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CHOLINERGIC ELEMENTS IN A HUMAN CHORIOCARCINOMA CELL LINE*

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Abstract—Components of the cholinergic system have been identified in the JEG choriocarcinoma cell line. [^{3}H]Quinuclidinyl benzilate (QNB) was used to identify high affinity muscarinic binding sites in a whole cell preparation. Specific binding was saturable with respect to QNB concentration and revealed a binding site density of 27 fmoles/mg protein. The bimolecular rates of association, 2.24×10^7 $M^{-1}\,min^{-1}$, and dissociation, $4.2 \times 10^{-3}\,min^{-1}$, revealed a dissociation constant (K_d) of 180 pM which agreed closely with that derived from saturation isotherms, 245 pM. Muscarinic antagonists and agonists were able to compete effectively for these binding sites, whereas non-muscarinic compounds were not. Cholinesterase activity was also demonstrated with substrate preference consistent with that of acetylcholinesterase (acetylcholine > acetyl-β-methylcholine > butyrylcholine) hydrolyzing $2.42 \pm 0.19 \times 10^{-3}$ μmoles acetylcholine $\cdot$ $min^{-1} \cdot$ (mg protein)$^{-1}$. No choline acetyltransferase activity was detected in these cells, however.

The cholinergic system found in tissues of neural origin has been extensively studied and characterized. Of particular interest in recent years has been its presence in non-neuronal tissue with its role there being a source of continued interest and controversy (see review in Ref. 1). Much of the work done in non-neuronal tissues has been conducted in the human placenta. Acetylcholine (Ach) and choline acetyltransferase (ChA) in human placental tissue have been demonstrated for some years [2–5]. The existence of human placental acetylcholinesterase (AChE) activity has been a subject of debate, although recent evidence seems to confirm the presence of syncytiotrophoblast specific AChE [6, 7]. Preliminary efforts to identify cholinergic receptors in human placental microvillous membrane using [^{125}I]-α bungarotoxin have been unsuccessful.‖ We have, however, recently been able to identify [^{3}H]quinuclidinyl benzilate (QNB) binding sites, in a placental membrane preparation, with properties consistent with muscarinic receptor function [8]. Since the human placenta lacks innervation [9] and Ach does not exert a significant effect on the placental vasculature [10], questions have arisen regarding the significance of a cholinergic system in the human placenta.

In seeking to develop a useful model by which we could examine the regulation and role of the placental cholinergic system, our studies were extended to identify and characterize components of the cholinergic system in the JEG choriocarcinoma cell line. The presence of cholinergic elements in a continuous trophoblastic cell line of human origin offers unique advantages as an experimental model and may be of great benefit when used in conjunction with data obtained from normal placental tissue.

MATERIALS AND METHODS

Compounds were obtained as follows. [3-^{3}H] Quinuclidinyl benzilate (sp. act. 43 Ci/mmole) was purchased from Amersham/Searle, (Arlington Heights, IL). [^{14}C]Acetylcholine, [^{14}C]acetyl-β-methylcholine, and [^{14}C]butyrylcholine were obtained from the New England Nuclear Corp. (Boston, MA). Oxotremorine and pilocarpine were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Scopolamine HCl, choline, nicotine sulfate, carbamylcholine, DL-carnitine and atropine sulfate were purchased from the Sigma Chemical Co. (St. Louis, MO). *dl*-Propranolol HCl was obtained from Ayerst Research Laboratories (Montreal, Canada), glycine from the Fisher Scientific Co. (Pittsburgh, PA), and γ-aminobutyric acid (GABA) from ICN Pharmaceuticals (Irvine, CA).

Cell culture. Culture of the JEG cell line was as described previously [11]. In brief, cells were maintained at 37° in a humidified atmosphere of 95% O_2 and 5% CO_2 in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY). The cells were obtained from culture following removal of medium and one rinse with phosphate buffered saline (PBS), pH 7.4, by scraping with a rubber policeman and resuspending in PBS. The cells were centrifuged at 900 *g* for 5 min and resuspended in isotonic MOPS

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‖ C. H. Smith, personal communication.

