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Non-genomic effects of tamoxifen on the activation of membrane-bound guanylate cyclase GC-A

Zi-Jiang Chen, Michael Vetter, Geen-Dong Chang, Shiguo Liu, Yaxian Ding and Chung-Ho Chang

Abstract

Oestrogen is known to exert both genomic and non-genomic effects on target tissues. Unlike the genomic effects, the identity of receptors mediating the non-genomic effects of oestrogen remains controversial. 17β-Estradiol has been shown to activate membrane-bound guanylate cyclase GC-A in PC12 cells in a non-genomic manner. To examine whether 17β-estradiol exerts a similar effect in other cell types, we measured the effect of 17β-estradiol and tamoxifen, an anti-oestrogen, on guanylate cyclase activity in porcine kidney proximal tubular LLC-PK1 cells. 17β-Estradiol increased cGMP levels in LLC-PK1 cells. Interestingly, addition of tamoxifen also increased cGMP levels in a concentration-dependent manner in LLC-PK1 cells. The effects of both 17β-estradiol and tamoxifen on guanylate cyclase activity were not additive, suggesting that oestrogen and tamoxifen activate the same enzyme. Similar phenomena were also observed in LLC-PK1 cell membrane preparation. LLC-PK1 cells do not express membrane-bound guanylate cyclase GC-B and express low levels of membrane-bound guanylate cyclase GC-C. Tamoxifen inhibited the activation of GC-A by atrial natriuretic factor (ANF). However, it did not affect membrane-bound guanylate cyclase GC-C stimulated by guanylin or *Escherichia coli* heat-stable toxin STa. These results indicate that 17β-estradiol and tamoxifen activate GC-A in LLC-PK1 cells. Thus, tamoxifen functions as an agonist rather than an antagonist for the membrane oestrogen receptor coupled to the activation of GC-A.

Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, USA

Zi-Jiang Chen, Michael Vetter, Shiguo Liu, Yaxian Ding, Chung-Ho Chang

Department of Medicine, Reproductive Research Center, Shandong Provincial Hospital, Shandong University, Jinan, P. R. China

Zi-Jiang Chen

Graduate Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan

Geen-Dong Chang

Correspondence: C.-H. Chang, Department of Medicine, Division of Hypertension, Case Western Reserve University School of Medicine, W 165, Cleveland, OH 44106 USA. E-mail: cxc13@po.cwru.edu

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Introduction

Besides its genomic effects, oestrogen exerts short-term non-genomic effects that are independent of gene transcription (for review, see Moss et al 1997; Nemere & Farach-Carson 1998; Kelley & Levin 2001; Nadal et al 2001). However, little is known about the molecular mechanisms by which oestrogen elicits its non-genomic effects. Several mechanisms have been proposed to account for the non-genomic effects of oestrogen. Classical oestrogen receptor ERα has been shown to mediate the effect of oestrogen on the activation of nitric oxide (NO) synthase (Chen et al 1999; Hong et al 1999) and mitogen-activated protein kinase ERK (Migliaccio et al 1996; Watters et al 1997). Besides ERα, GPR30, a G protein-coupled receptor homologue, is involved in oestrogen-mediated ERK activation (Filardo et al 2000). Oestrogen may also elicit its non-genomic effects by interacting non-specifically with other membrane receptors or channels (Moss et al 1997; Nemere & Farach-Carson 1998).

Recently, we have found that 17β-estradiol can interact with different guanylate cyclase isoforms in a non-genomic manner. 17β-Estradiol (Chen et al 1998) and resveratrol (Chen et al 2000), a phytoestrogen, activate membrane-bound guanylate cyclase GC-A in PC12 cells. On the other hand, 17β-estradiol acutely inhibits the activity of soluble guanylate cyclase through a protein tyrosine phosphatase in PC12 cells (Chen et al 2001a). Thus, 17β-estradiol exerts dual effects on the activity of different guanylate cyclase isoforms. In PC12 cells, the effect of soluble guanylate cyclase inhibition is larger than that of GC-A activation (Chen et al 2001a). Therefore, addition of 17β-estradiol leads to a decrease in cGMP levels in PC12 cells. To examine whether 17β-estradiol has a similar effect on cGMP levels in other cell types, we measured the effect of 17β-estradiol in porcine kidney proximal tubular LLC-PK1 cells.

