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Original Paper

Elevation of Protein Kinase A and Protein Kinase C Activities in Malignant as Compared with Normal Human Breast Tissue

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Because of their central role in the transduction of extracellular signals, protein kinases A (PKA) and C (PKC) are critical enzymes in the control of cellular proliferation and differentiation. We have measured the catalytic activity of PKA and PKC, as well as the regulatory subunit expression for PKA, in paired samples of normal and malignant breast tissue from 13 patients with breast cancer. Paired non-parametric (Wilcoxon) analysis revealed significantly higher values for both basal ($P = 0.0002$) and total ($P = 0.0002$) PKA catalytic activity in malignant compared with normal breast in all 13 paired tissue samples. Expression of both R_I- and R_{II}-PKA regulatory subunits were also higher in malignant tissue from 12 ($P = 0.0005$) and 9 ($P = 0.01$) of the 13 pairs, respectively. However, the degree of R_I-subunit overexpression in malignant tissue was greater than that of the R_{II}-subunit, as demonstrated by an increase in the R_I/R_{II} subunit ratio in 10 of the 13 paired samples ($P = 0.017$). Total PKC catalytic activity was elevated in 11 of the 13 malignant tissue specimens when compared with corresponding normal breast tissue ($P = 0.01$). This was accounted for by an increase in Ca^{2+}-dependent PKC activity ($P = 0.01$), there being no significant increase in Ca^{2+}-independent PKC activity. These data suggest that the activities of both PKA and PKC signalling pathways are intrinsically higher in malignant compared with normal breast tissue and these may therefore represent targets for interventive treatment of breast cancer. Copyright © 1996 Elsevier Science Ltd

Key words: protein kinase A, protein kinase C, normal breast, breast cancer

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INTRODUCTION

MALIGNANT TRANSFORMATION of normal cells is characterised by dysregulation of cellular proliferation and differentiation, which in turn has been associated with aberrations in cell signalling systems [1, 2]. PKA and PKC are critical intracellular components of two second messenger systems activated by the binding of extracellular ligands to cell-surface receptors [3, 4]. Evidence is accumulating of their involvement in the natural history of cancer, although concurrent levels of these kinases have not previously been studied in tissue specimens. For example, high tumour levels of cAMP binding proteins in women with breast cancer may correlate with a poor prognosis [5] and the proportions of R_I-subunit may vary between different tumours [6]. However, most of the currently published data relates to cAMP binding in breast cancer. A direct comparison of PKA kinase activity (or, indeed, PKA R-subunit expression) between normal and malignant tissue collected simultaneously from individual women's breasts has not been undertaken. Whilst a single report has also shown total PKC activity may be elevated in malignant breast relative to normal tissue from the same patient [7], this did not distinguish between Ca^{2+}-dependent and Ca^{2+}-independent components of PKC activity, although overexpression of the Ca^{2+}-dependent PKC-α isoform in a breast cancer cell line has recently been reported to induce an aggressive phenotype [8].

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Table 1. Clinical characteristics of patients

Patient no.	Age	Tumour histology*	Tumour grade	Oestrogen receptor status†
1	58	Invasive carcinoma NST	3	+ ve
2	60	Invasive carcinoma of mucoid type	1	+ ve
3	61	Invasive carcinoma NST	3	− ve
4	37	Invasive ductal carcinoma NST with DCIS	1	+ ve
5	38	Invasive ductal carcinoma NST with DCIS	2	− ve
6	50	Invasive carcinoma NST	3	− ve
7	48	Invasive carcinoma NST	2	− ve
8	71	Invasive carcinoma NST	2	+ ve
9	73	Invasive lobular carcinoma of classical type	2	+ ve
10	51	Invasive carcinoma NST	2	+ ve
11	33	Invasive carcinoma NST	3	− ve
12	52	Extensive DCIS and foci of invasive carcinoma NST	2	+ ve
13	33	Invasive and non-invasive carcinoma NST	3	+ ve

*Tumour grade histology routinely assessed postoperatively. NST, no special type; DCIS, ductal carcinoma *in situ*. †A level of 20 fmol receptor/mg protein or greater was considered positive for oestrogen receptors.

