Developmental changes of purinergic control of intestinal motor activity during metamorphosis in the African clawed frog, *Xenopus laevis*

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Sundqvist M. Developmental changes of purinergic control of intestinal motor activity during metamorphosis in the African clawed frog, *Xenopus laevis*. Am J Physiol Regul Integr Comp Physiol 292: R1916–R1925, 2007. First published February 1, 2007; doi:10.1152/ajpregu.00785.2006.—Little is known about the purinergic regulation of intestinal motor activity in amphibians. Purinergic control of intestinal motility is subject to changes during development in mammals. The aim of this study was to investigate purinergic control of intestinal smooth muscle in the amphibian *Xenopus laevis* and explore possible changes in this system during the developmental phase of metamorphosis. Effects of purinergic compounds on mean force and contraction frequency in intestinal circular muscle strips from prometamorphic, metamorphic, and juvenile animals were investigated. Before metamorphosis, low concentrations of ATP reduced mean motor activity, whereas the effects were reversed at higher concentrations. ATP-induced relaxation was not inhibited by the P2-receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) but was blocked by the ecto-nucleotidase inhibitor 6-N,N-diethyl-D-adenosine (PPADS) and 2-methylthio-n,N-diethyl-β,γ-dibromomethylene ATP (ARL67256), indicating that an ATP-derived metabolite mediated the relaxation response at this stage. Adenosine induced relaxation before, during, and after metamorphosis, which was blocked by the A1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). The stable ATP-analogue adenosine 5′-[(γ-thio)-triphosphate (ATPγS) and 2-methylthio-ATP (2-MeSATP) elicited contractions in the circular muscle strips from prometamorphic tadpoles. However, in juvenile frogs, 2-MeSATP caused relaxation, as did ATPγS at low concentrations. The P2Y1/P2X1-receptor antagonist NF157 antagonized the ATPγS-induced relaxation. The P2X-prefering agonist α,β-methyleneadenosine 5′-triphosphate (α,β-MeATP) evoked PPADS-sensitive increases in mean force at all stages investigated. This study demonstrates the existence of an adenosine A1-like receptor mediating relaxation and a P2X-like receptor mediating contraction in the *X. laevis* gut before, during, and after metamorphosis. Furthermore, the development of a P2Y1-like receptor-mediated relaxation during metamorphosis is shown.

DURING AMPHIBIAN METAMORPHOSIS, the gastrointestinal tract of the herbivorous tadpole goes through dramatic changes to adapt to the new feeding behavior as a carnivorous frog. These changes include considerable shortening of the intestinal length and remodeling of the gastric and intestinal mucosa (16, 29). The shortening of the anuran intestine occurs uniformly along the structure (27, 29), although the degree of shortening can vary between 60 and 90% depending on species. Our group has previously shown that the expression of neurotrophin-like receptors is increased in enteric neurons during metacorphic climax (30), suggesting that the enteric nervous system also undergoes changes at this stage. Moreover, previous studies indicate that the control of gastrointestinal motility during early developmental stages of the tadpole (31) differs compared with the adult gut, suggesting that regulatory changes may take place during metamorphosis. For example, the tonic nitrergic inhibition present in the adult *Xenopus* (25) is absent in the early tadpole gut (31).

The purinergic system influences motor function in the mammalian gut (7) and several other species as well (8), with various purine nucleotides and nucleosides mediating responses via purinoceptors. Adenosine mediates responses via metabotropic P1 receptors (A1, A2A, A2B, A3). ATP, uridine 5′-triphosphate (UTP), and ADP activate P2 receptors, which comprise two groups of subfamilies, the metabotropic P2Y [P2Y1, P2Y2, P2Y4, P2Y6, P2Y11–14 (found in human) and species-specific orthologs: p2y3 (chicken), p2y8 (*Xenopus*), p2y12 (turkey)] receptors and the ionotropic P2X (P2X1,7) receptors (6, 33). In mammals, adenosine has been shown to relax gastrointestinal smooth muscle in vitro, while ATP can mediate both relaxing and contracting responses depending on the receptor subtype expressed and the region of the gut (6). However, effects of ATP can be difficult to elucidate because of the rapid hydrolysis of the molecule by ectonucleotidases. In *Xenopus*, the A1 adenosine receptor (gene accession no. AJ249842) has been cloned along with several receptors for ATP including P2Y1, P2Y8, P2X3, P2X7 (2, 9, 19, 26), and P2Y11 (gene accession no. AM040941).

