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Short communication

Muscarinic cholinoceptor-mediated activation of JNK negatively regulates intestinal secretion in mice

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ABSTRACT

Regulation of intestinal secretion is important for body fluid homeostasis. We investigated the role of three MAP kinases (MAPKs) as negative regulators in muscarinic cholinoceptor (mAChR)-mediated intestinal secretion in mice. Electrophysiological analyses revealed that mAChR stimulation enhanced intestinal chloride secretion, which was further augmented by the inhibition of JNK but not by that of ERK or p38 with specific inhibitors SP600125, U0126 or SB203580, respectively. Immunoblot analyses in colonic mucosa showed that mAChR stimulation increased MAPKs phosphorylation that was suppressed by the specific inhibitor for each MAPK. This suggests that JNK is a major negative regulator in mAChR-induced intestinal secretion.

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One of the primary functions of the intestinal epithelium is to maintain the fluid and electrolyte balance by regulating absorption-secretion pathways. Intestinal fluid transport is driven by active ion transport with absorption by cations and secretion predominantly by chloride (Cl^-) ions. Acetylcholine (ACh) is a central molecule for the regulation of these epithelial functions. Although ACh is regarded as a classical neurotransmitter, numerous studies report the synthesis and release of ACh from non-neuronal epithelial cells (1–3), where it exerts its auto/paracrine effects via the stimulation of nicotinic or muscarinic cholinoceptors (mAChRs). In a previous study, we reported the expression of mAChRs in mouse intestinal epithelial cells which are involved in the regulation of MAP kinase (MAPK) signaling (4). Three members of MAPK family, ERK (5), JNK (6) and p38 (7), are reported to be responsible for the negative regulation of intestinal secretion, in a cell culture system. Thus in the present study, we aim to explore the contribution of each MAPK for the negative regulation of mAChR-mediated intestinal secretion in a conventional Ussing chamber system.

The experiments were reviewed by the ethics committee for animal experiments in compliance with the ethical guidelines of Asahikawa Medical University. Male BALB/c mice between 9 and 10 weeks of age were used. Compounds were purchased from commercial sources as follows: atropine sulfate, mecamylamine, tetrodotoxin and U0126 (U0) (Wako Pure Chemical Industries Ltd., Osaka, Japan); acetylcholine chloride (Daiichi Sankyo Co. Ltd., Tokyo, Japan); forskolin (Sigma–Aldrich, St. Louis, USA); SB203580 (SB), SP600125 (SP), all primary antibodies and HRP-labeled secondary antibody were purchased from Cell Signaling Technology Inc. (Massachusetts, USA).

In order to investigate the mAChRs-mediated MAPKs signaling, mouse mucosal fragments were used as a sample because the purified crypt epithelial cells underwent apoptosis as soon as the temperature was shifted to 25 °C (8), The mucosal fragments were scraped away from the membrane of a mouse colon as described in a previous report (4). The fragments were stimulated by ACh (100 μM) for 3 min with or without the pretreatment of inhibitors

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at 25 °C under the presence of a neuronal blocker, tetrodotoxin (1 μM) and a nicotinic AChR antagonist, mecamylamine (10 μM). The reaction was terminated by adding a SDS sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, 1% β-mercapto ethanol, and 0.1% bromophenol blue in the final concentration) and heated for 3 min at 100 °C. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was probed with an appropriate primary antibody. The immunoreactive proteins were detected by horseradish-peroxidase-labeled secondary antibody with Amersham ECL Select Western Blotting Detection Kit (GE healthcare, Buckinghamshire, UK). The ratio of intensities of signals was quantified by densitometry.

