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Calcitonin Gene-related Peptide-induced Relaxation of Isolated Human Colonic Smooth Muscle Cells through Different Intracellular Pathways

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ABSTRACT. Calcitonin gene-related peptide (CGRP) plays a significant role in the non-adrenergic non-cholinergic (NANC) regulation of intestinal tract motility. In this work, the contractile properties of enzymatically isolated circular smooth muscle cells (SMC) from human colon in response to CGRP were evaluated. Relaxation by CGRP (1 μM) was determined in cells maximally contracted by carbachol (CCh, 1 nM). Simultaneously, cGMP contents of SMC were measured by radioimmunoassay. CCh-induced contraction was inhibited by 1 μM CGRP (maximum: 69 ± 5% within 60 sec); similarly, exposure of cells to sodium nitroprussiate (SNP),1 μM, fully inhibited contraction (maximum: 89 ± 8% within 30 sec). In the same time-course as for relaxation, CGRP and sodium nitroprussiate caused significant increase in intracellular cGMP levels (2- and 10-fold that of the basal level, respectively, $P < 0.01$). The nitric oxide synthase (NOS) inhibitor, L-N5(I-iminoethyl)ornithine, dihydrochloride, (L-NIO), 1 μM, partly inhibited SMC relaxation induced by CGRP (78.26%); the protein kinase inhibitor, *N*-(2-aminoethyl)-5-isoquinolinesulfonamide hydrochloride (H9), 1 μM, and the selective cAMP-dependent protein kinase inhibitor, adenosine-3′,5′-monophosphorothioate triethylammonium salt, Rp isomer, (Rp-cAMP(S)), 1 μM, also caused inhibition of relaxation (70.30% and 28.6%, respectively). In parallel, the increase in cGMP caused by CGRP was partly reduced by L-NIO (65.47%) and by H9 (55%). In conclusion, the nitric oxide generation following exposure of human colonic SMC to sodium nitroprussiate causes relaxation through the cGMP pathway; on the other hand, exposure of SMC to CGRP causes relaxation in part by activation of nitric oxide synthase and guanylate cyclase and in part through the cAMP pathway. BIOCHEM PHARMACOL **56**;9:1097–1104, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. colon; smooth muscle; circular muscle layer; calcitonin gene-related peptide; sodium nitroprusside; relaxation

Many studies have emphasized the importance of the enteric nervous system in the control of gastrointestinal motility. This enteric nervous system, third component of the autonomic nervous system, comprises a large number of neurons that are organized in ganglionated plexuses: the myenteric plexus is embedded within the gastrointestinal wall between longitudinal and circular muscle layers. This system includes distinct types of neurons that release neurotransmitters different from noradrenaline and acetylcholine and are therefore called NANC.‖ NANC innervation of the circular smooth muscle of human colon has also been documented for many years [1].

CGRP is a 37 aminoacid neuropeptide found in two isoforms (α and β) in man and in the rat: the first one, α-CGRP, is encoded by the calcitonin gene, while the second form, β-CGRP, is a product of a separate gene [2, 3]. In the peripheral nervous system, CGRP was found in both sensory and motor nerves; it appeared postganglionically in various NANC nerves of the gastrointestinal tract [4]. In the rat, myenteric CGRP neurons issue projections within both the myenteric plexus and the circular muscle. In addition, in the guinea-pig intestine, circular muscles and the myenteric plexus were shown to contain numerous CGRP-immunoreactive nerve fibers [5].

In the gut, the predominant effect of CGRP is to inhibit gastrointestinal motor function [5–7]. In man, the lack of peristalsis in Hirschprung's disease due to an absence of NANC inhibitory innervation is emphasized by a reduction in CGRP-immunoreactive nerve fibbers of the ganglionic segment and by an absence of NOS in the neuronal system [8]. The NOS (constitutive cNOS and inducible iNOS), which have been evidenced throughout the gastrointestinal

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‖ *Abbreviations*: cAMP-PK, cyclic AMP-dependent protein kinase; cGMP-PK, cyclic GMP-dependent protein kinase; CCh, carbachol; CGRP, calcitonin gene-related peptide; H9, N-(2-aminoethyl)-5-isoquinolinesulfonamide hydrochloride; IBMX, 3 isobutyl-1-methyl xanthine; L-NIO, L-N5(I-iminoethyl)ornithine, dihydrochloride; NANC, non-adrenergic non-cholinergic; NOS, nitric oxide synthase; Rp-cAMP(S), adenosine-3′,5′-monophosphorothioate triethylammonium salt, Rp isomer; SMC, smooth muscle cells; SNP, sodium nitroprusside; and VIP, vasoactive intestinal peptide.