The role of purinergic receptors in the gut change during development of both mouse (13) and rat (4, 11, 15, 23), resulting in altered responses to purinergic ligands. Relaxing responses induced by adenosine can be seen in duodenal longitudinal segments isolated from rat *day 14* after birth in nonprecontracted tissue (11), and from *day 5* in tissue precontracted with carbachol (23). These effects are likely to be mediated via A2B receptors, which are present earlier in the postnatal period of the rat than, for instance, the A1 receptor, which is not expressed until *day 20* (3). In contrast to adenosine, ATP can elicit dual responses in rat and mouse gastrointestinal smooth muscle, depending on concentration and developmental age. Low doses of ATP were found to relax rat duodenal longitudinal smooth muscle during the early stages, while high concentrations elicited contraction. The contractile response to ATP was reduced during development (23). Similarly, relaxing responses to ATP in the murine stomach, duodenum, ileum, and colon seem to develop with age up to 20 days after birth, while the contractile responses decreased with age (13). Interestingly, these results suggest that P2Y1 recep-
tors in longitudinal smooth muscle of both rat and mouse switch from mediating contracting responses to relaxing responses around the time of weaning. This may be an adaptation to altered contents of nutrients in the food, from a lipid-rich diet to a more carbohydrate-containing variety (13–15).

Because our previous data indicate altered control of GI motility during development, it is hypothesized that there may also be developmental changes in the purinergic system during amphibian metamorphosis concurring with the change from herbivorous to carnivorous food intake and the simultaneous adaptive remodeling of the gastrointestinal tract. We therefore studied the effects of various purinergic ligands on intestinal smooth muscle isolated from X. laevis before, during, and after metamorphosis.

MATERIALS AND METHODS

Animals

Tadpoles from the African clawed frog, Xenopus laevis, were obtained by inducing adult animals to breed by injecting the animals with human chorionic gonadotropin (200 U/female and 150 U/male in the morning and 400 U/female and 300 U/male in the afternoon). The adults were kept overnight in a semidark aquarium, and removed in the morning leaving the fertilized eggs to develop into tadpoles. These were then reared in plastic aquariums until metamorphosis. They were fed 5 days a week with powdered Xenopus diet as tadpoles and with diet for small Xenopus frogs after metamorphosis. All experimental procedures were approved by the animal ethics committee of the city of Göteborg.

Drugs

Carbamoylcholine chloride (carbachol), ATP, adenosine hemisulfate salt (adenosine), adenosine 5'-[γ-thio]-triphosphate tetra-lithium salt (ATPγS) and α-β-methyleneadenosine 5'-triphosphate lithium salt (α-β-meATP) were purchased from Sigma-Aldrich (St. Louis, MO). 6-N,N-diethyl-2,3,6,7-dibromomethylene ATP trisodium salt (ARL67156), 2-methylthioadenosine triphosphate tetrasodium salt (2-MeATP), N6-cyclopentyladenosine (CPA), ABT-702, 4-(2,3,6,7-tetrahydro-2,6-dioxo-1-propyl-1H-purin-8-yl)-benzenesulfonic acid (PSB1115), 1,3-dipropyl-8-(p-sulphophenyl)xanthine (DPSPX), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), 8,8'-[carbonylbis[jimino]-3,1-phenylene]carbonylimino(4-fluoro-3,1-phenylene)carbonylimino]bis-1,3,5-naphthate nitr sofomic acid (NF157), and TTX were obtained from Tocris Cookson, Bristol, UK. Stock solutions of all compounds except ABT-702, PSB1115, and DPCPX were created by dissolving the compound in Millipore water and were then stored in aliquots at −20°C. ABT-702, PSB1115, and DPCPX were dissolved in DMSO. Further dilution was made in McKenzie’s amphibian Ringer solution.

Experimental Procedure

The animals were anesthetized and killed by immersion in a solution of 0.05% MS 222 (3-aminobenzoic acid ethyl ester; Sigma), and the developmental stage was determined according to Nieuwkoop and Faber (24). Tadpoles in stages 56 and 57 were used as prometamorphic animals, while animals in stages 61–63 were defined as metamorphic tadpoles. Animals after metamorphosis, in stage 66, were defined as juveniles. The abdomen of the animal was opened and the gastrointestinal tract was quickly removed and put in a silicon rubber-coated petri dish filled with modified McKenzie’s amphibian Ringer solution (in mM): 115 NaCl, 20 NaHCO3, 5.0 HEPES, 3.2 KCl, 1.4 MgSO4, 1.3 CaCl2, pH 7.8. The gut length was measured and ring-formed sections (~2 mm wide) were cut from the intestine at approximately one-third of the intestinal length. The circular muscle strips were then mounted in 10-ml organ baths containing 5 ml McKenzie’s amphibian Ringer solution bubbled with gas (0.3% CO2 in air). The temperature was kept at 23°C. Muscle strips from prometamorphic and metamorphic animals were stretched to a force of 2 mN, whereas the more developed and stronger juvenile muscle strips (see RESULTS, Spontaneous activity) were stretched to 5 mN. The Ringer solution was changed every 30 min and the muscle strips were allowed to rest for 1 h before experiments were started. The mean force developed was measured using Grass FT03 force transducers and recorded via a Grass amplifier coupled to a computer using the program LabView. The recordings were made with a sampling frequency of 2 Hertz. The spontaneous activity was first recorded for 5–10 min, after which drugs were added in a cumulative manner to construct a concentration-response curve. In TTX experiments, the agonist (ATP, adenosine, or α-β-meATP) was added as a single concentration and the response was recorded for 5 min or until a maximum response was obtained. The compound was then washed out and TTX was administered. TTX was allowed to equilibrate for 30 min before an identical concentration of the same agonist was added. Thus, the effects of agonists were tested in the absence and presence of TTX. Purinergic antagonists were administered in a similar manner with an incubation time of 20 min. For experiments with NF157 and ARL67156, the antagonist or enzyme inhibitor was added first and allowed to equilibrate for 20 min. Then a concentration-response curve for the agonist was constructed, and the results were compared with a curve constructed in the absence of antagonist or enzyme inhibitor. Potassium chloride (80 mM) was added to each muscle strip at the end of all experiments to check the viability of the tissue.

