InsP$_3$R-associated cGMP Kinase Substrate (IRAG) Is Essential for Nitric Oxide-induced Inhibition of Calcium Signaling in Human Colonic Smooth Muscle*

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Nitrergic smooth muscle relaxation is crucial for the maintenance of human gut function. The molecular mechanisms of NO-dependent smooth muscle relaxation involve cyclic GMP-mediated inhibition of store-dependent calcium signaling. Recently, IRAG (inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate) has been characterized as a novel target molecule of cGMP-dependent protein kinase (cGKI) mediating NO/cGMP-dependent inhibition of InsP$_3$- and cGMP-dependent calcium release in transfected COS cells. The aim of the present study was to characterize IRAG expression and its functional role in NO-dependent signaling in human colonic smooth muscle. Reverse transcriptase-PCR revealed IRAG mRNA expression in human colon, rectum, and cultured colonic smooth muscle cells. In cultured human colonic smooth muscle cells, bradykinin (BK) elicited InsP$_3$- and cGMP-dependent calcium transients that were repeatable and independent of extracellular calcium. The NO donor sodium nitroprusside and the specific cGK activator 8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP) significantly inhibited BK-induced increase in intracellular calcium. Cells transfected with antisense oligonucleotides against IRAG (IRAG-AS) showed strongly decreased IRAG protein expression. In these cells, sodium nitroprusside and 8-pCPT-cGMP both failed to modulate BK-induced calcium transients. Thus, endogenous IRAG appears to be essentially involved in the NO/cGK-dependent inhibition of InsP$_3$- and cGMP-dependent Ca$^{2+}$-signaling in colonic smooth muscle.

Gastrointestinal smooth muscle has been demonstrated throughout the gut of various species (4–9) and has been established as a crucial event contributing to the maintenance of normal gut function (10, 11).

The molecular target of NO in smooth muscle is the soluble guanylate cyclase, which is strongly activated by NO (12, 13). Increased levels of cGMP lead to smooth muscle relaxation predominately via activation of cGMP-dependent protein kinase (cGK) (14, 15). The crucial role of cGK for gastrointestinal smooth muscle function became apparent in cGKI-deficient mice showing a selective lack of NO-dependent smooth muscle relaxation associated with severe gastrointestinal dysfunction and marked hypertrophy of gastrointestinal smooth muscle (16). The mechanisms of cGKI-dependent smooth muscle cell relaxation, however, have not been fully understood (for a review, see Ref. 14).

In a recent study (see Ref. 26), a new protein has been identified as molecular target of cGKI in smooth muscle microsomal membranes and has been termed IRAG (Inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate). IRAG precipitated together with cGKβ and InsP$_3$ receptor type 1 in a complex which was found to be localized at the endoplasmic reticulum membrane. In transfected COS cells, the coexpression of IRAG with cGKI was essential for the cGMP-dependent inhibition of InsP$_3$-dependent calcium release. This inhibition has further been demonstrated to depend on the cGKI-mediated phosphorylation of IRAG at Ser$_{696}$. No interaction between cGKI and the InsP$_3$ receptor was observed when IRAG was absent (17).

To date, only little is known about IRAG expression and its function in gastrointestinal tissues. Furthermore, evidence for a functional role of IRAG in smooth muscle tissue is lacking. We show here that IRAG is expressed in human colon and that the suppression of IRAG protein expression in human colonic smooth muscle cells is sufficient to abolish the inhibitory effect of sodium nitroprusside and 8-pCPT-cGMP on bradykininin-induced calcium release in these cells.

**EXPERIMENTAL PROCEDURES**

**Tissue Preparation—** Tissues from human colon and rectum were obtained from surgical resections for colorectal malignant disease. The tissues were macroscopically and microscopically free of tumor. The muscle layer containing the nerve plexus was separated from the

InsP$_3$, inositol 1,4,5-trisphosphate; cGK, cGMP-dependent protein kinase; BK, bradykinin; IRAG-AS, IRAG antisense oligonucleotide; IRAG-IS, IRAG inverse sequence oligonucleotide; SNP, sodium nitroprusside; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate; Rp-8-pCPT-cGMP, guanosine, 3',5'-cyclic monophosphorothioate; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinazoline-1-one; 2-APB, 2-aminoethoxydiphenyl borate.

