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Contraction of human colonic circular smooth muscle cells is inhibited by the calcium channel blocker pinaverium bromide

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Summary The effects of L-type calcium channel blockers (CCBs) selective for the gastrointestinal tract (pinaverium) or non-selective (nicardipine and diltiazem), were investigated on CCK-, CCh- or KCl-induced contraction of smooth muscle cells (SMC) isolated from the circular muscle layer of normal or of inflamed human colons. In the normal tissue colon, whatever the contractile agent used, CCK-8 (1 nM), CCh (1 nM) or KCl (20 mM), a micromolar concentration of pinaverium significantly inhibited contraction (88.36%, 93.10%, 93.92% inhibition respectively); this effect was concentration-dependent for CCh ($IC_{50} = 0.73 \pm 0.08$ nM) and for CCK ($IC_{50} = 0.92 \pm 0.12$ nM). In parallel, both nicardipine and diltiazem inhibit significantly contraction of isolated SMC. In inflamed colons, pinaverium (1 μM) display a significant higher efficacy than diltiazem or nicardipine to reduce cell contraction induced by CCK-8 or by KCl. In addition, RT-PCR experiments were performed to evidence tissue specificity of the L-type calcium channel. They revealed the expression of the messenger of the α-1 subunit L-type calcium channel (binding site of such CCBs), consistent with the expression of the rbC-2 splice variant of the α_1-C gene.

In conclusion: (i) the inhibition by calcium channel blockers of agonist-induced contractile activity suggest a modulation of SMC contraction upon extracellular calcium via 'L-type' voltage-dependent calcium channel; (ii) this study provides a rationale for the clinical use of pinaverium in colonic motor disoders affecting the contractility of SMC, since it appeared to decrease the contraction even in pathological situation; and (iii) RT-PCR experiments confirms the presence in human colon SMC of the α-1 subunit mRNA of calcium channel. © 2001 Harcourt Publishers Ltd

INTRODUCTION

Current drug therapy for functional gastrointestinal (GI) motility disorders [e.g. irritable bowel syndrome (IBS), chronic constipation] is not effective in many cases. A new approach to such disorders is based on the fact that Ca^{2+} is involved in excitation-contraction coupling, the mechanism which directly or indirectly controls GI motility.

Such a finding has provided strong rationale for testing therapeutic approaches based on Ca^{2+}-antagonism in organs such as the small or large bowel. Therapy with Ca^{2+}-antagonists can be used upon considering that activation of Ca^{2+} channels represent the final 'common path' of all mechanisms that regulate GI motility.

All smooth muscles possess Ca^{2+} selective voltage-dependent calcium channels (VDCC), which open when the cell membrane is depolarized. They represent the main route of entry of inward current that is gated by potential in smooth muscle cells, particularly in the GI tract. To date, among the five different subtypes of VDCC, only two types (L and T) were reported in intestinal smooth muscle, but the T-type channel was not clearly identified, and seemed to be dependent on species and tissues preparations. Both L- and T-types have been identified electrophysiologically in the rat colon [1], in guinea-pig taenia-coli [2] and in human colonic circular smooth muscle [3], whereas the L-type was found in

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circular smooth muscle from canine colon [4–5], and from canine and human jejunum [6].

As classical cardiovascular Ca^{2+}-antagonists (e.g. nicardipine, diltiazem) were shown to modify GI activity, they could represent a good promise as therapy for GI disorders. However, their adverse cardiovascular side effects represent the major disadvantages of such antagonists, which were overcomed by using the atypical Ca^{2+}-antagonist pinaverium bromide [7].

This quaternary ammonium compound, used in the treatment of hypermotility disorders of the intestine (such as Irritable Bowel Syndrome), is a spasmolytic agent devoided of any systemic cardiovascular effect even when given intravenously [8], indicating that it may have some specificity for intestinal VDCC. Pinaverium, exhibited in-vitro inhibitory effects on contractions induced by cholecystokinin and carbachol, and inhibited in-vivo contractile activities of the colon in animals [9] and of the sigmoid in man [10]. From these findings, it may be supposed that the regulation of smooth muscle contraction could result from an activation of VDCC, highly sensitive to a specific class of calcium channel blockers (CCB).

The selectivity of action of the calcium antagonists observed in clinical use, may arise from a number of classical factors, such as pharmacokinetic characteristics of the drug, effective drug concentration at its site of action, physicochemical properties, etc …

To evaluate the rational use of calcium channel blockers in colonic motor activity affecting the contraction of smooth muscle and to increase our understanding of the physiopathology of such functional disorders, we have first investigated changes in contractile activity of smooth muscle cells isolated from normal human colon in the presence or in the absence of pinaverium bromide or of two other classical calcium channel blockers, nicardipine and diltiazem.

In addition, we have also analyzed the influence of some features of mucosal inflammation on the contractility of smooth muscle cells from these tissues; in the last part, in order to evidence some tissue specificity of the L-type calcium channels, we have characterized the isoform found in human circular colonic smooth muscle.

METHODS

Chemicals

Potassium chloride (KCl), carbachol, C-terminal octapeptide of cholecystokinin (CCK-8), collagenase from *Clostridium histolyticum*, pronase, soybean trypsin inhibitor (STI), streptomycin and penicillin G, diltiazem and nicardipine, mineral oil were purchased from Sigma Chemical Co (St Louis, USA). Pinaverium bromide was provided from Solvay-Pharma (Suresnes, France) Moloney murine leukemia virus reverse transcriptase, ribonuclease inhibitor were from (Gibco BRL) and Taq polymerase from Promega (Madison, USA). *Medium A:* 132 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM HEPES, 0.2% glucose, 0.2% BSA, 0.02% phenol red, pH 7.4. *Medium B:* Earle's balanced salt solution containing 10 mM HEPES, 0.1% BSA, pH 7.4. PBS: phosphate saline buffer pH = 7.4.

