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Hydrogen peroxide contributes to motor dysfunction in ulcerative colitis

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Cao, Weibiao, Matthew D. Vrees, Michael T. Kirber, Claudio Fiocchi, and Victor E. Pricolo. Hydrogen peroxide contributes to motor dysfunction in ulcerative colitis. *Am J Physiol Gastrointest Liver Physiol* 286: G833–G843, 2004. First published December 11, 2003; 10.1152/ajpgi.00414.2003.—Ulcerative colitis (UC) affects colonic motor function, but the mechanism responsible for this motor dysfunction is not well understood. We have shown that neurokinin A (NKA) may be an endogenous neurotransmitter mediating contraction of human sigmoid colonic circular muscle (HSCCM). To elucidate factors responsible for UC motor dysfunction, we examined the role of hydrogen peroxide (H_2O_2) in the decrease of NKA-induced response of HSCCM. As previously demonstrated, NKA-induced contraction or Ca^{2+} increase of normal muscle cells is mediated by release of Ca^{2+} from intracellular stores, because it was not affected by incubation in Ca^{2+}-free medium (CFM) containing 200 μM BAPTA. In UC, however, CFM reduced both cell contraction and NKA-induced Ca^{2+} increase, suggesting reduced Ca^{2+} release from intracellular stores. In normal Ca^{2+} medium, NKA and KCl caused normal Ca^{2+} signal in UC cells but reduced cell shortening. The decreased Ca^{2+} signal and contraction in response to NKA or thapsigargin were partly recovered in the presence of H_2O_2 scavenger catalase, suggesting involvement of H_2O_2 in UC-induced dysmotility. H_2O_2 levels were higher in UC than in normal HSCCM, and enzymatically isolated UC muscle cells contained much higher levels of H_2O_2 than normal cells, which were significantly reduced by catalase. H_2O_2 treatment of normal cells in CFM reproduced the reduction of NKA-induced Ca^{2+} release observed in UC cells. In addition, H_2O_2 caused a measurable, direct release of Ca^{2+} from intracellular stores. We conclude that H_2O_2 may contribute to reduction of NKA-induced Ca^{2+} release from intracellular Ca^{2+} stores in UC and contribute to the observed colonic motor dysfunction.

neurokinin A; calcium; smooth muscle; human; colon

ULCERATIVE COLITIS (UC) is a chronic inflammatory condition that affects the large bowel. Approximately half of UC patients have disease limited to the rectum and rectosigmoid. In 30–40% of the patients, the disease extends beyond the sigmoid without affecting the whole colon, and 20% of patients have pancolitis (20). Usually, histological inflammation is limited to the mucosal layer (31). We therefore examined contraction of the sigmoid colon, which is most commonly affected in UC.

We (5) have previously shown that neurokinin A (NKA) is an important excitatory neurotransmitter in human sigmoid circular muscle, because contraction induced by electrical field (i.e., neural) stimulation is abolished by NK-2 receptor antagonists and not by NK-1 antagonists or atropine. We therefore used NKA as an agonist to examine contraction of sigmoid circular muscle.

The pathogenesis of UC is not well understood. It is thought that UC may depend on inappropriate and ongoing activation of the mucosal immune system initiated by normal luminal flora or by their products (34). Genetic factors determine differential susceptibility to the development of the disease and proinflammatory cytokines, such as TNF-α and IL-1 and -6, and potentiate the inflammatory processes that eventually cause many of the clinical manifestations (33).

Inflammation in UC has been better characterized in the mucosa than in the muscularis propria. It has been reported that the production of proinflammatory cytokines, including IL-1β, TNF-α, IL-6, and IL-8, is increased in the colonic mucosa of patients with UC (6, 10, 18, 19, 25, 29, 51). Elevated levels of other inflammatory mediators, including lipid mediators such as platelet activating factor, prostaglandin E_2, leukotriene B_4, thromboxane B_2, and neuropeptides and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), have also been reported in mucosal tissue samples from patients with UC (37, 38). The diarrhea commonly observed in patients with UC is almost invariably related to the degree of activity of the disease. Motor dysfunction is another frequent abnormality associated with UC, and it has been described in patients as well as animal models of colonic inflammation (7, 22, 27, 45, 46). The effect of inflammatory mediators on human colonic motor function in UC, however, has not been examined.

Increased H_2O_2 production has been reported in colonic muscularis propria of dextran sodium sulfate-treated rats (11). In addition, H_2O_2 has been shown to consistently depress the Ca^{2+}-ATPase responsible for uptake of Ca^{2+} into the endoplasmic reticulum (12–14, 26, 35). In pig coronary artery smooth muscle, H_2O_2 damaged the sarcoplasmic reticulum Ca^{2+} pump, causing a decrease in the available Ca^{2+} stores (14). These data are consistent with our own findings in cat lower esophageal sphincter (LES) muscle in which we have shown that H_2O_2 depletes intracellular Ca^{2+} stores, reduces LES tone, and plays an important role in motor dysfunction of acute esophagitis (4). We therefore examined whether NKA-induced Ca^{2+} release from the intracellular Ca^{2+} stores are affected in UC and whether H_2O_2 has a role in the observed motor dysfunction and changes in Ca^{2+} signaling in this condition.

MATERIALS AND METHODS

Tissue specimens. Normal sigmoid colon was obtained from histologically normal margins of sigmoid tissues from cancer resections

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($n = 20$). Inflamed sigmoid colons were obtained from patients undergoing proctocolectomy for UC ($n = 19$). Fresh specimens were brought to the laboratory in oxygenated, chilled Krebs solution containing (in mM) 116.6 NaCl, 21.9 $NaHCO_3$, 1.2 KH_2PO_4, 5.4 dextrose, 1.2 $MgCl_2$, 3.4 KCl, and 2.5 $CaCl_2$. None of the control patients had any previous history of colonic motility disorder or evidence of diverticular disease. The experimental protocols were approved by the Human Research Institutional Review Committee at Rhode Island Hospital.

Preparation of circular muscle strips. After the mucosa was removed by sharp dissection under a microscope, consecutive circular muscle strips (10 mm long, 2 mm wide) of sigmoid colon were cut with razor blades held in a metal block 2 mm apart. The strips were mounted in separate 1-ml muscle chambers as previously described in detail (2). They were initially stretched to 2.5 g of force to bring them near conditions of optimum force development and equilibrated for an additional 30 min after continuous perfusion with oxygenated Krebs solution for 30 min. During the perfusion period, spontaneous phasic contractions developed gradually and stabilized after a 30-min period of equilibration.

Circular muscle strips from normal or UC colons were randomly divided into control and catalase groups, and a cumulative dose-response to NKA was obtained after strips were incubated with vehicles (Krebs solution) or Krebs solution containing catalase (78 U/ml for 50 min). Contractile forces measured in grams above the basal levels in response to NKA were compared between the two groups.

Isolation of smooth muscle cells. Sigmoid circular smooth muscle strips (~1 mm wide) were isolated by enzymatic digestion in HEPES-buffered collagenase solution, as described previously (5, 41). Briefly, the collagenase solution (pH 7.2) contained 0.5 mg/ml collagenase Sigma type F, 1 mg/ml papain, 1 mg/ml BSA, and in mM: 1 $CaCl_2$, 0.25 EDTA, 10 glucose, 10 HEPES (sodium salt), 4 KCl, 125 NaCl, 1 $MgCl_2$, and 10 taurine. The tissue was kept in enzyme solution at 4°C for ~16 h, warmed up at room temperature for 30 min, and incubated in a water bath at 31°C for ~30 min. At the end of the digestion period, the tissue was poured out over a 200-μm Nitex mesh (Tetko, Elmsford, NY), rinsed in collagenase-free HEPES-buffered solution to remove any trace of collagenase, and incubated in this solution at 31°C and gassed with 100% O_2. Collagenase-free HEPES-buffered solution (pH 7.4) contained (in mM) 112.5 NaCl, 3.1 KCl, 2.0 KH_2PO_4, 10.8 glucose, 24.0 HEPES (sodium salt), 1.9 $CaCl_2$, and 0.6 $MgCl_2$, with 0.3 mg/ml basal medium Eagle (BME) amino acid supplement and 0.08 mg/ml soybean trypsin inhibitor. Gentle agitation was used to release single cells.

