Epidermal Growth Factor Binding and Protein Kinase C Activities in Human Breast Cancer Cell Lines: Possible Quantitative Relationship

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ABSTRACT

Quantitative polyacrylamide gel electrophoresis analysis of Ca2+, phospholipid-dependent protein kinase (PKC) of human mammary tumor cell lines (MCF-7, ZR-75, T-47-D, MDA-MB-231, BT-20, and HBL-100) revealed that 80% of the total cellular PKC resided in the cytosol. The tumor cells with no detectable levels of estrogen receptors (MDA-MB-231, HBL-100, and BT-20 cells) exhibited significantly larger (P < 0.001) cytosolic PKC activities than those cells that contained estrogen receptors (MCF-7, T-47-D, and ZR-75 cells). In addition, in estrogen receptor-negative cell lines, relatively high levels of specific low-affinity (apparent Kd = 700 pm) epidermal growth factor (EGF) binding activities were found as compared with estrogen receptor-positive cells with significantly (P < 0.01) lower levels of specific high-affinity (apparent Kd = 90 pm) EGF binding. A significant positive correlation (P < 0.01) was observed between the number of EGF receptor (R0 = 0.50) and/or the EGF receptor dissociation constants (R0 = 0.78) with the cytosolic PKC activity levels. These data indicate that, in human breast cancer cells, a positive relationship may exist between PKC activity, estrogen, and EGF receptors.

INTRODUCTION

Tyrosine kinase activity appears to be an intrinsic property of polypeptide growth factor receptors such as for the epidermal growth factor, platelet-derived growth factor, insulin-like growth factor I, and insulin (1—4). The best characterized PGF3 receptor is the binding protein for EGF receptor, which is localized at the plasma membrane. The binding of EGF to an extracellular domain of the EGF receptor activates its cytoplasmic Tyr-PK which phosphorylates various cellular proteins and the EGF receptor itself (autophosphorylation) on tyrosine residue(s) (5, 6). Both the Tyr-PK and EGF-binding activities reside on the EGF receptor which is a single Mr 170,000 polypeptide chain spanning the plasma membrane (7). Both activities can be regulated by tumor promoters such as TPA, which not only reduces EGF receptor binding (8, 9) but also its activities can be regulated by tumor promoters such as TPA, or lacking (MDA-MB-231, HBL-100, and BT-20 cells) estrogen and progesterone receptors. The EGF receptor activity was measured using intact cells, while the determination of cytosolic PKC activity was performed by PAGE, under non-denaturing conditions, which allows a reliable quantitation of even small amounts of PKC activity.

MATERIALS AND METHODS

Chemicals. Tissue culture dishes were purchased from Falcon. Serum and culture medium were obtained from Gibco. Fatty acid-free BSA, lysine-rich histone H1 (calf thymus-type V-S), 1,2-diolein, L-α-phosphatidyl-L-serine, leupeptin, and aprotinin were from Sigma (St. Louis, MO). Spectrograde magnesium nitrate and riboflavine were bought from Merck (Darmstadt, Federal Republic of Germany). DEAE-cellulose (DE-52) was obtained from Whatman (Maidstone, England). γ[32P]ATP (specific activity, 10 Ci/mmol) and 125I-EGF (specific activity, 180 μCi/μg) were bought from New England Nuclear (Boston, MA). Unlabeled mouse maxillary gland EGF was purchased from Collaborative Research (Lexington, MA). Reagents for protein determination were obtained from Bio-Rad (Munich, Federal Republic of Germany). Acrylamide, N,N'-N'-tetramethylethylenediamine, N,N'-methylenebisacrylamide, and PHWP filters (0.45 μm) were bought from Serva (Heidelberg, Federal Republic of Germany) and Millipore (Bedford, MA), respectively. Instagel was from Packard (Warrenville, IL), and the RBY-dye was from Gelman (Ann Arbor, MI).

Cell Culture Conditions. The mammmary carcinoma cell lines MCF-7, T-47-D, ZR-75-1, MDA-MB-231, HBL-100, and BT-20 were obtained from the Mason Research Institute (Rockville, MD). The estrogen- and progesterone-receptor concentrations measured in our laboratory agreed well with earlier reports (26, 27). The 6 cell lines were routinely grown on plastic culture dishes at 37°C in IMEM-ZO (28), supplemented with 1-glutamine (2 mM), insulin (1 μg/ml), minocyclin (3 μg/ml), gentamycin (0.4 μg/ml), tylocin (45 μg/ml), 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.3 (10 mM), and 5% (v/v) fetal calf serum (Amimed, Basel, Switzerland). The cells were maintained at

Received 5/14/85; revised 9/12/85, 1/31/86; accepted 2/11/86.