Tamoxifen, an anti-oestrogen, has been used for the treatment of breast cancers that express classical oestrogen receptors (Gail et al 1999; Lippman & Brown 1999). It exerts biological responses that can be either oestrogenic or anti-oestrogenic depending on the target tissues or cells. Tamoxifen primarily functions through its ability to compete with oestrogen for binding to the classical oestrogen receptors. Although the identity of the membrane oestrogen receptor remains unclear, we examined whether tamoxifen can affect GC-A activity and 17β-estradiol-mediated GC-A activation in LLC-PK1 cells. The results indicate that 17β-estradiol and tamoxifen activate GC-A, but not membrane-bound guanylate cyclase GC-B or GC-C, in LLC-PK1 cells, and that tamoxifen functions as an agonist for the membrane oestrogen receptor coupled to the activation of GC-A.

Materials and Methods

Materials

RPMI medium was obtained from Gibco BRL (Gaithersburg, MD). LLC-PK1 cells were purchased from ATCC (Rockville, MD). 17β-Estradiol, tamoxifen and other chemicals were purchased from Sigma (St Louis, MO).

cGMP determination

LLC-PK1 cells were grown to near confluence in 12-well plates (35 mm). The cells were washed with 2 mL of serum-free RPMI medium, and then pre-incubated at 37 °C for 10 min with 1 mL RPMI medium containing 0.5 mM isobutylmethylxanthine, a phosphodiesterase inhibitor, to prevent the hydrolysis of cGMP. Cells were then incubated with various concentrations of tamoxifen for 10 min or various concentrations of 17β-estradiol (or a combination of these) for another 10 min at 37 °C. After incubation, the medium was aspirated and 0.75 mL cold 10% trichloroacetic acid was added to the plates. The cell extracts were scraped and centrifuged for 15 min at 2000 *g*, and the supernatant fractions were extracted with water-saturated ether to remove trichloroacetic acid. The cGMP levels in the supernatants were determined by radioimmunoassay (Chang & Song 1993; Chen et al 1998, 2000, 2001a, b).

Preparation of LLC-PK1 cell membranes

LLC-PK1 cells were grown to near confluence in 75 cm^2 flasks with RPMI medium containing 10% bovine fetal serum and 5% horse serum. Cultured cells were washed with cold PBS, and then resuspended in 25 mM Tris-HCl (pH 7.6) buffer containing 250 mM sucrose. The cells were frozen at −80 °C and thawed on ice to break down the cell membranes. The broken cells were then centrifuged (12 000 *g*, 4 °C) for 15 min. The membrane pellets were resuspended with 50 mM Tris buffer, pH 7.6, containing 250 mM sucrose. The membrane fractions were then assayed for guanylate cyclase activity.

Guanylate cyclase assay

Guanylate cyclase was assayed at 37 °C in the presence of 50 mM Tris, pH 7.6, 0.5 mM isobutylmethylxanthine, 1 mM GTP, 4 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin, 25 mM creatine phosphate, 55 U mL^{-1} creatine kinase (135 U (mg protein)$^{-1}$) and about 8 μg of membrane proteins with or without various doses of 17β-estradiol or tamoxifen in a final volume of 0.1 ml. Reactions were initiated by the addition of LLC-PK1 cell membranes or cytosolic proteins, incubated for 10 min and terminated by the addition of 0.5 ml 50 mM chilled sodium acetate, pH 4.0. Generated cGMP was quantified by the radioimmunoassay as previously described (Chang & Song 1993; Chen et al 1998, 2000, 2001b).

Statistical analysis

All error bars represent the standard deviation from the mean of four experimental replicates. A two-way analysis of variance was utilized to determine the significance of differences between treatment concentration–response curves. A P value less than 0.005 was considered statistically significant.

