ATP induces guinea pig gallbladder smooth muscle excitability via the P2Y$_4$ receptor and COX-1 activity

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ATP IS AN ABUNDANT SIGNALING molecule throughout the central and enteric nervous systems, where it can act as a neurotransmitter or long-term signaling molecule. ATP has been implicated in the production of prostaglandins in different cell types. For example, stimulation of P2Y receptors increases prostaglandin production in Madin-Darby canine kidney cells (43), astrocytes (7, 8), and airway epithelium (28). In rat aorta (17) and guinea pig uterine smooth muscle (2, 35), contractions in response to ATP application are a result of prostaglandin production. A similar mechanism may exist in the gallbladder. In the early 1980s ATP was shown to induce contractions of guinea pig gallbladder smooth muscle (19, 42). This contractile response was inhibited by prior incubation with the nonselective cyclooxygenase enzyme (COX) inhibitor indomethacin, suggesting the contraction to ATP is mediated by prostaglandin production (42). However, it is unknown whether ATP is acting on intrinsic gallbladder neurons or acting directly on the smooth muscle. Likewise, it is unclear the mechanism of actions of ATP in terms of receptor and COX subtype.

Prostaglandins are signaling molecules produced from arachidonic acid by COX (50). In the gallbladder, prostaglandins and COX expression is increased in patients with cholecystitis (29, 37, 38) and in animal models of gallbladder inflammation (40, 44). During gallbladder inflammation muscle contraction in response to excitatory agonists is greatly reduced (55). Prostaglandins contract gallbladder smooth muscle (GBSM) by acting on receptors expressed within the muscle (12, 53). Furthermore, prostaglandin-induced contractions are preserved during gallbladder inflammation and increase cytoprotective mechanisms within the GBSM (53, 55). Consequently, prostaglandins inhibit the reduction in agonist-induced GBSM contractions due to reactive oxygen species that may occur during gallstone disease (53). Therefore, it has been suggested that prostaglandins provide a protective role on GBSM function during gallbladder disease (31, 46, 53).

Considering the importance of ATP as a signaling molecule and the role of prostaglandins in gallbladder disease, we sought to elucidate the mechanisms by which ATP induces GBSM excitation. Using intracellular recording, we demonstrate that ATP induces membrane depolarization and an increase in action potential (AP) frequency of GBSM. Furthermore, we provide pharmacological and RT-PCR data that suggest that ATP stimulates P2Y$_4$ receptors that are linked to COX-1, resulting in GBSM excitation, likely through prostaglandin production.

MATERIALS AND METHODS

Animals and tissue preparation. Male adult guinea pigs (200–350 g) were exsanguinated under isoflurane anesthesia, according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Vermont. Gallbladders were removed and placed in an ice-cold Krebs solution (in mM: 121 NaCl, 5.9 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 25 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, and 8 glucose, pH 7.4). Gallbladders were cut open from neck to base, washed to remove bile, and pinned stretched mucosal side up in a Sylgard-coated dish (Dow Corning, Midland, MI). The mucosal layers were teased off with sharp forceps under stereoscopic microscopic observation to prepare whole mounts of muscularis propria. Samples not immediately used were kept in ice-chilled Krebs buffer for 2–4 h.

Intracellular recording. For intracellular recording, the gallbladders were cut in half to produce preparations suitable for recording. Preparations were stretched pinned in a small recording chamber (∼2.5 ml volume) under constant superfusion with heated Krebs (35–37°C) containing the myosin light chain kinase inhibitor wortmannin (0.5 μM). The recording chamber was placed onto a Nikon TMD inverted microscope (Nikon USA) fitted with a Hoffman filter.
and muscle bundles were identified under a ×10 objective. Sharp glass microelectrodes (80–200 MΩ) backfilled with 2 M KCl were used for GBSM impalements. Electrical activity and membrane potential were recorded with a negative-capacitance compensation amplifier (Axoclamp 2A, Axon Instruments, Union City, CA) with bridge circuitry. Electrical activity was analyzed by use of PowerLab/SP and Chart 5, v.5.01 software (AD Instruments, Colorado Springs, CO). To initiate spontaneous electrical activity tissue was equilibrated for a minimum of 15 min before impalements. After a 5–10 min basal recording period, experimental compounds were applied to preparations through the superfusion buffer throughout the recording time frame for a minimum of five min before application of ATP. All GBSM cells within a given bundle discharge APs simultaneously and at the same frequency (6); therefore, if an impalement was lost during recording a new cell was impaled within the same muscle bundle to allow for a more continuous timeframe of AP frequency.