Agonist-induced contraction of isolated muscle cells. Cell contraction was induced by exposure to NKA (10^{-13} to 10^{-9} M) for 30 s. For thapsigargin treatment, cells were exposed to HEPES-buffered solution without (control) or with thapsigargin (3 μM) for 15 s, 30 s, or 1, 5, 10, or 20 min. When the H_2O_2 scavenger catalase was used, cells from patients with UC were incubated in HEPES-buffered solution without (control) or with catalase 78 U/ml for 50 min before stimulation with NKA or thapsigargin. When the role of H_2O_2 in the intracellular Ca^{2+} stores was tested, normal muscle cells were incubated in HEPES-buffered solution without (control) or with H_2O_2 (70 μM) for 30 min.

After exposure to NKA or thapsigargin, the cells were fixed in acrolein at a 1% final concentration and kept refrigerated. For cell length measurement, a drop of the cell-containing medium was placed on a glass slide and 30 consecutive cells from each slide were observed through a phase-contrast microscope (Carl Zeiss) and a closed-circuit television camera (model WV-CD51; Panasonic, Secaucus, NJ) connected to a Macintosh computer (Apple, Cupertino, CA). An image software program (National Institutes of Health, Bethesda, MD) was used to acquire images and measure cell length. The average length of 30 cells, measured in the absence of agonists, was taken as the control length and compared with length measured after the addition of test agents. Shortening was defined as percent decrease in average length after agonists compared with the control length.

Cytosolic Ca^{2+} measurements. Freshly isolated cells were loaded with 1.25 μM fura-2 AM for 40 min and placed in a 5-ml chamber mounted on the stage of an inverted microscope (Carl Zeiss). The cells were allowed to settle onto a coverslip at the bottom of the chamber. The bathing solution was collagenase-free HEPES-buffered solution (normal Ca^{2+} medium) or the one without $CaCl_2$ but with 200 μM BAPTA (Ca^{2+}-free medium). When Ca^{2+}-free medium was used, after settling to the bottom of the chamber, the cells were rinsed twice with Ca^{2+}-free medium before the experiments.

NKA (1 μM), KCl (1 M), or H_2O_2 (100 mM) was applied directly to the cells by using a pressure ejection micropipette system. Solutions in the pressure ejection micropipettes were identical to the bathing solutions except for the addition of NKA or H_2O_2. When KCl was used, it was dissolved in distilled water.

Concentration of agents in the micropipette were considerably higher than those used in cell suspensions. The pipette tip was very small, and it was expected that the solution ejected from the tip may be diluted several times by the buffer surrounding the cells. Thus the concentration of the agonists reaching the cells was much lower than that present in the micropipette. For instance, to cause maximal cell shortening 10^{-9} M NKA was used for cell suspensions and 1 μM NKA was used in a puffing pipette. Thus the concentrations of NKA in these two preparations were 1,000 times different. Similarly, for H_2O_2 a 100-mM micropipette concentration was needed to elicit a measurable Ca^{2+} signal, because 5 and 10 mM H_2O_2 did not cause visible cytosolic Ca^{2+} changes.

Ca^{2+} measurements were obtained by using a modified dual excitation wavelength imaging system (IonOptix, Milton, MA). The Ca^{2+} concentrations were measured from the ratios of fluorescence elicited by 340-nm excitation to 380-nm excitation using standard techniques (15). Ratiometric images were masked in the region outside the borders of the cell, because low photon counts give unreliable ratios near the edges. We developed a method for generating an adaptive mask that follows the borders of the cell as Ca^{2+} changes and as the cell contracts. A pseudoisobestic image (i.e., an image insensitive to Ca^{2+} changes) was formed in computer memory from a weighted sum of the images generated by 340- and 380-nm excitation. This image was then thresholded, i.e., values below a selected level were considered to be outside the cell and assigned a value of zero. For each ratiometric image, the outline of the cell was determined, and the generated mask was applied to the ratiometric image. This method allows the simultaneous imaging of the changes in Ca^{2+} and in cell length. Our algorithm has been incorporated into the IonOptix software. After the experiment, the cell images were copied into a Microsoft Powerpoint file, which was converted into a .jpg file. The cell length was then measured by using NIH Image software.

H_2O_2 measurement. Sigmoid circular smooth muscle squares (100 mg) were homogenized in PBS. Homogenization consists of a 20-s burst with a Tissue Tearer (Biospec, Racine, WI) followed by 50 strokes with a Dounce tissue grinder (Wheaton, Melville, NJ). An aliquot of homogenate was taken for protein measurement. The homogenate was centrifuged at 15,000 rpm for 15 min at 4°C in a model J2–21 centrifuge with a fixed-angle model JA-20 rotor (Beckman, Palo Alto, CA), and the supernatant was collected.

H_2O_2 content was measured by Bioxytech H_2O_2-560 Quantitative Hydrogen Peroxide Assay Kit (Oxis International, Portland, OR). This assay is based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by H_2O_2 under acidic conditions. The ferric ion binds with the indicator dye xylenol orange 3,3′-bis[*N*,*N*-di(carboxymethyl)-aminomethyl]-*o*-cresoisulfone-phthalein sodium salt to form a stable, colored complex that can be measured at 560 nm.

Fluorescence microscopic measurement of intracellular ROS. Intracellular ROS were measured according to the methods described previously (28, 49). Briefly, freshly isolated muscle cells were incu-

bated in HEPES-buffered solution with or without catalase 78 U/ml for 50 min and then loaded with 2.5 μM 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) for 30 min at room temperature in the dark. During loading, the acetate groups on CM-H_2DCFDA are cleaved by intracellular esterase, trapping the probe inside the muscle cells. Several dihydrofluorescein derivatives have been used for measuring intracellular ROS generation (36, 43, 47). CM-H_2DCFDA was chosen, because it showed better retention in cells than other derivatives. After being loaded, the cells were fixed in PBS buffer containing 4% paraformaldehyde and kept at 4°C in the dark.

Production of ROS was measured by changes in fluorescence, because subsequent oxidation of CM-H_2DCFDA produced a fluorescent product in sigmoid muscle cells. The fluorescence was detected on a fluorescent microscope (Eclipse model E800; Nikon, Mellville, NY) at an excitation wavelength of 488 nm and emission at 520 nm, and the cell images were collected into a Macintosh computer with identical parameters for all samples. The intensity of fluorescence of each cell was measured by using the NIH image software. At least 10 cells from each patient were measured.

Drugs and chemicals. Soybean trypsin inhibitor was from Worthington Biochemicals (Freehold, NJ); fura-2 AM, CM-H_2DCFDA, and BAPTA were from Molecular Probes (Eugene, OR). NKA, H_2O_2, collagenase type F, papain, catalase, BME amino acid supplement, HEPES sodium, paraformaldehyde, and other reagents were purchased from Sigma (St. Louis, MO).

Statistical analysis. Data are expressed as means ± SE. Statistical differences between two groups were determined by Student's *t*-test. Differences among multiple groups were tested by using ANOVA and checked for significance using Fisher's protected least significant difference test.

RESULTS

KCl- and NKA-induced Ca^{2+} signal in UC. In fura-2 AM-loaded normal sigmoid circular smooth muscle cells, KCl caused a 312.7 ± 38.3 nM (n = 3 subjects, 11 cells) increase in cytosolic Ca^{2+} and 15.2 ± 1.7% cell shortening (n = 3 subjects, 11 cells). In UC cells, KCl caused a 336.9 ± 35.8 nM (n = 4 subjects, 32 cells) increase in cytosolic Ca^{2+} and 9.1 ± 1.8% cell shortening (n = 4 subjects, 27 cells). KCl-induced Ca^{2+} increase was not different in normal and UC cells (Fig. 1*A*); however, KCl-induced cell shortening was significantly lower in UC cells than in normal cells (Fig. 1*B*, $P < 0.02$, unpaired *t*-test), suggesting that the contractile signal transduction pathways may be impaired in UC.

After a 10-min incubation of UC cells in Ca^{2+}-free medium, the KCl-induced Ca^{2+} signal was reduced to 25.4 ± 3.6 nM (n = 3 subjects, 7 cells), significantly lower than in normal Ca^{2+} medium ($P < 0.01$, unpaired *t*-test) (Fig. 2). Because KCl-induced Ca^{2+} changes are mediated by Ca^{2+} influx (16, 21, 30, 39), our data establish a Ca^{2+}-free medium incubation protocol to selectively block influx of extracellular Ca^{2+} without affecting signals mediated by release of Ca^{2+} from intracellular stores, as shown in Fig. 3*A*.

The unstimulated length of normal muscle cells was 90.7 ± 5.6 μm (4 patients, 120 cells). Figure 3 shows that in normal sigmoid circular muscle cells in normal Ca^{2+} medium, NKA (1 μM) increased cytosolic Ca^{2+} levels by 336.1 ± 24.6 nM (n = 3 patients, 14 cells) and caused 26.5 ± 2.4% cell shortening. In Ca^{2+}-free medium, NKA (1 μM) caused a 326 ± 23.9 nM Ca^{2+} increase (n = 4 patients, 17 cells) and caused 26.1 ± 2.4% cell shortening.

