Mechanism of action of cholera toxin on the opossum internal anal sphincter smooth muscle

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Fan, Y-A-Ping, Sushanta Chakder, and Satish Rattan. Mechanism of action of cholera toxin on the opossum internal anal sphincter smooth muscle. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G152–G160, 1999.—Cholera toxin (CTX), an activator of Gs protein, is an important pharmacological tool in G protein research. The effect and the mechanism of action of CTX in the gastrointestinal smooth muscle, including the internal anal sphincter (IAS), are not known. The present investigation was carried out to examine the effects of CTX on the signal transduction associated with the adenylate cyclase (AC) pathway on the basal tone of the IAS smooth muscle. CTX caused a prompt and dose-dependent fall in the basal tone of the IAS that was not affected by the neurotoxins TTX and ω-conotoxin or the nitric oxide synthase inhibitor Nω-nitro-L-arginine. The cyclooxygenase inhibitor indomethacin, cAMP-dependent protein kinase inhibitor Rp-8-bromoadenosine 3′,5′-cyclic monophosphorothioate inhibited CTX-induced IAS smooth muscle relaxation. Furthermore, CTX caused a concentration-dependent relaxation of the isolated smooth muscle cells (SMC) of the IAS, which was blocked by Gsα antibody (Gsα-Ab). The IAS smooth muscle relaxation was accompanied with an increase in the GTPase activity that was also specifically blocked by Gsα-Ab. We conclude that a major part of the inhibitory action of CTX in the IAS is via the direct response of the SMC that is linked with Gs protein to the AC pathway. A part of the inhibitory action of CTX on the smooth muscle occurs via the activation of cyclooxygenase pathway. The relative contribution of such actions of CTX in the smooth muscle in the gastrointestinal motility disturbances following cholera infection remains to be determined.

Cholera toxin (CTX) is well known to activate Gs protein and serves as an important pharmacological tool in G protein research. Additionally, CTX causes a number of gastrointestinal disturbances, including severe diarrhea. The effects of CTX on changes in secretion and absorption being responsible for the diarrhea are well established (4). The effects of CTX on the changes in the gastrointestinal motility, except for a few studies, have not been investigated. From in vivo studies, Cowles and Sarna (8–10) suggested that the primary effect of CTX on the canine intestine was an increase in the frequency of migrating motor complexes in the fasted state. The secondary effect of CTX may be due to an increase in the fluid volume in the small intestinal lumen and comprise an increase in the phase II duration and inhibition of migrating clustered contractions during this phase of migrating motor complexes. These actions of CTX take from 1 to 6 h to develop.

The anorectal region, especially the internal anal sphincter (IAS), plays an important role in the anorectal continence and incontinence and anorectal reflex-mediated relaxation of the IAS (2, 11, 31, 36). The effects and mechanism of action of CTX on any part of the large intestine including the anorectum have not been investigated before. Such studies may provide important insights into the pathogenesis of cholera-induced symptoms related to the changes in gastrointestinal motility.

In other systems it has been suggested that CTX may activate a membrane receptor that is linked to the G protein (12, 20, 23, 32, 38). A specific Gs protein has been shown to be involved in the signal transduction in response to CTX (20, 26). The activation of Gs protein in turn causes the activation of adenylate cyclase (AC) that is responsible for the action of CTX at the target site. An increase in cAMP may be responsible for the relaxation of a variety of smooth muscles (16, 34).

The secretory effects of CTX are well established and have been suggested to occur via both the enteric nerves (22) and the direct stimulation of the mucosa (5). There are no studies that examine the site of action of CTX on the gastrointestinal smooth muscle.

In different systems investigated so far it has been shown that CTX takes from several hours to days for the expression of its actions. This includes the tissues that otherwise are known to respond to different agonists within a matter of seconds. A long latency of action makes it rather difficult to examine in detail the mechanisms of actions of CTX and may be the reason for the lack of significant data on the mechanism of action of CTX in the gastrointestinal smooth muscle. The purpose of the present investigation was therefore to examine the effects and mechanism of action of CTX on the gastrointestinal smooth muscle. The IAS smooth muscle was used as a model since this smooth muscle is tonic and allows the investigation of both the inhibitory and excitatory actions (11, 29, 37). Our preliminary experiments suggested that the actions of CTX in the IAS smooth muscle were prompt. This allowed us to examine the mechanism of action of CTX in the IAS smooth muscle. Furthermore, the studies were designed to investigate the nature of G protein involved in the gastrointestinal smooth muscle relaxation by CTX.
MATERIALS AND METHODS