**Drugs.** Adenosine-5-triphosphate (ATP), suramin, reactive blue 2, tetrodotoxin (TTX), pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid (PPADS), uridine-5'-triphosphate (UTP), and wortmannin were purchased from Sigma (St. Louis, MO). Indomethacin was obtained from Cayman Biochemicals (San Diego, CA). Wortmannin and indomethacin were dissolved in DMSO and TTX was dissolved in 0.1% acetic acid in dH2O. All other drugs were dissolved in KREBS. Diluents alone had no effect on GBSM AP frequency.

**RT-PCR.** Gallbladder muscularis preparations were placed in RNAlater (Ambion; Austin, TX) and stored at −20°C for a minimum of 48 h. Tissue was disrupted and homogenized by rotor-pestle and RNA was isolated by use of RNEasy Micro Kit (Qiagen; Valencia, CA). After elution of RNA, samples were incubated with DNase I (Ambion) for 20–30 min at 37°C to eliminate any traces of DNA. Complementary DNA (cDNA) was produced by use of reaction vials containing 1 μg RNA, 1 μM random decamers (Promega; Madison, WI), 1 mM dNTPs, RNase inhibitor (Promega), 1× Moloney murine leukemia virus (MMLV) buffer, and MMLV reverse transcriptase (200 units, Promega). The following protocol was used to produce cDNA: 20°C 10 min, 37°C 90 min, 70°C 15 min. For positive controls, liver samples (<20 mg) were incubated 5–10 min in 50 mM NaOH at 97°C to extract genomic DNA.

P2Y primers for RT-PCR were designed from mouse mRNA sequences identical to rat and human sequences (P2Y2 forward 5'-GCTATGGCTTTGGACCTCT-3', reverse 5'-GATGCAGGTGA-3'), P2Y4 forward 5'-GCTATGAGGACCTCTCAAGT-3', reverse 5'-GCAAGGTTGCTGCACAATG-3'), acc. no. NM_020621). Reaction mixtures contained 1× Taq polymerase buffer, 1 unit HotStrat Taq polymerase (USB; Cleveland, OH), 1 mM dNTPs, and 4 μl cDNA and were incubated in a thermocycler initially for 2 min at 94°C to activate the polymerase. Amplification occurred from 35 cycles of the following protocol: 94°C for 30 s, 55°C 30 s, 68°C 75 s. All products were run on 2% agarose gels at 80 V for 35 min. Amplified products were visualized under UV light with ethidium bromide by using ChemiDoc XRS (Bio-Rad; Hercules, CA) and images were captured with Quantity One software (Bio-Rad). Images were processed via Photoshop (Adobe; San Jose, CA).

**Data analysis.** Resting membrane potential was determined as the difference between bath potential and the lowest cellular potential during the recording trace. APs were defined as a rapid spike followed by a plateau phase (58), and frequency was calculated (as Hz) from a 1-min period at given time points during the recording. For the ATP and UTP dose-response curve, AP frequency was normalized to the basal frequency of the cell immediately prior to drug application. Statistical analysis (paired t-test, repeated-measures ANOVA, or one-way ANOVA with multiple comparisons vs. control) was performed by using Number Cruncher Statistical Systems (NCSS; Kaysville, UT) or MicrocalOrigin (Microcal Software; Northampton, MA). Data are expressed as means ± SE, and the difference was considered statistically significant at P < 0.05. The n value represents number of tissue preparations from different animals.