Data Analysis and Statistics

The responses (mean force and frequency) were measured during 200 s using a LabView-based analysis program. To normalize the mean force values in each experiment, the resting tension value between contractions for the control period were subtracted from all of the data points. The values were transported to Excel and GraphPad Prism 4.0 (GraphPad Software, San Diego, CA) for further calculations and statistics. EC50 values were calculated using a sigmoidal dose-response model in GraphPad Prism 4.0 but can in most cases only be regarded as tentative for the excitatory compounds, since the plateau level was never reached with the concentrations tested. This is also the reason why the maximal response of the compounds is not referred to as efficacy with the exception of carbachol. Experiments were statistically analyzed using repeated-measures one-way ANOVA followed by Dunnett’s post hoc test, one-way ANOVA followed by Bonferroni’s post hoc test, or Student’s t-test for unpaired or paired observations when appropriate. Results are presented as means ± SE and are considered significant when P < 0.05.

RESULTS

General Responses

Spontaneous activity. The mean force developed during basal conditions in intestinal muscle strips increased with age (Fig. 1A). The force developed in muscle strips from juvenile froglets was more than twofold higher (P < 0.05) compared with prometamorphic tadpoles (Fig. 1A). No difference was seen in the spontaneous contraction frequency of the intestine (data not shown).

Carbachol. The cholinesterase-insensitive contractile cholinergic agent, carbachol, was administered to investigate the responsiveness of the tissues before, during, and after metamorphosis. Carbachol (0.001–100 μM) elicited a concentration-dependent increase in the mean force developed (Fig. 1B) but did not affect contraction frequency (data not shown). The
potency for carbachol was similar at all stages (EC\textsubscript{50}: 1–8 
\text{\textmu}M). The maximal response to carbachol was 10-, 7- and 
39-fold over baseline in prometamorphic, metamorphic, and 
juvenile stages, respectively.

Responses to ATP

In muscle strips from the intestine of prometamorphic tadpoles, cumulative administration of ATP (0.01–1,000 \text{\textmu}M) produced a biphasic response consisting of an initial decrease in mean force and contraction frequency in response to low concentrations of ATP followed by an increase in the same parameters at higher concentrations. The resulting concentration-response curve has an inverted bell-shape (Fig. 2). In prometamorphic tadpoles, significant decrease was obtained with an EC\textsubscript{50}: 4.6 \pm 2.4 \text{\textmu}M (Fig. 2B). ATP also significantly decreased contraction frequency of the muscle strips, at some concentrations completely abolishing contractions (Fig. 2C, EC\textsubscript{50}: 1.9 \pm 0.5 \text{\textmu}M). The contractile effects of ATP were not dependent on prior relaxation produced in the cumulative administration protocol since, in a separate experiment, a single high dose of ATP (1,000 \text{\textmu}M, n = 5) evoked a 3.5-fold increase in developed mean force, indicating that the contractile effects of ATP could be evoked without prior relaxation (data not shown).

Intestinal muscle strips from tadpoles in metamorphic climax also responded to ATP with an inverted bell-shaped concentration-response curve with a significant decrease in mean force at 0.1–300 \text{\textmu}M (EC\textsubscript{50}: 2.9 \pm 1.9 \text{\textmu}M) and in contraction frequency at 1–300 \text{\textmu}M (EC\textsubscript{50}: 2.2 \pm 1.2 \text{\textmu}M, Fig. 3). An increase in both parameters were seen at the higher concentrations but did not differ from the control values at the beginning of the experiment.

