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Human HT-29 colon carcinoma cells contain muscarinic M_3 receptors coupled to phosphoinositide metabolism

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Five different muscarinic receptor subtypes can be distinguished by the differences in their amino acid sequence, the coupled signal transduction system, pharmacological binding properties and activation of ionic fluxes. The present study served to characterize the binding profile of muscarinic receptors in human colon carcinoma cells (HT-29) using selective muscarinic antagonists. The affinities of the compounds were compared with their potency to inhibit cholinergically-activated phosphoinositide metabolism. Pirenzepine displaced [^{3}H]N-methyl-scopolamine binding and inhibited inositolphosphate (IP) release with potencies typical of those of non-M_1 receptors. The M_3 subtype-selective antagonists sila-hexocyclium and hexahydro-sila-difenidol had high affinity to the muscarinic receptors in HT-29 cells (K_D = 3.1 nM and 27 nM, respectively) and inhibited IP release at nanomolar concentrations. The M_2 receptor antagonists, AF-DX 116 and methoctramine, had low antimuscarinic potencies. Our results demonstrate that HT-29 human colon carcinoma cells contain an apparently pure population of M_3 receptors. These cells could serve as a model system for further investigations concerning regulatory and signal transduction mechanisms associated with glandular muscarinic M_3 receptors.

Muscarinic M_3 receptor subtypes; HT-29 colon carcinoma cells; Phosphatidylinositol metabolism; AF-DX 116; Sila-hexocyclium; Hexhydro-sila-difenidol; Methoctramine

Introduction

The activation of muscarinic acetylcholine receptors (mAChR) in a wide variety of tissues induces hydrolysis of polyphosphoinositides and subsequently increases the release of the second messengers, inositoltrisphosphate (IP_3) and diacylglycerol (DAG) (Nathanson, 1987). This results in mobilization of intracellular calcium stores and activation of the calcium-sensitive, phospholipid-dependent protein kinase C family (Berridge, 1987; Nishizuka, 1986).

The development of antagonists which have different affinities for subtypes of muscarinic receptors permitted their pharmacological classification. The M_1-selective antagonist, pirenzepine, allowed M_1 and M_2/M_3 receptors (terminology used according to Bonner et al., 1988) to be differentiated based on a higher affinity to M_1 receptors (Hammer et al., 1980). Furthermore, the discovery of selective antagonists permitted a distinction between cardiac (M_2; previously termed $M_{2\alpha}$ by us) and glandular or smooth muscle-typical (M_3; previously termed $M_{2\beta}$ by us) receptor subtypes. Antagonists such as sila-hexocyclium (SiHC)

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and hexahydro-sila-difenidol (HHSiD) bound potently to glandular M_3 receptors at nanomolar concentrations, while methoctramine or AF-DX 116 showed higher affinities for cardiac M_2 receptors (Mutschler and Lambrecht, 1984; Lambrecht and Mutschler, 1985; Giachetti et al., 1986; Hammer et al., 1986; Melchiorre et al., 1987; Lambrecht et al., 1988; Waelbroeck et al., in press).

The existence of at least five muscarinic receptor subtypes has now been confirmed in molecular terms (Kubo et al., 1986a,b; Bonner et al., 1987; Peralta et al., 1987a,b). Experiments on expression of cloned receptors in neuroblastoma cells and CHO-K1 cells proved the activity of these receptors and showed their specific coupling to different second messenger systems and ionic currents (Fukuda et al., 1988; Peralta et al., 1988). Although the terminology for the different receptor subtypes is still confusing, binding studies indicate that muscarinic receptors with low affinities for pirenzepine are best represented by the M_3 receptor subtype. These receptors bind HHSiD and SiHC with high affinity and are coupled to the phosphoinositide system. This receptor subtype is identical with the mAChRIII or M_4 receptor subtype, as described by Fukuda et al. (1988) and Peralta et al. (1988), respectively (for overview see Barnard, 1988).

We recently reported that glandular non-M_1 muscarinic receptors of rat parietal cells mediate cholinergically stimulated acid secretion by activation of the phosphoinositide system (Pfeiffer et al., 1988). Current investigations indicate that this hydrogen ion production is mediated by M_3 receptors (Pfeiffer et al., submitted). Additionally, we reported that muscarinic M_3 receptors in porcine gastric mucosa also showed nanomolar affinities for HHSiD and differed significantly from muscarinic subtypes in gastric muscularis (Herawi et al., 1988).