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tract, catalyze a sequence of oxidative reactions leading to NO generation.

Furthermore, *in vitro* studies using isolated gastric SMC from various animal species, devoid of neural elements, demonstrated the ability of some neuropeptides, like VIP, to induce relaxation through stimulation of NO generation [9–11]. In addition, by blocking NOS activity, the involvement of NO in the relaxant effect of CGRP on substance *P*-induced uterine contraction was demonstrated [12]. It has also been reported that CGRP caused both cGMP production and relaxation of vascular smooth muscles [4] and induced relaxation by modulation of NO release in the guinea-pig gallbladder [13]. Nevertheless, the mechanism by which CGRP causes colonic relaxation in human tissues is not clearly defined.

In this work, the involvement of intracellular pathways in the action of CGRP on isolated SMC muscle cells from human colons was studied, and the effects of CGRP on both carbachol-induced contraction and cGMP contents as compared to those of SNP were evaluated. In addition, in order to assess the intracellular signaling of the NO pathway, the effects of pharmacological inhibitors of this NO pathway on cell contraction and cGMP contents were studied.

MATERIALS AND METHODS

Chemicals

Human CGRP, SNP, CCh, collagenase from *Clostridium histolyticum*, pronase, soybean trypsin inhibitor (STI), streptomycin, penicillin G, IBMX and glutaraldehyde were purchased from Sigma; H9 and L-NIO were obtained from RBI and Rp-cAMP(S) was from Alexis Corp.; cGMP radioimmunoassay kit was from Immunotech. Medium A: 132 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1 mM of $CaCl_2$, 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.

Clinical Details

This study was performed in agreement with the guidelines of the ethical committee of the Centre Hospitalier Universitaire de Nimes (France). Resection specimens were obtained from distal colon of patients undergoing colon surgery for adenocarcinoma (N = 17) or diverticular diseases (N = 7). The average age was 73 ± 2 years (14 men and 10 women), with a range of 90–48 years.

Selection of Tissues for Study

The muscle tissue used in these studies came from a colonic area macroscopically free of carcinoma or polyp. Tissues from patients with large bowel obstruction were discarded because of the incidence of obstruction on the contractile properties of intestinal muscles.

The muscle tissue was removed immediately after ligature and rapidly (5 min) transported to the laboratory in a dry bag stored on ice. After washing of the sample with PBS (3 times), a tissue fragment from the area used for contraction studies was removed for histologic examinations after H&E staining. All slides were examined in a blinded manner and histologic data were collected and used a posteriori. In this study, only the results obtained from normal tissues are presented: the existence of anything other than small numbers of vascular features of inflammation (oedema and vascular congestion confined to the mucosa) or discrete infiltration of mucosa or lamina propria by polymorphonuclear cells (plasma cells, lymphocytes, histiocytes, eosinophils or rare neutrophils) was considered as abnormal.

Isolation of SMC

Mucosa and submucosa were removed by microdissection without stretching or damaging the underlying muscle. The circular muscle layer was then separated from longitudinal muscle and serosal layers. SMC from the circular muscle layer (ca. 2 grams) were then isolated by a two-step enzymatic dissociation, adapted from the method previously described for animal tissues [14]. Briefly, muscle strips (2–3 mm [2] were incubated for 10 min at 37° in medium A supplemented with antibiotics (100 UI/mL of penicillin G and 50 μg/mL of streptomycin), containing 0.27% collagenase, 0.03% pronase, 0.01% soybean trypsin inhibitor, 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. Cells were then harvested by filtration through a 500-μm nylon mesh, diluted in fresh medium A and centrifuged (150g, 4 min). The cell pellet was then diluted in medium B. This method yielded about 10^6 isolated circular SMC per gram of wet tissue. Purity in circular muscle (95 to 99%) of the resulting preparation was evaluated by histologic examination. Viability (estimated by trypan blue exclusion) was always greater than 95%. It is to be noted that only those cells that dissociated in enzyme-free medium were used for subsequent studies.

Contraction/Relaxation Measurements

An aliquot (0.45 mL) of the cell suspension (10^4 cells/mL) was added to 50 μL of 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 of medium B were used instead of the agent solution. An aliquot of the cell suspension was placed on a Thoma slide and the length of the first 100 whole cells encountered randomly in successive microscopic fields was measured. Cell lengths were

evaluated with a scale mask directly on the video screen (NIKON optiphot-2 microscope with a Hitachi KP C-501 camera). All measurements were performed at the time of maximal contraction (statistically evaluated on 100 cells), which occurred by 30 sec after addition of the contractile agent CCh.

