

Interaction Between Acetylcholine And ATP in Controlling The Longitudinal Smooth Muscle of Chicken Anterior Mesenteric Artery.

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Summary

The role of ATP in excitatory neurotransmission and its possible modulating role on acetylcholine (ACh) action were examined in the longitudinal muscle of the chicken anterior mesenteric artery (AMA) using tension recording technique.

Electrical field stimulation (EFS) caused frequency-dependent contractions that were sensitive to tetrodotoxin (1 μ M) and completely abolished by atropine (1 μ M) in 63% of the preparations but in 36% of the preparations an atropine resistant component remained and was blocked by the non-specific purinergic antagonist, suramin (200 μ M). Application of suramin alone from the beginning significantly and markedly decreased the EFS- induced contraction in a dose-dependent manner. Preparations contracted to exogenous applications of ATP and 2-methylthio ATP (2-MeSATP) in dose-dependent manners. Desensitization of P2X receptor with its putative receptor agonist α,β -MeATP (1 μ M, for 30 min) has no or insignificant enhancing effect on the contractile response. In contrast, desensitization of P2Y receptor with its putative agonist 2-MeSATP (1 μ M, for 30 min) abolished the contractile response, indicating that this response is mediated through P2Y but not P2X receptor. In atropine-pretreated preparations, the contractile response to 2-MeSATP was significantly inhibited. The obtained findings of EFS and exogenous applications together indicate that ATP produces its action, at least partly, via presynaptic enhancement of the release of ACh and this occurs through activation of facilitatory P2Y receptor; this is in addition to the direct action of ATP on P2Y receptors being expressed on smooth muscle cells of the longitudinal muscle of chicken AMA.

Introduction

Avian mesenteric artery differs greatly from morphological and innervation pattern points of view from that of mammals. Early

morphological studies conducted by (2) revealed that in addition to the circularly oriented smooth muscle layer common to mammalian mesenteric and other arteries (22), there is a well-developed outer longitudinal muscle layer characteristic to the chicken anterior mesenteric artery (AMA). This arrangement may be analogous to that of *muscularis externa* of the intestinal wall and venous wall (44; 30; 36). The longitudinal muscle layer of chicken AMA however, decrease in thickness along the course of the artery where it disappear in the small side branches and be replaced by collagen fibres.

In addition to the artery structural difference, chicken anterior AMA receives unusual innervation pattern that differ from that of most mammalian arteries which receive excitatory adrenergic nerves (29); in chicken AMA, adrenergic fibres and varicose terminals are found at the adventitial-medial border in this as in other arteries, however, these elements also extend into the longitudinal muscle itself. Longitudinal muscle of this vessel also receives cholinergic innervation (9; 4; 5). Adrenergic nerve stimulation produces contraction of the circular muscle, which is mimicked and blocked by α -adrenergic agonists and antagonists, respectively (4; 10; 26). In contrast, the longitudinal muscle is relaxed by adrenergic nerve stimulation, acting through β -adrenergic receptors; however, stimulation of cholinergic nerve fibres caused the longitudinal muscle to contract (10; 4). In mammals, in contrast, acetylcholine is well established to relax blood vessels only when endothelium is intact (23). Also vascular smooth muscle membranes are hyperpolarized by ACh and related drugs like carbachol and by substance P and bradykinin in an endothelium-dependent manner (11; 6). ACh may hyperpolarize and relax the circular smooth muscle of AMA.

Adenosine 5'-triphosphate (ATP) is well documented to colocalize with norepinephrine (NE) in sympathetic nerves in variable proportions depending on the tissue and the species (16). It is also reported that in the longitudinal muscle of the rabbit portal vein (30) and in the longitudinal muscle of the portal vein

of the guinea pig (36), ATP is mediating relaxation and contraction, respectively.

The receptors mediating responses to ATP have been characterized as P2 purinoceptors; while P1 receptors are for adenosine (17). P2 purinoceptors have been subdivided into two major classes: P2X and P2Y purinoceptors (1). P2X and P2Y receptors are ligand-gated ion channels (13; 41) and G-protein-coupled receptors (8), respectively. P2X purinoceptors were proposed to mediate contractile effects of ATP on smooth muscle. P2Y purinoceptors were proposed to mediate the relaxant effects of ATP on smooth muscle and/or endothelium (38). Recently, (31) reported that ATP is the NANC excitatory mediator involved in the generation of EFS-evoked depolarization in chicken AMA, and this excitatory action may be mediated via P2Y2 receptor.

