British Journal of Pharmacology (2002) 137, 1134–1142 © 2002 Nature Publishing Group All rights reserved 0007–1188/02 $25.00

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The colon-selective spasmolytic otilonium bromide inhibits muscarinic M_3 receptor-coupled calcium signals in isolated human colonic crypts

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1 Otilonium bromide (OB) is a smooth muscle relaxant used in the treatment of irritable bowel syndrome. Otilonium bromide has been shown to interfere with the mobilization of calcium in intestinal smooth muscle, but the effects on other intestinal tissues have not been investigated. We identified the muscarinic receptor subtype coupled to calcium signals in colonic crypt derived from the human colonic epithelium and evaluated the inhibitory effects of OB.

2 Calcium signals were monitored by fluorescence imaging of isolated human colonic crypts and Chinese hamster ovary cells stably expressing the cloned human muscarinic M_3 receptor subtype (CHO-M_3). Colonic crypt receptor expression was investigated by pharmacological and immunohistochemical techniques.

3 The secretagogue acetylcholine (ACh) stimulated calcium mobilization from intracellular calcium stores at the base of human colonic crypts with an EC_{50} of 14 μM. The muscarinic receptor antagonists 4-DAMP, AF-DX 384, pirenzepine and methroctamine inhibited the ACh-induced calcium signal with the following respective IC_{50} (pK_b) values: 0.78 nM (9.1), 69 nM (7.2), 128 nM (7.1), and 2510 nM (5.8).

4 Immunohistochemical analyses of muscarinic receptor expression demonstrated the presence of M_3 receptor subtype expression at the crypt-base.

5 Otilonium bromide inhibited the generation of ACh-induced calcium signals in a dose dependent manner (IC_{50} = 880 nM).

6 In CHO-M_3 cells, OB inhibited calcium signals induced by ACh, but not ATP. In addition, OB did not inhibit histamine-induced colonic crypt calcium signals.

7 The present studies have demonstrated that OB inhibited M_3 receptor-coupled calcium signals in human colonic crypts and CHO-M_3 cells, but not those induced by stimulation of other endogenous receptor types. We propose that the M_3 receptor-coupled calcium signalling pathway is directly targeted by OB at the level of the colonic epithelium, suggestive of an anti-secretory action in IBS patients suffering with diarrhoea.

British Journal of Pharmacology (2002) **137,** 1134–1142. doi:10.1038/sj.bjp.0704942

Keywords: Colon; crypt; otilonium bromide; acetylcholine; muscarinic; calcium

Abbreviations: 4-DAMP, 4-diphenyl acetoxy-methyl piperidine methiodine; ACh, acetylcholine; AF-DX 384, [(+/−)-5, 11-dihydro-11-[-(-2-[(dipropylamino)methyl]-piperidinylethyl) amino]-carbonyl-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one]; BSA, bovine serum albumin; CHO, Chinese hamster ovary; GPCR, G protein-coupled receptor; HBS, HEPES-buffered saline; OB, otilonium bromide; OCT, optimum cutting temperature; PBS, phosphate-buffered saline

Introduction

The quaternary ammonium derivative otilonium bromide (OB) relieves the abdominal pain/discomfort symptoms exhibited by irritable bowel syndrome (IBS) sufferers (Battaglia *et al*., 1998). Otilonium bromide acts by inhibiting the contraction of intestinal smooth muscle initiated by a variety of spasmogenic stimuli (Narducci *et al*., 1986; Sutton *et al*., 1997). Various modes of action are thought to underlie this behaviour. In particular, OB has been shown to interfere with the mobilization of calcium from intra- and extracellular sources *via* muscarinic- and tachykinin-receptor antagonist actions, and by blocking voltage-operated calcium channels (Maggi *et al*., 1983; Gandia *et al*., 1996; Evangelista *et al*., 1998; Santicioli *et al*., 1999). Significantly, when orally administered at doses that produce intestinal spasmolytic effects in humans, OB was devoid of both central and peripheral atropine-like side effects. This may be explained by clinical and *in vitro* studies which demonstrated that, following oral administration, OB was able to effectively

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penetrate into the large intestinal walls, but was poorly absorbed systemically (Signorini *et al*., 1984; Sutton *et al*., 1997). Although there is minimal systemic absorption, the general nature of the above modes of drug action raises the intriguing possibility that OB may also modulate physiologically active calcium signalling pathways of other large intestinal tissues, such as the rapid renewing colonic epithelium.

The epithelial lining of the colonic mucosa is composed of a carpet of flask-like invaginations called crypts. These are thought of as the unitary structures of the colonic mucosa since each crypt houses the different cell types that are found within the heterogeneous epithelium. Collectively, the cells lining the crypt fulfil the absorptive and secretory functions of the colon (Chang & Leblond, 1971). Studies performed on cell lines and intact mucosal preparations strongly implicate acetylcholine in the control of large intestinal fluid secretion *via* activation of the calcium signalling pathway (Barrett & Keely, 2000; Hardcastle *et al*., 1984). In support of these findings, work in our laboratory has recently demonstrated the presence of acetylcholine-induced rat colonic crypt calcium waves (Lindqvist *et al*., 1998) that exhibit a strong spatial correlation with the site of intestinal fluid secretion in the lower half of the crypt (Greger *et al*., 1997). Furthermore, the pharmacological profile of a range of muscarinic receptor antagonists suggested the M_3 muscarinic receptor subtype to be responsible for activation of colonic crypt calcium signals (Lindqvist *et al*., 1998). This is in keeping with previous findings, which show that M_3 muscarinic receptor activation stimulates chloride-driven fluid secretion *via* a calcium-dependent mechanism (Dickinson *et al*., 1992; O'Malley *et al*., 1995; Uribe *et al*., 1996).