Relaxation was determined as previously described [14]: cells were preincubated with CGRP or SNP for various periods of time, then CCh (1 nM) was added for an additional 30 sec; the reaction was stopped by fixation with 2% (vol/vol) glutaraldehyde.

In some experiments, smooth muscle cells were first incubated for 10 min with the NOS inhibitor L-NIO or with the cAMP-dependent protein kinase inhibitor Rp-cAMP(S), or for 5 min with the protein kinase inhibitor H9. In order to determine the maximally effective concentration of the various inhibitors, dose-response effects were evaluated in the range 1 nM–10 μM.

Cyclic GMP Content Determination

Intracellular cGMP contents of isolated circular SMC were measured by radioimmunoassay as follows: a threshold concentration of IBMX (1 μM) was added to each aliquot (0.25-mL aliquots of the cell suspension containing 10^4 cells/mL) at the time of addition of relaxant agents. For measurements of cGMP contents, the same time sequence as previously indicated for contraction/relaxation studies was used for incubation of cells and addition of relaxant agents; the reaction was stopped by addition of 60% ice-cold perchloric acid (v/v). Then, the suspension was centrifuged (2000 *g*, 15 min, 4°). The resulting supernatant was recovered for cyclic GMP determinations in duplicate by radioimmunoassay. Samples were neutralized then succinylated. Fifty μL of aliquots from each succinylated sample were incubated for 22 hr at 4° in antibody-coated tubes in the presence of 504.5 Bq [I^{125}]-ScGMP-TME. Then, the content of the tubes was discarded and bound radioactivities were measured in a gamma counter. The amount of cGMP was obtained by interpolation on a standard curve and was expressed as pmol/10^6 cells or as percent increase above basal levels.

Calculations and Statistical Analysis

Contractile responses were expressed as the percentage of decrease in mean cell length in the presence of the contractile agent as compared to control cells. For each experimental point, the decrease (or increase) in mean cell length was determined using the formula [(Lo-Lx)/Lo] × 100, in which Lo is the mean length of cells in a resting state and Lx is the mean length of treated cells.

Relaxation was expressed as percent variation in the mean length of CCh-contracted muscle cells or as percent inhibition of contraction.

Statistical analyses were performed using Student's *t*-test for paired or unpaired values. Data were expressed as mean ± SEM from N different samples.

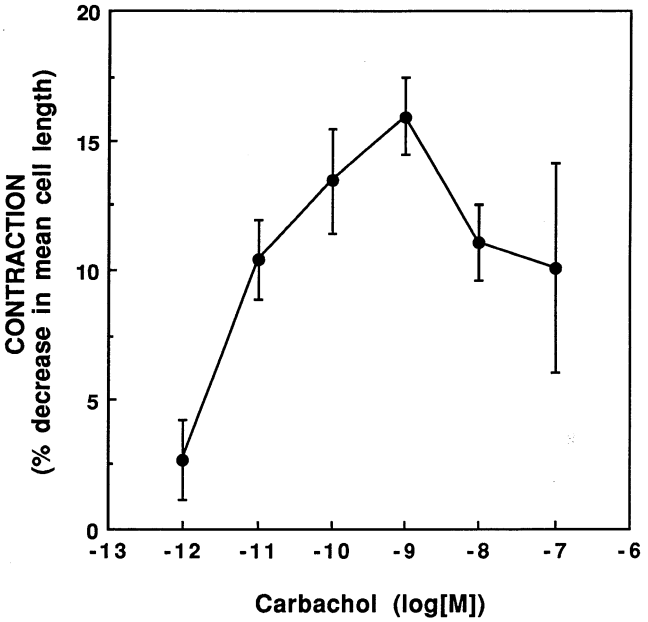

FIG. 1. Dose-response curve for effects of carbachol on colonic SMC. Cells were suspended and incubated at 37° with various CCh concentrations for 30 sec. After fixation, as described in the Methods section, the lengths of 100 cells were measured. Results (mean ± SEM from five separate experiments) were expressed as % decrease in mean cell length.