In muscle strips from juvenile intestines, ATP decreased mean force and contraction frequency only at 300–1,000 \text{\textmu}M, with the highest concentration completely abolishing contractions (Fig. 4). The potency of ATP was \textasciitilde 50–100 times lower after metamorphosis (EC\textsubscript{50}: 212 \pm 90 \text{\textmu}M) compared with prometamorphosis.

To elucidate the location of the receptors mediating the ATP-induced relaxation the Na\textsuperscript{+}-channel blocker TTX was administered, thus blocking neuronal activity. TTX (1 \text{\textmu}M) did not affect ATP-dependent relaxation in either prometamorphic (10 \text{\textmu}M ATP, n = 7) or juvenile animals (1,000 \text{\textmu}M ATP, n = 7, data not shown) (metamorphic tadpoles were not tested).

\textit{P1-receptor-mediated responses}

ARL67156. ARL67156 inhibits the metabolism of ATP by ecto-nucleotidases. Administration of ARL67156 (100 \text{\textmu}M), 20 min before generating a cumulative concentration-response curve to ATP, blocked the ATP-induced decrease in mean force in prometamorphic tadpoles and instead unmasked ATP-dependent contractions (EC\textsubscript{50}: 259 \pm 138 \text{\textmu}M, Fig. 2B). The inhibitory effect of ATP on contraction frequency was attenuated in the presence of ARL67156 (Fig. 2C). In preparations from juvenile froglets, ARL67156 significantly attenuated the inhibitory effect of ATP on mean force (Fig. 4B) and on contraction frequency (Fig. 4C), although a slight response to ATP still remained at the highest concentration (1,000 \text{\textmu}M ATP).

\textit{Adenosine.} Adenosine (0.01–1,000 \text{\textmu}M) produced a concentration-dependent decrease in the mean force in all three stages tested. In prometamorphic intestine, a significant decrease in mean force was achieved with an EC\textsubscript{50} of 32 \pm 17 \text{\textmu}M (Fig. 5A) and the contraction frequency was abolished (EC\textsubscript{50}: 1.5 \pm 0.7 \text{\textmu}M, Fig. 5B). A similar decrease in mean force (EC\textsubscript{50}: 85 \pm 56 \text{\textmu}M) and in contraction frequency (EC\textsubscript{50}: 0.5 \pm 0.1 \text{\textmu}M) was seen in metamorphic intestine (Fig. 5, A and B). In juvenile intestine, higher concentrations of adenosine (\geq 100 \text{\textmu}M) were required for significant reduction of mean force (EC\textsubscript{50}: 194 \pm 76 \text{\textmu}M) and frequency (EC\textsubscript{50}: 205 \pm 86 \text{\textmu}M, Fig. 5, A and B).

The relaxing responses to a single concentration of adenosine (1,000 \text{\textmu}M) were not blocked by TTX (1 \text{\textmu}M) either in prometamorphic (n = 8) or juvenile (n = 10) intestinal muscle strips (metamorphic tadpoles were not tested).

\textit{CPA.} The selective adenosine A\textsubscript{1} receptor agonist CPA (0.0001–100 \text{\textmu}M) produced a significant, concentration-dependent decrease in mean force developed in intestinal muscle strips from prometamorphic (EC\textsubscript{50}: 0.12 \pm 0.11 \text{\mu}M), metamorphic (EC\textsubscript{50}: 0.04 \pm 0.03 \text{\mu}M) and juvenile stage animals (EC\textsubscript{50}: 0.26 \pm 0.11 \text{\mu}M, Fig. 5C). In prometamorphic muscle strips, contraction frequency was significantly attenuated at 0.1 \text{\mu}M and almost completely abolished at higher concentrations, while in juvenile muscle strips, the contraction frequency was...
only attenuated by 50% (Fig. 5D). However, the potency of CPA was similar before (EC50: 0.31 ± 0.26 μM) and after metamorphosis (EC50: 0.09 ± 0.06 μM). During metamorphosis, the potency of CPA at inhibiting contraction frequency was slightly less (EC50: 3.6 ± 1.1 μM) than during other stages, although in contrast to juveniles, contractions were almost completely abolished at the highest concentration (100 μM). That CPA was an effective relaxation agent at all stages suggests that there is an A1-like receptor present in the tissue.

ABT-702. ABT-702 inhibits the enzyme adenosine kinase, which metabolizes adenosine. Administration of ABT-702 (1 μM) caused a significant decrease in mean force and contraction frequency in intestinal muscle strips from prometamorphic tadpoles (Fig. 6), suggesting that endogenous adenosine may mediate relaxation in the tissue. The juvenile froglets also responded with a significant decrease in mean force, although no significant decrease in contraction frequency was seen. The vehicle used to solubilize ABT-702 (0.01% DMSO) had no effect on the muscle strip preparations.

