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Characterisation of Muscarinic Cholinergic Receptors in Human Ovaries, Ovarian Tumours and Tumour Cell Lines

Satish Batra, Lars D. Popper and Constantin S. Iosif

Using [^{3}H]quinuclidinyl benzilate (QNB) as radioligand, muscarinic cholinergic receptor sites in isolated plasma membrane fractions from human ovarian tumours, cultured tumour cells, and normal ovarian tissue were characterised. QNB binding to all preparations, except from poorly differentiated tumour, was specific, saturable, and of high affinity. In contrast to normal ovaries, benign tumours, well differentiated adenocarcinoma and OVCAR-3 cells, the poorly differentiated adenocarcinoma and SKOV-3 cells completely lacked specific QNB binding. The muscarinic receptor densities and the K_d values in preparation from ovaries, receptor-positive tumours and OVCAR-3 cells were similar. QNB binding was strongly inhibited by the classical muscarinic receptor antagonist atropine, but poorly by the agonist carbachol. In contrast to atropine, inhibition by pirenzepine and AF-DX 116 was relatively low. These data suggest that muscarinic receptors in ovaries and ovarian tumours are of m3 type.
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INTRODUCTION

A GREAT deal of interest has recently been focused on the role of neurotransmitters in the regulation of cell growth. It has been reported that neurotransmitter receptors can regulate cell proliferation and neoplastic transformation [1, 2]. Some evidence has also been presented for the growth regulating effects mediated by histaminergic, adrenergic, dopaminergic, serotinergic, and muscarinic cholinergic receptors in certain cell systems. In some cell lines, receptors for some of the above neurotransmitters have been reported [3–6].

The presence of cholinergic receptors has been reported in human lung tumours and in cultured human pancreatic carcinoma cells [7, 8]. In a recent study we presented data on the presence and characterisation of muscarinic cholinergic receptors in different types of rat prostatic tumours (Dunning) and in normal prostatic tissue [9]. Among the two types of tumours used, whereas receptors were present in one that was well differentiated and androgen dependent, they were completely absent in the hormone-independent and anaplastic tumour. The extent to which the origin type and degree of differentiation can influence the density of membrane neurotransmitter receptors is not known. It is also not known whether the normal human ovary contains muscarinic cholinergic receptors although data are available showing that ovarian follicles obtained from ovaries of different animal species can produce a contractile response upon stimulation of the muscarinic cholinergic receptor [10]. Cholinergic stimulation is also able to induce a secretory response in granulosa cells [11]. In addition, ovaries of many species are supplied with adrenergic and cholinergic nerves [12].

In the present study we have identified and partially characterised muscarinic cholinergic receptors in human ovary, ovarian tumours and in cultured human ovarian carcinoma cells. We now report that human ovaries contain muscarinic cholinergic receptors of m3 type which are also present in human ovarian tumours and in one of the two cultured human ovarian cancer cell lines examined.

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MATERIALS AND METHODS

Chemicals

Tritiated 1-quinuclidinyl benzilate ([^{3}H]QNB) with a specific activity of 1.22 TBq/mmol was purchased from New England Nuclear (Boston, Massachusetts). Authentic DL-QNB was a gift from Hoffman-La Roche. The radiochemical purity of [^{3}H]QNB was checked by thin-layer chromatography (Merck Silica Gel 60 plates) in a solvent system of chloroform, methanol, and ammonia solution (80:20:1 by vol.). Over 985 of the radioactivity migrated as a single peak as revealed by Berthold Radiochromatogram scanner model LB 2723. Pirenzepine and AF-DX 116 were obtained from Karl Thomae (Germany). All other drugs were purchased from the Sigma Chemical Co.

Tissues, tumours and tumour cell culture

Ovarian tissue and tumour material. Specimens from untreated human ovarian carcinoma (n = 6) and benign cystadenoma (n = 2) were obtained from patients undergoing gynaecological operation for removal of the tumour. In addition, for comparison, normal ovarian tissue specimens (n = 6) were obtained from patients, 43–71 years old, operated for adenomyosis and myomata of the uterus. 4 of the patients were postmenopausal, 1 was premenopausal, and 1 was perimenopausal. The whole ovarian tissue including stroma was used for membrane preparation.

