

Chronic regulation of colonic epithelial secretory function by activation of G protein-coupled receptors.

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Chronic Regulation of Colonic Epithelial Secretory Function by activation of G Protein-Coupled Receptors.

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ABSTRACT

Background: Release of neurotransmitters from secretomotor neurons in the intestinal mucosa is well known to acutely promote intestinal Cl^- and fluid secretion through activation of epithelial G protein-coupled receptors (GPCRs). In the present study we examined if GPCR activation might also have more long-term consequences for epithelial secretory function. **Methods:** Cl^- secretion was measured as changes in short-circuit current across voltage-clamped T_{84} colonic epithelial cells. Protein expression was measured by western blotting and intracellular Ca^{2+} levels by Fura-2 fluorescence. **Key Results:** Treatment of T_{84} cells with the cholinergic G_q PCR agonist, carbachol (CCh; $100\mu\text{M}$), rapidly stimulated Cl^- secretory responses. However, 24 hrs after acute treatment with CCh, responses to subsequent challenge with the agonist were attenuated by $43.1 \pm 5.5\%$ ($n = 29$; $p < 0.001$). CCh-pretreatment did not alter responses to another G_q PCR agonist, histamine, or to thapsigargin or forskolin which elevate intracellular Ca^{2+} and cAMP respectively. This chronically-acting antisecretory mechanism is not shared by neurotransmitters that act at G_s PCRs. Conditioned medium from CCh-pretreated cells mimicked the chronic antisecretory action of the agonist, suggesting the involvement of an epithelial-derived soluble factor. Acute exposure to agonist did not chronically alter surface expression of muscarinic M_3 receptors but inhibited intracellular Ca^{2+} mobilization upon subsequent agonist challenge. **Conclusions and Inferences:** These data reveal a novel, chronically-acting, antisecretory mechanism that serves to downregulate epithelial secretory responses upon repeated exposure to agonists of G_q PCRs. This mechanism involves release of a soluble factor that uncouples receptor activation from downstream prosecretory signals.

INTRODUCTION

The intestinal epithelium functions to provide a barrier against the uptake of harmful substances from the lumen into the body and to transport fluid and electrolytes to and from the intestinal contents. Fluid absorption is driven by cation absorption while secretion is driven primarily by Cl⁻ ion secretion. Absorption normally predominates, allowing conservation of the large volumes of fluid that enter the intestine each day, but secretion is also ongoing and is necessary for normal hydration of the mucosal surface and maintenance of epithelial barrier function. However, in many pathological conditions the delicate balance between absorptive and secretory processes can be disrupted leading to the clinical manifestation of diarrhea (1).

The enteric nervous system (ENS) plays a critical role in regulating intestinal secretory function (2-4). Secretomotor nerve terminals originating in the submucosal plexus are found closely opposed to the epithelium throughout the intestinal tract. Upon activation these nerves release an array of neurotransmitters capable of evoking secretory responses, most of which act by binding to cell surface G protein coupled receptors (GPCRs). Activation of these receptors in turn brings about stimulation of intracellular signalling cascades which ultimately interact with the epithelial transport machinery to induce Cl⁻ secretion. The most important receptors with respect to stimulation of Cl⁻ secretion are G_qPCRs which are linked to activation of phospholipase C, elevations in intracellular Ca²⁺ and activation of protein kinase C (PKC) and G_sPCRs which stimulate cAMP accumulation (5). In animal and cell culture models Cl⁻ secretory responses to neurotransmitters that act at G_qPCRs, for example acetylcholine, are typically rapid in onset and transient while those to G_sPCRs agonists, such as vasoactive intestinal polypeptide (VIP), are more gradual in onset and sustained. This suggests that mechanisms exist within epithelial cells

that serve to limit the extent and duration of responses to agonists of G_q PCRs and indeed a number of such antiseecretory effectors have been identified including PKC (6, 7), inositol tetrakisphosphate (8), and mitogen-activated protein kinases (9, 10). In addition, *in vivo*, specific re-uptake mechanisms and the actions of metabolising enzymes, such as acetylcholine esterase, ensure that the half life of transmitters within the neuroepithelial junction is kept short. Thus, the combined actions of intracellular and extracellular regulatory mechanisms dictate the extent and duration of epithelial secretory responses to neurotransmitters that act at G_q PCRs.

While the mechanisms underlying the immediate actions of neurotransmitters on epithelial transport have been extensively investigated over the years, to date, there have been no reported studies of long-term consequences of neurotransmitter exposure on epithelial transport function. To address this gap in our knowledge we sought to investigate if acute exposure to neurotransmitters that act at GPCRs has long-term effects on intestinal epithelial secretory function. We employed a reductionist approach in which the long-term effects of acute exposure to prototypical G_q and G_s PCR agonists on Cl^- secretion across cultured colonic epithelial cells were investigated. Our data reveal a novel, chronically acting, antiseecretory mechanism that downregulates epithelial responsiveness to repeated exposure to agonists of G_q PCRs.

