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Pharmacological characterization and distribution of muscarinic receptors in human placental syncytiotrophoblast brush-border and basal plasma membranes

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Abstract

Based on the existence of choline acetyltransferase and acetylcholine in human placenta, we have investigated the presence of muscarinic acetylcholine receptors in brush-border and basal plasma membranes from human term placenta. Radioligand binding assay, using [^{3}H]*N*-methyl-scopolamine as tracer, showed the existence of acetylcholine muscarinic receptors in brush-border (K_d 0.28 ± 0.04 nM; B_{max} 9.4 ± 1.6 fmol/mg protein) and basal plasma membranes (K_d 0.24 ± 0.05 nM; B_{max} 34.3 ± 6.3 fmol/mg protein). In order to perform a pharmacological characterization of these receptors, competition binding experiments were carried out using the muscarinic receptor antagonists pirenzepine, (11(2-diethyl-amino)methyl)-1-piperidinylacetyl-5-11-dihydro-6*H*-pyrido(14)benzodiazepine (AF-DX 116), himbacine, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), dicyclomine and hexahydro-sila-difenidol (HHSD). The results obtained showed that the muscarinic receptors in brush-border and basal plasma membranes belong to different subtypes. In brush-border membranes, the receptor found match in terms of affinity for the antagonists with the muscarinic M_1 receptor subtype (K_i pirenzepine, 13.6 ± 8.2 nM; K_i AF-DX 116, 1680 ± 271 nM; K_i himbacine, 212 ± 6.5 nM; K_i 4-DAMP, 1.5 ± 0.4 nM; K_i dicyclomine, 5.1 ± 0.8 nM; K_i HHSD, 34.3 ± 7.3 nM), whereas the receptor in basal plasma membrane seems to be of the muscarinic M_2 receptor subtype (K_i pirenzepine, 202 ± 48 nM; K_i AF-DX 116, 124 ± 60 nM; K_i himbacine, 20.6 ± 4.8 nM; K_i 4-DAMP, 4.5 ± 1.2 nM; K_i dicyclomine, 54.6 ± 22 nM; K_i HHSD, 89.2 ± 15.8 nM). The results obtained show the existence of muscarinic acetylcholine receptors in brush-border and basal plasma membranes from human term placenta with a different distribution pattern in terms of number of receptors and distribution of different subtypes. The functional significance of these findings is as yet unknown, but these receptors probably mediate different functions as they belong to different subtypes and are coupled to different second messengers.

Keywords: Muscarinic receptor; Muscarinic receptor subtype; Placenta; (Human)

1. Introduction

During the last two trimesters of gestation the human placenta is an organ that occupies a unique physiological position, and is able to play an important endocrine role. This organ synthesizes a large variety of hormones, such as progesterone, estradiol and estriol from precursors obtained from the mother or fetus which are secreted in both maternal and fetal blood compartments (Bourget et al., 1995).

The human syncytiotrophoblast is known to serve several roles in pregnancy. It mediates the transport of nutrients, exchange of minerals and immunoglobulins from the maternal to fetal circulation. This polarized epithelium has a cell layer with a microvillous apical plasma membrane (brush-border) facing maternal blood, containing filamentous internal actin structure and rich in alkaline phosphatase activity, and a smooth basal membrane facing fetal circulation rich in adenylate cyclase and Na^+,K^+-ATPase activities.

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Although it has been demonstrated that human placenta is able to synthesize and secrete acetylcholine (Sastry et al., 1976; Olubadewo and Sastry, 1977), the function of this neurotransmitter in a non-innervated organ has not yet been clearly defined. It has been suggested that acetylcholine could regulate placental amino-acid transport (Rowell and Sastry, 1980; Yudilevich and Sweiry, 1985), blood flow (Boura et al., 1986), the release of placental hormones, such as chorionic somatomamotrophin, and/or regulate prostaglandin output from placental tissue (King et al., 1991). However, the existence of muscarinic cholinergic receptors in human placental tissues has not been clearly demonstrated. The first report on the existence of muscarinic receptors in human placenta (Fant and Harbison, 1977) was not confirmed in further studies by other authors (Welsch and Wennerberg, 1978), and no additional evidence on this field has been produced since then.