RESULTS

Effects of CGRP and SNP on Carbachol-induced Contraction

As shown recently [15], CCh causes dose-dependent contraction of isolated SMC from the human colon with a maximal effect at 1 nM (Fig. 1). In order to determine the optimal time of incubation for the action of CGRP and SNP on CCh-induced contraction, we examined the time courses of CGRP- and SNP-induced relaxations following stimulation of SMC by a maximal concentration (1 nM) of CCh.

As illustrated in Fig. 2, CCh-induced contraction (22.3 ± 1.7% decrease in mean cell length) was significantly inhibited by a 10 sec preincubation with 1 μM CGRP (45%, $P < 0.01$). Maximal inhibition (69 ± 5%) occurred within 60 sec, while increasing the incubation time did not increase CGRP-induced inhibition (basal: 22.3 ± 1.7% contraction; at 10 sec: 12.3 ± 1.3% contraction; at 60 sec: 6.9 ± 2.2% contraction). When resting cells were incubated with 1 μM CGRP, the mean length of the cells was not altered.

Exposure of cells to SNP (Fig. 3) for at least 5 sec before CCh addition led to significant inhibition (35%) of the contractile response to carbachol; maximal inhibition (89 ± 8%) was obtained by 30 sec (basal: 18.8 ± 3.1% of contraction; at 5 sec: 12.2 ± 4.7% of contraction; at 30 sec: 2.09 ± 1.7% of contraction). From these curves, it can be

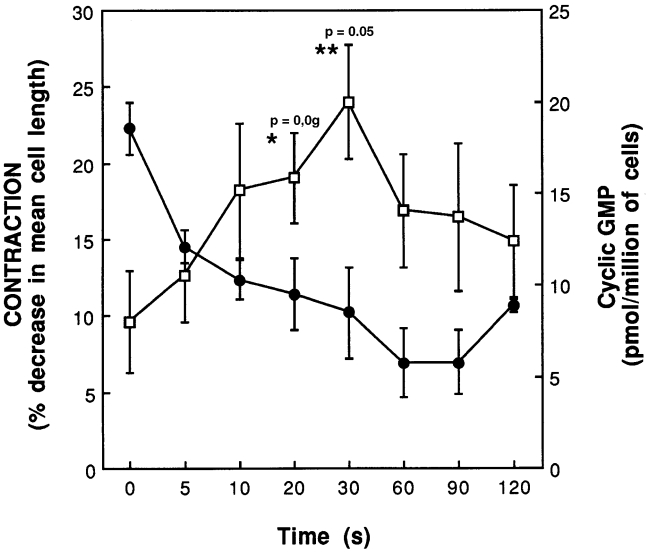

FIG. 2. Time course for effects of CGRP on carbachol-induced contraction and intracellular cGMP levels in human colonic SMC. Cells were suspended and preincubated at 37° for indicated times alone or with 1 μM CGRP. Then CCh was added for an additional 30 sec and the lengths of 100 cells were measured. Results (mean ± SEM from five separate experiments) were expressed as % decrease in mean cell length (●). Cells were preincubated, for indicated periods of time, in the presence of a threshold concentration of IBMX (1 μM) with CGRP (1 μM). Then, after CCh (1 nM) exposure for 30 sec, the reaction was stopped as described in the Methods section and cGMP concentrations were determined by radioimmunoassay. Results are expressed as pmoles cGMP per million cells (□). Values are means ± SEM from five separate experiments (basal value: 8.04 ± 2.76 pmoles/million cells). Significantly different from controls at *P < 0.05.

seen that the time required to achieve the half-maximal response to SNP was approximately 9 sec for SNP and 17 sec for CGRP.

To further evaluate the concentration-dependence of the inhibition of CCh-induced contraction caused by CGRP, cells were incubated with various CGRP concentrations for 60 sec prior to CCh addition. As illustrated in Fig. 4, a significant inhibition of contraction (35.7 ± 2.4%, $P < 0.01$) was obtained with concentrations of CGRP as low as 1 nM, and when concentrations of CGRP increased, relaxation became maximal at 1 μM, then decreased to reach a plateau at 0.1 mM CGRP or greater. The concentration of CGRP required to obtain 50% of the maximal relaxation was in the range of 2 nM (2 ± 1.8 nM; N = 5).