MATERIALS AND METHODS:

Materials: Carbachol, forskolin, histamine, thapsigargin, phorbol myristyl acetate, vasoactive intestinal polypeptide and HRP-conjugated secondary antibodies were obtained from Sigma-Aldrich, UK. Tyrphostin AG1478, anti-TGF- α antibody and GM6001 were obtained from Calbiochem, San Diego, CA. Anti-muscarinic M₃ receptor antibody was from Santa Cruz Biotechnology, Santa Cruz, CA. All other reagents were of analytical grade and were obtained commercially.

Cell Culture: The colonic epithelial cell line, T₈₄, was cultured on petri dishes in DMEM/Hams F12 media (1:1) (Sigma-Aldrich, UK) supplemented with 5% newborn calf serum (HyClone, Logan, Utah, USA). Cells were cultured in an atmosphere of 5% CO₂ at 37°C with medium changes every 3 - 4 days. For Ussing chamber/voltage clamp studies, approximately 5 x 10⁵ cells were seeded onto 12 mm Millicell-HA Transwells (Millipore, Bedford, MA). For western blotting/biotinylation experiments, approximately 10⁶ cells were seeded onto 30 mm Millicell-HA Transwells. Cells seeded onto Millicell filters were cultured for 10 - 15 days prior to use. Under these conditions T₈₄ cells develop the polarized phenotype of native epithelial cells and are widely considered to be among the best models for reductionist studies of Cl⁻ epithelial secretion.

Electrophysiological Measurements: After culture for 10 – 15 days on filter supports, T₈₄ cell monolayers were washed in serum free medium and allowed to equilibrate for 30 min. Cells were then treated on the basolateral side with agonists at various concentrations and for various periods of time as noted in the figure legends. After agonist-treatment monolayers were mounted in Ussing

chambers (aperture = 0.6 cm²), voltage-clamped to zero potential difference, and monitored for changes in I_{sc} (ΔI_{sc}) using a VCC MC8 Voltage Clamp (Physiological Instruments, San Diego, CA). Under such conditions secretagogue-induced changes in I_{sc} across T₈₄ monolayers are wholly reflective of changes in electrogenic chloride secretion (11). I_{sc} measurements were carried out in Ringer's solution containing (in mM): 140 Na⁺, 5.2 K⁺, 1.2 Ca²⁺, 0.8 Mg²⁺, 119.8 Cl⁻, 25 HCO₃⁻, 2.4 H₂PO₄²⁻, and 10 glucose. Results were normalized and expressed as ΔI_{sc} (μA/cm²).

Western Blotting: T₈₄ cell monolayers were washed (x3) in serum free medium and allowed to equilibrate for 30 min at 37°C. Cells were then treated with agonists as noted in the figure legends after which they were washed and allowed to recover in serum-free medium. Monolayers were then washed (x2) with ice-cold PBS and lysed in ice-cold lysis buffer (500 μl) (consisting of 1% Triton-X-100, 1 mM NaVO₄, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 mM NaF, 1 mM EDTA, and 100 μg/ml phenylmethylsulfonyl fluoride in PBS). Cells were then incubated at 4°C for 45 min, scraped into microcentrifuge tubes, and centrifuged at 12,000 *rpm* for 10 min. After centrifugation, the pellet was discarded and samples were adjusted so that they contained equal amounts of protein. Samples were then mixed with 2X gel loading buffer (50 mM Tris, pH 6.8, 2% SDS, 100 mM dithiothreitol, 0.2% bromophenol blue, 20% glycerol), boiled for 3 min, and proteins separated by SDS-PAGE. Separated proteins were transferred to PVDF membrane after which the membrane was washed in 1% blocking buffer for 30 min, followed by incubation with the appropriate dilution of primary antibody in 1% blocking buffer for 60 min. This was followed by washing (x5) in Tris buffered saline with 1% tween (TBST). HRP-conjugated secondary antibodies were then added to the membrane in 1% blocking buffer, and allowed to incubate for an additional 30 min. After further washing (x5) in TBST, immunoreactive proteins were detected using an ECL

detection kit (Amersham Lifesciences, UK) and exposure of the membrane to X-ray film. Quantitation of protein phosphorylation was determined by densitometry using Scion image software.

Cell Surface Biotinylation: The protocol used was based on one previously described (7). Following treatment the cells were washed three times in ice-cold PBS. Freshly prepared biotinylation buffer (1mg/ml Sulfo-NHS-Biotin (Pierce) in PBS) was added to the basolateral side of the cells. Cells were then incubated at 4°C for 15 minutes on a rotating platform after which the buffer was removed and a replaced with a second fresh aliquot. After an additional 15 minutes incubation, the cells were washed twice with PBS and then incubated with a quenching reagent (100 mM glycine in PBS). The cells were then washed with PBS and lysed in Triton lysis buffer for 45 minutes on ice. The lysate was centrifuged at 14,000 rpm for 6 minutes and the protein concentration of the supernatant was determined and normalized. The samples were then precipitated on a rotator overnight at 4°C with 100 µl of streptavidin-agarose beads (Pierce). The beads were then washed three times in lysis buffer and 40 µl of 2X laemmli buffer (Sigma-Aldrich, UK) was added. The samples were boiled at 95°C for 5 minutes and subjected to SDS-PAGE analysis. M₃R was detected by western blotting as described above.