Results

Effects of 17β-estradiol and tamoxifen on guanylate cyclase activity in intact LLC-PK1 cells

We have shown that addition of 17β-estradiol to PC12 cells leads to a decrease in cGMP levels (Chen et al 2001a). To examine whether the effect of 17β-estradiol on cGMP formation is conserved in other cell types, we measured the effect of 17β-estradiol in porcine kidney proximal tubular LLC-PK1 cells. Figure 1 shows that, unlike PC12 cells, 17β-estradiol increased the cGMP levels in a concentration-dependent manner with an EC50 (concentration producing half-maximal activation) of 100 μM in LLC-PK1 cells. Tamoxifen has been shown to be an antagonist or partial agonist for the classical oestrogen receptors, ERα and ERβ (for review, see Clarke et al 2001; Lonard & Smith 2002). Although the receptor mediating the effects of 17β-estradiol on the activation of GC-A remains unknown, we examined whether tamoxifen affects guanylate cyclase activity or blocks the effect of 17β-estradiol on the activity of GC-A in LLC-PK1 cells. Interestingly, tamoxifen increased cGMP formation more effectively than 17β-estradiol, with a maximal activation about 4.7-fold and an EC50 of around 50 μM (Figure 1). To examine whether tamoxifen and 17β-estradiol activate the same guanylate cyclase isoform, we measured the effect of 17β-estradiol on cGMP levels in the presence of 50 μM tamoxifen in LLC-PK1 cells. Tamoxifen inhibited 17β-estradiol-stimulated guanylate cyclase activity (Figure 2), suggesting that tamoxifen and 17β-estradiol activate the same guanylate cyclase isoform.

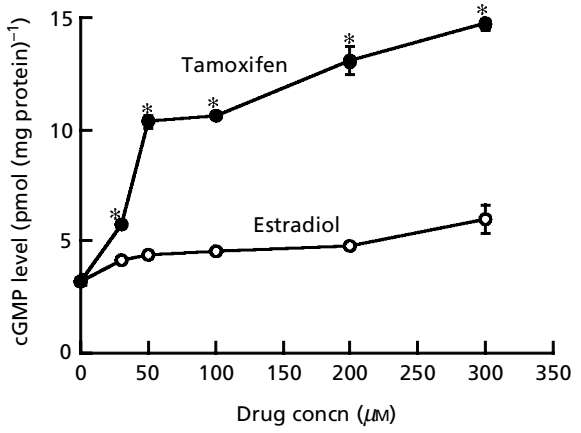

Figure 1 Effect of 17β-estradiol and tamoxifen on cGMP levels in LLC-PK1 cells. LLC-PK1 cells were exposed to 0.5 mM isobutylmethylxanthine at 37 °C for 10 min, and then various concentrations of 17β-estradiol and tamoxifen for another 10 min. Generated cGMP was measured by radioimmunoassay. The error bar represents the deviation from the mean of four replicates. 17β-Estradiol and tamoxifen increased cGMP level in LLC-PK1 cells. *$P < 0.005$, tamoxifen vs 17β-estradiol.

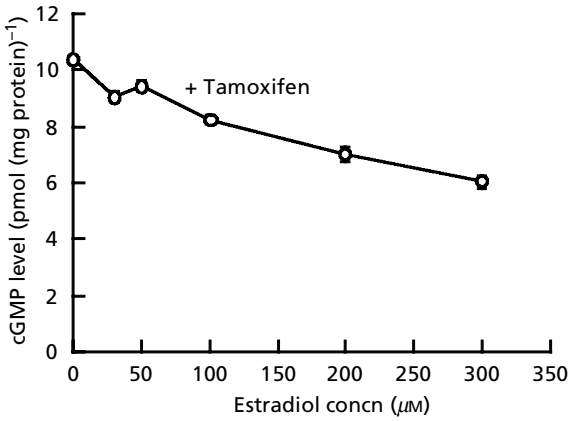

Figure 2 Competition of tamoxifen with 17β-estradiol on cGMP formation by tamoxifen in LLC-PK1 cells. LLC-PK1 cells were exposed to 0.5 mM isobutylmethylxanthine at 37 °C for 10 min, 50 μM tamoxifen for 10 min and then various concentrations of 17β-estradiol for another 10 min. Generated cGMP was measured by radioimmunoassay. The error bar represents the deviation from the mean of four replicates.