Clinical design

This study was performed in accordance with the guidelines of the ethical committee of the Centre Hospitalier Régional et Universitaire de Nimes (France). Resection specimens were obtained from colon of patients undergoing surgery, after general anesthesia, for adenocarcinoma ($n = 34$), diverticular diseases ($n = 19$), ulcerative colitis (UC) ($n = 1$) and Crohn's disease (CD) ($n = 1$). Patients were operated for failure of medicinal therapy or development of chronic complications. Patients had been affected with CD or UC for at least 3 years. The average age was 66.24 ± 4.53 years with a range of 25–89 years in experiments with pinaverium, and 64.19 ± 4.98 years with a range of 43–79 with others calcium channel blockers. No statistical association were found between contraction values and the age of the patient in each group.

Selection of tissues for the study

The colonic circular smooth muscle used in these studies was surgically removed from a non-necrotic area, macroscopically free of carcinoma or polyps. Tissues from patients with large bowel obstruction were not used because of the impact of obstruction on mechanical and contractile properties of the smooth muscle. Only distal colons, corresponding to left colon and the adjacent half part of the transverse colon were used. The distance between the sigmoid and the resection tissues varied from one sample to another, the minimal distance being 3 cm. The excised tissue was then removed immediately after ligature and rapidly (5 min) transported to the laboratory in a dry bag stored on ice. After 3 time washing of the sample with PBS, part of the area selected for contraction studies, was used for histological examination: a full thickness sample was fixed in 10% formaldehyde for 24 h, embedded in paraffin, sectionned (5 μm), and stained with haematin and eosin; slides were examined in a blinded manner. An evaluation for the presence of inflammatory cells in the mucosa was achieved: the presence of anything other than scarce of vascular features of inflammation (oedema and vascular congestion confined to the

mucosa) or than discrete infiltration of polymorphonuclear cells in mucosa or lamina propria (plasma cells, lymphocytes, histiocytes, eosinophils or rare neutrophils) was considered as abnormal. The abundance of inflammatory cells and other features such as granuloma, muscular layer hypertrophy, neural hyperplasia, ulceration and crypt abcesses were recorded. The histological data were collected and used *a posteriori* with the functional data.

Isolation of smooth muscle cells

Mucosa and submucosa were removed by sharp microdissection. The mucosa was removed without stretching or damaging the underlying muscle; the circular muscle was then separated from longitudinal muscle and serosal layer. Muscle cells were isolated from the circular muscle layer (about 2 g) by a two-step enzymatic dissociation, following an adaptation of the method previously described in the case of animal tissues [11]. Briefly, muscle strips (2–3 mm^2) were incubated for 30 min at 37°C in medium A supplemented with antibiotics (100 UI/ml penicillin G and 50 μg/ml streptomycin), containing 0.27% collagenase, 0.03% pronase, 0.01% STI and gassed with 100% O_2. The incubation medium was diluted in medium A and filtered through a nylon mesh. Remaining tissues were washed with 20 ml of enzyme-free medium A, and muscle cells were allowed to disperse spontaneously for 30 min in medium A. Cells were then harvested by filtration through a 500 μm nylon mesh, diluted in fresh medium A and centrifuged at 150 g for 4 min. The cell pellet was then diluted in medium B. Viability (estimated by trypan blue exclusion) was always greater than 90%. This method yielded about 10^6 isolated circular smooth muscle cells per gram of wet tissue. It is emphasized that only those cells that dissociated in enzyme-free medium were used for subsequent studies.

Contraction studies on isolated smooth muscle cells were usually performed within 30 min after dissociation. An aliquot (0.45 ml) of cell suspension (10^4 cells/ml) was added to 50 μl medium B containing the contractile agent to be tested, thereby ensuring rapid mixing, and the reaction was stopped by adding 2% (vol/vol) glutaraldehyde. In control experiments, 50 μl medium B were used instead of the agent solution. For studies with calcium channel blockers, cells were first incubated for 10 min with the calcium channel blockers and then the contractile agent was added. The length of 100 cells was measured in sequential microscopic field by scanning micrometry (NIKON microscope with a JVC video-camera) .

Measurements were performed at the time of maximal contraction (statistically evaluated on 100 cells) which occurred by about 30 s after addition of the contractile agent (data not shown).

Calculations and statistical analysis

The contractile response was expressed as the per cent of decrease in mean cell length of a population of 100 SMC in the presence of the contractile agent as compared to control cells. At each experimental point, the decrease in mean cell length was determined using the formula $[(Lo - Lx)/Lo] \times 100$, in which Lo is the mean length of cells in the unstimulated state and Lx is the mean length of treated cells.

In statistical analysis, because data were not normally distributed, non-parametric tests were applied. A Kruskall–Wallis test was used to evaluate the effect of treatment with CCB by comparing each distribution of quantitative values (percent decrease of mean cell length), for each kind of tissue's population (global, normal, inflamed).

Amplification of calcium channel sequences

Total RNA was isolated by the guanidinium thiocyanate procedure from normal human colonic circular muscle homogenate or isolated cells. First strand cDNA synthesis was carried out on 5 μg of total RNA according to the following procedure: reverse primers used in the PCR (0.5 μg) together with the RNA sample were incubated for 10 min at 70°C. Then Moloney murine leukemia virus reverse transcriptase (200 U), deoxynucleotide triphosphates (1mM) and dithiothreitol (1mM) were added in a final volume of 20 μl. The mixture was incubated for 30 s at 42°C, then for 30 s at 50°C and finally for 15 min at 55°C. Then, ribonuclease inhibitor was added (10 U) and the preparation was incubated for further 20 min at 37°C. 100 pmoles of each specific primer were added to 5 μl of the first-strand synthesis mixture with the following: 5 U Taq polymerase, deoxynucleotide triphosphates (10 mM), 5 μl 10X PCR buffer, 2.5 mM $MgCl_2$ and H_2O to bring the final volume to 50 μl. The mixture was overlaid with 50 μl mineral oil and incubated in a MJ Research thermal cycler for 30 cycles each, consisting of 1 min denaturation at 94°C, 2 min hybridization at 56°C, and 90 s polymerization at 72°C. Primers hybridize to conserved regions of the canine and rabbit calcium channel alpha-1 subunit and are designed to amplify nucleotide sequences between the IVS1 and the IVS6 transmembrane segments. The forward primer (5′GTCACCCTTCC-AGGAGCAGGGGGA-3′) was chosen 942 bp upstream from the reverse primer (5′-GTCAAAGTTGTCCATGAT-GACAGC-3′).