In the present study we have used fluorescence imaging techniques to investigate the effects of OB on human colonic crypt calcium signals. Functional pharmacology and immunohistochemistry studies demonstrated the presence of M_3 muscarinic receptors that are coupled to calcium mobilization in human colonic crypts. Otilonium bromide inhibited M_3 receptor-coupled calcium signals in human colonic crypts and CHO-M_3 cells, but not those induced by stimulation of other endogenous receptor types. We propose OB antagonizes acetylcholine-induced colonic crypt calcium signals by blocking M_3 muscarinic receptor activation and suggest that this may confer an anti-secretory action in IBS patients suffering with diarrhoea.

Methods

Tissue samples and human colonic crypt isolation

Biopsy tissue samples were obtained from the sigmoid colon during colonoscopy of patients subsequently found to have no apparent pathology of the large intestine (Norfolk and Norwich Health Care Trust, Ethics committee approval). Human colonic crypts were isolated in a similar fashion to that previously applied to rodent tissue (Doolan & Harvey, 1996; Lindqvist *et al*., 1998). Briefly, the biopsies were washed in HEPES-buffered saline (HBS): (mM): NaCl 140, KCl 5, HEPES 10, D-glucose 5.5, Na_2HPO_4 1, $MgCl_2$ 0.5, $CaCl_2$ 1, and placed in HBS, devoid of both calcium and magnesium, and supplemented with EDTA (1 mM), for 1 h at room temperature. Single crypts were liberated by vigorous shaking and were affixed to non-fluorescent glass coverslips coated with collagen (BDH). The CHO-M_3 cell line, a stable transfection of the parental cell line with the human recombinant M_3 muscarinic receptor (Buckley *et al*., 1989), was a kind gift from Professor Noel Buckley (University of Leeds, U.K.). CHO-M_3 cells were grown in Dulbecco's modified Eagles's medium supplemented with 5% foetal bovine serum, 1% non-essential amino acids, proline, streptomycin (5 mg ml^{-1}) and penicillin (5000 units ml^{-1}), in a humidified 95% air, 5% CO_2 incubator at 37°C. All tissue culture media and supplements were from Life Technologies (Paisley, Scotland, U.K.). For calcium imaging experiments CHO-M_3 cells were seeded onto glass coverslips 24–48 h before use.

Fluorescence calcium imaging

In order to monitor agonist-induced changes in intracellular calcium, a similar approach to that described by Lindqvist *et al*. (1998) for rat colonic crypts was adopted. In brief, isolated human colonic crypts were loaded with the dual excitation, single emission ratiometric dye Fura-2/AM (3 μM). Crypts were incubated with the esterified form of the dye for 45 min and washed for a further 30 min to allow de-esterification of the dye. The loaded specimen was placed in an experimental chamber located on the stage of an inverted epifluorescence microscope ($\times 40$ neofluor Nikon objective). Crypts were continuously perifused with HBS (35°C). The exchange half-time of the bathing solution within the perfusion chamber was 2 s. Experimental solutions were administered *via* a two-way tap and changes in intracellular calcium were monitored using digital calcium imaging techniques (Riach *et al*., 1995). The intracellular dye was excited alternately with light of 340 and 380 nm wavelengths and the emitted fluorescence (510 nm) was detected by a 12-bit intensified CCD camera. The background-corrected F_{340}/F_{380} ratio was calculated and monitored in real time and digital images sampled every 2 s (Image Master, Photon Technology International, Surbiton, U.K.) Over the physiological range, this ratio was demonstrated to be linearly proportional to the free ion concentration in the specimen (data not shown).

At the beginning of each experiment, a region of interest, ROI, was located at the crypt base in the field of view. The average 340/380 ratio within the ROI was monitored simultaneously with respect to time, and the effects of OB (Spasmomen®, Menarini Ricerche spa, Florence, Italy) on acetylcholine- and histamine-induced colonic crypt calcium signals were studied. The relative potencies of the muscarinic receptor antagonists 4-DAMP (Research and Diagnostics, U.K.), AFDX-384 (Boehringer Ingleheim, Germany), methroctamine and pirenzepine were assessed to identify the receptor subtype coupled to colonic crypt calcium signals. A similar approach was adopted for the CHO-M_3 cells in order to study the effects of OB on calcium signals induced by acetylcholine and ATP. However, due to prolonged receptor desensitisation, CHO cell experiments were conducted using an unpaired design, i.e. the control and experimental responses were conducted on separate preparations. All the above experiments were performed at least in triplicate and single representative traces are shown.

Immunocytochemistry

Human biopsies were immediately placed in fixing solution (4% paraformaldehyde in phosphate buffered saline (PBS)) for 15 min and then washed in PBS. The fixed tissue was frozen in OCT (BDH) using dry ice and left to incubate at −20°C inside a cryostat (Bright, U.K.) for 1 h. Longitudinal sections (10 μm) were cut and attached to poly-L-lysine coated slides (BDH), left to dry for 30 min and then washed in PBS prior to use for immunohistochemistry. Sections were labelled with primary polyclonal antibodies raised against each of the five muscarinic receptor subtypes (Research Diagnostic Antibodies, CA, U.S.A.). Since the antigen epitope is located on the cytosolic side, crypts were permeabilized with 0.1% TritonX-100 for 30 min and then washed before treating with blocking solution (buffer I: 10% goat serum, 1% BSA in PBS) overnight at 4°C. The following day, polyclonal subtype-specific primary antibodies were added to the sections for 24 h at 4°C. For negative controls, one preparation in each group was left in buffer II (1% goat serum, 0.1% BSA in PBS) only, and another incubated with a solution containing the primary antibody pre-adsorbed with the immunizing peptide. After 24 h the primary antibody and the negative controls were washed for 24 h in buffer II to ensure removal of unbound antibody. The specimens were incubated with a secondary goat anti-rabbit antibody (Research and Diagnostics Antibodies, CA, U.S.A.) for 2 h at room temperature. The second antibody was conjugated to FITC and the labelled receptors were visualized by viewing the associated epifluorescence (Nikon, Eclipse E800).

