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Short communication

Interaction of antiestrogens with binding sites for muscarinic cholinergic drugs and calcium channel blockers in cell membranes

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Summary. The interaction of tamoxifen and clomifene with membrane binding sites for the cholinergic ligand quinuclidinyl benzilate (QNB) and the dihydropyridine calcium antagonist nitrendipine was studied. Both tamoxifen and clomifene competed with [^{3}H]-QNB and [^{3}H]-nitrendipine for their binding to the receptor in the membrane fractions from the urinary bladder and myometrium. The extent of inhibition as judged by the K_i values for both antiestrogens was similar at both receptor sites. The data suggest that the antiproliferative effects of tamoxifen may involve not only the intracellular estrogen-receptor system but also receptors for neurotransmitters and membrane calcium channels

Introduction

The antiestrogen tamoxifen has been proven to be a useful clinical agent in the palliative treatment of advanced breast cancer in postmenopausal women. Its competition for the intracellular estrogen receptor is the general accepted mode of action of tamoxifen. More recently, reports on several other interesting pharmacological effects of tamoxifen have been published. Some evidence has been presented that the drug is a calmodulin antagonist [14], a calcium-channel blocking agent [17] and, more recently, even a histamine antagonist [13]. It therefore appears that, in addition to interfering in the normal processing of intracellular events triggered by estrogen, this drug may interact with cell membrane components. This is compatible with recent evidence of specific binding of tamoxifen in the isolated microsomal fractions [21].

In the present study we looked at the interaction of tamoxifen and clomifene with membrane binding sites for both anticholinergic drugs and dihydropyridine calcium antagonist in different tissues. Membrane preparations from both rabbit and human tissues were used. Interactions with the muscarinic receptor binding site were examined in the human urinary bladder and in rabbit myometrium, whereas human as well as rabbit myometrium was used to study interactions with calcium antagonist binding sites. Data on the characterization of membrane muscarinic cholinergic receptor binding using quinuclidinyl benzilate (QNB) or nitrendipine binding in these tissues have recently been reported [1–3].

Materials and methods

Tissues. Samples of human myometrium were obtained from women who underwent hysterectomy and urinary bladder tissue was obtained from patients undergoing surgery for bladder malignancy or ureter re-implantation. After excision, the tissue was immediately placed in ice-cold saline (0.9%) and transported to the laboratory, which took only a few minutes. Rabbit myometrial tissues were obtained from mature estrogenized females weighing 2.7–3.2 kg. Estrogenization was accomplished by a single i.m. injection of 1 mg/kg estradiol polyphosphate [4]. This was done particularly to see whether endogenous estrogen had any effect on the binding of tamoxifen to membrane sites.

Membrane preparation. Tissues were immediately trimmed of excess fat and connective tissue and the mucosal layer from each tissue was removed by scraping. Samples were then blotted dry and placed in ice-cold sucrose (0.25 *M*), HEPES (10 m*M*), and phenylmethylsulfonyl fluoride (0.5 m*M*) buffer (pH 7.2). After being weighed, the tissue was homogenized in about 5 vol. sucrose-HEPES buffer with a Polytron homogenizer (PGA 10–35) for three periods of 10 s/g tissue, with intermittent cooling pauses of 20 s. The homogenate was centrifuged at 1,000 *g* for 10 min and the pellet was discarded. The supernatant was filtered through three layers of gauze and centrifuged at 12,000 *g* for 15 min and the pellet was discarded. The above supernatant was centrifuged at 40,000 *g* for 1 h to obtain the microsomal pellet. The microsomal pellet was suspended in the above buffer and centrifuged again. The resulting pellet was suspended in sucrose-HEPES solution to give a protein concentration of 1–2 mg/ml and was stored frozen at –80° C until used for ligand binding studies. The protein concentration in particulate fractions was determined by the method of Peterson [18].