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¶ The abbreviations used are: NO, nitric oxide; IRAG, InsP$_3$R-associated cGMP kinase substrate; InsP$_3$R, inositoltrisphosphate receptor.

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mucosa by sharp dissection and prepared as described previously (18, 19).

RNA Isolation and RT-PCR—Liquid nitrogen-frozen colon, rectum and colonic smooth muscle layer preparations, and cultured colonic smooth muscle cells (harvested after splitting the cells three and six times) were homogenized with a Polytron homogenizer (Kinematica), and total RNA was isolated using the guanidine isothiocyanate/phenol/chloroform extraction method as described previously (18, 20).

Subsequently, reverse transcription and PCR amplification were carried out as described before (19). RT-PCR was performed with specific intron spanning primer pairs for human IRAG (Table I) for 35 cycles using random hexamer primed cDNA from human colon, rectum, colon smooth muscle cells, and as negative control, HeLa cells (annealing 60°C, 30 s; extension 72°C, 30 s; denaturation 94°C, 15 s). Amplification products were cloned into pCRII plasmid (Invitrogen) and subjected to DNA sequence analysis (GATC, Konstanz, Germany).

Cell Culture—An established culture of human colonic smooth muscle cells (Clonetics Normal Human Cell Systems, San Diego, CA) was used for calcium imaging experiments and Western blot analysis. Cells had been isolated from a single donor, brought into cell culture, and shipped after two splittings. The cell culture has been shown to retain smooth muscle phenotype for at least 15 population doublings by positive staining for α-smooth muscle actin and negative staining for factor VIII by the provider. To minimize dedifferentiation, we did not use cells from passages higher than the 5th and 6th passage, although comparable cultures of primary cells have been used beyond that (21, 22). When used for experiments, cells preserved the typical morphology of smooth muscle cells, and characteristic calcium transients were elicited by high potassium depolarization or cholinergic stimulation with carbachol (data not shown). Furthermore, these cells expressed cGKI β as demonstrated by Western blot analysis (not shown) (23).

Cells were grown in smooth muscle growth medium containing 5% fetal bovine serum, 0.5 μg/liter human recombinant epidermal growth factor, 5 mg/liter insulin, 0.39 mg/liter dexamethasone, 50 mg/liter gentamicin, and 50 μg/liter amphotericin B (all Biowhittaker, Baltimore, MD) in humidified 95% air, 5% CO2 atmosphere. Medium was replaced every other day. Cells were grown to 80% confluence before they were used for calcium imaging experiments.

Western Blot Analysis—For Western blot analysis, cultured human colonic myocytes were harvested using a cell scraper, washed twice in phosphate-buffered saline buffer, and suspended in protein lysis buffer (0.5 mM EDTA, 0.5 mM EGTA, 25 mM Tris-HCl, and 1% Triton X-100, 0.2% protease inhibitor mixture). Cells were then freeze-thawed, treated with ultrasound (four strokes of 15 s, cooled on ice between treatments), and lysed using a 24-gauge needle. The suspension was centrifuged at 10,000 × g for 5 min, and protein concentration in the supernatant was determined using the BCA protein assay kit (Pierce). Western blot analysis was performed as described previously (24). In brief, proteins were separated by SDS-PAGE on 7.5% slab gels using a brief procedure of Laemmli (25). Proteins were blotted onto a polyvinylidene difluoride membrane (Bio-Rad) using Tris-glycine-SDS buffer (50 mM Tris, 500 mM glycine, 0.05% SDS, and 20% methanol). After blocking the membrane with 5% dry milk, we probed blots with a selective antibody for IRAG (diluted 1:2000). Purified protein was used as a positive control as described previously (5). The antibody was raised against the common sequence of IRAG (17, 26). Detection was performed using horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescence (ECL system; Amersham Biosciences).

For comparison of IRAG protein expression in cells transfected with antisense oligonucleotides (IRAG-AS) and cells transfected with IRAG-IS, IRAG immunoreactivity was detected at ~125 kDa, with a nonspecific band at ~135 kDa indicating equal protein loading. B, statistical comparison of integrated density of IRAG protein bands showing reduction of IRAG band intensity to an average of 17% of controls (percentage of integrated density, arbitrary units ± S.E.; n = 4, p < 0.001).

