid-lactating rats were incubated under autophosphorylating conditions and analysed as described in the Experimental section. Parallel incubations were performed with authentic isoenzymes from the sources indicated. The positions of α , β and γ isoenzymes are indicated on the autoradiographs shown. Calibration markers are $M_r (\times 10^{-3})$. The activity (μ -units) of each isoenzyme used in these incubations was as follows: brain α , 151; brain β , 109; spleen α , 76; spleen β , 52; COS-7 α , 22; mammary I, 0.9; II, 2.1; III, 2.2; IV, 1.8; V, 5.8. Exposure times were adjusted to achieve acceptable signal intensities. A representative set of results, out of a total of three experiments, is shown.

phosphorylated. Representative autoradiographs are shown in Figure 7. The γ and β isoenzymes gave relatively 'clean' signals, each dominated by one major phosphoprotein, of M_r 90000 and 80000 respectively. The α isoenzymes from spleen and COS-7 cells also gave a major phosphoprotein of M_r 90000 (one among several in the case of the spleen preparation), although the brain α isoenzyme yielded a much more diverse phosphoprotein composition (not shown). One major phosphoprotein, which corresponded in M_r to the autophosphorylated PK-C α subunit, was present after autophosphorylation of mammary isoenzyme V. Similarly, a single major autophosphorylated protein was present in mammary isoenzyme I which corresponded to the principal radiolabelled product (M_r 90000) in the brain PK-C γ isoenzyme preparation. Weak signals relative to other phosphoproteins in mammary isoenzymes III and IV corresponded to the autophosphorylated β isoenzyme subunit.

Two major autophosphorylation products (of M_r 58000 and 49000) were found in mammary isoenzyme II. The component at M_r 49000 was, to a variable extent, also present in all other mammary isoenzyme fractions and probably represents the calpain-clipped 'core' enzyme, PK-M (Kishimoto et al., 1989), generated during sample handling and incubation. The other component was unique to isoenzyme II. Of the PK-C isoenzymes described, it most nearly corresponds in M_r to that of the ζ isoenzyme. Furthermore, the effector-responses of isoenzyme II are more similar to those reported for PK-C ζ than for other PK-C isoenzymes (Ono et al., 1989).

Recognition of mammary PK-Cs by isoenzyme-specific antisera

Western-blot analysis of soluble extracts from Triton X-100-treated post-mitochondrial supernatants, using the entire panel of isoenzyme-specific antisera, revealed positive signals in mammary tissue for the presence of only the α isoenzyme. However, the immunodevelopment technique used throughout most of this study (second antibody/PAP-sandwich with chromogenic substrate) was insufficiently sensitive to detect other isoenzymes known to be present in the unfractionated extracts of other

Figure 8 Western blotting of mammary tissue and mammary PK-C V

Authentic α and β isoenzymes from rat brain were probed with their respective antibodies, yielding a major specific signal at an M_r of around 80000. Calibration markers are $M_r (\times 10^{-3})$. A major signal also around M_r 80000 was detected by anti- α isoenzyme antiserum in brain and mid-lactating mammary tissue (L10) extracts, but in extracts from mid-pregnant mammary tissue (P8), a doublet signal was found. Abbreviation: P.A., peptide antigen.

tissues, including rat brain. Applying the same Western-blot analysis to authentic isoenzymes (Figure 8), individually resolved from rat brain, spleen and COS-7 cells, allowed the detection of α , β_1 and β_{11} isoenzymes so long as not less than 25 μ -units of activity (before disaggregation in SDS-containing buffer) was loaded on the gel per cm (width) of sample track. The inability of γ -isoenzyme-specific anti-peptide antiserum to detect the γ isoenzyme remains unexplained at present. Brain β isoenzyme fraction reacted with both anti- β_1 and anti- β_{11} serum, whereas the spleen β isoenzyme was recognized only by the anti- β_1 serum. The specificity of positive reactions was confirmed in all cases by competition with the antigen peptide. The M_r of the Western-blot signals elicited from β isoenzymes was 80000. The α isoenzyme from brain, spleen and COS-7 cells was recognized by anti- α serum, the positive signal in each case migrating with an M_r of 80000. It is noteworthy that this M_r is substantially smaller than that of the autophosphorylated α isoenzyme (90000); see Figure 7. Evidently, phosphorylation induced a slowing of the migration of the PK-C α protomer through SDS gels, a property not characteristic in this study of the β protomers. Such behaviour has been previously reported for γ and β isoenzymes (Huang et al., 1986b) and for the α isoenzyme (Mitchell et al., 1989; Pears et al., 1992), although Parker and colleagues claim that transphosphorylation as well as autophosphorylation is involved in the case of the α isoenzyme. Among the resolved mammary isoenzymes, after extensive concentration before SDS/PAGE, V was recognized by anti- α serum, giving a signal at M_r 80000 (results not shown). In contrast, when probing whole mammary tissue extracts, the M_r of the major positive Western-blot signal from pregnant rats was 86000, substantially greater than that from the authentic α isoenzymes from other cell/tissue types (Figure 8), which only a minor signal at 80000, whereas from lactating animals this latter signal predominated. These results suggest that mammary PK-C α is more highly phosphorylated in pregnant than in lactating animals. Corroboration for this proposal was sought by incubating Triton X-100-treated extracts of mammary tissue from 8-day-pregnant rats with phosphatases, then assessing PK-C α mobility in SDS gels by Western blotting. ECL-development was used in these preliminary experiments to enhance detection sensitivity. Treatment with potato acid phosphatase (2 units/ml; 4 mg of extract protein/ml) for 30 min at 30 °C quantitatively converted PK-C α from the slow-migrating into the normal form: no conversion occurred in