Semi-quantitative image analysis

The immunofluorescence images were acquired using a computer-linked video CCD camera (JVC colour video camera, Victor Co. Ltd., Japan) and the image acquisition software LUCIA (version 3.5 colour image analysis, Laboratory Imaging). The indirect fluorescence was analysed by using Scion image-analysis software and the relative amounts of each muscarinic receptor subtype were determined by adopting a procedure described previously by Ndoye *et al*. (1998). Briefly, the total fluorescence intensity (arbitrary units) within a region of interest (ROI), of fixed area covering the basal cell membranes at the crypt-base was measured and the average background, obtained from parallel analysis of images obtained from a protocol including a blocking immunization peptide, was subtracted. Tissue samples derived from at least three patients were utilised in the immunocytochemical analysis of receptor subtype expression. For each preparation five different ROIs from at least four separate sections were selected at random.

Curve fitting and statistics

The initial rate of rise of the calcium signal at the crypt base was quantified by fitting a linear regression line of best fit to the first 5-20 data points. A dose-response curve for a single ligand-binding site was fitted to the acetylcholine data

$$R = \left(\frac{A}{A + EC_{50}}\right) \quad (1)$$

where R is the normalized response, the initial rate of calcium increase at a given agonist concentration, A, divided by the rate of calcium increase in response to a maximal agonist concentration. EC_{50} is the concentration of agonist required to elicit 50% of the maximal response, which is given by the curve of best fit. Similarly an equation of the form:

$$R = 1 - \left(\frac{B}{B + IC_{50}}\right) \quad (2)$$

was fitted to the muscarinic receptor and OB inhibition data, where R is the initial rate of calcium increase observed in the presence of the antagonist divided by the initial rate of calcium increase in the absence of the antagonist, B is the concentration of the antagonist, and IC_{50} is the concentration of the antagonist required to inhibit the response by 50%. Curves of best fit were fitted using an algorithm in the Mathcad software that minimised the sum of the squares of the error.

An antagonist equilibrium constant, K_b, was calculated using the functional equivalent of the Cheng–Prussoff equation:

$$K_b = \frac{IC_{50}}{\left(1 + \frac{A}{EC_{50}}\right)} \quad (3)$$

and

$$pK_b = -\log(K_b) \quad (4)$$

where A is the concentration of ACh, 10 μM, used to stimulate the crypt in the presence of the antagonist.

Significant differences in calcium responses between paired and unpaired experiments were detected by performing the appropriate t-test and the level of the P value indicated. All traces are representative of at least three experiments. Dose response and bar chart data points are plotted as the mean ± s.e. (n number).

Materials

All chemicals were purchased from Sigma (U.K.) unless otherwise stated.

Results

Dose-dependency of ACh-induced calcium signal generation at the colonic crypt base

The crypt isolation procedure yielded intact human colonic crypts from biopsy tissue samples. The crypts exhibited a distinctive lumen bounded by a monolayer of epithelial cells (Figure 1a). Epithelial cells lining the crypt loaded efficiently with Fura2 and maintained a constant ratio for a number of hours in the resting condition (data not shown). The fluorescence ratio, and thus intracellular calcium, increased dramatically at the crypt base upon stimulation with ACh (10 μM). The calcium responses were reproducible and did not show significant desensitization in response to successive exposures to the agonist, each exposure separated by a recovery period of 30 min (Figure 1b,c). Analysis of five typical paired responses yielded values for the normalized initial rate of calcium increase, peak amplitude and peak area of 1.04 ± 0.08, 1.05 ± 0.01