Transfection with Antisense Oligonucleotides—A mixture of two an-

**TABLE I**

| Name        | Sequence                                                                 |
|-------------|--------------------------------------------------------------------------|
| IRAG (S)    | 5'-CAA GTT GGT GAG TGA GCG ATT C-3'                                     |
| IRAG (AS)   | 5'-TGC TTC GAG AGG ATG TAG GAG AA-3'                                     |

**TABLE II**

| Name          | Sequence                                                                 |
|---------------|--------------------------------------------------------------------------|
| IRAG-IS 1    | 5'-CsAsGsCGACCTTCTTCAGCaAAsCsA-3'                                       |
| IRAG-IS 2    | 5'-CsTsCcCAGTGCACCAAGAAsCsAsA-3'                                        |
| IRAG-AS 1    | 5'-CsAsCaAGCAGCTGACCAsGAsTcC-3'                                         |
| IRAG-AS 2    | 5'-AsAsCsTtGgCtCTTtCtGCAsAsGcC-3'                                       |

**FIG. 1.** IRAG mRNA expression in human colon, rectum, and colonic smooth muscle cells. RT-PCR for IRAG using total RNA isolated from the muscle layers of human colon (lane 1) and rectum (lane 2), cultured human colonic smooth muscle cells after passage three (lane 3) and six (lane 4) and HeLa cells (lane 5). Single bands with the expected size of ~125 bp confirmed the expression of IRAG in the investigated tissues and human colon smooth muscle cells, whereas HeLa cells (lane 5) and the negative control (lane 6, RT-control) showed no specific PCR product. M, base pair ladder.

**FIG. 2.** Transfection of cultured human colonic smooth muscle cells with antisense oligonucleotides raised against IRAG-mRNA (IRAG-AS). A, representative Western blot comparing IRAG protein expression in controls transfected with inverse sequence oligonucleotides (IRAG-IS) and cells transfected with IRAG-AS. IRAG immunoreactivity was detected at ~125 kDa, with a nonspecific band at ~135 kDa indicating equal protein loading. B, statistical comparison of integrated density of IRAG protein bands showing reduction of IRAG band intensity to an average of 17% of controls (percentage of integrated density, arbitrary units ± S.E.; n = 4, p < 0.001).
Expression of IRAG mRNA in Human Colon and Cultured Colonic Smooth Muscle Cells—The presence of IRAG mRNA in human colon and rectum muscle layers and in cultured human colonic smooth muscle cells was determined by RT-PCR using specific intron spanning primer pairs (Table I). A single band at the expected size of $-125$ bp was obtained in the investigated...
tissues and cultured colonic smooth muscle cells after passage three and six, respectively, whereas no PCR product was seen for HeLa cells (Fig. 1). Sequencing of all cloned RT-PCR products confirmed IRAG mRNA expression.

Transfection of Human Colonic Smooth Muscle Cells with Antisense Oligonucleotides Raised against IRAG mRNA—To determine the functional role of IRAG in human colonic smooth muscle, IRAG protein expression was inhibited by transfection of cultured colonic myocytes with antisense oligonucleotides raised against IRAG mRNA (IRAG-AS, Table II). Cells for control experiments were transfected with inverse sequence oligonucleotides (IRAG-IS, Table II). Intracellular delivery of oligonucleotides was facilitated using the FuGENE 6 transfection reagent. In pilot experiments, cultured cells were transfected with fluorochrome-labeled oligonucleotides. Eight hours after transfection, a cytoplasmatic fluorescence signal could be detected in virtually all cells in culture (not shown).

Western blot analysis using a polyclonal antiserum raised against IRAG (17) demonstrated IRAG protein expression in cultured human colonic smooth muscle cells. A protein band corresponding to IRAG was detected at 125 kDa. A less intense band detected at 135 kDa was not seen when the antibody was probed with purified IRAG (not shown) and most likely represents a nonspecific band. Comparison of IRAG protein expression was performed using equal amounts of protein extracted from cells transfected with IRAG-AS and from controls transfected with IRAG-IS. Cells transfected with antisense oligonucleotides raised against IRAG showed a significantly lower intensity of the IRAG protein band as compared with controls transfected with IRAG-IS (Fig. 2A). Densitometric analysis of bands revealed a reduction of IRAG band intensity to 17% of controls (Fig. 2B; n = 4; p < 0.001), indicating a strongly decreased IRAG protein expression in cells transfected with IRAG-AS.

