The Inhibitory Effect of Eupatilin on the Intestinal Contraction Induced by Carbachol

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Abstract — This study was conducted to determine whether treatment with the anti-inflammatory eupatilin influences intestinal smooth muscle contraction stimulated by carbachol and, if so, to investigate the related mechanism. Denuded ileal or colonic muscles from Sprague-Dawley rats were used for the study and measurements of isometric contractions were obtained using a computerized data acquisition system; this data was also combined with results from molecular experiments. Eupatilin from Artemisia asiatica Nakai significantly decreased carbachol-induced contractions in both ileal and colonic muscles. Interestingly, eupatilin decreased carbachol-induced phosphorylation of ERK1/2 more significantly than that of MYPT1 at Thr855 in ileal and colonic muscles. However, eupatilin significantly decreased phosphorylation of MYPT1 at Thr855, but only in ileal muscle. Therefore, thin filament regulation, including MEK inactivation and related phospho-ERK1/2 decrease, is mainly involved in the eupatilin-induced decrease of intestinal contraction induced by carbachol. In conclusion, this study provides the evidence and a possible related mechanism concerning the inhibitory effect of the flavonoid as an antispasmodic on the agonist-induced contractions in rat ileum and colonic muscles.

Keywords: Colon, ERK, Eupatilin, Ileum, MYPT1, Smooth muscle

INTRODUCTION

The coordinated contractions and relaxations of intestinal smooth muscles produce the motor activities of gastrointestinal tracts and are important functions for the optimal transport and digestion of the ingested foods. Regulation of intestinal motility is mediated by several different pathways including extrinsic autonomic nerves, intrinsic neurons and intestinal hormones, in addition to the central nerve system (Mizuta et al., 2006). These regulatory mechanisms can be classified according to their main activities such as stimulation and inhibition of intestinal motility. The excitatory neurotransmitters specifically interact with their neuronal receptors, and ligand-receptor interactions cause depolarization of cellular membranes and activation of voltage-dependent Ca²⁺ channels, resulting in contractions of intestinal smooth muscles (Caulfield and Birdsall, 1998; Murthy, 2006). Acetylcholine- and serotonin-mediated signaling pathways are well known as excitatory stimulatory mechanisms for intestinal motility. Among five different subtypes of acetylcholinergic muscarinic receptors, the M₂ and M₃ receptors are predominantly distributed in the smooth muscles throughout gastrointestinal tracts and primarily induce the excitation of gastrointestinal smooth muscles by acetylcholine (Ehler, 2003; Unno et al., 2005). However, the stable acetylcholine analogue carbamoylcholine (carbachol) is commonly used in experiments, since acetylcholine itself is unstable.

The smooth muscle contractile system is basically regulated by myosin light chain (MLC) phosphorylation, which is driven by the balance between MLCK activity and myosin phosphatase activity. During receptor agonist-induced contractions, contractile elements are sensitized to Ca²⁺ and induce a greater MLC phosphorylation and greater force at a given cytosolic Ca²⁺ level (Somlyo and Somlyo, 2000; Pfitzer, 2001), referred to as Ca²⁺ sensitization. Recent studies have shown that a small GTP-binding protein, RhoA, and RhoA-dependent coiled-coil serine/threo-
nine kinases play major roles in Ca\(^{2+}\) sensitization. The activated RhoA activates the Rho-kinase, which in turn phosphorylates a noncatalytic subunit of myosin phospha-
tase (MYPT1), inactivating the myosin phosphatase activity. PKC is also activated by receptor stimulation. The activated PKC phosphorylates MEK, which in turn phos-
phorylates ERK1/2, attenuating the effect of caldesmon.

**MATERIALS AND METHODS**

**Tissue preparation**

Male Sprague-Dawley rats, weighing 320-350 g, were acclimated to the laboratory environment for several days before entering the study. During the experimental period, animals were housed in standard plastic cages with wood chip bedding and were kept in temperature-controlled (22 ± 2°C), relative humidity-controlled (55 ± 15%), and 12-h light/dark cycle-controlled rooms with free access to food and water. Animals were anesthetized using sodium pen-
tobarbital (50 mg/kg i.p., supplemented if required) fol-
lowed by cervical dislocation, in agreement with proce-
dures approved by the Institutional Animal Care and Use Committee. The intestine, including ileum and colon, was quickly removed and immersed in oxygenated (85% \(O_2\)/
5% \(CO_2\)) physiological saline solution composed of (mM): 118.0 NaCl, 4.8 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 24.0 NaHC\(_2\)O\(_3\), 1.2 KH\(_2\)PO\(_4\) and 11.0 dextrose (pH 7.4). The intestine was then cleaned of all adherent connective tissue, and the mucosa was removed by gentle abrasion with a cell scraper.

**Contraction measurements**

Intestinal ileal or colonic circular strips (8 mm wide) were prepared, suspended in a water-jacketed organ bath (10 ml) maintained at 37°C, and aerated with a mixture of 95% \(O_2\) and 5% \(CO_2\). The strips were attached to a force trans-
ducer and passively stretched by applying an optimal rest-
tension of 1.0 g, which was maintained throughout the ex-
periment. Strips were allowed to equilibrate at 37°C for at least 1 h and then challenged with a depolarizing sol-
ution containing 72 mM KCl. Muscle strips were then
washed and allowed to equilibrate for another 1 h before
beginning the experiment. To study pretreatment effects,
eupatilin was applied 30 min before the addition of 10 \(\mu\)M
carbachol.

**Western blot analysis**

Muscle strips were quick frozen by immersion in a
dry-ice/acetone slurry containing 10% trichloroacetic acid
(TCA) and 10 mM dithiothreitol (DTT). Muscles were also
washed in an acetone/DTT mixture and stored at −80°C until use. Samples were brought to room temperature and
homogenized in a buffer containing 20 mM mops, 4% SDS, 10% glycerol, 10 mM DTT, 20 mM \(\beta\)-glycerophos-
phate, 5.5 \(\mu\)M leupeptin, 5.5 \(\mu\)M pepstatin, 20 KIU aproti-
nin, 2 mM Na\(_2\)VO\(_4\), 1 mM NaF, 100 \(\mu\)M ZnCl\(_2\), 20 \(\mu\)M
4-(2-aminopheny) benzenesulphonyl fluoride (AEBSF) and
5 mM EGTA. Protein-matched samples (modified Lowry
protein assay, DC Protein Assay Kit, Bio-Rad) were elec-
trophoresed on SDS-PAGE (Protogel, National Diagno-
tics), transferred to PVDF membranes and subjected to
immunostaining and densitometry using the appropriate
antibody. The success of protein matching was confirmed
by Naphthol Blue Black staining of the membrane and
densitometry of the actin band. Any mismatch of lane loading was
corrected by normalization to actin staining. Each set of
samples from an individual experiment was run on the
same gel and densitometry was performed on the same
film. The densitometry was performed using KODAK Mole-
cular Imaging Software, Version 4.0 (Woodbridge, CT,
USA) and corrected by normalization to background inten-
sity.