Intracellular Ca²⁺ Imaging: After pretreatment with CCh (100 µM; 15 min) T₈₄ cells grown on glass coverslips were allowed to recover for 24 hrs in serum free medium. The cells were then washed and loaded with 5 µM Fura-2/AM (dissolved in 0.01% Pluronic F-127 plus 0.1% DMSO in physiological salt solution (140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 10 mM D-glucose, and 10 mM HEPES-trimethylamine, pH 7.4) at room temperature for 30 min. Coverslips were then

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3 washed and mounted in a perfusion chamber on a Nikon microscope stage. Cells were perfused
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5 with normal physiological salt solution for 5 min before adding CCh (100 μ M) to the perfusing
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7 solution. The ratio of Fura-2 fluorescence with excitation at 340 or 380 nm ($F_{340/380}$) was
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9 measured every 3 s and images were captured using an intensified CCD camera (ICCD200) and a
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11 MetaFluor Imaging System (Molecular Devices Corporation, Sunnyvale, CA, USA).
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17 *Statistical Analysis:* All data are expressed as mean \pm sem for a series of n experiments. Students' t-
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19 tests were used to compare paired data. One-way analysis of variance with the Student-Neuman-
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21 Keuls post-test was used when three or more groups of data were compared. p values ≤ 0.05 were
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23 considered to be statistically significant.
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RESULTS

Acute stimulation with CCh chronically inhibits secretory responses to subsequent agonist exposure. We first investigated acute and long-term actions of CCh on epithelial secretory responses. CCh is an analog of acetylcholine that acts at G_q-coupled M₃ receptors on intestinal epithelial cells. In line with several previous studies we found that acute exposure of voltage-clamped T₈₄ cell monolayers to CCh evoked rapid onset and transient I_{sc} responses (Figure 1A). These responses have previously been characterized to be wholly due to electrogenic Cl⁻ secretion (12). We next examined if acute exposure to CCh might have more long-term consequences for epithelial secretory function. T₈₄ cell monolayers were stimulated with CCh (100 μM) for 15 min after which time they were washed and allowed to recover for 24 hrs in serum free medium. Monolayers were then mounted in Ussing chambers and I_{sc} responses to a 2nd challenge with CCh (100 μM) were measured. As shown in Figure 1A, acute pretreatment with CCh significantly downregulated I_{sc} responses to subsequent challenge with the agonist 24 hrs later. Control responses to CCh were 37.3 ± 2.5 μA/cm² compared to 19.6 ± 1.7 μA/cm² in cells that were pretreated with the agonist (n = 25; p < 0.001). The concentration-dependence for CCh-pretreatment in chronically downregulating secretory responses to subsequent agonist challenge was similar to that for its effects in acutely stimulating Cl⁻ secretion (Figure 1B) with the threshold for both effects occurring between 1 – 10 μM and maximal responses being observed for both effects at 1 mM. We next analyzed the time-course over which acute (15 min) stimulation with CCh alters subsequent secretory responses to agonist exposure. Pretreatment with CCh acutely inhibited subsequent secretory responses to the agonist when measured after 1 hr. However, within 6 hrs after initial challenge with CCh, secretory responses to a second challenge had recovered almost to control levels. This was then followed by a prolonged loss of

secretory responsiveness to subsequent CCh challenge that was maximal after 24 hrs. In all subsequent experiments pretreatment of the cells with CCh (100 μ M) was carried out for 15 min with secretory responses being measured 24 hrs later. Under these conditions, pretreatment with CCh did not alter basal I_{sc} or conductance. The basal conductance of T₈₄ cell monolayers 24 hrs after pretreatment with CCh was 1.40 ± 0.15 mS/cm² compared to 1.37 ± 0.13 mS/cm² in control monolayers. Pretreatment with CCh (100 μ M; 15 mins) did not inhibit secretory responses to either thapsigargin (TG; 2 μ M) or forskolin (FSK; 10 μ M) when measured 24 hrs later. Responses to TG and FSK in CCh-pretreated cells were 143.8 ± 32.9 μ A/cm² (n = 8) and $134.5 \pm 18.5\%$ (n = 8) μ A/cm² of those in control cells, respectively.