Therefore, in normal sigmoid circular muscle cells, shortening and Ca^{2+} signals were not different in normal Ca^{2+} and in Ca^{2+}-free medium, confirming previously reported findings (5, 17) that an NKA-induced calcium signal is mainly due to calcium release from intracellular calcium stores.

In UC muscle cells, the resting cell length was 98.7 ± 7.6 μm (3 patients, 90 cells). On average, the UC muscle cells were slightly longer than normal, but the difference was not statistically significant. NKA (1 μM) caused a 372.2 ± 42.7 nM Ca^{2+} increase (n = 5 patients, 30 cells) and 18.1 ± 1.3% cell shortening in normal Ca^{2+} medium. Thus in UC cells, NKA-induced shortening was significantly lower than in normal cells, but the Ca^{2+} increase was not different from normal cells.

In UC cells in Ca^{2+}-free medium, however, NKA (1 μM) caused a 187.4 ± 30 nM Ca^{2+} increase (n = 5 patients, 35 cells) and a 7.6 ± 1.7% cell shortening. Thus in UC cells in Ca^{2+}-free medium, the Ca^{2+} signal was significantly lower than in normal Ca^{2+} medium and in normal cells (Fig. 3). Similarly, in UC cells in Ca^{2+}-free medium shortening was significantly lower than in normal Ca^{2+} medium and in normal cells in Ca^{2+}-free medium (Fig. 3).

The data suggest that in normal cells, the NKA-induced Ca^{2+} signal depends only on release of Ca^{2+} from intracellular

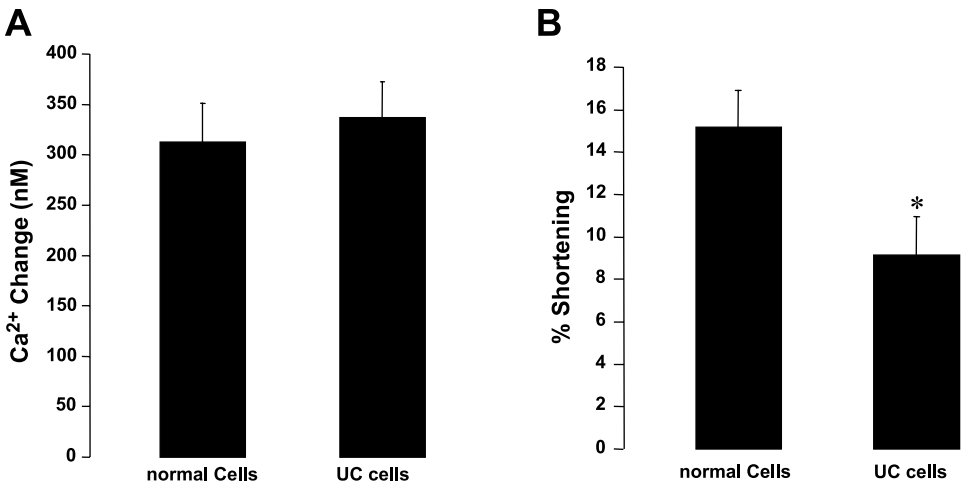

Fig. 1. Ca^{2+} signaling and cell shortening in response to KCl in normal Ca^{2+} medium in fura-2 AM-loaded sigmoid circular smooth muscle cells from normal colon (n = 3 patients, 11 cells) and from patients with ulcerative colitis (UC; n = 4 patients, 27–32 cells). *A*: KCl-induced Ca^{2+} signal increase had no significant difference between normal and UC cells. *B*: KCl-induced cell shortening was significantly lower in UC cells than in normal cells (*$P < 0.02$, unpaired *t*-test), suggesting that the contractile signal transduction pathways may be impaired in UC. Ca^{2+} change was the difference between the basal Ca^{2+} value and the peak value.

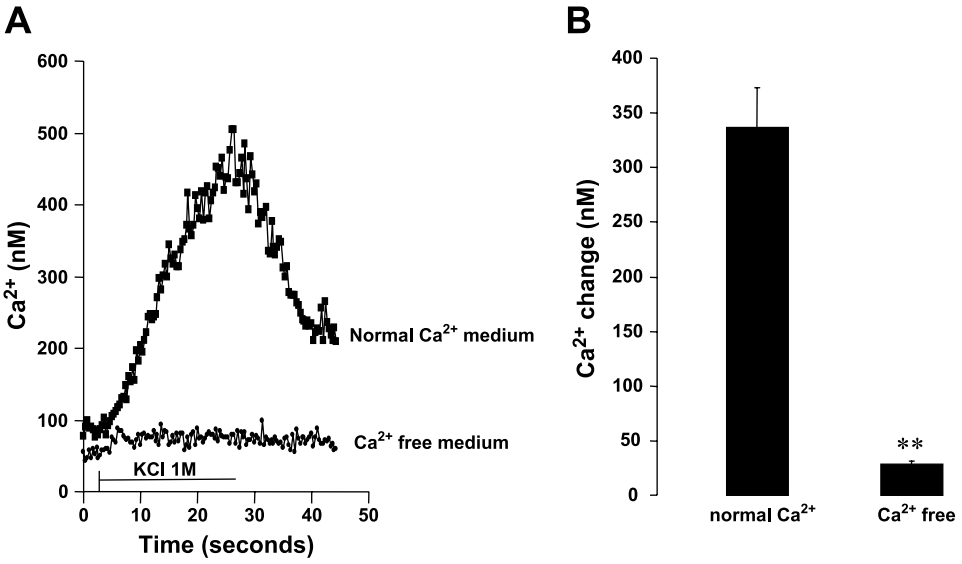

Fig. 2. Ca^{2+} signaling in fura-2 AM-loaded sigmoid circular smooth muscle cells in response to KCl. Cells were kept in normal Ca^{2+} medium or Ca^{2+}-free medium with 200 μM BAPTA to inhibit influx of extracellular Ca^{2+}, and 1 M KCl was applied directly to the cells by using a pressure ejection micropipette system. *A*: typical Ca^{2+} tracing graph. *B*: summarized data showed that KCl caused an increase in intracellular Ca^{2+} in normal Ca^{2+} medium but not in Ca^{2+}-free medium. Because KCl-induced Ca^{2+} changes are mediated by Ca^{2+} influx, this figure shows that Ca^{2+}-free medium with 200 μM BAPTA selectively blocked influx of extracellular Ca^{2+}. $^{**}P < 0.001$, unpaired *t*-test; $n = 4$ patients, 32 cells (normal Ca^{2+}); $n = 3$ patients, 7 cells (Ca^{2+} free).

stores, because it is not affected when influx is abolished by incubation in Ca^{2+}-free medium.

In contrast, in UC cells the NKA-induced Ca^{2+} signal depends in part on influx of extracellular Ca^{2+} and in part on release of Ca^{2+} from intracellular stores, because the Ca^{2+} signal is diminished when influx is abolished by incubation in Ca^{2+}-free medium. In normal Ca^{2+}, however, the Ca^{2+} signal is the same in UC as in normal cells.

In UC cells, influx of extracellular Ca^{2+} must compensate for the reduction in release of Ca^{2+} from intracellular stores. The finding that in normal Ca^{2+} medium, contraction of UC cells is less than in normal cells despite equal amplitude of

