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Is acetylcholine an autocrine/paracrine growth factor via the nicotinic α7-receptor subtype in the human colon cancer cell line HT-29?

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ABSTRACT

We used immunochemistry to demonstrate expression of acetylcholine's nicotinic α7-receptor subtype in human colon cancer cell line HT-29. Moreover, RT-PCR and immunochemistry showed that choline acetyltransferase and acetylcholine esterase, the enzymes responsible for acetylcholine synthesis and degradation, respectively, localise in HT-29 cells. Bromoacetylcholine bromide, an inhibitor of choline acetyltransferase, significantly attenuated basal cell growth. Our findings suggest that acetylcholine might serve as an autocrine/paracrine–or speculatively, even intracrine–signalling molecule in cell line HT-29, thus contributing to carcinogenesis/cancer progression.

1. Introduction

Acetylcholine, a phylogenetically old signalling molecule present in vertebrates and invertebrates as well as plants, fungi, and bacteria (Horiuchi et al., 2003), is synthesised from choline and acetyl-CoA by choline acetyltransferase in the central nervous system and by both choline acetyltransferase and carnitine acetyltransferase in the periphery (cf. Horiuchi et al., 2003). Acetylcholine esterase degrades acetylcholine at neuroeffector junctions, and butyrylcholine esterase degrades acetylcholine in plasma and the liver (Hoffman and Taylor, 2001). The literature increasingly acknowledges acetylcholine as an ubiquitous signalling molecule in neuronal as well as non-neuronal tissues. A previous report demonstrated choline acetyltransferase and acetylcholine expression in several non-neuronal cell types, e.g., endothelial, mesothelial, epithelial, immune, and muscle cells (Wessler et al., 2003). This widespread localisation suggests that acetylcholine plays many roles, possibly involving gene expression, proliferation, differentiation, intercellular communication, cell motility, electrical activity, secretion, absorption, and immune functions (Wessler and Kirkpatrick, 2001; 2008). Acetylcholine conveys its biological effects by binding to either of two membrane receptor classes, i.e., nicotinic (ion channels) and muscarinic (G-protein coupled) acetylcholine receptors of which their respective classical prototype ligands are nicotine and muscarine (Hoffman and Taylor, 2001).

Nicotinic acetylcholine receptors form two classes of ligand-gated ion channels in skeletal muscle and neuronal tissues, respectively (Taylor, 2001). Interestingly, several studies have demonstrated non-neuronal expression of neuronal nicotinic acetylcholine receptors (Wessler et al., 2003; Grando, 2008). Irrespective of localisation, nicotinic acetylcholine receptors consist of homo- or heteropentamers that contain α (α1–α10), β (β1–β4), γ, δ, or ε subunits (Millar and Harkness, 2008). Recent attention has focussed on the α7 homopentamer, which conveys cholinergic anti-inflammatory effects in for instance macrophages of secondary lymphoid organs (Wang et al., 2003; de Jonge and Ulloa, 2007), human colon cancer cell line HT-29 (Summers et al., 2003), or microglia of the central nervous system (Giunta et al., 2004).

Recent evidence suggests that acetylcholine might serve as an autocrine/paracrine growth factor in several types of tumour or tumour cell lines, e.g., lung (Trombino et al., 2004), breast (Espanol et al., 2007), and colon cancer (Cheng et al., 2008). Several studies have focussed on the muscarinic acetylcholine receptors. For example, mRNA for the M_3 subtype of this receptor localises in several colon cancer cell lines, including HT-29 (Kopp et al., 1989; Frucht et al.,

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1999). Compared with normal tissue, overexpression of the M_3 muscarinic acetylcholine receptor occurs in the tumour cells of colon cancer (Yang & Frucht, 2000), and stimulation with carbamylcholine, a muscarinic cholinergic agonist, albeit with some action on the nicotinic acetylcholine receptors (see Brown and Taylor, 2001) enhances tumour cell proliferation (Frucht et al., 1999). Similarly, Cheng et al. (2003) showed that acetylcholine-stimulation of the M_3 muscarinic acetylcholine receptor resulted in calcium-dependent phosphorylation of p44/42 mitogen-activated protein kinase and p90 ribosomal S6 kinase, which accelerate proliferation in human colon cancer cell line H508. Similar studies of the nicotinic acetylcholine receptors in human colon cancer or colon cancer cell lines are sparse. However, nicotine modulates the innate immune functions of human colon cancer cell line HT-29 (Gregory and Gfell, 1996), suggesting the presence of functional nicotinic acetylcholine receptors. On the level of mRNA, Summers et al. (2003) demonstrated expression of nicotinic acetylcholine receptor subunits α4, α5, α7, and β1 in HT-29 cells. Interestingly, only the α7 subunit forms a functional receptor in this cell line (cf. Summers et al., 2003). To the best of our knowledge, protein expression of the α7 nicotinic acetylcholine receptors in HT-29 cells remains undetermined. Recently, Wong et al. (2007) confirmed that HT-29 cells express mRNA for α7 nicotinic acetylcholine receptors, and also demonstrated that nicotine administration (10–1000 nM) in HT-29 cells markedly increases proliferation, concentration dependently. Methyllycaconitine, an antagonist of the α7 nicotinic acetylcholine receptors, reversed this effect.