For the electrophysiological study, the mucosal-submucosal preparation as a sheet from each mouse (middle-to-distal colon) was separated as described in a previous report (4) and mounted in Ussing chambers that provided an exposed area of 0.2 cm^2. The volume of the bathing solution on each side was 5 ml, and the solution temperature was maintained at 37 °C in a water-jacketed reservoir. The bathing solution was composed of NaCl, 119 mM; $NaHCO_3$, 21 mM; K_2HPO_4, 2.4 mM; KH_2PO_4, 0.6 mM; $CaCl_2$, 1.2 mM; $MgCl_2$, 1.2 mM; and glucose 10 mM, which was bubbled with a mixture of 95% O_2 and 5% CO_2 gas. The active ion transport as a short-circuit current (I_{sc}) across the epithelium was measured by using an automatic voltage-clamping device (CEZ 9100; Nihon Kohden, Tokyo, Japan). After a 30 min equilibration period, the baseline I_{sc} was recorded. Tissues were then challenged with ACh (100 μM) under the presence of a neuronal blocker, tetrodotoxin (1 μM) and a nicotinic AChR antagonist, mecamylamine (10 μM). The response to ACh was recorded as the maximum change in I_{sc} to occur within 10 min of the treatment. At the end of each experiment, all tissues were challenged with forskolin (10 μM) to test for viability and to ensure that the tissue had been mounted in the correct orientation in the Ussing chamber. Data were analyzed using PRISM software (Version 5.01, Graph Pad Software, La Jolla, USA). In immunoblots, the signal intensity was calculated using Image J software. Statistical significance was evaluated using Student's *t*-test and was considered to be significant when *p* values were less than 0.05. Data were represented as the mean ± SEM.

Stimulation of mucosal fragments with ACh resulted in significant increases in phosphorylation of ERK, JNK and p38 (Fig. 1). These increases in phosphorylation were completely inhibited by the addition of atropine (10 μM) prior to the stimulation, suggesting that the ACh-induced phosphorylation of MAPKs is elicited by mAChRs. We employed mecamylamine and tetrodotoxin in all sample tubes to avoid the possible involvement of nicotinic AChRs and neuronal components.

We tested the effect of selective inhibitors of MAPKs upon ACh-induced phosphorylation. We used U0, SP and SB as a selective inhibitor for ERK, JNK and p38, respectively. Pretreatment of mucosal fragments with the selective inhibitor (1–30 μM), canceled the mAChR-mediated phosphorylation of the respective MAPKs in a concentration-dependent manner as shown in Fig. 2. Based on our analyses we also assumed that each MAPK inhibitor is specific to the respective MAPK in the concentration range we employed.

Next, we examined the ACh-induced electrophysiological response of colonic epithelial cells in the Ussing chamber. After the base line I_{sc} was established, tissues were challenged with ACh (100 μM) under the presence of mecamylamine and tetrodotoxin in the serosal side. The transient increase in I_{sc} confirmed the viability and proper setting of the mucosal fragment in the Ussing chamber. After washing the tissues by changing the buffer solution several times, tissues were again challenged with ACh under the presence of mecamylamine and tetrodotoxin and the transient increase of I_{sc} was recorded. Tissues were washed again and a third challenge was performed with ACh with or without pretreatment with various MAPK inhibitors (U0, SP, or SB). The change of I_{sc} in the third ACh challenge was normalized with that of the second challenge as 100%. The magnitudes of I_{sc} change in the third challenge were 108.6 ± 3.9 (control), 111.4 ± 13.0 (SP 3 μM), 131.4 ± 9.6 (SP 10 μM), 194.5 ± 19.3 (SP 30 μM), 118.6 ± 14.2 (U0 30 μM) and 106.3 ± 10.2% (SB 30 μM) (Fig. 3A), showing that SP significantly enhanced the ACh-induced Cl^- secretion in a concentration-dependent manner. However, U0 and SB, even at a high concentration (30 μM), did not enhance the ACh-induced Cl^- secretion, suggesting that mAChR-mediated JNK signaling is the main driver for the negative