Preparation of smooth muscle strips. Opossums (Didelphis virginiana) of either sex were used for the present studies. The animals were anesthetized with pentobarbital (40–50 mg/kg ip). Laparotomy was performed, and the entire anal canal was isolated and transferred to oxygenated (95% oxygen plus 5% carbon dioxide) Krebs physiological solution of the following composition (in mM): 118.07 NaCl, 4.69 KCl, 2.52 CaCl2, 1.16 MgSO4, 1.01 NaH2PO4, 25 NaHCO3, and 11.10 glucose. The anal canal was carefully freed of all the extraneous structures, including the striated muscle fibers, the adventitia, and the large blood vessels, and was opened and pinned flat with the mucosal side up on a dissecting tray containing oxygenated Krebs physiological solution. The mucosal and submucosal layers were removed by sharp dissection, and IAS circular smooth muscle strips (~1–10 mm) were prepared for the recording of isometric tension as described previously (21, 30).

Measurement of isometric tension. The smooth muscle strips were secured at both ends with silk sutures and transferred to 2-ml muscle baths containing oxygenated Krebs solution (37°C). One end of the muscle strip was anchored at the bottom of the muscle bath and the other end was attached to a force transducer (model FTO3; Grass Instruments, Quincy, MA) for the measurement of isometric tension on a Dynograph recorder (model R411; Beckman Instruments, Schiller Park, IL). The muscle strips were stretched initially with 10 mN of force and then allowed to equilibrate for at least 1 h with regular washings at 20-min intervals. Only the strips that developed spontaneous steady tension and relaxed in response to electrical field stimulation (EFS) were used. The optimal length and the baseline of the smooth muscle strips were determined as described previously (21). At the conclusion of the experiments, all the tissues were cut below the suture material, blotted dry, and accurately weighed. The force of all the smooth muscle tissues was calculated in millinewtons as described before (19). The changes in the force, whether the relaxation or contraction, were expressed as percent of maximal changes in the basal tension at the end of the experiment with 5 mM EGTA or 3 × 10^{-6} M phenylephrine, respectively.

Nonadrenergic noncholinergic nerverelaxation stimulation with EFS. EFS was delivered from a Grass stimulator (model S88, Grass Instruments) connected in series to a Med-Lab Stimu-splitter II (Med-Lab Instruments, Loveland, CO). The Stimu-splitter was used to amplify and measure the stimulus intensity using the optimal stimulus parameters for the nonadrenergic noncholinergic (NANC) neural stimulation (12 V, 0.5 ms pulse duration, 200–400 mA, 4-s train) at varying frequencies of 0.5 to 20 Hz. The electrodes used for the EFS consisted of a pair of platinum wires that were secured at both sides of the smooth muscle strip. Neurally mediated relaxation of the IAS smooth muscle was quantified in response to different frequencies.

Isolation of smooth muscle cells from IAS. The smooth muscle cells (SMC) from the IAS region were isolated following the method previously used in our laboratory (6). Briefly, the respective smooth muscle tissues were cleared from the adjoining blood vessels, serosa, and mucosa and cut into small pieces (1-mm cubes). The small pieces of tissues were incubated at 31°C in oxygenated Krebs solution containing 0.1% collagenase (CLS II, 140 U/mg), 0.01% soybean trypsin inhibitor, and mixtures of amino acids and vitamins for two successive 60-min periods and then filtered through 500-µm Nitex mesh. The tissues trapped on the mesh were rinsed with 50 ml of collagenase-free Krebs solution and incubated for another 30 min in oxygenated Krebs solution. The dispersed cells were harvested by filtration through 500-µm Nitex mesh, centrifuged at 350 g for 10 min, and resuspended as needed. For some experiments employing the permeabilized SMC, the enzymatically digested smooth muscle tissues were washed with an oxygenated cytosolic buffer of the following composition (in mM): 20 NaCl, 100 KCl, 5.0 MgSO4, 0.96 NaH2PO4, 25 NaHCO3, 1.0 EGTA, 0.48 CaCl2, and 1% BSA and were allowed to dissociate in this medium for 30 min.