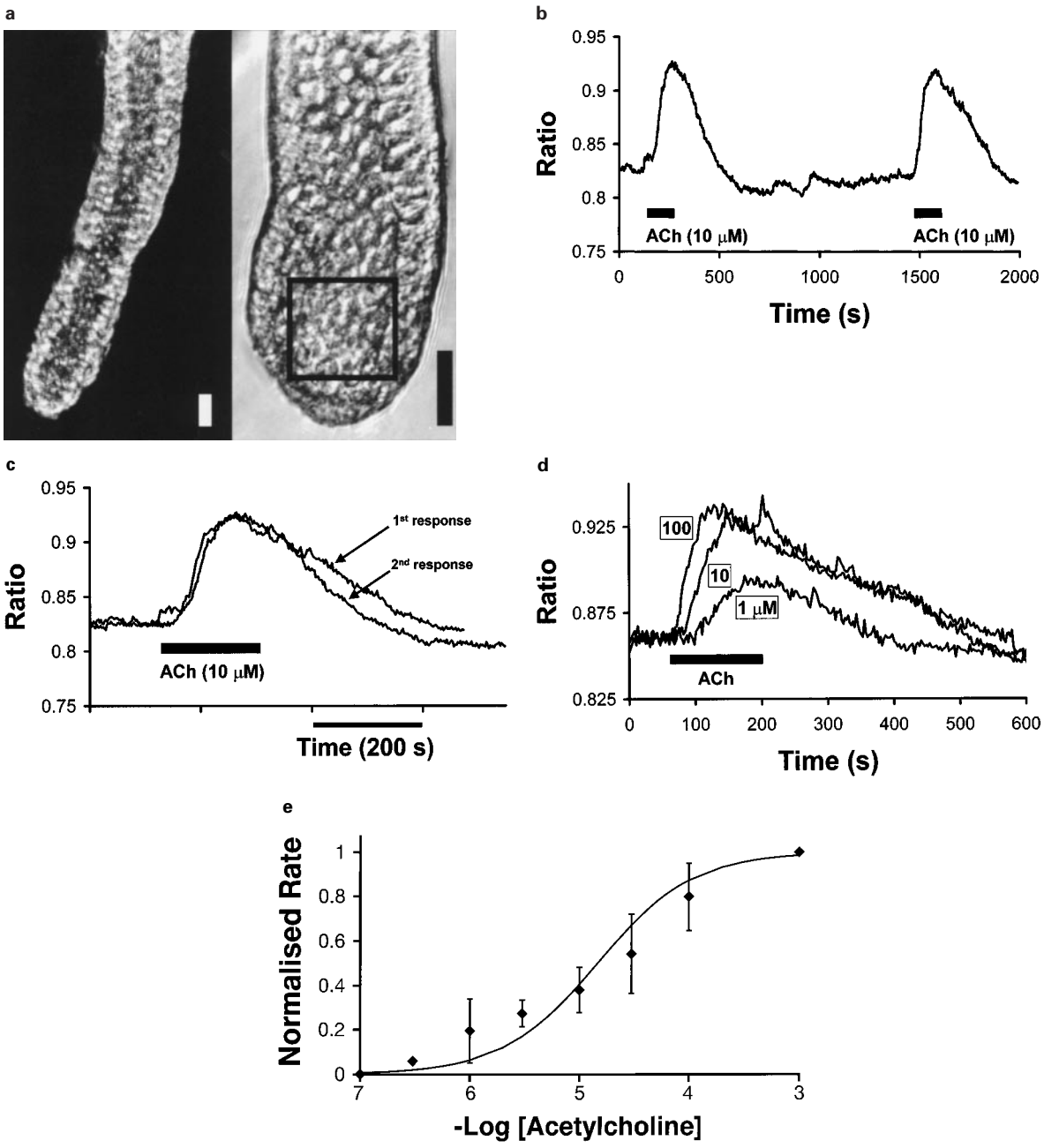

Figure 1 Reproducibility and concentration dependency of calcium signal initiation at the base of human colonic crypts. (a) Phase microscopy of isolated human colonic crypts bounded by a monolayer of non-contaminated epithelial cells that surround the distinctive crypt lumen (left hand panel: objective. ×20; scale bar = 30 μm. Right hand panel: objective ×40; scale bar = 30 μm). A typical region of interest within which changes in intracellular calcium were monitored with respect to time is indicated by the open black box placed at the crypt-base in the right hand panel. (b) Calcium responses to successive pulses of stimulation by ACh (10 μM) are reproducible. The individual responses are shown to be superimposable in (c), see text for statistical details. (d) Illustration of the concentration dependency of initial rate, amplitude and latency of the ACh-induced calcium response. At the end of each crypt experiment a maximal response was invoked (achieved with 1 mM ACh), and the preceding responses normalised according to the corresponding parameter. Data from individual crypts are shown collated in the form of a concentration–normalized rate of response curve (e) (mean ± s.e., $n > 3$, absence of error bars indicates that magnitude of s.e. is less than the size of the data symbol). The data were best-fitted by a curve of first order kinetics and an EC_{50} value of 14 μM.

and 1.14 ± 0.13 (mean ± s.e.mean, $P > 0.05$ in each case), respectively.

An initial objective was to characterize the efficacy with which ACh stimulates calcium signals in the stem cell region located at the base of human colonic crypts. At least three crypts from eight different patients were stimulated with various ACh concentrations. The rate of calcium signal increase at each concentration was normalized to the rate obtained in response to a maximal ACh concentration (1 mM) for each crypt (Figure 1d). The normalized concentration-response data is plotted in Figure 1e and the points were fitted to equation 1, giving an EC_{50} of 14 μM.

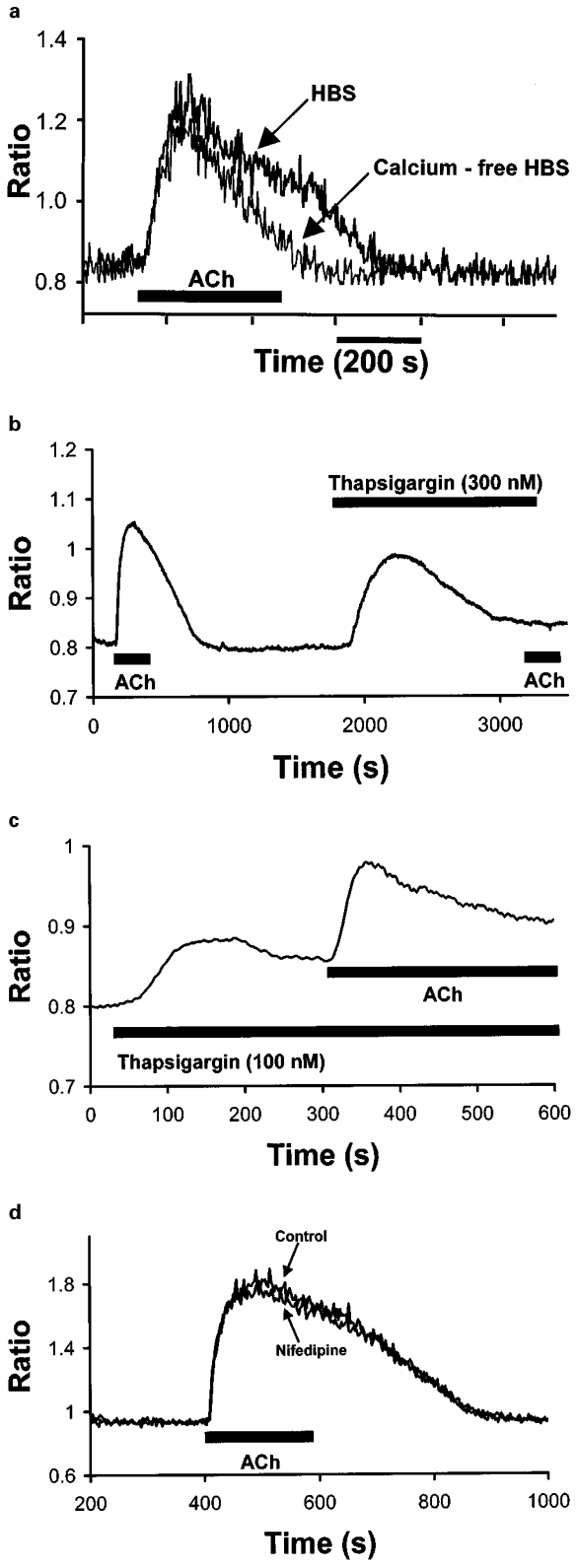

Figure 2 ACh-induced colonic crypt calcium signals are initiated by mobilization of calcium from intracellular stores. (a) The rate and amplitude of calcium peak formation were superimposable in the presence or absence of calcium in the bathing medium (see text for statistical details). (b) Chronic (>1000 s exposure) thapsigargin (300 nM)-induced depletion of intracellular calcium stores ablated the response to the second exposure to ACh (10 μM). (c) Acute exposure to a lower concentration of thapsigargin (100 nM) generated a moderate increase in the intracellular calcium level, which did not inhibit the ACh response. (d) Successive ACh (10 μM)-induced calcium responses conducted in the absence (bold trace) or presence (light trace) of the calcium channel blocker nifedipine (1 μM, 15 min pre-incubation). The responses are of similar rate and amplitude (see text for statistical details).