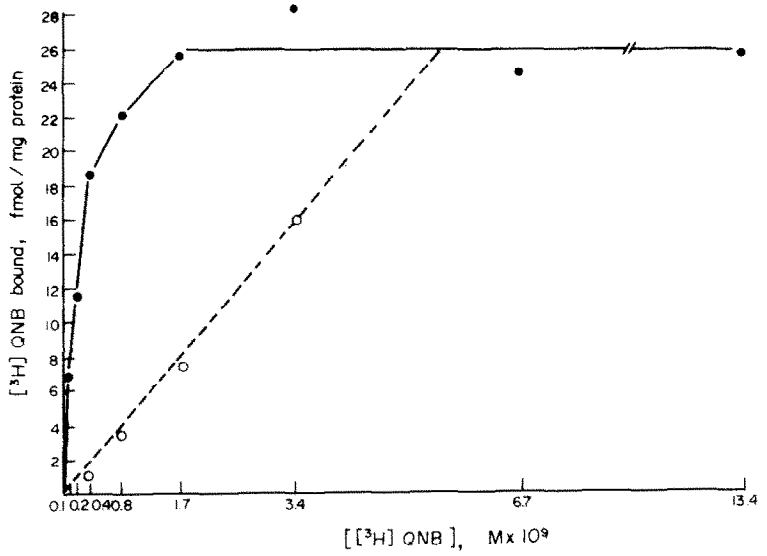

Fig. 1. Specific [³H]QNB binding as a function of ligand concentration. Receptor concentration was 18 pM. Specific binding was determined by the difference between total binding and non-specific binding, in tandem tubes in the absence and the presence of 1 μM scopolamine. All points were determined in triplicate. Data are representative of typical saturation isotherms. Key: (●——●) specific [³H]QNB binding, and (⊙——⊙) non-specific [³H]QNB binding.

(β-*N*-morpholino-propane sulfonic acid) buffer (10 mM NaMOPS, 2.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 134.2 mM NaCl), pH 7.4.

This procedure was repeated twice to thoroughly wash the cell preparation. The cells were resuspended in MOPS buffer, pH 7.4, at a concentration of 7.0 mg protein/ml, rapidly frozen, and stored at −20° until assayed.

To assay specific binding of [³H]QNB, 100 μl aliquots of the cell preparation were incubated in a total volume of 500 μl Na–MOPS buffer (pH 7.4, at 37°) containing various amounts of [³H]QNB. After incubation for 90 min, 3 ml of ice-cold Na–MOPS buffer was added to the incubation tube, and the contents were poured over a GF/F glass fiber filter positioned on a vacuum filtration manifold (Hoeffer); the filters were washed three times with 3 ml of ice-cold buffer. Every concentration point in the binding assay was done in triplicate along with triplicate samples containing 1 μM scopolamine to determine non-specific [³H]QNB binding. The filters were dried and placed in 12 ml of a Triton X-100–toluene–phosphor liquid scintillation mixture and maintained at room temperature for 16 hr. Radioactivity was then determined using a Packard Tricarb liquid scintillation spectrometer at a counting efficiency of 40 per cent. Variability within and between experiments was always less than 10 per cent and usually less than 5 per cent.

Choline acetyltransferase and acetylcholinesterase activities were assayed by radiometric assays described previously [12, 13]. Protein was determined by the method of Lowry *et al.* [14], using bovine serum albumin as a standard.

RESULTS

Saturability of [³H]QNB binding to JEG cells. Specific [³H]QNB binding was saturable with increasing concentrations of ligand (Fig. 1). The half-maximum binding concentration was 245 pM (by Scatchard analysis). Non-specific binding, however, increased linearly with increasing QNB concentration. Typical binding experiments were done with a receptor con-

Table 1. Comparison of enzyme activities in JEG cells with those found in normal human placenta and rat brain*

	Cholinesterase substrates (ChE)			
	Acetylcholine	Acetyl-β-methylcholine	Butyrylcholine	Choline acetylase (ChA)
JEG cells	2.42 ± 0.19	0.34 ± 0.05	<0.05	<0.05
Rat brain†	11.7–354.0			0.8–111.0
Placental vesicles†	13.8 ± 0.9	2.7 ± 0.4	0.1 ± 0.1	0.15 ± 0.03

* All enzyme activity is expressed as nmoles substrate hydrolyzed (ChE), or product formed (ChA), per min per mg protein. JEG cell and placental vesicle data are means ± S.E.M., N = 3.

† Data abstracted from literature values [7, 16].