Amplification products were analyzed on 1% agarose. DNA fragments were excised and purified using Wizard PCR Preps (Promega, Madison USA). Sequencing reactions were performed by ESGS (Montigny le Bretonneux, France).

RESULTS

Effect of pinaverium bromide on contraction of human smooth muscle cells

When incubating SMC with contractile agonist (1 nM CCh and 1 nM CCK) in the presence of increasing concentrations of pinaverium, a dose-dependent inhibition of agonist-induced contractions was observed. IC_{50} values, deduced from the previous curve, were about 0.73 ± 0.08 nM for CCh and of 0.92 ± 0.12 for CCK. This inhibitory effect was significant for at least 0.1 nM pinaverium (22% inhibition for CCh and 38% inhibition for CCK as shown in Fig. 1).

At 1 μM concentration the ability of pinaverium bromide to inhibit CCh-, CCK-8- and KCl-induced contraction of SMC was first evaluated (normal group). As some reduction in colonic contraction were generally observed in patients with inflammatory disease, we then analyzed SMC responsiveness with regard to the inflammatory status of the colon (inflamed group): SMC were incubated with an agonist concentration that caused maximal contraction (1 nM for CCh and CCK-8, 20 mM for KCl) as previously described [12].

In the normal group, significant changes in the mean cell length were observed after treatment with pinaverium ($P<0.001$), whatever the contractile agent used: it significantly reduced contraction, as expressed by the per cent of inhibition of the maximal response to CCh (88.36%), CCK-8 (93.10%), and KCl (93.92%) (Fig. 2, Table 1) .

In the inflamed group, although, the respective contractile effects of CCh, CCK-8 and KCl were lower in inflamed tissues than in normals as shown previously [12], pinaverium (1 μM) caused significant ($P<0.01$) reduction of contraction of isolated SMC (76.72% for CCh, 74.27 % for KCl) excepted for CCK (59.31%, $P=0.128$) (Fig. 2, Table 1).

It must be noted that the cell length of SMC in a resting state was found to be similar in inflamed and normal conditions (length in μm: global: 67.19 ± 3.52; normal: 67.46 ± 3.10; inflamed: 66.78 ± 3.52).

Effect of nicardipine and diltiazem on agonist-induced contraction of SMC

To compare the effect of pinaverium on agonist-induced contraction of SMC to that of other CCBs, we have tested in parallel compounds from the two main pharmacological classes of antagonists, the dihydropyridine class (nicardipine) and the benzothiazepine class (diltiazem). The CCB concentration used (1 μM) was that required to get maximal inhibition of contraction of isolated SMC as described previously [13].

In the normal group, both antagonists (nicardipine and diltiazem) inhibit significantly ($P<0.05$) contraction induced either by CCK or KCl (Tables 2 & 3, Figs 3 & 4).

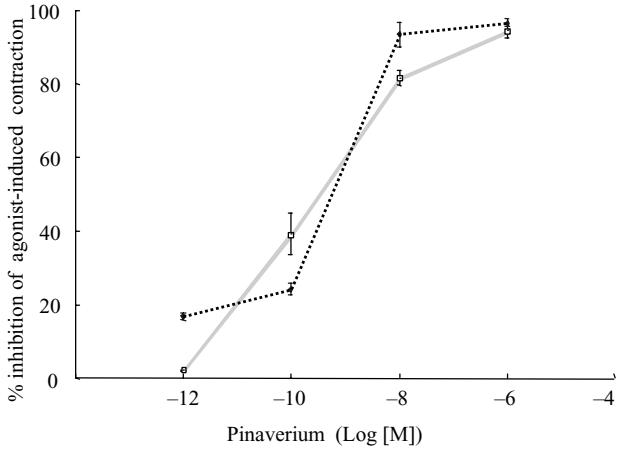

Fig. 1 Inhibition by pinaverium bromide of agonist-induced contraction. Cells (100 000 in 0.5 ml) are preincubated in medium B with various concentrations of pinaverium for 10 min, then CCh (1 nM) [solid line] or CCK (1 nM) [dotted line] were added for further 30 s at 37°C. After fixation, the length of 100 cells was measured (see Materials and Methods section). Each value is the mean ± SEM from six separate experiments. Contraction was expressed as the % of reduction of the mean cell length as compared to the mean cell length of unstimulated cells. Results are presented as % of inhibition of the maximal response to agonist.

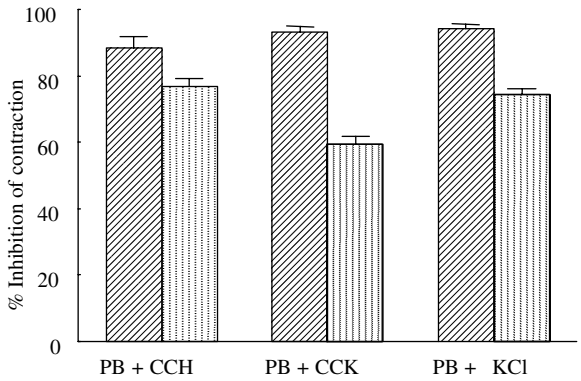

Fig. 2 Comparative inhibitory effect of pinaverium on hormone- and depolarization-induced contraction. Cells (100 000 in 0.5 ml) (normal group: solid bar and inflamed group: dotted bar) are preincubated in medium B with 1 μM pinaverium for 10 min at 37°C. Various agonists, carbachol (CCh), cholecystokinin (CCK) and potassium chloride (KCl) were then added for further 30 s. Contraction was expressed as the % of decrease of the mean cell length as compared to mean cell length of unstimulated cells. Inhibitory effect was expressed as the % of inhibition of the maximal response. Each value is the mean ± SEM from *n* separate experiments (see Table 1).