Chronic downregulation of secretory responses upon repeated agonist exposure is specific to G_qPCRs. We next went on to examine if the chronic downregulation of secretory responses seen upon repeated agonist stimulation is specific for muscarinic M₃ receptors or if it is phenomenon common across the GPCR superfamily. Thus, we examined acute and chronic actions of the immune cell mediator, histamine, on secretory function. Similar to CCh, we found that in cells acutely pretreated histamine (100 μ M; 15 min), subsequent secretory responses to the agonist were significantly attenuated when measured 24 hrs later. Control responses to histamine were 13.5 ± 1.3 μ A/cm² compared to 9.3 ± 1.0 μ A/cm² in cells that were pretreated with the agonist (n = 8; p < 0.001). However, in similar experiments acute exposure to vasoactive intestinal polypeptide (VIP; 100 nM; 15 min), a neurotransmitter that acts at G_sPCR receptors, did not chronically alter subsequent responses to VIP exposure (Figure 2B). Furthermore, we found that in cells acutely pretreated with CCh, subsequent responses to histamine were unaltered (Figure 2C), while in cells pretreated with histamine, subsequent responses to CCh were not affected when measured 24 hrs later (Figure 2D).

Elevations in intracellular Ca^{2+} or activation of PKC does not chronically alter epithelial secretory responses. Ligand binding to G_q PCRs is linked to activation of phospholipase C which cleaves membrane phospholipids to yield IP_3 and diacylglycerol. IP_3 then releases Ca^{2+} from intracellular stores while diacylglycerol serves to activate various isoforms of PKC. To begin to elucidate mechanisms underlying chronic downregulation of secretory responses, we examined if activation of either of these signaling pathways alone could mimic the antiseecretory effects observed upon repeated G_q PCR stimulation. Thapsigargin (TG), which inhibits the endoplasmic reticulum Ca^{2+} ATPase pump, was used to elevate intracellular Ca^{2+} and the phorbol ester, phorbol myristyl acetate (PMA) was used to activate PKC. We found that acute pretreatment with either TG (2 μ M; 30 min) or PMA (100 nM; 30 min) alone did not alter subsequent responses to CCh 24 hrs later (Figure 3). In further experiments we found that pretreatment of the cells with a general inhibitor of PKC, chelerythrine chloride (10 μ M), did not restore secretory responses after repeated exposure to CCh.

The chronic antiseecretory action of G_q PCR activation is mediated by a soluble factor but not by EGFr transactivation. We next investigated if the loss of epithelial secretory responsiveness that occurs upon repeated G_q PCR agonist exposure could be mediated by the production of an epithelial-derived soluble factor. In these experiments cells were pretreated with CCh (100 μ M) for 15 min and after allowing them to recover for 24 hrs the basolateral culture medium was collected and I_{sc} responses to a second challenge with CCh were measured in Ussing chambers. Virgin monolayers of cells were then exposed to the basolateral media collected from control and CCh-pretreated cells. After 24 hrs incubation, these conditioned medium-treated cells were also voltage clamped and I_{sc} responses to CCh were measured. As shown in figure 4A, conditioned

medium from CCh-pretreated cells was equally effective to the agonist itself in inhibiting subsequent secretory responses to CCh challenge. Since, these data suggest that an epithelial-derived soluble factor is involved we went on to investigate if ligand-dependent transactivation of the EGF might play a role in mediating the antiseecretory effects of G_q PCR activation. However, as shown in Figures 4 B - C. Neither the EGFr inhibitor, AG1478 (100 nM), the general metalloprotease inhibitor, GM6001 (10 μ M), nor a blocking antibody to TGF- α (5 μ g/ml), could restore secretory responses to CCh in monolayers that had been acutely pretreated with the agonist 24 hrs previously.

Acute stimulation with CCh does not chronically alter M_3 receptor expression but uncouples the receptor from elevations in intracellular Ca^{2+} . Finally, we investigated if altered cellular or surface expression of the M_3 receptor or if altered coupling of the receptor to downstream effectors might underlie the attenuation of secretory responses that occurs with repeated agonist exposure. Using western blotting and cell surface biotinylation we found that in cells pretreated with CCh neither total cellular nor basolateral surface expression of the M_3R was altered after 24 hrs (Figure 5A - B). As a measure of receptor coupling to prosecretory signaling pathways we analyzed intracellular Ca^{2+} levels by Fura2 fluorescence. These experiments revealed that, similar to its effects on Cl^- secretion, acute pretreatment of the cells with CCh chronically attenuated their capacity to mobilize intracellular Ca^{2+} upon subsequent agonist exposure (Figure 5C).

DISCUSSION

Upon activation the enteric nervous system releases an array of neurotransmitters that exert their effects through activation of GPCRs on the basolateral side of the epithelial cells. Extensive research has been carried to characterize the roles that these receptors play in regulating epithelial functions, including cell growth and cytokine synthesis (13-15). Neurotransmitters that act at either G_q or G_sPCRs have also been well-documented to rapidly induce transpeithelial Cl⁻ secretion, an effect that *in vivo* causes enhanced fluid secretion into the intestinal lumen (5). Despite their well-established roles in acutely promoting intestinal secretory function, potential long-term consequences of neurotransmitters that act at GPCRs on epithelial secretory function are unknown. In the current study we have begun to address this gap in our knowledge and present data to show that acute exposure to GPCR agonists chronically inhibits epithelial secretory responses to subsequent agonist exposure. Furthermore, these effects are specific for agonists that act at the G_q class of GPCR.