The current study aimed to investigate protein expression of the α7 subunit of the nicotinic acetylcholine receptors in human colon cancer cell line HT-29. To determine a possible role of acetylcholine as an autocrine/paracrine mediator in this cell line (a role recently refuted by Cheng et al., 2008), we sought to demonstrate mRNA and protein expression of choline acetyltransferase and acetylcholine esterase, enzymes that play a key role in synthesis and degradation, respectively, of acetylcholine. We further investigated whether pharmacological interference with choline acetyltransferase or cholinoceptors affects basal cell growth. A preliminary report was presented previously (Pettersson et al., 2008).

2. Materials and methods

2.1. Cell culture

HT-29 (ATCC HTB 38; a kind gift from Prof. K. Lundholm, Department of Surgery, Sahlgrenska University Hospital, Gothenburg, Sweden) is a human colon cancer cell line derived from adenocarcinoma. We maintained the cells in culture in McCoy's 5a medium (Invitrogen, Stockholm, Sweden) supplemented with 1% L-glutamine (Bio Whittaker Europe, Verviers, Belgium) and 1% penicillin–streptomycin (Invitrogen) in the presence of 4% fetal calf serum (Invitrogen). We selected a split ratio of 1:8 once weekly and changed the medium in between (McCoy's 5a supplemented with glutamine and antibiotics as above, plus 2% fetal calf serum). Cells were renewed after approximately 22 passages. For the experiments, we seeded the cells in 96-well plates (1000 cells/well) for the growth study or in chamber slides (200,000 cells) for immunocytochemistry. On the fifth day following seeding, we used the cells for Western blotting, immunocytochemistry, or growth studies (for PCR, see below). We performed all experiments on at least three different cell batches.

2.2. cDNA preparation and RT-PCR analysis

We seeded HT-29 cells overnight in 96-wells (20,000 cells/well). cDNA extracted from the cells using FastLane Cell cDNA kit (Qiagen, Solna, Sweden) according to the manufacturer's instructions, was stored at −80 °C until use. We performed Lightcycler Q-PCR (Roche Diagnostics AB, Stockholm, Sweden) using FastStart DNA Master SYBR Green I (Roche Diagnostics AB). Polymerase chain reaction was performed using 2 µl of each cDNA sample. To obtain the highest signal intensity and the lowest background, we optimised the concentration of $MgCl_2$ to 4 mM at 50 cycles. TAG Copenhagen (Copenhagen, Denmark) synthesised the specific sense and anti-sense primers used for RT-PCR analyses of choline acetyltransferase, acetylcholine esterase, and glyceraldehyde-3P-dehydrogenase (house-keeping gene); the primers were previously designed by Yang et al. (2002; acetylcholine esterase), Song et al. (2003; choline acetyltransferase), and Kawashima et al. (2007; glyceraldehyde-3P-dehydrogenase), respectively (Table 1). We then separated 10 µl of the PCR product according to size on 1.0% agarose gels and conducted visualisation using ethidium bromide staining. The ladder was Direct-Load, Low Ladder (these latter three products were obtained from Sigma-Aldrich, St. Louis, MO). To determine the respective RT-PCR-product, we used QuantityOne_4.0.2 software (Bio-Rad Laboratories, Hercules, CA).

2.3. Protein extraction

We removed the culture medium and rinsed the cell culture with phosphate-buffered saline (PBS) prior to incubation with 0.175 ml of ice-cold homogenization buffer (150 mM NaCl, 0.1% sodium deoxycholate, 1% Igepal CA-630 [a detergent], 0.5% deoxycholate, all from Sigma-Aldrich) and 4% Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) for 30 min. Following incubation, we scraped the cells into a tube. After centrifugation at 220 *g* for 20 min (4 °C), we collected the supernatant. Protein concentration was determined using Quick Start Bradford Protein Assay (Bio-Rad Laboratories) according to the manufacturer's instructions.

2.4. Western blotting

The method has been described previously (Jacobsson et al., 2006). Briefly, we added a lysate volume obtained from the HT-29 cells corresponding to 20 µg of total protein to each well. The proteins were separated by SDS-PAGE for 50 min at 200 V, using NuPAGE 4–12% Bis–Tris gel (Invitrogen) and MOPS SDS running buffer supplemented with NuPAGE antioxidant (Invitrogen). We used Western blotting (30 V for 1 h) to transfer protein in buffer supplemented with NuPAGE antioxidant and 10% ethanol. The nitrocellulose membrane was rinsed with distilled water. We blocked the membrane for 1 h with a blocking solution (5% non-fat milk in TBST, i.e., 50 mM Tris–HCl, 150 mM NaCl, and 0.5% Tween 20, pH 7.5, all chemicals from Sigma-Aldrich) and then probed it overnight with either a goat polyclonal antibody against the α7 nicotinic acetylcholine receptor mapping the C-terminus; a goat polyclonal anti-acetylcholine esterase antibody mapping the N-terminus (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA), diluted following optimisation to 1:400 and 1:1600, respectively; or with a mouse monoclonal anti-choline acetyltransferase antibody (Millipore, provided by Chemicon, Chandlers Ford, UK) diluted following optimisation to 1:1600. After rinsing the membrane with TBST, we applied the secondary antibody, a donkey anti-goat or goat anti-mouse AP-