The aim of the work presented in this paper was to compare components of PKA and PKC systems from paired samples of normal and malignant tissue from the breasts of patients with breast cancer.

MATERIALS AND METHODS

Chemicals

Reagents were obtained from Sigma (Poole, Dorset, U.K.) unless otherwise stated; malantide (PKA peptide substrate) was obtained from Ocean Biologics, Inc. (Edmonds, WA, U.S.A.). Peptide inhibitor of PKA (PKI) [9], GS peptide (i.e. [$Ala^{9,10}$, $Lys^{11,12}$] GS^{1-12}, modified from residues 1–12 of glycogen synthase) and PsI (i.e. pseudosubstrate inhibitor peptide of PKC (PKC^{19-31})) peptide were kindly synthesised by Dr Jim Beattie (Hannah Research Institute, Ayr, U.K.). Analysis by reversed phase HPLC demonstrated that they were all $\geqslant 90\%$ pure and the authenticity of the major component was confirmed by mass spectrometry.

Patient material

Breast tissues were collected from patients undergoing surgery for breast cancer at the Breast Unit, Western General Hospital, Edinburgh, U.K. Cancer material was taken from histologically proven malignant tumours. Normal breast tissue was removed at least 1 cm away from overt cancer, and confirmed to contain parenchymal elements without evidence of malignant transformation. Samples were snap-frozen and stored in liquid nitrogen until used. Tumour oestrogen receptor (OR) status and grading were routinely evaluated as part of the patients' assessment. Patient and tumour details are summarised in Table 1. The study formed part of a research programme for which ethical approval had been obtained.

Tissue sampling and fractionation

All procedures were performed at 4°C. Approximately 200 mg of tissue was homogenised using a standard laboratory Silverson homogeniser at maximum speed for 20 s in 10 vol. buffer A (20 mM Tris–HCl, 1 mM EDTA, 5% glycerol, 250 mM sucrose, 50 mM NaCl, 0.2 mM IBMX (isobutylmethylxanthine), pH 7.5, containing 15 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulphonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin and 1 μg/ml antipain). Further processing was by a modification of methods previously described [10]. Briefly, homogenates were centrifuged at 6000*g* for 10 min. The supernatant was retained and divided into two parts. One aliquot was centrifuged at 100 000*g* in a Beckman TL-100 benchtop ultracentrifuge for 15 min and the cytosolic supernatant was used for PKA C- and R-subunit determinations; the other aliquot was stirred with 1% v/v Triton X-100 for 1 h and subsequently centrifuged at 100 000*g* for 1 h. Of the resultant clear supernatant, 240 μl was loaded on to a 0.3 ml DEAE cellulose column equilibrated with buffer B (20 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA (ethyleneglycolbis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid), 50 mM 2-mercaptoethanol, pH 7.5). Columns were washed with 2.5 ml buffer B; PKC was then eluted by a single step of salt concentration using buffer C (20 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 400 mM NaCl, 50 mM 2-mercaptoethanol, pH 7.5) and immediately assayed for PKC activity.

Protein content of respective PKA and PKC containing fractions was determined by the Bradford method [11] using reagents supplied by Bio-Rad (Hemel Hempstead, U.K.). Paired samples of normal and malignant breast tissue were simultaneously processed and assayed.