**RESULTS.** GBSM had a resting membrane potential of −46.7 ± 2.3 mV and discharged APs at a frequency of 0.34 ± 0.04 Hz with average amplitude of 43.4 ± 3.6 mV (n = 13), consistent with our previous reports on spontaneous activity in GBSM (4, 6, 58).

**ATP induces a delayed excitatory response via a nonneuronal mechanism.** Application of ATP (100 μM) caused a delayed (30–60 s) excitatory response in GBSM (Fig. 1A) which was characterized by a 5.3 ± 1.1 mV membrane depolarization (P = 0.007) along with a significant increase in AP frequency (0.66 ± 0.02 Hz ATP 1 min vs. 0.33 ± 0.06 Hz basal; P < 0.001). This excitatory response was transient, lasting ~4 min (0.38 ± 0.08 Hz ATP 4 min vs. 0.33 ± 0.06 Hz basal; P = 0.47). In some cases, AP frequency in the continued presence of ATP fell below the control levels (Fig. 1B). In the presence of the Na+ channel blocker, TTX (0.5 μM), the excitatory response to ATP persisted (0.64 ± 0.09 TTX+ATP vs. 0.26 ± 0.04 Hz TTX control; P = 0.02; figure not shown) and was not significantly different from ATP alone (0.64 ± 0.09 TTX+ATP vs. 0.66 ± 0.02 Hz ATP; P > 0.05), indicating the actions of ATP on GBSM are via a nonneuronal mechanism.

**Evidence of P2Y4 receptor-mediated excitatory response.** ATP is known to act on two different classes of P2 receptors, P2X and P2Y receptors. Therefore, experiments were conducted to determine which class of P2 receptor mediates the excitatory response to ATP on GBSM. Application of ATP depolarized GBSM membrane and increased action potential (AP) frequency. Resting membrane potential of this cell was −55.79 mV. After 1 min AP frequency demonstrating the excitatory effects of ATP. ATP after 1 min significantly increased AP frequency in GBSM compared with basal frequency (*P < 0.001, repeated-measures ANOVA; n = 8) but after 4 min AP frequency was not significantly different from the basal period (P > 0.05).
excitatory response to ATP in GBSM. Suramin (100 μM), a nonspecific P2 antagonist, significantly reduced the ATP-evoked membrane depolarization and increase in AP frequency (0.12 ± 0.05 suramin+ATP vs. 0.28 ± 0.05 Hz suramin control; P = 0.009; Fig. 2) (−52.3 ± 4.9 mV suramin control vs. −51.5 ± 6.1 mV suramin+ATP; P = 0.67). In the presence of the nonspecific P2X antagonist PPADS (30 μM), ATP induced membrane depolarization (5.66 ± 2.1 mV) and a significant increase in AP frequency (0.33 ± 0.06 Hz PPADS control vs. 0.51 ± 0.04 Hz PPADS+ATP; P = 0.03; Fig. 3). This increase in frequency was not significantly different from ATP alone (0.66 ± 0.02 Hz; P > 0.05, ANOVA). Suramin (0.19 ± 0.03 Hz basal vs. 0.28 ± 0.05 Hz suramin, P = 0.30) and PPADS (0.29 ± 0.08 Hz vs. 0.33 ± 0.06 Hz PPADS, P = 0.68) alone did not significantly alter basal AP frequency.