Table 1
Primers used in RT-PCR investigation of choline acetyltransferase, acetylcholine esterase, and glyceraldehyde-3P-dehydrogenase mRNA expression in HT-29 cells.

Name	Forward sequence (5′->3′)	Reverse sequence (5′->3′)	Length (bp)	Reference
Choline acetyltransferase	GGAGATGTTCTGCTGCTA	ACGGTGATCCAAAGTGGAGG	280	Song et al. (2003)
Acetylcholine esterase	TACGCCTACGTCTTTGAAACACCGTGCTTC	ACAGGTCTGAGCAGCGATCCTGCTTGCT	475	Yang et al. (2002)
Glyceraldehyde-3P-dehydrogenase	CGTATTGGGCGCCTGGTCACCAG	GACCTTGCCCACAGCCTTGGCAGC	624	Kawashima et al. (2007)

conjugated antibody (Santa Cruz), at a dilution of 1:10,000 for 1 h. Finally, we visualised immunoreactivity with a chemiluminescent detection system that utilises enzyme-linked immunodetection (Assay Buffer and CDP-Star, both from Tropix, Bedford, MA) according to the manufacturer's instructions. We used QuantityOne_4.0.2 software (Bio-Rad Laboratories) to determine the molecular weight of the α7 nicotinic acetylcholine receptor, acetylcholine esterase, and choline acetyltransferase, respectively. Negative controls were performed in one experiment by either excluding the primary, or the secondary antibody, or by applying the primary antibody following its pre-absorption overnight with its control peptide, when such was commercially available (i.e. for the anti-α7 nicotinic acetylcholine receptor antibody, and the anti-acetylcholine esterase antibody, respectively, both obtained from Santa Cruz). Either procedure resulted in no immune reaction, thus demonstrating the specificity of the respective antibody used.

2.5. Immunocytochemistry

Unless specified otherwise, we conducted all procedures at room temperature. Before fixing the cells with phosphate-buffered formaldehyde, pH 7.4 (Substratavdelningen, Sahlgrenska University Hospital, Gothenburg, Sweden) for 25 min, we washed the cells twice with PBS. Following fixation, we rinsed the cells in tris-buffered saline (TBS) for 3–5 min. We blocked endogenous peroxidase with 0.3% hydrogen peroxidase (VWR International, Stockholm, Sweden) in methanol (Merck, Darmstadt, Germany) for 30 min, and then blocked unspecific protein binding with 2% normal horse serum PK6200 (ImmunKemi, Järfälla, Sweden) for 1 h in a moist chamber. The primary antibody—polyclonal goat anti-α7 nicotinic acetylcholine receptor antibody mapping the C-terminus (Santa Cruz; 1:100; 1:200; 1:400), monoclonal mouse anti-choline acetyltransferase antibody (Millipore; 1:250, 1:500; 1:1000), and polyclonal goat anti-acetylcholine esterase antibody mapping the N-terminus (Santa Cruz: 1:100; 1:200; 1:400), respectively was added and incubated overnight in a moist chamber at 4 °C. The next day, we washed the slides in TBS (5 min) and then incubated them with the secondary antibody–biotinylated anti-goat IgG (ImmunKemi) and biotinylated universal anti-mouse IgG (ImmunKemi), respectively–for 30 min in a moist chamber, followed by a second wash in TBS (5 min). For staining, we incubated the slides with the ABC-reagents (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA) for 30 min in a moist chamber and rinsed them again with TBS (5 min) before adding the DAB solution (DakoCytomation, Solna, Sweden). Positive immunoreactivity manifested as a brown staining. We stopped the colour reaction after 2–4 min by rinsing the slides in water for 10 min. The cells were usually counterstained with Mayer's hematoxylin (Histolab, Gothenburg, Sweden) for 2 min and finally rinsed in water for 5 min. We mounted all slides with Faramount Aqueous Mounting Medium (DakoCytomation) before photographing them under a light microscope (Nikon Eclipse E400 & Nikon Digital Camera DXM 1200; Upplands Väsby, Sweden). Negative controls were performed by either excluding the primary antibody and incubating the cells instead with horse serum, resulting in no brown staining, or incubating the cells with the primary antibody following pre-absorption by its control peptide overnight (anti-α7 nicotinic acetylcholine receptor and anti-acetylcholine esterase [Santa Cruz]). The latter procedure resulted in markedly attenuated immunoreactivity.