control experiments lacking the phosphatase (results not shown). These differences in phosphorylation status and consequent mobility in SDS gels were lost, presumably owing to dephosphorylation, on extensive handling of the resolved isoenzymes.

DISCUSSION

Mammary tissue PK-C from lactating rats can be resolved into five isoenzyme fractions (I–V). Isoenzymes I–III are more abundant during pregnancy than during lactation. Isoenzyme IV may be absent during pregnancy.

The evidence presented suggests that mammary PK-C II may be ζ , and PK-C III may be β_1 ; these identifications are supported by Western blotting with appropriate antibodies using the more sensitive ECL detection system (Figure 9). Although mammary PK-C isoenzymes I and IV share some properties with authentic γ and β_{II} respectively, these identities could not be confirmed by Western blotting (see Figure 9). The M_r of the subunit cross-reacting with PK-C ζ -specific antibodies was significantly greater than the value of 58000 found for one of the major autophosphorylatable subunits in mammary isoenzyme II preparations. Thus, although mammary isoenzyme II contains PK-C ζ , it cannot be ruled out that other PK-C isoenzymes are also present.

By several independent criteria, isoenzyme V is PK-C α . However, it differs from the α isoenzymes derived from brain, spleen and COS-7 cells in several respects: (i) its sensitivity to Ca^{2+} shows plasticity, decreasing between pregnancy and lactation; (ii) even at its most sensitive, its EC_{50} for Ca^{2+} remains substantially above that of the control α isoenzyme preparations; (iii) its subunit in extracts from mammary tissue of pregnant rats, as detected by Western blotting, migrates more slowly in SDS gels than control α isoenzyme preparations. Covalent attachment of phosphate during autophosphorylation causes an analogous slowing of migration of control α isoenzymes in SDS gels. A constitutively higher level of phosphorylation may explain the slower migration of the mammary enzyme from pregnant animals; this could result from enhanced autophosphorylation or transphosphorylation activity (Pears et al., 1992). It has been reported that autophosphorylation of rat brain PK-C leads to

an increase in its sensitivity to Ca^{2+} (Huang et al., 1986a). Although the present findings have established a link between phosphorylation status and mobility on SDS/PAGE, further experimentation will be required to reveal whether there is a connection between the phosphorylation status of mammary PK-C α and changes in its sensitivity to Ca^{2+} . In this context, it is interesting that changes in the phosphorylation status of PK-C α in MDA-MB-231 human breast cancer cells have been correlated with inhibition of their proliferation by treatment with tumour-promoting phorbol esters (Borner et al., 1989). The diminution in the Ca^{2+} -sensitivity of mammary PK-C α around parturition may reflect an adaptation to the altered Ca^{2+} economy characteristic of mammary secretory cells during lactation. The unchanged tissue content of this isoenzyme throughout pregnancy and lactation suggests that it serves cellular 'housekeeping' functions, unrelated specifically to proliferation or secretion. Conversely, the behaviour of isoenzymes I, II and III implicates them in cell proliferation and growth, whereas that of isoenzyme IV suggests an involvement in secretion.

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Figure 9 Western blotting of the minor mammary PK-C isoenzymes I–IV

Resolved mammary isoenzymes were concentrated by centrifugal ultrafiltration, by using M_r 10000 cut-off membranes, before electrophoresis and Western blotting with isoenzyme-specific antibodies as shown. Negative controls for anti-peptide antibodies were conducted in the presence of excess peptide antigen (P.A.) where indicated. As positive and negative controls for the mouse monoclonal antibody 36G9, brain extract was electrophoresed beside PK-C isoenzyme I and probed with MC5 (which recognizes both α and β isoenzymes) and non-immune (n.i.) IgG respectively. The following activities of PK-C isoenzymes (μ -units) were loaded per electrophoretic track (20 mm wide): I, 170; II, 195; III, 340; IV, 52. Immunoprobe were applied to four separate lanes in each track. Calibration markers are $M_r (\times 10^{-3})$. Abbreviation: Abs, antibodies.

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