In order to clarify these controversial observations, the aim of this study was focused on investigating the presence and distribution of muscarinic receptors in brush-border and basal plasma syncytiotrophoblast membranes from human term placenta.

2. Material and methods

2.1. Membrane preparation

Human term placentas were obtained within 30 min after normal delivery and placed on ice-cold 0.9% NaCl. All the procedures were carried out at 4°C. The umbilical cord, amnion, chorion and fibrous membranes were removed and the syncytiotrophoblastic tissue was cut into small pieces with scissors. Brush-border membranes were prepared using a homogenization and Mg^{2+} precipitation technique (Glacier et al., 1988) and basal plasma membranes were obtained according to a method described previously (Kelley et al., 1983). Both syncytiotrophoblast basal plasma and brush-border membrane preparations were resuspended in 5 mM HEPES/Tris, pH 7.4, containing proteinase inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1 μg/ml bacitracin and 0.1 μg/ml phenylmethanesulphonyl fluoride) and were frozen at −80°C until use for muscarinic receptor binding assay. The purity of preparations was assessed by measuring the enrichment of the brush-border membrane enzyme alkaline phosphatase (Lansing et al., 1967) and the basal plasma membrane marker, [^{3}H]dihydroalprenolol binding (Kelley et al., 1983), as compared with the homogenate.

2.2. Drugs and ligands

[^{3}H]*N*-Methyl-scopolamine (specific activity 80–84 Ci/mmol) was purchased from Amersham (Amersham, UK). HEPES, Tris and other chemicals were purchased from Sigma (st. Louis, MO, USA). 4-Diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), dicyclomine and hexahydro-sila-difenidol (HHSD) were from Research Biomedicals International (Natick, MA, USA). Atropine sulphate was a generous gift from B. Braun Medical (Barcelona, Spain). Pirenzepine dihydrochloride and (11(2-diethyl-amino)methyl)-1-piperidinylacetyl-5-11-dihydro-6*H*-pyrido(14)benzodiazepine (AF-DX 116) were generous gifts from Dr. Karl Thomae (Biberach, Germany). Himbacine was a generous gift from Dr. W.C. Taylor (Sydney, Australia).

2.3. Binding assays

Membranes were resuspended in 20 mM HEPES buffer, pH 7.4, containing 50 mM NaCl and 10 mM $MgCl_2$, to achieve the final protein concentration of approximately 300 μg/ml (296 ± 15) for brush-border membranes and 140 μg/ml (140 ± 12) for basal plasma membranes. Samples were incubated for 45 min at 30°C in the presence of the radioligand ([^{3}H]*N*-methyl-scopolamine) in a final volume of 1 ml, in the absence or presence of 10 μM atropine to define the specific binding. Binding reactions were stopped by the addition of 4 ml of ice-cold 20 mM Tris buffer, pH 7.4, and then bound and free ligands were separated by rapid filtration under vacuum through Whatman GF/B glass filters, presoaked with 0.1% polyethylenimine for 1 h before filtration using a Brandel filtration manifold. The filters were washed three times with 4 ml of ice-cold distilled water, and placed in vials containing 4 ml of scintillation liquid (Formula 989 Liquid Scintillation Cocktail, DuPont NEN, Wilmington, DE, USA). The radioactivity was measured by liquid scintillation counting (Wallac 1410) with 50% counting efficiency. Saturation experiments were carried out with eight concentrations of [^{3}H]*N*-methyl-scopolamine, ranging from 0.05×10^{-9} M to 2×10^{-9} M. Inhibition studies of the binding of 2×10^{-9} M [^{3}H]*N*-methyl-scopolamine with the muscarinic receptor antagonists pirenzepine, AF-DX 116, himbacine, 4-DAMP, dicyclomine and HHSD were carried out with 22 concentrations of these drugs, ranging from 6×10^{-10} M to 8×10^{-5} M.

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.4. Data analysis

All binding data were analyzed by iterative curve fitting procedure using the LIGAND program modified by McPearson (1985). In saturation experiments, the statistical analysis was performed by the analysis of covariance test; statistical significance was set at $P < 0.05$. All data represent the mean ± S.E.M. from three to five experiments performed in triplicate.