PKA C-subunit activity

The activity of PKA C-subunit (the catalytic subunit of PKA) was measured by incorporation of the radiolabel from [γ-^{32}P]ATP (Amersham, Little Chalfont, Bucks, U.K.) into the synthetic PKA substrate, malantide, during a 5 min assay at 30°C as described previously [12]. The final assay mixture (total volume 80 μl) contained 50 mM sodium phosphate, 10 mM $MgCl_2$, 262 μM [γ-^{32}P]ATP, 0.01% Tween 20, 20 μM malantide, 0.2 mM IBMX and approximately 5 μg cytosolic protein. The reaction was initiated by addition of [γ-^{32}P]ATP and terminated with 1 M HCl (10 μl) before spotting an aliquot (30 μl) onto Whatman P81 phosphocellulose papers (Whatman, Maidstone, Kent, U.K.). These were then washed free of non-bound radioactivity in wash buffer (0.05% w/v polyphosphoric acid, 25 mM H_3PO_4) before Cerenkov counting. Fractions were assayed (1) in the presence of 20 μM cAMP, (2) in the pre-

sence of 1.25 μg/ml PKI peptide, and (3) in the absence of both these agents. This enabled calculation of basal ((3) minus (2)) and total ((1) minus (2)) PKA activities. Mean values of triplicate determinations (which varied by <10%) are shown. One unit of PKA (or PKC) catalytic activity represents that which catalyses the transfer of 1 μmol of phosphate/min to acceptor substrate.

PKA R-subunit determination

The expression of PKA R-subunits was determined by photoaffinity labelling with [^{32}P]-8-azido-cAMP (ICN Flow, Thame, Oxon, U.K.) as previously described [6, 13]. Briefly, cytosolic fractions, standardised for protein content (1.5 mg/ml), were incubated for 1 h in the dark at room temperature in 96-well plates. The assay mixture (total volume 80 μl) contained 0.6 μM [^{32}P]8-azido-cAMP, 50 mM sodium morpholine ethane sulphonic acid and 10 mM $MgCl_2$, pH 6.2. Assay mixtures were then irradiated for 60 s with 254 nm UV light held directly over the plate, using a Mineralight UVS-II hand held lamp (Camlab, Cambridge, U.K.) and the reaction stopped by disaggregation in SDS-containing buffer. Samples were then analysed by SDS–PAGE [14] on 12.5% gels followed by autoradiography at −80°C. Paired samples were co-electrophoresed and co-autoradiographed. R_I and R_{II} subunit expression were measured by computer-assisted densitometry as a percentage of the total gel scan for each track. For comparison of relative expression between paired normal and malignant samples, values were then expressed relative to the total scan value of the normal tissue sample of each pair.

Protein kinase C activity

PKC activity was measured by incorporation of the radiolabel from [γ-^{32}P]ATP into GS peptide for 10 min at 30°C, essentially as described [10]. The use of GS peptide gave significantly greater rate values than, for instance, Histone III-S; however, it should be noted that the specific activity of Ca^{2+}-dependent isoforms in the phosphorylation of GS peptide are greater than those of Ca^{2+}-independent isoforms. Furthermore, it has been reported that PKC-ε does not catalyse the phosphorylation of GS peptide [15]. PKC activity was measured in (1) the presence of added Ca^{2+}; (2) the absence of Ca^{2+}; (3) the presence of added Ca^{2+} and the presence of PsI; and (4) the absence of Ca^{2+} and the presence of PsI. L-α-Phosphatidylserine (PS) was present in all assay conditions, and where no Ca^{2+} was added, EGTA was added in its place. This enabled values for "Total" (assay condition (1) minus assay condition (3)), "Ca^{2+}-independent" (assay condition (2) minus assay condition (4)) and "Ca^{2+}-dependent" (Total minus Ca^{2+}-independent) PKC activities to be obtained. There was no difference in values obtained for assay conditions (3) and (4) indicating that there was no non-PKC-inhibited Ca^{2+}-dependent kinase activity. The final assay mixture (60 μl, pH 7.0) contained 13.3 mM 3-[N-morpholino] propanesulphonic acid/KOH, 10.7 mM magnesium acetate, 66.7 μM [γ-^{32}P]ATP, 6.7 mM dithiothreitol, 50 μg/ml PS, 200 μg/ml of GS peptide, 0.83 μg/ml PKI peptide inhibitor of PKA, 0.5 mM $CaCl_2$ (or 1 mM EGTA) and approximately 5 μg enzyme fraction (420 μg/ml PsI was present in assay conditions (3) and (4)). The reaction was initiated by addition of [γ-^{32}P]ATP and terminated by spotting an aliquot (30 μl) on to Whatman P81 phosphocellulose papers, which were washed free of non-bound radioactivity in wash buffer (0.05% w/v polyphosphoric acid, 25 mM H_3PO_4) before Cerenkov counting. Mean values of triplicate determinations (which varied by <10%) are shown.