1 This work was supported in part by the Swiss Cancer League, Grant FOR-249-A.K.83(6), and by the Swiss National Science Foundation, Grant 3.409.83, as well as by CIBA-GEIGY Ltd., Basel, Switzerland.
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3 The abbreviations used are: PGF, polypeptide growth factor; EGF, epidermal growth factor; Tyr-PK, tyrosine kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, Ca2+, phospholipid-dependent protein kinase; TGF, transforming growth factor; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; IMEM-ZO, improved minimal essential medium zinc option; EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TCA, trichloroacetic acid; HBSS, Hanks’ balanced salt solution; cAMP, cyclic AMP; R0, significant positive correlation.
PROTEIN KINASE C AND EGF RECEPTOR OF HUMAN MAMMARY TUMOR CELL LINES

37°C in a humidified atmosphere of 95% air with 5% CO2 and harvested during the last period of exponential growth. Culture media were routinely changed every 3 days and 24 h before each experiment. All cell lines were periodically screened for Mycoplasma.

Preparation of Cell Extracts. Cell extracts were prepared from confluent cells (150-mm cell culture dish). The grown medium was discarded, and the cell monolayer was rapidly washed 3 times with 10 ml of ice-cold wash buffer [20 mM Tris-HCl-150 mM NaCl-5 mM glucose, leupeptin (20 μg/ml)-aprotinin (2 μg/ml), pH 7.4]. All subsequent steps were carried out at 4°C. The wash buffer was decanted, and attached cells were scraped into a plastic tube and resuspended in homogenization buffer containing 1% Nonidet P40 (w/v) at a concentration of 3 mg of protein per ml, sonicated for 10 s as described, and centrifuged at 100,000 × g for 1 h, yielding a crude soluble membrane extract (MIC-E) and a residual pellet which was discarded. The cytosol and MIC-E were subsequently assayed for PKC activity.

DEAE-Cellulose Chromatography. About 2–5 mg of cytosol protein were applied onto a column (2 x 4 cm) of DEAE-cellulose, equilibrated with 10 mM Tris-HCl (pH 7.4)-10 mM β-mercaptoethanol, and washed with 50 ml of the above buffer. Elution was carried out with a linear gradient (0–400 mM NaCl) collecting 1-mI fractions at a flow rate of 20 ml/h.

PKC Assay. Protein kinase C was assayed by measuring 32P incorporation from γ-[32P]ATP into lysine-rich histone (histone H1) essentially as described by Takai et al. (14). The assay was carried out in a total volume of 250 μl containing 50 μg of histone H1, 20 mM Tris-HCl, 10 mM Mg(NO3)2, 100 μg of L-a-phosphatidyl-L-serine, 10 μg of diolein, 24 μM [γ-32P]ATP (0.5 μCi), and 300 μM CaCl2, pH 7.4. Aliquots of phosphatidylserine (10 mg/ml) and diolein (10 mg/ml) were dissolved in chloroform and evaporated under nitrogen. The residues were resuspended in ice-cold 20 mM Tris-HCl, pH 7.4, and sonicated for 5 min. Phospholipids and enzyme were mixed in 200-μl volume, and incubations were carried out for 3–15 min at 32°C. The reaction was stopped with 2 ml of ice-cold 20% TCA. The precipitate was collected on Millipore filters which were washed 5 times with 2 ml of ice-cold 20% TCA, dissolved in 8 ml of Instagel, and counted for radioactivity. Basal PKC activity was obtained with 0.5 mM EGTA in the absence of CaCl2, phosphatidylserine, and diolein. Endogenous phosphorylating activity was determined in the absence of histone H1 and served as blank. The PKC assay was performed under conditions in which the reaction velocity was constant with respect to time and proportional to the enzyme concentration. One unit of PKC activity is defined as the amount of enzyme transferring 1 pmol of γ-[32P]ATP to histone H1 during 1 min under assay conditions.