Binding of QNB and nitrendipine to plasma membrane fractions. The standard binding assay was carried out in KCl (100 n*M*) HEPES (20 n*M*) medium (pH 7.2) in a total volume of 0.5 ml for each assay tube. The protein concentration in each assay tube was approximately 50 μg and the [^{3}H]-QNB or [^{3}H]-nitrendipine concentration in various assays

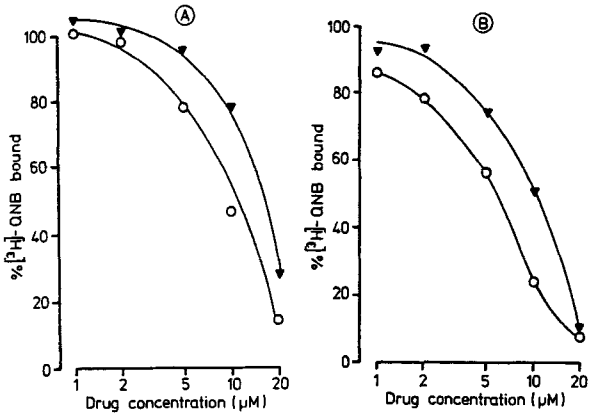

Fig. 1 A, B. Representative curves for inhibition of [^{3}H]-QNB binding by tamoxifen (▼) and clomifene (○) in preparations from **A** human urinary bladder and **B** rabbit myometrium. Incubations were carried out at 37° C for 60 min. The [^{3}H]-QNB concentration in the medium was 0.2 n*M*, and the [^{3}H] nitrendipine concentration in the medium was 1 n*M* respectively

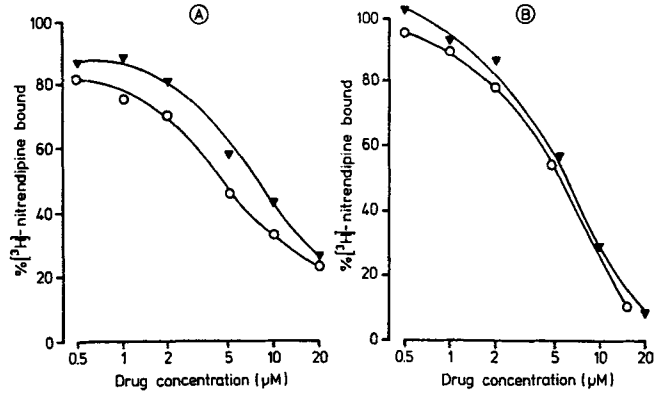

Fig. 2 A, B. Representative curves for inhibition of [^{3}H]-nitrendipine binding by tamoxifen (▼) and clomifene (○) in preparations from **A** human myometrium and **B** rabbit myometrium. Incubations were carried out at 25° C for 30 min. The medium [^{3}H]-nitrendipine concentration was 1 n*M*

varied between 0.05 and 1 n*M*. A second set of tubes containing 10 μ*M* atropine for [^{3}H]-QNB binding or 0.25 μ*M* nitrendipine for [^{3}H]-nitrendipine binding assays was run to measure non-specific binding. The binding reaction was initiated by the addition of membrane protein, and incubation was allowed to proceed for 60 min at 37° C for [^{3}H]-QNB binding or for 30 min at 25° C for [^{3}H]-nitrendipine binding assays. The reaction was terminated by filtration of 0.4 ml incubation mixture through Whatman GF/F glass fiber filters. Other details have previously been described [1, 2].

Chemicals. Tritiated quinuclidinyl benzilate (specific activity, 33 Ci mmol^{-1}) and tritiated nitrendipine (specific activity, 72 Ci mmol^{-1}) were purchased from New England Nuclear Corporation. Authentic Dl-QNB and nitrendipine were a gift from Hoffman-La Roche & Co (Switzerland) and Bayer (Sweden), respectively. The radiochemical purity of [^{3}H]-QNB and [^{3}H]-nitrendipine was checked by thin-layer chromatography HPLC as previously described [1, 2]. Estradiol polyphosphate (Estradurin) was obtained from AB Leo (Helsingborg, Sweden).

Results

Inhibition by tamoxifen and clomifene of specific QNB binding to membrane preparations from human urinary bladder and estrogenized rabbit myometrium are shown in Fig. 1. Each drug was tested at several concentrations and the IC_{50} value was determined from the respective curve.

A similar experiment was done for studying inhibition by antiestrogens of nitrendipine binding to membrane preparations from human and rabbit myometrium (Fig. 2). The effect of both estradiol-17β and diethylstilbestrol was also tested. Whereas up to 20 μ*M* estradiol-17β had no effect on either QNB or nitrendipine binding, diethylstilbestrol (20 μ*M*) caused 10%–20% inhibition of nitrendipine binding (data not shown).