Statistical analysis

Statistical comparison of values for normal and malignant samples was by a paired Wilcoxon non-parametric test.

RESULTS

Paired samples of PKA C-subunit activity for each of 13 normal and malignant breast tissue samples are shown in Figure 1. Higher levels of both basal and total PKA C-subunit activity were observed in all 13 malignant specimens when compared with paired normal breast tissue. By Wilcoxon paired anaysis, these differences were highly sig-

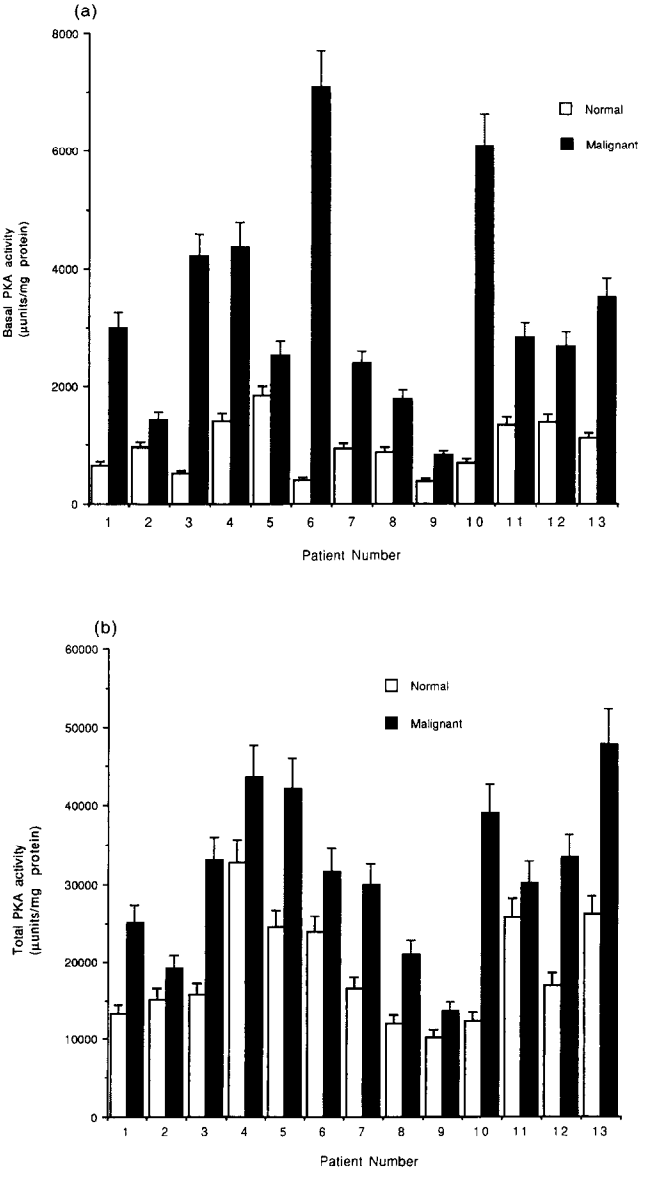

Figure 1. PKA C-subunit activity in normal and malignant breast tissue: μ units/mg protein represents pmol of ^{32}P transferred from [^{32}P]ATP to malentide/min/mg cytosolic protein. (a) Basal activity. (b) Total (cAMP stimulated) activity.