In the present study, using tension recording technique, we further investigated the contributing role of ATP in the control of longitudinal muscle of AMA and the possible interaction that might occur between ATP and ACh in regulating the contraction of such muscle layer that is unique to chicken.

Material and Methods

Tissue preparation

Male white leghorn chicken aged 10-14 weeks old were killed by dislocation of the neck. The anterior mesenteric artery was severed at its origin from the aorta and at the region of the sub-branches supplying the intestine, and placed in a physiological salt solution (PSS) at room temperature as previously described. The anterior mesenteric artery and its branches were cleaned from connective tissue and the vessels were cannulated at the proximal end with a glass micropipette (200 μm tip diameter) attached to the gravity-driven perfusion apparatus to perfuse the vessels with warmed (37°C) PSS to remove blood from the vessels. A segment of 2 cm long was cut from the distal portion of the main artery and mounted longitudinally in an organ bath of 10 ml capacity. The bath was filled with PSS that was constantly warmed at 37°C and aerated with carbogen mixture. An initial tension of 0.7 g was applied and the arterial segment was left for equilibration for 1

hour before recordings were taken. The segment was washed every 15 min without any changes in the initial tension.

Mechanical recordings

Mechanical changes of the longitudinal direction of the preparations were recorded isometrically with a force displacement transducer (Orientic T7-30-240, Japan), an AC amplifier (AS1202, Nihon Kohden, Tokyo, Japan), and a potentiometric pen recorder (Hitachi, 561, Japan). EFS was carried out by means of two platinum-wire rings which were connected to an electronic stimulator.

EFS was used to deliver rectangular pulses at different frequencies (1, 2, 5, 10 and 20 Hz) for 5s. The pulse duration was fixed at 0.5 ms for stimulation of intramural nerves. The stimulation intensities were used as supramaximal voltages at any particular frequency. Contractions were expressed as percentages of those produced by high (60 mM) K^+ concentration. Contractions were recorded either in the absence or in the presence of appropriate receptor blockers. Desensitization experiments are thought to be more reliable than effects of PPADS and CBF3GA because they exclude the nonspecific effects that may be exerted on other systems by these antagonists. Tissues, therefore, were incubated for 30 min either in α,β -MeATP (P2X receptor agonist; 100 μ M) to desensitize P2X receptors or in 2-MeSATP (P2Y receptor agonist; 100 μ M) to desensitize P2Y receptors and then EFS-induced tissue contractions were recorded.

Drugs

The following drugs have been used in the present study: atropine, acetylcholine (ACh), suramin, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic (PPADS), adenosine 5'-triphosphate (ATP), α,β -methylene ATP (α,β -MeATP), 2-methylthio ATP (2-MeSATP), tetrodotoxin (TTX). All drugs have been purchased from Sigma (St. Louis, MO, USA) and were serially diluted in the PSS to the required final concentration just before the experiments.

Statistics

Data are expressed as a mean \pm s.e.; (*n*) indicates the number of arteries in which electrical events were recorded. Statistical analysis was performed with student's *t*-test, and *P*-values < 0.05 were considered to be statistically significant (39).

Results

General observations and control responses of longitudinal muscle of AMA

Longitudinal muscle of chicken AMA is not quiescent muscle where it showed spontaneous activity in most of preparations especially at the start of experiments; however, when present, this activity stops after sometime. EFS of the muscle resulted in monophasic contractile responses whose amplitudes were dependent on stimulus frequency (Fig. 1a). Increasing the strength of the stimulus (more than 5 s at 20Hz) delayed the return of the muscle to its basement resting line after the end of the monophasic contraction but didn't affect its amplitude. (*n* = 5 for each).

Effects of TTX, atropine, suramin, PPADS and CBF3GA

Fig. 1b shows that the contractile responses at all recorded frequencies were sensitive to the neuronal blocker TTX (1 μ M). Prior incubation with the universal muscarinic receptor antagonist, atropine (0.5 μ M) resulted in either partial (50% inhibition of the control; 35.2% of preparations used) or complete (64.7% of preparations used) inhibition of EFS-evoked contractile response. In case of partial inhibition, the remaining component after atropine was markedly inhibited by the universal purinergic antagonist, suramin (200 μ M). However, and this is the most important finding in this study, application of suramin from the beginning resulted in marked inhibition of the response in spite of absence of atropine. As shown in Fig 2a and 2b, PPADS, the P2X receptor antagonist (50 μ M) resulted in insignificant inhibition of the response; while CBF3GA, the P2Y receptor antagonist, (200 μ M) resulted in marked inhibition of the contractile response. (*n* = 3 for each).