DPCPX, DPSPX, and PSB1115 To further investigate the P1 receptor involved in the relaxation of intestinal muscle strips,
produced a significant concentration-dependent increase in mean force developed in muscle strips from prometamorphic intestine (EC\textsubscript{50}: 111 ± 52 μM, Fig. 7, A and D) but had no effect on contraction frequency (Fig. 7E). Muscle strips from metamorphic tadpoles responded to ATP\textsubscript{γS} with a significant increase in mean force (EC\textsubscript{50}: 188 ± 89 μM, Fig. 7, B and D), while the contraction frequency decreased in a bell-shaped manner with the maximal effect occurring at 1–10 μM (EC\textsubscript{50}: 3.7 ± 1.5 μM, Fig. 7E). Interestingly, ATP\textsubscript{γS} caused an inverted bell-shaped dose-response curve (i.e., an initial decrease in mean force followed by an increase in the same parameter at higher concentrations) for the mean force developed (Fig. 7, C and D) in intestinal muscle strips from juvenile froglets. The decrease in mean force was maximal at 10 μM (EC\textsubscript{50}: 62 ± 40 μM) and was followed by an increase in mean force at the highest concentrations. The contraction frequency in juvenile muscle strips was dose-dependently attenuated by ATP\textsubscript{γS} (EC\textsubscript{50}: 56 ± 49 μM, Fig. 7E).

NF157 and MRS2179. To characterize the ATP\textsubscript{γS}-induced relaxation only found in juvenile animals, the P2Y\textsubscript{1}P2X\textsubscript{1} receptor antagonist NF157 (100 μM) was administered 20 min before constructing a concentration-response curve of ATP\textsubscript{γS} (0.1–100 μM). NF157 shifted the inhibitory response of ATP\textsubscript{γS} to the right (Fig. 7, F and G) with respect to both mean tension and contraction frequency suggesting the involvement of a P2Y\textsubscript{1}P2X\textsubscript{1} receptor. The P2Y\textsubscript{1}-selective antagonist MRS2179 was tested in an identical protocol but did not inhibit ATP\textsubscript{γS}-induced relaxation (n = 4, data not shown), suggesting that the receptor involved is not P2Y\textsubscript{1}.

2-MeSATP. To further investigate P2-mediated relaxation in the tissue, the P2Y\textsubscript{1}, P2Y\textsubscript{11}, P2X\textsubscript{1}, and P2X\textsubscript{3} (17) agonist 2-MeSATP were tested. Administration of 2-MeSATP (0.01–100 μM) elicited a concentration-dependent increase in mean force developed in intestinal muscle strips from both prometamorphic (EC\textsubscript{50}: 9.9 ± 3.1 μM) and metamorphic (EC\textsubscript{50}: 23 ± 9.4 μM) tadpoles (Fig. 8A), although the responses were relatively small compared with the effects evoked by other agents. The only change in contraction frequency was a small but significant dip at 1 μM 2-MeSATP in metamorphic tadpoles (Fig. 8B). By contrast, intestinal muscle strips from juvenile tadpoles responded with a decrease in mean force (EC\textsubscript{50}: 95 ± 44 μM) to 2-MeSATP (Fig. 8A). The intestinal contraction frequency was only attenuated at the highest dose (EC\textsubscript{50}: 70 ± 14 μM, Fig. 8B). These results further support the development of a P2 receptor-mediated relaxation after metamorphosis.

α-β-MEATP. To determine the role of P2X receptors in ATP-evoked contractions in the intestine, the P2X\textsubscript{1}, P2X\textsubscript{3}, P2X\textsubscript{14}, P2X\textsubscript{2/3}, and P2X\textsubscript{3/5} agonist (12, 22) α-β-MEATP was applied. In the intestinal muscle strips from all three stages, administration of α-β-MEATP (0.01–100 μM) elicited a concentration-dependent increase in the mean force developed (EC\textsubscript{50}: 21 ± 6.4, 26 ± 8.4, and 94 ± 26 μM in prometamorphic, metamorphosis, and juveniles, respectively, Fig. 8C), suggesting the presence of a P2X-like receptor. No significant changes in the contraction frequency were seen in prometamorphic tadpoles or tadpoles in metamorphic climax, although a small increase in frequency was seen in juvenile froglets (Fig. 8D).

TTX (1 μM) was ineffective (data not shown) in blocking the response to α-β-MEATP in either prometamorphic tadpoles (10 μM α-β-MEATP, n = 6) or juvenile froglets (30 μM

three adenosine receptor antagonists were evaluated on muscle strips from prometamorphic and juvenile animals. The A\textsubscript{1}-selective antagonist, DPCPX (1 μM), inhibited adenosine-induced relaxation in both prometamorphic and juvenile animals (Fig. 5E). The vehicle (0.01% DMSO) had no effect (data not shown). Neither the adenosine receptor antagonist DPCPX (3 μM, slightly A\textsubscript{1}-selective) nor the A\textsubscript{2B} preferring PSB1115 (1 μM) had any effect on adenosine-induced relaxation of the intestine (data not shown).

