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Progress in understanding of inhibitory purinergic neuromuscular transmission in the gut

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Abstract

Recent studies with genetic deletion of P2Y1 receptor (P2Y1−/−) have clinched its role in enteric purinergic inhibitory neurotransmission and suggested that β-NAD may be the purinergic inhibitory neurotransmitter in the colon. In this issue of the Journal, Gil and colleagues extend their earlier observations to the cecum and gastric antrum, showing that P2Y1 receptor mediated purinergic inhibition may be a general phenomenon in the gut. However, the authors made an unexpected observation in contrast with their earlier findings in the colon that neither the selective P2Y1 receptor antagonist MRS2500, nor P2Y1 receptor deletion, blocked the hyperpolarizing action of β-NAD in the cecum. These observations suggest that β-NAD may be the purinergic inhibitory neurotransmitter in the colon, but not in the cecum. This group had previously reported that the selective P2Y1 receptor antagonist MRS 2179 suppressed the hyperpolarizing action of ATP or ADP. Further studies are now needed to determine whether the hyperpolarizing actions of ATP and ADP are suppressed by the more potent P2Y1 antagonist MRS2500, and in P2Y1−/− mutants to test the intriguing possibility that different purines serve as purinergic inhibitory neurotransmitters in the colon and cecum and perhaps in different parts of the gut. Studies in P2Y1−/− mice will resolve other issues in purinergic neurotransmission including cellular localization of the β-NAD or ATP-activated P2Y1 receptors on either smooth muscle cells or PDGFRα+ fibroblast-like cells, relationship of purinergic to nitrergic neurotransmission and understanding the physiological and clinical importance of purinergic transmission in gastrointestinal motility and its disorders.

Keywords

ATP; exocytosis from nerve terminals; myosin Va; P2Y1 receptor; P2Y1−/− mice; PDGFRα+ fibroblast-like cell; SLC17A9; β-NAD

Studies in the 1960s showed that nerve-mediated inhibition (membrane hyperpolarization or mechanical relaxation) of the intestinal smooth muscle was not blocked by

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DISCLOSURE

None.

either cholinergic or adrenergic antagonists and the inhibitory transmitter was termed nonadrenergic noncholinergic (NANC) neurotransmitter (reviewed in Burnstock 2008).[1] In 1970, Burnstock *et al.* reported that stimulation of vagal non-adrenergic inhibitory nerves in a variety of intestinal preparations released adenosine triphosphate (ATP).[2] This study also confirmed that ATP causes inhibition of the smooth muscles, and suggested that ATP may be the NANC inhibitory neurotransmitter (Burnstock *et al.* 1970).[2] ATP-induced hyperpolarization was suppressed by ATP tachyphylaxis, apamin (slow conductance potassium (SK) channel blocker) and the dye reactive blue.[3] The treatments that blocked the effect of exogenous ATP in gastrointestinal smooth muscle strips also blocked the nerve-mediated hyperpolarization (inhibitory junction potential, IJP). Later, it was shown that in the presence of apamin, another IJP was revealed and the IJP was distinguished into two components: a prominent, fast and an inconspicuous, slow component. The fast IJP (fIJP) was shown to be mediated by ATP, whereas the slow IJP (sIJP) was mediated by VIP and nitric oxide.[4] Therefore, the fIJP and the sIJP are also called the purinergic IJP and the nitrergic IJP, respectively. These observations strengthened the case for ATP as one of the inhibitory neurotransmitters. However, unequivocal support for the candidacy of ATP as a NANC agent required evidence of the cognate receptor and demonstration that the nerve-mediated purinergic IJP was absent in tissues lacking P2Y1 receptor and was blocked by highly selective antagonists of P2Y1 receptors.

A major advance in the field occurred with cloning of the P2Y1 purinergic receptor[5,6] and development of a selective agonist, {[[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS2365)} and antagonists, {2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS2179), (1*R**,2*S**)-4-[2-Chloro-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester diammonium salt (MRS2279) and (1R, 2S, 4S, 5S)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy) bicycle [3.1.0] hexane-1-methanol dihydrogen phosphate ester tetra ammonium salt (MRS2500)}.[7] These selective antagonists of P2Y1 receptors have potency order as: MRS2500 > MRS 2279 > MRS2179).[8] In 2006, Gallego and colleagues reported that MRS2179 treatment suppresses the purinergic fast IJP.[9] These observations provided strong support for the P2Y1 receptor as mediator of the purinergic IJP in the gut.