The aim of the present study was to characterize the muscarinic receptor subtype, in a human colon carcinoma cell line HT-29, found to potently activate the phoshoinositide system in response to cholinergic stimulation (Kopp et al., 1988). We therefore compared the binding properties of N-methyl-scopolamine (NMS), carbachol, pirenzepine and atropine to HT-29 cells to their potency to stimulate the release of inositol phosphates. To characterize the muscarinic receptor subtype involved, we determined the binding profile of the subtype-selective antagonists HHSiD, SiHC, AF-DX 116 and methoctramine and their inhibitory potency on inositol phosphate release.

The results indicated that muscarinic receptors coupled to the phosphoinositide system in HT-29 cells are most likely of the M_3 receptor subtype. Thus, HT-29 cells may be used as a well defined model system for further investigations of receptor regulation and signal transduction mechanisms of human intestinal muscarinic receptors. The lower affinity of HHSiD we now observed, compared to previous results obtained in rat parietal cells and porcine gastric mucosa, suggests the existence of species-related heterogeneity in muscarinic receptor subtypes.

2. Materials and methods

2.1. Cell culture

Cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.), subcultured with 0.05% trypsin/1 mM EDTA in Dulbecco's modified Eagle's medium (DMEM), containing 6% fetal calf serum (FCS), 1 mM pyruvic acid, 2 mM glutamine, 100 U/ml penicillin, 50 U/ml streptomycin and 200 I.U./ml nystatin in 75 cm^2 disposable tissue culture flasks in a humified atmosphere of 5% CO_2/95% air. For the experiments, cells were seeded at a density of 2×10^5 cells in 24-well flat bottom tissue culture plates (Falcon, Heidelberg, F.R.G.) and grown as monolayers until subconfluency ($0.7\text{-}1.0 \times 10^6$ cells/well).

2.2. Determination of [^{3}H]inositol phosphates

For measurements of inositol phosphate release, cells were preincubated with [^{3}H]myo-inositol (2 μCi/ml) for 48 h when complete isotopic equilibrium was achieved. Accumulation of inositol phosphates was measured in the presence of LiCl (10 mM), known to inhibit the degradation of inositol monophosphates (Berridge et al., 1982).

The cells were then stimulated for 30 min with carbachol (0.1 mM). Phospholipase C activity, as represented by inositol phosphate release, was determined as described by Pfeiffer et al. (1988), using the Dowex anion exchange technique (Downes and Michell, 1981). Inositol phosphates were eluted with 1 M ammonium formate/0.1 M formic acid. Antimuscarinic compounds were added to the incubation medium 45 min prior to stimulation with carbachol to allow complete equilibrium to be reached. The pA_2 values for pirenzepine were obtained from dose-response curves repeated in the presence of various concentrations of pirenzepine and the dose ratios at IC_{50} values were determined (Arunlakshana and Schild, 1959).

2.3. Binding studies

The cells were incubated in the presence of 0.25 nM [^{3}H]NMS (specific activity 72 Ci/mmol) for 60 min in Hanks' balanced salts solution (HBSS), containing HEPES 20 mM, $NaHCO_3$ 3.7 g/l, glutamine 2 mM, pyruvic acid 2 mM, at room temperature. The cells were then washed rapidly with ice-cold HBSS, dissolved in 0.5 M NaOH and radioactivity was measured by liquid scintillation spectrophotometry. Specific binding was calculated after subtraction of non-specific binding in the presence of 1 μM atropine. Specific receptor binding was $82 \pm 5\%$ of the total bound radioactivity.

2.4. Data analysis

The data are reported as means $\pm$ S.E.M. from three or more independent experiments, each performed in triplicate. The data were analyzed with the computerized non-linear least-squares regression program (LIGAND and ALLFIT (De Lean et al., 1978; Munson and Rodbard, 1980).

2.5. Drugs and radiochemicals

All reagents were of analytical grade and purchased from Merck, Darmstadt, F.R.G., unless indicated otherwise. HEPES was from Serva (Heidelberg, F.R.G.), DMEM, HBSS and FCS were from Gibco (Karlsruhe, F.R.G.) and [^{3}H]NMS, [^{3}H]myo-inositol from Amersham Buchler, (Dreieich, F.R.G.). Pirenzepine dihydrochloride was kindly supplied by Dr. Hammer, Boehringer (Ingelheim, F.R.G.) and methoctramine tetrahydrochloride by Dr. Melchiorre, University of Bologna. HHSiD and SiHC were synthesized as described (Tacke et al., 1985, 1989). AF-DX 116 was a kind gift from Dr. A. Zimmer and Dr. G. Trummlitz, Dr. Karl Thomae GmbH, Biberach, F.R.G. Unlabeled NMS and carbachol were from Sigma (Taufkirchen, F.R.G.).

