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Review

Ionic Conductance(s) in Response to Post-junctional Potentials

Sang Don Koh[1] and Poong-Lyul Rhee[2]*

[1]Department of Physiology and Cell Biology, University of Nevada, School of Medicine, Reno, NV, USA; and [2]Division of Gastroenterology, Department of Medicine, Sungkyunkwan University School of Medicine, Samsung Medical Center, Seoul, Korea

The gastrointestinal motility is regulated by extrinsic and intrinsic neural regulation. Intrinsic neural pathways are controlled by sensory input, inter-neuronal relay and motor output. Enteric motor neurons release many transmitters which affect post-junctional responses. Post-junctional responses can be excitatory and inhibitory depending on neurotransmitters. Excitatory neurotransmitters induce depolarization and contraction. In contrast, inhibitory neurotransmitters hyperpolarize and relaxe the gastrointestinal smooth muscle. Smooth muscle syncytium is composed of smooth muscle cells, interstitial cells of Cajal and platelet-derived growth factor receptor α-positive (PDGFRα$^+$) cells (SIP syncytium). Specific expression of receptors and ion channels in these cells can be affected by neurotransmitters. In recent years, molecular reporter expression techniques are able to study the properties of ion channels and receptors in isolated specialized cells. In this review, we will discuss the mechanisms of ion channels to interpret the post-junctional responses in the gastrointestinal smooth muscles.
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Key Words
Gastrointestinal motility; Ionic conductance; Post-junctional potentials

Introduction

Stimulation of enteric motor neuron releases many neurotransmitters and neuropeptides. To evoke post-junctional electrical responses, many ion channels in smooth muscle cells (SMCs) or specialized cells (e.g., interstitial cells of Cajal [ICC] and platelet-derived growth factor receptor α-positive [PDGFRα$^+$] cells) can be activated.[1,2] Post-junctional responses can be categorized by 2 components: excitatory junction potentials (EJPs) and inhibitory junction potentials (IJPs). EJPs are mediated by acetylcholine (ACh) and neurokinins (NKs). Muscarinic receptors respond to ACh released from cholinergic neurons. Muscarinic receptors (M2 and M3) are expressed in the gastrointestinal (GI) smooth muscle. Three neurokinins (NKs) are substance P, neurokinin A and neurokinin B. These NKs are mediated by activation of neurokinin receptors (NK1-3). IJPs are mediated by purines, nitric oxide (NO), vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP). Purines bind to purinergic receptors, in particular P2Y receptors. NO directly activates soluble guanylate cyclase. VIP and PACAP act through VPAC1 and VPAC2. Besides NO, most

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*Correspondence: Poong-Lyul Rhee, MD
Division of Gastroenterology, Department of Medicine, Sungkyunkwan University School of Medicine, Samsung Medical Center, 81, Irwon-ro, Gangnam-gu, Seoul 135-710, Korea
Tel: +82-2-3410-3409, Fax: +82-2-3410-6983, E-mail: plrhee@skku.edu
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neurotransmitters or neuropeptides are coupled to G-proteins. These G protein coupled receptors have a unique relationship with specific G-proteins and thus activate ion channels in unique ways. Recently the post-junctional responses are focused on the roles of intermediary cells between neurons and SMCs. These cells are ICC and PDGFRα^+ cells.[1,2] Thus, in this review, we will discuss the ion channel candidates with cell-specific roles which can be modulated by neurotransmitters or neuropeptides.

Cholinergic Excitatory Response

ACh is the major excitatory neurotransmitter[3] and plays a primary role in increasing the contractile force in GI motility. Cholinergic excitatory responses are mediated by 2 types of muscarinic receptors (M2 and M3).[4] M2 receptors are highly expressed in SMC. M2 receptors act via $G_{i/o}$ proteins which decrease the production of cAMP. ICC expresses mainly M3 receptors.[5,6] M3 receptors are coupled to $G_{q/11}$ which activates phospholipase C (PLC) and its downstream signaling pathways. Activation of PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol and inositol-1,3,4-triphosphate (Ins-1,4,5-IP_3).[7,8] The PLC blocker U-73122 and the anti-$G_{q/11}$ antibody inhibit muscarinic activation of non-selective cation currents (mI_{CAT}) in murine gastric myocytes.[9] The effects of inhibiting PLC on mI_{CAT} were found to be independent of triphosphage (IP_3), diacylglycerol or Ca^{2+} store depletion in guinea pig ileal myocytes[10] and in murine gastric myocytes.[11] One interpretation of this finding is that activation of PLC is coupled to M2 receptors by β dimers released from $G_{i/o}$ proteins.[12,13] However, there is no direct evidence of $G_{i/o}$-mediated regulation of mI_{CAT} in GI smooth muscle to date. In studies of the IP_3 mediated pathway, flash photolysis of "caged" IP_3 augmented mI_{CAT} in guinea-pig ileal cells suggesting that IP_3 receptor-mediated release plays a central role in modulation of mI_{CAT}.[14] Intracellular Ca^{2+} has been shown to facilitate mI_{CAT} in certain species.[15,16] Interestingly, the inhibitory effect of Ca^{2+}-dependent PKC on mI_{CAT} suggests that endogenous stimulation of PKC by ACh might be responsible for desensitization of mI_{CAT}.[17]