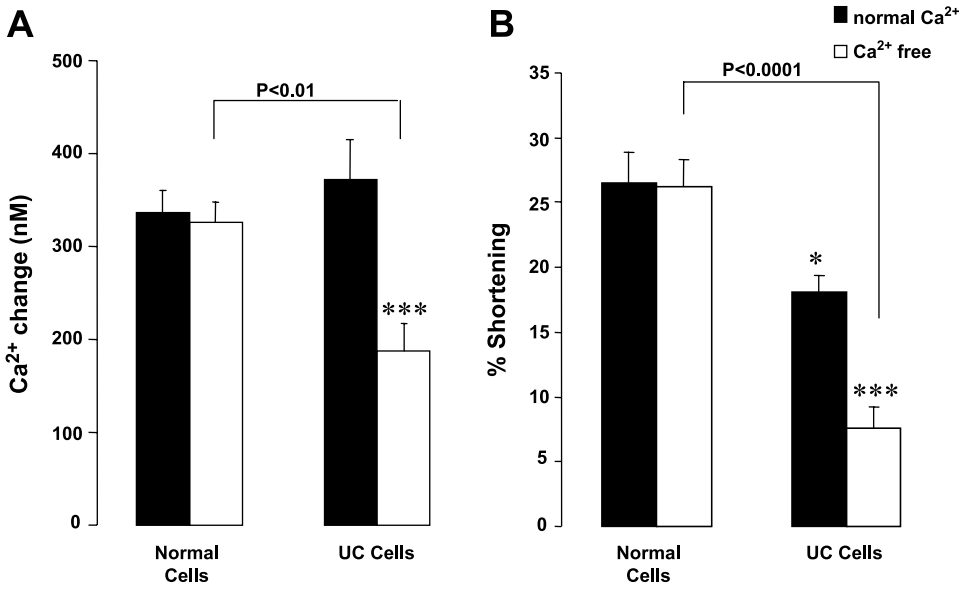

Fig. 3. *A*: neurokinin A (NKA)-induced Ca^{2+} signal in fura-2 AM-loaded sigmoid muscle cells. In normal cells, the intracellular Ca^{2+} change in response to NKA (1 μM) was the same in normal Ca^{2+} medium ($n = 3$ patients, 14 cells) and in Ca^{2+}-free medium with 200 μM BAPTA ($n = 4$ patients, 17 cells), suggesting that NKA-induced contraction in normal cells is mediated by Ca^{2+} release from intracellular stores. In cells from patients with UC, however, NKA-induced intracellular Ca^{2+} change was significantly lower in Ca^{2+}-free medium ($n = 35$ cells of 5 patients) than in normal Ca^{2+} medium ($n = 30$ cells of 5 patients), suggesting that in UC the NKA-induced Ca^{2+} signal depends in part on influx of extracellular Ca^{2+} and in part on release of Ca^{2+} from intracellular stores. Reduction in the NKA-induced Ca^{2+} signal in Ca^{2+}-free medium in UC suggests that release of Ca^{2+} from the intracellular stores may be impaired. *B*: NKA-induced cell shortening in fura-2 AM-loaded intact sigmoid muscle cells. Cell images were continuously recorded into a computer before and after applying NKA (1 μM) directly to cells by using a pressure ejection micropipette system. Shortening was defined as percent decrease in cell length 40 s after application of NKA, compared with control length at 0 s. In UC cells, NKA-induced cell shortening was significantly less than in normal cells in normal Ca^{2+} medium (UC, $n = 19$ cells of 3 patients; normal, $n = 13$ cells of 3 patients) and in Ca^{2+}-free medium with 200 μM BAPTA (UC, $n = 20$ cells of 3 patients; normal, $n = 17$ cells of 4 patients), indicating motor dysfunction in UC. $^{*}P < 0.01$, ANOVA, UC cells compared with normal cells in normal Ca^{2+} medium; $^{***}P < 0.0001$, ANOVA, UC cells compared with normal cells in Ca^{2+}-free medium.

Ca^{2+} signal suggests that in addition to alteration of Ca^{2+} release, other mechanisms of contractile signal transduction might also be affected in UC.

H_2O_2 and UC motor dysfunction. Elevated levels of H_2O_2 have been demonstrated in colonic mucosa of patients with UC (9, 40). To test whether H_2O_2 may also be present in the circular smooth muscle and contribute to motor dysfunction, UC muscle strips and cells were exposed to the H_2O_2 scavenger catalase.

Figure 4*A* shows that NKA caused concentration-dependent contractions of normal and UC circular sigmoid muscle strips. Consistent with the cell data shown in Fig. 3, UC strips contracted less than normal strips at all NKA concentrations tested ($P < 0.0001$) (Fig. 4*A*), and incubation in catalase (78 U/ml for 50 min) significantly increased contraction of UC strips ($P < 0.05$) but did not increase contraction of normal strips.

Similarly, in enzymatically isolated circular muscle cells, NKA induced a concentration-dependent contraction (Fig. 4*B*) that was significantly lower in UC than in normal cells. Incubation in catalase (78 U/ml for 50 min) significantly increased contraction of UC cells ($P < 0.0001$, ANOVA). The resting cell length after catalase treatment was 104.9 ± 8.1 μm (3 patients, 90 cells), and this length was not significantly different from untreated UC cells. In normal cells, catalase had no effect on cell shortening. These data strongly suggest that H_2O_2 is present in the circular smooth muscle, because preparations of both strips and isolated cells are free of mucosa.

Because NKA-induced release of Ca^{2+} from intracellular stores may be impaired in UC (Fig. 3), we then examined thapsigargin-induced contraction of circular muscle cells (Fig. 5). Thapsigargin inhibits uptake of Ca^{2+} into stores, causing a net release of Ca^{2+} and contraction. Continuous release of Ca^{2+} in the absence of Ca^{2+} uptake results eventually in depletion of stores.

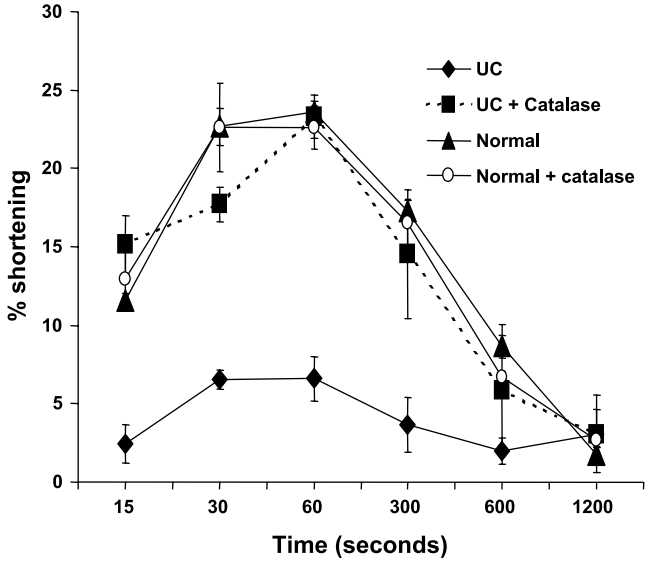

Fig. 5. Isolated sigmoid circular smooth muscle cells were treated with thapsigargin (3 μM) for the indicated times. Thapsigargin inhibits uptake of Ca^{2+} into stores, causing a net release of Ca^{2+} and contraction. Thapsigargin-induced contraction was significantly less in sigmoid circular smooth muscle cells from patients with UC ($P < 0.0001$, ANOVA, $n = 3$) compared with normal sigmoid cells. Incubation of isolated UC sigmoid cells in the H_2O_2 scavenger catalase (78 U/ml for 50 min) augmented thapsigargin-induced contraction ($P < 0.001$, ANOVA, $n = 3$). Catalase treatment had no effect on thapsigargin-induced contraction in normal cells ($n = 3$).

Similarly to NKA-induced contraction, thapsigargin induced a time-dependent contraction (Fig. 5) that was significantly lower in UC than in normal cells, supporting the proposition that UC reduces releasable Ca^{2+} stores. Incubation in catalase (78 U/ml for 50 min) did not affect contraction of normal cells but significantly increased contraction of UC cells ($P < 0.001$,

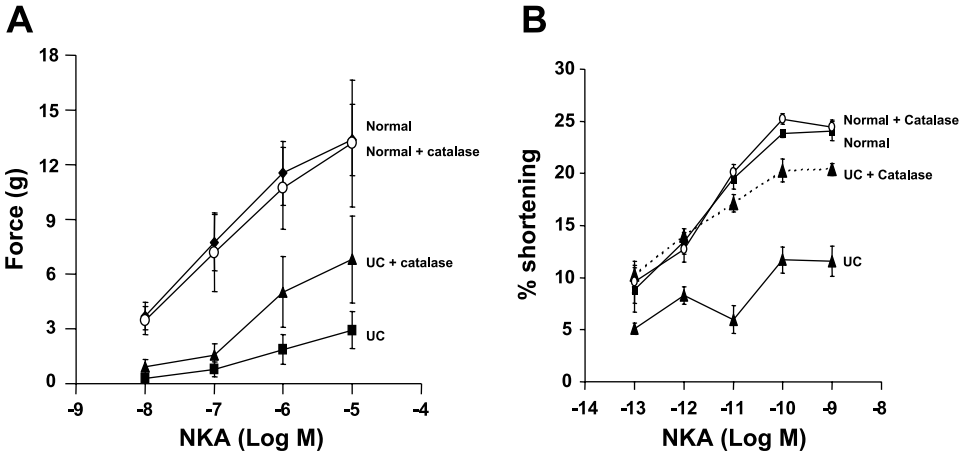

Fig. 4. *A*: in human sigmoid circular muscle strips, the NKA-induced contraction was significantly decreased in UC ($P < 0.0001$, ANOVA, $n = 3$ patients, 6 strips) when compared with normal strips ($n = 4$ patients, 13 strips). After UC strips were treated with catalase 78 U/ml for 50 min, the NKA-induced contraction was significantly augmented ($P < 0.05$, ANOVA, $n = 3$ patients, 6 strips) when compared with UC strips, suggesting that H_2O_2 may contribute to the motor dysfunction in UC. However, catalase treatment didn't affect the NKA-induced contraction in normal strips ($n = 3$ patients, 6 strips). *B*: the NKA-induced contraction was significantly reduced in isolated sigmoid circular smooth muscle cells from patients with UC ($P < 0.0001$, ANOVA, $n = 6$) when compared with normal sigmoid cells ($n = 6$). This reduction was restored by catalase treatment (78 U/ml, 50 min) ($P < 0.0001$, ANOVA, $n = 3$). Catalase had no effect on the NKA-induced contraction in normal cells ($n = 3$). The average of resting cell length was 90.7 μm (normal), 98.7 μm (UC), and 104.9 μm (UC + catalase).