P2-receptor-mediated responses. ATP\textsubscript{γS}. ATP\textsubscript{γS} is a relatively stable ATP analog and is presumably not converted to adenosine during the time period of the experiment (~40 min). ATP\textsubscript{γS} is particularly potent at P2Y\textsubscript{2}, P2Y\textsubscript{11}, and P2X\textsubscript{3} but is also an agonist at P2Y\textsubscript{1}, P2X\textsubscript{1,2,3,4,6} P2X\textsubscript{1/5}, and P2X\textsubscript{2/3} purinoceptors (1, 22). Administration of ATP\textsubscript{γS} (0.1–300 μM)
α-β-MeATP, \( n = 7 \)), suggesting that contracting P2X-like receptors are situated directly on the smooth muscle both in tadpoles before metamorphosis and in froglets.

PPADS. Administration of the P2 receptor antagonist PPADS [effective at homomeric P2X\(_{1,2,3,5}\), heteromeric P2X\(_{2/3}\), and P2X\(_{1/5}\), and P2Y\(_{1,6,13}\) receptors (1, 17)] at 30-|H9252|/H9251| MeATP, tadpoles before metamorphosis and in froglets. receptors are situated directly on the smooth muscle both in |H9252|/H9251| MeATP-induced contractions in prometamorphic tadpoles. PPADS also inhibited α-β-MeATP-induced contractions in juvenile animals, although the effective concentration had to be increased to 100 |H9252|/H9251| (metamorphic animals were not tested). PPADS (100 |H9252|/H9251|) did not inhibit ATP-dependent relaxation in either prometamorphic (10 |H9252|/H9251| ATP, \( n = 6 \)) or juvenile animals (1,000 |H9252|/H9251| ATP, \( n = 5 \), data not shown) (metamorphic tadpoles were not tested).

Rank order of potency summary. In prometamorphic and metamorphic animals, the potency order for relaxation in intestinal muscle strips was CPA > ATP > adenosine although with respect to contraction frequency, it was CPA > ATP = adenosine in prometamorphic animals and adenosine > ATP = CPA in metamorphic tadpoles. In juvenile animals, the potency order for relaxation was CPA > ATP\(_{\gamma S} = 2\)-MeSATP > ATP = adenosine.

Before and during metamorphism, the potency order for contractile effects was 2-MeSATP = α-β-MeATP > ATP\(_{\gamma S} > ATP and in juvenile froglets, the potency order was α-β-MeATP > ATP\(_{\gamma S} > ATP.

**DISCUSSION**

This is, to my knowledge, the first study examining the changes in purinergic modulation of gastrointestinal motility during metamorphosis in an anuran species.

The activity of untreated preparations was examined to determine the general changes in spontaneous activity during metamorphosis. The increase in force developed by the smooth muscle preparation found in the intestine after metamorphosis can probably be explained by the increase in thickness in the circular muscular layer during this time (21, 30). Carbachol had approximately the same potency in all preparations, but the efficacy was increased after metamorphosis. This increase in efficacy may be due to an increase in the number of muscarinic receptors, a more efficient signaling transduction pathway, or simply that an increased muscle thickness can exert higher levels of mean force in response to cholinergic signaling.

The experiments using ATP revealed a dual response in intestinal muscle strips isolated before and during metamorphosis. Low concentrations of ATP elicited relaxation, while
P2Y1-receptor-mediated response. The ATP-induced relaxation was blocked using the ecto-nucleotidase inhibitor ARL67156, indicating that a dephosphorylated metabolite derived from ATP metabolism mediates the relaxing response. In contrast, an ATP-induced decrease in contraction frequency was still remaining after treatment with ARL67156, suggesting that the inhibitory response of ATP on contraction frequency is at least partly mediated by ATP via a P2 receptor. However, it is also possible that higher concentrations of ARL67156 are required to completely block metabolite production. The P2-receptor antagonist PPADS did not affect ATP-induced relaxation, indicating that the relaxation is less likely to be mediated by any of the P2Y-like receptors that are PPADS-sensitive (for example P2Y1). Further, administration of the stable ATP-analog ATPγS did not cause relaxation in prometamorphic tadpoles, supporting the view that a degraded product from ATP metabolism mediates the relaxing effect.

