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Characterization of ibodutant at NK_2 receptor in human colon

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ABSTRACT

We have characterized the pharmacological profile of the nonpeptide tachykinin NK_2 receptor antagonist ibodutant (MEN15596) through radioligand binding and contractility assays in the human colon smooth muscle. The antagonist affinity of ibodutant was evaluated through concentration-dependent inhibition curves at the [^{125}I]NKA specific binding by using membranes prepared from human colon smooth muscle. In this assay the affinity of ibodutant (pK_i 9.9) was compared to that of other two selective NK_2 receptor antagonists, nepadutant (pK_i 8.4) and saredutant (pK_i 9.2). The antagonist potency of ibodutant was evaluated towards the [βAla^8]NKA(4-10)-mediated contractions of human colon smooth muscle strips. In this assay ibodutant (3, 10, 30 and 100 nM) induced a concentration-dependent rightward shift of the [βAla^8]NKA(4-10) concentration-response curves without depressing the maximal contractile effect. The analysis of the curves yielded a Schild-plot linear regression with a slope not different from unity (1.02), thus indicating a surmountable antagonist behavior. The calculated apparent antagonist potency as pK_B value was 9.1. No sex related differences were observed in NK_2 receptor pharmacology for [βAla^8]NKA(4-10) or ibodutant in colonic strips obtained from male or female patients. Reversibility experiments of tachykinin NK_2 receptor blockade indicated that the inhibition of the agonist-induced contractions in preparations pre-exposed to ibodutant, and afterwards subjected to repeated washing cycles remained almost constant showing no sign of recovery during the 3 h observation period. Overall, the present study indicates ibodutant as a potent tachykinin NK_2 receptor antagonist in the human colon tissue, also endowed with a persistent duration of action.

1. Introduction

Tachykinin receptors, namely NK_1, NK_2 and NK_3, are seven transmembrane class-A (rhodopsin-like) G-protein coupled receptors, widely expressed in the central and peripheral mammal tissues (Shimizu et al., 2008). In the mammalian intestine, tachykinins play a role as excitatory transmitters that mediate the ascending excitatory reflex and atropine-resistant peristalsis (Barthó and Holzer, 1985; Costa et al., 1985; Maggi et al. 1994) either directly on muscle cells or indirectly by activating intramural neurons (Barthó and Holzer, 1985; Holzer and Holzer-Petsche, 1997a, b). In human intestine tachykinin-like immunoreactivity originates primarily from intrinsic neurons but also from the peripheral endings of capsaicin-sensitive afferent neurons (Holzer and Holzer-Petsche, 1997a). In the human colon, the tachykinin receptors mediating smooth muscle contraction belong, for the most part, to the NK_2 type (Maggi et al., 1993) although radioligand binding studies (Warner et al., 1999) have revealed the presence of a small population of NK_1 receptors, whose functional role remains to be elucidated.

In this view, tachykinin NK_2 receptor antagonists are regarded as possible candidates for counteracting altered smooth muscle motility and visceral hypersensitivity present in pathological conditions characterized by an inflammatory state and impaired motility such as irritable bowel syndrome (IBS) (Lecci et al., 2004 for review), and represent potential innovative therapeutic drugs (Lecci et al., 2006; Quartara et al., 2009).

We recently developed a new potent tachykinin NK_2 receptor-selective nonpeptide antagonist, ibodutant (previously named MEN15596) that is presently undergoing Phase-IIb clinical trial for treatment of diarrhea-predominant IBS. The pharmacological outlines of ibodutant have indicated its high affinity and selectivity for the human tachykinin NK_2 receptor over the NK_1 and NK_3 and subnanomolar antagonist potency in human, guinea-pig, and minipig *in vitro* bioassays (Cialdai et al., 2006). Moreover, ibodutant displays a long duration of action both *in vivo* and *in vitro*, due to its bioavailability, metabolic resistance, and long residence

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time on the tachykinin NK_2 receptor (Cialdai et al., 2006; Meini et al., 2009).