In some experiments using guanosine 5’-O-(2-thiodiphosphate) (GDPβS), G protein antibodies, or other larger molecules that do not readily diffuse across the intact SMC plasma membranes, the SMC were permeabilized as described by Sohn et al. (33). Permeabilization was accomplished by incubation of the freely dispersed SMC in the cytosolic buffer containing saponin (75 µg/ml) for 3 min. The SMC suspensions were spun at 500 g for 5 min, and the pellets were resuspended in the saponin-free modified cytosolic buffer containing antimycin A (10 µM), ATP (15 mM), phosphocreatine (5 mM), and creatine phosphokinase (10 U/ml). The procedure was repeated twice to remove the saponin, and SMC were resuspended in the modified cytosolic buffer.

Measurement of changes in SMC lengths (contraction or relaxation) by scanning micrometry. Individual SMC lengths were measured by scanning micrometry as described before (28). An 0.1-µl aliquot of suspension containing 1 × 10^7 to 3 × 10^8 SMC/ml was treated with bethanechol (1 × 10^{-7} or 1 × 10^{-6} M), and the response was terminated after 30 s with 1% acrolein. The mean SMC length of 50 cells was measured randomly by micrometry using phase contrast microscopy, and the percent shortening of the SMC length in the presence of bethanechol compared with the vehicle-treated SMC was calculated. To examine the effect of CTX (50, 100, or 200 ng/ml), GDPβS (1 mM), or Gαi antibody (Gαi-Ab; 1:200 dilution) on the SMC contraction caused by bethanechol, the SMC were first exposed to these reagents for 1, 10, or 60 min, respectively, followed by the bethanechol treatment in the manner similar to that previously described.

Preparation of SMC membranes. SMC membranes were prepared according to the method of Pomerantz et al. (25). Briefly, the dispersed SMC were washed in PBS containing 10 µM phenylmethylsulfonyl fluoride and snap-frozen at -70°C. The SMC were resuspended in 20 mM HEPES buffer (pH 7.2) containing 2 mM MgCl2, 1 mM EDTA, 1 mM benzamidine, and 10 µg each of leupeptin and pepstatin A, lysed by passing through a 25-gauge needle, and then homogenized using a teflonized glass homogenizer. Membrane fractions were isolated by centrifugation at 75,000 g at 4°C for 60 min (Beckman L8-70M Ultracentrifuge, Beckman Instruments, Palo Alto, CA) through 40% sucrose. The pellets were rehomogenized in the HEPES buffer and reisolated by centrifugation (110,000 g at 4°C for 60 min). The final pellets were suspended in the HEPES buffer and stored at -70°C. The protein concentrations of the membrane suspensions were determined by the method of Lowry et al. (18) using BSA as the standard.

Measurement of GTPase activity. GTPase activity was determined by the method of Pomerantz et al. (25). Briefly, the dispersed SMC were washed in PBS containing 10 µM phenylmethylsulfonyl fluoride and snap-frozen at -70°C. The SMC were resuspended in 20 mM HEPES buffer (pH 7.2) containing 2 mM MgCl2, 1 mM EDTA, 1 mM benzamidine, and 10 µg each of leupeptin and pepstatin A, lysed by passing through a 25-gauge needle, and then homogenized using a teflonized glass homogenizer. Membrane fractions were isolated by centrifugation at 75,000 g at 4°C for 60 min (Beckman L8-70M Ultracentrifuge, Beckman Instruments, Palo Alto, CA) through 40% sucrose. The pellets were rehomogenized in the HEPES buffer and reisolated by centrifugation (110,000 g at 4°C for 60 min). The final pellets were suspended in the HEPES buffer and stored at -70°C. The protein concentrations of the membrane suspensions were determined by the method of Lowry et al. (18) using BSA as the standard.
kinase, and 1,000,000 dpm of \( \gamma^{32}\text{P} \text{GTP} \) (5,000 Ci/mmol). After 10 min of incubation at 37°C, 0.8 ml of ice-cold acid charcoal solution (1 N HCl containing 10% Norit A) was added and mixed well, and the samples were centrifuged at 14,000 g for 4 min. Radioactivity was counted in 0.45 ml of supernatant plus 5 ml of scintillation liquid. To examine the effects of CTX, GDP\( \beta \)S, GDP\( \alpha \)S, or GTP\( \gamma \)S, the mixtures of membrane protein and assay buffer without \( \gamma^{32}\text{P} \text{GTP} \) were pretreated with GDP\( \beta \)S or antibodies for 10 or 60 min, respectively, and then incubated with CTX and \( \gamma^{32}\text{P} \text{GTP} \). The GTPase activity was calculated as femtomoles per minute per milligram protein and expressed as percent of control. The control values were determined in the absence of any treatment except \( \gamma^{32}\text{P} \text{GTP} \).