Bradykinin-induced Calcium Transients in Human Colonic Smooth Muscle Cells—Ratiometric measurements of changes in \([\text{Ca}^{2+}]_i\) in cultured human colonic smooth muscle cells were performed using the dual wavelength microfluorescence technique. Low resting Fura-2 ratios were maintained for more than 2 h during experiments.

The addition of bradykinin (10^{-6} M) to the perifusate evoked a rapid increase in intracellular calcium concentration followed by a slightly delayed decrease back to baseline. Allowing 300 s of washout between stimulation episodes, a minimum of three subsequent stimulations of cells with bradykinin resulted in virtually identical calcium signals without significant variation (not shown). In a typical experiment, 60 and 70% of all cells visualized simultaneously within a microscopic field were reactive to bradykinin (10^{-6} M) and underwent further analysis as described under “Experimental Procedures.”

To determine the source of calcium mobilized by bradykinin, extracellular calcium was removed from the perifusate after the first stimulation episode. Fig. 3, A and B, shows a representative tracing and the statistics of bradykinin-induced calcium transients in the presence and absence of extracellular calcium. There was no significant change of peak ratios during stimulation of cells with bradykinin in the presence or absence of extracellular calcium (n = 7, not significant), indicating \(\text{Ca}^{2+}\)-release from intracellular stores.

To further characterize bradykinin-induced calcium release,
cells were incubated with 2-APB (75 μM) after the first stimulation period. 2-APB has been shown to inhibit InsP₃-dependent calcium release when used at this concentration range (29) and has widely been used as an inhibitor of InsP₃-sensitive calcium release in intact cells (28). Application of 2-APB (75 μM) did not result in any detectable changes in [Ca²⁺]ᵢ. However, bradykinin-induced calcium transients were almost completely abolished when cells were incubated with 2-APB (5% of control peak ratios, n = 10, p < 0.001), indicating that bradykinin induced calcium release from InsP₃-dependent stores (Fig. 4).

Inhibition of Bradykinin-induced Calcium Transients by NO-dependent Pathways—To characterize the effect of NO-dependent pathways on bradykinin-induced calcium transients in hu-
human colonic smooth muscle cells, the NO donor SNP was added to the perifusate after the first stimulation period. Increasing concentrations of SNP inhibited bradykinin-induced calcium release in a dose-dependent manner with a maximum effect observed at a concentration of 5 x 10^{-4} M (38% of control peak ratios, n = 41, p < 0.001; Fig. 5A). To ensure that the effect of SNP was mediated by cGMP-dependent mechanisms, cells were coincubated with ODQ, a selective and membrane-permeable inhibitor of soluble guanylate cyclase. ODQ (2.5 x 10^{-6} M) completely reversed the inhibition of bradykinin-induced calcium release induced by 5 x 10^{-4} M SNP (116% of control peak ratios, n = 9, not significant, Fig. 5A), indicating that the SNP-induced inhibition of bradykinin-induced calcium transients was mediated by soluble guanylate cyclase. Similarly, when cells were incubated with Rp-8-pCPT-cGMPs (5 x 10^{-5} M), a selective and membrane-permeable inhibitor of cGMP-dependent protein kinase (cGK), 5 x 10^{-3} M SNP failed to inhibit bradykinin-induced calcium transients and induced a slight increase of bradykinin-induced calcium release (112% of control peak ratios, n = 20, p < 0.05, Fig. 5A), whereas Rp-8-pCPT-cGMPs had no effect on bradykinin-induced calcium transients in the absence of SNP (not shown).

To further support these findings, we co-incubated cells with 8-pCPT-cGMP, a membrane-permeable and direct activator of cGMP-dependent protein kinase. 8-pCPT-cGMP induced a dose-dependent inhibition of bradykinin-induced calcium transients with a maximum effect observed at 10^{-4} M (54% of control peak ratios, n = 19, p < 0.001, Fig. 5B). Taken together, these data indicate that, in cultured human colonic smooth muscle cells, the activation of the NO/cGK-signaling pathway induces a significant inhibition of bradykinin-induced calcium transients.