The Rho-kinase (RhoK) pathway is a major signaling cascade that controls GI smooth muscle contraction. Recently there have been many reports about the importance of this pathway in GI muscle.[18-21] The initiating step in this pathway is the small GTPase, RhoA that is activated by receptors coupled to $G_{12/13}$. M3 receptors also couple through $G_{12/13}$ and G_q/G_{11} can also rapidly activate RhoA.[22] In the active GTP-bound state, RhoA associates with its main downstream effector, RhoK and inhibits myosin light-chain phosphatase (Fig. 1), thus increasing the phosphorylation state of myosin and the contractile responses to intracellular Ca^{2+} $[Ca^{2+}]_i$. However, it is important to note the non-specificity of RhoK inhibitors. Studies of GI muscle have neglected the fact that RhoK may also be coupled to membrane excitability mechanisms. In addition recent studies have indicated that the RhoA signaling modulates a growing number of ion channels.[23-25] RhoK has also been suggested to affect Ca^{2+} influx through inhibition of non-selective cation channels (NSCC).[26,27] It is worthwhile to note that the pharmacology of native NSCC is complicated and there are no specific blockers for these channels. Therefore, studying the role of NSCC in tissue experiments is still problematic.

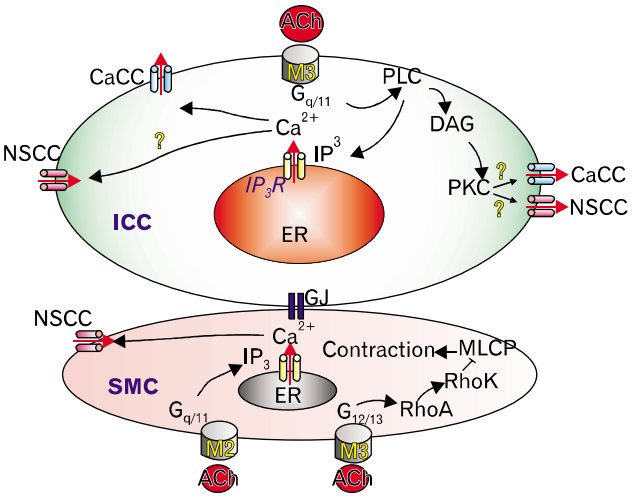

Figure 1. Possible post-junctional mechanisms responsible for cholinergic excitation. Acetylcholine (ACh) is coupled to $G_{q/11}$ protein and activates conductance(s) through inositol 1,4,5-triphosphate receptor (IP_3R) in interstitial cells of Cajal (ICC) and smooth muscle cells (SMC). ACh might also be coupled to $G_{12/13}$ protein and activate Rho-Kinase (RhoK) pathway to induce contraction in SMC. ER, endoplasmic reticulum; PLC, phospholipase C; DAG, diacyl glycerol; PKC, protein kinase C; CaCC, Ca^{2+}-activated Cl^- channels; NSCC, non-selective cation channels; GJ, gap junction; MLCP, myosine light chain phosphatase.