Drugs and chemicals. The following chemicals were used in the study: CTX (Research Biochemicals International, Natick, MA); bethanechol chloride, isoprotrenol hydrochloride, phenylephrine, sodium nitroprusside, N\( \text{O} \)-nitro-L-arginine (L-\( \text{NNA} \)), TTX, indomethacin, GDP\( \beta \)S (Sigma Chemical, St. Louis, MO); \( \alpha \)-conotoxin GVIA, (Bachem Bioscience, Torrance, CA); 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP), Rp-8-bromoadenosine 3',5'-cyclic monophosphorothioate (Rp-8-BrcAMPs), and Rp-8-bromoguanosine 3',5'-cyclic monophosphorothioate (Rp-8-BrcGMPS) (Biolog Life Sciences Institute, La Jolla, CA); EDTA tetrasodium, and EGTA (Fisher Scientific, Pittsburgh, PA).

G protein antibodies (G\( \alpha \)-Ab and G\( \alpha_{1-3} \)-Ab) raised against synthetic peptides corresponding to the amino acid sequence of the COOH-terminal of the G protein \( \alpha \)-subunits were purchased from Calbiochem-Novabiochem (San Diego, CA); collagenase type II from Worthington Biochemical (Lake-wood, NJ); amino acid solution from GIBCO Life Technologies (Grand Island, NY); benazidine and dithiothreitol from Fisher Scientific; \( \gamma^{32}\text{P} \text{GTP} \) from Amersham Life Sciences (Arlington Heights, IL).

All chemicals except indomethacin were dissolved and diluted in Krebs physiological solution and prepared fresh on the day of the experiment. Indomethacin was dissolved initially in a small volume of ethanol and then diluted in Krebs solution. Krebs solution containing the corresponding volumes of ethanol that served as a solvent for the indomethacin solvent produced no significant effect on the resting tone of the IAS.

All the glassware used for studies of the SMC were siliconized with "Sigmacote" solution to prevent the adherence of the SMC to the surface. The muscle baths were washed at least six times and the resting tension was allowed to recover to the preinjection levels.

To examine the effect of prostaglandins on the fall in the IAS tension by CTX, the smooth muscle strips were pretreated with indomethacin (3 \( \times \) 10\(^{-6}\) and 1 \( \times \) 10\(^{-5}\) M) for 30 min before testing the effect of CTX. Likewise, other inhibitors or antagonists were given at least 15 min before testing their effects on their respective selective agonists and CTX. The responses of different agents in the isolated SMC were determined as explained above in the section that deals with the measurements of changes in SMC lengths.

To examine the role of different mediators on the effect of different stimuli and agonists, the smooth muscle strips and SMC were pretreated with the respective and selective antagonists and inhibitors in the concentrations that have been previously established to be maximally effective.

Data analysis. The data were represented as means \( \pm \) SE of different experiments. The basal tone and changes in the IAS smooth muscle tension in response to different agonists, stimuli, and CTX in control vs. different inhibitor and antagonist pretreatments as the case may be, were calculated as the force in millinewtons as previously described. The maximal fall (or the passive force) in the basal tension in each smooth muscle strip was determined at the end of each experiment by the addition of excess EGTA (usually 5 mM) until there was no further fall in the basal force. The data showing fall in the basal tension with the agonists and stimuli were expressed with reference to percent maximal (E\( \text{max} \)) fall in the presence of EGTA. Percent fall in the basal tension was calculated by the ratio of decrease in force by the stimuli to the basal tone or active tone. The basal tone was calculated by subtracting the passive force from the total tension (15). The passive force was the residual force in the presence of EGTA.