Due to the presence of specific reuptake mechanisms and metabolic enzymes, released neurotransmitters do not persist in the neuroepithelial junction *in vivo* and their contact time with epithelial cells is likely to be brief. With this in mind, we adopted the approach of investigating the long-term consequences of relatively acute agonist exposure on epithelial secretory function. We found that acute treatment of colonic epithelia with CCh, a stable analog of the most prominent intestinal neurotransmitter, acetylcholine, induced a time-dependent biphasic decrease in responsiveness to subsequent agonist exposure. An early attenuation of secretory responses occurred up to 3 hrs after initial agonist exposure. Such early inhibitory effects have been previously described and are due to depletion of intracellular second messengers and activation of

antisecretory mechanisms, such as transport protein internalization and the generation of inositol tetrakisphosphate (7, 8, 16). However, within 6 hrs these relatively short-term antisecretory signals had abated as evidenced by the observation that responses to a second challenge with CCh were comparable to those in control cells. Intriguingly, at time points after 6 hrs we observed a second, slow onset, and sustained loss of epithelial responsiveness to a second challenge with agonist. This chronic antisecretory effect was not due to a loss of cell viability since responses to thapsigargin and forskolin, which are receptor independent activators of the Ca^{2+} and cAMP-dependent secretory pathways respectively, were not inhibited. Similarly, basal electrical parameters of epithelial monolayers were not chronically altered in cells pretreated with CCh.

The generation of chronically acting antisecretory signals after G_qPCR activation appears to be highly specific. Similar to our observations with CCh, an M_3R agonist, pretreatment of T_{84} cells with an immune cell-derived G_qPCR agonist, histamine, also attenuated subsequent responses to histamine challenge. In contrast, pretreatment with a neurotransmitter that acts at G_sPCRs , VIP, did not chronically alter responses to subsequent challenge with this agonist. Thus, the attenuation of epithelial secretory responsiveness that occurs upon repeated agonist exposure appears to be specific for those that act at the G_qPCR subclass. Interestingly, pretreatment of epithelial monolayers with histamine did not chronically alter responses to CCh, or *vice versa*, suggesting that this antisecretory mechanism exerts its actions in a receptor specific manner and that heterologous receptor desensitization, which can be a feature of GPCR activation in some systems (17, 18), does not occur. Thus, on the basis of these data one would expect that *in vivo*, release of acetylcholine from secretomotor neurons would render the epithelium chronically refractory to subsequent cholinergic stimulation, whereas responses to other G_qPCR agonists, such as histamine released from mast cells, would remain unaltered.

Agonists that act at G_qPCRs classically initiate cellular signaling through activation of phospholipase C and generation of IP₃ and DAG from membrane phospholipids. These messengers then go on to bring about elevations in intracellular Ca²⁺ and activation of PKC, respectively. Since PKC has been shown to be important in other systems in mediating receptor desensitization to GPCR agonists (19-21), we examined if activation of either of these pathways alone could induce long-term attenuation of epithelial secretory capacity. For these experiments we used thapsigargin and PMA, which act in a receptor-independent manner to elevate intracellular Ca²⁺ and PKC, respectively. Although both agonists were used under conditions we have previously shown to effectively elevate intracellular Ca²⁺ and to activate PKC (9, 22), neither altered subsequent secretory responses to CCh. Furthermore, a general inhibitor of PKC, chelerythrine chloride, did not prevent the attenuation of responses that occurs upon repeated CCh exposure. These data suggest that neither depletion of Ca²⁺ stores, elevation of cytosolic Ca²⁺ or activation of PKC alone are sufficient signals to induce a chronic hyposecretory phenotype.

Our previous studies have identified a G_qPCR-induced antiseecretory signaling mechanism that is mediated by EGFr and MAPK activation and that serves to acutely downregulate the extent of Ca²⁺-dependent secretory responses (9, 23). Transactivation of the EGFr is mediated by Src-induced activation of a metalloprotease which cleaves TGF- α from the membrane with the growth factor then acting in an autocrine fashion to activate the EGFr. We hypothesized that this pathway might also be involved in the more long-term antisecretory action that occurs upon repeated agonist exposure. Thus, to determine if an epithelial-derived soluble factor is involved,

we analyzed secretory responses to CCh in cells treated with conditioned medium collected from monolayers that were acutely treated with the agonist. Interestingly, prolonged exposure to conditioned medium from CCh-pretreated cells was as effective as the agonist itself in attenuating the subsequent responses to CCh. Thus, our data support the idea that an epithelial-derived soluble factor is involved. To determine if this factor acts through EGFr activation we used a range of inhibitors that we have previously shown to effectively inhibit EGFr transactivation in response to CCh (9, 23). However, in these experiments we found that neither the EGFr inhibitor, tyrphostin AG1478, the metalloprotease inhibitor, GM6001, nor a blocking antibody to TGF- α could reverse the attenuation of epithelial secretory responsiveness that occurs upon repeated agonist exposure. Thus, although colonic epithelial cells have the capability to produce soluble EGFr ligands, such as TGF- α , in response to G_qPCR agonists (23, 24), our data suggest that the epithelial-derived factor that chronically inhibits responses to repeated agonist challenge acts independently of the EGFr. Experiments are currently underway to determine the identity of the soluble factor involved.