Lack of Inhibition of Bradykinin-induced Calcium Transients by NO-dependent Pathways in Colonic Smooth Muscle Cells with Decreased IRAG Expression—To test whether IRAG is involved in the NO/cGK-dependent inhibition of bradykinin-induced calcium release in cultured human colonic smooth muscle cells, we used phosphorothioate-stabilized antisense oligonucleotides to suppress IRAG protein expression (Fig. 2) and determined the effect of SNP and 8-pCPT-cGMP on bradykinin-induced calcium release in these cells. Both compounds were used at concentrations that had been found to be highly effective in native cells (Fig. 5). To rule out nonspecific effects of the transfection procedure, controls were transfected with inverse sequence oligonucleotides (IRAG-IS, Table II) following an identical protocol. Fig. 6, A and B, shows a representative tracing and the statistics obtained from controls transfected with IRAG-IS, confirming the significant inhibition of bradykinin-induced calcium transients by SNP (5 x 10^{-4} M) found in native cells (38% of control peak ratios, n = 11, p < 0.05). Similarly, 8-pCPT-cGMP (10^{-4} M) induced an inhibition of bradykinin-induced calcium release in controls transfected with IRAG-IS that was comparable with the inhibition observed in native cells (46.5%, n = 17, p < 0.001, Fig. 7, A and B).

Parallel experiments were performed using cultured colonic smooth muscle cells transfected with IRAG-AS. Decreased IRAG protein expression in these cells was demonstrated by Western blot (Fig. 2). To ensure that the calcium imaging data obtained from the three differently treated groups of cells were comparable, peak ratios measured during stimulation with bradykinin (10^{-8} M) were compared between native

Fig. 6. SNP inhibits calcium transients in controls. A, representative cytosolic fura-2 ratiometric measurement from a single cultured human colonic smooth muscle cell (control cell population transfected with IRAG-IS) stimulated with BK 10^{-6} M twice, before and during incubation with the NO donor SNP (5 x 10^{-4} M). B, SNP attenuates BK-induced calcium signaling to 38% of control (Δ average peak ratio ± S.E., n = 11, p < 0.05).

Fig. 7. 8-pCPT-cGMP inhibits calcium transients in controls. A, representative cytosolic fura-2 ratiometric measurement from a single cultured human colonic smooth muscle cell (control cell population transfected with IRAG-IS) stimulated with BK 10^{-6} M twice, before and during incubation with 8-pCPT-cGMP (10^{-4} M), a direct activator of cGK. B, 8-pCPT-cGMP (10^{-4} M) attenuates BK-induced calcium signaling to 46.5% of control (Δ average peak ratio ± S.E., n = 17, p < 0.001).
cells, cells transfected with IRAG-AS, and cells transfected with IRAG-IS. Between these three groups, peak ratios did not differ significantly (p > 0.1 for each comparison between single groups).

However, in cells transfected with IRAG-AS, the NO donor SNP (5 \times 10^{-4} M) failed to significantly modulate bradykinin-induced calcium release (99.6% of control peak ratios, n = 19, not significant). Fig. 8, A and B, shows a representative tracing and the statistics of the effect of SNP (5 \times 10^{-4} M) on bradykinin-induced calcium transients in cultured colonic smooth muscle cells transfected with IRAG-AS.

Similarly, when cells transfected with IRAG-AS were exposed to bradykinin in the presence of the cGMP-analogue and direct activator of cGK, 8-pCPT-cGMP (10^{-4} M), no significant effect on bradykinin-induced calcium release was observed (102% of control peak ratios, n = 22, not significant). Fig. 9, A and B, shows a representative tracing and the statistics of bradykinin-induced calcium transients in cultured colonic smooth muscle cells transfected with IRAG-AS before and during incubation with 8-pCPT-cGMP (10^{-4} M).

In summary, the inhibition of bradykinin-induced calcium release by SNP and the cGK activator 8-pCPT-cGMP in cultured human colonic smooth muscle cells was abolished when the expression of the recently discovered cGKI substrate protein IRAG was substantially suppressed. These data suggest a crucial role for endogenously expressed IRAG in the interaction between NO-dependent signaling and IP_3-dependent calcium release in human colonic smooth muscle.