3. Results

3.1. Membrane purity

In order to obtain a correct interpretation of the binding data, the purity of the membrane preparation was previously analyzed. Alkaline phosphatase activity in syncytiotrophoblast brush-border membranes was on average enriched 18-fold compared to the homogenate (homogenate: 1.1 ± 0.2; brush-border membranes: 20.4 ± 4.8; basal plasma membranes: 2.1 ± 0.9 μmol/min per mg protein), while [^{3}H]dihydroalprenolol binding in basal plasma membranes was more than 30-fold greater than the binding in the homogenate (homogenate: 90.5 ± 8.1; brush-border membranes: 87.4 ± 7.2; basal plasma membranes: 2843.1 ± 313.8 fmol/mg protein).

3.2. Binding assays

To perform a pharmacological characterization of the muscarinic cholinergic receptors in human placental syncytiotrophoblast brush-border and basal plasma membranes, saturation and competition binding experiments were carried out. Specific [^{3}H]*N*-methyl-scopolamine binding reached the equilibrium at 20 min of incubation at 30°C and was stable for up to 90 min (data not shown). The results obtained in saturation experiments with [^{3}H]*N*-methyl-scopolamine to placental membranes (Table 1) revealed the existence of a single population of muscarinic receptors in both brush-border and basal plasma membrane preparations (Fig. 1) with similar affinities (K_d 0.28 ± 0.04 nM and 0.24 ± 0.05 nM, respectively ($P = 0.51$)). Nevertheless, the total number of muscarinic receptors was higher in brush-border than in basal plasma membranes (B_{max} 34.3 ± 6.3 and 9.4 ± 1.6 fmol/mg protein, respectively ($P < 0.05$)), with the Hill coefficients in both locations close to 1 (0.93 ± 0.04 and 1 ± 0.02).

Table 1
Results from [^{3}H]*N*-methyl-scopolamine saturation experiments on syncytiotrophoblast brush-border and basal plasma membranes from human term placenta

	Brush-border membranes	Basal plasma membranes
K_d (nM)	0.28 ± 0.04	0.24 ± 0.05
B_{max} (fmol/mg protein)	9.4 ± 1.6	34.3 ± 6.3 [a]
n_H	0.93 ± 0.04	1.0 ± 0.02

Means ± S.E.M. from five experiments performed in triplicate are shown.
[a] $P < 0.05$ versus brush-border membranes.

Table 2
Results from competition experiments of the binding of [^{3}H]*N*-methyl-scopolamine versus pirenzepine, AF-DX 116, himbacine, 4-DAMP, dicyclomine and HHSD on syncytiotrophoblast brush-border and basal plasma membranes from human term placenta

Drugs	Brush-border membranes		Basal plasma membranes	
	K_i (nM)	n_H	K_i (nM)	n_H
Pirenzepine	13.6 ± 8.2	0.87 ± 0.07	202 ± 48	0.84 ± 0.06
AF-DX 116	1680 ± 271	0.98 ± 0.06	142 ± 78	0.85 ± 0.07
Himbacine	212 ± 6.5	0.87 ± 0.03	20.6 ± 4.8	0.85 ± 0.05
4-DAMP	1.5 ± 0.4	1.05 ± 0.2	4.5 ± 1.2	0.85 ± 0.05
Dicyclomine	5.1 ± 0.8	0.91 ± 0.1	54.6 ± 22	1.1 ± 0.1
HHSD	34.3 ± 7.3	0.98 ± 0.1	89.2 ± 15.8	0.87 ± 0.1

Means ± S.E.M. from three experiments performed in triplicate are shown.