3. Results

3.1. Binding affinities of muscarinic antagonists

The binding profiles of muscarinic receptors on HT-29 cells were investigated in binding assays with tritiated NMS and various selective muscarinic antagonists. [^{3}H]NMS labeled a single population of muscarinic receptors on HT-29 cells with an affinity of 0.15 nM (K_D). The receptor capacity was 5.35 fmol/10^6 cells, corresponding to 3300 binding sites per single cell. Atropine displaced [^{3}H]NMS in a dose-dependent fashion with a K_D of 0.79 nM. The M_1-selective antagonist, pirenzepine, competed with [^{3}H]NMS for a single binding site with a low affinity of 279 nM (n = 3), indicating the presence of M_2 or M_3 muscarinic receptors in HT-29 cells (fig. 1 and table 1). The agonist carbachol bound to a single binding site with an affinity of 15 μM (table 1).

To further investigate the muscarinic receptor subtypes present in HT-29 cells, we characterized the binding profile of the subtype selective antagonists HHSiD, SiHC and AF-DX 116. HHSiD, SiHC and AF-DX 116 completely displaced tritiated NMS from a single binding site with affinities of 27 ± 4.5 nM for HHSiD (n = 5), 3.1 ± 0.6 nM for SiHC (n = 3) and 7564 ± 879 nM for AF-Dx 116 (n = 5, fig. 2). The binding data for the three antagonists did not permit better fits to two-site models.

Although the relatively low binding affinity of AF-DX 116 did not suggest the presence of M_2 receptors in HT-29 cells, we also investigated the

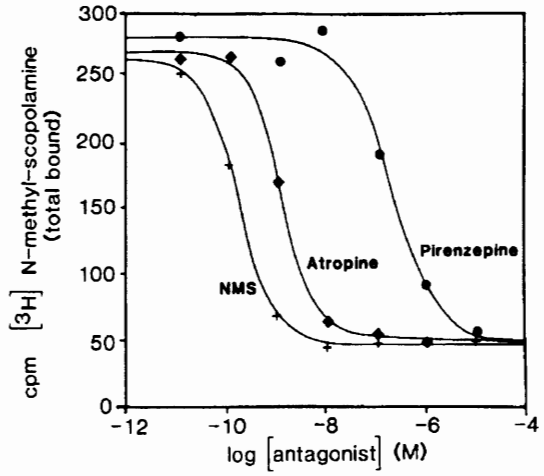

Fig. 1. Displacement of [^{3}H]NMS (0.25 nM) by unlabeled NMS, atropine and pirenzepine. Curves represent one-site fits analyzed with the LIGAND program. The data points are from three independent experiments for each compound, performed in triplicate.

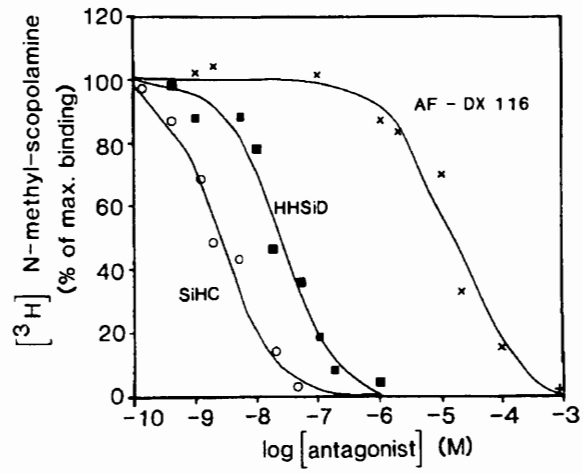

Fig. 2. Displacement of [^{3}H]NMS by the muscarinic M_3 ($M_{2\beta}$)-selective antagonists, SiHC and HHSiD, and by the cardioselective (M_2) antagonist, AF-DX 116. The data points are means of three independent experiments for each compound and were analyzed with the LIGAND program. The values for specifically bound radioactivity correspond to 320 ± 39 cpm/10^6 cells.

binding profile of the highly M_2 selective antagonist, methoctramine (Melchiorre et al., 1987). As expected, methoctramine displaced 90% of [^{3}H]NMS binding from a single binding site with an extremely low binding affinity (K_D: 2022 ± 262 nM, n = 3) (fig. 3). This permitted us to exclude the possible existence of even a small population of M_2 binding sites. Attempts to analyze the displacement curve of methoctramine using a model of two binding sites did not reveal a significant increase in the 'goodness of fit'.