Table 2. Summary of means of PKA and PKC activities for normal and malignant breast tissue

	Normal*	Tumour*
PKA		
C-subunit activity (μunits/mg protein)		
Basal	891 ± 127 (390–1840)	3230 ± 496 (820–7090)
Total	18870 ± 1920 (10250–32680)	31530 ± 2810 (13550–47850)
R-subunit expression (% total scan to normal breast value)		
R_{II}	34.7 ± 1.96 (22.4–50.6)	71.7 ± 13.56 (14.8–176.9)
R_I	31.58 ± 2.51 (15.3–43.1)	122.2 ± 27.52 (36.0–373.5)
R_I/R_{II}	0.97 ± 0.11 (0.40–1.56)	1.74 ± 0.18 (0.93–3.03)
PKC activity (μunits/mg protein)		
Total	441 ± 60 (156–856)	722 ± 114 (173–1803)
Ca^{2+}-independent	188 ± 27 (71–393)	309 ± 87 (86–1298)
Ca^{2+}-dependent	252 ± 37 (84–486)	411 ± 52 (63–685)

*Values shown are mean ± SEM of values for 13 paired normal and malignant breast tissue specimens. The range of values is shown in parentheses.

nificant ($P = 0.0002$ for both comparisons). Interestingly, the patterns of increase in basal and total C-subunit activities were contrasting. Basal PKA C-subunit activity was below 2000 μunits/mg protein for normal breast samples, whereas corresponding malignant tissue values had a larger range, with a maximum value in excess of 7000 μunits/mg protein (see Table 2 for a summary of means and ranges of values). Hence, whilst some of the values obtained for malignant tissue overlapped with those for normal breast, others were clearly abnormally elevated. In contrast, for total PKA activity, there was a generalised elevation of values in malignant compared with normal tissue, with considerable overlap of the ranges. Photoaffinity labelling with [^{32}P]8-azido-cAMP revealed binding proteins migrating with molecular weights of 67, 54, 52, 48, 39 and 37 kDa by SDS–PAGE (see Figure 2). These were present in both normal and malignant breast tissue samples. The aim of this study was to examine the relative expression of the major cAMP binding proteins, rather than characterise these proteins, but others have suggested that the minor cAMP binding protein bands of $M_r = 39\,000$ and $M_r = 37\,000$ may be proteolytic products of R_I- and R_{II}-subunit breakdown [16], and the $M_r = 67\,000$ band may comprise an albumin-like protein [6]. Of the major cAMP binding proteins, the $M_r = 48\,000$ band corresponds to R_I-subunit expression, and the $M_r = 52\,000$ and $M_r = 54\,000$ bands to R_{II}-subunit expression; these bands may represent phosphorylation isoforms of the R_{II}-subunit, or alternatively, $R_{II\alpha}$-dephosphorylated and $R_{II\beta}$-subunits [17]. Interestingly, whilst 52 kDa bands were detected in normal and malignant tissue samples, the 54 kDa band would appear to be present in the majority of normal but not in corresponding malignant tissue specimens. R_I-subunit expression, as measured by scanning densitometry of the $M_r = 48\,000$ band, was higher in 12 of the 13 malignant samples compared with paired normal breast tissue values ($P = 0.0005$, see Figure 2b). R_{II}-subunit expression, as measured by scanning densitometry of $M_r = 52\,000$ and $M_r = 54\,000$ bands, was increased in 9 of the 13 patients' samples ($P = 0.01$, see Figure 2c). However, the relative increase in R_I-expression appeared to be greater than that for R_{II}-subunit expression as reflected by an increase in the R_I/R_{II} subunit ratio in 10 of the 13 paired tissue specimens ($P = 0.017$).

Total PKC activity was elevated in 11 of the 13 malignant samples compared with corresponding normal breast tissue ($P = 0.01$, see Figure 3). Ca^{2+}-independent PKC activity was elevated in 8 of the 13 malignant samples compared with corresponding normal breast samples, but this was not statistically significant ($P = 0.27$). In contrast, Ca^{2+}-dependent PKC activity was significantly elevated ($P = 0.01$, see Figure 3b) being greater in 10 of the 13 malignant specimens versus corresponding normal breast tissue. These results indicate that the increase in total PKC activity was largely due to an elevation in its Ca^{2+}-dependent component.