Competition experiments with the muscarinic receptor antagonists pirenzepine, AF-DX 116, himbacine, 4-DAMP, dicyclomine and HHSD are summarized in Table 2, and the competition curves for these antagonists in brush-border and basal plasma membranes are shown in Figs. 2 and 3, respectively. Data show clear differences in terms of affinities between syncytiotrophoblast brush-border and basal plasma membranes from human placenta, with the rank potency of inhibition (K_i): 4-DAMP > dicyclomine >

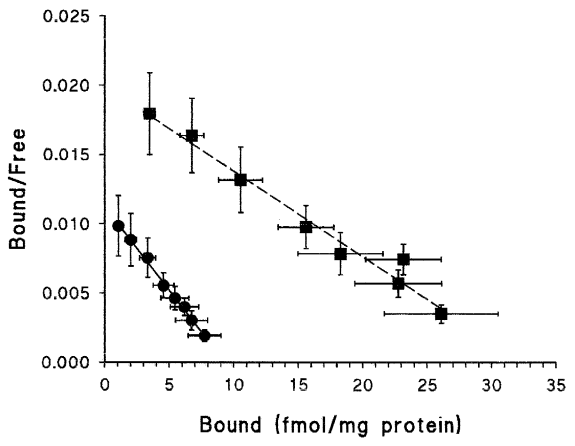

Fig. 1. Scatchard representation from [^{3}H]*N*-methyl-scopolamine saturation experiments in syncytiotrophoblast brush-border (●) and basal plasma membranes (■) from human term placenta. Means ± S.E.M. from five experiments performed in triplicate are plotted.

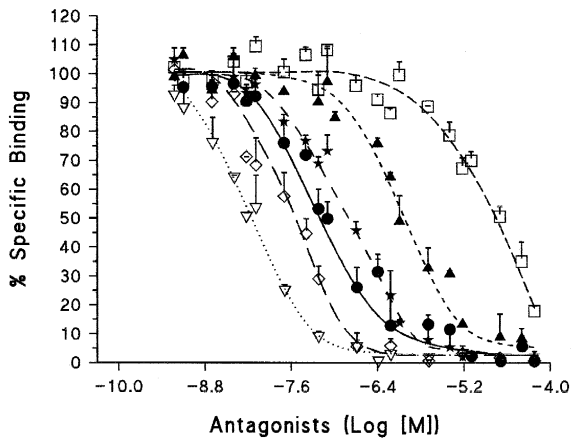

Fig. 2. Competition experiments of [^{3}H]*N*-methyl-scopolamine versus the antagonists pirenzepine (●), AF-DX 116 (□), himbacine (▲), 4-DAMP (▽), dicyclomine (◇) and HHSD (★) in syncytiotrophoblast brush-border membranes from human term placenta. Means ± S.E.M. from three experiments performed in triplicate are plotted.

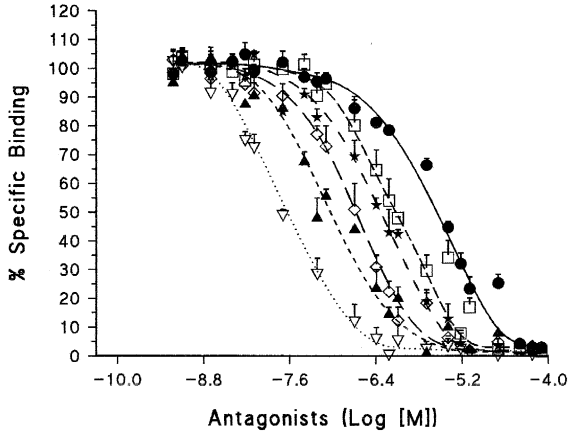

Fig. 3. Competition experiments of [^{3}H]*N*-methyl-scopolamine versus the antagonists pirenzepine (●), AF-DX 116 (□), himbacine (▲), 4-DAMP (▽), dicyclomine (◇) and HHSD (★) in syncytiotrophoblast basal plasma membranes from human term placenta. Means ± S.E.M. from three experiments performed in triplicate are plotted.

pirenzepine > HHSD > himbacine > AF-DX 116 for brush-border membranes, and 4-DAMP > himbacine > dicyclomine > HHSD > AF-DX 116 > pirenzepine for basal plasma membranes. In the analysis of all competition experiments with the above antagonists the Hill coefficients were close to 1, suggesting the existence of a homogeneous population of binding sites in both brush-border and basal plasma membranes. Moreover, in all the experiments performed, the best fit was to the one-site model. All these data, taken together, suggest that the muscarinic receptors expressed in syncytiotrophoblast brush-border and basal plasma membranes from human term placenta belong to different subtypes.