Smooth muscle preparations of the human colon have been previously used to characterize both the potency and affinity of different tachykinin NK_2 receptor antagonists (Giuliani et al., 1991; Advenier et al., 1992; Warner et al., 1999; Patacchini et al., 2000). Therefore, the present investigation was undertaken to characterize the pharmacological properties of ibodutant at the tachykinin NK_2 receptor in the circular muscle of the human colon. Radioligand binding experiments using iodinated neurokinin-A (NKA) and smooth muscle membranes were performed to assess ibodutant affinity in comparison to that of other tachykinin NK_2 receptor antagonists (nepadutant and saredutant, formerly known as MEN11420 and SR48968; Catalioto et al., 1998; Emonds-Alt et al., 1993). Smooth muscle contractility experiments were performed to evaluate the ibodutant antagonist potency and the reversibility of receptor blockade towards the responses produced by the tachykinin NK_2 receptor selective agonist [βAla^8]NKA(4-10). Moreover, since some sex-related variations in NK_2 receptor pharmacology in human colon have been described (Burcher et al., 2008) we assessed whether the response to NK_2 receptor agonist and antagonist differs, at some extent, in colonic strips from male and female patients.

2. Materials and methods

2.1. Patients and specimens

All the procedures used in the present study were approved by the Ethics Committee of the Medical Faculty of Florence University. Written informed consent was obtained from all patients.

Segments of human colon, approximately 10 cm in length, were taken from grossly normal margins of surgical resections from 16 patients (7 males and 9 females, age range 47–84 years) undergoing partial colectomy for adenocarcinoma. Most segments were taken from the descending colon (9) and some from sigmoid (3), ascending (2) and transverse (2) colon.

Immediately after resection, colonic segments were placed in ice-cold Ringer-lactate solution and quickly transported to the laboratory. No patient received radio or chemotherapy before intervention. All specimens appeared macroscopically normal, with no signs of tumor or inflammation.

The tissue was transferred into fresh oxygenated (95% O_2 and 5% CO_2) ice-cold Krebs solution of the following composition (mmol/l): NaCl 119; $NaHCO_3$ 25; KH_2PO_4 1.2; $MgSO_4$ 1.5; $CaCl_2$ 2.5; KCl 4.7 and glucose 11, cleaned from serosal fat, mucosal layer and tenia coli leaving the smooth muscle bands. Part of this muscle was weighed, frozen and stored in liquid nitrogen and then used to prepare membranes for radioligand binding experiments. The remainder part of the tissue was used for functional studies by dissecting strips (3 mm wide by 10–15 mm long; 5–8 from each segment) of muscular tissue in the direction of circular muscular fibers. All strips for functional studies were stored overnight at 4 °C in oxygenated (95% O_2 and 5% CO_2) Krebs solution.

2.2. Chemicals

Ibodutant (MEN15596; 6-methyl-benzo[*b*]thiophene-2-carboxylic acid [1-(2-phenyl-1*R*-{[1-(tetrahydropyran-4-yl)methyl-piperidin-4-ylmethyl]-carbamoyl}-ethylcarbamoyl)-cyclopentyl]-amide, batch L3/08) and nepadutant (MEN11420; (cyclo-{[Asn(β-DGlcNAc)-Asp–Trp–Phe–Dpr–Leu]cyclo(2β-5β)}), batches L1/04 and L1/08) were synthesized at Lusochimica (Menarini Group, Lomagna, Italy). Saredutant (SR48968; (S)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) and the nonpeptide NK_1 receptor antagonist SR140333 ([(S)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2,2,2]octane chloride]) were a kind gift of Sanofi Aventis (Montpellier, France); NKA and [βAla^8]NKA(4-10) were from EspiKem (Firenze, Italy); atropine sulfate, indomethacin and nifedipine were from Sigma-Aldrich (Milano, Italy). [^{125}I]NKA (NEX252, specific activity 2200 Ci · mmol^{-1}) was from PerkinElmer (Boston, MA, U.S.A.).

All salts used were of analytical grade and purchased from Merck (Darmstadt, Germany). All other materials were from Sigma-Aldrich (Milano, Italy).