Prolonged exposure to agonists of G_qPCRs typically leads to their desensitization through receptor internalization and degradation (25-27). However, previous studies have not addressed whether acute stimulation of G_qPCRs in epithelial cells might also lead to a prolonged loss of receptor expression, thereby leading to an attenuation of agonist-induced responses. Here we found that acute pretreatment of epithelial monolayers with CCh did not chronically alter either total cellular or surface expression of the muscarinic M₃R. This suggests that, in contrast to the alterations in receptor trafficking and expression that occur with prolonged GPCR exposure, acute exposure to G_qPCRs agonists does not chronically alter receptor expression in intestinal

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3 epithelia. They also suggest that the loss of secretory responsiveness that occurs upon repeated
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5 agonist exposure is not due to a loss of cell surface receptor expression but rather to an
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7 uncoupling of receptors from downstream prosecretory signaling pathways. This hypothesis is
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9 supported by our observations that in cells acutely pretreated with CCh, mobilization of
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11 intracellular Ca^{2+} in response to subsequent agonist challenge was significantly attenuated. This
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13 inhibition of Ca^{2+} release is not due to depletion of internal stores since secretory responses to
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15 other Ca^{2+} -dependent agonists, such as TG and histamine, are not altered by prior treatment with
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17 CCh. Thus, our data suggest that the chronically acting antiseecretory mechanism impinges on
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19 G_qPCR -induced prosecretory pathways at a point distal to agonist/receptor binding but proximal
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21 to release of Ca^{2+} from stores.
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29 In summary, we have identified a novel, chronically acting, antiseecretory mechanism that limits
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31 the capacity of epithelial cells to evoke secretory responses upon repeated exposure to
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33 neurotransmitters that act at G_q , but not G_sPCRs . This mechanism is not due to receptor
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35 downregulation or sequestration but is mediated by an epithelial-derived soluble factor that
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37 uncouples receptor activation from downstream prosecretory signals. The
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39 physiological/pathophysiological significance of our findings is unclear and raises several
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41 interesting questions. Perhaps, most importantly why do epithelial cells respond in such a
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43 complex manner to agonists of G_qPCRs ? At this point we can only speculate on the answer to this
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45 question but it seems likely that *in vivo* such a chronically-acting antiseecretory mechanism would
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47 serve to limit epithelial secretory capacity therefore preventing the onset of diarrhea in conditions
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49 where enteric neuronal activity is enhanced. Indeed, previous studies *in vivo* support this
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51 hypothesis since colonic tissues from mice with IBD are hyporesponsive to cholinergic
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53 stimulation without alterations in M_3R expression (28-30). In future studies we aim to more fully
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investigate the molecular mechanisms involved in chronic downregulation of epithelial responsiveness by G_qPCR agonists with the hope that this will yield a greater understanding of the mechanisms by which enteric neurotransmitters regulate intestinal fluid transport in health and disease.

For Peer Review

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The contributions of each author to the paper were as follows:

Ferial Toumi – Carried out research, designed experiments analyzed data, prepared manuscript

Michelle Frankson – Carried out research.

Magdalena Mroz – Carried out research and analyzed data

Orlaith Kelly – Carried out research and analyzed data

Joseph Ward – Carried out research and analyzed data

Lone Bertelsen – Carried out research and analyzed data

Stephen Keely – Carried out research, designed experiments, analyzed data, prepared manuscript

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ABBREVIATIONS

CCh; carbachol, EGFr, epidermal growth factor receptor, ENS; enteric nervous system, FSK; forskolin; GPCR; G protein-coupled receptor, PKC; protein kinase C, TG; thapsigargin, VIP; vasoactive intestinal polypeptide, TGF- α ; transforming growth factor- α

FIGURE LEGENDS

Figure 1: Acute exposure to CCh chronically inhibits secretory responses to subsequent agonist exposure. **A)** Cells were acutely treated with basolateral CCh (100 μ M for 15 mins) and were then washed and allowed to recover in serum free medium for 24 hrs. Cells were then mounted in Ussing chambers and Cl^- secretory responses to a second challenge with CCh (100 μ M) were measured as changes in I_{sc} (n = 25). **B)** Cells were acutely exposed to different concentrations of CCh (1 μ M - 1 mM; 15 mins) and Cl^- secretory responses to a second challenge with CCh (100 μ M) were measured 24 hrs later (n = 3). The concentration-dependence of the chronic antisecretory effect of CCh is plotted alongside that of its acute prosecretory actions for comparison (n = 3). **C)** Cells were acutely treated with CCh (100 μ M; 15 min) and were then allowed to recover for various periods (1 - 24 hrs) in serum free medium before Cl^- secretory responses to a second challenge with CCh were measured (n = 3 - 8 for each time point; * p < 0.05, ***p < 0.001 compared to control cells).