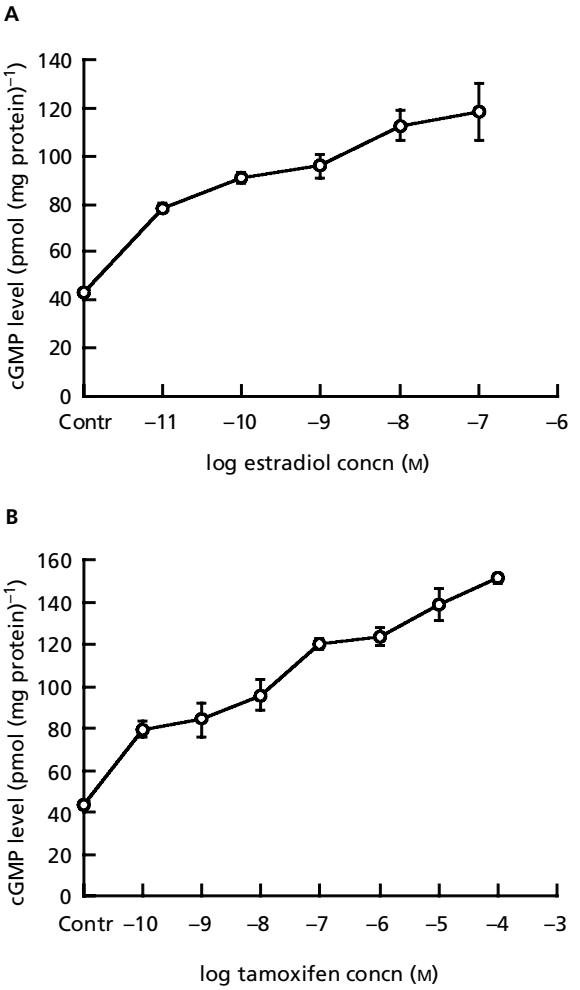

Figure 3 Effect of 17β-estradiol and tamoxifen on guanylate cyclase activity in LLC-PK1 cell membranes. Guanylate cyclase assay were performed at 37 °C for 15 min in the presence and absence of various concentrations of 17β-estradiol (A) or tamoxifen (B) using LLC-PK1 cell membranes. Generated cGMP was measured by radioimmunoassay. The error bar represents the deviation from the mean of four replicates. Both 17β-estradiol and tamoxifen activated membrane-bound guanylate cyclase in LLC-PK1 cell membranes.

Effects of tamoxifen and 17β-estradiol on guanylate cyclase activity in LLC-PK1 cell membranes

In a previous study, we found that 17β-estradiol and resveratrol, a phytoestrogen, activate membrane-bound guanylate cyclase GC-A in PC12 cell membranes (Chen et al 1998, 2000). To examine whether tamoxifen affects membrane-bound guanylate cyclase activity, we measured the effect of tamoxifen and 17β-estradiol on guanylate cyclase activity in LLC-PK1 cell membranes. Figure 3A shows that 17β-estradiol activated GC-A in LLC-PK1 cell membranes in a concentration-dependent manner with an EC50 of around 10^{-11} M as previously described in PC12 cell membranes (Chen et al 1998). Tamoxifen also increased membrane-bound guanylate cyclase about 3.5-fold with an EC50 of around 10^{-8} M (Figure 3B). Like that in intact LLC-PK1 cells, 17β-estradiol did not enhance the effects of 10^{-4} M tamoxifen on guanylate cyclase activity, suggesting that tamoxifen and 17β-estradiol share the same pathway in the activation of membrane-bound guanylate cyclase (Figure 4). To rule out the possibility that tamoxifen may activate soluble guanylate cyclase, we measured the effect of