2.6. Cell growth

We selected six wells for each treatment and repeated each experiment five times (i.e., six different cell batches), unless specified otherwise. The following treatments were undertaken for 24 h (all chemicals obtained from Sigma-Aldrich):

1. Controls, incubated with McCoy's 5a.
2. Nicotine tartrate (1, 10, 100, or 1000 nM).
3. α-Bungarotoxin (a preferential antagonist of α7 nicotinic acetylcholine receptors [Chen and Patrick, 1997]; 0.1, 1, or 10 μM), undertaken in 3 experiments.
4. Nicotine tartrate (10 nM) + α-Bungarotoxin (1 μM).
5. Atropine sulphate (a non-selective antagonist of muscarinic acetylcholine receptors [Hoffman and Taylor, 2001]; 1 μM).
6. Hexamethonium bromide (a non-selective antagonist of nicotinic acetylcholine receptors [Hoffman and Taylor, 2001]; 100 μM).
7. Atropine sulphate (1 μM) + hexamethonium bromide (100 μM).
8. Bromoacetylcholine bromide (an inhibitor of choline acetyltransferase [Sastry and Sadavongvivad, 1978; Klapproth et al., 1997]; 0.1, 10, or 1000 nM).
9. Nicotine tartrate (10 nM) + bromoacetylcholine bromide (1 μM), undertaken in 3 experiments.

We removed the medium after 24 h of challenge and deep-froze the 96-wells plates (−80 °C). We used the CyQuant® Cell Proliferation Assay Kit (Invitrogen), including the construction of a standard curve for each experiment, according to the manufacturer's instructions.

2.7. Statistics

Data is presented as means ± S.E.M., where $n = 1$ signifies one cell batch. Statistical analyses were performed on normalised data (% of control). We compared the various treatment groups to the control group, or with each other, using the non-parametric, Mann–Whitney *U*-test. We used the non-parametric, Spearman rank correlation test to investigate a concentration-dependent effect of bromoacetylcholine bromide. The correlation coefficient, ρ, is presented. A P-value <0.05 was considered statistically significant. The standard curve for the CyQuant proliferation kit was analysed by parametric correlation.

3. Results

3.1. α7 nicotinic acetylcholine receptors are expressed in HT-29 cells

Immunochemistry: Immunocytochemistry demonstrated that the anti-α7 nicotinic acetylcholine receptor antibody produced varying intensities of immunoreactivity in the cells, seemingly localised in the

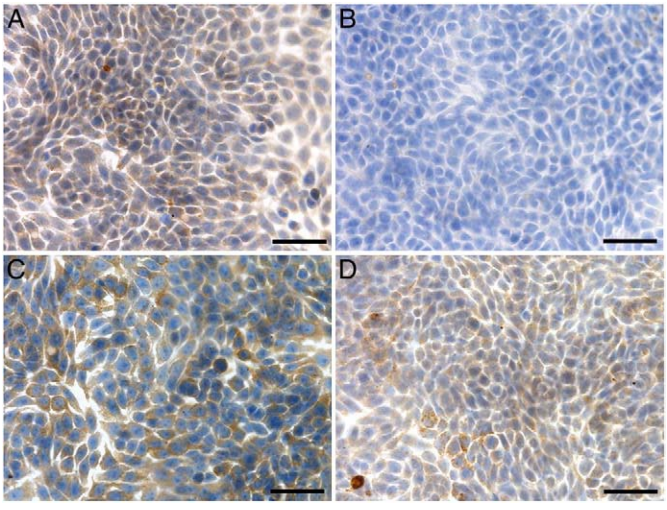

Fig. 1. Immunoreactivity in HT-29 human colon cancer cell line. A. Anti-α7 nicotinic acetylcholine receptor antibody at 1:200, showing immunoreactivity seemingly localised in the cytoplasm. B. Pre-treatment of the anti-α7 nicotinic acetylcholine receptor antibody with its blocking peptide results in markedly attenuated immunoreactivity, thus demonstrating the specificity of the antibody. C. Anti-choline acetyltransferase antibody at 1:250, showing immunoreactivity seemingly localised in the cytoplasm. D. Anti-acetylcholine esterase antibody at 1:200. The immunoreactivity is localised mainly in the cytoplasm, but a patchy nuclear localisation, as well, cannot be excluded. Bars are 25 μm.