In 1999, Fabre *et al.* generated mice lacking P2Y1 receptors by engineered deletion of P2Y1 receptors (P2Y1−/− mice).[10] Use of $P2Y1^{-/-}$ mice clinched the involvement of P2Y1 receptor in purinergic inhibition in the gut.[11,12] Two different groups (Barcelona group in Spain and Reno group in Nevada, USA) showed that the purinergic fIJP was lost in the colon of P2Y1 receptor knockout ($P2Y1^{-/-}$) mice.[11,12] The accompanying report by the Barcelona group (Gil *et al.*, 2013) further shows that purinergic inhibitory responses are also lost in gastric antrum and cecum in these knockout mice, suggesting that P2Y1 receptors may be mediators of the purinergic inhibition in other parts of the gut.[13] There is now general agreement that P2Y1 receptor is the mediator of purinergic inhibitory neurotransmission in the gut.[14]

Although the receptor responsible for the purinergic inhibitory neurotransmission in the gut is now established with reasonable certainty, several important issues in purinergic

neurotransmission remain unresolved. Firstly, there is uncertainty regarding the cellular localization of the purinergic receptor that mediates the IJP. It has been suggested that the purinergic receptor mediating the purinergic IJP is localized on the PDGFRα fibroblast-like cells that may transduce purinergic IJP to the smooth muscle and that a different set of purinergic receptors are localized to the smooth muscles.[15,16] This view is supported by the observations that the purinergic agonists cause apamin sensitive outward currents in the isolated PDGFRα fibroblast-like cells.[15] However, ATP-mediated apamin sensitive K current has also been reported in isolated smooth muscles[17] and P2Y1 receptors have been localized to the smooth muscles with immunohistochemical techniques.[9] Cell-specific P2Y1R$^{-/-}$ knockout may be valuable in defining cellular location of the P2Y1 receptors on PDGFα-fibroblast-like cells or the smooth muscle that mediates the purinergic IJP.

Another fundamental issue is whether β-NAD and ATP are purinergic neurotransmitters in different parts of the gut.[18] In the accompanying report, Gil and colleagues addressed this issue and found that in the cecum, β-NAD-mediated hyperpolarization was not suppressed by either MRS2500 or in P2Y1$^{-/-}$ mutant.[13] These observations do not support the proposal that β-NAD acts via P2Y1 receptor and that β-NAD is the inhibitory purinergic neurotransmitter in the cecum. Contrasting reports from studies in colonic tissues show that hyperpolarization by β-NAD or ADPR was abolished by the selective P2Y1 receptor antagonist MSR2500, as well as in P2Y1$^{-/-}$ mice.[11,12,16] The reason for these opposing results is not clear. It may indicate either the unlikely possibility that different purines serve as inhibitory purinergic transmitters in the colon and the cecum or that there may be some technical reason for these confusing results. In this regard, it is of interest to note that the enteric ganglionic cellular network in the colon including the cecum is derived from the same embryonic source.[19] This makes it unlikely that the colon and cecum will have different purine neurotransmitters.

As an alternative to β-NAD, the effects of ATP and ADP on P2Y1 receptors have been examined, but results with ATP have also been contradictory. It was reported that the hyperpolarizing effect of ATP or ADP was not suppressed by the selective P2Y1 receptor antagonist MRS2500 or in P2Y1$^{-/-}$ mutant.[12,16] On the other hand, Gil and colleagues have reported that the hyperpolarizing action of ADP is suppressed by the P2Y1 receptor antagonist MRS2179.[8,9] Other studies have also shown that ATP causes P2Y1 receptor mediated hyperpolarization that is blocked by selective P2Y1 receptor antagonist MRS 2179.[20] The reasons for the contrasting results of antagonism of the selective P2Y1 receptor antagonists on β-NAD/ADPR and ATP/ADP are not clear.