Stimulation of cGMP Production by SNP and CGRP in Circular Colonic SMC

The ability of CGRP or SNP to stimulate cGMP production in circular muscle cells was then studied. The time-courses for effects of CGRP or SNP on cGMP levels are shown in Figs. 2 and 3. Incubation of cells with 1 μM CGRP (Fig. 2) caused a significant increase in cGMP (basal value: 8.04 ± 2.76; maximum:19.98 ± 3.09 pmol/million cells). The time course of the increase in cGMP levels reached a maximum by 30 sec, then declined subsequently. Similarly, after addition of 1 μM of SNP (Fig. 3), cGMP levels increased maximally within 5 sec (basal: 7.86 ± 1.13; maximum: 79.72 ± 3.2 pmol/million cells), then decreased by 20 sec (17.52 ± 2.56 pmol/million cells). It must be noted that, as previously shown [16], the inhibition of cAMP-dependent phosphodiesterases by 0.1 μM IBMX was without any effect on the length of the unstimulated cells. As compared to that observed with CGRP, the time-course for the effect of SNP was faster (t1/2: 10 sec for CGRP and 2.5 sec for SNP) and the peak level of cGMP was higher (19.98 ± 3.09 and 79.72 ± 3.2 pmol/million cells for CGRP and SNP, respectively). Maximal changes in cGMP levels with both agents were achieved at least 25 sec prior to maximal changes in contractility.

Effect of L-NIO and H9 on Relaxation Induced by CGRP

In order to study the involvement of the NO pathway in the response of SMC to CGRP, we evaluated the effects of an NOS inhibitor (L-NIO) and of a protein kinase inhibitor (H9) on both relaxation of SMC and intracellular cGMP

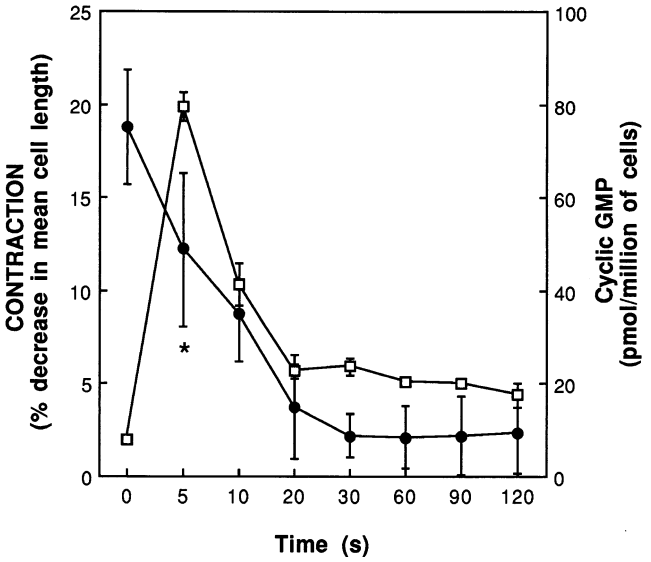

FIG. 3. Time-course for effects of SNP on carbachol-induced contraction and intracellular cGMP levels in human colonic SMC. Cells were suspended and preincubated at 37° for indicated times alone or with 1 μM SNP. Then CCh was added for an additional 30 sec and the lengths of 100 cells were measured. Results (mean ± SEM from five separate experiments) were expressed as % decrease in mean cell length (●). Cells were preincubated, for indicated periods of time, in the presence of a threshold concentration of IBMX (1 μM) with SNP (1 μM). Then, after CCh (1 nM) exposure for 30 sec, the reaction was stopped, as described in the Methods section, and cGMP concentrations were determined by radioimmunoassay. Results are expressed as pmoles cGMP per million cells (□). Values are means ± SEM from five separate experiments (basal value: 8.04 ± 2.76 pmoles/million cells). Significantly different from controls at *P < 0.05. (Basal value for cGMP levels: 7.86 ± 1.13 pmoles/million cells). Significantly different from controls at *P < 0.05, **P < 0.01.