DISCUSSION

By comparing paired samples of normal and malignant tissue from the breasts of women with breast cancer, it has been possible to show that both the regulatory and catalytic subunits of the PKA system and the Ca^{2+}-dependent component of PKC activity are significantly elevated in malignant samples (data summarised in Table 2), although potential changes in PKC-ε activity would not have been detected by the methods that we have used. Whilst certain aspects of these findings can be inferred from previous studies that have measured some of the components of one of these signalling systems in either normal or malignant breast tissue, to our knowledge the results of this study present the first direct comparison in normal and malignant tissue from individual breasts in which the various components and subtypes of PKA and PKC systems have been measured.

The observed elevations in PKA C-subunit activity are of interest, particularly since these have not been determined before in normal breast tissue samples. Our data indicate that the total pool of PKA C-subunit (representing the potential total kinase activity) as well as the basal activity (representing the kinase activity which is not bound to R-subunits and freely available to catalyse protein phosphorylation, without the requirement for cAMP binding and dissociation from R-subunits for expression of catalytic activity) are increased in malignant samples. Since alterations in endogenous substrate phosphorylation are likely to accompany these differences in basal PKA C-subunit activity, this may suggest an alteration in the tonic regulation of PKA-mediated cellular events in malignant tissue. In the case of total C-subunit measurements, the physiological consequences of increases in this parameter are less evident, since they represent potential catalytic activity, which is realised only upon exposure to pharmacological concentrations of cAMP. Furthermore, although it may be inferred from separate studies [5, 6, 18] that PKA R-subunit expression is elevated in malignant breast tissue, there has been no direct comparison with normal breast. The present