To test whether a P2Y receptor was involved in the excitatory response the pyrimidine nucleotide UTP was used. UTP is an agonist of specific subtypes of the P2Y receptor (10, 47). Similar to ATP, application of UTP (100 μM) to GBSM induced membrane depolarization (9.8 ± 4.7 mV) and an increase in AP frequency (0.4 ± 0.09 Hz basal vs. 0.66 ± 0.06 Hz; P = 0.03, n = 5). Concentration effect curves for ATP (EC50 31 μM) and UTP (EC50 62 μM) indicate comparable potencies and efficacies for these agonists (Fig. 4). Of the seven P2Y receptor subtypes known to exist, two match the present data of being sensitive to UTP and blocked by suramin: P2Y2 and P2Y4 (1, 9, 10). To determine which of these subtypes are expressed in guinea pig gallbladder muscularis, we used RT-PCR with specific primer pairs to determine which of the two subtypes is expressed in the gallbladder muscularis. Both P2Y2 and P2Y4 primer pairs amplified appropriate-sized fragments from genomic DNA (334 and 127 bp, respectively); however, using guinea pig gallbladder muscularis cDNA, amplification was only obtained by using P2Y4 primers (Fig. 5).

Fig. 2. Suramin blocks the excitatory response to ATP. A: representative trace demonstrating that ATP reduces AP frequency in GBSM when preincubated with the nonselective P2 antagonist suramin. Resting membrane potential for this cell was −55.58 mV. B: data summarizing AP frequency. After 1 min ATP significantly reduced AP frequency in the presence of suramin (*significantly different from suramin, P = 0.009, paired t-test; n = 5).

Fig. 3. Pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid (PPADS) does not alter the excitatory response to ATP. A: representative trace demonstrating that ATP induces membrane depolarization and increased AP frequency in the presence of the nonselective P2X antagonist PPADS. Resting membrane potential for this cell was −50.12 mV. B: data summarizing the changes in AP frequency. After 1 min ATP significantly increased AP frequency in the presence of PPADS (*significantly different from PPADS, P = 0.03, paired t-test; n = 5).

Fig. 4. ATP and UTP concentration-response curve. Concentration-response curve demonstrating that both ATP (EC50 32 μM) and UTP (EC50 62 μM) have similar order of potency and efficacy in increasing AP frequency in GBSM. Normalized frequency in response to agonists is expressed as percent of basal frequency.
Therefore, we determined the effect of RB2 (100 nM) on ATP-induced excitation. In the presence of RB2, ATP-induced membrane hyperpolarization (10.8 ± 2.2 mV) and spontaneous APs in two of three cells investigated were eliminated whereas AP frequency was dramatically reduced in the third cell (0.10 ± 0.10 Hz RB2+ATP vs. 0.68 ± 0.13 Hz RB2 control; n = 3). Membrane hyperpolarization was observed in all three cells. This block of the ATP-induced excitatory response by RB2 is consistent with our RT-PCR data, suggesting involvement of P2Y4 receptors.

COX-1 activity is necessary for the excitatory ATP response. Previous studies have shown that indomethacin blocks ATP-induced contractions of GBSM (42). In the present study, indomethacin similarly abolished the excitatory response to ATP (0.14 ± 0.06 Hz indomethacin+ATP vs. 0.32 ± 0.05 Hz indomethacin control; P = 0.005). There are two types of COX enzymes that can be constitutively active in smooth muscle, COX-1 and COX-2 (39, 45). In the presence of the COX-1 inhibitor, SC-560 (300 nM), application of ATP completely eliminated GBSM APs and induced membrane hyperpolarization (9.7 ± 2.9 mV) in all preparations (n = 3), indicating a complete block of the excitatory response (Fig. 6). Conversely, the COX-2 inhibitor nimesulide did not alter the excitatory response (Fig. 7). In the presence of nimesulide (500 nM), ATP caused membrane depolarization (8.3 ± 2.4 mV) and significantly increased AP frequency (0.72 ± 0.08 Hz nimesulide+ATP vs. 0.30 ± 0.02 Hz nimesulide control; P = 0.03, n = 3). This increased AP frequency was significantly different from ATP alone (0.66 ± 0.02 Hz; P > 0.05, ANOVA). Indomethacin and COX inhibitors alone did not significantly altered basal AP frequency (0.40 ± 0.08 Hz basal vs. 0.32 ± 0.05 Hz indomethacin, P = 0.12; 0.35 ± 0.05 Hz basal vs. 0.32 ± 0.003 Hz SC560, P = 0.66; 0.27 ± 0.04 Hz basal vs. 0.30 ± 0.02 Hz nimesulide, P = 0.48).