Cell cultures. The established cell lines used in these experiments were human ovarian carcinoma cells (OVCAR-3 and SKOV-3) and Chinese hamster ovary cells (CHO). These were obtained from American Type Culture Collection (Rockville, Maryland). Cells were grown in plastic flasks (Falcon) as mono-

layer cultures in RPMI 1640 medium containing 10% fetal calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and 2 mmol/l L-glutamine (GIBCO, Grand Island, New York). The culture medium was changed every 2 days. After 5–6 days (at near confluence) cells from 8–10 flasks were harvested by trypsinisation and dispersed into single cell suspension with calcium-free Hank's balanced salt solution (HBSS) (GIBCO). Cells from each flask were thereafter pooled and transferred to centrifuge tubes for isolation of a membrane fraction.

Membrane preparations

Ovarian tissue and tumours. After trimming off any excess fat and adhering connective tissue, the remaining tissue or tumour was thoroughly washed in ice-cold sucrose (0.25 mol/l)-Hepes (10 mmol/l) buffer, blotted dry, and weighed. Each tumour or tissue was then homogenised in about 5 vol. of the buffer with a Polytron homogeniser (PGA 10–35) for three periods of 10 s/g tissue with intermittent cooling pauses of 20 s. The homogenate was centrifuged at 1000 *g* for 10 min and the pellet was discarded. The supernatant was filtered through three layers of gauze and centrifuged at 12000 *g* for 15 min and the pellet was discarded. The supernatant was centrifuged at 40000 *g* for 60 min to obtain the microsomal pellet. The microsomal pellet was suspended in the above buffer and centrifuged again. The resulting pellet, which is enriched with plasma membrane, was suspended in sucrose–Hepes buffer to give a protein concentration of 1–2 mg/ml and was stored frozen at −70°C until used for ligand binding studies. The protein concentration in particulate fractions was determined by the method of Peterson [13].

Cell culture. Cells were washed three times with ice-cold sucrose–Hepes buffer. They were then homogenised with an all-glass Potter homogeniser and centrifuged at 1000 *g* for 10 min. The supernatant was decanted and saved, while the pellet was again homogenised and recentrifuged. The supernatants were pooled and centrifuged at 40000 *g* for 60 min to obtain the membrane pellet. After washing once, the pellet was suspended in the above buffer to give a protein concentration of 1–2 mg/ml.

Binding of [^{3}H]QNB to plasma membrane fractions

The standard binding assay was performed in KCl (100 mmol/l)-Hepes (20 mmol/l) 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 concentration in various assays varied between 0.1 and 1 nmol/l. A second set of tubes containing 10 μmol/l atropine was included in order to measure non-specific binding. The binding reaction was initiated by the addition of membrane protein and incubation was allowed to proceed, unless otherwise stated, for 60 min at 37°C. The reaction was terminated by filtration of 0.4 ml of the incubation mixture through Whatman GF/F glass fibre filters. Other details have been previously described [9, 14].