2.3. Membranes preparation

Crude membranes were prepared as described in Burcher et al. (1986) and Warner et al. (1999) with minor modifications. All of the following procedures were performed at 4 °C. Tissues were thawed on ice and then placed in 15 volumes of 50 mM Tris containing 120 mM NaCl and 5 mM KCl (pH 7.4). It was first minced by using fine scissors and then homogenized by using a homogenizer (Ultra-Turrax T25, IKA Labortechnik) set at 20,500 loads min^{-1}. The homogenate was centrifuged at 48,000 g for 20 min and the pellet was re-homogenized and re-suspended in 50 mM Tris HCl (pH 7.4) containing 10 mM EDTA and 300 mM KCl for 1 h. After centrifugation as before, the membranes were washed twice in 50 mM Tris HCl. Before the last centrifugation the protein content was determined according to Bradford (1976). The membranes were finally re-suspended in binding buffer.

2.4. Radioligand binding studies

The binding buffer was 50 mM Tris HCl (pH 7.4) containing 0.02% bovine serum albumin (BSA), 3 mM $MnCl_2$, 100 μg/ml bacitracin, 10 μg/ml chymostatin, 5 μg/ml leupeptin, and 2.5 μg/ml thiorphan. Non-specific binding was defined as the amount of radiolabelled ligand bound in the presence of unlabeled NKA (1 μM). Preliminary experiments were performed to verify the protein concentration to be used (about 70–100 μg/ml, final concentration) and the time to reach the radioligand binding steady state (60 min). Competing ligands (NKA, ibodutant, saredutant and nepadutant) were tested in a wide range of concentrations (1 pM–10 μM, and serial dilutions were performed with binding buffer. [^{125}I]NKA concentration was in the range between 0.066 and 0.093 nM. Binding reaction (at room *T*) started at the time of membranes addition (final volume of 500 μl) and stopped 60 min later by rapid filtration through UniFilter-96 plates (Packard Instrument Company), pre-soaked overnight in BSA 0.5%, and using a MicroMate 96 Cell Harvester (Packard Instrument Company). The tubes and filters were then washed five times with 0.5 ml aliquots of Tris buffer (50 mM, pH 7.4, 4 °C) containing 3 mM $MnCl_2$ and 0.02% BSA. Filters were dried and soaked in Microscint 40 (50 μl/well, Packard Instrument Company), and bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Packard Instrument Company).

2.5. Functional studies

The strips (15–20 h after excision) were placed in 5-ml organ baths filled with oxygenated Krebs solution at 37 °C and connected to isometric force transducers (Ugo Basile, Varese, Italy) under an initial tension of 20 mN. Mechanical activity was amplified and digitally recorded by an Octal Bridge Amplifier connected to PowerLab/8sp hardware system and analyzed using the Chart 4.2 software (AD Instruments, Australia).

The activity of ibodutant at the human tachykinin NK_2 receptors was evaluated against the selective NK_2 receptor agonist [βAla^8]NKA(4-10) (Maggi et al. 1993) in the presence of atropine (1 μM), indomethacin (3 μM) and the selective tachykinin NK_1 receptor antagonist SR140333 (0.1 μM).

After 60 min stabilization period two reproducible responses to 80 mM KCl were established at 45 min intervals to assess tissue viability. After a further 45 min equilibration period, during which the medium was renewed every 15 min, nifedipine (0.3 μM) was added to the Krebs solution and there was left in all subsequent experimental phases to eliminate spontaneous activity (Maggi et al. 1989; Zagorodnyuk et al. 1994). After 30 min of incubation in the presence of nifedipine the preparations were challenged with 1 μM [βAla^8]NKA(4-10) to evaluate the contractile response of each preparation.