3.2. Inhibitory effects of muscarinic antagonists on carbachol-stimulated inositolphosphate release

To assess the second messenger system involved in the signal transduction activated by

TABLE 1

Affinity of muscarinic antagonists for [^{3}H]N-methyl-scopolamine (NMS) binding sites in comparison to their inhibitory potencies on 0.1 mM carbachol-stimulated inositol phosphate release in HT-29 cells. Each number represents the result from 3-5 experiments, each performed in triplicate. The binding data were analyzed with the LIGAND program, while the IC_{50} values of inositol phosphate accumulation were calculated with the ALLFIT program. N.D. = not determined.

Ligand	[^{3}H]NMS binding (K_D, nM)	IC_{50} values of inositol phosphate accumulation (nM)
NMS	0.15 ± 0.03	N.D.
Atropine	0.79 ± 0.07	11.22 ± 1.61
Pirenzepine	279 ± 34	1836 ± 159
Carbachol	15100 ± 2100	-

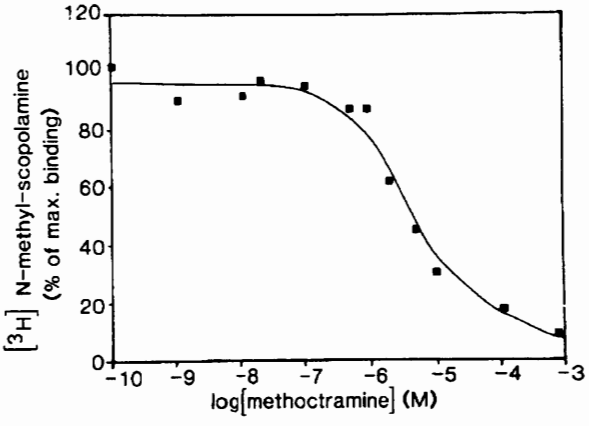

Fig. 3. Displacement of [^{3}H]NMS by the M_2 ($M_{2\alpha}$)-selective antagonist methoctramine. The curve corresponds to data of three independent experiments and was fitted by means of the LIGAND program. Specifically bound radioactivity was 389 ± 46 cpm/10^6 cells.

muscarinic receptors we investigated the cholinergic activation of the phosphoinositide system by determination of inositol phosphate release in the presence of LiCl (10 mM). The carbachol-stimulated inositol phosphate release (30 min, 0.1 mM) was inhibited dose dependently by atropine and pirenzepine with IC_{50} values of 11.2 and 1836 nM (n = 3), respectively (table 1). Since the inhibitory effects of antagonists are dependent on the concentrations of agonist used for stimulation of phosphoinositide metabolism, the IC_{50} values exceeded the K_D values, due to a parallel shift to the right of the dose-response curves. Schild analysis was performed to obtain a more precise estimate of the affinity of pirenzepine to muscarinic receptors mediating the increase in inositol phosphates. The dose-response relationships for stimulation of IP release are shown in fig. 4A. Increasing concentrations of pirenzepine caused parallel shifts in the dose-response curves for carbachol, typical for competitive antagonism. Arunlakshana and Schild analysis (1959) indicated a pA_2 value of 6.73. The slope of the Schild plot (0.95 ± 0.03, fig. 4B) was not significantly different from unity ($P > 0.05$).

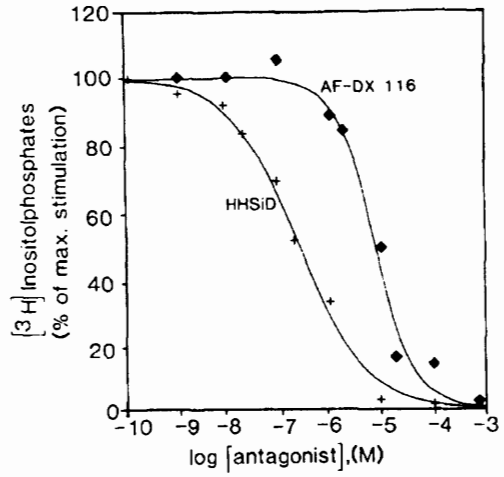

Fig. 5. Dose-response curve for the inhibition of carbachol-activated (30 min, 0.1 mM) inositol phosphate release by HHSiD and AF-DX 116. The data points represent means from three independent experiments for each compound. The basal and stimulated values for inositol phosphate release correspond to 172 ± 26 and 818 ± 87/10^6 cells, respectively. The curves were analyzed with the ALLFIT program.