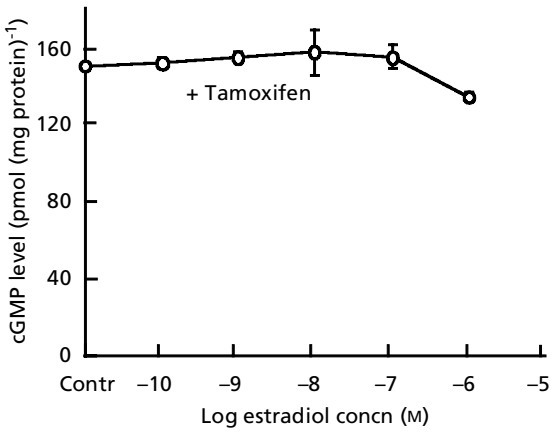

Figure 4 Effect of 17β-estradiol on tamoxifen-stimulated membrane-bound guanylate cyclase activity in LLC-PK1 cell membranes. Guanylate cyclase assay was performed at 37°C for 15 min in the presence and absence of various concentrations of 17β-estradiol and 10^{-4} M tamoxifen using LLC-PK1 cell membranes. Generated cGMP was measured by radioimmunoassay. The error bar represents the deviation from the mean of four replicates. 17β-Estradiol and tamoxifen did not have additive effects on membrane-bound guanylate cyclase in LLC-PK1 cell membranes.

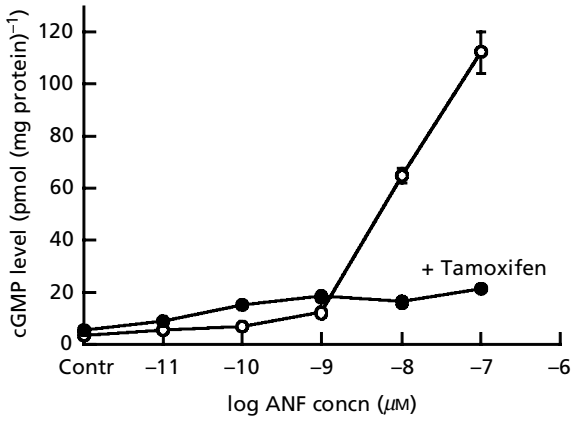

Figure 6 Effect of tamoxifen on ANF-stimulated GC-A activity in LLC-PK1 cells. LLC-PK1 cells were exposed to 0.5 mM isobutylmethylxanthine at 37°C for 10 min, and 30 μM tamoxifen for 10 min and then various concentrations of ANF for another 10 min. Generated cGMP was measured by radioimmunoassay. The error bar represents the deviation from the mean of four replicates. Tamoxifen inhibited ANF-stimulated GC-A activity in LLC-PK1 cells.

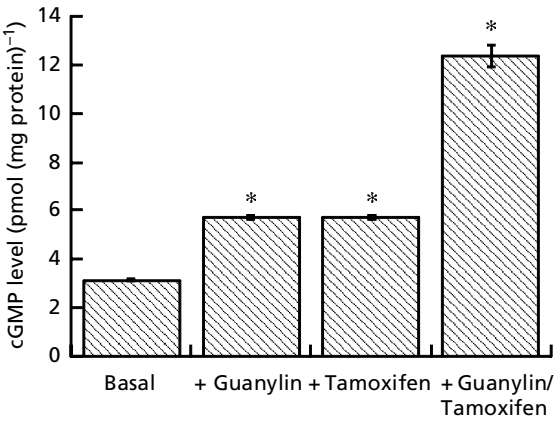

Figure 5 Effect of guanylin and tamoxifen on cGMP levels in LLC-PK1 cells. LLC-PK1 cells were exposed to 0.5 mM isobutylmethylxanthine at 37°C for 10 min, and 30 μM tamoxifen for 10 min and then 10^{-7} M guanylin for another 10 min. Generated cGMP was measured by radioimmunoassay. The error bar represents the deviation from the mean of four replicates. Tamoxifen did not inhibit guanylin-stimulated GC-C activity in LLC-PK1 cells. *$P < 0.001$ vs control by unpaired t-test.

tamoxifen on soluble proteins prepared from LLC-PK1 cells. The results indicated that tamoxifen did not affect the activity of soluble guanylate cyclase (data not shown).

Effects of tamoxifen on guanylate cyclase activity stimulated by atrial natriuretic factor, C-natriuretic peptide, guanylin and *Escherichia coli* heat-stable toxin STa in LLC-PK1 cells