Fig. 1

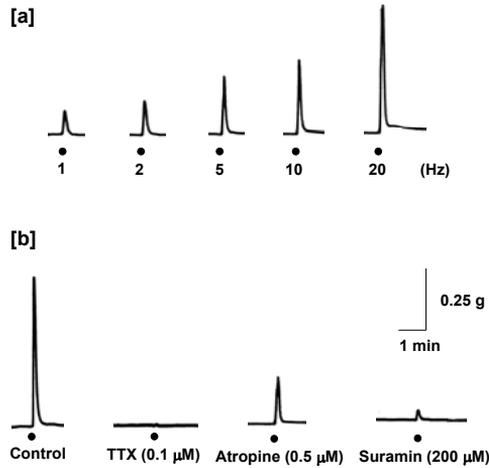


Fig. 1

[a] Contraction produced by EFS for 5s at 1, 2, 5, 10 and 20 Hz., pulse duration of 0.5 ms, supramaximal voltage. [b] the inhibitory effects of tetrodotoxin (TTX, 0.1 μM), atropine (0.5 μM) and suramin (200 μM). ($n = 5$ for each).

Fig. 2

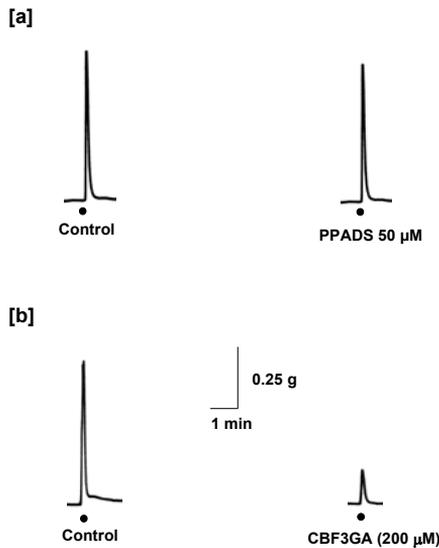


Fig.2

[a] The non-effect of the P2X purinoceptor blocker PPADS (50 μM); [b] The inhibitory effect of the P2Y purinoceptor blocker CBF3GA (200 μM). ($n = 3$ for each).

Effects of purinergic receptor desensitization

To confirm the involvement of P2 receptors and to determine which its subtype (X or Y) mediated the EFS-evoked contractile response, desensitization experiments were carried out using P2X and P2Y agonists. As shown in (Fig. 3a), after desensitization of P2X receptor, the amplitude of the contraction was insignificantly increased. ($n = 3$).

While desensitization of P2Y receptor (Fig. 3b) completely abolished the EFS-evoked contraction ($n = 3$). Fig. 3c shows a histogram summarizing the desensitization experiments.

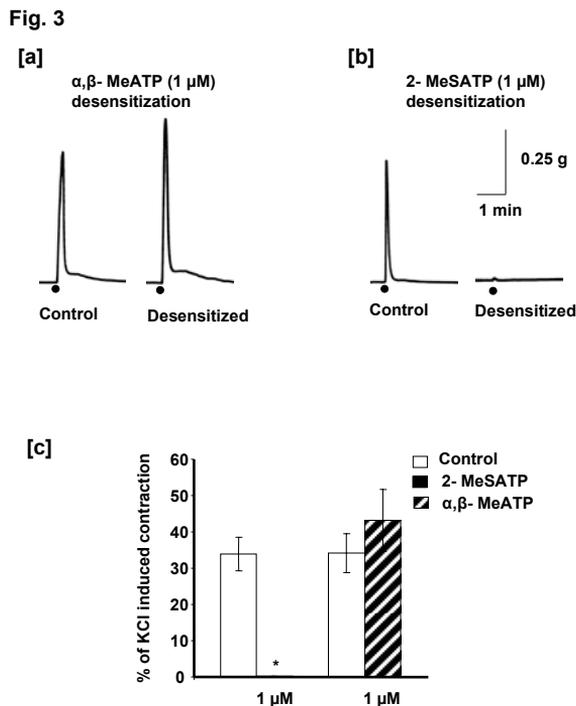


Fig. 3

[a] The non-effect of P2X purinoceptor desensitization by α, β -MeATP (1 μ M for 30 min); [b] The abolishing effect of P2Y purinoceptor blocker 2-meSATP (1 μ M for 30 min); [c] histogram summarizing desensitization experiment. ($n = 3$ for each).