ANOVA), confirming presence of H_2O_2 in circular smooth muscle cells from UC patients.

To directly examine Ca^{2+} release from intracellular stores, enzymatically isolated circular muscle cells were loaded with the calcium indicator fura-2 AM, and NKA-induced calcium signal and shortening were examined in Ca^{2+}-free medium (i.e., in the absence of Ca^{2+} influx) in the same cells. Ca^{2+} signal and shortening of the sigmoid circular muscle cell in Ca^{2+}-free medium are shown in Fig. 6. The figure shows that in Ca^{2+}-free medium, a normal cell exhibits a strong Ca^{2+} signal and shortens ~34.6%. In contrast, a UC cell in Ca^{2+}-free medium exhibits little or no Ca^{2+} signal and shortening, but both Ca^{2+} signal and shortening are augmented after the cell is incubated in catalase. Although in UC cells incubated in catalase the Ca^{2+} signal is greater than in normal cells, the cell shortening is slightly lower than in normal cells. Average values for shortening and amplitude of Ca^{2+} signal are shown in Fig. 7. However, catalase treatment had no effect on NKA-induced Ca^{2+} signal and cell contraction in normal cells.

The finding that the H_2O_2 scavenger catalase increases amplitude of NKA-induced Ca^{2+} signal and shortening in UC circular muscle suggests that H_2O_2 is produced by and present in circular muscle and that it contributes to motor dysfunction in UC by reducing NKA-induced Ca^{2+} release from intracellular stores.

H_2O_2 and intracellular calcium stores in normal sigmoid circular smooth muscle cells. To test the hypothesis that H_2O_2 may be present in the circular muscle layer and responsible for impaired release of Ca^{2+} from intracellular stores, we measured H_2O_2 content in normal and UC sigmoid circular muscle.

H_2O_2 levels were significantly elevated in UC, compared with normal muscle (Fig. 8), supporting the possibility that H_2O_2 may be involved in the observed changes in muscle contraction. To test whether isolated sigmoid muscle cells contain H_2O_2, we measured intracellular H_2O_2 by using fluorescence of CM-H_2DCFDA (Fig. 9). The cells were loaded with 2.5 μM CM-H_2DCFDA for 30 min at room temperature in the dark. During loading, the acetate groups on CM-H_2DCFDA are removed by intracellular esterase, trapping the probe inside the muscle cells. When oxidized in situ by ROS, DCF generates a signal that can be visualized by using a fluorescent microscope (1). A stronger oxidant signal was detected by DCF fluorescence in UC muscle cells than in normal cells. This oxidant signal was significantly reduced by

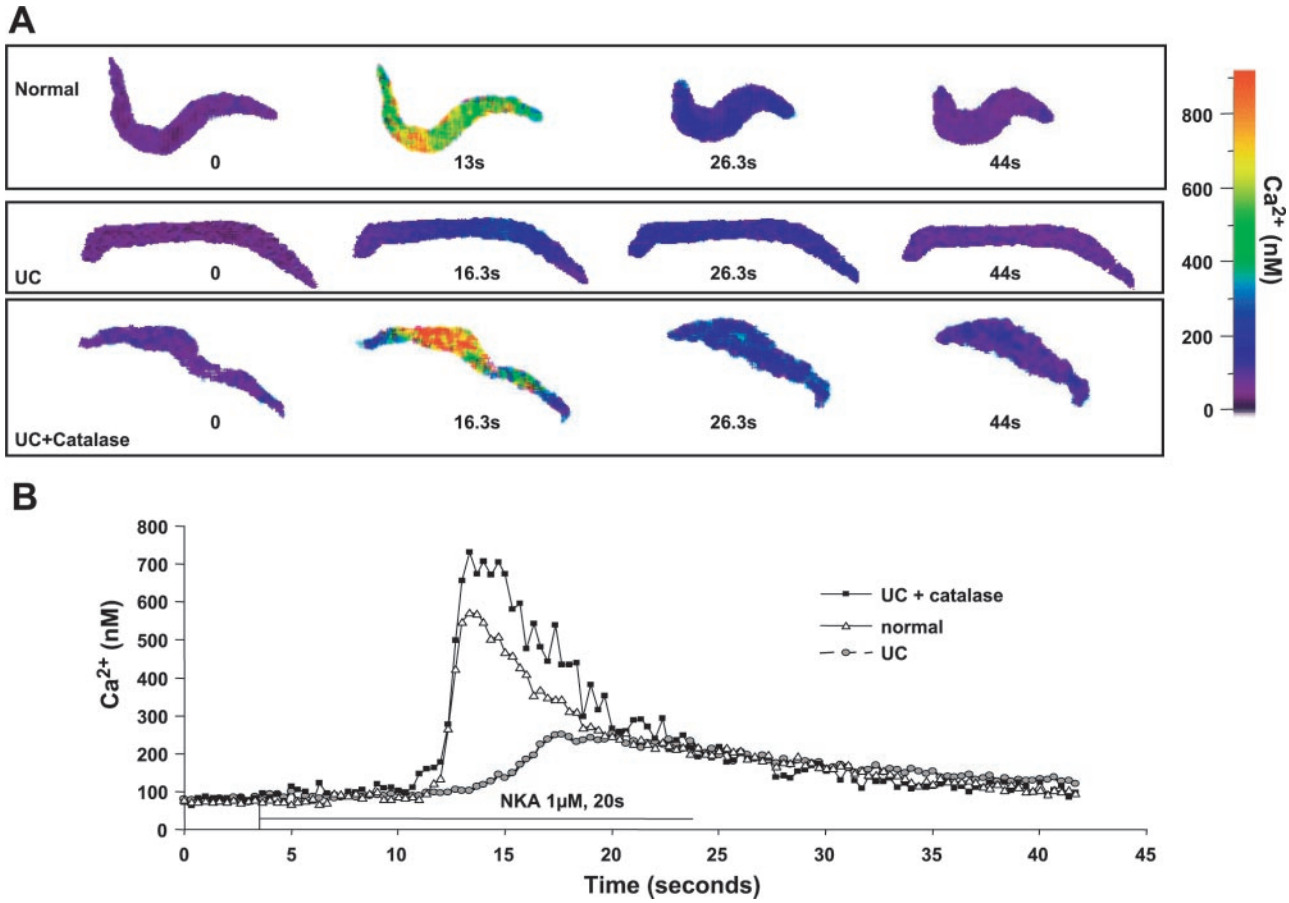

Fig. 6. *A*: Ca^{2+} signaling in response to NKA in fura-2 AM-loaded sigmoid circular smooth muscle cells from normal colon and from patients with UC. Cells were kept in Ca^{2+}-free medium with 200 μM BAPTA to inhibit influx of extracellular Ca^{2+}, and 1 μM NKA was applied directly to the cells by using a pressure ejection micropipette system. The numbers below the images indicate the point in time at which the images were acquired. In sigmoid circular smooth muscle cells from normal colon, NKA caused cell shortening (34.6%) and Ca^{2+} increase, which usually rose from one site and then spread to the whole cell. Sigmoid muscle cells from patients with UC did not contract in response to NKA, although there was Ca^{2+} increase in this cell. After catalase treatment (78 U/ml, 50 min) of UC cells, NKA induced significant Ca^{2+} increase and cell shortening (24.3%). *B*: time course of Ca^{2+} transients of the above cells is shown in the traces. The bar indicates the time when 1 μM NKA was applied via a pressure ejection micropipette.