Relative roles of intracellular and extracellular calcium mobilization in colonic crypt calcium signal initiation

Initially, two simple protocols were performed to assess the relative contribution of calcium mobilization from intracellular and extracellular sources to the ACh response. Firstly, consecutive ACh responses performed in the presence and absence of calcium in the bathing medium gave rise to calcium signals at the crypt base that were initially superimposable (Figure 2a). The normalized values for the rate of calcium increase and peak amplitude derived from paired responses conducted in the presence and absence of extracellular calcium were 1.01 ± 0.07 and 0.93 ± 0.07 (mean $\pm$ s.e.mean, $n=5$, $P>0.05$ in each case), respectively. However, in the sustained presence of the agonist the elevated plateau phase of the response was attenuated in the absence of calcium from the bathing medium (Figure 2a). A pivotal role for an intracellular calcium store mobilization in the formation of the ACh induced calcium signal at the crypt-base was further probed by using the sarcoplasmic/endoplasmic calcium (SERCA) pump inhibitors, thapsigargin (Thastrup *et al*., 1990) and cyclopiazonic acid (CPA, Seidler *et al*., 1989). Following a control ACh response, chronic exposure to either of these inhibitors (CPA data not shown) depleted the intracellular calcium store at the crypt-base, as evidenced by an increase in intracellular calcium (Figure 2b). Subsequent stimulation with the agonist failed to elicit a further increase in cytoplasmic calcium suggesting that calcium release from intracellular stores is responsible for the generation of the colonic crypt calcium signal. Note that after thapsigargin treatment the crypt cells are unable to regulate intracellular calcium levels down to resting levels. To confirm that this elevated level of calcium was not in itself negating the ACh-induced calcium response, crypts were pre-treated with a lower concentration of thapsigargin (100 nM) for a shorter duration to generate a similar elevated level of cytosolic calcium (i.e. the fluorescence ratio at 3000 s in Figure 2b is approximately the same as that at 300 s in Figure 2c), but without chronic depletion of intracellular calcium stores (data not shown). Subsequent application of ACh generated a further increase in intracellular calcium (Figure 2c). Nevertheless, a role for voltage-gated calcium channels in colonic crypt calcium signal generation has recently been suggested by the recent demonstration of L-type channel protein and mRNA expression in the colonic epithelium. We pursued this possibility by investigating the effects of the L-type calcium channel blocker nifedipine (1 μM) on calcium signal generation. Figure 2d and the associated analyses illustrate that ACh-induced calcium signals in the crypt base were not significantly affected by the presence of nifedipine (normalized rate $=0.89 \pm 0.13$, normalized amplitude $=0.9 \pm 0.11$, mean $\pm$ s.e.mean, $n=7$, $P>0.05$).

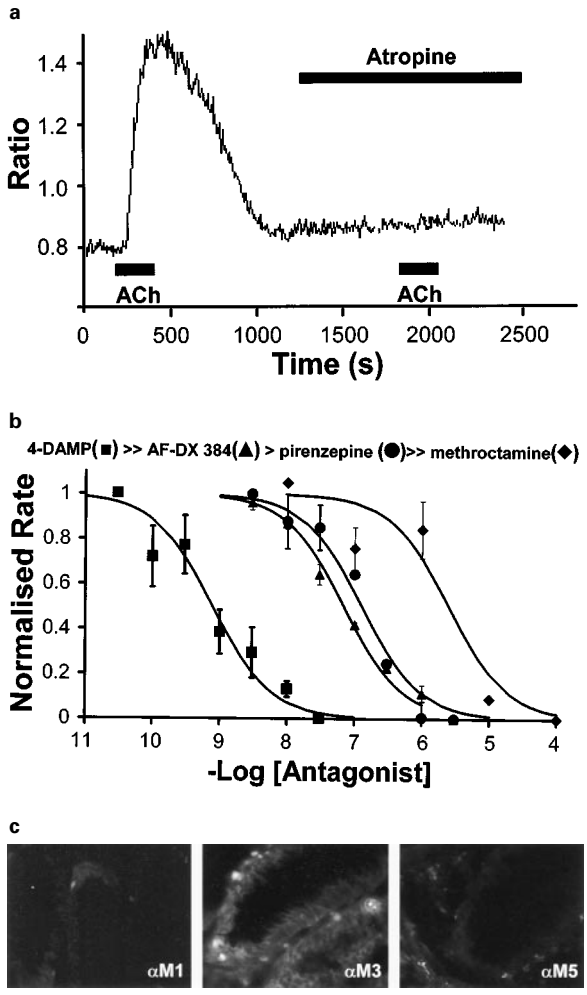

Figure 3 Functional and immunohistochemical characterization of calcium-coupled muscarinic receptor subtypes in human colonic crypts. (a) Inhibition of ACh (10 μM) induced calcium response by the muscarinic receptor antagonist atropine (100 nM). (b) mAChR subtype selective antagonists inhibited the rate of calcium signal initiation in a dose dependent manner 4-DAMP <AF-DX 384 <pirenzepine <methroctamine. Normalized rate data were best fitted by inhibition curves (see equation 2, Methods) with IC_{50} values (nM) of 0.78, 69, 128, 2510, respectively. Data points represent the mean$\pm$s.e.mean ($n>3$). (c) Immunohistochemical labelling of M_1, M_3 and M_5 muscarinic receptor subtypes. M_3 receptor labelling was evident along the basolateral membranes of cells located at the crypt base. See text for background corrected fluorescence intensities associated with the labelling of each receptor subtype.