NSCC is important conductance in understanding the fundamental excitatory pathway in GI SMC, but evidence to date suggests that cholinergic activation of these channels is unlikely to occur to any great extent in vivo. In *W/W*^{v} murine fundus which ICCs were ablated, the EJP was abolished suggesting that cholinergic activation of the gut appears to occur primarily through activation of M3 receptors in ICC.[28,29] Recently, many

studies reported that ICC uniquely express the *Ano1* (*Tmem16a*) transcript and protein.[30-32] *Ano1* is a molecular candidate for Ca^{2+}-activated Cl^- channels (CaCC) which could be another candidate conductance in response to ACh (Fig. 1). Activation of M3 receptors by ACh in ICC increases intracellular Ca^{2+} through the PLC-downstream pathway. Thus, an increase in Ca^{2+} can activate Cl^- conductance. However, this hypothesis has not been carefully studied. Interestingly, mice which express copGFP constitutively only in ICC displayed functional expression of ANO1 in small intestinal smooth muscle.[31,32] Using isolated ICC cells from these mice, the characterization of activated currents by muscarinic agonists will be important to interpret the ionic conductance responsible for EJP. Another strong approach will be generation of *Ano1* knockout (KO) mice. Unfortunately, the conventional *Ano1* KO mouse dies within 20 days after birth. It is necessary to generate an inducible *Ano1* KO mouse to elucidate the functional role of CaCC in ICC in response to EJP.

Peptidergic Excitatory Response

It has been suggested that high frequency stimulation of electrical field stimulation (EFS) (> 10 Hz) releases neuropeptides. NKs and tachykinins are the candidates for excitatory peptides. Substance P binds to neurokinin 1 (NK1) receptors, neurokinin A (NKA) binds to neurokinin 2 (NK2) receptors and neurokinin B (NKB) binds to neurokinin 3 (NK3) receptors.[33] Activation of these receptors induces activation of PLC and produces IP_3. Thus, we speculate that the functional role of NKs is not much different from ACh. Activation of these receptors induces depolarization and contraction. The distribution of NK receptors is interesting. The NK1 receptor is mainly expressed in ICC and NK2 receptors are expressed in SMC.[34,35] Application of NKA and substance P in canine colonic SMC activates NSCC similar to mI_{CAT}.[36] In tissue experiments, *W/W*^{v} and *Ws/Ws* fundus revealed that substance P-mediated excitation with the marked spontaneous phasic contraction was augmented compared to wild type. These data suggest that the absence of ICC would give the musculature unmasked access to substance P since fundic ICC are innervated by dominantly inhibitory neurotransmitter (e.g., NO). Although there is no report about the effects of NKs on ICC conductance, it will be worthwhile to characterize the ionic conductance activated by NKs in comparison with the ionic conductance in SMC. It might be possible to activate CaCC through the PLC-downstream pathway with an increase in intracellular Ca^{2+} by NKs in ICC.

Purinergic Inhibitory Response

EFS evoked a EJP followed by a fast hyperpolarization (fast IJP) in GI smooth muscle. The phenomenon resulted from activation of P2Y receptors by purines (mainly ATP or β-NAD).[37-41] There are eight identified human P2Y receptors: $P2Y_{1,2,4,6,11,12,13,14}$.[42] The $P2Y_1$-$P2Y_{11}$ receptors are coupled via $G_{q/11}$ and $P2Y_{12}$-$P2Y_{14}$ receptors are coupled via $G_{i/o}$.[42] Recent evidence showed that P2Y1 receptor has the most prominent role in fast IJP. MRS2500, a specific blocker for the P2Y1 receptor, completely abolished fast IJP.[37-40] Furthermore, *P2ry1* KO mice showed the absence of fast IJP.[39,40] P2Y1 receptors are coupled to $G_{q/11}$ and activate PLC downstream signaling. An increase in IP_3 production and in turn, release of intracellular Ca^{2+} from IP_3 Ca^{2+} store may be the key component. Ca^{2+}-dependent K^+ conductance(s) is the main candidate to generate hyperpolarization. Apamin, a blocker of small-conductance Ca^{2+}-activated K^+ (SK) channels, inhibits partially the fast IJP.[41,43,44] Thus, activation of SK channels coupled to P2Y1 receptor could be one of the main