A likely candidate for mediating the inhibitory effects of ATP before metamorphosis is adenosine. Adenosine per se induced relaxing effects in all stages tested. The involvement of adenosine receptors was further supported by administration of the adenosine A1 receptor agonist CPA, which was more potent than either adenosine or ATP in relaxing intestinal muscle strips. Also, the adenosine kinase inhibitor ABT-702 inhibited spontaneous activity in the intestine, suggesting that there is an endogenous pool of adenosine that can inhibit motor activity unless phosphorylated. The potency of CPA in relaxing the intestinal muscle strips was similar before and after metamorphosis, suggesting that the decrease in potency seen for adenosine after metamorphosis could reflect an increase in adenosine kinase enzyme levels or activity in the tissue, rather than changes in receptor expression. The A1-specific antagonist DPCPX blocked adenosine-induced relaxation, further supporting the evidence suggesting that the P1-receptor mediating adenosine-induced relaxation in the Xenopus intestinal circular muscle is A1-like. The A2B-receptor antagonist PSB1115 had no effect. It is interesting to note, however, that another P1 antagonist, DPPX, which is somewhat similar to DPCPX, did not inhibit adenosine-induced relaxation. Thus, there seem to be potential differences in antagonist affinity to adenosine receptors in amphibians compared with mammals. Adenosine has been found to relax vessels in a preparation of isolated aorta from the amphibian Rana temporaria (20) and in this study, the effects of adenosine were not affected by the nonselective adenosine receptor antagonist 8-p-sulphophenylthetheophylline. Also, in the study by Knight and Burnstock (20), the A1 receptor agonist CPA was without effect, suggesting that the receptors in the amphibian vessels and intestine differ or that the receptors diverge between the amphibian species Rana and Xenopus.

P2Y1-receptor-mediated response. In contrast to prometamorphic intestine, ATPγS elicited a dual response in juvenile intestinal muscle, indicating the presence of two types of functional P2 receptors after metamorphosis, one receptor mediating relaxation and the other mediating contraction. In addition, 2-MeSATP attenuated both mean force and contraction frequency at this stage. Furthermore, during metamorphosis, there was a marked decrease in contraction frequency at a specific dose-interval of ATPγS (1–10 μM) and 2-MeSATP (1 μM), which was not present prior to metamorphosis. The fact that ARL67156 could not completely block ATP-evoked relaxation in juvenile tissues lends further support to the development of a P2Y-mediated relaxing response during metamorphosis. Moreover, the ATPγS-induced relaxation seen in juveniles could be antagonized using the P2Y1/P2X1 receptor agonist NF157. Because ATPγS is potent at P2Y11 receptors, it seems plausible that the developing P2Y receptor found is P2Y11-like. Although this receptor has not been identified in rodents, it has recently been cloned in Xenopus (gene accession no. AM040941). ATP has a lower potency for the P2Y11 receptor than ATPγS (1), which is consistent with the potency order seen in juveniles in the present study. Although ATPγS also has been shown to bind to P1 receptors (see Ref. 28), it seems less likely that the ATPγS-evoked relaxation is mediated by P1 receptors since the response was blocked by NF157. That PPADS did not block at least part of the ATP-induced relaxing response in juveniles further supports the existence of a P2Y11-like receptor, since this receptor is PPADS insensitive (32). Changes in the purinergic system have been reported to occur in both murine and rat gut at the time of weaning. Weaning occurs around day 20 in the life of a rat and is coupled to a number of other developmental changes in the gastrointestinal tract adapting the animal to ingestion of a more carbohydrate-
rich diet. For example, the intestinal levels of enzymes like maltase and sucrase, relating to the degradation of carbohydrates, increase dramatically during weaning, while lactase levels decrease. These developmental changes are regulated by thyroid hormones and cortisone (14, 35). Similarly, the metamorphic remodeling of the gastrointestinal tract in amphibians is regulated by thyroid hormones (10) and might also be influenced by corticosteroids (34). Although the...

Fig. 7. Responses to ATP$\gamma$S in intestinal smooth muscle in prometamorphic, metamorphic, and juvenile Xenopus. For concentration-response curves, statistical analysis was performed using repeated-measures one-way ANOVA followed by Dunnett’s post hoc test. A: representative tracing of the excitatory response to ATP$\gamma$S in prometamorphic tadpoles. B: representative tracing of the biphasic response to ATP$\gamma$S in metamorphic tadpoles. C: representative tracing of the biphasic response to ATP$\gamma$S in juvenile froglets. D: concentration-dependent increase in mean force was obtained with ATP$\gamma$S ≥ 10 μM in prometamorphic tadpoles (n = 10), ≥ 100 μM in metamorphic tadpoles (n = 10) and in juvenile froglets at 300 μM (n = 18), P < 0.05. For metamorphic tadpoles a slight decrease in mean force was seen at 3 μM (nonsignificant) and for juvenile froglets at 10 μM, P < 0.05. E: in metamorphic tadpoles a decrease in contraction frequency was obtained with ATP$\gamma$S at 1–10 μM and in juvenile froglets a decrease in contraction frequency was obtained ≥ 3 μM, P < 0.05. F: NF157 antagonizes the ATP$\gamma$S concentration-dependent decrease in mean force in froglets (n = 6). G: NF157 antagonizes the ATP$\gamma$S concentration-dependent decrease in contraction frequency in juvenile froglets (n = 6). Statistical significance for NF157 effects (F and G) was calculated using an unpaired Student’s t-test for each concentration. *P < 0.05. C1 and C2 on the x-axis denote control values before and after antagonist administration.
change in nutrient contents of the food for the developing frog differs from that of the rat, metamorphosis is still a transition time to solid food, and it is possible that similar changes in the intestinal purinergic system occur to regulate motor activity.