The relaxation of the isolated SMC was determined by decrease in the percent maximal contraction caused by bethanechol as described before (28). Likewise, the contraction of the SMC was expressed as the percent decrease in the length of the SMC. Both of these responses were expressed as percent of maximal change as previously explained.

Statistical significance of the differences between different groups was determined by Student’s t-test. Two-way ANOVA was used for the comparison of the entire concentration or frequency response curve before and after the treatment.

**RESULTS**

Effect of CTX on basal tone of IAS. As shown in Fig. 1, CTX caused a prompt and dose-dependent fall in the basal tone of the IAS. The threshold concentration of 10 ng/ml caused 15.7 \( \pm \) 2.3% fall in the basal tension of the IAS. The maximal response of 88.6 \( \pm \) 4.0% fall was observed with 500 ng/ml of CTX in the muscle bath. The relaxation of the IAS smooth muscle in response to 10 ng/ml CTX.
ng/ml CTX began within 15.8 ± 1.5 s and peaked within 91.3 ± 8.8 s. Likewise, the latency of onset and peak of the fall in the basal tension of the IAS following 500 ng/ml were 14.3 ± 1.7 and 86.5 ± 9.1 s, respectively. Such an immediate response of CTX in the smooth muscle in comparison to its actions in other systems was new and unexpected. The relaxation caused by CTX in the IAS smooth muscle persisted for 1–2 h. The actions of CTX were fully reversible on washing of the smooth muscles. An example of the typical tracing showing the effect of different concentrations of CTX on the basal tone of the IAS is shown as an inset in Fig. 1.

Influence of nitric oxide synthase inhibitor L-NNA and neurotoxins TTX and ω-conotoxin on CTX-induced fall in basal tone of IAS. To determine the site of action of CTX in causing the relaxation of the IAS smooth muscle, first we investigated the influence of the neurotoxin TTX on the IAS smooth muscle relaxation by CTX. TTX had no significant effect on the fall in the IAS tension caused by CTX (Fig. 2). In these experiments, in control studies, 250 ng/ml of CTX produced 81.9 ± 1.8% fall in the basal tone of the IAS. In the presence of TTX, the fall in the IAS tension by CTX was 80.0 ± 2.0% (P > 0.05, n = 4). It has been shown previously that the neurally mediated relaxation of the IAS smooth muscle is mediated primarily by the nitric oxide synthase (NOS) pathway (27). There are examples where the IAS smooth muscle relaxation in response to an agonist may be neurally mediated at the nerve terminal site via the activation of NOS and is resistant to TTX (29). Such smooth muscle relaxation is sensitive to ω-conotoxin and the NOS inhibitor. To examine those possibilities for the CTX-induced IAS smooth muscle relaxation, we tested the effects of ω-conotoxin and the NOS inhibitor L-NNA on the relaxant effect of CTX in the IAS smooth muscle. Neither ω-conotoxin nor L-NNA were found to have any significant effect on the IAS smooth muscle relaxation caused by CTX (P > 0.05, n = 4; Fig. 2). In some experiments, we also examined the effect of ω-conotoxin plus L-NNA on CTX-induced fall in the basal tension of the IAS smooth muscle. The combination had no significant effect on the inhibitory effect of CTX on the basal IAS tone. In these experiments, 250 ng/ml of CTX caused 85.2 ± 3.0 and 81.3 ± 6.0% fall in the IAS tension before and after the combination of ω-conotoxin plus L-NNA, respectively (P > 0.05, n = 4). The comparison of entire dose-response curve in control vs. in the presence of ω-conotoxin plus L-NNA experiments also revealed no significant effect of the combination (data not shown). The findings suggest that the major site of the relaxant action of CTX in the IAS smooth muscle is directly at the SMC.