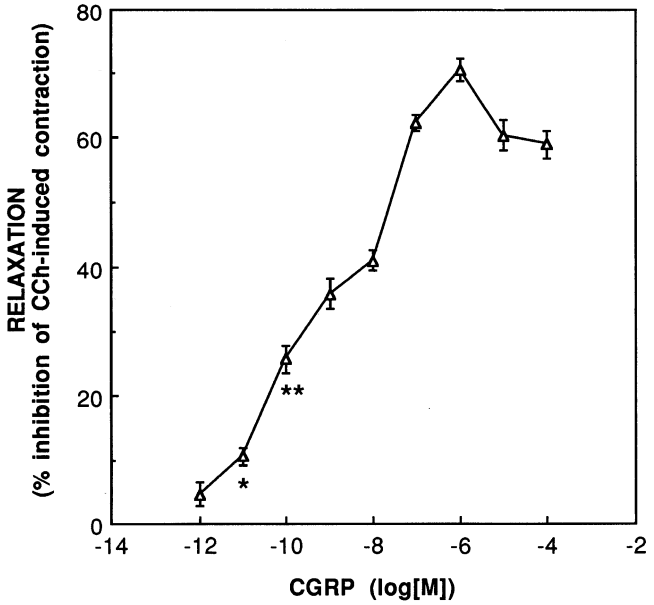

FIG. 4. Dose-response curve for effects of CGRP on CCh-induced contraction of human SMC. Cells were preincubated in the presence of increasing concentrations of CGRP for 1 min at 37°. Then 1 nM CCh was added for an additional 30 sec and the lengths of 100 cells were measured. Results (means ± SEM from five separate experiments) are expressed as percent inhibition of CCh-induced contraction.

levels caused by CGRP; furthermore, the possible implication of the cAMP pathway in the relaxation of SMC caused by CGRP was analyzed by studying the effects of selective Rp-cAMP(S). As expected, H9, Rp-cAMP(S), and L-NIO had no effect on the length of either unstimulated or CCh-treated SMC.

Figure 5 shows that L-NIO reduced CGRP-induced relaxation of colonic circular SMC, and this effect was concentration-dependent with a maximally effective concentration at 1 μM (not shown); at this concentration, L-NIO reduced CGRP-induced relaxation of colonic circular SMC (with CGRP: 95.01% relaxation; with CGRP + L-NIO: 20.65% relaxation, $P < 0.01$). Similarly, H9 (1 μM) caused a reduction in CGRP-induced relaxation (with CGRP: 95.01% relaxation; with CGRP+H9: 28.2% relaxation, $P < 0.01$) (Fig. 5). In addition, the reduction in relaxation observed with 1 μM L-NIO was fully reversed by a 10-min preincubation with L-arginine (100 μM) (94.3% relaxation). Finally, Rp-cAMP(S) at 1 μM only partly reversed the relaxing effect of CGRP (with CGRP: 95.01% relaxation; with CGRP + Rp-cAMP(S): 71.8% relaxation, $P < 0.05$).

Effect of L-NIO and H9 on cGMP Production Induced by CGRP

In freshly isolated circular SMC from the human colon, CCh did not significantly modify the level of intracellular cGMP levels. Figure 6 shows that addition of 1 μM CGRP caused an increase in cGMP levels (CCh: 1.63 ± 0.78% above basal; with CGRP: 24.93 ± 3.2% above basal) and this effect was inhibited by 1 μM L-NIO (with CGRP + L-NIO: 9.13 ± 1.02% above basal). Similarly, with a maximally effective concentration of H9 (1 μM), the CGRP-induced rise in cGMP levels was inhibited (with CGRP+H9: 10.98 ± 1.8% above basal) (Fig. 6).

DISCUSSION

Neuropeptides are thought to play an important role in the regulation of gut motility. The role of CGRP in intestinal smooth muscles has been difficult to establish in studies performed on intact tissue where diffusion barriers and tissue heterogeneity, particularly the presence of neural tissue, impose limitations on the interpretation of the results. In this work, we have attempted to overcome these limitations by performing studies on isolated SMC from human colon, which provide a homogenous cell system that is also free of diffusion barriers. Compared to strips, this model allowed us to investigate direct effects of drugs at the post-synaptic level.

SMC were isolated from the circular layer of human colon. The circular smooth muscle layer was chosen because previous studies had indicated that circular and longitudinal muscles differed in their sensitivity to pharmacological agents and that the circular layer played a predominant role in the contractile activity of the gut. Microscopic examination of the cell preparation showed a homogenous suspension of isolated SMC. Contraction by