After a further 90 min equilibration period, concentration-response curves to the tachykinin NK_2 receptor selective agonist [βAla^8]NKA(4-10), were cumulatively constructed. In each experiment one strip was pretreated with the vehicle (DMSO; 1–3 μl/ml) and used to perform the control curve to [βAla^8]NKA(4-10), while the other strips, obtained from the same specimen, were pretreated with ibodutant (3, 10, 30 and 100 nM) added to the organ bath 60 min before the concentration-response curve to [βAla^8]NKA(4-10). In each preparation only one cumulative concentration-response curve to [βAla^8]NKA(4-10) was carried out and only one concentration of the antagonist was tested.

In a separate series of experiments the reversibility of tachykinin NK_2 receptors blockade produced by ibodutant was evaluated in the human colon preparations using the technique as described by Patacchini et al. (2000) and by Meini et al. (2009). These experiments were also performed in the presence of atropine (1 μM), indomethacin (3 μM), SR140333 (0.1 μM) and nifedipine (0.3 μM). After a stabilization period of 60 min, preparations were exposed twice, every 30 min, at a submaximal concentration (171 nM) of [βAla^8]NKA(4-10), calculated from control concentration response curve of the agonist, as the one producing 90% of its maximal contractile effect. After further 30 min, ibodutant (10, 30, 100 and 300 nM) or the vehicle (DMSO, 1–3 μl/ml) were added to the bath solution and incubated for 60 min before the next challenge with the agonist (Time 0). After the agonist had produced its maximum contractile effect, the preparations were subjected to a washing protocol of the agonist and the antagonist consisting of three washing periods lasting 10 s each every 10 min during which the volume of the organ bath was renewed five times for each washing period (15 renewals in all). Thereafter the administration of the agonist was repeated every 30 min in antagonist-free solution to measure the reversibility of antagonist action for 180 min.

2.6. Data evaluation and statistical analysis

All data in the text or figures are expressed as mean ± standard error of the mean (S.E.M.) or 95% confidence limits (95% c.l.).

Data from radioligand binding experiments were fitted by nonlinear regression using GraphPad Software Prism 4.02 to determine the equilibrium dissociation constant (K_d) from homologous competition experiments performed with NKA, and the ligand concentration inhibiting the radioligand binding of the 50% (IC_{50}) from heterologous competition experiments (ibodutant, saredutant and nepadutant). K_d values were calculated as IC_{50}—[radioligand]. K_i values were calculated from IC_{50} using the Cheng-Prusoff equation ($K_i = IC_{50}/(1+[\text{radioligand}]/K_d)$) according to the used concentration and the obtained K_d value of the radioligand (Cheng and Prusoff, 1973) in each experimental section (using tissue from the same donor). For graphical presentation data obtained at each concentration of competing ligand, were normalized as percentage of specific binding as follows: [(bound-nonspecific)/specific] × 100.

Functional data were fitted by sigmoidal nonlinear regression (Prism 4.02, GraphPad Software) to determine the agonist concentration producing the 50% (EC_{50}) of the maximal response from the concentration-response curves. Differences in the maximum contractile effects between controls and ibodutant-pretreated preparations were evaluated by one-way Analysis of Variance (ANOVA) and the Dunnett Multiple Comparison Test.

The antagonist potency of ibodutant was expressed in terms of pK_B estimated as the mean of the individual values obtained with the Gaddum equation: $pK_B = \log(CR-1) - \log[B]$ were CR is the concentration-ratio calculated from equieffective concentrations of agonist (EC_{50}) obtained in the presence and in the absence of antagonist and B is the used antagonist concentration (Kenakin, 2006).

Competitive antagonism was checked by the Schild regression analysis by plotting the estimates of $\log(CR-1)$ against log[B] to determine the slopes of linear regression: a plot with linear regression line and slope not significantly different from unity was considered as proof of competitive antagonism (Arunlakshana and Schild, 1959).

In reversibility experiments of tachykinin NK_2 receptor blockade produced by antagonists performed in the human colon preparation responses obtained in each strip at different time were percentaged towards the basal response obtained in each preparation (before antagonist treatment), and compared to those obtained in control time-matched preparations. Data obtained were analyzed by two-way analysis of variance (ANOVA) for repeated measures followed by the Bonferroni post-test.