**P2X-receptor-mediated response.** The excitatory effect of high doses of ATP was only seen before and during metamorphosis and was enhanced by administration of ARL67156 in metamorphic tadpoles, suggesting that this effect was indeed evoked by ATP per se. ATPyS also elicited increased mean force in these stages, as did the P2X-selective (22) compound α-β-MeATP, suggesting that the receptor mediating ATP-evoked contractions is likely to be P2X1- or P2X3-like.

That the P2 receptor antagonist PPADS blocked α-β-MeATP-induced contractions in both prometamorphic and juvenile animals further supports the existence of a P2X1- or P2X3-like receptor. It is, however, difficult to pharmacologically distinguish between the different P2X receptors, and it should also be taken into account that the receptors can form heteromers of different subunits in vivo. Therefore, the receptor found in this study will be referred to as P2X-like. Previously, a contracting P2X-like receptor has been found on the isolated aorta of the amphibian *Rana temporaria* (20). 2-MeSATP also elicited contractions before and during metamorphosis in muscle strips with a similar potency as α-β-MeATP. Recent data indicate that 2-MeSATP is not only an agonist at P2Y-receptors but also acts as an agonist at P2X-receptors (17), which might explain the results achieved in the current study. In juvenile froglets, 2-MeSATP primarily evoked relaxation but also acts as an agonist at P2X-receptors (17), which could be blocked by TTX at a concentration previously shown to be effective in gastrointestinal tissue isolated from *Xenopus* (18). Thus, P1 and P2 receptors appear to be situated directly

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**Fig. 8.** Responses to 2-MeSATP and α-β-MeATP in intestinal smooth muscle in prometamorphic, metamorphic, and juvenile *Xenopus*. For concentration-response curves, statistical analysis was performed using repeated measures one-way ANOVA followed by Dunnett’s post hoc test. A: concentration-dependent increase in mean force was obtained with 2-MeSATP ≥ 30 μM in prometamorphic (n = 9) and metamorphic (n = 5) tadpoles, P < 0.05. In juvenile froglets (n = 11) a concentration-dependent decrease in mean force was obtained with 2-MeSATP ≥ 30 μM (n = 11), P < 0.05. B: in metamorphic tadpoles and juvenile froglets, a decrease in contraction frequency was obtained at 1 μM and 100 μM, respectively, P < 0.05. C: concentration-dependent increase in mean force was obtained with α-β-MeATP ≥ 10 μM in prometamorphic (n = 10), metamorphic (n = 6), and juvenile (n = 9) *Xenopus*, P < 0.05. D: An increase in contraction frequency was obtained in juvenile froglets with α-β-MeATP ≥ 10 μM, P < 0.05.

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**Fig. 9.** Responses to α-β-MeATP in the absence and presence of PPADS. Minus signs indicate no compound added and plus signs indicate the presence of compound. Statistical significance was calculated using a one-way ANOVA followed by Bonferroni’s post hoc test. A: significant effect of PPADS on α-β-MeATP-induced contractions. For 30 μM PPADS prometamorphic animals, n = 6, juveniles n = 7, and for 100-μM PPADS prometamorphic animals n = 5, juveniles n = 8.
on the muscle fibers although the potential involvement of TTX-insensitive nerves cannot be excluded (5).

In conclusion, this study presents evidence that adenosine mediates relaxation via an A<sub>1</sub>-like receptor in Xenopus intestinal smooth muscle both before and after metamorphosis. The existence of a P2X-like receptor mediating ATP-evoked contractions is also demonstrated. Furthermore, the results also show the ontogeny of a P2Y-mediated relaxing response at metamorphosis. The finding that a P2Y-receptor-mediated relaxing mechanism develops during the transition from liquid to solid food intake seems to be evolutionary conserved, indicating the importance of purinergic control of intestinal motility as a means to adapt to new physiological situations. In contrast to similar conditions in rodents, the developing receptor in Xenopus seem to be P2Y<sub>11</sub>-like rather than P2Y<sub>1</sub>-like.

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