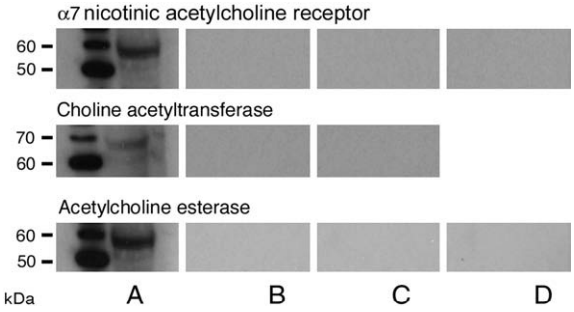

Fig. 2. Western blot analysis of HT-29 cells: α7 nicotinic acetylcholine receptor (55.17 ± 0.42 kDa, mean ± S.E.M.; $n = 3$; antibody at 1:400). Choline acetyltransferase (67.93 ± 0.15 kDa; $n = 3$; antibody at 1:1600). Acetylcholine esterase (54.87 ± 0.19 kDa; $n = 3$; antibody at 1:1600). A. Primary and secondary antibodies applied. B. Negative control: Primary antibody excluded. C. Negative control: Secondary antibody excluded. D. Negative control: Primary antibody pre-absorbed by its control peptide. The results obtained demonstrate the specificity of the antibodies used.

cytoplasm (Fig. 1A). Pretreating the antibody with its blocking peptide resulted in markedly attenuated immunoreactivity, thus demonstrating specificity of the antibody (Fig. 1B). Western blotting showed protein expression of the α7 nicotinic acetylcholine receptor, indicated by a single band at 55.17 ± 0.42 kDa ($n = 3$; Fig. 2), the same size reported by Wang et al. (2003) in lysates from human macrophages. The control experiment (as described in Methods) demonstrated the specificity of the antibody used (Fig. 2).

3.2. Choline acetyltransferase is expressed in HT-29 cells

3.2.1. RT-PCR

HT-29 cells expressed mRNA for choline acetyltransferase (280.93 ± 2.81 bp; $n = 3$; Fig. 3B).

3.2.2. Immunochemistry

The antibody produced immunoreactivity that seemingly localised in the cytoplasm (Fig. 1C), concentration-dependently. In addition, Western blotting showed protein expression of choline acetyltransferase, indicated by a single band at 67.93 ± 0.15 kDa ($n = 3$; Fig. 2), cf. Klapproth et al. (1997). The control experiment (as described in Methods) demonstrated the specificity of the antibody used (Fig. 2).

3.3. Acetylcholine esterase is expressed in HT-29 cells

3.3.1. RT-PCR

HT-29 cells expressed mRNA for acetylcholine esterase (475.23 ± 2.95 bp; $n = 3$; Fig. 3C).

3.3.2. Immunochemistry

Immunocytochemical staining of cell line HT-29 with the anti-acetylcholine esterase antibody showed positive immunoreactivity (Fig. 1D) in a concentration-dependent fashion. The immunoreactivity was localised mainly in the cytoplasm, but a patchy nuclear localisation,

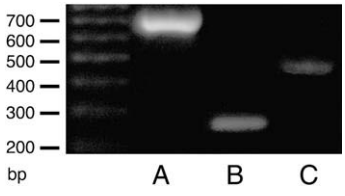

Fig. 3. Demonstration of gene expression of glyceraldehyde-3P-dehydrogenase, choline acetyltransferase, and acetylcholine esterase by RT-PCR in HT-29 cells: A. Glyceraldehyde-3P-dehydrogenase (house keeping gene): 623.96 ± 3.75 bp ($n = 3$). B. Choline acetyltransferase: 280.93 ± 2.81 bp ($n = 3$). C. Acetylcholine esterase: 475.23 ± 2.95 bp ($n = 3$).

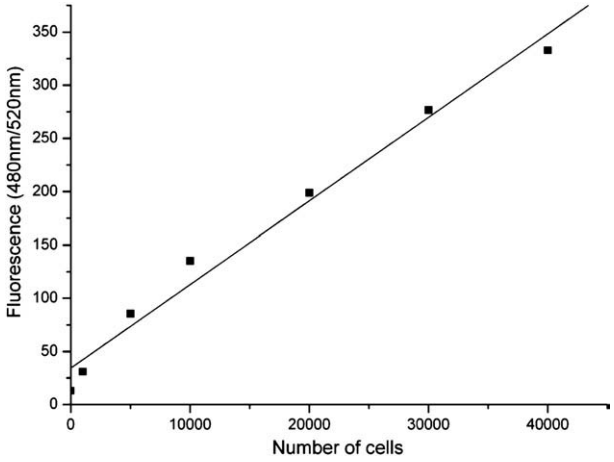

Fig. 4. Representative standard curve for the CyQuant® Cell Proliferation Assay Kit, showing a linear relationship between cell number (i.e., HT-29 cells) and fluorescence, with the equation $Y = 34.511 + 0.00785X$. $r^2 = 0.9823$; $P < 0.0001$.

as well, cannot be excluded. Pretreating the antibody with its blocking peptide resulted in markedly attenuated immunoreactivity (not shown), thus demonstrating the specificity of the antibody. Western blotting showed protein expression of acetylcholine esterase, indicated by a single band at 54.87 ± 0.19 kDa ($n = 3$; Fig. 2); see also Discussion. The control experiment (as described in Methods) demonstrated the specificity of the antibody used (Fig. 2).