**DISCUSSION**

In this study, we demonstrate for the first time that IRAG, a recently discovered cGKI substrate protein, is expressed in the human gastrointestinal tract. To characterize the functional role of IRAG in NO-dependent signal transduction in human gastrointestinal smooth muscle, calcium imaging studies using cultured colonic smooth muscle cells were performed. Bradykinin was used to induce IP_3-dependent calcium transients in these cells. Bradykinin has successfully been used as a tool for store-dependent calcium release in gastrointestinal smooth muscle (30) and other cell types (26, 31). The persistence of bradykinin receptors under culture conditions has been reported in primary cultured rabbit colonic smooth muscle (32). Bradykinin-induced calcium transients in cultured colonic smooth muscle cells were unaffected by the removal of extracellular calcium but virtually completely abolished in the presence of 2-APB, indicating calcium mobilization from InsP_3-sensitive calcium stores.

The nitric oxide donor SNP significantly inhibited the bradykinin-induced increase in intracellular calcium. This inhibition was dose-dependent and reached statistical significance at 5 \times 10^{-5} M SNP with a maximum effect observed at 5 \times 10^{-4} M. To make sure that the effects observed with these relatively high concentrations of SNP used in this experimental setting were mediated by activation of the cGMP/cGK pathway, we used two specific blockers of downstream targets of the NO/cGK pathway. Low concentrations of ODQ, a specific inhibitor of soluble guanylate cyclase (33), and the cGK-inhibitor, Rp-8-
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8-pCPT-cGMP (10^{-6} M) before and during incubation with 8-pCPT-cGMP (10^{-4} M) raised against IRAG mRNA (IRAG-AS). Two periods of stimulation with BK 10^{-6} M, before and during incubation with 8-pCPT-cGMP (10^{-4} M) are shown. 8-pCPT-cGMP (10^{-4} M) fails to attenuate BK-induced calcium signaling in transfected cells with decreased IRAG expression (Δ average peak ratio ± S.E., n = 22, not significant).

An inhibition of InsP_{3}-dependent calcium signaling by NO-dependent pathways and InsP_{3}-dependent calcium signaling in human colonic smooth muscle. Therefore, we inhibited IRAG protein expression by transfection of cells with phosphorothioate-stabilized antisense oligonucleotides raised against IRAG-mRNA (IRAG-AS). Cells used in control experiments were transfected with inverse sequence oligonucleotides to exclude nonspecific effects. Exposure of cells with strongly diminished IRAG expression to bradykinin evoked calcium transients that were undistinguishable from those seen in native cells and controls. This indicates that the suppression of IRAG is not associated with an increase in bradykinin-induced calcium release in these cells. However, the suppression of IRAG protein expression was sufficient to abolish the inhibition of bradykinin-induced calcium release induced by SNP and 8-pCPT-cGMP. This is in good agreement with the findings using Rp-8-pCPT-cGMPs to inhibit cGMP-dependent protein kinase in these cells since Rp-8-pCPT-cGMPs (5 × 10^{-5} M) entirely blocked the SNP-induced inhibition of bradykinin-induced calcium release but had no effect on bradykinin-induced calcium release when applied in the absence of SNP. Taken together, these data suggest that, in human colonic smooth muscle cells, IRAG is essentially involved in the cGK-dependent inhibition of InsP_{3}-dependent calcium transients. However, our findings cannot rule out the functional involvement of other mechanisms of interaction between the NO/cGK pathway and InsP_{3}-dependent calcium release in mediating the observed effects. Furthermore, since smooth muscle tissues show significant heterogeneity, conclusions based on these data have to be limited to the type of smooth muscle tissue used in the experiments, and gene knockout models will be required to clarify demonstrated in vitro and in vivo (38, 39). Finally, an increased uptake of calcium ions into sarcoplasmatic stores achieved by cGK-dependent activation of sarcoplasmatic Ca^{2+}-ATPase pumps has been demonstrated in vascular smooth muscle (40–42). However, which of these mechanisms significantly contribute to the NO/cGK-mediated inhibition of InsP_{3}-dependent calcium signaling in gastrointestinal smooth muscle has not been clearly established. Some functional evidence has been obtained recently indicating a principal role of the newly discovered cGKI-substrate protein IRAG in linking cGKI with InsP_{3}-dependent calcium release (26). IRAG has been found to complex together with cGKI and InsP_{3} receptor type I in smooth muscle membranes. In transfected COS cells, this complex was found to be localized at the endoplasmic reticulum membrane. Functional evidence for the specific interaction of cGKI, IRAG, and InsP_{3} receptor type I has been obtained from experiments using a two-hybrid screen and transfected COS cells. In these cells, the interaction between cGKI and the InsP_{3} receptor has been shown to depend on the coexpression of IRAG. Similarly, a cGMP-dependent inhibition of InsP_{3}-dependent calcium release has only been observed in the presence of both cGKI and IRAG. The mechanisms of interaction between cGKI and IRAG have been characterized by mutation analysis showing that mutation of IRAG at the phosphorylation site at Ser^{696} abolished the cGK-dependent inhibition of InsP_{3}-dependent calcium release (17). These findings are of special interest since they suggest a central role for IRAG in linking NO-dependent signaling to InsP_{3}-dependent calcium signaling. However, most of the functional data were obtained from experiments using COS cells in which cGKI and IRAG had been overexpressed, a nonphysiologic condition bearing the risk of artifacts. Up to now, no data existed providing evidence for a functional role of endogenous IRAG in smooth muscle tissue.