In a similar manner, in the inflamed group, addition of nicardipine or diltiazem did not significantly affect the contraction due to 1 nM CCK-8 or KCl ($P>0.05$) (Tables 2 & 3; Figs 3 & 4).

Table 1 Comparative inhibitory effect of pinaverium on hormone- and depolarization-induced contraction in normal and in inflamed colons.

		Normal					Inflamed				
		% Contraction			% Inhibition		% Contraction			% Inhibition	
		n	m	SEM	m	SEM	*n*	m	SEM	m	SEM
CCh	Alone	12	18.05	5.4			7	11.77	3.35		
	+PB	12	2.10	1.32	88.36	3.36	7	2.74	1.56	76.72	2.45
	KW		$P<0.01$					$P<0.01$			
CCK	Alone	15	18.81	1.9			10	11.33	2.62		
	+PB	15	1.3	1.25	93.10	1.58	10	4.61	2.03	59.31	2.33
	KW		$P<0.01$					$P<0.05$			
KCl	Alone	15	18.23	1.94			10	10.38	2.09		
	+PB	15	1.29	1.24	93.92	1.52	10	2.67	1.83	74.27	1.96
	KW		$P<0.01$					$P<0.03$			

Cells (100 000 in 0.5 ml) were preincubated in medium B with 1 μM pinaverium (PB) for 10 min at 37°C. Various agonists, carbachol (CCh), cholecystokinin (CCK) and potassium chloride (KCl) were then added for further 30 s. After fixation, the length of 100 cells was measured (see Materials and Methods section). Contraction was expressed as the % of reduction of the mean cell length as compared to mean cell length in the absence of stimulant, and inhibitory effect as the % inhibition of the maximal contractile response. Each value is the mean ± SEM from *n* separate experiments. A Kruskall–Wallis test (KW) was performed for each group of tissues to compare the contraction in absence (alone) or in presence of pinaverium (+PB). The signifiance is presented as *P*-value.
m: mean

Table 2 Comparative inhibitory effect of diltiazem on depolarization- and hormone- induced contraction in normal and inflamed colons.

		Normal					Inflamed				
		% Contraction			% Inhibition		% Contraction			% Inhibition	
		n	m	SEM	m	SEM	*n*	m	SEM	m	SEM
KCl	Alone	10	18.36	5.02			13	16.93	7.61		
	+DTZ	10	3.48	1.2	81.05	3.11	13	12.01	3.48	29.02	5.55
	KW		$P<0.05$					$P=0.686$			
CCK	Alone	10	18.48	5.74			14	15.17	4.65		
	+DTZ	10	2.14	1.36	93.10	1.58	14	11.48	5.8	24.32	5.23
	KW		$P<0.05$					$P=0.893$			

Cells (100 000 in 0.5 ml) were preincubated in medium B with 1 μM diltiazem (DTZ) for 10 min at 37°C. Various agonists, potassium chloride (KCl) and cholecystokinin (CCK) were then added for further 30 s. After fixation, the length of 100 cells was measured (see Materials and Methods section). Contraction was expressed as the % of reduction of the mean cell length as compared to mean cell length in the absence of stimulant, and inhibitory effect as the % inhibition of the maximal contractile response. Each value is the mean ± SEM from n separate experiments. A Kruskall–Wallis test (KW) was performed to compare the contraction in absence (alone) or in presence of diltiazem (+DTZ) for each group of tissues. The signifiance is presented as *P*-value.
m: mean

To address our hypothesis suggesting that agonist-induced contraction is modulated upon extracellular Ca^{2+} in circular layer of the colonic smooth muscle, we studied the mRNA expression of the L-type calcium channel in circular SMC from human colon.

Expression of the α_1-subunit

To assess the presence of the CCB binding site on SMC from the circular layer of human colon, molecular amplification technique was used by expression of the IVth repeating motif of the calcium channel α_1-subunit corresponding to this binding site. Because the initiating event for smooth muscle contraction is a rise in cytosolic calcium concentration, such investigation could confirm the participation of an extracellular route for the rise in intracellular calcium, through L-type calcium channel.

cDNA for α_1-subunit was obtained by reverse transcription of total RNA extracted either from the whole circular smooth muscle layer or from isolated SMC. Reverse

Table 3 Comparative inhibitory effect of nicardipine on depolarization- and hormone-induced contraction in normal and in inflamed colons.

		Normal					Inflamed				
		% Contraction			% Inhibition		% Contraction			% Inhibition	
		n	m	SEM	m	SEM	*n*	m	SEM	m	SEM
KCl	Alone	9	17.65	3.64			13	16.24	8.63		
	+NC	9	3.29	1.19	81.36	2.41	13	13.67	4.18	15.83	6.4
	KW		$P>0.10$					$P=0.788$			
CCK	Alone	9	18.20	4.29			13	15.56	7.41		
	+NC	9	2.73	0.42	85.00	2.36	13	5.10	3.32	67.22	5.37
	KW		$P<0.05$					$P=0.071$			

Cells (100 000 in 0.5 ml) were preincubated in medium B with 1 μM nicardipin (Nc) for 10 min at 37C. Various agonists, potassium chloride (KCl) and cholecystokinin (CCK) were then added for further 30 s. After fixation, the length of 100 cells was measured (see Materials and Methods section). Contraction was expressed as the % of reduction of the mean cell length as compared to mean cell length in the absence of stimulant, and inhibitory effect as the % inhibition of the maximal contractile response. Each value is the mean ± SEM from n separate experiments. A Kruskall–Wallis test (KW) was performed to compare the contraction in absence (alone) or in presence of nicardipin (+Nc) for each group of tissues. The significance is presented as *P*-value.
m: mean