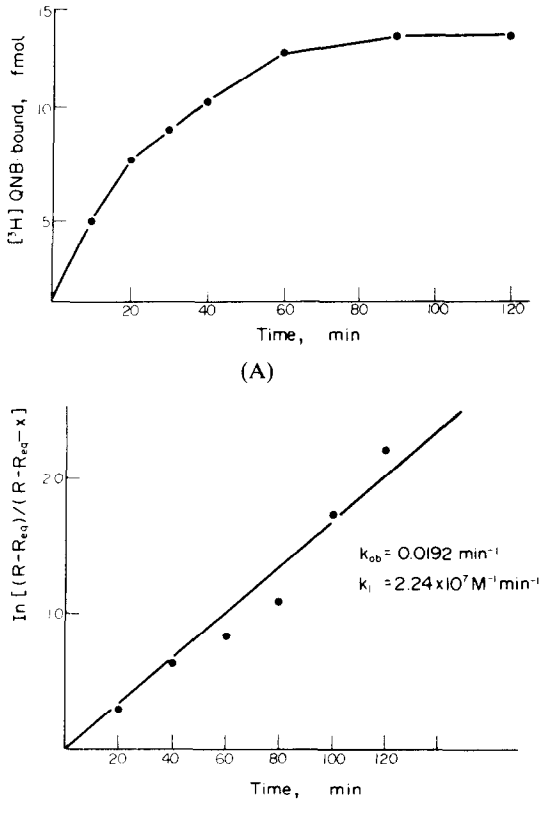

Fig. 2. Panel A. Rate of association of [^{3}H]QNB binding as a function of time. Incubation was performed at 37°. Non-specific binding was determined in the presence of 1 μM scopolamine. The concentration of [^{3}H]QNB was 0.67 nM; the receptor concentration was 36 pM. Each point represents the average of triplicate determinations. Panel B: Pseudo first-order rate plot of [^{3}H]QNB binding. Data were taken from panel A. Abbreviations: R, total concentration of receptors (R = 36 pM); R_{eq}, number of free receptors at equilibrium; x, concentration of bound [^{3}H]QNB at each time; and k_1 rate constant for the association of receptor and ligand. The slope of the line (k_{ob}), determined by linear regression analysis, estimates the observed rate constant for the pseudo first-order reversible reaction. The treatment of the data as a pseudo first-order reaction is valid [15] since $[R]/[QNB] = 0.05$.

centration at which specific QNB binding was linear with varying protein concentrations. Non-specific binding in a typical binding experiment ([^{3}H]QNB, 670 pM) was less than 20 per cent of the total binding.

Association and dissociation of specific [^{3}H]*QNB binding.* At 37°, specific [^{3}H]QNB binding was approximately half-maximum at about 20 min and was complete at about 90 min (Fig. 2A). The bimolecular rate constant (k) for association was $2.24 \times 10^7\,M^{-1}\,min^{-1}$ (Fig. 2B). The rate of dissociation of the QNB–receptor complex was determined at 37° (Fig. 3). The rate constant for dissociation (k_2) was $4.2 \times 10^{-3}\,min^{-1}$ with a half-life of about 2.5 hr. The dissociation constant, k_2/k_1, was 180 pM, which agrees closely with the K_d value derived from saturation isotherms, 245 pM.

Effects of various drugs on specific [^{3}H]*QNB binding.* The muscarinic antagonists—scopolamine and

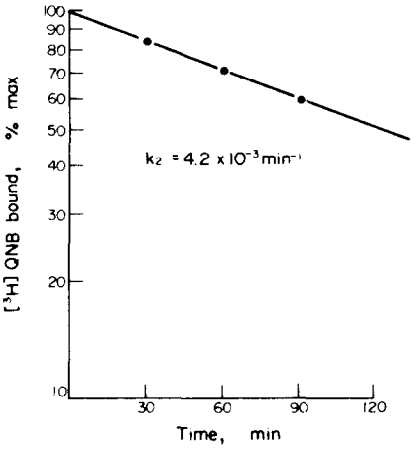

Fig. 3. Dissociation of [^{3}H]QNB from JEG cells. The cells were incubated with 0.67 nM QNB for 90 min at 37°. Scopolamine (1 μM) was added to the incubate at t = 0. The slope of the line provides an estimate of the dissociation rate constant, k_2.