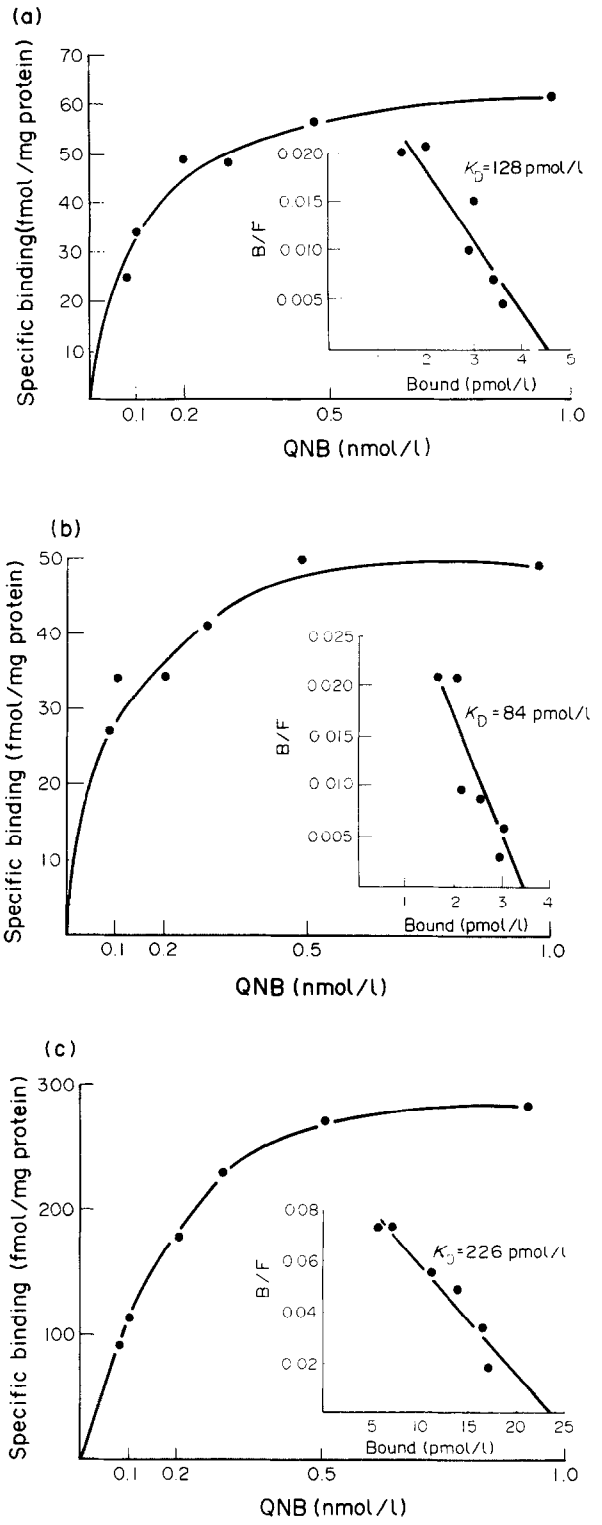

Fig. 1. Representative saturation and Scatchard analysis of [^{3}H]QNB binding to the membrane fraction from ovaries (a), ovarian tumour (b) and cultured OVCAR-3 cells (c).

RESULTS

Preliminary experiments showed that at 37°C half-maximum binding occurred at about 10 min for all preparations with complete saturation achieved within 30–60 min.

The data in Fig. 1 show specific binding of QNB to membrane preparations from human ovaries, ovarian tumours and from cultured ovarian tumour cells, OVCAR-3. Non-specific binding was very low (< 10%) in each case. The specific binding sites were saturated with low (0.5–1.0 nmol/l) concentrations of QNB in the medium. The Scatchard analysis of these data (inset Fig. 1) shows a single class of binding sites having a very high affinity in all three preparations. Nearly identical saturation curves were obtained for the two benign tumours analysed.

Similar experiments performed on tumour preparations showed no specific binding in three of the eight tumours. Non-

Table 1. Muscarinic cholinergic receptor density and apparent dissociation constant (K_d) in ovaries, ovarian tumour and cultured OVCAR-3 cells

Tumour/tissue	Receptor density (fmol/mg protein)	K_d (pmol/l)
Ovary	82.9 ± 5.4	230.4 ± 71.9
Tumour	76.3 ± 25.7	233.8 ± 93.0
OVCAR-3	273.2 ± 56.7	184.3 ± 16.5

Values are means ± S.E.M. of 6, 4 and 5 preparations from ovaries, ovarian tumours and OVCAR-3 cells, respectively.

specific binding in these was similar to that found in the receptor-positive preparations. Likewise, no specific binding occurred in preparations from cultured ovarian tumour cells from another cell line, SKOV-3, used in this investigation or CHO cells (data not shown).