EGF Receptor. The same cell passages as for the PKC determination were used to investigate EGF receptor levels. EGF-binding studies were carried out on intact mammary tumor cells grown in 16-mm multiddishes (24 wells). The cell monolayer grown to a density of 1-5 × 10^6 cells per well was washed twice with 1 ml of HBSS containing 1 mg of BSA. In order to determine total binding, the cells were incubated for 3 h at 25°C with 1.0 ml of HBSS-BSA supplemented with 0.2 ng of [125I]-EGF per ml and 0.4–30 ng of unlabeled EGF per ml. Nonspecific binding was estimated in the presence of a 100-fold excess of unlabelled EGF (40–3000 ng/ml). Kinetic studies showed that equilibrium of EGF binding was reached within 3 h. After the incubation medium was removed, the cells were washed 3 times with 1 ml of ice-cold HBSS-BSA and dissolved in 0.1 N NaOH containing 0.5% Triton X-100 (w/v), and radioactivity was determined (60% efficiency). Cells in parallel wells were trypsinized to determine the cell number. All determinations were carried out in triplicate.

Analytical PAGE. Polya crylamide gels were prepared 2% cross-linked with N,N'-methylene bisacrylamide at a total monomer concentration of 10% (resolving gel). Stacking (upper) gels were formed at 3.5% total gel concentration and cross-linked with 20% N,N'-methylene bisacrylamide. Polymerization and electrophoresis were performed as described by Chrambach et al. (29). Multiphasic zone electrophoresis was carried out with Buffer System B (30). Triton X-100 (0.2%) was included in polyacrylamide gels, and cathode buffer, the moving front, was marked with RBY-dye. After electrophoresis, gels were quickly frozen and sliced into 1-mm sections. Protein kinase C was eluted from the slices overnight with 300 μl of 20 mM Tris-HCl-50 mM β-mercaptoethanol-0.1 mM EGTA-50 mM NaCl, pH 7.4. The eluates were subsequently assayed for PKC activity, which was quantitated by the integration of the specific Ca2+-sensitive and phospholipid-dependent protein kinase activity peak resolved by PAGE.

RESULTS

Quantiﬁcation of Cytosolic PKC Activity of Mammary Tumor Cells by PAGE: Comparison to DEAE-Cellulose Chromatography. PKC activity was difﬁcult to assess in mammary tumor cell cytosols since the enzyme was stimulated only 20–50% in the presence of Ca2+, phosphatidylserine, and diolein. However, DEAE-cellulose chromatography or PAGE of cytosols resulted in a 10–30-fold stimulation of protein kinase activity by Ca2+ and phospholipids (data not shown). It should be mentioned that the diﬃculties in measuring PKC of crude cellular extracts might be due to the interference of other protein kinases present, such as cAMP-dependent and Ca2+- and calmodulin-dependent protein kinases as well as the presence of small quantities of phospholipids. The concentrations of cytosolic PKC activity, analyzed by PAGE of mammary tumor cells, were comparable to PKC levels resolved by DEAE-cellulose chromatography (Fig. 1). The PKC activity was eluted from the DEAE-cellulose column with 100 mM NaCl as shown in Fig. 1B. As previously reported, cAMP-dependent protein kinase type 1 coeluted with PKC from the DEAE-cellulose column (32). In the cell lines investigated, this enzyme can be found in different amounts and may interfere with the PKC assay. In contrast, PAGE analysis of mammary tumor cytosols exhibited a single peak of phospholipid-sensitive Ca2+-dependent protein kinase activity (Fig. 1A) without any cAMP-dependent protein kinase (data not shown). By applying this method, PKC activity could accurately be detected in less than 30 μg of cytosol protein. The relationship between PKC activity and the amount of protein was linear within a range of 30–1000 μg of cytosol protein, enabling the estimation of PKC of rather low specific activity (about 30 units/mg of protein). In order to obtain maximal recovery of PKC activity, 0.2% Triton X-100 (w/v) was included in the stacking and resolving gels as well as in the cathode buffer. Such conditions allowed an 80–90% recovery of PKC activity as estimated using puriﬁed PKC.

Subcellular Distribution and Quantiﬁcation of PKC Activity. Subcellular fractionation yielded 70–90% PKC activity in the cytosol and only 10–30% in the corresponding membrane fractions (Table 1). The extraction conditions for the crude membranes have been shown to ensure maximal solubilization of PKC activity (33). Since a similar subcellular distribution of PKC was observed in all investigated cell lines, the cytosolic...
Table 1 Subcellular distribution of PKC activity in human mammary tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cytosol (1 ml)</th>
<th>MIC-E (0.5 ml)</th>
<th>Cytosol % of distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>2123 ± 190</td>
<td>260 ± 120</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>ZR-75</td>
<td>292 ± 15</td>
<td>92 ± 18</td>
<td>77 ± 5</td>
</tr>
<tr>
<td>T-47-D</td>
<td>155 ± 30</td>
<td>65 ± 25</td>
<td>72 ± 11</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>4155 ± 452</td>
<td>278 ± 55</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>HBL-100</td>
<td>1140 ± 156</td>
<td>313 ± 54</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>BT-20</td>
<td>767 ± 159</td>
<td>113 ± 5</td>
<td>76 ± 2</td>
</tr>
</tbody>
</table>

Mean ± SD of 3 different experiments carried out in triplicate.