ACh muscarinic receptor subtype identification at the crypt-base

The odd numbered members of the G protein coupled muscarinic receptor family M_1, M_3 and M_5 are classically coupled to the release of calcium from intracellular stores in a number of cell types (Eglen *et al*., 1994; Caulfield, 1993). Atropine, a non-selective muscarinic receptor antagonist, completely abolished the response to ACh at the base of human colonic crypts (Figure 3a). In the absence of muscarinic receptor subtype specific inhibitors, a range of subtype selective antagonists (Caulfield, 1993) was employed to characterize the subtype coupled to the generation of colonic crypt calcium signals. A similar protocol to that of the atropine experiments was used to investigate the potency with which 4-DAMP AF-DX 384, methroctamine and pirenzepine inhibited the formation of acetylcholine-induced calcium signals at the crypt-base. Inhibition curves for all four antagonists are shown in Figure 3b, with the following order of potency: 4-DAMP>>AF-DX 384>pirenzepine

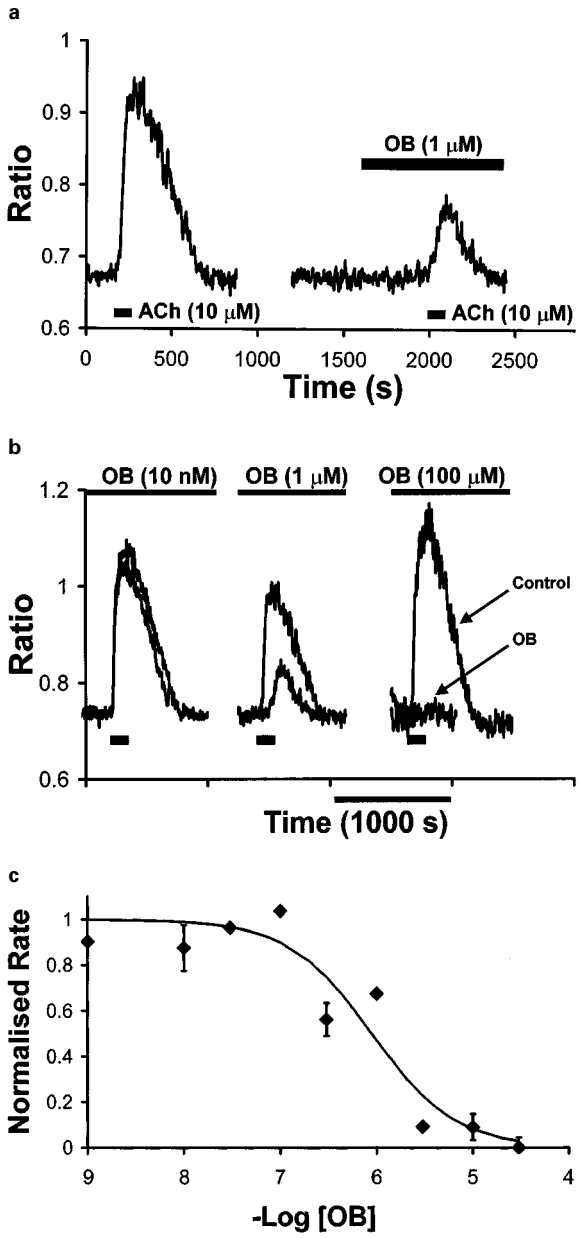

Figure 4 Otilonium bromide inhibits acetylcholine-induced colonic crypt calcium signals in a dose dependent manner. (a) A real-time example of the control and experimental acetylcholine-induced calcium responses conducted in the absence and presence of OB, respectively. (b) Progressive inhibition of ACh (10 μM)-induced calcium responses at increasing concentrations of OB. Traces for the paired control and OB pre-treatment (10 nM, 1 μM, 100 μM) are shown superimposed. (c) For each concentration of OB the normalized rate of calcium increase, with respect to the paired control response, is plotted in the form of an inhibition curve. The data were fitted by equation 2 ($IC_{50}=880$ nM). Data points represent the mean$\pm$s.e.mean ($n>3$) and the illustrated traces are representative of at least four independent experiments.