Effect of exogenous application of ATP and 2-MeSATP

The inhibition of EFS-evoked contraction by the P2 purinoceptor antagonist, suramin and the complete block of the response by 2-MeSATP desensitization suggested that ATP, acting through P2Y purinoceptor was involved in the generation of EFS-evoked contraction. Therefore, the effects of exogenous ATP and 2-MeSATP were tested to determine if exogenous application of drugs would mimic the EFS-induced response. As shown in Fig. 4a and 4b, either ATP (100 μM) or 2-MeSATP (100 μM) resulted in contraction of the longitudinal muscle segment of chicken AMA ($n = 3$ for each). There was no significant difference between ATP- and 2-MeSATP-induced contractile responses.

Fig. 4

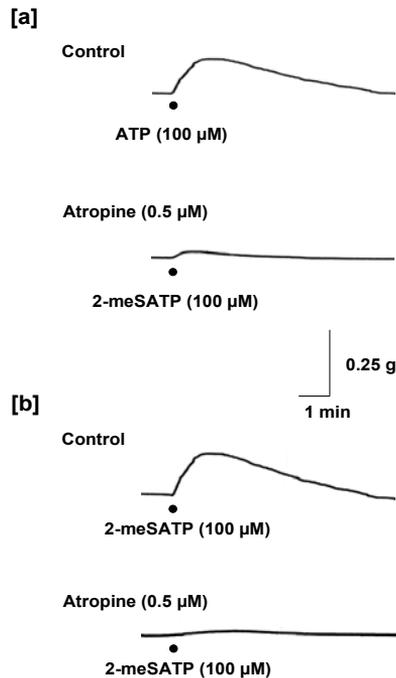


Fig 4.

[a] The contractile effect of exogenous application of ATP (100 μM) and the blocking effect of prior application of atropine (0.5 μM). [b] The contractile effect of exogenous application of 2-meSATP (100 μM) and the blocking effect of prior application of atropine (0.5 μM). ($n = 3$ for each).

Importantly, the effects of ATP and 2-MeSATP were also examined in the presence of atropine, PPADS and suramin. Prior application of atropine (0.5 μ M) or suramin (200 μ M) significantly inhibited and blocked the effects of either ATP or MeSATP, respectively. While prior application of PPADS (50 μ M) was of no effect ($n = 3$ for each).

Discussion

The potent actions of purine nucleotides and nucleosides in the cardiovascular system were first recognized by (19). Long time later, (17) proposed two distinguished types of purinoceptors, P1 for adenosine and P2 for ATP. He added that purinergic neurotransmission is mediated largely by postjunctional P2 receptors, while inhibition of release of the transmitter from nerve terminals is mediated largely by prejunctional P1 (A1) receptors. ATP was documented to produce either contraction, as in case of the longitudinal muscle of the portal vein of the guinea pig (36), or relaxation, as in case of the longitudinal muscle of the rabbit portal vein (30), of vascular smooth muscle. The different effects of ATP occur through two major families of P2 purinoceptors: a P2X family of ligand-gated ion channel receptors; and a P2Y family of G protein-coupled receptors (1). Recently, in the longitudinal muscle of chicken AMA, (31) proved that ATP is responsible for excitatory junction potential (EJP) evoked by EFS under the NANC condition and this effect is achieved via P2Y not P2X receptor. However, there were no data shown about the contracting effect of ATP that are presented here.

From another aspect of view, ACh is well proved to mediate vasorelaxation. In 1980, (23) described a mediator that is released by the vascular endothelium in response to ACh and causes vascular smooth muscle relaxation. This mediator, originally called endothelium derived relaxation factor (EDRF) and is released upon activation of endothelial muscarinic receptor by ACh. Five molecularly distinct muscarinic receptor subtypes have been identified and designated M1–M5 (42). Previous studies using pharmacological approaches with muscarinic receptor

agonists and antagonists suggest that M3 receptors mediate the vasodilatory actions of ACh in several vascular beds, including rat aorta (12), rabbit aorta (27), rat renal artery (21), guinea pig ileum submucosal artery (14), rabbit pial artery (25) and rat pulmonary artery (34). However, the subtype selectivity of muscarinic receptor agonists and antagonists is often marginal, raising concern about conclusions drawn from these earlier studies. In addition, investigators have demonstrated that other muscarinic receptor subtypes, including M1, M2 and M5, are expressed in various vascular beds (20; 37) and may be involved in the vasodilatory response. The situation is completely different in the longitudinal muscle of chicken AMA; where ACh was reported by (10) and (4) to cause contraction not relaxation of the muscle probably by a direct action on muscarinic receptors on the surface of longitudinal smooth muscle cells.