Influence of cyclooxygenase inhibitor indomethacin on CTX-induced fall in basal tone of IAS. Indomethacin was used as a cyclooxygenase inhibitor in concentrations that have been shown to be effective before (17). Indomethacin caused a significant and concentration-dependent suppression of the IAS smooth muscle relaxation by CTX (P < 0.05, n = 6; Fig. 3A). In control experiments, 100 ng/ml CTX caused 68.2 ± 6.2% fall in the basal tone of the IAS. The fall in the IAS tone by the same concentration of CTX in the presence of 3 × 10⁻⁶ and 1 × 10⁻⁵ M indomethacin were 48.9 ± 5.1 and 39.4 ± 5.1%, respectively. The comparison of the latencies of the onsets of the relaxant actions of CTX before and after indomethacin also provided important information. The cyclooxygenase inhibitor caused a significant delay in the inhibitory action of CTX (500 ng/ml) in the IAS, from 18.4 ± 2.0 s (observed in control experiments) to 36.0 ± 3.7 s (in the presence of indomethacin; P < 0.05, n = 6). Indomethacin in the concentrations used does not appear to have neural actions because it had no significant effect on the IAS smooth muscle relaxation by neural stimulation with EFS as shown in Fig. 3B (P > 0.05, n = 4). The data suggest that the fall in the basal tone of the IAS by CTX is via its action directly at the smooth muscle cell. However, in the IAS SMC, the inhibitory action of CTX is partly mediated via the cyclooxygenase pathway.

Effect of permeant cyclic nucleotides 8-BrcAMP on basal tone of IAS: influence of cAMP-dependent protein kinase inhibitor Rp-8-BrcAMPS on fall in basal IAS tension by CTX. It is well known that CTX in a number of systems causes an activation of Gs protein, leading to an activation of AC and an increase in intracellular cAMP (20, 26). In the IAS SMC, AC pathway has been shown to play a significant role in the relaxation of the smooth muscle (7, 14). To investigate the signal transduction involved in the CTX-induced relaxation of the IAS smooth muscle, we examined the role of AC pathway in the relaxation of the IAS smooth muscle. In this process, we examined the actions of 8-BrcAMP before and after Rp-8-BrcAMPS (cAMP-dependent protein kinase inhibitor) (1) on the basal IAS tone. Then we examined the influence of the specific G protein antibody (Gαα-Ab) on the smooth muscle relaxation by CTX.
8-BrcAMP caused a concentration-dependent fall in the basal tone of the IAS (Fig. 4A). Previous studies in our laboratory have shown that the fall in the basal tension of the IAS in response to the cyclic nucleotides is due to their direct effect on the SMC (21). The present data further show that the IAS smooth muscle relaxation by 8-BrcAMP was antagonized specifically, dose dependently, and significantly (\(P < 0.05, n = 6\)) by \(Rp\)-8-BrcAMPS. The IAS smooth muscle relaxation by 8-BrcAMP was not affected by \(Rp\)-8-BrcGMPS (\(P > 0.05, n = 6\)). The data in Fig. 4B show that \(Rp\)-8-BrcAMPS caused a significant attenuation of the CTX-induced fall in the basal tone of the IAS (\(P < 0.05, n = 6\)). The concentrations of \(Rp\)-8-BrcAMPS used have previously been shown to cause the specific blockade of the cAMP-dependent protein kinase (I).

Effect of CTX on isolated SMC of IAS: influence of \(G_{s\alpha}\)-Ab. CTX caused a concentration-dependent relaxation of the isolated SMC of the IAS in a manner similar to that in the IAS smooth muscle strips (Fig. 5). Figure 5A shows that \(1 \times 10^{-6}\) M bethanechol caused maximal contraction (\(E_{\text{max}}\) or 100%) in the intact SMC of the IAS. CTX produced a concentration-dependent relaxation of the SMC (Fig. 5B). CTX (250 ng/ml) produced 76.6 ± 1.6% relaxation of the SMC maximally contracted by bethanechol.

Because some of the experimental protocols required the use of certain agents that work inside the SMC and do not permeate the cell wall readily, the studies were performed in the permeabilized SMC as outlined in MATERIALS AND METHODS. The data in the permeabilized SMC suggest that the permeabilization procedure had no significant effect on the integrity of the SMC because

Fig. 3. A: influence of prostaglandin synthetase inhibitor indomethacin on fall in IAS tension by CTX. Data show that entire concentration-effect curve was attenuated by indomethacin in concentration-dependent manner (*\(P < 0.05, n = 6\)). Asterisks on right side of curve in this and subsequent figures suggest that entire curve was significantly different. B: indomethacin in concentrations that caused significant attenuation of fall in basal tone of IAS by CTX was found to have no significant effect on neurally mediated relaxation of IAS smooth muscle. EFS, electrical field stimulation.