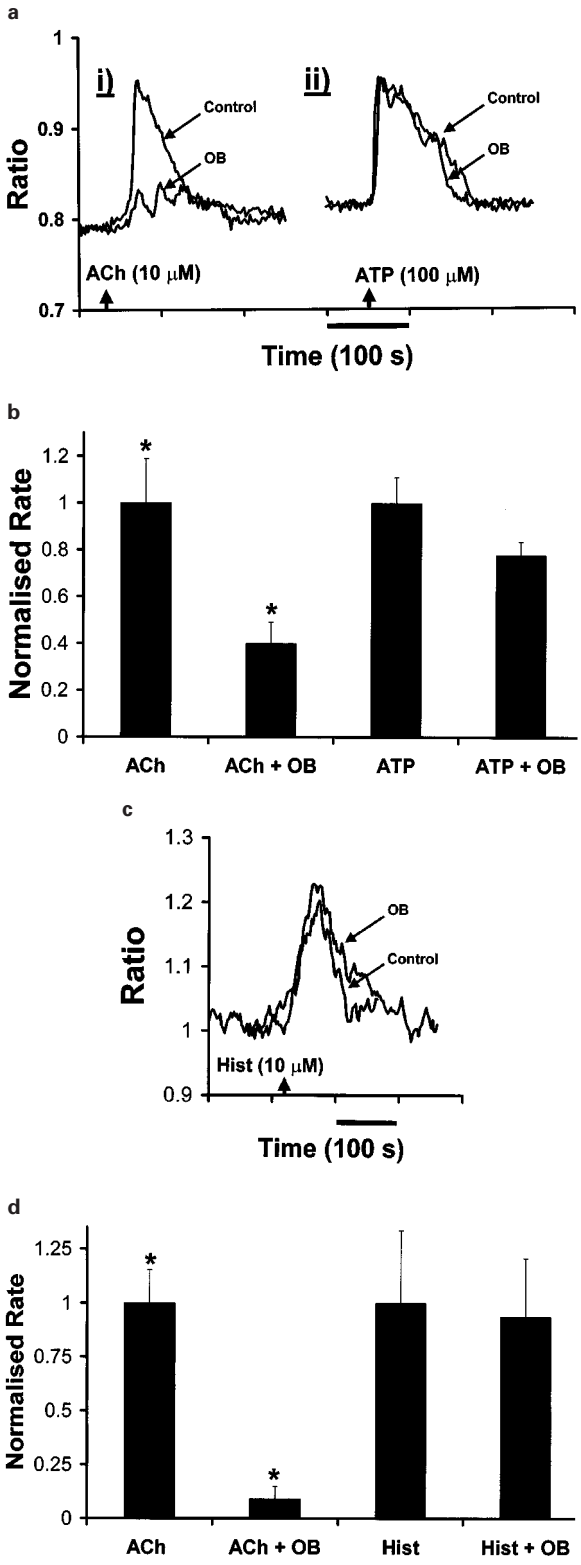

Figure 5 Otilonium bromide inhibits recombinant M_3AChR coupled calcium signals, but not those induced by stimulation of endogenous purinergic and histaminic receptors. (a) Otilonium bromide (10 μM) ameliorated ACh (10 μM)-induced calcium signals in CHO-M_3 cells (i) but not those induced by activation of endogenous P_2Y_2 receptors (ii). (b) A bar chart of the initial rate of cytosolic calcium increase induced by each agonist in the presence of OB, normalized to the unpaired control rate in each case. (c) Histamine-induced human colonic crypt calcium responses were not inhibited by OB (10 μM). (d) Mean agonist (ACh and histamine both 10 μM)-induced rate of calcium increase in the presence of OB (10 μM) normalized to the paired control (*$P<0.05$, unpaired t-test, $n\geqslant 3$ in each case).

>>methroctamine. The IC_{50} values used for the best fit inhibition curves (equation 2) were calculated to be 0.78, 69, 128 and 2510 nM, respectively, and the Cheng–Prussoff equation gave corresponding pKb values of 9.1, 7.2, 7.1 and 5.8 (equation 4). This pharmacological profile is consistent with the functional expression of the M_3 subtype at the human colonic crypt-base (Caulfield, 1993). These functional pharmacology data were complemented by immunocytochemical analyses of muscarinic receptor protein expression. Labelling of the M_1, M_3 and M_5 receptor subtypes, which are classically coupled to calcium mobilization (Caulfield, 1993; Eglen *et al*., 1994), is shown in Figure 3c. The fluorescence obtained following pre-adsorption of the primary antibody with the immunizing peptide (i.e., the background level of fluorescence) was similar to that observed by omitting the primary antibody from the procedure. The background corrected immunofluorescence labelling intensity (arbitrary fluorescence units) for the M_1, M_3 and M_5 receptor subtypes were 0.03 ± 3.03, 33.74 ± 4.38, 0.08 ± 3.43 (mean$\pm$s.e.mean), respectively, and confirms the presence of the M_3 receptor subtype.

Concentration dependency of OB inhibition on ACh-induced calcium signals in the crypt base

Pre-incubation with the spasmolytic OB also diminished the calcium response evoked by ACh at the crypt-base (Figure 4a). Given that OB has been documented to interfere with the mobilization of calcium from intra- and extra-cellular sources by inhibiting membrane receptor activation and/or blocking voltage operated calcium channels (Maggi *et al*., 1983; Gandia *et al*., 1996; Evangelista *et al*., 1998; Santicioli *et al*., 1999), we embarked on a series of experiments to study the mode of action by which OB suppresses the initiation of ACh-induced colonic crypt calcium waves. Firstly, we characterized the dose-dependency of the inhibitory action of OB on the generation of ACh-induced calcium signals in the crypt-base.

Figure 4b illustrates that at low concentrations of OB (e.g. 10 nM), the calcium responses in the presence and absence of OB were typically very similar (all representative of four separate experiments). However, at increasing doses of OB (e.g. 1 and 100 μM) both the amplitude and duration of the calcium responses were reduced (Figure 4b). The OB concentration-dependent decrease in the initial rate of the ACh-induced calcium signal (with respect to paired control responses) is presented in the form of an inhibition curve in Figure 4d. The data was fitted to equation 2 and gave an IC_{50} value of 880 nM. From equation 3 and 4 the corresponding apparent *pKb* value was 6.3.

Effect of OB on M_3 muscarinic receptor-induced calcium signals and those activated by other endogenous receptor types

Considering that OB has been demonstrated to affect various aspects of calcium signal generation, the final series of

experiments was designed to descriminate between specific actions of OB at the M_3 receptor subtype versus the possible effects of OB on other components of the calcium signalling pathway. The effects of OB on calcium signals induced by acetylcholine in CHO-M_3 cells were studied and compared with those invoked by ATP. Acetylcholine-induced calcium signals in CHO-M_3 cells (Edelman *et al*., 1994) were inhibited by OB (Figure 5a). Significantly, OB did not inhibit calcium signals induced by ATP activation of endogenous CHO cell P_2Y_2 receptors (Dickinson *et al*., 1998) (Figure 5b). Histamine-induced colonic crypt calcium signals were similarly unaffected by OB (Figure 5c,d).