There are three membrane-bound guanylate cyclases whose ligands are known. GC-A, GC-B and GC-C are the receptors for atrial natriuretic factor (ANF), C-natriuretic peptide (CNP) and guanylin (or *E. coli* heat stable toxin STa) (for review, see Wedel & Garbers 2001), respectively. We examined whether GC-A, GC-B and GC-C are expressed in LLC-PK1 cells by incubating LLC-PK1 cells with various concentrations of ANF, CNP and guanylin. CNP had no effect on cGMP levels, indicating that LLC-PK1 cells do not express GC-B (data not shown). On the other hand, guanylin and STa (data not shown) slightly increased cGMP levels (about 80%) (Figure 5), indicating that LLC-PK1 cells express low levels of GC-C. In contrast, ANF effectively increased cGMP levels about 35.4-fold in LLC-PK1 cells (Figure 6). We then examined whether tamoxifen affects ANF-stimulated GC-A and guanylin-stimulated GC-C activity in LLC-PK1 cells. Addition of 30 μM tamoxifen decreased ANF-stimulated GC-A activity (Figure 6), but not guanylin-stimulated GC-C activity (Figure 5). These results indicate that tamoxifen activates GC-A, but not GC-B or GC-C in LLC-PK1 cells.

Discussion

We have found that 17β-estradiol exerts a dual effect on the activity of guanylate cyclase isoforms in a non-genomic manner. 17β-Estradiol stimulates GC-A activity (Chen et al 1998), but inhibits soluble guanylate cyclase activity (Chen et al 2001a). The extent of 17β-estradiol-mediated GC-A activation and soluble guanylate cyclase inhibition varies with cell types. For instance, in PC12 cells, addition of 17β-estradiol decreases overall cGMP levels (Chen et al 2001a), indicating that the effect of 17β-estradiol-mediated inhibition of soluble guanylate cyclase dominates over that

of 17β-estradiol-mediated activation of GC-A. However, 17β-estradiol-mediated GC-A activation is the prevailing pathway in LLC-PK1 cells (Figure 1). Thus, the overall effects of 17β-estradiol on cGMP levels depend on the balance of these two signaling pathways. Therefore, 17β-estradiol may increase or decrease the intracellular cGMP levels in different cell types.

Tamoxifen is a selective oestrogen receptor modulator (SERM) (for review, see Kauffman & Bryant 1995; Kaye et al 2001; Lonard & Smith 2002). To examine whether tamoxifen affects guanylate cyclase activity, we measured the effect of tamoxifen on guanylate cyclase activity in LLC-PK1 cells. Tamoxifen increased cGMP levels more effectively than 17β-estradiol in LLC-PK1 cells. Moreover, the effects of tamoxifen and 17β-estradiol on guanylate cyclase activity were not additive, suggesting that they activate the same enzyme. These results indicate that tamoxifen is an oestrogen agonist rather than an oestrogen antagonist in the activation of guanylate cyclase, and that tamoxifen and 17β-estradiol activate the same guanylate cyclase isoform.

The stimulatory effect of tamoxifen on cGMP levels could be due either to tamoxifen activating soluble guanylate cyclase or membrane-bound guanylate cyclase GC-A. Using soluble and membrane fractions from LLC-PK1 cells, we found that tamoxifen activates membrane-bound, but not soluble, guanylate cyclase. Currently, there are only three membrane-bound guanylate cyclase isoforms with known ligands: GC-A, GC-B and GC-C. Two lines of evidence suggest that tamoxifen activates membrane-bound guanylate cyclase GC-A. First, we and others have shown that 17β-estradiol (Chen et al 1998) and resveratrol (Chen et al 2000; El-Mowafy 2002), a phytoestrogen, activate GC-A. Since competition experiments suggest that tamoxifen and 17β-estradiol activate the same guanylate cyclase isoform, tamoxifen likely activates GC-A; and second, LLC-PK1 cells do not express GC-B because CNP has no effect on cGMP formation. LLC-PK1 cells express low level of GC-C since guanylin and *E. coli* heat-stable toxin STa have a small effect on cGMP levels. In contrast to CNP and guanylin, ANF activates GC-A very potently in LLC-PK1 cells, indicating that GC-A is the predominant membrane-bound guanylate cyclase isoform in LLC-PK1 cells. We found that guanylin does not compete with tamoxifen for guanylate cyclase activation, indicating that tamoxifen does not activate GC-C. However, tamoxifen inhibits ANF-stimulated GC-A activity in LLC-PK1 cell membranes, suggesting that tamoxifen activates GC-A. Thus, these results indicate that tamoxifen functions as an agonist for the membrane oestrogen receptor involved in the non-genomic activation of GC-A.