To investigate whether the cholinergic activation of phosphoinositide metabolism is mediated by the above described receptor subtype in HT-29

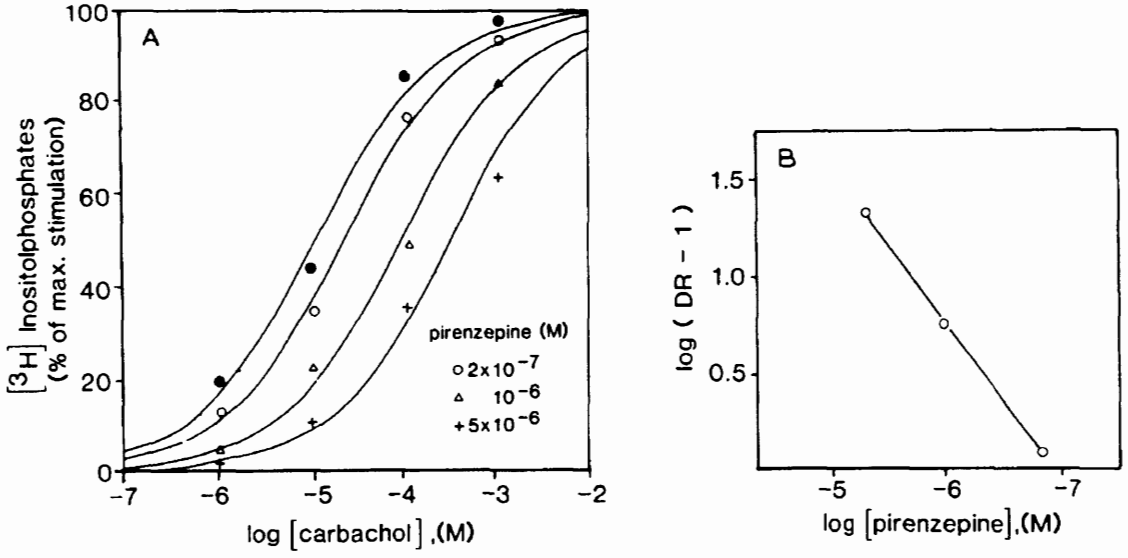

Fig. 4. Dose-response curves for the stimulation of inositol phosphate release by carbachol (30 min, 0.1 mM) either alone, or in the presence of indicated concentrations of pirenzepine (A) and Schild plot (B) from the data shown in (A). The basal and stimulated values for inositol phosphate accumulation were 142 ± 8 and 466 ± 22 cpm/10^6 cells, respectively. The curves were fitted with the ALLFIT program. The Schild plot was calculated according to Arunlakshana and Schild (1959).

cells, we determined the inhibitory effects of hexahydro-sila-difenidol and AF-DX 116 on carbachol-stimulated inositol phosphate release. Incubations with HHSiD and AF-DX 116 alone did not increase inositolphosphate accumulation, excluding the possibility of partial agonism for these compounds. As shown in fig. 5, both antagonists dose dependently reduced carbachol-stimulated inositol phosphate release with half-maximal concentrations of 270 ± 52 nM for HHSiD and 9200 ± 180 nM ($n = 3$) for AF-DX 116. These results show clearly that muscarinic M_3 receptor subtypes mediate the stimulation of phosphoinositide metabolism in HT-29 cells.

4. Discussion

Human HT-29 colon carcinoma cells have been described to contain binding sites for various hormones and peptides including insulin (Forgue-Lafitte et al., 1979), epidermal growth factor (Kitabgi et al., 1979), vasoactive intestinal polypeptide (Laburthe et al., 1978) and neurotensin (Kitabgi et al., 1980) and the α_2-adrenoceptor (Bouscarel et al., 1985). Incubation of HT-29 cells with neurotensin has been shown to result in activation of the phosphoinositide system (Amar et al., 1986).