The mean concentration (± S.E.M.) of muscarinic receptors as well as the K_d values for the membrane preparations from normal ovaries, ovarian tumours and OVCAR-3 cells are shown in Table 1. The receptor concentration in OVCAR-3 cells was significantly higher than that in the ovaries or receptor positive tumours ($P < 0.05$) whereas no significant difference in the K_d values in preparations from ovaries, receptor positive tumours and OVCAR-3 cells was found. In normal ovaries the variation in receptor density was relatively little compared to that among tumours. There was no relationship with the age of the patient and muscarinic receptor density in normal ovaries. Table 2 shows receptor density and K_d values obtained for different tumours along with histological findings. No specific binding could be detected in three of the eight ovarian tumours examined. The K_d values for tumours were very similar to those of the normal ovarian tissue and whereas in two malignant tumours the receptor density was lower, in the third it was considerably higher than that in the ovaries. Similarly, in the two benign tumours, although receptors were present in both, the amounts varied considerably. In patient no. 4 both the primary tumour and the metastasised tumour were obtained and both were receptor-positive. The values in Table 2 are from the primary tumours.

Table 2. Patient information, histological diagnosis and muscarinic receptor density of human epithelial ovarian tumours

Patient	Age	Stage*	Histology	Differentiation (grade)	Receptor density (fmol/mg protein)	K_d (pmol/l)
1	60		Benign cystadenoma		77.9	218.6
2	66		Benign cystadenoma		183.2	207.0
3	76	III	Serous papillary adenocarcinoma	Low	N.S.	
4	54	III	Serous papillary adenocarcinoma	High	55.6	84.4
5	58	III	Serous papillary adenocarcinoma	Low	N.S.	
6	42	I	Mucinous cystadenocarcinoma	High	57.8	97.9
7	75	III	Mucinous cystadenocarcinoma	Middle High	152.5	271.0
8	62	III	Endometrioid adenocarcinoma	Low	N.S.	

* According to FIGO; N.S. = No specific binding.

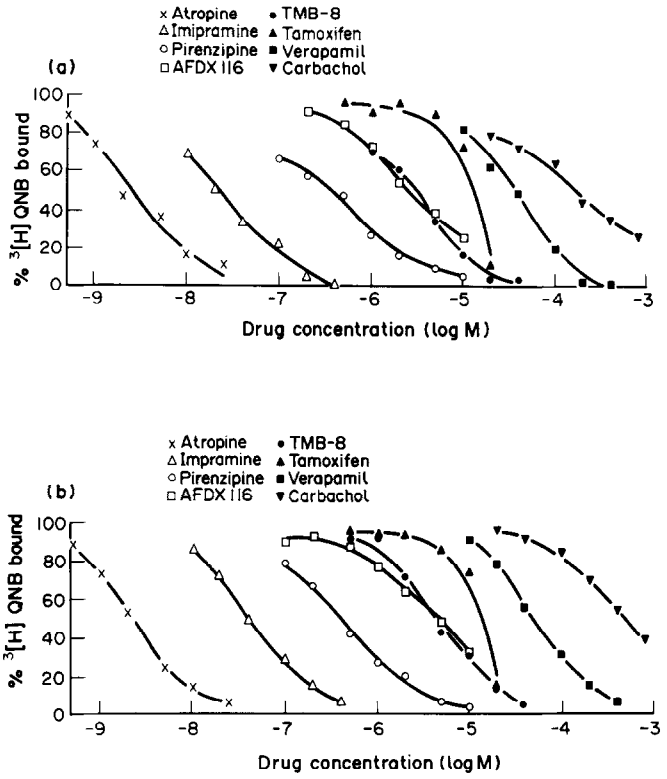

Fig. 2. Inhibition by various drugs of [³H]QNB binding in membrane preparation from cultured OVCAR-3 cells (a) and ovarian tissue (b).