An understanding of the nature of the purine localized to the prejunctional nerve varicosities and identification of the purine released from the neurosecretory vesicles may help define the chemical nature of the purine neurotransmitter. The purines are widely distributed in all cell types and in the nerves, they may be contained in neurosecretory vesicles.[21] With nerve stimulation, the purines are released from the nerve terminals by exocytosis. Release of purine derivative after nerve stimulation in nerve muscle preparations has been examined in overflow solution collected during the nerve stimulation.[16] β-NAD and its metabolites, ADPR and adenosine, and ATP and its metabolites ADP, AMP, and adenosine were detected in the overflow solution. ATP and β-NAD represent primary purines and are

released from the nerves, although some ADP and ADPR may also be released from the nerves. The metabolites of the primary purines are thought to be produced in the junctional space by the action of ectonucleotidases and ectonucleosidases that are widely distributed in tissues.[22] Many of these secondary metabolites are bioactive on different purinergic receptors including P2Y1 receptors. This makes it difficult to determine whether a primary purine or a breakdown derivative mediates the purinergic IJP.

Consideration of the time course of the fIJP and availability of different purines in the junctional space at the time of the fIJP may provide additional insight into the nature of the purinergic transmitter candidate. The purinergic fast IJP can be elicited with nerve stimulation of a single millisecond pulse.[11] The fIJP starts almost instantaneously and reaches a peak in fraction of a second. Therefore, only the purine that is present within milliseconds of nerve stimulation is the relevant inhibitory purinergic neurotransmitter candidate. It has not been shown that the secondary metabolites can accumulate fast enough to initiate the fast IJP that starts almost instantaneously. Given the very fast time scale of purinergic IJP, it is most likely that the purines that are released from the nerve terminals are neurotransmitter candidate for the purinergic fast IJP. The vesicular release of β-NAD directly from isolated nerve varicosities has not been demonstrated, but ATP has been shown to be released from the myenteric varicosities upon nerve stimulation.[23]

Identification of the purine derivative in purinergic vesicles may provide further evidence for the nature of the purinergic varicosities. According to a new model, β-NAD [and perhaps, adenosine 5′-diphosphate-ribose (ADPR)] is contained in the purinergic vesicles in the enteric varicosities.[24] However so far, β-NAD has not been demonstrated in the enteric purinergic vesicles and no vesicular transporter of β-NAD/ADPR has been identified. Moreover, the release of β-NAD has not been identified from the isolated enteric nerve terminals. In contrast, ATP has been shown to be contained in the enteric varicosities. As early as 1978, Burnstock *et al.* demonstrated the presence of ATP in the enteric varicosities using quinacrine staining as a surrogate for the presence of ATP.[25] ATP is widely distributed in the cytosol as well as the neurosecretory vesicles. Vesicular loading of ATP as well as ADP is thought to involve vesicular nucleotide transporter (VNUT). However, the identity of VNUT has been elusive, thus impeding progress in the understanding of purinergic neurotransmission. Following the identification of SLC17A9 (solute carrier family 17, member 9), a member of the major facilitator superfamily involved in anion/phosphate transport, it was demonstrated that it transports the highly charged ATP or ADP inside a membrane bound vesicle.[26] We have provided the first demonstration of the presence of the ATP/ADP transporter SLC17A9 in isolated enteric varicosities.[27] Although ATP is widely distributed in the cytosol as well as the neurosecretory vesicles and is released from both these sites, vesicular release by exocytosis is tightly regulated and is the primary source of purines during neurotransmission (reviewed in Lazarowski).[28]

Important advances have also been made in understanding the process of exocytosis of ATP containing purinergic vesicles in enteric varicosities. Purinergic vesicular exocytosis, like exocytosis of other vesicular neurotransmitters, may be regulated by a complex cascade of intraneuronal events and participation of intracellular molecular motors. We have recently shown that in isolated enteric varicosities, SCL17A9 stained varicosities also contain the

motor protein myosin Va and the two are colocalized.[27] Stimulation of the varicosities results in exocytosis of the vesicles. In the 'dilute' (DBA, dilute, brown, non-agouti) mutant mice lacking myosin Va, exocytosis of the neurosecretory vesicles is impaired. Moreover in DBA mice, purinergic IJP is markedly reduced. These observations are consistent with the view that myosin Va motor may be involved in transport of the vesicles to the varicosity membrane for exocytosis.[27]