4. Discussion

Human placenta express a variety of different receptors, among them endothelin (McQueen et al., 1993), dopamine D_2 and 5-HT_2 (Vaillancourt et al., 1994), epidermal growth factor (Duello et al., 1994), estrogen and progesterone (Patricio, 1994), calcitonin (Lafond et al., 1994), angiotensin II (Jiménez et al., 1996) and α- and β-adrenoceptors (Falkay et al., 1994). Furthermore, several mRNA encoding for different receptors have been found in human placenta, such as human κ-opioid receptor (Mansson et al., 1994) and histamine H_1 receptor (Fukui et al., 1994). However, contradictory data on the existence of muscarinic acetylcholine receptors in this tissue exist. Whereas some authors have suggested the presence of binding sites for muscarinic ligands (Fant and Harbison, 1977), this finding has not been confirmed by other authors (Welsch and Wennerberg, 1978).

Based on the existence of choline acetyltransferase and acetylcholine in human placenta, we have investigated the presence of muscarinic cholinergic receptors in syncytiotrophoblast brush-border and basal plasma membranes from human term placenta.

The results of the present study indicate the presence of specific and saturable binding sites for [^{3}H]*N*-methyl-scopolamine in both brush-border and basal plasma membranes from human term placenta that label a single population of muscarinic receptors. The dissociation constant (K_d) for the tracer as determined by saturation experiments was in agreement with previously reported data obtained in several tissue preparations (Waelbroeck et al., 1990; Lazareno et al., 1990; Pavía et al., 1991) and in transfected cell lines expressing different muscarinic receptor subtypes (Dörje et al., 1991). The number of binding sites per mg of protein which can be labeled by the tracer in basal plasma membranes was comparable to that reported for human submandibular gland (Martos et al., 1985), and was greater than in brush-border membranes. These findings unequivocally show the existence of muscarinic cholinergic receptors in human term placenta.

At present, at least three pharmacologically different muscarinic receptor subtypes have been defined: M_1 acetylcholine receptors which have high affinity for pirenzepine, and are preferentially localized in nervous tissue; M_2 acetylcholine receptors which have a high affinity for AF-DX 116, and are preferentially localized in cardiac tissue, and M_3 acetylcholine receptors which have a high affinity for both HHSD and 4-DAMP, and are localized preferentially in glandular tissue (Hulme et al., 1990). A fourth subtype (M_4) has been suggested in rabbit lung, chicken heart and NG108-15 cells (Lazareno et al., 1990). Moreover, five different molecular subtypes have now been delineated: m_1, m_2, m_3, m_4 and m_5. There seems to be a general agreement that the molecular subtypes m_1, m_2 and m_3 represent the pharmacological muscarinic M_1, M_2 and M_3 receptor subtypes, respectively (Goyal, 1989).

In order to pharmacologically characterize the above described binding sites, several antagonists competing with [^{3}H]*N*-methyl-scopolamine were used in this work. The inhibition constant (K_i) values obtained in brush-border membrane preparation with pirenzepine, a selective muscarinic M_1 receptor antagonist, were closer to the values found for the muscarinic M_1 than for the M_3 receptor subtype in mammalian tissues (Hulme et al., 1990; Delmedo et al., 1989) and than for the muscarinic m_3 or m_4 receptor subtypes when expressed in transfected cell lines (Buckley et al., 1989; Dörje et al., 1991). These results, in terms of high affinity for pirenzepine, were in agreement with those obtained when using AF-DX 116, a selective muscarinic M_2 receptor antagonist, as the competing drug which showed low affinity for the receptors found in brush-border membranes. The K_i values for this antagonist were similar to those found in transfected cell lines expressing the muscarinic m_1 receptor subtype (Buckley et al., 1989). These findings strongly suggest that the receptor in brush-border membranes belongs to the muscarinic M_1 receptor subtype.