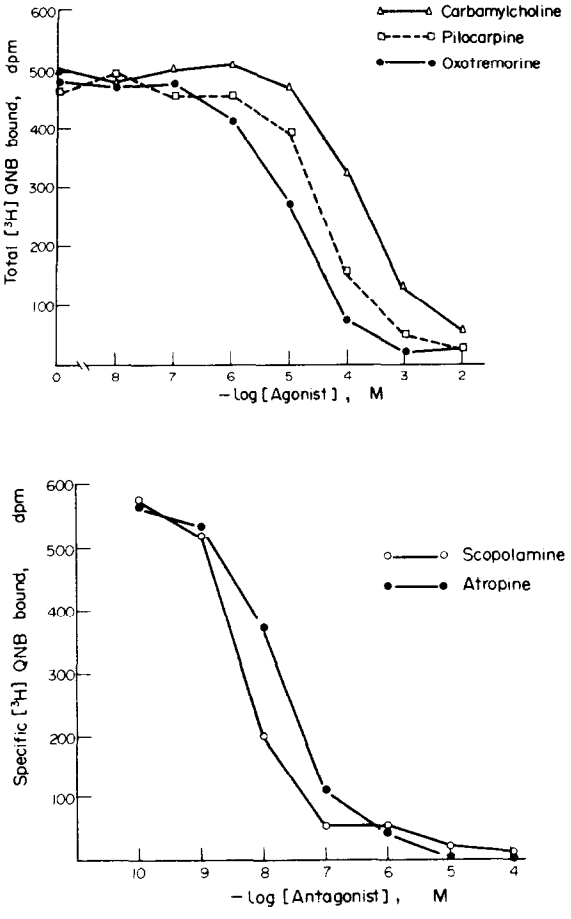

Fig. 4. Inhibition of specific [^{3}H]QNB binding to JEG cells by muscarinic agonists (A) and antagonists (B). [^{3}H]QNB concentration was 0.67 nM; [R] (agonist) = 18 pM, and (R) (antagonist) = 9 pM.

atropine—showed considerable ability to compete with [^{3}H]QNB for the binding sites (K_i = 1.2 nM and 3.4 nM respectively), whereas the agonists—oxotremorine, carbamylcholine and pilocarpine—possessed several orders of magnitude less affinity (K_i = 9.2 μM, 0.13 mM and 12 μM respectively) (Fig. 4, A and B). Nicotine was able to compete to a limited extent, whereas compounds devoid of any cholinergic activity, i.e. propranolol, GABA, glycine, carnitine and choline, were ineffective at 100 μM.

Choline acetyltransferase and cholinesterase activity in JEG cells. The JEG cells contained cholinesterase activity that showed a substrate specificity consistent with acetylcholinesterase (acetylcholine > acetyl-β-methylcholine > butyrylcholine) (see Table 1). No choline acetyltransferase activity was detected. All enzyme assays were performed in the presence of 0.5% Triton X-100; approximately 63 per cent of the AchE remained in the absence of the detergent, suggesting that at least part of the enzyme activity was on the external surface of the cell.

DISCUSSION

The data reported here suggest that the QNB binding sites represent muscarinic cholinergic receptors. The binding saturated a small population of sites, 27 fmoles per mg protein, with an affinity comparable to that reported for rat brain and guinea pig ileum [16, 17]. The K_d values determined from both kinetic data and saturation isotherms, however, show that these sites had a K_d that was 5- to 10-fold greater than that found in ileum and brain. It was much lower than the dissociation constants reported for several non-neuronal tissues, however—i.e. lymphocytes, leukocytes and erythrocytes [18–20]—and the same as that seen with the neuroblastoma clone N1E-115 [21]. This variation may reflect differences in methodology and/or species. The existence of regional differences in muscarinic receptors in rat brain has been reported [22]. It therefore appears that the concept of muscarinic receptors representing a heterogenous population of binding sites may be a very reasonable one. Whether major differences can be shown to be a characteristic of receptors of neuronal or non-neuronal origin or simply reflect differences in mechanisms of action regardless of tissue of origin remains to be seen. The binding in these cells reached equilibrium at about 90 min at 37° and was reversible with a half-life of about 2.5 hr. This is consistent with its relatively slow rates of association and dissociation seen in rat brain. The binding of [^{3}H]QNB revealed specificity expected of muscarinic receptors. Atropine and scopolamine were very effective competitors in the nanomolar range, whereas the classic muscarinic agonists were several orders of magnitude less effective. The order of agonist effectiveness, in decreasing order, was oxotremorine > pilocarpine > carbamylcholine. This is the same order of specificity seen in muscarinic receptors of rat brain and the neuroblastoma NIE-115 cell line [16, 21]. The nicotinic agent nicotine was able to reduce specific binding 22 per cent at 10^{-4} M, although several non-cholinergic agents were ineffective competitors at this concentration. These observations, when viewed collectively, suggest that, with respect to affinity, specificity and density, these [^{3}H]QNB binding sites are consistent with muscarinic cholinergic receptors.

Enzymatic analysis of this cell line revealed that although no choline acetyltransferase could be detected, acetylcholinesterase was present in amounts comparable to those reported in other tissues.

We have previously demonstrated the presence of cholinergic elements including muscarinic binding sites, in a microvillous membrane vesicle preparation from human placental tissue [7]. The role of acetylcholine in placental function and, consequently, fetal growth and development is unknown. We have developed the placental vesicle model to study further cholinergic-specific membrane interactions. The use of membrane vesicles has obvious limitations in examining cholinergic function in regard to general cellular metabolism. The JEG cells, which represent a choriocarcinoma cell line of human origin, should prove a valuable adjunct which can extend our studies of placental cholinergic function and regulation.

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