Inhibition by anticholinergic drugs, cholinergic agonist, verapamil and TMB-8 of specific QNB binding to membrane preparations from OVCAR-3 cells and human ovaries is shown in Fig. 2. Each drug was tested at several concentrations and the inhibition constant K_i for each was determined from the IC_{50} value derived from the respective curve. The mean values obtained from the inhibition curves in two separate experiments for the different drugs tested are compiled in Table 3. These

Table 3. Mean IC_{50} and K_i values for various drugs competing with [³H]QNB binding in membrane preparation from ovaries and OVCAR-3 cells

	IC_{50}		K_i	
Drug	Ovary	OVCAR-3	Ovary	OVCAR-3
Atropine	2.18 nmol/l	2.45 nmol/l	0.90 nmol/l	1.18 nmol/l
Imipramine	21.9 nmol/l	41.3 nmol/l	9.82 nmol/l	18.8 nmol/l
Pirenzepine	0.33 μmol/l	0.44 μmol/l	0.14 μmol/l	0.21 μmol/l
AF-DX 116	2.58 μmol/l	4.73 μmol/l	1.06 μmol/l	2.25 μmol/l
TMB-8	3.11 μmol/l	4.81 μmol/l	1.17 μmol/l	1.25 μmol/l
Tamoxifen	12.6 μmol/l	14.0 μmol/l	4.83 μmol/l	6.74 μmol/l
Verapamil	33.4 μmol/l	52.6 μmol/l	12.9 μmol/l	23.6 μmol/l
Carbacholine	0.18 mmol/l	0.48 mmol/l	0.07 mmol/l	0.22 mmol/l

IC_{50} values were calculated from the dose–response curves (see figures), K_i, values were calculated from the relation $K_i = IC_{50}/(1 + (L)/K_d)$, where (L) is the radioligand concentration and K_d the radioligand equilibrium dissociation constant [9]. Results are means of two separate determinations, run in triplicate.

data showed that atropine competed most strongly for the receptor having a K_i value in the nanomolar range. In contrast to atropine, inhibition by both pirenzepine and the cardioselective agent AF-DX 116 was relatively low. Inhibition by the agonist carbachol was much weaker, the K_i value being several orders of magnitude higher than that of the classical antagonist atropine. The extent of inhibition by the calcium channel blocker verapamil was greater than that of carbachol. The so-called intracellular calcium antagonist TMB-8 inhibited QNB binding with a potency similar to AF-DX 116. Although the pattern of inhibition by the various agents in the preparations from ovaries and OVCAR-3 cells was very similar, the K_i values in OVCAR-3 cells in most instances were found to be higher than in ovarian tissue.

DISCUSSION

Although there is much anatomical evidence for the innervation of the ovary by adrenergic and cholinergic nerves, the exact role of these nerves in the regulation of ovarian function is not known. Cholinergic stimulation has recently been shown to regulate hormone secretion in bovine ovarian cells [11]. Practically no information with regard to the role of cholinergic neuroeffector mechanisms in human ovaries is available. Indeed, there is no information on whether muscarinic cholinergic receptors are present on the human ovarian cells.

The present study describes for the first time, specific, saturable, high affinity muscarinic cholinergic receptors in human ovaries and ovarian carcinoma cells. The data showed that pirenzepine which has a high affinity for m1 but not m2 receptors competed weakly for the QNB binding. This indicates that the cholinergic receptor in the ovaries was not of the m1 type [15]. On the basis of the degree of affinity for AF-DX 116, the m2 type can be discriminated from cardiac (m2) and the glandular (m3) tissues [16]. The data showing a poor competition by both pirenzepine and AF-DX 116 for QNB binding indicate that the receptor is of the m3 type, which is consistent with the suggested localisation of this subclass of muscarinic receptors in glandular tissue. The relatively poor competition by the cholinergic agonist in contrast to the antagonists is in agreement with previous studies on exocrine glands and in prostatic carcinoma [9, 14]. The extent of competition by the calcium channel blocker verapamil for muscarinic receptor binding sites is in good agreement with previously published data [9, 17]. TMB-8, which has been generally used as a inhibitor of intracellular calcium release, exhibited a fair degree of competition for muscarinic receptor binding. This appears to be in agreement with our recent observations on the inhibition by this drug and by verapamil of a cholinergic stimulated increase in intracellular Ca^{2+} in OVCAR-3 cancer cells (unpublished data). The IC_{50} value for TMB-8 was 3–4 μmol/l which is in the range of concentrations used to specifically block the release of intracellular calcium [18, 19]. Caution is, therefore, indicated when using this agent as a specific tool for the inhibition of the release of intracellular calcium. Data showing competition by the anti-oestrogen tamoxifen at muscarinic receptor sites are in agreement with the data presented previously [20].