3.4. Pharmacological analysis of nicotine effect on growth of HT-29 cells

Fig. 4 shows a representative standard curve for the CyQuant® Cell Proliferation Assay Kit. Cell numbers varied greatly in the controls (i.e., unchallenged cells) between the different experiments (range of fluorescence: 213.84–328.95). Therefore, we normalised the results obtained after challenging with the drugs under investigation as % of control. We established a concentration-response relationship for nicotine (1, 10, 100, or 1000 nM) in three experiments and demonstrated that peak effect occurred at 10 nM (Fig. 5). Accordingly, we chose this concentration of nicotine for subsequent analyses. Furthermore, three experiments investigated whether the preferential antagonist of the α7 nicotinic acetylcholine receptors, α-Bungarotoxin (0.1, 1, or 10 μM), by itself affected cell growth. Fig. 6 shows a very minor increase in cell growth but no apparent difference between the concentrations used.

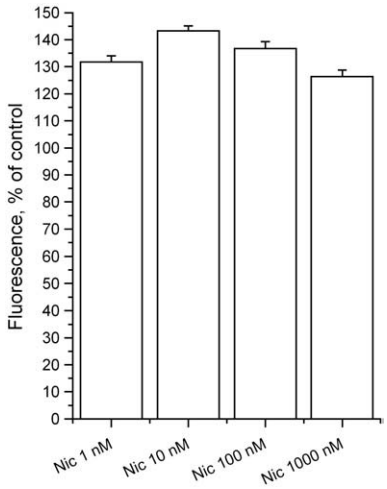

Fig. 5. Effect on HT-29 cell growth caused by challenging cells for 24 h with nicotine tartrate (Nic) in concentration: 1–1000 nM ($n = 3$), resulting in a bimodal effect on growth with a peak at 10 nM.

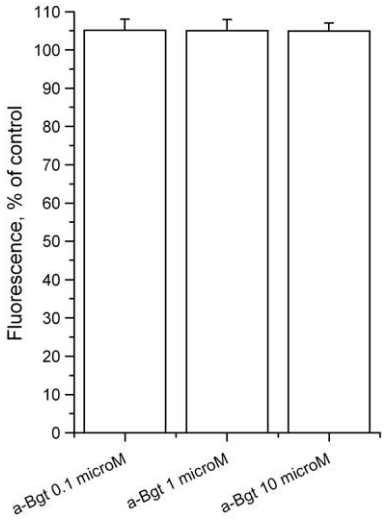

Fig. 6. Effect on HT-29 cell growth by challenging cells for 24 h with α-Bungarotoxin (a-Bgt) in concentration: 0.1–10 μM ($n=3$). There is a minimal effect of the toxin on cell growth, which is not concentration dependent.

Nicotine increased cell growth significantly, which effect was blunted by concomitant treatment with α-Bungarotoxin (Fig. 7), strongly suggesting an involvement of α7 nicotinic acetylcholine receptors. Singly or in combination, the cholinoceptor antagonists, atropine and hexamethonium, caused small but significant increases in cell growth, suggesting that these receptors participate in tonic inhibition of cell growth. Finally, the challenge using the choline acetyltransferase inhibitor, bromoacetylcholine bromide, resulted in concentration-dependent inhibition of cell growth ($\rho=-0.515$). In three separate experiments, we, moreover,