In this study, we aimed to determine whether endogenously expressed IRAG is involved in the signaling cascade linking NO-dependent pathways and InsP_{3}-dependent calcium signaling in human colonic smooth muscle. Therefore, we inhibited IRAG protein expression by transfection of cells with phosphorothioate-stabilized antisense oligonucleotides raised against IRAG-mRNA (IRAG-AS). Cells used in control experiments were transfected with inverse sequence oligonucleotides to exclude nonspecific effects. Exposure of cells with strongly diminished IRAG expression to bradykinin evoked calcium transients that were undistinguishable from those seen in native cells and controls. This indicates that the suppression of IRAG is not associated with an increase in bradykinin-induced calcium release in these cells. However, the suppression of IRAG protein expression was sufficient to abolish the inhibition of bradykinin-induced calcium release induced by SNP and 8-pCPT-cGMP. This is in good agreement with the findings using Rp-8-pCPT-cGMPs to inhibit cGMP-dependent protein kinase in these cells since Rp-8-pCPT-cGMPs (5 × 10^{-5} M) entirely blocked the SNP-induced inhibition of bradykinin-induced calcium release but had no effect on bradykinin-induced calcium release when applied in the absence of SNP. Taken together, these data suggest that, in human colonic smooth muscle cells, IRAG is essentially involved in the cGK-dependent inhibition of InsP_{3}-dependent calcium transients. However, our findings cannot rule out the functional involvement of other mechanisms of interaction between the NO/cGK pathway and InsP_{3}-dependent calcium release in mediating the observed effects. Furthermore, since smooth muscle tissues show significant heterogeneity, conclusions based on these data have to be limited to the type of smooth muscle tissue used in the experiments, and gene knockout models will be required to clarify...
the functional consequences of a lack of IRAG for the development and function of smooth muscle in various organ systems. Since the interaction of cGKI and IRAG has been found to be limited to the cGKI isoform (17), the functional involvement of IRAG suggests that the cGKI-dependent inhibition of bradykinin-induced calcium transients in human colonic smooth muscle cells is mediated by the cGKI isoform. This is in contrast to recent findings obtained from murine vascular smooth muscle cells deficient for both cGKI and cGKIβ (43). In those cells, the NO-dependent inhibition of noradrenaline-induced calcium transients could be rescued by expression of cGKIα, but not cGKIβ (43). However, besides differences in the experimental approaches leading to these divergent results, smooth muscle tissues from various organ systems and different species may show considerable variation in the specific cellular function of the two cGKI isoforms.

In summary, these data point to a physiologic role of IRAG in human gastrointestinal smooth muscle. They support the idea that IRAG is considerably involved in mediating NO-dependent inhibition of calcium signaling in gastrointestinal smooth muscle contributing to NO-dependent relaxation.

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