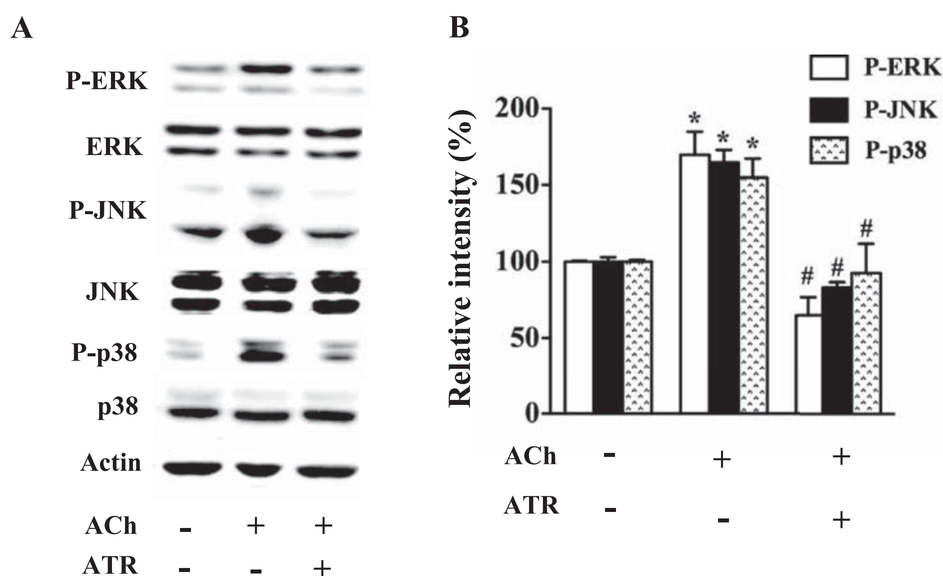

Fig. 1. Stimulation of mAChRs phosphorylates MAPKs in mouse mucosal fragments. (A) The representative immunoblots of MAPKs activation are shown. Mouse mucosal fragments were stimulated for 3 min by none (−), by 100 μM ACh (+), and by plus 10 μM atropine (+ATR). The tissues were dissolved, separated in SDS-PAGE, blotted and probed with anti-phospho-ERK1/2 (P-ERK) and anti-ERK1/2 (ERK) antibodies. The ratio of intensities of P-ERK and ERK was quantified by densitometry and was normalized to that without stimulation as 100%. Other MAPKs, JNK and p38 were also analyzed as above. (B) There was a significant increase in the phosphorylation of MAPKs by ACh (*$p < 0.05$), which was completely inhibited by ATR. Data were obtained from five independent experiments.