Interestingly, pirenzepine showed low affinity for the muscarinic receptor subtype present in basal plasma membranes. This affinity was similar to that found in rat heart, a tissue that mainly expresses the muscarinic M_2 receptor subtype (Delmedo et al., 1989; Lazareno et al., 1990), and closer to the muscarinic m_2 than to the m_4 receptor subtype when expressed in transfected cell lines (Dörje et al., 1991). Again, the behavior of the antagonist AF-DX 116 was the opposite, showing high affinity for the muscarinic receptor subtype in basal plasma membranes. The K_i values in this preparation were similar to the values found for the muscarinic M_2 receptor subtype expressed in rat heart (Delmedo et al., 1989) and in transfected cell lines expressing the muscarinic m_2 receptor subtype (Buckley et al., 1989). These findings strongly suggest that the receptor in basal plasma membranes belong to the muscarinic M_2 receptor subtype.

As we started to perform the competition experiments using the two antagonists with the highest selectivity (pirenzepine – M_1, AF-DX 116 – M_2), and based on the K_i values found, it was highly tempting to think that the receptors found in brush-border and basal plasma membranes correspond to the muscarinic M_1 and M_2 receptor subtypes respectively. In order to confirm this possibility and to establish a difference between muscarinic M_1-M_3 and M_2-M_4 receptor subtypes, further competition experiments using the muscarinic receptor antagonists HHSD, 4-DAMP, himbacine and dicyclomine were carried out.

Regarding brush-border membranes from human placenta, HHSD showed K_i values closer to the muscarinic m_1 than to the m_3 receptor subtype expressed in transfected cell lines (Buckley et al., 1989). Moreover, K_i values for 4-DAMP in the same membranes were closer to the muscarinic M_1 than to the M_3 receptor subtype (Lazareno et al., 1990), whereas both himbacine and dicyclomine showed K_i values similar to the values found in transfected cell lines expressing the muscarinic M_1 receptor subtype (Dörje et al., 1991). When the same experiments were performed in basal plasma membranes from human placenta, himbacine showed K_i values closer to the muscarinic m_2 than to the m_1 receptor subtype (Dörje et al., 1991), while the K_i values for dicyclomine were closer to the values for the muscarinic M_2 than for the M_4 receptor subtype expressed in rat heart and rabbit lung, respectively (Lazareno et al., 1990). HHSD and 4-DAMP showed K_i values similar to those found for the muscarinic m_2 receptor subtype when expressed in transfected cell lines (Dörje et al., 1991) or rat heart (Lazareno et al., 1990).

Competition experiments show therefore clear differences in terms of affinities between brush-border and basal plasma membranes from syncytiotrophoblast, supporting the idea that the receptors found in brush-border and basal plasma membranes can be preliminarily classified as the muscarinic M_1 and M_2 receptor subtypes respectively. Interestingly, the muscarinic M_2 receptor subtype was expressed in 4-fold higher number than the muscarinic M_1 receptor subtype in human term placenta. Our observations demonstrating a polar distribution of different subtypes of muscarinic acetylcholine receptors in human syncytiotrophoblast tissues are in agreement with our previous findings for angiotensin II receptors (Jiménez et al., 1996).

In summary, the major findings of this study are as follows. Firstly, specific [^{3}H]*N*-methyl-scopolamine binding to muscarinic acetylcholine receptors has been for the first time clearly identified in syncytiotrophoblast brush-border and basal plasma membranes from human term placenta. Secondly, the muscarinic receptors in syncytiotrophoblast brush-border and basal plasma membranes from human term placenta showed a different pattern in terms of the number of receptors expressed. Thirdly, a preliminary muscarinic receptor subtype classification in this tissue has been performed, showing that the receptors in brush-border and basal plasma placenta membranes belong respectively to the muscarinic M_1 and M_2 receptor subtypes.

The functional significance of these findings in terms of the number of receptors and distribution of different subtypes in this tissue is as yet unknown, but these receptors probably mediate different functions as they belong to different subtypes and are coupled to different second messengers. Several possibilities have been proposed for the effect of placental acetylcholine, such as a role in the control of amino-acid transport, in the regulation of placental blood flow, an effect on the release of placental hormones, and/or an involvement in the mechanism of human parturition (King et al., 1991).

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