Discussion

The acetylcholine-signalling axis is thought to be upregulated in a range of gastrointestinal diseases such as colorectal cancer (Yang & Frucht, 2000) and irritable bowel syndrome (Evangelista, 1999). Otilonium bromide has recently been reported to interfere with the mobilization of calcium from intra- and extra-cellular sources *via* muscarinic- and tachykinin-receptor antagonist actions, and by blocking voltage-operated calcium channels (Maggi *et al*., 1983; Gandia *et al*., 1996; Evangelista *et al*., 1998; Santicioli *et al*., 1999). Moreover, orally administered OB was localized to the intestine where it has been demonstrated to exert anti-spasmolytic effects on colonic smooth muscle (Narducci *et al*., 1995; Sutton *et al*., 1997). In the present study we have identified the muscarinic receptor subtype coupled to the calcium signalling pathway in the colonic epithelium and we have investigated the effects of OB on acetylcholine-induced human colonic crypt calcium signals.

The relative effects of muscarinic receptor selective antagonists have previously been used to characterize the functional expression of muscarinic receptor subtypes in native tissue preparations and cells expressing recombinant receptor protein (reviewed in Caulfield, 1993; Eglen *et al*., 1994). The order of potency of muscarinic receptor antagonists (Figure 3b) and immunocytochemical analyses (Figure 3c) demonstrated that M_3 receptors were responsible for ACh-stimulated calcium signals at the human crypt-base (Figure 1d,e). M_3 receptors are coupled to the $InsP_3$-calcium signalling pathway in a number of systems (Caulfield, 1993; Eglen *et al*., 1994), including intestinal epithelial cells (Dickinson *et al*., 1992), and the efficacy of calcium mobilization was similar to that observed in rodent tissue (Lindqvist *et al*., 1998). This finding is consistent with the functional expression of M_3 receptors in rat colonic crypts (Lindqvist *et al*., 1998) and the expression of mRNA for the M_3 receptor in the human colonic mucosa (Yang & Frucht, 2000).

Calcium responses to ACh are similar in the presence or absence of extracellular calcium (Figure 2a). The response is abolished by chronic depletion of intracellular calcium stores (Figure 2b), which in itself cannot be explained by an inhibitory elevation of cytosolic calcium (Figure 2c). These observations strongly suggest that ACh generates the calcium response by mobilizing calcium from intracellular calcium stores.

Otilonium bromide was observed to inhibit the initial rate calcium signal increase in the colonic crypt base in a dose dependent manner (Figures 1b and 2). The potency of inhibition ($IC_{50} = 880$ nM) was similar to values reported previously for binding to muscarinic receptors and L-type calcium channels (Evangelista *et al*., 1998; Gandia, 1996). Although voltage-operated calcium channels have been demonstrated recently to be expressed in the apical membranes of colonic epithelial cells (Wang *et al*., 2000), the generation and propagation of the ACh-induced colonic crypt calcium waves is independent of the presence of extracellular calcium (Figure 2a, Lindqvist *et al*., 1998). Moreover, the formation of the ACh-induced calcium was not affected by the L-type calcium channel blocker nifedipine (Figure 2d). It is therefore more likely that OB is acting at the muscarinic receptor subtype coupled to calcium signal generation.

The antagonistic effects of OB on the M_3 coupled calcium signalling pathway was also evident in CHO-M_3 cells where acetylcholine-induced calcium signals are inhibited by OB (Figure 4). It was conceivable that OB could exert its effect at any point along the signal transduction cascade that couples M_3 receptors to the mobilization of intracellular calcium (Berridge, 1993). However, OB did not influence the mobilization of calcium induced by the activation of purinergic receptors endogenous to CHO-M_3 cells or histaminic receptors endogenous to colonic crypts (Kahn *et al*., 1995). The inefficacy of OB on histamine-induced calcium signals (Figure 5c,d) is entirely in keeping with data demonstrating that OB does not inhibit binding to either H_1 or H_2 receptor subtypes (Evangelista *et al*., 1998). Similarly, the lack of effect on ATP generated signals (Figure 5a,b) suggests that OB does not bind to P_2Y_2 receptors (Dickinson *et al*., 1998). These observations suggest that OB does not act at a downstream site in the signalling pathway to inhibit the generation of calcium signals regardless of the nature of the stimulus and the activated receptor. However, evidence is beginning to emerge that different GPCRs can differentially activate/recruit diverse calcium signalling pathways (e.g. Strassheim & Williams, 2000). It is therefore concluded that OB may target the M_3AChR receptor directly and/or a downstream signalling component specific to the ACh-induced calcium signalling pathway.

Taken together, these data demonstrate that OB suppresses the generation of ACh-induced human colonic crypt calcium signals. This is the first evidence of OB action at an intestinal tissue aside from the documented spasmolytic effects at smooth muscle. In the latter setting, OB has been demonstrated to bind to the M2 receptor subtype and voltage-operated calcium channels. However, we have demonstrated that ACh-induced colonic crypt calcium signals are a consequence of M_3 receptor activation and that their generation is independent of L-type calcium channel activity. Moreover, we have not found any immunocytochemical evidence for the presence of colonic crypt M_2 receptor expression (data not shown). Otilonium bromide abrogated ACh-induced calcium signals in CHO-M3 cells and colonic crypts, but not those induced by purinergic or histaminic receptor activation. We therefore presently suggest that the M_3 receptor coupled calcium signalling pathway is directly targeted by OB at the level of the colonic epithelium. Given that M_3 receptor coupled calcium signals have been strongly implicated in promoting intestinal fluid secretion (Hardcastle & Hardcastle, 1984; Barrett & Keeley, 2000), treatment of

IBS patients with OB may also provide anti-secretory therapeutic value in addition to the well documented spasmolytic actions. Future work will identify the molecular target of OB in the large intestinal epithelium.

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The authors would like to thank Professor Noel Buckley for the CHO cells transfected with the human muscarinic receptor genes. We are grateful to Professor Alan Dawson for helpful discussion. This work was supported by grants from the Royal Society, Wellcome Trust (vacation studentship to N. Johns), Humane Research Trust, Mason Medical Trust, and Menarini Ricerche spa.

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(Received April 5, 2002
Revised June 19, 2002
Accepted August 27, 2002)