Data of the present study confirmed the excitatory role of ACh and provided a new evidence for the contracting role of ATP in the longitudinal muscle of chicken AMA. Furthermore, it provided an interesting relationship between the two neurotransmitters in regulating the contractile activity of the muscle layer under investigation. In the present study, ATP was proved to mediate fast monophasic contraction of the longitudinal muscle layer of chicken AMA, and the amplitude of contraction was directly proportional to the frequency of the stimulus. Contractions produced by EFS at all frequencies were sensitive to TTX indicating the neuronal origin of ATP. The involvement of ATP in mediating this contraction was concluded because of the following observations: i) blockade of purinergic receptors by suramin markedly inhibited or abolished the EFS-evoked response; ii) desensitization of purinergic P2Y but not P2X receptors by 2-MeSATP also abolished the response; iii) exogenous application of either ATP or 2-MeSATP resulted in contraction, and these contractions were blocked by corresponding purinergic blockers.

ATP is thought, in this study, to evoke its response indirectly via enhancing the release of ACh via activating P2Y receptors present at the presynaptic cholinergic varicosities, the released ACh, in

turn, activates muscarinic receptors present on the surface of longitudinal smooth muscle cells of chicken AMA. This conclusion has been derived from the following observations: i) blockade of muscarinic receptors by atropine resulted in either complete or partial inhibition of the EFS-evoked response; ii) the contractile response evoked by exogenous application of either ATP or 2-MeSATP was markedly inhibited by the muscarinic antagonist atropine. However, the direct action of ATP couldn't be completely excluded because sometimes we have recorded only partial blockade of the EFS- and ATP-mediated contractile responses in the presence of atropine, and the remaining component was abolished by suramin. It remains to be explained why atropine produces sometimes partial and other times complete blockade of the response, although individual variation between arterial samples (where every sample represents a bird) could be a reason.

Although it is firstly reported in chicken in this study, the interaction between ATP and ACh was recorded in different tissues of different animals including guinea-pig ileum where (3) found that both PPADS and suramin depressed the EFS-induced ileal contractions whereas they were without effect on contractions evoked by exogenous ACh and atropine-resistant contractions due to EFS. They concluded that the depressant action of P2 antagonists is probably due to blockade of the receptors located presynaptically. Physiologically, it is proposed that activation of such receptors may enhance ACh release. This idea is supported by (35) and (43) who reported that exogenous ATP seems to activate cholinergic neurons in the gut wall, in addition to its known direct contractile effect on the smooth muscle.

The interaction between ATP and ACh raises one of two hypotheses in chicken AMA; the first is that the purinergic and cholinergic neurons are located close to each other, while the second is that ATP and ACh are colocalized in a single neuron. The first possibility is the classical old concept which states that each type of neurons contains only a single type of transmitter. However, the second concept that is firstly postulated by (15) is

now generalized and is evidenced by immunostaining in more than one tissue and species. As for purinergic and cholinergic cotransmission, (18) reported that ATP coexists with ACh in motor nerves to skeletal muscle and, indeed, in developing myotubes ATP, acting through P2X receptors, is equally effective with ACh, acting via nicotinic receptors. However, in adults, the cotransmitter role of ATP is lacking, but the ATP released acts both as a postjunctional potentiator of the nicotinic actions of ACh and as a prejunctional modulator of ACh release following its breakdown to adenosine and action on prejunctional P1 receptors.

Studies by (24) and (33) in ileum and colon indicated that all neurons with cell bodies in the myenteric plexus whose axons make synapses with other neurons in the plexus express cholinergic phenotype (ChAT-immunoreactive). Therefore, purinergic inputs of myenteric origin presumably come from neurons that are cholinergic, suggesting that ACh and ATP are likely to be cotransmitters to myenteric neurons. Functionally, ATP was reported to mediate excitatory neuro-neuronal transmission in descending pathways in guinea-pig ileum (28; 32; 7). However, experimental data on reflexes from the mucosa demonstrated that both ACh and ATP act as excitatory neuro-neuronal transmitters in ascending and descending pathways (40). Abolishing the EFS-induced contractile response after desensitization of P2Y receptor by 2-meSATP suggests that the facilitatory modulation exerted by ATP on ACh action is mediated via P2Y receptor. However, the none or slight enhancing effect after desensitization of P2X receptor by α,β -meATP suggests no role for P2X receptor or probably an inhibitory role, although insignificant.

In conclusion, this study indicates that there is an interaction between ATP and ACh in controlling the contractile activity of longitudinal smooth muscle of chicken AMA. This interaction occurs in terms of enhancement of ACh action by ATP via probably a presynaptic P2Y receptor.

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