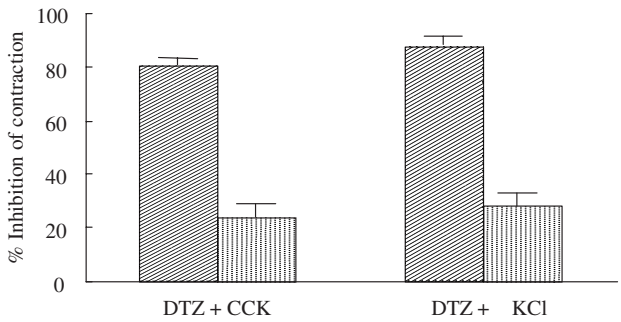

Fig. 3 Comparative inhibitory effect of diltiazem on hormone- and depolarization-induced contraction. Cells (100 000 in 0.5 ml) (normal group: solid bar and inflamed group: dotted bar) are preincubated in medium B with 1 μM Diltiazem (DTZ) for 10 min at 37°C. Various agonists, carbachol (C), cholecystokinin (CCK) and potassium chloride (KCl) were then added for further 30 s. Contraction was expressed as the % of decrease of the mean cell length as compared to mean cell length of unstimulated cells. Inhibitory effect was expressed as the % of inhibition of the maximal response. Each value is the mean ± SEM from *n* separate experiments (see Table 1).

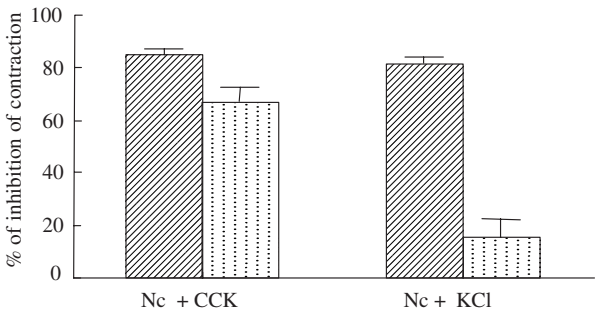

Fig. 4 Comparative inhibitory effect of nicardipin on hormone- and depolarization-induced contraction. Cells (100 000 in 0.5 ml) (normal group: solid bar and inflamed group: dotted bar) are preincubated in medium B with 1 μM nicardipin (Nc) for 10 min at 37°C. Various agonists, carbachol (C), cholecystokinin (CCK) and potassium chloride (KCl) were then added for further 30 s. Contraction was expressed as the % of decrease of the mean cell length as compared to mean cell length of unstimulated cells. Inhibitory effect was expressed as the % of inhibition of the maximal response. Each value is the mean ± SEM from *n* separate experiments (see Table 1).

primers used were the same oligonucleotides as in PCR procedure (see materials and methods section). Synthetic oligonucleotides were selected as primers in regions that are conserved between rabbit and canine intestine isoforms of the α_1-subunit. Whatever the source of RNA, a 942-bp fragment was easily identified after a 35 cycles-PCR, by ethidium bromide staining (Fig. 5A)

The amplicon specificity was established by the size of the fragment (about 942 bp), and confirmed by sequencing of the first 340 bp. The corresponding sequence was compared to that of other α_1-subunits in the NCBI database (BLAST network service) and 100% identity with the human cardiac L-type VDCC (gb[M57971]) was found.

We have further identified in the human colonic smooth muscle the presence of the rbC-2 gene isoform (Fig. 5B).

DISCUSSION

This work demonstrates that calcium channels blockers inhibit both neurohormonal- and depolarization-induced

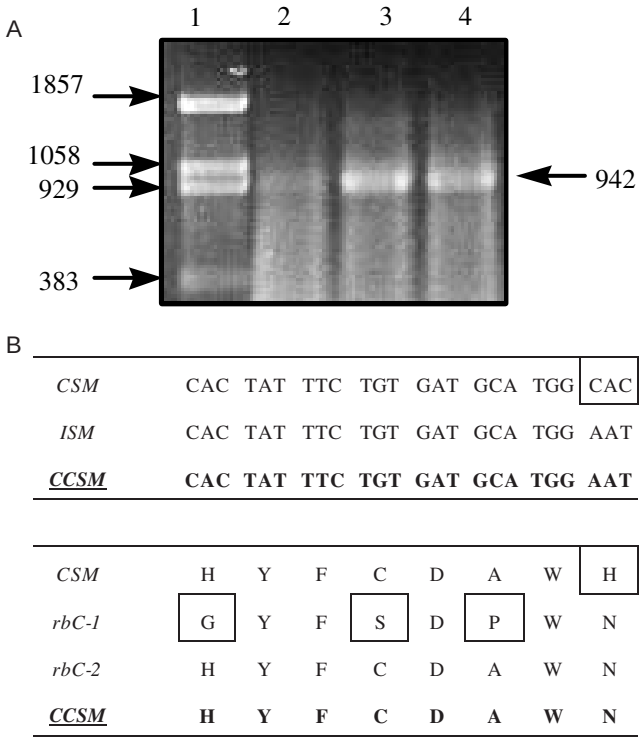

Fig. 5 Amplification of a specific cDNA fragment from the α_1-subunit of the intestinal smooth muscle calcium channel. 5A – amplification products (arrow on the right hand side) derived from mRNA of freshly isolated colonic smooth muscle cells (lane 2) or whole circular muscle layer (lane 3 & 4). Amplification products were separated on 1% agarose and stained with ethidium bromide. The size of molecular mass markers are indicated on the left hand side (lane 1). 5 B – Nucleotide and amino acid sequence of polymerase chain reaction product. Region of alternative splicing is shown in comparison with that of rbC-1 and rbC-2. (CSM: cardiac smooth muscle [gb M57971]; ISM: intestine smooth muscle [emb X78078]; rbC-1 and rbC-2 see reference [20]; CCSM: colonic circular smooth muscle)

contractions of SMC isolated from the circular layer of the human colon particularly, through an interaction with the α_1-subunit of the L-type calcium channel.