In the present study we also demonstrated the presence of muscarinic receptors in well differentiated and benign human ovarian tumours and in cultured human ovarian cells, OVCAR-3. The characteristics of these receptors were very similar to those found in ovarian tissue. In contrast to benign or well differentiated tumours, no receptors could be detected in tumours with a low degree of differentiation (Table 2) or in the human ovarian cancer cell line SKOV-3 (data not shown). In contrast to OVCAR-3 cells, SKOV-3 cells are thought to be poorly differentiated [21, 22] which is consistent with the above finding of the absence of receptors in poorly differentiated human ovarian tumours. The present data for the first time also provide evidence for a relationship between the disappearance of neurotransmitter receptors and the loss of differentiation. Muscarinic receptor assay could, therefore, be of use to determine the degree of differentiation in characterising tumour cell lines. In ovaries no relationship was found between receptor density (range = 68–97 fmol/mg protein) and age (range 43–71 years) of the women. Interestingly, in spite of the wide range of the age of the patients and the possible heterogeneity in the ovarian specimens analysed, there was relatively little variation in the receptor density. On the contrary, as can be expected, the variation in receptor density in different tumours was relatively large.

In a previous study on human lung tumours, although the authors found lower amounts of cholinergic receptors in tumours than in healthy lungs, receptors were found in all 11 tumours examined irrespective of different degrees of differentiation [7]. Since the authors did not find in these tumours any structural organisation comparable to lung, they considered the receptors as newly formed in tumour cell membranes. The results of the present study in contrast suggest that the receptors are lost with the loss of differentiation and do not seem to reappear. In a study on histamine receptors on cultured tumour cells the authors reported the presence of histamine receptors on the poorly differentiated human gastric cell line MKN-45 [23].

In conclusion, the results of the present study show that muscarinic receptors of type m3 are present in ovarian tissue, and in ovarian tumours depending on the degree of differentiation. They are also present in cultured ovarian cancer cells OVCAR-3 but absent in SKOV-3 cells, the former in contrast to the latter cell line is well differentiated as epithelial cells [21, 22]. The complete absence of receptors in poorly differentiated ovarian tumours or cultured cells with allegedly poor differentiation can be of prognostic value in patients with ovarian cancer. However, collection of data from a large number of patients with varying degree of differentiation is needed to support this view.

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Expression of O^6-Alkylguanine-DNA-Alkyltransferase *in situ* in Ovarian and Hodgkin's Tumours

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The cellular expression of O^6-alkylguanine-DNA-alkyltransferase (ATase) may be an important factor in determining tumour sensitivity to certain alkylating agents. In a comparative study, we have examined the inter- and intracellular distribution of ATase in tumour biopsies of a series of patients with Hodgkin's disease and ovarian cancer using a rabbit antihuman ATase antiserum. The antibody recognises the ATase protein on western blots of cell-free extracts of a number of ovarian tumours with ATase activities varying from 20 to 420 fmol/mg protein as determined by *in vitro* assay and there was a linear correlation between ATase activity and the intensity of the band on western blots ($r = 0.993$). Immunohistochemical staining was seen in all of the ovarian tumours examined and was confined to the nucleus. This is in contrast to the Hodgkin's tissue, where staining was much reduced and present in both nuclei and cytoplasm. The results suggest that in ovarian tumours the general resistance to nitrosourea chemotherapy may be related to the high cellular expression of ATase protein: this is in contrast to the more chemosensitive Hodgkin's disease. This raises the possibility that it might be feasible to predict sensitivity or resistance to these alkylating agents by immunohistochemical staining of tumour or tissue specimens.
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INTRODUCTION

THE RESPONSE rate of ovarian cancer following treatments with chloroethylating nitrosoureas, dacarbazine and procarbazine is low in comparison with the mustard-type alkylating agents: published reports of more than 1000 patients treated with either melphalan, chlorambucil, thio-tepa or cyclophosphamide have produced objective response rates of 35–65% compared to less than 6% response with nitrosoureas [1]. This is in contrast to Hodgkin's disease where single-agent therapy with either 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-