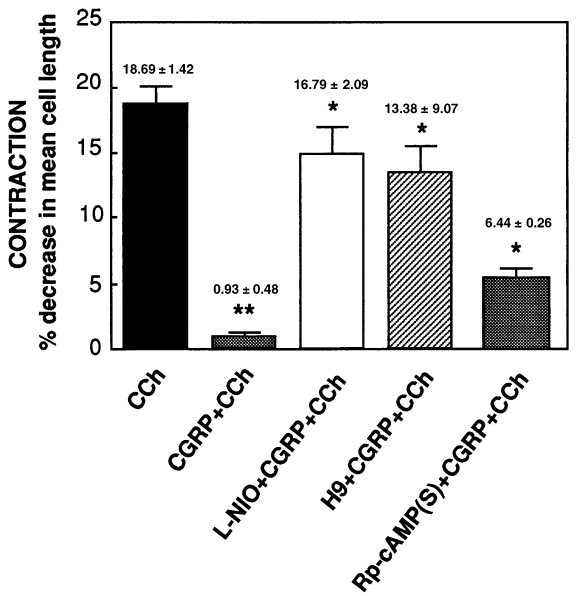

FIG. 5. Effects of L-NIO, H9 and Rp-cAMP[S] on CGRP-induced relaxation of colonic smooth muscle cells. Contraction (1 nM CCh) was measured alone (black bar), or in the presence of CGRP (1 μM) for 60 sec (grey bar), after exposure to L-NIO (open bar) or to Rp-cAMP(S) (grey bars) for 10 min, or to H9 (hached bar) for 5 min. Results are expressed as percent decrease in mean cell length. Values are means ± SEM from six separate experiments. Significantly different from CCh alone at *$P < 0.05$, **$P < 0.01$.

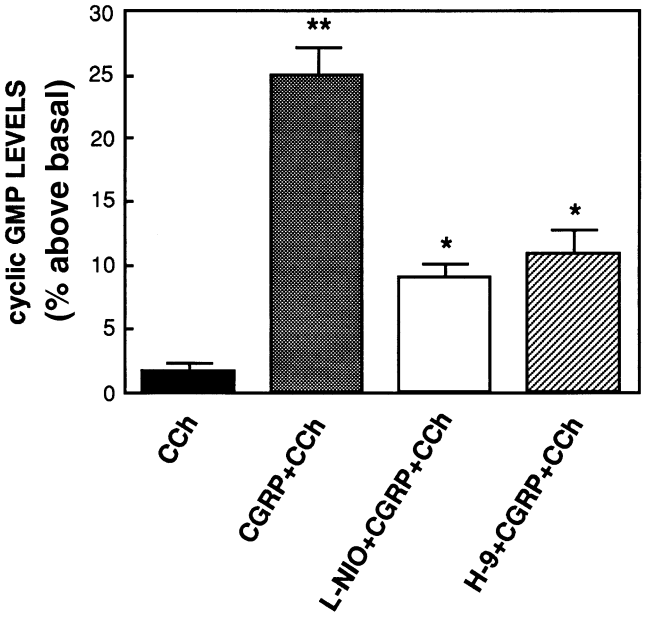

FIG. 6. Effects of L-NIO and H9 on cGMP levels of colonic smooth muscle cells. cGMP contents were determined after addition of 1 nM CCh for 30 sec, alone or in the presence of CGRP (1 μM) for 60 sec. After exposure to L-NIO for 10 min or to H9 for 5 min, the same time sequence as in Fig. 4 for incubation of cells and addition of CCh and CGRP used; the reaction was stopped after 30 sec by adding 60% cold perchloric acid (vol/vol). Results are expressed as cGMP levels in % above basal value (9.71 ± 0.8 pmol/million cells). Values are means ± SEM from six separate experiments. Significantly different from CCh alone at *$P < 0.05$, **$P < 0.01$.

carbachol was associated with the appearance of a large number of protrusions of the cell surface, due to the small decrease in volume occurring during contraction and to changes in the orientation of myofilaments within the cell [17]. This contraction may be limited by the maximal shortening rate of the cells (22.3% decrease in mean cell length). The rate of the response observed in this study was very similar to that reported on SMC isolated from other animal species (such as rabbit) [18] or from gastric antrum [11]. Carbachol-induced contraction is mainly mediated through activation of M3 muscarinic receptor subtypes [19, 20], involving an IP_3-sensitive increase in $[Ca^{2+}]_i$ [18].

Our data show that carbachol-induced contraction of isolated SMC from the circular layer of the human colon was time- and dose-dependently reduced by CGRP, with a maximal effect at 1 μM, confirming previous data obtained on guinea-pig gastric SMC [7]. SNP caused full relaxation of the cells (89 ± 8%) and CGRP only partial relaxation (69 ± 5%, also shown in Fig. 3). In terms of efficacy, there was less relaxation with CGRP (*ca.* 23%) and its time course of action was two-fold slower.