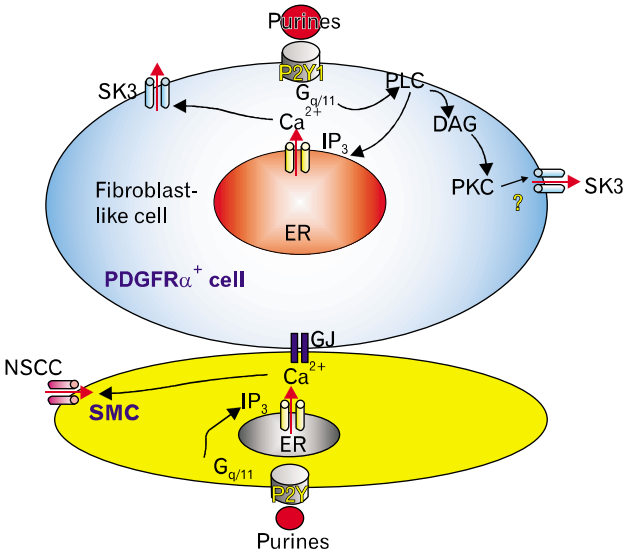

Figure 2. Possible post-junctional mechanisms responsible for purinergic inhibition. Purines (ATP and β-NAD) are coupled to $G_{q/11}$ protein and activate conductance(s) through inositol 1,4,5-triphosphate receptor (IP_3R) in platelet-derived growth factor receptor α-positive cells (PDGFRα$^+$ cell) and smooth muscle cells (SMC). ER, endoplasmic reticulum; PLC, phospholipase C; DAG, diacyl glycerol; PKC, protein kinase C; SK3, small-conductance Ca^{2+}-activated K^+ channels type 3; NSCC, non-selective cation channels; GJ, gap junction.

responses to generate fast IJP. It is important to discuss the specialized cell in response to fast IJP. Previously, the purinergic inhibitory response was regarded to result from the activation of SK channel in SMC.[45,46] However, recently the fibroblast-like cells were identified as PDGFRα immunoreactive positive cell, confirmed by using transgenic mice which expressed eGFP in nuclei (PDGFRα$^+$ cell).[47-49] PDGFRα$^+$ cell under patch clamp displayed a large outward current which was inhibited by apamin.[49] The current density of PDGFRα$^+$ cells is much higher than in SMC. Thus, there is strong possibility that fast IJP responses evoked by purines are mediated through P2Y1 receptor and SK channels in PDGFRα$^+$ cells (Fig. 2). This hypothesis still needs to be confirmed with inducible *Pdgfrα* KO mice since conventional *Pdgfrα* KO mice are not viable.

Nitrergic Inhibitory Response

Enteric nitric oxide synthase (NOS) containing inhibitory neurons releases NO.[50] NO induced slow hyperpolarization (slow IJP) and relaxed GI smooth muscle by neural stimulation.[51-55] NO activates soluble guanylate cyclase, produces 3',5'-guanosine cyclic monophosphate (cGMP), and activates protein kinase G (PKG). NOS inhibitors (e.g., L-NNA) and soluble guanylate cyclase inhibitors (e.g., ODQ) abolish slow IJP.[56] Firstly, slow IJP could be due to activation of K^+ conductance. Functional presence of stretch-dependent K^+ (SDK) channels has been reported in colonic myocytes.[57,58] SDK channels are activated by NO, a membrane-permeable analogue of cGMP and PKG. L-methionine and its derivatives inhibit SDK channels and decrease the evoked slow IJP.[59] TREK-1 channel has been found to be a molecular candidate for native SDK channels in murine colonic myocytes.[60] TREK-1 channel has a similar single channel conductance and regulatory properties including the effects of NO and membrane permeable analogue of cGMP. Secondly, slow IJP could also be due to inhibition of inward conductance. There are reports that slow IJP, particularly in esophageal smooth muscle, is due to inhibition of CaCC in tissue experiments.[61,62] As is known, CaCC blockers are notorious by non-specificity. The inhibition of CaCC in SMC by NO has not been reported to date. Thus, the candidate of ionic conductance for slow IJP is still controversial. It is important to note that the NO component of IJP (sIJP) was abolished in ICC ablated mice (*W/W^v* and *Sl/Sl^d*) and rat (*Ws/Ws*).[63-65] Recently, an ICC-specific deletion of PKG decreased the slow IJP[66] (Fig. 3). These data suggest that the slow IJP may be evoked by PKG activation