**DISCUSSION**

Prior studies have shown that exogenous application of ATP induces GBSM contraction (19, 42). The focus of this study was to elucidate the mechanisms by which ATP stimulates GBSM excitation by using intracellular recording. We demonstrate, consistent with contractile studies, that ATP induces excitation of the GBSM characterized by membrane depolarization and increased AP frequency. Both of these responses were inhibited by suramin (nonselective P2 antagonist), indomethacin (nonselective COX inhibitor), and SC-560 (COX-1 inhibitor) and mimicked by UTP (selective P2Y agonist), indicating that ATP binds P2Y receptors and, in turn, stimulates prostanoid production.

ATP is known to bind to two classes of P2 receptors: ionotropic P2X receptors and metabotropic P2Y receptors. Our data from experiments involving suramin, PPADS, and UTP support the conclusion that ATP in the guinea pig gallbladder muscularis stimulates P2Y receptors and not P2X receptors. Three rodent P2Y receptors are sensitive to UTP: P2Y2, P2Y4, and P2Y6 (10, 11, 47). However, P2Y2 and P2Y4 but not P2Y6 are sensitive to suramin (1, 52), suggesting that ATP is acting on P2Y2 and/or P2Y4 receptor subtypes in GBSM. Our RT-PCR data indicate that P2Y4, not P2Y2, receptors are expressed...
whereas P2Y4, compared with P2Y2, is more sensitive to block of the P2Y4 receptor.

excitatory effects of ATP in GBSM are mediated via activation of the P2Y2 receptor. Our pharmacological and RT-PCR data indicate that the excitatory response in GBSM originates from ICC-like cells. ICC in the mouse antrum express prostaglandin receptors, which, when stimulated, increase slow-wave activity (21). Therefore, along with possible influence of membrane channels, prostanoids may alter pacemaker activity in GBSM, resulting in the observed increase in AP frequency after application of ATP.

Prostanoids are synthesized from arachidonic acid by the enzyme COX (50). In the present study, inhibition of COX activity by indomethacin blocked the excitatory response elicited by ATP, suggesting that production of prostanoids is necessary for this response. The specific COX-1 inhibitor SC-560, but not the COX-2 inhibitor nimesulide, also blocked the excitatory response to ATP in the GBSM. Although both COX-1 and COX-2 may be constitutively active in smooth muscle (12, 27, 39), our data suggest that COX-1 is responsible for the production of prostanoids in response to ATP. This is consistent with other studies demonstrating that ATP-induced contractions of the uterus (2, 35) and arterial smooth muscle (17, 24, 48) are mediated by prostanoids via activation of P2Y receptors. In Madin-Darby canine kidney cells, P2Y receptor activation leads to activation of phospholipase A2 (PLA2) (43). PLA2 then cleaves arachidonic acid from membrane phospholipids, leading to prostaglandin production via COX. It is possible that prostanoids are produced in a similar mechanism after stimulation of G protein-coupled P2Y receptors in the gallbladder. This is supported by the decrease in prostanoid production in guinea pig GBSM after PLA2 inhibition (12) and the increase in prostaglandin levels detected in the human gallbladder following PLA2 stimulation (18).

Prostanoids can induce contractions of GBSM via activation of G protein-coupled receptors (12, 53). In guinea pig GBSM, COX-1 activity has been shown to affect thromboxane A2 (TXA2) levels but not prostaglandin E2 levels (12). TXA2-receptor stimulation can increase intracellular Ca2+ levels within smooth muscle via extracellular Ca2+ influx through voltage-dependent Ca2+ channels (VDCC) and inositol 1,4,5-trisphosphate (IP3)-mediated Ca2+ release from intracellular stores (13). Increases in intracellular Ca2+ levels after opening of VDCC or IP3-mediated store release could account for the observed membrane excitation of GBSM in response to ATP (6, 20, 33). TXA2 receptors can also activate protein kinase C (PKC) within smooth muscle (3). PKC is known to regulate KATP channels within smooth muscle, possibly by modulating cAMP levels, which influence KATP activity (36). Decreased KATP activity could explain the observed membrane depolarization of GBSM in response to ATP. Although PKC has been shown to decrease KATP activity in GBSM (34, 57), KATP channels in the gallbladder appear to be less sensitive to PKC regulation than those in other types of smooth muscle (16). This limited sensitivity may explain the modest level of depolarization of GBSM that was observed in response to ATP. Further investigation is needed to identify prostanoid(s) that are produced and mediate excitation of GBSM in response to ATP.