3. Results

3.1. Radioligand binding inhibition experiments

The characterization of [^{125}I]NKA binding sites was made by means of NKA homologous competitive inhibition curves (Fig. 1). NKA K_d value was 1.77 ± 0.20 nM ($n=7$). All tested NK_2 receptor antagonists completely inhibited the [^{125}I]NKA specific binding, and the rank order of potency was ibodutant > saredutant > nepadutant. The obtained pK_i values were 9.9 ± 0.14 ($n=7$) for ibodutant, 9.2 ± 0.06 ($n=7$) for saredutant and 8.4 ± 0.15 ($n=7$) for nepadutant (Table 1). The analysis of inhibition curves

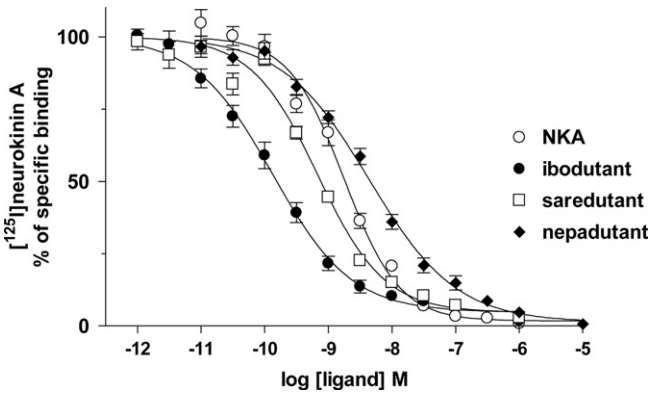

Fig. 1. Inhibition curves of NKA, ibodutant, saredutant and nepadutant at the [^{125}I]NKA specifically bound to the human tachykinin NK_2 receptor expressed in membranes of human colon circular smooth muscle. Each point represents the mean ± S.E.M of seven experiments, each one performed in duplicate. Six membrane preparations were used, each one obtained with the tissue from different donors.

Table 1
Comparison of ibodutant and tachykinin NK_2 receptor antagonists affinity and potency values detected in native and recombinant bioassays.

Binding affinity (pK_i)	**Ibodutant**	**Nepadutant**	**Saredutant**
Human colon smooth muscle	9.9	8.4	9.2
CHO/hNK_2R	10.8[a]	8.5[b]	9.8[b]
Antagonist potency (pK_B)			
Human colon smooth muscle	9.1	8.3[c]	insurm.[c]
CHO/hNK_2R	10.3–10.6[a]	8.3[a]	9.8 insurm.[a]

insurm. = insurmountable.
Data are from the present study or from previous measurements performed in our laboratories, as indicated.
The affinity (pK_i) was evaluated in inhibiting the [^{125}I]NKA specific binding to membranes prepared from smooth muscle of human colon or from CHO cells stably expressing the human tachykinin NK_2 receptor (CHO/hNK_2R).
The antagonist potency (pK_B) was evaluated in antagonizing contractile responses induced by [βAla^8]NKA(4-10) in the human colon smooth muscle or the inositol phosphates accumulation induced by NKA in CHO/hNK_2R.

[a] Meini et al., 2009.
[b] Renzetti et al., 1999.
[c] Patacchini et al., 2000.

indicated that NKA best fitted according to a one-binding site model, whereas inhibition curves of the three antagonists did not follow the law of mass action, and data were best fitted by a variable slope regression with Hill slope values significantly less than unity: −0.70 (95% c.l. −0.80 to −0.61) for ibodutant, −0.82 (95% c.l. −0.93 to −0.72) for saredutant, and −0.63 (95% c.l. −0.71 to −0.56) for nepadutant.

3.2. Antagonism toward concentration-dependent contractions produced by [βAla^8]NKA(4-10)

In the presence of atropine (1 μM), indomethacin (3 μM), SR140333 (0.1 μM) and nifedipine (0.3 μM) the selective NK_2 receptor agonist [βAla^8]NKA(4-10) produced slowly developing, concentration-dependent, tonic contractions of the human isolated circular colon: the E_{max} was 34.6 ± 2.6 mN (n=13). The EC_{50} value calculated from control (vehicle-treated) concentration-response curves to [βAla^8]NKA(4-10) was 12.4 nM (95% c.l. 11.1 − 13.9; n=13).