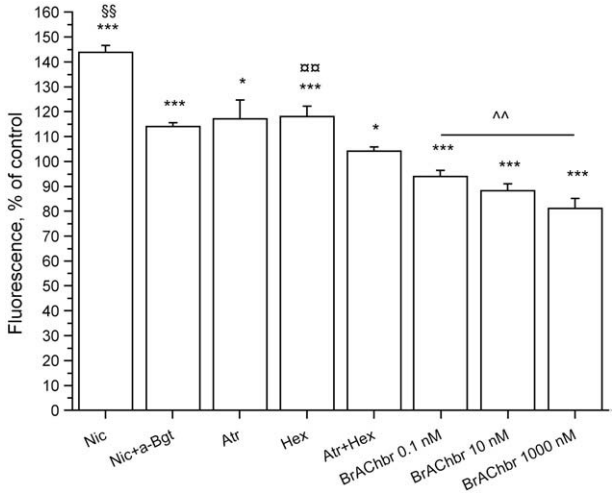

Fig. 7. Effect on HT-29 cell growth by challenging cells for 24 h as follows: nicotine tartrate (Nic; 10 nM). Nicotine tartrate (10 nM) + α-Bungarotoxin (a-Bgt; 1 μM). Atropine sulphate (Atr; 1 μM). Hexamethonium bromide (Hex; 100 μM). Atropine sulphate (1 μM) + hexamethonium bromide (100 μM). Bromoacetylcholine bromide (BrAChbr; 0.1 nM, 10 nM, or 1000 nM). Six wells were chosen for each treatment, and each experiment was repeated 5 times (i.e., $n=6$). Data is presented as means ± S.E.M. (calculated as % of control). Statistical analyses were performed on such normalized data. The various treatment groups were compared to the control group or with each other by the non-parametric, Mann–Whitney *U*-test. Nicotine elicits increased cell growth. This effect is blunted by α-Bungarotoxin, suggesting the involvement of α7-nicotinic acetylcholine receptors. Cell growth is also increased by blockade of muscarinic, and/or nicotinic acetylcholine receptors (with atropine, and/or hexamethonium). A concentration-dependent effect of inhibition of choline acetyltransferase by bromoacetylcholine bromide was investigated by the non-parametric, Spearman rank correlation test. The correlation coefficient $\rho=-0.515$. *$P=0.040$ vs. control. ***$P=0.0021$, vs. control. §§$P=0.0039$ vs. nicotine tartrate + α-Bungarotoxin. ¤ ¤$P=0.0039$ vs. atropine sulphate + hexamethonium bromide. ^^$P=0.0129$, correlation analysis for bromoacetylcholine bromide.

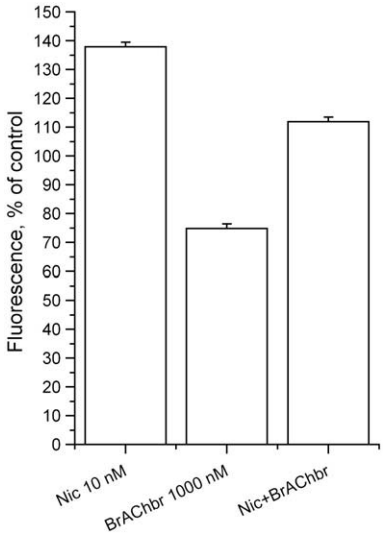

Fig. 8. Effect on HT-29 cell growth by challenging cells for 24 h with either medium (control), nicotine tartrate (Nic, 10 nM), bromoacetylcholine bromide (BrAChbr; 1 μM), or nicotine tartrate (10 nM) + bromoacetylcholine bromide (1 μM), as performed in 3 experiments. Challenge with nicotine increased cell growth by about 38% of control (i.e. unstimulated cells). The effect of nicotine obtained in the presence of bromoacetylcholine bromide (calculated as % of cells treated with bromoacetylcholine bromide only) was about 47%, suggesting that bromoacetylcholine bromide did not depress the sensitivity of the cells to nicotine.

investigated whether bromoacetylcholine bromide exerted inhibitory effects on cell signalling, in general. The cells were, therefore, challenged for 24 h with either nicotine (10 nM) alone, bromoacetylcholine bromide (1 μM) alone, or nicotine (10 nM) in combination with bromoacetylcholine bromide (1 μM), whereupon cell proliferation was assessed, as above. As can be noticed in Fig. 8, an hypothetical depressant action of the latter compound can be ruled out, since the increase in cell growth (calculated as % of control, i.e. unstimulated cells) as caused by nicotine alone was similar in magnitude to the effect of nicotine obtained in the presence of bromoacetylcholine bromide (calculated as % of cells treated with bromoacetylcholine bromide only).

4. Discussion

4.1. α7 nicotinic acetylcholine receptors in HT-29 colon cancer cells

To the best of our knowledge, we show here for the first time the protein expression of the α7 subunit of the nicotinic acetylcholine receptor in a colon cancer cell line, thus confirming results obtained previously in mRNA studies (Summers et al., 2003; Wong et al., 2007). Wong et al. (2007) showed that a selective α7 nicotinic acetylcholine receptor antagonist (methyllycaconitine) effectively reverses increased (concentration-dependent) cell proliferation resulting from nicotine challenge (10, 100, or 1000 nM for 5 h) of HT-29 cells. After challenging HT-29 cells with nicotine (1, 10, 100, or 1000 nM) for 24 h, our study yielded a different concentration–response relationship (Fig. 5). Interestingly, the magnitude of the peak effect (at 1000 nM) determined by Wong et al. (2007) was very similar to that in our study (obtained at 10 nM). Hypothetically, long-lasting stimulations with nicotine could induce cell desensitization, a phenomenon well known for nicotine as a ligand (Wang and Sun, 2005). Interestingly, Summers et al. (2003), also investigating cell line HT-29, reported that an 8 h nicotine challenge (0.01–1,000 nM) inhibits the release of interleukin 8 in response to stimulation with tumour necrosis factor-α (optimal nicotine concentration, 10–100 nM, which range is similar to our own finding).

We showed the inhibitory effect of α-Bungarotoxin on nicotine-induced cell growth which suggests the involvement of α7 nicotinic acetylcholine receptors. We have good reason to assume that α7 is the only subtype of the nicotinic acetylcholine receptors that could be

functional in cell line HT-29. α-Bungarotoxin binds to muscle nicotinic receptors (Picciotto et al., 2001) and also to α7- and α9 nicotinic receptors (Chen and Patrick, 1997). Summers et al. (2003) showed mRNA expression of subunits α4, α5, α7, and β1 in HT-29 cells. Since muscle nicotinic receptors consist of α1 subunits (Picciotto et al., 2001), this subtype does not exist in HT-29. Moreover, α7 is the only subtype that can homo-oligomerise and form a functional unit; other subunits demand a structural β-subunit different than β1 (cf. Summers et al., 2003). Therefore, we assume here that subtype α7 is the only functional nicotinic receptor subtype in HT-29. In addition, we assume that interference with α7 nicotinic acetylcholine receptors affects cell growth in challenges using nicotine, α-Bungarotoxin, or hexamethonium.