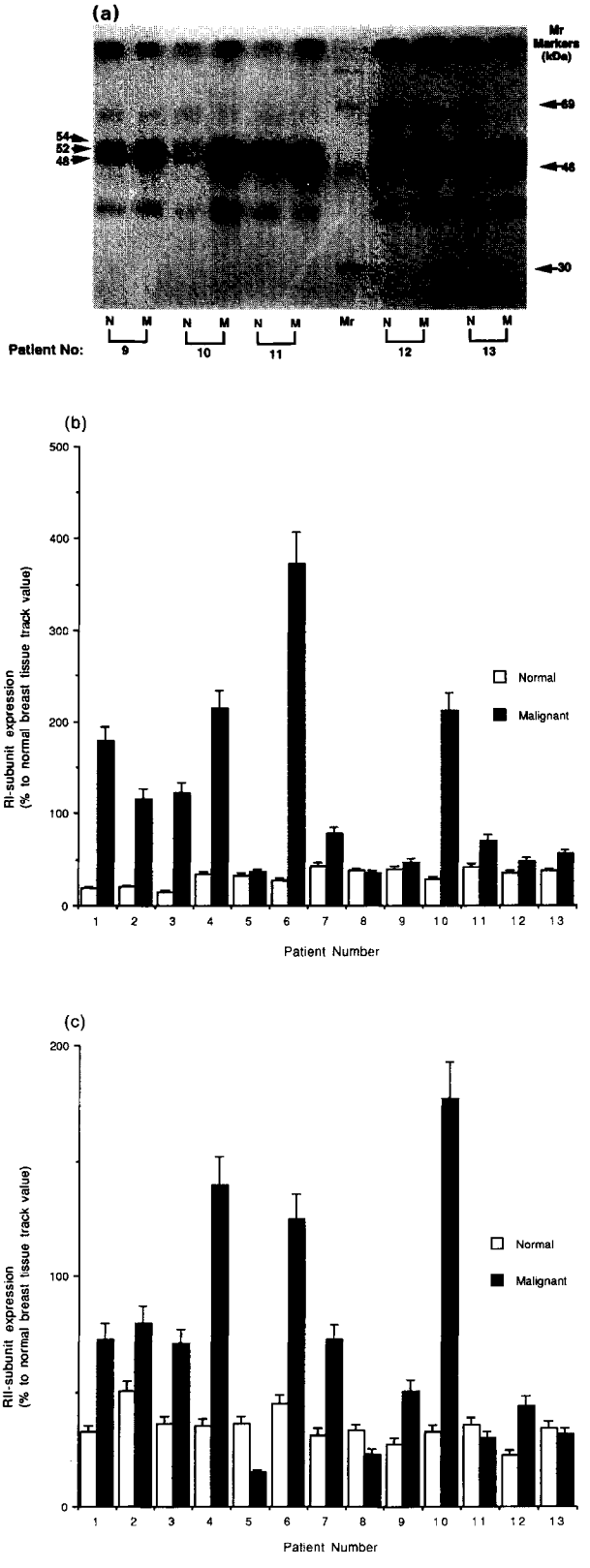

Figure 2a. PKA R-subunit expression in normal and malignant breast tissue. (a) Typical autoradiograph of PKA R-subunit expression, photoaffinity labelled with [^{32}P]8-azido-cAMP. Paired normal (N) and malignant (M) breast tissue samples from patients 9, 10, 11, 12, 13 are shown. Arrows on the left indicate position of cAMP binding proteins, of M_r = 48000 Da (R_I-subunit), M_r = 52000 Da and M_r = 54000 Da (R_{II}-subunit) by comparison with position of ^{14}C-methylated molecular weight markers (Amersham, U.K.), shown on the right. (b) R_I-subunit expression. Graph of densitometric scan of autoradiographs showing percentage of total track scan of R_I-subunit, relative to normal breast scan for each individual patient. (c) R_{II}-subunit expression. Graph of densitometric scan of autoradiographs showing percentage of total track scan of R_{II}-subunit, relative to normal breast scan for each individual patient.

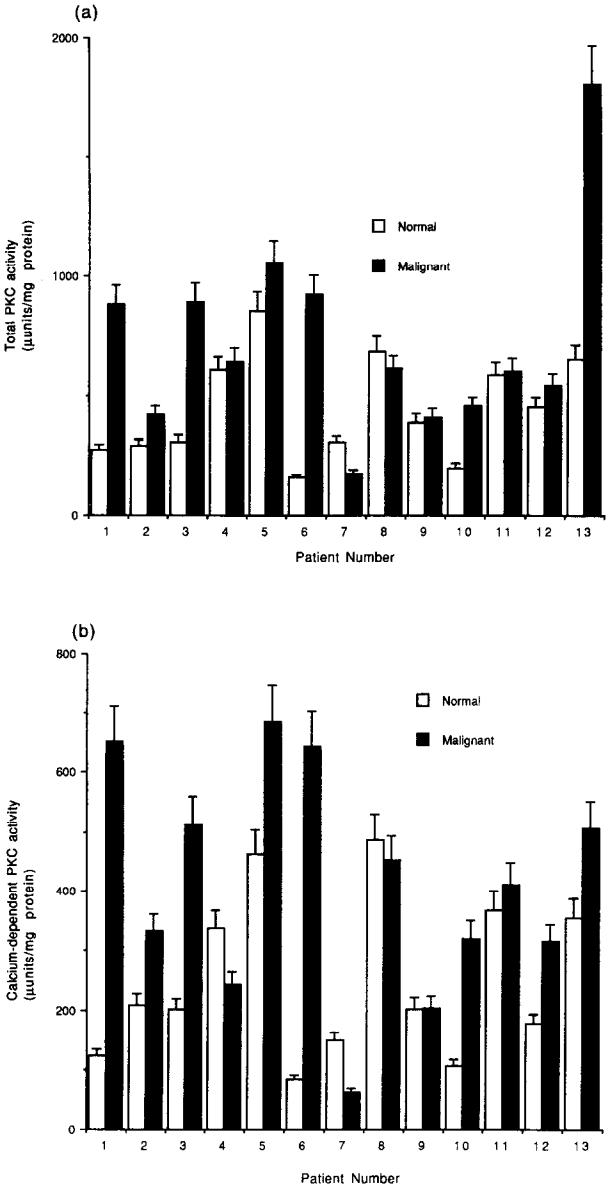

Figure 3. PKC catalytic activity in normal and malignant breast tissue; μunits/mg protein represents pmol of ^{32}P transferred from [^{32}P]ATP to GS peptide/min/mg protein. (a) Total PKC activity. (b) Ca^{2+}-dependent PKC activity.