Ibodutant (3, 10, 30 and 100 nM) was devoid of any effect on the resting tension of the preparation whereas it concentration-dependently and with high potency antagonized [βAla^8]NKA (4-10) -induced contractile responses producing a parallel rightward shifts (Fig. 2A) of the agonist response curves without depressing the agonist E_{max} (34.6 ± 2.6; 33.2 ± 2.9; 31.1 ± 2.9; 33.3 ± 4.9 and 37.0 ± 1.7 mN in controls and in the presence of 3, 10, 30 and 100 nM of ibodutant, respectively). Schild plot analysis was consistent with competitive antagonism (slope = 1.02, 95% c.l. 0.85–1.19) and a pK_B value of 9.1 ± 0.05 was calculated (Fig. 2B).

No gender differences were seen in response to the NK_2 receptor-agonist [βAla^8]NKA(4-10) nor to the antagonist activity of ibodutant. In particular the E_{max} to [βAla^8]NKA(4-10) averaged 30.8 ± 3.7 mN and 37.0 ± 3.4 mN (n=5 and n=8, respectively, n.s.) in strips from male and female, respectively. Likewise the potency of [βAla^8]NKA(4-10) averaged 15.4 nM (95% c.l. 12.6–18.9) and 10.3 nM (95% c.l. 9.3–11.5) (n=5 and n=8, respectively, n.s.) in strips from male and female, respectively.

With regard to ibodutant the analysis of its antagonist potency toward [βAla^8]NKA(4-10) yielded an apparent pK_B value of 9.0 ± 0.1 and 9.1 ± 0.05 (n=18 and n=31, respectively, n.s.) in colonic strips from male and female patients, respectively.

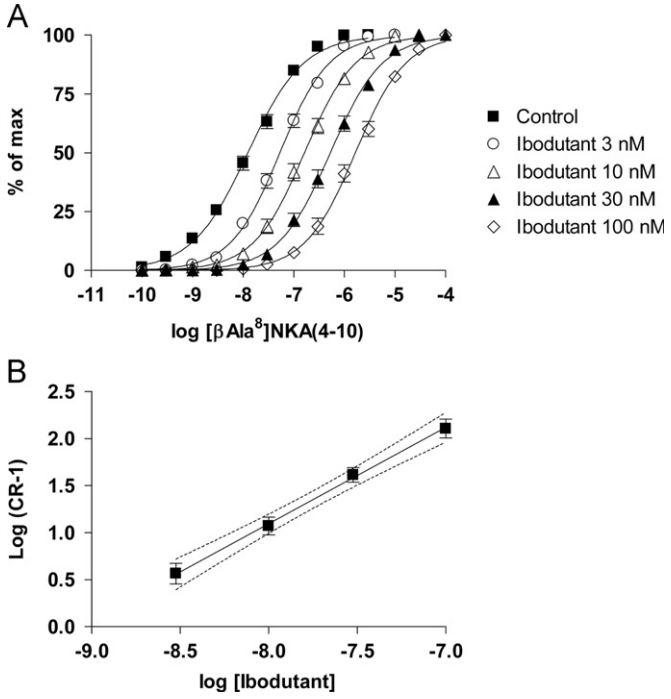

Fig. 2. (A) Antagonism by ibodutant towards the contractile responses induced by [βAla^8]NKA(4-10) in the circular muscle strips of human colon. Concentration-response curves for [βAla^8]NKA(4-10) were constructed as described in Section 2 in the absence (control) and presence of the indicated concentrations of ibodutant in the legend. Each value is the mean ± S.E.M. of 10–13 experiments. (B) Schild plot of agonist concentration ratios vs. ibodutant concentrations (Slope = 1.02, 95% c.l. 0.85–1.19; pK_B = 9.1 ± 0.05). Each value is the mean ± S.E.M. of 10–13 experiments.