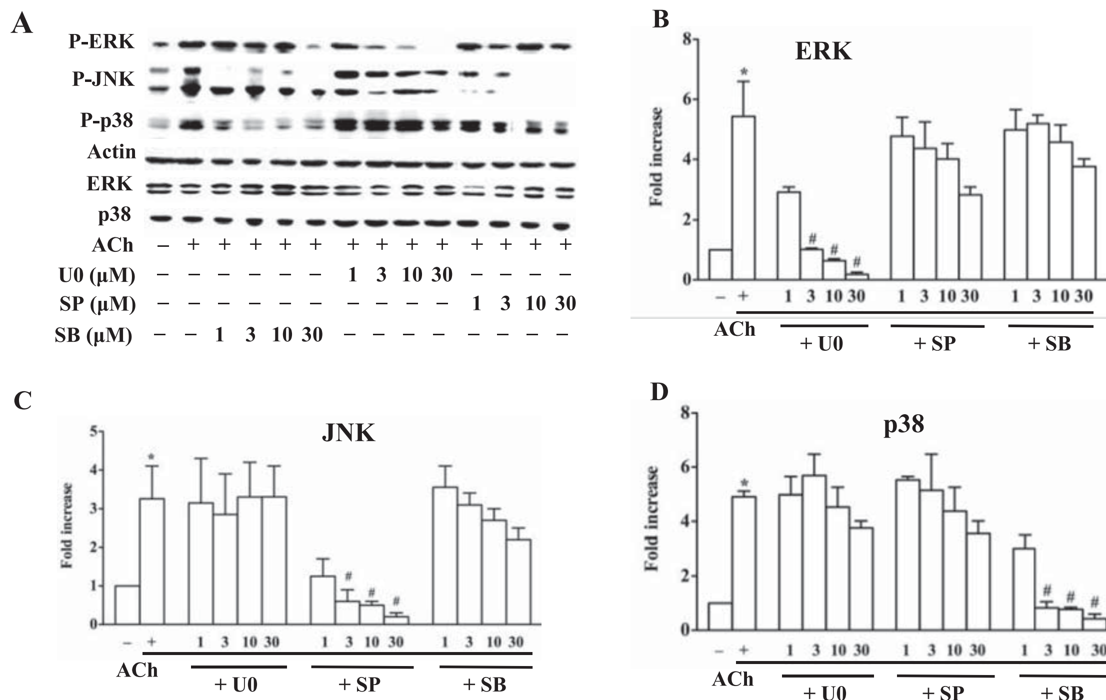

Fig. 2. Inhibition of MAPKs by specific kinase inhibitor. (A) Mucosal fragments were stimulated by ACh as described in Fig. 1 under the presence of the specific MAPK inhibitor, U0 (ERK), SP (JNK) or SB (p38) respectively at 1–30 μM concentration. (B–D) Each inhibitor suppressed the ACh-induced phosphorylation of the respective MAPK in a concentration-dependent manner without interfering each other in the concentration range we employed. Data were obtained from three independent experiments.

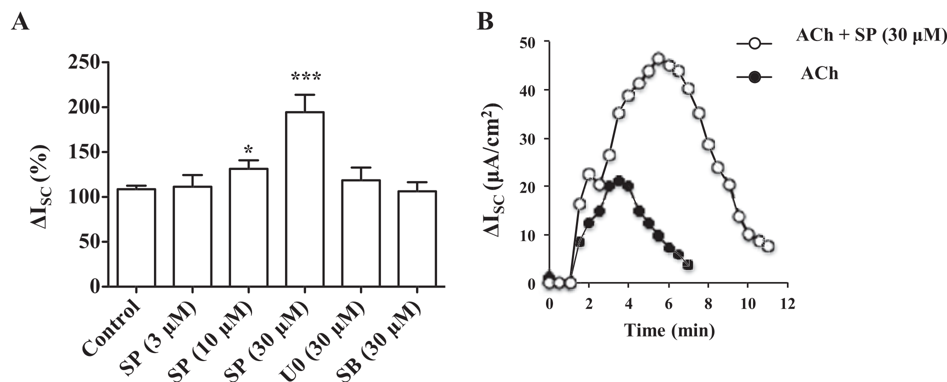

Fig. 3. Changes in I_{sc} in colonic segments after challenges with ACh (100 μM) with or without the pretreatment of various MAPK inhibitors. (A) The pretreatment with JNK inhibitor, SP (10–30 μM) significantly ($p < 0.05$) enhanced the ACh-induced I_{sc}. The change of I_{sc} in the third ACh challenge was normalized with that of the second challenge as 100%. (B) The representative recording of ACh-induced ΔI_{sc} with the pretreatment of SP (30 μM) (*white circle*) or without (*black circle*). Data were obtained from at least five independent experiments.

regulation of Cl^- secretion in mouse intestinal epithelial cells. The representative recording of ACh-induced Cl^- secretion under the presence of SP (30 μM) is shown in Fig. 3B.

Intestinal epithelial cells maintain body fluid as well as electrolytes homeostasis by regulating the balance of absorption and secretion (2). Numerous reports have established that cholinergic stimulation of mAChRs enhances the secretory functions of the colonic epithelium (9,10). However, in order to maintain homeostasis there must be antisecretory signaling along with secretory signaling. Barrett has proposed that there is a negative signaling pathway in the downstream of mAChR, in which ERK or p38 (11,12) is the responsible signaling molecule, uncoupling an agonist-stimulated increase in intracellular calcium from the following response of Cl^- secretion. Donnellan et al. also demonstrated that secretagogues-induced activation of JNK limits the Ca^{2+}-dependent Cl^- secretion in T84 human intestinal cells (6). Our data showed that inhibition of mAChR-mediated activation of JNK by the pharmacological inhibitor SP, but not that of ERK by U0 or that of p38 by SB, has significantly enhanced the ACh-induced Cl^- secretion in mouse intestinal epithelium. It is, thus, possible to speculate that JNK as a major signaling molecule in the MAPK family negatively regulates cholinergic intestinal secretion. Since receptor-mediated activation of MAP kinases is a complicated mechanism (13), further studies are required to elucidate the regulation of intestinal secretion by mAChR via MAP kinases.

In conclusion, stimulation of mAChRs in mouse intestinal epithelial cells regulates ERK, JNK and p38 MAPKs phosphorylation in which JNK signaling negatively regulates the secretagogue-induced Cl^- secretion, presumably to optimize intestinal fluid secretion.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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