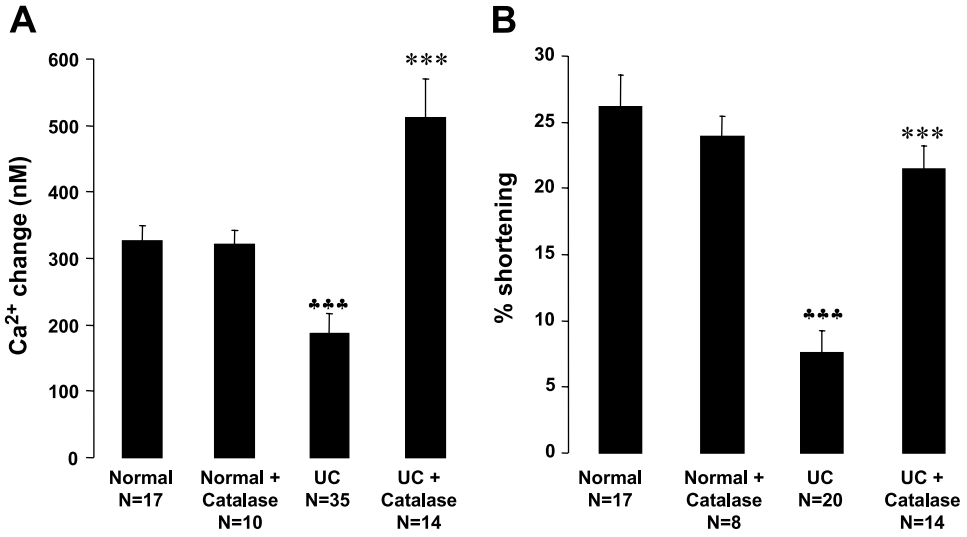

Fig. 7. Ca^{2+} signaling and contraction in response to NKA in fura-2 AM-loaded sigmoid circular smooth muscle cells from normal colon and from patients with UC. Cells were kept in Ca^{2+}-free medium with 200 μM BAPTA, to inhibit influx of extracellular Ca^{2+}, and 1 μM NKA was applied directly to the cells by using a pressure ejection micropipette system. *A*: catalase treatment (78 U/ml, 50 min) of UC cells completely restored the reduced Ca^{2+} signal and had no effect on NKA-induced Ca^{2+} signal in normal cells. *B*: catalase treatment significantly increased UC cell shortening but not back to completely normal ($P = 0.09$, ANOVA, when compared with normal cells). Catalase treatment had no effect on NKA-induced cell contraction in normal cells. ♣♣♣$P < 0.01$, ANOVA, compared with normal cells, ***$P < 0.0001$, ANOVA, compared with UC group.

preincubation of the muscle cells with catalase (78 U/ml, 50 min), suggesting that H_2O_2 is the main oxidant generated (Fig. 9). The data suggest that isolated UC muscle cells contain excess H_2O_2, which may affect cell contraction. Catalase did not completely remove the oxidant signal, suggesting that other ROS may be present in UC muscle cells.

To test whether H_2O_2 may induce release of Ca^{2+} from intracellular stores, a high concentration of H_2O_2 was directly applied to fura-2 AM-loaded cells (Fig. 10) through a pressure ejection micropipette to permit visualization of Ca^{2+} release.

Application of a high-concentration H_2O_2 to enzymatically isolated smooth muscle cells caused a gradual Ca^{2+} increase, which reached a stable level in ~1 min. The rate of Ca^{2+} increase was much lower than that of NKA-induced Ca^{2+} increase where peak Ca^{2+} concentration was reached in a few seconds, as shown in Fig. 6*B*. Figure 10 shows that in normal calcium, H_2O_2 caused a 194 ± 11.6 nM Ca^{2+} increase (from 96.6 ± 4.1 to 290.5 ± 9.5 nM, $n = 10$). In Ca^{2+}-free medium, H_2O_2 caused a 190.2 ± 23.7 nM Ca^{2+} increase (from 72.3 ± 6.2 to 262.6 ± 25.1 nM, $n = 6$) (Fig. 10). There was no difference in H_2O_2-induced Ca^{2+} increase in normal Ca^{2+} medium and in Ca^{2+}-free medium, indicating that H_2O_2 causes direct release of Ca^{2+} from intracellular stores. The recorded Ca^{2+} changes were not associated with any change in cell length within 3 min and were consistent with the finding that a similar Ca^{2+} increase was insufficient to cause contraction (Fig. 6).

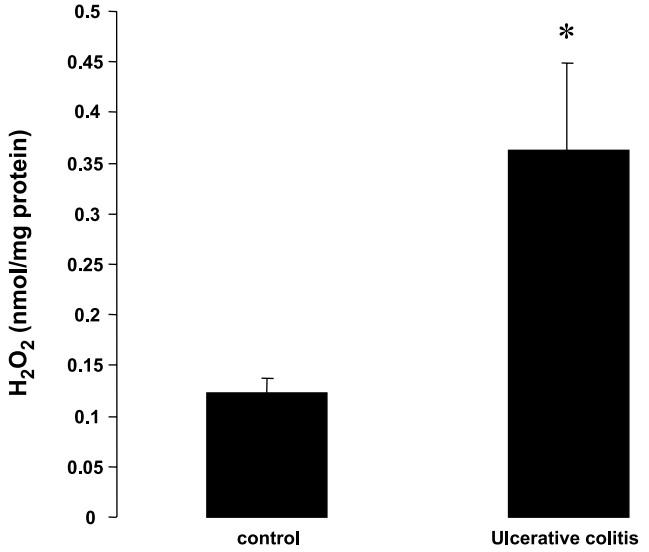

Fig. 8. H_2O_2 content of sigmoid circular muscle from normal colon (control, $n = 6$) and from patients with UC ($n = 7$). H_2O_2 was measured by a colorimetric assay. H_2O_2 production was significantly increased in UC (*$P < 0.05$, unpaired *t*-test).

To test whether prolonged exposure of normal cells to a relatively low concentration of H_2O_2 may result in depletion of intracellular Ca^{2+} stores, we examined NKA-induced Ca^{2+} signal in fura-2 AM-loaded normal cells incubated with 70 μM H_2O_2 for 30 min. The cells were placed in Ca^{2+}-free medium with 200 μM BAPTA to ensure that the Ca^{2+} signal was entirely produced by release of Ca^{2+} from intracellular stores.

H_2O_2 treatment did not change resting calcium levels in unstimulated cells but significantly decreased the NKA-induced Ca^{2+} signal (78.6 ± 73.3 nM) when compared with untreated cells (326 ± 24 nM) (Fig. 11), demonstrating that H_2O_2 may be directly responsible for decreasing releasable Ca^{2+} from intracellular stores and for the reduced Ca^{2+} signal in response to NKA.

DISCUSSION

We have previously shown that NKA is an important excitatory neurotransmitter in human sigmoid circular muscle (5). NKA-induced contraction occurs through activation of G_q-linked NK-2 receptors and release of calcium from intracellular stores (5), which is consistent with a previous finding in rat intestinal muscle cells (17). We have also shown that in UC, NKA-induced contraction is significantly reduced (48). In the present study, we examined a possible role of H_2O_2 as an agent responsible for decreasing contraction and release of Ca^{2+} from intracellular stores in sigmoid circular muscle from patients with UC.

To distinguish the contribution of Ca^{2+} release in the overall Ca^{2+} signal, we developed a protocol to ensure abolition of Ca^{2+} influx without affecting Ca^{2+} release. Isolated smooth muscle cells were incubated in Ca^{2+}-free medium containing