Figure 2: Chronic downregulation of secretory responses upon repeated agonist exposure is specific to G_q PCRs. **A)** Monolayers of T_{84} cells were pretreated with histamine (100 μ M) for 15 mins, washed and allowed to recover for 24 hrs before Cl^- secretory responses to a second challenge with histamine (100 μ M) were measured (n = 8). **B)** T_{84} cells were pretreated with VIP (100 nM) for 15 mins and after recovery for 24 hrs Cl^- secretory responses to a second challenge with VIP (100 nM) were measured (n = 3). **C and D)** T_{84} cell monolayers were acutely pretreated with histamine or CCh (100 μ M; 15 min) and after recovery in serum free medium for 24 hrs, I_{sc} responses to either **C)** CCh (100 μ M; n = 6) or **D)** histamine (100 μ M; n = 6) were measured in

Ussing chambers. Asterisks denote significant differences from control cells; * $p < 0.05$, *** $p < 0.001$.

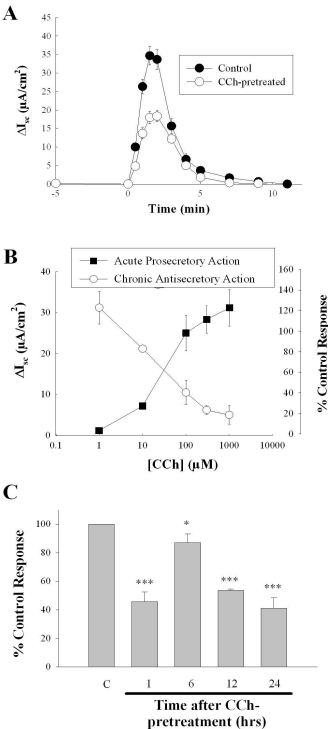
Figure 3: Elevations in intracellular Ca^{2+} or activation of PKC does not chronically alter secretory responses to CCh. Monolayers of T₈₄ cells were acutely pretreated with either CCh (100 μM ; 15 min), thapsigargin (2 μM ; 30 min), PMA (100 nM; 30 min) or a combination of TG and PMA. After washing and recovery for 24 hrs in serum free medium, cell monolayers were mounted in Ussing chambers and secretory responses to CCh (100 μM) were measured ($n = 3$). **B)** Cells were pretreated with CCh (100 μM ; 15 mins) either in the absence or presence of the general PKC inhibitor, chelerythrine chloride (10 μM), or vehicle for controls. Cells were then washed and maintained in chelerythrine chloride for 24 hrs before secretory responses to a second challenge with CCh (100 μM) were measured in Ussing chambers ($n = 5$). Asterisks denote significant differences from control cells; ** $p < 0.01$.

Figure 4: The chronic antiseecretory effects of G_qPCR activation are mediated by a soluble factor but not by EGFr transactivation. **A)** Monolayers of T₈₄ cells were treated with CCh (100 μM ; 15 min), washed, and after 24 hrs Cl^- secretory responses were measured as changes in I_{sc} in Ussing chambers. Naive monolayers of cells were then treated for 24 hrs basolaterally with conditioned medium from the basolateral side of control and CCh-pretreated cells ($n = 6$). **B - D)** T₈₄ cell monolayers were pretreated with CCh (100 μM ; 15 min) in the absence or presence of **B)** an EGFr inhibitor, tyrphostin AG1478 (100 nM; $n = 5$), **C)** a blocking antibody to TGF- α (5 mg/ml; $n = 3$) or **D)** a general inhibitor of metalloproteases, GM 6001 (10 μM ; $n = 4$). After treatment with CCh, cells were maintained in the presence of inhibitors alone, or vehicle, for 24

hrs before I_{sc} responses to a second challenge with CCh were measured in Ussing chambers. Data are expressed as % of vehicle- or inhibitor-treated controls as appropriate. Asterisks denote significant differences from control cells that were not pretreated with CCh; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