PKC activity was taken as a measure for total cellular PKC activity (Table 1). The MDA-MB-231 cells had the highest PKC activity as illustrated in Fig. 2. Furthermore, the estrogen receptor-negative cells (MDA-MB-231, BT-20, and HBL-100) displayed approximately 5-fold higher PKC activity (P < 0.001) as compared with the estrogen receptor-positive cell lines (MCF-7, T-47-D, and ZR-75-1), although BT-20 and MCF-7 cells exhibited similar PKC levels.

125I-EGF Binding. Time courses of 125I-EGF binding demonstrated that equilibrium was reached after 3 h at 25°C. The internalization and degradation of EGF bound to the cells were negligible under these conditions (34, 35). Scatchard plots of 125I-EGF binding were linear in all cell lines and consistent with a single class of noncooperative binding sites (Fig. 3). In contrast to other reports our binding data were reasonably well-fitted by a one-site binding model (36) resulting in linear Scatchard plots. The apparent KdS ranged from 10–1000 μM (Table 2), and maximum-binding capacities were from 3–500 x 10^6 molecules of EGF bound per cell (Fig. 4). A significant difference (P < 0.001) is evident with respect to 125I-EGF-binding capacities and the presence of estrogen and progesterone receptors in the respective cell lines (Table 2). The estrogen receptor-lacking cell lines exhibited a 50-fold higher binding capacity than the estrogen receptor-positive breast cancer cells (Fig. 4). In contrast, the estrogen receptor-negative mammary tumor cells revealed a significant (P < 0.001) higher Kd as compared with the estrogen receptor-positive cells (Table 2). In conclusion, breast tumor cells (BT-20, MDA-MB-231, HBL-100) without estrogen and progesterone receptors bound approximately 50-fold more EGF than the estrogen receptor-positive cells (MCF-7, T-47-D, ZR-75-1), and their EGF recep-
Table 2  Dissociation constants ($K_d$) of EGF receptor, estrogen receptor, and progesterone receptor levels in human breast tumor cells

The binding assays were performed as described in "Materials and Methods." Dissociation constants ($K_d$) were determined by Scatchard analysis. Estrogen receptor and progesterone receptor concentrations were measured as reported previously (35).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$K_d$ (pM)</th>
<th>Estrogen receptor (fmol/10^9 cells)</th>
<th>Progesterone receptor (fmol/10^9 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>965 ± 207</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HBL-100</td>
<td>295 ± 102</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BT-20</td>
<td>878 ± 361</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ZR-75</td>
<td>73 ± 42</td>
<td>25</td>
<td>45</td>
</tr>
<tr>
<td>MCF-7</td>
<td>190 ± 67</td>
<td>45</td>
<td>75</td>
</tr>
<tr>
<td>T-47-D</td>
<td>28 ± 13</td>
<td>19</td>
<td>623</td>
</tr>
</tbody>
</table>

*Mean ± SD of 6 determinations.

Fig. 5. Correlation between cytosolic PKC activity and apparent $K_d$ of $^{125}$I-EGF binding to its receptor (EGF receptor) in mammary tumor cell lines. Parallel cell cultures were assayed for PKC activities, and $K_d$ of EGF binding by Scatchard plot were analyzed as described in "Materials and Methods." Results represent 6 independent determinations for each cell line: ■, T-47-D; ●, ZR-75; ●, MCF-7; ○, BT-20; ◊, HBL-100; and †, MDA-MB-231. Line of best fit (---).

Fig. 3. Analysis of EGF binding in MDA-MB-231 and MCF-7 cells. Cultures of MDA-MB-231 and MCF-7 cells were grown to a density of 0.5 x 10⁶ cells/well, respectively. After the cells were washed with binding buffer, they were incubated with 0.2 g of $^{125}$I-EGF per ml (45 x 10⁶ cpm) mixed with increasing concentrations of unlabeled EGF (0.4—30 g/ml) in 1.0 ml of binding buffer. After a 3-h incubation at room temperature (25°C), the wells were washed, and the cells were lysed as described. Inset, Scatchard plot analysis of the binding data.