As previously described [12], cells isolated from the circular colonic smooth muscle were fully functional: a significant contraction (% decrease in mean cell length) was obtained for physiological concentrations of neurotransmitters (carbachol) or hormone (CCK), and these cells were shown to contain electrophysiologically active VDCCs [3]. We previously showed [14], that CCK-8-induced contraction of rabbit colonic cells, was dependent upon an increase in $[Ca^{2+}]_i$ concentrations. This increase may partly result from an influx of extracellular Ca^{2+} through voltage-sensitive or voltage-insensitive channels. Farrugia et al. [6], using single-channel and whole cell-patch clamp in freshly dissociated human and canine jejunal circular smooth muscle cells, have shown that the $[Ca^{2+}]_i$ level increase by acetylcholine (Ach) is mainly due extracellular Ca^{2+}. Indeed, ACh (1 mM) evoked an increase in inward current through an effect on DHP-sensitive channel: application of ACh (1 μM) to bath containing barium (as permeant ion) increased whole-cell inward current by 42%, which was blocked by nifedipine (1 μM). In addition, Sato et al., showed that about 80% of the contractile response to ACh of muscle strips from the circular layer of canine proximal colon was blocked by nicardipine [15].

We tested the effect of a quaternary ammonium compound, pinaverium bromide, which acts as a CCB [16,17]. In SMC isolated from the gastric antrum of the rabbit, pinaverium inhibited hormone-dependent contractions, with IC 50 values of 1nM for gastrin or CCK, and 20 nM for motilin as shown by Bobo et al. [13]. In SMC from normal human colon, this compound was as potent to inhibit CCK-induced contraction as to inhibit carbachol- or KCl-induced contraction (IC_{50} about 0.60–0.70 nM). As some reports showed reduction of colonic contraction in muscle strips from patients with inflammatory bowel diseases, we then analyzed data obtained with cells isolated from tissues, taking into account the inflammatory status of the colon (normal and inflamed groups). In tissues from inflamed colon, we found significant differences between the efficacies of the different CCB on agonist-induced contractions: in our experimental conditions, pinaverium was more efficient than the other CCBs. Little is known about the specific effects of CCB on contraction of colonic smooth muscles in inflammatory tissues. Colonic inflammation is accompanied by enhanced expression of interleukine-1β, a proinflammatory cytokine [18], that may influence synthesis or regulation of VDCC, as shown recently in cardiac myocytes [19]. Furthermore, the existence of three states of calcium channel, resting, open and inactivated, which can display different affinities for a given drug (the 'modulated receptor hypothesis') provides another explanation for tissue selectivity (e.g.: normal or inflamed tissue):

- Dihydropyridine bind more readily to the inactivated state, benzothiazepine to the resting state, and pinaverium combine with all three states with similar affinity [20].
- We postulate that inflammatory reaction affect the proportion of calcium channels in each state:

activated (open) ⟷ inactivated (closed) ⟷ resting

This change in the ratio of the three states could explain part of the differencies of effect between the three CCB in normal and in inflamed conditions.

Whatever the pathological status (inflamed or normal) of the tissue or the CCB used, contraction was not fully abolished: it is well established that another pathway, different from extracellular calcium entry, is responsible for a rise in intracellular Ca^{2+} and contraction, in this cell-type. Grider and Makhlouf [21] showed that both circular and longitudinal small intestine muscle layers (guinea-pig ileum and human jejunum) are able to mobilize Ca^{2+} by two different ways during agonist-induced contraction (ACh or CCK-8): Ca^{2+} was released from intracellular store in the circular layer; Ca^{2+} came from extracellular medium in the longitudinal layer. Contraction and increase in $[Ca^{2+}]_i$ were abolished by CCB in longitudinal, but not in circular SMC. Finally, Kuemmerle et al. [22] showed that, in longitudinal, but not in circular SMC, the agonist-mediated Ca^{2+} influx activated sarcoplasmic ryanodine-sensitive IP3-insensitive Ca^{2+} release. These findings suggest that L-type calcium channels are present only in the longitudinal layer.

These studies were performed in animals: as noted by Publicover et al. [23], the ratio between the type of calcium channels may vary between species and between distal and proximal localization on the colon: in these experiments, the CCB D600 was added simultaneously with agonists and, therefore, cannot exert the same effect as in our experiments where a 10 min preincubation period with pinaverium before addition of agonists was performed. Indeed, this clearly demonstrated that a given CCB interacts preferentially with a given state of the Ca^{2+} channel.

To address our hypothesis suggesting that agonist-induced contraction is modulated upon extracellular Ca^{2+} in circular layer of the colonic smooth muscle, we studied the expression of the L-type calcium channel in circular SMC from human colon. Biochemical and molecular biology studies have established that calcium channels are multi-subunit proteins composed of an ion conducting subunit (a large membrane-spanning protein) named the α1-subunit, and smaller accessory subunits. Concerning the α_1-subunit, which is the binding site of calcium channel blockers, six genes have been cloned, to date, and three of them (α_1-S, α_1-C, α_1-D) characterize the L-type; the α_1-C gene (CACH 2) is expressed in number of other tissues (vessels, heart, brain…) [For review, 24]. Using specific primers for the IVth domain of the α-1subunit, we amplified a 942 bp cDNA fragment from both isolated SMC or from the whole circular muscle layer. These observations support the following conclusion that L-type calcium channel is present in the circular layer of human colon.