observations of R-subunit expression indicate that levels of both R_I- and R_{II}-subunits are indeed elevated and that this increase is predominantly associated with the R_I-type. Interestingly, elevated R_I-subunit expression has previously been proposed to accompany increased cellular proliferation, whereas increased R_{II}-subunit expression has been proposed to accompany differentiation (reviewed in [2]). Comparison of the fold increase of R-subunit expression with that of C-subunit activity between paired normal and malignant specimens does not reveal a concomitant increase (data not shown). Others [19] have also observed non-uniformity in PKA parameters, notably R/C subunit ratio, measured in mammary tumours of both control (untreated) and ovariectomised rats. Thus, the *in vivo* consequence(s) of an aberrant R/C ratio are unclear, although it has been proposed that an increased R/C ratio may accompany increased epithelial cell growth of both normal and malignant cells ([19], and see [20] for a theoretical discussion of the effects of altered R/C ratio).

The present results confirm the previous findings of O'Brian and co-workers [7] regarding the increase in PKC activity in malignant breast relative to normal tissue. To our knowledge, it has not previously been shown that this elevation of PKC activity resides in the Ca^{2+}-dependent component of total cellular PKC. Measurement of the levels of individual PKC isoforms by, for example, Western blotting would be informative. However, whilst we are currently developing such assays, the low level of activity in breast tissues and their limited availability have precluded routine analysis, but our preliminary data on a limited set of paired samples would suggest that at least PKC-α is elevated in malignant compared with normal tissue. Similarly, it was not feasible to analyse cytosolic and membrane fractions in order to determine subcellular distribution of individual isoforms due to a lack of tissue and low activity.

It should be noted that, although there are statistically significant differences in levels of both PKA and PKC systems between normal and malignant tissue, identified by paired comparison, the range of values obtained in both groups was large. The factors that influence these values are still to be defined. Indeed, it is possible that there may be inter-dependence (or "cross-talk") between these two systems.

One possible explanation for the rise in levels of both PKA and PKC activities in malignant tissue could be that the elevations are related to a generalised increase in the cellularity of the malignant phenotype. This, however, is unlikely since measurements were equalised for protein content of respective normal and malignant paired samples. Furthermore, others [21, 22], who have also equalised tissue for protein content, have observed decreases in PKC expression in colorectal cancer when compared to normal colorectal mucosal tissue. This suggests that alterations in activity levels are not due to differences in cellularity, and implies a degree of tissue specificity in alterations of PKC expression. Hence, it would appear that transition from a normal to a malignant phenotype is not accompanied by a generalised elevation of PKC.

In this paper, we have described elevations of both PKA and PKC cellular signalling systems in malignant compared with normal breast tissue. In order to elucidate the involvement of these systems in the process of breast carcinogenesis, it would be useful to study premalignant conditions. However, such precursor lesions have not readily been defined within the breast. The present study does not discriminate between the possibilities that elevated levels of PKA and PKC messenger systems in malignant breast tissue are either coincidentally associated with the cancerous phenotype or programme cells for their malignant behaviour. However, there is some evidence in support of the latter. For example, transfection and overexpression of PKC-α in a breast cancer cell line is associated with the acquisition of a more aggressive phenotype [8]. Conversely, the anti-oestrogen, tamoxifen, which has been shown to inhibit PKC *in vitro* [23, 24], has antiproliferative properties against breast cancer cells [25]. Similarly, antiproliferative effects on breast cancer cells may also result from antisense strategies against the R_I-subunit of PKA [26]. It may therefore be that PKA

and PKC signalling cascades represent targets for therapeutic intervention in breast cancer.

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