Simultaneously with a decrease in contractility, CGRP (or SNP) caused an increase in intracellular cGMP levels. SNP predominantly activated guanylate cyclase, leading to production of high levels of cGMP (80 pmol/million cells) and, in turn, full relaxation (89 ± 8%) of cells contracted by carbachol; in contrast, at the same concentration of 1 μM of CGRP, the generation of cGMP was lower (20 pmol/million cells), in accordance with a partial relaxation (69 ± 5%). In terms of the time-courses of both cGMP production and relaxation, SNP caused a rapid and large increase in cGMP levels which durably activate cGMP-PK, leading to full relaxation. In contrast, with CGRP the time course of production of cGMP was slower; hence, there was a better adequation between cGMP production and relaxation.

The NOS inhibitor L-NIO partly inhibited (78%) relaxation, suggesting an implication of NO in this event. NO is probably produced by SMC itself via activation of an NOS. A similar conclusion was drawn from studies performed on guinea-pig gastric fundus SMC, in which an NO generation from target cells by the action of VIP was found [9]. In gastric circular muscle layer of the rabbit, Murthy *et al.* [11] have reported that VIP-specific receptors are coupled to activation of a constitutive NOS. This hypothesis was supported by recent data from Teng *et al.* [21] who showed, in rabbit and human gastrointestinal SMC, expression of a constitutive endothelial NOS; in addition, by using reverse-transcriptase polymerase chain reaction (PCR) (RT-PCR) with specific primers for iNOS, they failed to evidence such isoenzymes in human cells either freshly isolated or in primary culture. Moreover, as indicated by Rekik *et al.* [22], intestinal SMC could constitutively express an isoform of NOS, the pharmacological properties of which are similar to those previously reported for the inducible form. However, neurons and interstitial cells of Cajal from the circular smooth muscle layer, which could be present to a very low extent in our preparation, may also produce NO. Nevertheless, in this isolated cell system, if NO is released, its concentration in the medium would probably be too low to activate SMC guanylate cyclase.

The NOS inhibitor L-NIO partly reduced (65.5%) cGMP generation, suggesting that the CGRP-induced increase in cGMP levels, following activation of a guanylate cyclase by NO, may in part account for relaxation. These data are consistent with the hypothesis that an increase in cGMP could account for the decreased contractility by lowering cytoplasmic $[Ca^{2+}]i$, as shown on vascular and tracheal smooth muscles stimulated by NO-donors (such as nitroglycerine) [23]. Nevertheless, the lack of a complete relaxation following incubation with L-NIO further suggests that CGRP acts on muscle cells through another pathway.

Cyclic GMP is known to activate selective cGMP-PK, which in turn phosphorylate regulatory proteins of the contractile process. Used at 1 μM, the protein kinase inhibitor H9 is an inhibitor of cGMP-PK (respective affinities: cGMP-PK > cAMP-PK > PKC [24]). Our results show that H9 is able to reverse the relaxant effect of CGRP to the same extent as L-NIO (71.8% vs 78.3%), in agreement with an involvement of cGMP-PK in this effect. To explain the inhibition of CGRP-induced cGMP generation by H9, we can suppose an indirect effect of H9 on GC through PKC: some studies have shown that PKC may

activate GC [25]; as H9 was shown to inhibit PKC, a reduction in cGMP levels may be obtained as a result of PKC/GC inhibition.

The partial blockade of cell relaxation by L-NIO and H9 (ca. 25% remains unaffected) is in agreement with the involvement of another pathway in this effect. The inhibition of cell relaxation by selective Rp-cAMP(S) supports the participation of the cAMP pathway; other studies have also reported that relaxation by CGRP is associated with a cAMP generation in isolated gastric SMC [4] or in longitudinal muscle of guinea-pig ileum [23]. Other mechanisms may also be evoked, such as a participation of K^+-dependent Ca^{2+} channels linked to CGRP receptor, as suggested by Raybould *et al.* [6], or the involvement of nonreceptor pathways. Furthermore, in longitudinal muscle of guinea-pig ileum, Sun *et al.* [26] have reported changes in the sensitivity of smooth muscle contractile elements to Ca^{2+} as a primary mechanism for relaxation by CGRP.

Finally, our study on isolated human colonic SMC has shown that CGRP receptors mediate relaxation and induce an increase in cGMP; this CGRP-induced relaxation, partly inhibited by the NOS inhibitor L-NIO, supports the ability of CGRP to generate NO in these cells. In addition, the partial inhibition of relaxation by both H9 and selective cAMP-PK inhibitor Rp-cAMP(S) suggests the involvement of cGMP and cAMP signaling systems. This conclusion, together with the finding that CGRP and NO are inhibitory NANC neurotransmitters in the human colon, should lead to a better understanding of gastrointestinal motility and finally to the development of new therapeutical approaches to motility disorders.

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