Tamoxifen and clomifene inhibited QNB binding in the human urinary bladder to the same extent, the K_i values being very similar (Table 1). Although the K_i for tamoxifen in the rabbit myometrium was slightly higher than that for clomifene, the difference was not statistically significant, nor was the small difference between the values obtained for human urinary bladder and rabbit myometrium. Corresponding data on nitrendipine binding showed that the K_i values for both tamoxifen and clomifene were comparable with those obtained using QNB binding in the human myometrium, whereas in the rabbit myometrium they were lower for both antiestrogens ($P < 0.05$).

The Hill coefficients (^nH) calculated from Hill plots showed that the values for clomifene at nitrendipine binding sites were close to and not significantly different from unity. The value for tamoxifen was 1.5. At QNB binding sites the values for both antiestrogens were close to 2.

Table 1. Inhibition by tamoxifen and clomifene of [^{3}H]-QNB and [^{3}H]-nitrendipine (NT) binding to membrane preparations from human and rabbit tissues

Tissue (ligand)	Tamoxifen:		n_H	Clomifene:		n_H
	IC_{50} (μ*M*)	K_i (μ*M*)		IC_{50} (μ*M*)	K_i (μ*M*)	
Human urinary bladder (QNB)	13.6 ± 0.88	1.45 ± 0.09	2.6	11.9 ± 0.60	1.2 ± 0.06	2.3
Rabbit myometrium (QNB)	10.7 ± 2.2	2.35 ± 0.40	2.3	6.2 ± 0.84	1.3 ± 0.16	1.9
Rabbit myometrium (NT)	6.0 ± 0.22	0.83 ± 0.03*	1.5	5.0 ± 0.32	0.69 ± 0.05*	0.95
Human myometrium (NT)	6.3 ± 0.73	1.05 ± 0.12	1.5	7.4 ± 1.02	1.2 ± 0.23	1.1

IC_{50} values were calculated from the dose-response curves (see Figs. 1, 2). K_i values were calculated from the equation
$K_i = IC_{50}/(1+(L)/K_D)$,
where (L) represents the radioligand concentration and K_D, the radioligand equilibrium dissociation constant [6]. The mean value for the Hill coefficient (n_H) is also shown. Values represent the means ± SE of determinations from 5–6 individual preparations
* Statistically significant difference ($P < 0.05$) in K_i for QNB and NT binding in the rabbit myometrium

Discussion

Although many previous studies have shown that the antiproliferative action of tamoxifen involves the estrogen-receptor system [12], the pharmacology of this drug is probably highly complex since the antiproliferative effects are in many instances not explainable on the basis of antiestrogen action [9, 19]. The results of the present study indicate that tamoxifen not only competes for dihydropyridine binding sites, as reported recently by Greenberg et al. [9], but also binds to muscarinic cholinergic receptor to about the same extent.

The anomalous binding behaviour of antiestrogens at QNB binding sites as characterized by the n_H value (being close to 2) would suggest positive cooperativity. However, the possibility that the antiestrogens may also have a membrane perturbing effect cannot be completely ruled out by the present data

Results showing that antiestrogens inhibited ligand binding for both [^{3}H]-QNB and [^{3}H]-nitrendipine in the estrogenized rabbit uterus to the same degree as in the urinary bladder (a non-target organ for estrogen) and in the unestrogenized human uterus not only suggest that the binding sites are not influenced by estrogen treatment but also rule out any contamination in the microsomal preparation by intracellular estrogen receptors.

A number of recent studies suggest that calcium plays an important role in tumour cell growth [7, 10]. Thus, the calcium-channel blocking effects of tamoxifen might well contribute to its antiproliferative action in estrogen-responsive tissues and could be an important mechanism of action in cancer cells unresponsive to estrogen.

Neurotransmitters and neuropeptides may exert their effects on tumour cell growth through an action initiated by growth factors [8, 20]. It has recently been shown that endocrine secretion of epidermal growth factor in the prostate was stimulated by acetylcholine, which could be blocked by atropine, indicating the involvement of a cholinergic receptor mechanism [11]. The anticholinergic properties of tamoxifen would therefore lead to an inhibitory action on the secretion of growth factors in organs responsive to cholinergic stimulation, such as the urinary bladder, prostate and uterus. Recently, models based on autocrine mechanisms involving growth factors for the action of sex steroids in target cells have been proposed [16].

Although the clinical significance of the calcium-channel-antagonistic and anticholinergic properties of tamoxifen cannot be determined from the present data, micromolar concentrations of tamoxifen are required to inhibit the growth of cancer cells in vitro [15], and serum tamoxifen levels can be as high as 1.4 μM in patients receiving the drug for the treatment of breast cancer [5].

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