3.3. Reversibility of functional tachykinin NK_2 receptor blockade

Reversibility of functional tachykinin NK_2 receptor blockade by ibodutant was evaluated by measuring the capability of the human colon muscle to recover the control contractile response produced by a single submaximal concentration (171 nM) of [βAla^8]NKA(4-10) which induced about 90% of its maximum contractile effect and amounted to 29.7 ± 3.2 mN (n=15).

After the incubation period of 60 min, ibodutant (10, 30, 100 and 300 nM) produced a significant concentration-dependent inhibition of the contractile effect produced by [βAla^8]NKA(4-10). At this time (Time 0) the inhibitory effect induced by ibodutant was 23 ± 9, 55 ± 12, 73 ± 6 and 84 ± 8% at 10, 30, 100 and 300 nM, respectively (Fig. 3). The inhibition remained constant for all concentrations tested with no recovery of the subsequent responses to the agonist obtained in drug-free medium for 180 min.

4. Discussion

In this study the pharmacological characterization of the tachykinin NK_2 receptor antagonist ibodutant (MEN15596) is presented in the circular smooth muscle of human colon, and the high affinity and antagonist potency, besides the long duration of action of this antagonist, proved also in this tissue.

The determination of ibodutant affinity through radioligand binding experiments indicate that this antagonist recognizes the NKA binding sites present in the human colon with a significant high affinity (pK_i 9.9). In the same experiments the affinity of the others tachykinin NK_2 receptor antagonists, nepadutant and saredutant, was evaluated as well. Present results indicate that overall the rank order of affinity values for the three antagonists,

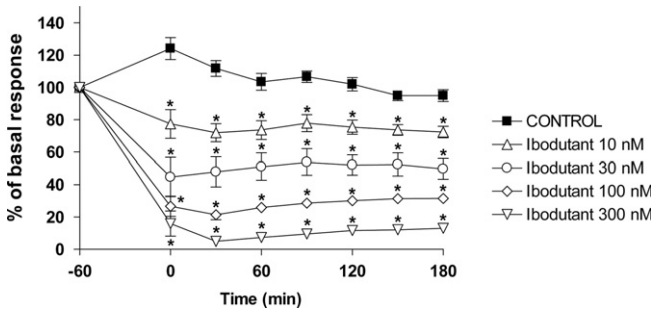

Fig. 3. Reversibility of tachykinin NK_2 receptor blockade induced by ibodutant in the isolated human colon. The values represent contractile responses to single submaximal concentration (171 nM) of [βAla^8]NKA(4-10) obtained in the absence (control) or in the presence (time 0) of the indicated concentrations of ibodutant administered 60 min before. Responses to the agonist were obtained every 30 min during which three wash cycles were performed as described in Section 2. Data are expressed as percentage of the basal response to [βAla^8]NKA(4-10) obtained in each preparation in the absence of the antagonist (or vehicle). Each value is the mean ± S.E.M of 3 experiments. * Significantly different toward the corresponding control responses: $p < 0.05$.

i.e. ibodutant (pK_i 9.9) > saredutant (pK_i 9.2) > nepadutant (pK_i 8.4), well matches with that previously obtained at the human recombinant tachykinin NK_2 receptor (ibodutant pK_i 10.8, saredutant pK_i 9.8, nepadutant pK_i 8.5; Meini et al., 2009; Renzetti et al., 1999). The affinity of saredutant and nepadutant was previously shown in the human colon also by Warner et al. (1999) and the calculated pK_i values are in a similar range (saredutant 9.5 and nepadutant 9.1).

The antagonist potency of ibodutant estimated at the NK_2 receptors of the human colon in the present study was similar (pK_B 9.1 ± 0.05) to that found in human urinary bladder (pK_B=9.2), guinea-pig colon (pK_B=9.3) and minipig urinary bladder (pK_B=8.8) NK_2 receptor smooth muscle preparations (Cialdai et al., 2006), but also in the inositol phosphates accumulation induced by NKA in CHO cells expressing the human NK_2 receptor (pK_B 10.3–10.6) (Meini et al., 2009).