The data we now obtained indicate that the HT-29 cell line contains an apparently pure population of muscarinic receptors of the glandular M_3 subtype and demonstrate their coupling to the phosphoinositide second messenger system. Incubations with the M_1-selective antagonist, pirenzepine, revealed a more than 100-fold lower binding affinity as compared to atropine, typical for the presence of M_2 or M_3 receptors in HT-29 cells (table 1). The low affinities of AF-DX 116 and methoctramine allowed us to exclude the presence of M_2 receptor subtypes, while the high-affinity binding of SiHC and HHSiD characterized the muscarinic receptor in HT-29 cells as an M_3 subtype (figs. 1–3). The low affinity of carbachol in HT-29 cells is in agreement with previous results obtained with porcine gastric mucosa (Herawi et al., 1988).

It is interesting that the binding affinity of the M_3-selective antagonist hexahydrosila-difenidol to HT-29 cells was about 5-10-fold lower than the K_D values reported for rat gastric cells (Pfeiffer et al., 1988) or porcine gastric cells (Herawi et al., 1988). These results could indicate that there is species-specific heterogeneity related to differences in the primary structure, differences in receptor-associated G proteins or in the microenvironment. The high affinity of SiHC, however, confirmed the presence of M_3 receptors in HT-29 cells.

The suggestion of a species-dependent heterogeneity of human muscarinic receptor subtypes is supported by results from our laboratory for the binding profiles of human gastric mucosa. In these experiments HHSiD also displaced [^{3}H]NMS with an affinity approximately 10-20 fold lower than in control experiments using porcine gastric mucosa (Pfeiffer et al., submitted for publication). Additionally, Baudiere et al. (1987) characterized the muscarinic binding profile for HHSiD in rabbit gastric fundic cells and concluded from the K_D values around 200 nM that the muscarinic receptor subtype mediating acid secretion may be different from the glandular subtype. Although the different binding profiles for muscarinic antagonists may result from species heterogeneity of muscarinic receptors, the authors did not investigate the binding profile of a second M_3-selective antagonist such as SiHC.

Several studies investigating muscarinic receptor subtypes in freshly isolated intestinal smooth muscle preparations (Giraldo et al., 1987; Michel and Whiting, 1988) evidenced a heterogenous population of M_2 and M_3 receptors, as identified by different binding affinities for methoctramine. This heterogeneity of muscarinic receptors in smooth muscle has recently been confirmed by tissue distribution studies of mRNAs encoding M_2 and M_3 receptors (Maeda et al., 1989). Attempts to correlate receptor subtypes to smooth muscle contraction indicate that M_3 receptors mediate this physiological response, while the function of M_2 receptors is unknown. Since heterogenous populations of muscarinic receptors are undesirable for investigations on receptor specific phenomena, the remarkably pure population of

M_3 receptors in HT-29 cells may serve as an important model system.

Binding profiles have now been reported for pirenzepine, HHSiD, SiHC, AF-DX 116 and methoctramine at cloned muscarinic receptor subtypes (M_1-M_5) (Akiba et al., 1988; Buckley et al., 1989). Comparison of the binding values obtained from these studies with our results indicates that the receptor present on HT-29 cells is best represented by the M_3 receptor subtype.

Bonner et al. (1988) demonstrated the existence of a new muscarinic receptor subtype in neuronal tissue, designated M_5, which seems to be coupled to the phosphoinositide system as also postulated for the M_3 subtype. The receptor subtype described in this study seems unlikely to resemble the neuronal M_5 receptor since methoctramine has a relatively high affinity in cells transfected with the M_5 subtype (Buckley et al., 1989). This is in contrast to our results, where methoctramine behaved as a low-affinity antagonist.

As mentioned above, the concentrations of atropine, pirenzepine and HHSiD required to obtain half-maximal inhibitory effects on stimulated IP-release exceeded K_D values about 10-fold. The concentrations of AF-DX 116 necessary to inhibit carbachol-mediated IP accumulation were within the range of the K_D value for this compound, suggesting additional effects of AF-DX 116 on M_3 muscarinic receptor interaction. The most likely explanation is a positive allosteric effect occurring with several M_2 antagonists, including AF-DX 116, as reported by Roffel et al. (1989).

In summary, the muscarinic binding profile of HT-29 cells, as now described, indicates a relatively pure population of M_3 (previously termed $M_{2\beta}$) receptors in this cell line, which may be used as a well defined model system to investigate the regulation of M_3 receptors and the details of the receptor coupling system. Kunysz et al. (1989) have very recently provided evidence for the existence of non-cardiac and non-M_1 muscarinic receptors in a human astrocytoma cell line (1321 N_1), which are also linked to phosphoinositide turnover and are likely to be of the M_3 receptor type.

Acknowledgements

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