4.2. Expression of choline acetyltransferase in HT-29 colon cancer cells

In the current study, RT-PCR, immunocytochemistry, and Western blotting showed that cell line HT-29 expresses the choline acetyltransferase enzyme. Recently, Cheng et al. (2008) used RT-PCR and immunofluorescence confocal microscopy to demonstrate choline acetyltransferase expression in several colon cancer cell lines. Notably, however, choline acetyltransferase was absent in HT-29 cells. The reason for this discrepancy is obscure. Since we used well-established primers in our study (Song et al., 2003), it is reasonable to assume that HT-29 cells may vary between different laboratories.

4.3. Expression of acetylcholine esterase in HT-29 colon cancer cells

Falugi et al. (1986) used histochemistry to demonstrate acetylcholine esterase expression in SW311 human colon carcinoma cells, with a partially nuclear localisation. Deng et al. (2006) reported that very low mRNA and protein expression of acetylcholine esterase in human colon cancer cell line SW620 increased markedly when challenged with apoptotic stimuli. In our study, Western blotting showed that protein expression for acetylcholine esterase was not remarkably low compared to that of choline acetyltransferase. Three mature mRNA transcripts from the acetylcholine esterase gene can be spliced (Deng et al., 2006). Recently, Santos et al. (2007) reported expression of two different proteins (approximately 55 and 70 kDa, respectively) in human endothelial cells, as recognised by different anti-acetylcholine esterase antibodies. Interestingly, the 55 kDa acetylcholine esterase protein localised in the nucleus and cytoskeleton. Our study used the antibody that recognised this acetylcholine esterase-variant, producing a band at approximately 55 kDa in lysate from the HT-29 cells. Thus, it is likely that the localisation of acetylcholine esterase in our study is identical to that reported by Santos et al. (2007), using the same antibody.

4.4. Pharmacological analysis of acetylcholine induced effects on growth of HT-29 cells

Our results show that nicotine increases cell growth, which was blunted by an antagonist to the α7 nicotinic acetylcholine receptor, and confirm the results reported by Wong et al. (2007) in the same cell line. We further demonstrate that choline acetyltransferase likely is functionally active in HT-29 cells, since inhibition of this enzyme with bromoacetylcholine bromide decreased cell growth. However, this compound may influence other intracellular signalling systems (cf. Sastry and Sadavongvivad, 1978). In the current study, nicotine induced increased proliferation was maintained in the presence of bromoacetylcholine bromide (Fig. 8), which may suggest that growth regulating signalling mechanisms were unimpaired.

In addition to α7 nicotinic acetylcholine receptors, HT-29 cells express muscarinic acetylcholine receptors of the M_3 subtype (Kopp et al., 1989), whose stimulation likely increases cell proliferation (cf. Kopp and Pfeiffer, 1990). It may appear surprising that challenging these cells with either atropine or hexamethonium elicited a minor, but significant, increase in cell growth. Following interference by a combination of atropine and hexamethonium, there was still a slight increase in cell growth, while, as mentioned, inhibition of choline acetyltransferase with bromoacetylcholine bromide resulted in diminished cell growth. This discrepancy could be explained by the hypothesis that acetylcholine could affect cell growth in not only an autocrine fashion via membrane receptors, but also as an intracrine mediator. Thus, increasing evidence suggests that endogenous ligands activate genomic regulation by G-protein coupled receptors with a nuclear localisation (Goetzl, 2007). Moreover, other studies have demonstrated muscarinic receptors with nuclear localisation in rabbit corneal epithelial and endothelial cells (Lind and Cavanagh, 1993), but not, to our knowledge, in gut epithelia, so far. Choline acetyltransferase likely is overexpressed in human colon cancer (Cheng et al., 2008). Whether such a putative intracrine signalling pathway is biologically significant to the explanation of our findings and also participates in carcinogenesis/cancer progression will require further investigation.

5. Conclusion

Our study demonstrates protein expression of α7 nicotinic acetylcholine receptors in human colon cancer cell line HT-29. Since this is the only subunit of the nicotinic acetylcholine receptors that can be functional in this cell line (cf. Summers et al., 2003), we assume that effects obtained by their challenging with nicotine result from activation of this subtype. Our demonstration of choline acetyltransferase and acetylcholine esterase in HT-29 cells by RT-PCR and immunochemistry may suggest production and degradation of acetylcholine, which then could serve as an autocrine/paracrine, or even intracrine, signalling molecule that might promote carcinogenesis/cancer progression in the large intestine.

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