The roles of purinergic and nitrergic neurotransmissions has now been established by studies on P2Y1$^{-/-}$ and nNOS$^{-/-}$ mutant mice.[11–13,29] These mutants may help define non-purinergic inhibitory transmission and interaction of the purinergic and nitrergic inhibitory transmitters. In addition to the transport of purinergic vesicles to the varicosity membrane for exocytosis, the same motor protein myosin Va has also been shown to be involved in membrane localization of nNOSα and nitrergic neurotransmission.[30] Earlier studies had shown quinacrine labeling of the enteric nitrergic varicosities.[31] Thus, SLC17A9 staining can be used for labeling purinergic nerves and terminals. We have recently demonstrated colocalization of SLC17A9 in nNOSα positive varicosities,[27] suggesting that both purinergic and nitrergic inhibitors are released from the same 'inhibitory' nerve terminal.

The relationship of the purinergic and nitrergic inhibitory junction potentials to mechanical relaxation is not very clear. Purinergic IJP is prominent, fast and short whereas the nitrergic IJP inconspicuous, slow and long lasting. However, nerve-mediated mechanical relaxation cannot be easily distinguished into fast and slow components, correlating with fast and slow IJPs. Studies of neurally mediated junction potentials and mechanical relaxation in P2Y1$^{-/-}$ and nNOS$^{-/-}$ mutants, and double knockouts may provide better understanding of relative roles of purinergic and nitrergic inhibition in the gut.

The physiological importance of purinergic inhibitory neurotransmission is also not well understood. The current reports involving the P2Y1 knockout mice do not show a frank gastrointestinal phenotype. The lack of clinical manifestations may be due to low relative importance of the purinergic neurotransmission or upregulation of compensatory mechanisms. It is well known that major symptomatic or structural abnormality may not be obvious in disorders of gastrointestinal motility. However, functional motor disorders may be demonstrated by specific physiologic studies. P2Y1 receptor inhibitors have been shown to increase nerve stimulated spontaneous contractions in colonic tissues.[9] Delayed colonic transit has been reported with blockers of P2Y1 receptors and genetic deletion of P2Y1 receptors.[12] Propulsive motility of epoxy-coated artificial pellets have been shown to be significantly slower *invitro* in guinea pig colon in the presence of MRS2179.[32] These observations, however, may not reflect loss of purinergic inhibitory neuromuscular neurotransmission. P2Y1 receptors, apart from neuromuscular neurotransmission, are also involved in neurotransmission in the enteric nervous system and other regulators of gastrointestinal motility.[33] Therefore, in intact tissues or *in vivo*, P2Y1 knockout mice will reveal the effect of deficiency of P2Y1 receptors at all the sites where they are present. It is clear that P2Y1 receptor deficiency may lead to significant gastrointestinal motility disorders. It is also of interest to note that purinergic neurotransmission may be impaired in the ulcerated regions of the colon.[32] Further studies are needed to fully define

these abnormalities and to dissect out the roles of impaired purinergic enteric reflexes and inhibitory purinergic neuromuscular neurotransmission in the gut.

In summary, studies in P2Y1 knockout mice have confirmed the certainty of P2Y1 receptor in purinergic neurotransmission.[11–13] However, debate persists among investigators regarding many other aspects of purinergic inhibitory transmission. Most importantly, the very nature of the purinergic inhibitory neurotransmitter, ATP/ADP *vs* β-NAD/ADPR remains unsettled and the intriguing possibility that different purines may serve as the neurotransmitters in different parts of the gut requires rigorous examination. Other related topics of disagreement apart from the identity of the putative purine transmitter stored and released from the purinergic varicosities include the cellular localization of P2Y1 receptors and the role of PDGFRα+ fibroblasts in transducing purinergic signals to smooth muscle. There is also limited understanding of the importance of purinergic inhibitory neurotransmission to overall gastrointestinal motility and of the gastrointestinal phenotypes of P2Y1 receptor deficiency. Some of the key controversies may be resolved by reevaluation of the original data to identify any technical reasons for the divergent results. Others would require careful studies to obtain additional evidence. P2Y1$^{-/-}$ mice will play an important role in proper understanding of the inhibitory purinergic neurotransmission in the gut.

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