Fig. 4. A: influence of cAMP-dependent protein kinase inhibitor \(Rp\)-8-bromoadenosine 3',5'-cyclic monophosphorothioate (\(Rp\)-8-BrcAMPS) on fall in IAS tension by 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP). Protein kinase inhibitor caused specific and concentration-dependent antagonism of IAS smooth muscle relaxation by 8-BrcAMP (*\(P < 0.05, n = 6\)). \(Rp\)-8-BrcAMPS on the other hand had no significant effect on the smooth muscle relaxation by 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP, not shown). B: in same concentrations, protein kinase inhibitor also caused significant blockade of IAS smooth muscle relaxation by CTX (*\(P < 0.05, n = 6\)). \(Rp\)-8-BrcGMPS, \(Rp\)-8-bromoguanosine 3',5'-cyclic monophosphorothioate.

8-BrcAMP caused a concentration-dependent fall in the basal tone of the IAS (Fig. 4A). Previous studies in our laboratory have shown that the fall in the basal tension of the IAS in response to the cyclic nucleotides is due to their direct effect on the SMC (21). The present data further show that the IAS smooth muscle relaxation by 8-BrcAMP was antagonized specifically, dose dependently, and significantly (\(P < 0.05, n = 6\)) by \(Rp\)-8-BrcAMPS. The IAS smooth muscle relaxation by 8-BrcAMP was not affected by \(Rp\)-8-BrcGMPS (\(P > 0.05, n = 6\)).
the responses to bethanechol and CTX in the permeabilized SMC were similar to that in the intact cells (Fig. 5, C and D).

Interestingly, the relaxation of the IAS SMC was specifically inhibited by Gsα-Ab as shown in Fig. 6. The relaxation of the SMC in response to CTX was not modified by normal serum and by the antibodies raised against G proteins other than Gsα (not shown). In control experiments, the percent shortening of the isolated SMC by CTX (100 ng/ml) was 52.7 ± 5.7, and in the presence of Gsα-Ab it was 78.9 ± 6.3% (P < 0.05, data from 4 animals). In these experiments, percent maximal shortening (100%) of the isolated SMC was obtained with 1 × 10^−6 M bethanechol.

Effect of CTX on GTPase activity: influence of Gsα-Ab.

To examine and compare the role of specific G protein on the CTX-induced changes in the GTPase responsible for the relaxation of the IAS SMC, we first determined the presence of GTPase activity in the IAS smooth muscle in the basal state and then the effect of different concentrations of CTX on the GTPase activity. This was followed by the determination of GTPase activity in the presence of CTX (100 ng/ml) with or without Gsα-Ab. The data show the presence of GTPase activity in the IAS and that CTX causes a concentration-dependent increase in the GTPase activity (Fig. 7A). Furthermore, the GTPase activity in the basal state and the increased GTPase activity in the presence of CTX were dose dependently inhibited by the universal G protein inhibitor GDPβS (data not shown). We then examined the influence of CTX (100 ng/ml) on the GTPase activity before and after Gsα-Ab. To determine the specificity of Gsα-Ab in inhibiting the action of CTX, we also examined the effect of Gi1-3-Ab. The data show that the
DISCUSSION

These studies for the first time show a prompt and direct relaxation of gastrointestinal smooth muscle of the IAS in response to CTX. Furthermore, the studies show that the relaxant response of CTX on the IAS smooth muscle occurs via two pathways: 1) the activation of Gs protein that is linked to the AC and 2) the activation of cyclooxygenase pathway in the SMC.