The Schild analysis yielding to a slope (1.02) not significantly different from unity clearly indicates the competitive antagonist behavior of ibodutant in antagonizing the NK_2 receptor-mediated motor responses produced by the application of the selective NK_2 receptor agonist [βAla^8]NKA(4-10). In a previous study Burcher et al. (2008) reported a significantly higher B_{max} value for NK_2 receptor in male as compared to female human colon, although no differences in NK_2 receptor mRNA were observed as well as no difference in potency and maximal responses to NKA or [Lys^5, MeLeu^9, Nle^{10}]-NKA(4-10) were detected. Likewise, we failed to detect any gender differences in the contractile response to [βAla^8]NKA(4-10) nor in the antagonist potency of ibodutant. Overall the results of Burcher et al. (2008) and the present results offer little ground to speculate for a gender-related differential role of NK_2 receptors in the genesis of symptoms of IBS.

We have previously shown (Cipriani et al., 2011) that ibodutant prevents internalization of NK_2 receptors induced by [βAla^8]NKA(4-10) in human colon. The receptor internalization is unlikely to be relevant for present findings because: (a) the techniques used to highlight receptor internalization requires a prolonged exposition to the agonist at very low temperature followed by a rapid heating, far different in terms of kinetics from the present experimental conditions; (b) the protocol used in this study enables reproducible responses to NK_2 receptor agonists to be performed at relatively short (30 min) time intervals thus indicating that receptors internalization, if any, is negligible.

The kinetic profile of ibodutant was previously observed at molecular level and its fast associating and slow dissociating properties in the interaction with the human tachykinin NK_2 receptor assessed (Meini et al., 2009). In the current investigation, the kinetic of ibodutant interaction at the NK_2 receptor was evaluated by using a functional experimental approach. Present data indicate that ibodutant persistently binds to the human tachykinin NK_2 receptor expressed in the human colon smooth muscle, as the inhibition of the motor response induced by [βAla^8]NKA(4-10) does not recover during the 3h observation period in drug-free medium. These data obtained with ibodutant resemble those previously observed with the tachykinin NK_2 receptor antagonist saredutant in the same experimental model (Patacchini et al., 2000). On the other hand, although the functional receptor blockade exerted by ibodutant (present study) or by saredutant (Patacchini et al., 2000) in the present assay appear to be similar, analogs experiments performed in a cell system (Meini et al., 2009) indicated that despite of their slow dissociation, both antagonists exerted a reversible functional blockade. The different behavior obtained in the cell system and in the smooth muscle tissue can be ascribed to the different experimental parameters used, such as the concentrations of used agonist and antagonist and the kinetics of the measured response. On the other hand, despite the very slow dissociation property of both ibodutant and saredutant they are endowed of a different antagonist behavior. In particular although ibodutant slowly reverts from the receptor compartment it exerts a surmountable competitive antagonism type (present study, Meini et al., 2009), whereas saredutant was reported to display an insurmountable antagonism both in contractility smooth muscle (Patacchini et al., 2000) and cell system assays (Meini et al., 2009).

NKA, via NK_2 receptors, has been already documented to be a major mediator of the non-adrenergic non-cholinergic (NANC) excitatory imput to the circular muscle of human ileum (Maggi et al., 1992) and colon (Cao et al., 2000; Aulí et al., 2008). It appears therefore that the role of NKA as excitatory NANC enteric neurotransmitter, as widely documented to exist in various mammalian species (Holzer and Holzer-Petsche, 1997a,b), is largely maintained in humans and there is abundant evidence associating tachykinins with altered gastrointestinal motility, secretion and visceral sensitivity (Lecci et al., 2004 for review) thus making the field of tachykinins antagonists an appealing target for the development of a new pharmacological treatment of IBS.

The present findings, which document the potent and long lasting NK_2 receptor antagonist activity of ibodutant in human colon, support the concept that this molecule is suitable candidate for therapeutic strategies aiming at a control of exaggerated intestinal motility. Ibodutant is currently undergoing Phase II clinical trial in IBS patient with predominant diarrhea.

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