It should be noted that the EC50 of 17β-estradiol on the activation of GC-A in cell membranes (5×10^{-11} M) is much lower than that (100 μM) in intact cells. The lower EC50 of 17β-estradiol in LLC-PK1 cell membranes than that in intact cells is probably due to the greater accessibility of 17β-estradiol in the membrane system. Recently, we have shown that 17β-estradiol also inhibits the activity of soluble guanylate cyclase (Chen et al 2001a). Therefore, the loss of soluble guanylate cyclase in the membrane preparation may also shift the EC50 of 17β-estradiol to the lower concentration. The same reasoning may also account for the lower EC50 of tamoxifen in the membrane system than that in intact cells. It is known that the steady serum concentration of tamoxifen during endocrine therapy is in the range of 1.1–4 μM (Etienne et al 1989; Trump et al 1992). However, the concentration of tamoxifen in the breast tumour can reach up to 14 μM (Daniel et al 1981). We have found that the EC50 of tamoxifen for GC-A activation in the membrane preparation in this study is 10^{-8} M. This concentration is much lower than those found in serum and breast tumour. Therefore, GC-A activation may contribute to the beneficial effects of tamoxifen on breast cancer therapy.

Tamoxifen normally functions through its ability to compete with the available oestrogens for binding to classical oestrogen receptors ERα and ERβ (Clarke et al 2001; Lonard & Smith 2002). The identity of the membrane receptor mediating the non-genomic effects of oestrogen remains unclear. Both ERα (Migliaccio et al 1996; Watters et al 1997; Chen et al 1999; Hong et al 1999) and GPR30 (Filardo et al 2000), a G protein-coupled receptor homologue, have been suggested to be the membrane oestrogen receptor or one component of the receptor complex. Our results showed that, similar to 17β-estradiol, tamoxifen can activate GC-A in a non-genomic manner. These results suggest that tamoxifen functions as an agonist for the membrane oestrogen receptors coupled to GC-A activation. Precedents that tamoxifen can exert a non-genomic effect have also been reported in other systems. For instance, tamoxifen has been shown to induce apoptosis in neuronal cells in which the classical oestrogen-receptor-mediated genomic pathway is non-functional (Hashimoto et al 1997). Tamoxifen has also been shown to activate adenylate cyclase (Aronica et al 1994) and mitogen-activated protein kinase (Migliaccio et al 1996) in breast cancer cells in a non-genomic manner. In addition, like oestrogen, tamoxifen activates the BK channel in smooth muscle (Dick et al 2001) and a large conductance chloride channel in NIH3T3 fibroblast cells (Valverde et al 2002).

Tamoxifen has been documented to exert an antioxidant function. It has been shown to inhibit lipid peroxidation in phospholipid liposomes, rat liver microsomes (Wiseman et al 1990, Wiseman 1995), sarcoplasmic reticulum membranes (Custodio et al 1994) and in postmenopausal women with breast cancer (Thangaraju et al 1994). Furthermore, tamoxifen also inhibits the oxidation of low-density lipoprotein (Wiseman 1995, Kuohung et al 2001). Like tamoxifen, we have found that other antioxidants, such as 17β-estradiol, resveratrol, dithiothreitol and vitamin C, also activate GC-A (Chen et al 1998, 2000, 2001b). Transgenic and gene disruption studies have shown that the ANF/GC-A system is involved in the regulation of blood pressure and cardiac hypertrophy (Steinhelper et al 1990; John et al 1995; Lopez et al 1995; Oliver et al 1997, 1998). Therefore, it is possible that GC-A may mediate some of the antioxidant and cardioprotective effects of tamoxifen.

Conclusions

Our study indicates that GC-A is the predominant membrane-bound guanylate cyclase isoform in LLC-PK1 cells.

17β-Estradiol and tamoxifen increase cGMP levels in LLC-PK1 cells in a non-genomic manner by the activation of membrane-bound guanylate cyclase. The effect of tamoxifen on guanylate cyclase activation is decreased by 17β-estradiol and ANF, indicating that tamoxifen and 17β-estradiol activate the same enzyme, GC-A. Thus, tamoxifen can function as an agonist for the membrane estrogen receptor coupled to the activation of GC-A.

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