Interstitial cells of Cajal (ICC) generate pacemaker currents that are responsible for spontaneous slow-wave activity of gastrointestinal smooth muscle (22, 49). Our laboratory has previously demonstrated that ICC-like cells exist in the guinea pig gallbladder (25). In that study, gap junction uncouplers eliminated spontaneous activity in gallbladder smooth muscle cells but not in ICC-like cells, suggesting spontaneous activity in GBSM originates from ICC-like cells. ICC in the mouse antrum express prostaglandin receptors, which, when stimulated, increase slow-wave activity (21). Therefore, along with possible influence of membrane channels, prostanoids may alter pacemaker activity in GBSM, resulting in the observed increase in AP frequency after application of ATP.

![Diagram](AJP-Gastrointest Liver Physiol • VOL 294 • JUNE 2008 • www.ajpgi.org)

**Fig. 7.** Nimesulide does not alter the excitatory response to ATP. A: representative trace demonstrating that in the presence of the COX-2 inhibitor nimesulide ATP induces membrane depolarization and increased AP frequency. The resting membrane potential for this cell is −53.68 mV. B: data summarizing the effect of ATP on AP frequency. After 1 min ATP significantly increased AP frequency in the presence of nimesulide (*significantly different from nimesulide, \( P = 0.03, n = 3 \)).
The physiological source of ATP in the gallbladder is unknown. It is possible that ATP functions as a neurotransmitter. Takahashi et al. (51) demonstrated an increase in ATP concentrations in response to nerve stimulation in the guinea pig gallbladder. Therefore, ATP may be released from nerves within the gallbladder to stimulate GBSM contraction. In the urinary bladder, ATP is released by the urothelium in response to stretch and distortion (15, 23, 26). Inflammation may also induce ATP release from epithelial cells in the mucosa. Another possibility is that gallbladder mucosa releases ATP to maintain or increase spontaneous activity in GBSM. This is supported by the AP characteristics that we have observed in GBSM preparations containing mucosa vs. muscularis preparations devoid of mucosa (5). Cholangiocytes of the liver release ATP to stimulate local P2Y receptors that regulate ion transport and fluid secretion (14, 32). Nathanson et al. (41) demonstrated that the hydrophilic bile salt ursodeoxycholate (UDC) stimulates ATP release from cholangiocytes, resulting in increased ATP concentrations within bile. UDC is a therapy for gallstone disease and can prevent or reverse the adverse effects of hydrophobic bile salts on smooth muscle function (30, 54, 56). It has been suggested that prostaglandins provide a protective role of smooth muscle function during gallstone disease (31, 46, 53). It is possible that UDC mediates its effects of hydrophobic bile salts on smooth muscle function in GBSM. Stimulation of the P2Y4 receptor and resulting activation of COX-1, which depends on COX-1 and P2Y4 receptors coupled to rises in intracellular calcium in mouse cultured urinary bladder epithelial cells by hydrostatic pressure changes—a possible sensory mechanism? J Physiol: 503–511, 1997.

In conclusion, ATP increases GBSTM excitability through activation of the P2Y4 receptor, which depends on COX-1 activity. Our results are consistent with the concept that activation of P2Y4 receptors by ATP leads to activation of COX-1, which in turn increases the production of prostanooids that may alter the function of membrane channels and/or pacemaker activity within the gallbladder muscularis.

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