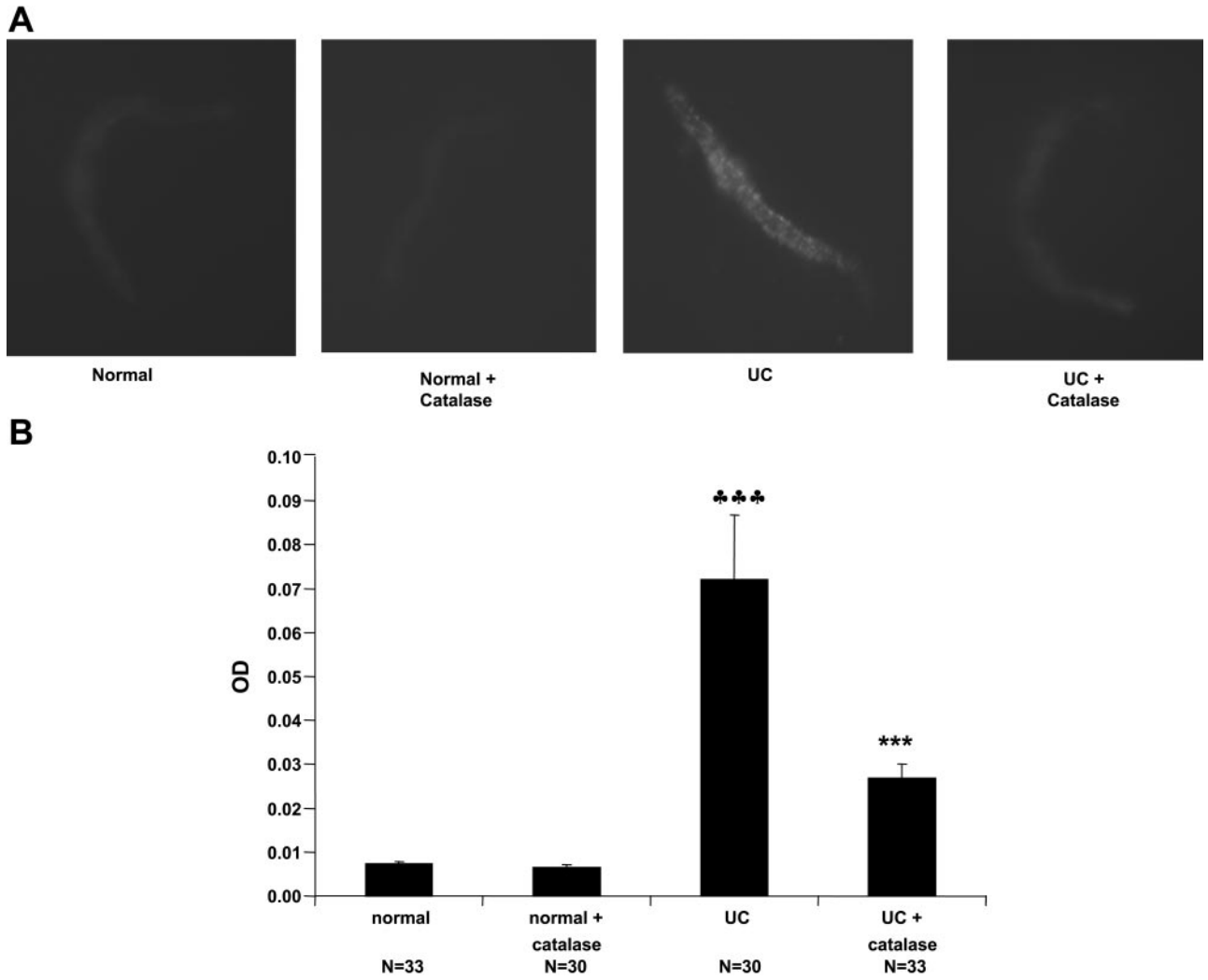

Fig. 9. Intracellular H_2O_2 was detected by using fluorescence of 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). Freshly isolated sigmoid muscle cells from normal or UC sigmoid colon were incubated in HEPES-buffered solution with or without catalase (78 U/ml for 50 min) and then loaded with CM-H_2DCFDA for 30 min at room temperature in the dark. The fluorescence was detected on a fluorescent microscope at an excitation wavelength of 488 nm and emission at 520 nm, and the cell images were collected into a Macintosh computer with identical parameters for all samples. Intensity of fluorescence of each cell was measured by using the NIH image software. Typical cell images (*A*) and summarized data (*B*) show that a stronger oxidant signal was detected by fluorescence in UC muscle cells than in normal cells. This oxidant signal was significantly reduced by preincubation of the muscle cells with catalase, suggesting that H_2O_2 is the main oxidant generated. The data suggest that isolated UC muscle cells contain excess H_2O_2, which may affect the cell contraction and that smooth muscle cells might be one source of H_2O_2. Catalase could not completely remove the oxidant signal, suggesting that other reactive oxygen species might be present in UC muscle cells. The number of cells (from three patients) used to obtain the averages in each group is shown in the figure. ♣♣♣$P < 0.0001$, ANOVA, compared with normal cells; ***$P < 0.0001$, ANOVA, compared with catalase-free UC group.

200 μM BAPTA for no longer than 10 min before starting the experiment. This protocol abolished KCl-induced contraction, which is mediated by influx of extracellular Ca^{2+} without affecting NKA-induced contraction that is mediated by release of Ca^{2+} from intracellular stores. Establishing an appropriate protocol is important, because longer incubations or higher BAPTA concentrations may cause depletion of intracellular stores.

Using this protocol, we confirmed that, although in normal cells the NKA-induced Ca^{2+} signal is mediated entirely by the release of Ca^{2+} from intracellular stores (Fig. 3), in UC cells the Ca^{2+} signal is only in part mediated by Ca^{2+} release and releasable Ca^{2+} stores are reduced. In UC cells, the Ca^{2+} signal was reduced when the cells were incubated in Ca^{2+}-free medium, supporting damage to Ca^{2+}-release mechanisms. In normal Ca^{2+}, however, the Ca^{2+} signal was normal, indicating that Ca^{2+} influx compensated for the reduced Ca^{2+} release. Despite the normal Ca^{2+} signal in normal Ca^{2+} medium, however, contraction in response to NKA was reduced. Similarly, KCl-induced Ca^{2+} signal in UC cells was not significantly different from that in normal cells, whereas KCl-induced contraction was reduced in UC cells, which is consistent with the literature (42). The data suggest that mechanisms of Ca^{2+} release and other contractile signal transduction pathways might be impaired in UC. KCl-induced cell shortening in normal cells is lower than NKA despite a similar Ca^{2+} signal.

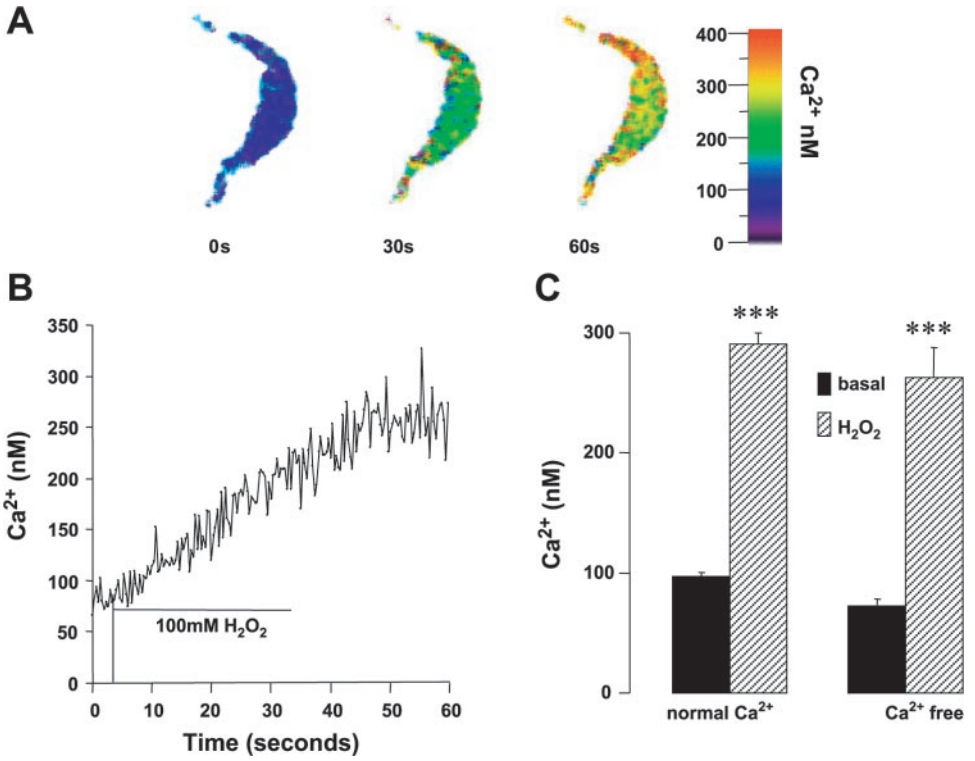

Fig. 10. Ca^{2+} signaling in response to H_2O_2 in fura-2 AM-loaded sigmoid circular smooth muscle cells from normal colon. *A*: normal sigmoid smooth muscle cells were kept in Ca^{2+}-free medium with 200 μM BAPTA, to inhibit influx of extracellular Ca^{2+}, and 100 mM H_2O_2 was applied directly to the cell by using a pressure ejection micropipette system. A series of images of a typical cell taken before applying H_2O_2 (0 s) and 30 and 60 s after H_2O_2 are shown. *B*: H_2O_2 application caused a gradual increase in intracellular Ca^{2+} levels, suggesting that H_2O_2 can release Ca^{2+} from intracellular stores. *C*: normal sigmoid smooth muscle cells were kept in normal Ca^{2+} medium or Ca^{2+}-free medium with 200 μM BAPTA. H_2O_2 significantly increased the Ca^{2+} signal in normal and Ca^{2+}-free medium. There was no difference in H_2O_2-induced Ca^{2+} increase in normal Ca^{2+} medium and in Ca^{2+}-free medium, indicating that H_2O_2 causes direct release of Ca^{2+} from intracellular stores. ***$P < 0.001$, paired *t*-test, $n = 6$.