The relaxation of the IAS smooth muscle in response to CTX was found to occur within a matter of seconds. This rapid response of CTX, especially on the smooth muscle, has not been shown before. Most of the actions of CTX in different systems have been shown to take from two to several hours, and this includes changes in the gastrointestinal motility (8, 9, 13). The fastest action of CTX so far reported has been on the rabbit vascular smooth muscles of the ear artery, thoracic aorta, and saphenous vein (35). Such actions of CTX on the vascular smooth muscles occurred on the order of 10 min and were seen only in the smooth muscles that were precontracted with an agonist. These blood vessels are otherwise well known to relax immediately with agonists other than CTX. Because of the long latencies of the action, the exact mechanism of the smooth muscle relaxation by CTX might have been difficult to ascertain. It is possible that the vascular smooth muscle relaxation by CTX is the result of the interference of the toxin with the contractile agonist at the receptor level or at the intracellular level. The immediate relaxation of the IAS smooth muscle is a novel finding, and the responses were highly reproducible and concentration dependent. Moreover, the smooth muscle relaxation by CTX was also observed in the isolated SMC via the mechanisms similar to those proposed earlier for the other secretory and nonsecretory cells (20, 26).

The relaxation of the IAS smooth muscle caused by CTX was found to be by its action directly at the SMC since it was modified by neither of the neurotoxins TTX and ω-conotoxin. Furthermore, the CTX-induced relaxation of the IAS smooth muscle was independent of the NOS pathway and was not modified by the NOS inhibitor L-NNA. These inhibitors used in the same concentrations have been shown before to block the IAS smooth muscle relaxation caused by the NANC nerve stimulation (27, 29). Additionally, CTX also caused a relaxation of the SMC isolated from the IAS.

A major component of the CTX-induced relaxation of the IAS SMC seems to involve the activation of G protein linked to AC. There were several lines of evidence to that effect. The relaxation of the IAS SMC by CTX was blocked specifically by Gsα-Ab and was not affected by similar pretreatment of the cells with antibodies against other G proteins. The activation of G protein in causing the smooth muscle relaxation was also reflected by an increase in the GTPase activity following CTX pretreatment in the time frame that causes the smooth muscle relaxation.

The relaxation of the IAS smooth muscle by CTX was found to be by its action directly at the SMC since it was modified by neither of the neurotoxins TTX and ω-conotoxin. Furthermore, the CTX-induced relaxation of the IAS smooth muscle was independent of the NOS pathway and was not modified by the NOS inhibitor L-NNA. These inhibitors used in the same concentrations have been shown before to block the IAS smooth muscle relaxation caused by the NANC nerve stimulation (27, 29). Additionally, CTX also caused a relaxation of the SMC isolated from the IAS.

The studies show that the major part of the IAS smooth muscle relaxation in response to CTX occurs via the AC pathway. However, the studies suggest an additional signal transduction pathway involving cyclooxygenase in partial response to the toxin. The relaxation of the IAS smooth muscle by CTX was significantly attenuated by the cyclooxygenase inhibitor indomethacin. Similar concentrations of indomethacin were found to have no significant effect on the NANC nerve-mediated relaxation of the IAS smooth muscle. The dual pathway in the relaxation of the smooth muscle has not been shown before. However, this effect is similar to that observed in the murine...
smooth muscle-like cells (BC3H1) (24). The role of prostaglandins in the mediation of pathogenesis of secretory actions leading to diarrhea is well known (3). Whether there is an association or an overlap between these two pathways in the IAS smooth muscle remains to be determined. The present studies also did not examine the role of guanylate cyclase pathway in the CTX-mediated relaxation of the IAS smooth muscle.

The immediate and direct relaxation of the IAS smooth muscle provides significant new information and will facilitate the understanding of basic mechanisms underlying CTX. The studies also provide important insights into the mechanism of the inhibitory action of CTX in the gastrointestinal smooth muscle. The actions of CTX on the gastrointestinal smooth muscles that are not spontaneously active are not known. The relaxant actions of CTX on the IAS smooth muscle were immediate and reversible. Because of these characteristic responses, the IAS smooth muscle may serve as an important model to investigate the mechanism of actions of CTX. A considerable delay in the order of hours for the action of CTX has been reported in other cell types. In-depth studies in the IAS smooth muscle may help in defining the intracellular mechanisms for the differences in the signal transduction of CTX in different cell types. Moss and Vaughan (20) have reviewed a number of possible mechanisms for the delay in the action of CTX in different systems.

In summary, CTX exerts potent, prompt, and direct inhibitory actions on the IAS smooth muscle via the activation of G protein-linked AC and cyclooxygenase pathways. The actions of CTX on the IAS smooth muscle may provide further insights into the basic mechanisms of the pathophysiology of CTX-associated gastrointestinal motility disorders.

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