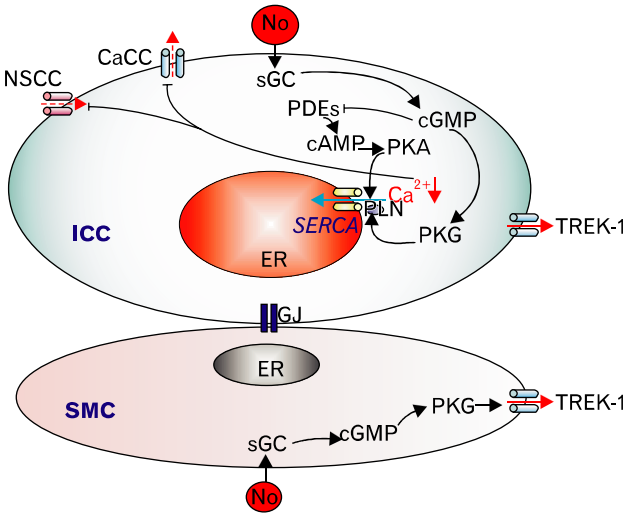

Figure 3. Possible post-junctional mechanisms responsible for nitrergic inhibition. Nitric oxide (NO) directly activates soluble guanylate cyclase (sGC) and activates conductance(s) through various possible mechanisms in interstitial cells of Cajal (ICC) and smooth muscle cells (SMC). ER, endoplasmic reticulum; cGMP, 3',5'-guanosine cyclic monophosphate; PKG, protein kinase G; PDEs, phosphodiesterases; cAMP, 3'5'-adensosine cyclic monophosphate; PKA, protein kinase A. SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; CaCC, Ca^{2+}-activated Cl^- channels; NSCC, non-selective cation channels.

and may not be due to the activation of ion channels in SMC but in ICC. We need to consider that phosphodiesterase 3a is highly expressed in ICC.[6] This enzyme is inhibited by cGMP. Inhibition of phosphodiesterase 3a can increase the concentration of cAMP and activity of protein kinase A (PKA). PKA including PKG might involve the phosphorylation of phospholamban in sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in ICC, and in turn Ca^{2+} influx into the endoplasmic reticulum might be augmented (Fig. 3). In addition to activation of K^+ conductance by PKG, it is possible to inhibit Ca^{2+}-activated inward conductance in ICC during NO release. Thus, it will be very important to investigate ionic conductance(s) evoked by NO and its intracellular signaling mechanisms in freshly dispersed ICC.

Peptidergic Inhibitory Response

VIP and PACAP are known to be enteric inhibitory peptides.[67] These peptides induce hyperpolarization and relaxation of the GI smooth muscle.[68] In rat colonic smooth muscle, the VIP antagonist (VIP10-28) blocked the inhibitory response elicited by EFS.[69] Two VIP receptors, VPAC1 and VPAC2 are activated by both peptides. VPAC2 is predominantly expressed in the GI

tract.[68,70] This receptor is coupled to Gs and increases the production of cAMP. VIP activates delayed rectifying K^+ currents (K_{DR}) via PKA activation in SMC.[71] However, K_{DR} currents are voltage-dependent and thus have a threshold for activation (~40 mV). The activation of K_{DR} currents cannot undergo further hyperpolarization from the resting membrane potentials. In contrast, PACAP induced-hyperpolarization was inhibited by apamin suggesting that activation of SK channel may be mediated through VPAC1 receptors which are coupled to $G_{q/11}$.[67,69] However there is no clear study of ion channels regarding how VIP and PACAP can induce hyperpolarization. Also, no study has shown what types of cells (ICC or PDGFRα^+ cell) are mediated by peptidergic inhibitory responses.

In conclusion, it is not clear what types of receptors, ion channels and cells stimulated by enteric motor neurons are involved in post-junctional responses. Studies with animal models (e.g., *W/W*^{v}, *Ws/Ws* and *Sl/Sl*^{d} etc) suggested that specialized cells are involved in post-junctional responses. For instance, ICC are coupled to SMC through gap junction. PDGFRα^+ cells have a similar electrical coupling to SMC. Thus it is possible that neurotransmitters and possibly peptides can bind to the receptors in these specialized cells, generate electrical events and conduct these electrical events to the SMC. Three types of cells (SMC, ICC and PDGFRα^+ cell) can be candidates in response to neurotransmitters and neuropeptides. Many studies in tissue experiments have relied on pharmacology. Many receptor antagonists and some ion channel blockers are non-specific. This non-specificity can be solved by direct investigation of functional expression of ion channels in these cell types. Since there was only a limited approaches to isolate and separate the specialized cells (e.g., ICC and PDGFRα^+ cell), the characterization of ionic conductance(s) in SMCs has been studied extensively. Recent transgenic approaches make it possible to identify ICC and PDGFRα^+ cells. Characterization of the ion channels in these cells activated by neurotransmitters and neuropeptides will elucidate new concepts of electrophysiology of GI smooth muscle. Finally we have to consider the difference of electrical responses in the human GI smooth muscle. Although many transgenic animals will be generated and developed for the future, studies on ionic conductance activated by transmitters or peptides using human smooth muscle should be emphasized.

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