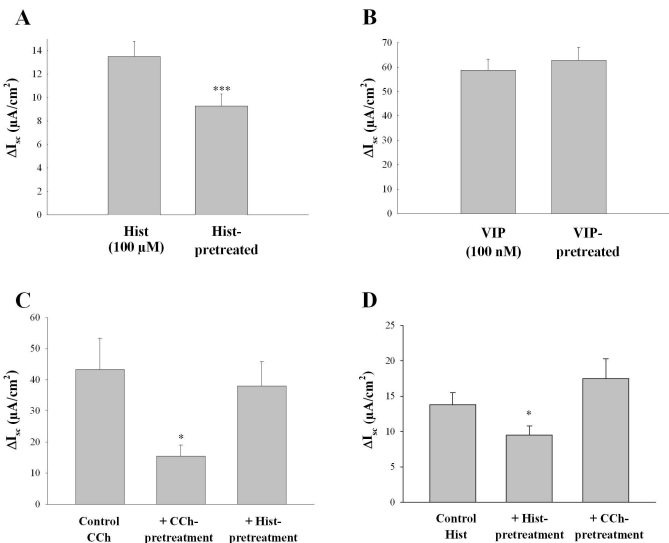
Figure 5: Acute stimulation with CCh does not chronically alter M_3 receptor expression but uncouples the receptor from elevations in intracellular Ca^{2+} . A) Monolayers of T_{84} cells were pretreated with CCh (100 μM) for 15 mins and 24 hrs later cells were lysed and cellular M_3 receptor expression was analyzed by western blotting. Actin expression was measured as a loading control (n = 6). B) Cells were pretreated with CCh as described above and M_3 receptor surface expression was analyzed by basolateral cell surface biotinylation (n = 3). C) T_{84} cells grown on glass coverslips were pretreated with CCh (100 μM) for 15 mins and after 24 hrs CCh-induced increases in cytosolic Ca^{2+} were measured by Fura-2 fluorescence. Data are expressed as mean fluorescence ratio at 340 and 380 nM (n = 6 coverslips for each condition). The inset shows the net change in mean fluorescence ratio ($\Delta F_{340/380}$) for these experiments. Asterisks denote significant differences from control cells that were not pretreated with CCh; * $p < 0.05$.

Figure 1



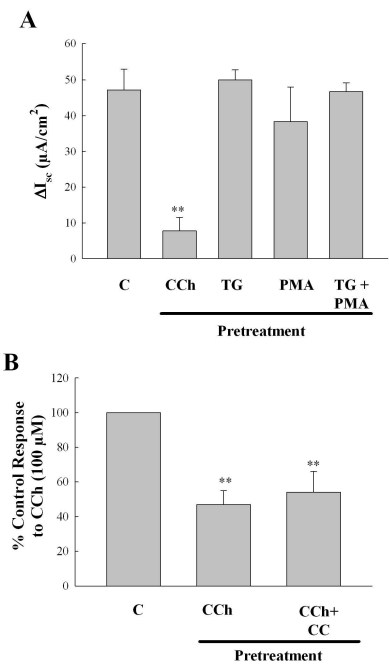
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Figure 2



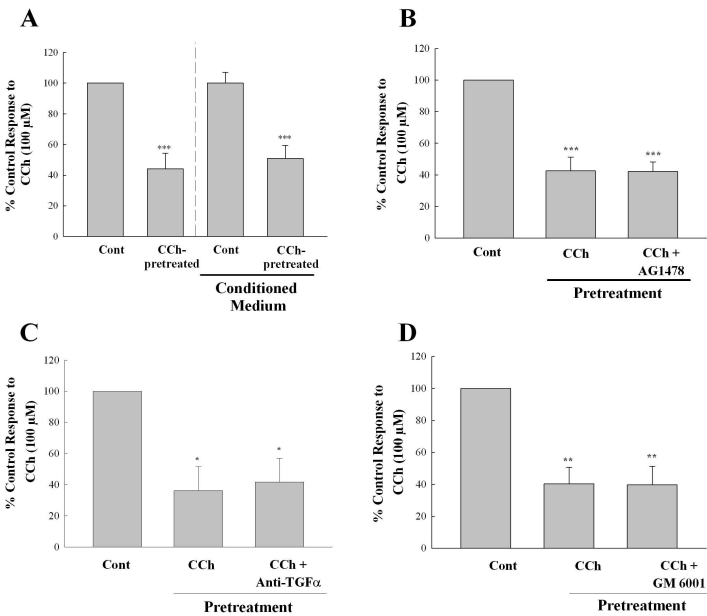
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Figure 3



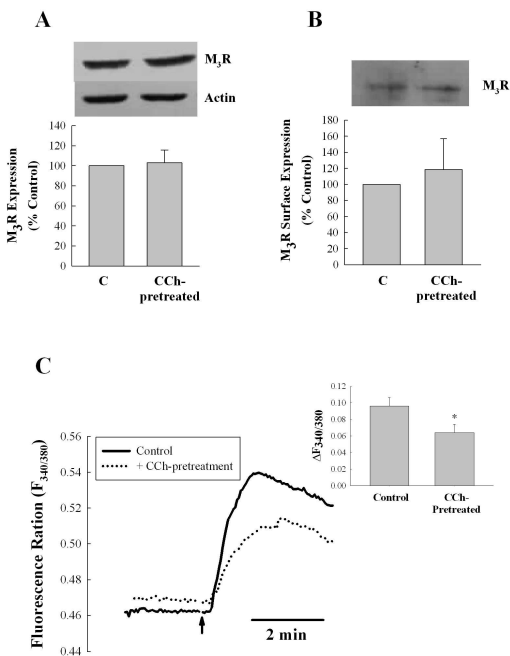
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Figure 4



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Figure 5



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