The first amplified 340 bp cDNA fragment (following the sens primer) of the 940 bp of the IVth domain, encodes part of the human colonic α-1subunit. The comparison of published amino-acid sequences (between IVS1 and IVS2) reveals that this gene belong to the α_1-C class (CACH 2): the similarity with the α_1-C cardiac subunit is highly preserved [26]. These results are consistent with the inhibition of agonist-induced contraction by CCB. Indeed, dihydropyridine (DHP) binding site is located close to the SS1-SS2 region (P-loop) of repeat III [27,28] and to a sequence following the IV S6 segment [29,30]. Diltiazem binding site is defined in the SS1 IV segment; finally, Feron et al. [31] have characterized in isolated intestinal SMC the interaction of pinaverium bromide with specific binding site for DHP.

Alternative splicing has been shown to contribute to the molecular diversity of calcium channels. The α_1-C gene is alternatively spliced in at least six regions. One location of them, was found in the IV S3 as noted previously [31,32]. Snutch et al. [33] have determined two splice variants in the IV S3 segment refered as rbc-1 and rbc-2 (IV S3A and IV S3B), resulting in several amino-acid substitutions. Although our experiments cannot rule out the presence of another type of calcium channel, we showed the presence of rbc-2 form in the human colonic smooth muscle, as previously evidenced in canine colonic smooth muscle by Rich et al. [5], or in heart by Diebold et al. [34]. In rabbit colonic smooth muscle, Feron et al. [31] reported that both rbc-1 and rbc-2 (IV S A and IV S B) variants are equally expressed, but in this last study the authors used the whole smooth muscle layer of the small intestine, which could enhance the probability to amplify some other Ca^{2+}channels from their smooth muscle preparations (e.g. Ca^{2+} channel from interstitial cells of Cajal or from neurons that are more prevalent than in isolated circular smooth muscle cells). Our results indicate that rbc-2 is the major splice variant in smooth muscle of human colon. Other sites of alternative splicing have been reported in the extracellular loop between IV S3 and IV S4 segments [31], in the C-terminal tail [35,36] and in the IS6, IIS1 loop [35,37]. The functional significance of each of splice sites has not been determined. Alternative splicing could be differentially regulated in various human tissues. It is well established, however, that S4 segment in each domain contains a positively charged amino acid (arginine or lysine) at every third or fourth position and that it is believed to function as a voltage sensor for voltage-gated Ca^{2+} channels [38].

In conclusion, in this study CCB were shown to inhibit neurohormonal- and depolarization-induced contraction of smooth muscle cells isolated from the circular layer of human colon, suggesting that L-type calcium channels may contribute to the rise in $[Ca^{2+}]_i$ and to the contractile responses to such agonists as CCh, CCK-8, or KCl. Molecular data support this point of view by suggesting that, agonist regulation of contraction, in isolated circular SMC from human colon, might occur by a product of the α_1-C gene, as we showed the expression of the rbc-2

splice variant. However, these studies cannot rule out the presence of another type of calcium channel or another signalling pathway involving different second messengers.

Finally, our work provide a rationale for clinical use of pinaverium in colonic motor disorders affecting the contractility of SMC since pinaverium appeared to be very efficient to decrease the contraction of these cells.