This is probably due to the fact that KCl and NKA activate different contractile signal transduction pathways. For instance, NKA activates G protein-coupled receptors that may magnify the signal but KCl does not.

Because reduction of releasable Ca^{2+} and cell shortening is a feature of UC, we tested H_2O_2 as a possible factor contributing to depletion of releasable Ca^{2+} stores and sigmoid motor dysfunction. ROS have been shown to consistently depress the Ca^{2+}-ATPase responsible for uptake of Ca^{2+} into the endoplasmic reticulum (12–14, 26, 35). In addition to inhibiting Ca^{2+} uptake into the endoplasmic reticulum, ROS cause release of Ca^{2+} stores through both ryanodine- and inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channels (24). It was recently reported (32) that H_2O_2 may inhibit actomyosin ATPase, and thus H_2O_2 might inhibit NKA-induced contraction despite a normal Ca^{2+} signal in normal Ca^{2+} medium in UC.

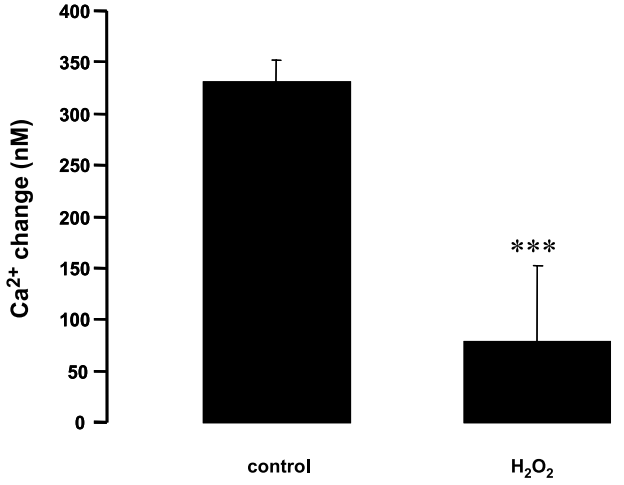

Fig. 11. Ca^{2+} signaling in response to NKA in fura-2 AM-loaded untreated sigmoid circular smooth muscle cells and H_2O_2-treated cells. Normal cells were kept in Ca^{2+}-free medium with 200 μM BAPTA, to inhibit influx of extracellular Ca^{2+}, and 1 μM NKA was applied directly to the cells by using a pressure ejection micropipette system. H_2O_2 treatment (70 μM, 30 min) of normal sigmoid cells significantly decreased NKA-induced Ca^{2+} signal, suggesting that H_2O_2 may inhibit NKA-induced Ca^{2+} release from the intracellular calcium stores. (***$P < 0.001$, unpaired *t*-test; control, $n = 17$; H_2O_2, $n = 6$).

We found that UC sigmoid circular muscle contains three times as much H_2O_2 as normal muscle, which is consistent with findings in dextran sodium sulfate-treated rats that H_2O_2 production increases in the muscularis of the inflamed colon (11). We also found that UC sigmoid muscle cells had much higher levels of intracellular H_2O_2 than normal cells and that this intracellular H_2O_2 can be removed by extracellular catalase (Fig. 9). This observation is consistent with the literature showing that extracellular catalase can neutralize intracellular H_2O_2 in adipocytes and HepG2 cells (28). The mechanism of removal of intracellular H_2O_2 by catalase is not clear. Because H_2O_2 can diffuse across biological membranes (44), it is possible that removal of extracellular H_2O_2 by catalase may facilitate diffusion of intracellular H_2O_2 into the extracellular medium, resulting, eventually, in removal of both intracellular and extracellular H_2O_2.

Catalase treatment significantly increased NKA-induced contraction in UC cells and muscle strips (Fig. 4) and restored NKA-induced Ca^{2+} signal in Ca^{2+}-free medium, indicating that H_2O_2 is present both in circular muscle strips and in enzymatically isolated smooth muscle cells and contributing to UC-associated motor dysfunction.

H_2O_2-associated depletion of releasable Ca^{2+} was confirmed by testing thapsigargin-induced contraction of isolated

smooth muscle cells. Thapsigargin inhibits uptake of Ca^{2+} into stores (8), shifting the uptake-release balance toward a net release of Ca^{2+} and contraction. The finding that thapsigargin-induced contraction is reduced in UC confirms again an UC-associated depletion of releasable Ca^{2+} stores. Neutralization of H_2O_2 by catalase restored thapsigargin-induced contraction, indicating that the presence of H_2O_2 in enzymatically isolated smooth muscle cells may be directly responsible for inhibiting the refilling of Ca^{2+} stores and that Ca^{2+} stores quickly return to normal when H_2O_2 is neutralized.

Catalase-induced restoration of the Ca^{2+} stores is directly demonstrated in Fig. 6 in which cytosolic Ca^{2+} can be visualized before and after application of catalase to an enzymatically isolated circular muscle cell.

These data suggest that H_2O_2 contributes to motor dysfunction and to reduced intracellular Ca^{2+} signal in UC. Catalase or related compounds may be useful tools in the treatment of UC, as reported by others (3, 50) who have shown that pretreatment with catalase decreased the extent of colonic inflammation in a rat model. It is likely that removal of intracellular H_2O_2 may allow replenishment of intracellular Ca^{2+} stores. In fact, after muscle cells from UC patients were exposed to catalase, NKA-induced Ca^{2+} changes were higher than in normal cells, even in Ca^{2+}-free medium (Fig. 7). The mechanism responsible for this Ca^{2+} rebound remains to be explored.

Much of the H_2O_2 produced by eukaryotic cells is derived from reduction of the superoxide anion O_2^-, normally produced in the respiratory process (44). O_2^- in aqueous solution is short-lived and rapidly reduced to the much more stable molecule H_2O_2. Thus in most biological systems, generation of O_2^- usually results in the formation of H_2O_2. However, the source of excess H_2O_2 in the circular muscle layer in UC is not clear.

The findings that intracellular H_2O_2 is present at much higher levels in UC smooth muscle cells than in normal cells and that catalase restores NKA-induced calcium signal and cell shortening in UC cells suggest that UC muscle cells may also be a source of H_2O_2. H_2O_2 production has been previously reported in rat aortic smooth muscle cells (23).

To demonstrate that H_2O_2 may directly cause release of Ca^{2+} from intracellular stores, H_2O_2 was directly applied by a pressure ejection micropipette onto fura-2 AM-loaded cells. Applying H_2O_2 directly onto the cells caused a gradual increase of cytosolic Ca^{2+} even when cells were maintained in Ca^{2+}-free medium, demonstrating direct H_2O_2-induced release of Ca^{2+} from intracellular Ca^{2+} stores, consistent with previous data (4) in acute esophagitis. Although H_2O_2 caused Ca^{2+} increase, it did not cause cell contraction. This may be due to a slower rate of Ca^{2+} increase and to possible inhibition of actomyosin ATPase (32) induced by H_2O_2.

To produce a measurable Ca^{2+} release in a short time, we used a relatively high concentration of H_2O_2. Over a prolonged period, however, lower H_2O_2 concentrations are sufficient to cause a reduction in releasable Ca^{2+} in normal muscle cells, as shown in Fig. 11, where 30-min incubation with 70 μM H_2O_2 almost abolished NKA-induced Ca^{2+} release in Ca^{2+}-free medium, suggesting that over a long period, exposure to H_2O_2 may cause a reduction in releasable Ca^{2+} stores as previously demonstrated (12–14, 26, 35). Thus the presence of increased levels of H_2O_2 may explain the reduced intracellular calcium release observed in UC.

The finding that catalase only in part restored muscle contraction may perhaps be explained by the existence of other inflammatory mediators such as nitric oxide and arachidonic acid metabolites (prostaglandins, leukotrienes, isoprostanes, etc.) that are not neutralized by catalase.

In conclusion, our data clearly demonstrate that H_2O_2 is produced not only in the mucosa as generally thought, but also in the muscle layer of UC colon. H_2O_2 produced in the muscle layer may account for at least some of the motor disturbances observed in UC, and neutralization of H_2O_2 may result in improvement of UC-associated dysmotility.

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DISCLOSURE

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