Fig. 4. $^{125}$I-EGF binding of intact mammary tumor cells. Binding assay, Scatchard plot analysis, and maximal binding capacities of $^{125}$I-EGF of hormone-dependent (■) and hormone-independent (□) cells were performed as described in "Materials and Methods." Columns, mean; bars, SD from 6 independent measurements.

Controversial results have been reported concerning the mitogenic action of EGF in human breast tumor cells (35, 37, 38), although preliminary data indicate an autocrine regulation of hormone-dependent growth mediated by an estrogen-promoted secretion of α-TGF (22, 23). It appears that EGF does not enhance growth of hormone-independent, estrogen receptor-negative breast cancer cells (37, 38), whereas the mitogenic action of EGF in hormone-dependent (i.e., estrogen receptor-positive) cells seems to depend on experimental conditions (35, 37, 38). A large number of EGF receptors is apparently not
mandatory for the growth response by EGF as reported for the human epidermoid carcinoma cell line A431, because concentrations of about 1 nm EGF prevented cellular proliferation (39, 40). This growth inhibition of A431 cells correlates with the large number of EGF receptors per cell (1—3 × 10^6) (41), since selected A431 variants which are not inhibited by nanomolar EGF doses (42) contained lower numbers of EGF receptors. However, stimulation of growth of A431 cells can be achieved by picomolar concentrations of EGF if unoccupied EGF receptor sites are simultaneously blocked by monoclonal antibodies which specifically inhibit the EGF binding to A431 cells (43). These findings have been explained by the presence of high- and low-affinity EGF-binding sites on A431 cells (12).

In our cell lines investigated, only one EGF receptor population was found using the isotopic dilution assay (34). High-affinity EGF receptor with apparent \( K_d \)s between 20 and 200 pm were only present in the hormone-dependent cell lines (T-47-D, MCF-7, and ZR-75-1) as compared with low-affinity \( K_d \)s (300—1000 pm) of hormone-independent cell lines (HBL-100, BT-20, and MDA-MB-231). Therefore one may speculate about the presence of low- and high-affinity receptor populations distributed on 2 different types of human mammary tumor cells (i.e., estrogen receptor positive or negative) being reminiscent of the 2 receptor classes detected on A431 cells (12). The significantly decreased \( (P < 0.001) \) number of EGF receptors observed on estrogen-receptor-positive breast cancer cell reconfirms reports on an inverse relationship between estrogen receptor levels and EGF receptor-binding activities in human mammary biopsies (44, 45).

Furthermore, a correlation \((R = 0.78)\) between cytosolic PKC activities and EGF receptor affinities (Fig. 4) and a weaker association between PKC and the number of EGF receptors \((R = 0.50)\) were found in the 6 human mammary carcinoma lines. The large number of EGF receptors on BT-20 cells biases the statistical analysis of the data, resulting in a less significant correlation \((R < 0.01)\) between EGF receptor and PKC activity.

The reason for the high EGF receptor number in this particular cell line (BT-20) might rely, like in the A431 cells, on the amplification or the overexpression of the EGF receptor gene, although more experimental work is required to support such a hypothesis.

With respect to hormone-dependent growth of mammary tumors, these results might be very valuable, since protein kinase C is known to down-regulate the EGF receptor as well as its intrinsic tyrosine kinase (10—12). Protein kinase C is predominantly localized in the cytosol (Table 2), but it should also be emphasized that TPA and (or) its naturally occurring agonist diacylglycerol are able to "dock" the PKC to the plasma membrane (33), allowing a direct interaction between the PKC and EGF receptor activation. The quantitation of cytosolic PKC activity was considerably improved by PAGE under non-denaturing conditions, as compared with previously applied techniques (33, 46, 47), and enables a reliable quantitative estimation of PKC activity in crude cellular extracts (48).

Although the number \((n = 6)\) of the cell lines investigated does not allow a definite conclusion, this study gives first evidence that, in human breast cancer cells, quantitative relation may exist between PKC activity and EGF receptor. Hormone-dependent, estrogen receptor-positive mammary tumor cells that contain low levels of PKC correlate with low numbers of high-affinity EGF receptors, whereas the hormone-independent, estrogen receptor-negative cells exhibit higher PKC activities and higher levels of low-affinity EGF receptors. Further investigations of PKC and EGF receptors on other established mammary tumor cell lines as well as on hormone-dependent and independent human breast tumor biopsies will eventually validate our findings.
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