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REFERENCES

1. Xiong ZL, Sperelakis N, Noffsinger A, Fenoglio-Preiser C. Change in calcium channel current densities in rat colonic smooth muscle cells during development and aging. *Am J Physiol* 1993; **265**: C617–C625.
2. Yoshino A, Someya A, Nishio A, Yazawa K, Usuki T, Yabu H. Mutiple types of voltage-dependent calcium channels of chick heart and skeletal muscle. *Pflugers Arch* 1989; **414**: 401–409.
3. Xiong ZL, Sperelakis N, Noffsinger A, Fenoglio-Preiser C. Ca^{2+} currents in human colonic smooth muscle cells. *Am J Physiol* 1995; **269**: G378–G385.
4. Ward SM, Sanders KM. Upstroke component of electrical slow waves in canine colonic smooth muscle due to nifedipine resistant calcium current. *J. Physiol* 1992; **455**: 321–337.
5. Rich A, Kenyon JL, Hume JR, Overturf K, Horowitz B, Sanders KM Dihydropyridine-sensitive calcium channel expressed in canine colonic smooth muscle cells. *Am J Physiol* 1993; **264**: C745–C754.
6. Farrugia G, Rich A, Rae JL, Sarr MG, Szurszewski JH. Calcium currents in human and canine jejunal circular smooth muscle cells. *Gastroenterology* 1995; **109**: 707–717.
7. Christen MO. Pinaverium bromide: a calcium antagonist with selectivity for the gastrointestianl tract. *Today's Therapeutic Trends* 1995; **13**: 47–62.
8. Guerot C, Khemache A, Sebbah J. and Noël B. Electrophysiological study of intravenous pinaverium bromide in cardiology. *Curr Med Res Opin* 1988; **11**: 73–79.
9. Fioramonti J, Christen MO, Dupre I. and Bueno L. Involvement of a CCK-dependent capsaicin-sensitive afferent pathway in the inhibitory effect of pinaverium bromide on the colonic motor response to eating in rats. *Fundam Clin Pharmacol* 1997; **11**: 231–236.
10. Passaretti S, Sorghi M, Colombo E, Mazotti G, Tittobello A and Guslandi M. Motor effects of locally administered pinaverium bromide in the sigmoid tract of patients with irritable bowel syndrome. *Int J Clin Pharmacol Ther Toxicol* 1989; **27**: 47–50.
11. Bitar KN, and Makhlouf GM. Receptors on smooth muscle cells: Characterization by contraction and specific antagonists. *Am J Physiol* 1982; **242**: G400–G407.
12. Boyer JC, Guitton C, Pignodel C, et al. Differential responsiveness to contractile agents of isolated smooth muscle cells from human colons as a function of age and inflammation. *Dig Dis Sci* 1997; **42**: 2190–2196.
13. Bobo MH, Magous R, Christen MO and Bali JP. Effect of pinaverium and other calcium channel blockers on contraction of isolated gastric antral smooth muscle cells caused by gastrointestinal hormones. *Life Sci* 1994; **54**: 1947–1954.
14. Moummi C, Magous R and Bali JP. Gastrointestinal hormones receptors on isolated smooth muscle cells from gastric antrum of the rabbit. *Biochem Pharmacol* 1989; **38**: 2895–2901.
15. Sato K, Sanders KM, Gerthoffer WT, and Publicover N. Sources of calcium utilised in cholinergic responses in canine colonic smooth muscle. *Am J Physiol* 1995; **267**: C1666–C1673.
16. Beech DJ, McKenzie I, Bolton TB and Christen MO. Effects of pinaverium on voltage-activated calcium channel currents of single smooth muscle cells isolated from the longitudinal muscle of the rabbit jejunum. *Br J Pharmacol* 1990; **99**: 374–378.
17. Malysz, J, Farraway, LA, Christen, MO and Huizinga, JD. Pinaverium acts as L-type calcium channel blocker on smooth muscle of colon. *Can J Physiol Pharmacol* 1997; **75**: 969–975.
18. Khan I, Al-Awadi. Colonic muscle enhances the production of interleukin-1beta messenger RNA in experimental colitis. *Gut* 1997; **40**: 307–312.
19. Thaik CM, Calderone A, Takashi N, and Colucci W S. Interleukin-1 beta modulates the growth and the phenotype of neonatal rat cardiac myocytes. *J Clin Invest* 1995; **96**: 1093–1099.
20. Christen MO. *In* Calcium Atagonism in Gastroenterology. Research and Perspectives Christen MO, and Paoletti R. eds, Kluwer, pp. 53–63, 1990.
21. Grider JR, and Makhlouf GM. Contraction mediated by Ca^{2+} release in circular and Ca^{2+} influx in longitudinal intestinal muscle cells. *J Pharmacol Exp Ther* 1988; **244**: 432–437.
22. Kuemmerle JF, Murthy KS and Makhlouf GM. Agonist activated ryanodine-sensitive, Ins(1,4,5)P_3-insensitive Ca^{2+} release channels in longitudinal muscle. *Am J Physiol* 1994; **266**: C1421–C1431.
23. Publicover NG, Horowitz NN, and Sanders KM. Calcium oscillation in freshly dispersed and cultured intestinal cells from canine colon. *Am J Physiol* 1992; **262**: C589–C597.
24. Perez-Reyes E, and Schneider T. Calcium channels: structure, function and classification. *Drug Develop Res* 1994; **33**: 295–318.
25. Feron O, Wibo M, Christen MO and Godfraind T. Interaction of pinaverium (a quaternary ammonium compound) with 1,4-dihydropyridine binding sites in rat ileum smooth muscle. *Br J Pharmacol* 1992; **105**: 480–484.
26. Perez-Reyes E, Wei XY, Castellano A, and Birbaumer, LJ. Molecular diversity of L-type calcium channels. Evidence for alternative splicing of the transcripts of three non allelic genes. *J Biol Chem* 1990; **265**: 20430–20436.
27. Striessnig J, Murthy JB and Catterall WA. Dihydropyridine receptor of L-type Ca^{2+} channel: identificatuion of binding domains for [^{3}H](+)-PN-200-110 and [^{3}H]azidopine within the α_1-subunit. *Proc Natl Acad Sci* 1991; **88**: 10769–10773.
28. Nakayama H, Taki M, Striessnig J, Glossmann H, Caterall WA and Kanaoka, Y. Identification of 1,4-dihydropyridine binding regions within the alpha1 subunit of skeletal muscle Ca^{2+} channels by photoaffinity labeling with diazipine. *Proc Natl Acad Sci* 1991; **88**: 9203–9207.
29. Regulla S, Schneider T, Nastainczyk W, Meyer HE, and Hofmann F. Identification of the site of interaction of the dihydropyridine channel blockers nitrendipine and azodipine with the calcium channel α_1-subunit. *EMBO J* 1991; **10**: 45–49.
30. Schuster A, Lacinova L, Klugbauer N, Ito H, Birnbaumer L and Hofmann F. The IV S6 segment of the L-type calcium channel is critical for the action of dihydropyridines and phenylalkylamines. *EMBO J* 1996; **15**: 2365–2370.

31. Feron O, Octave JN, Christen MO and Godfraind, T. Quantitative study of two splicing events in the L-type calcium channel α_1-subunit of intestinal smooth muscle and other tissues. *Eur J Biochem* 1994; **222**: 195–202.
32. Koch WJ, Ellinor PT and Schwartz A. Cloning of a dihydropyridine-sensitive calcium channel from rat aorta. *J Biol Chem* 1990; **265**: 17786–17791.
33. Snutch TP, Tomlinson WJ, Leonard JP and Gilbert MM. Distinct calcium channels are generated by alternative splicing and are differentially expressed in mammalian CNS. *Neuron* 1991; **7**: 45–57.
34. Diebold RJ, Koch WJ, Ellinor, PT et al. Mutually exclusive exon splicing of the cardiac calcium channel alpha 1 subunit gene generates developmentally regulated isoforms in the rat heart. *Proc Natl Acad Sci* 1992; **891**: 497–1501.
35. Hui A, Ellinor PT, Krizanova O, Wang JJ, Diebold RJ. and Schwartz A. Molecular cloning of multiples subtypes of a novel rat brain isoform of the alpha-1 subunit of the voltage-dependent calcium channel. *Neuron* 1991; **7**: 35–44.
36. Mori Y, Friedrich T, Kim MS et al. Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature* 1991; **350**: 398–402.
37. Biel M, Ruth P, Bosse E et al. Primary structure and functionnal expression of a high voltage activated calcium channel from rabbit lung. *F.E.B.S. Lett* 1990; **269**: 409–412.
38. Catterall WA. Structure and function of voltage-sensitive ion channels. *Science* 1988; **242**: 50–61.