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Changes in the control of gastric motor activity during metamorphosis in the amphibian *Xenopus laevis*, with special emphasis on purinergic mechanisms

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SUMMARY

The stomach of the amphibian *Xenopus laevis* is subject to extensive remodelling during metamorphosis. We investigated the changes in gastric activity control during this period using *in vitro* circular smooth muscle preparations mounted in organ baths. The nitric oxide synthase inhibitor L-NAME increased mean force in metamorphic and juvenile frogs but not in prometamorphic tadpoles. Serotonin (5-HT) relaxed stomach muscle prior to metamorphosis but elicited a biphasic response in juveniles consisting of contraction at low concentrations and relaxation at high concentrations. The effects of 5-HT were blocked by methysergide. In the prometamorphic tadpole, ATP elicited relaxation that was blocked by the ectonucleotidase inhibitor ARL67156 and the adenosine A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), suggesting adenosine as the mediator. Exogenous adenosine and the A_1 receptor agonist N^6-cyclopentyladenosine (CPA) induced relaxation at all stages. After metamorphosis, the potency of ATP decreased and neither DPCPX nor ARL67156 could block ATP-induced relaxation. Uridine 5′-triphospate (UTP) induced relaxation prior to metamorphosis, but caused contraction of muscle strips from metamorphosing tadpoles. Single doses of UTP blocked phasic contractions in juveniles in a tetrodotoxin (TTX)-sensitive manner while the simultaneous increase in muscle tension was TTX insensitive. The $P2X_1/P2X_3$ receptor agonist α-β-MeATP elicited pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS)-sensitive contractions at all stages investigated. These results indicate the development of an inhibitory nitrergic tonus during metamorphosis and a 5-HT receptor involved in muscle contraction. Also, the development of UTP receptors mediating increased tension and neural UTP receptors decreasing contraction frequency in juveniles is indicated. An adenosine A_1-like receptor mediating relaxation and a P2X-like receptor mediating contraction is demonstrated at all stages.

Key words: metamorphosis, development, amphibian, *Xenopus laevis*, 5-HT, nitric oxide, adenosine, UTP, ATP, purinergic.

INTRODUCTION

During amphibian metamorphosis, the gastrointestinal tract is subject to dramatic changes as the herbivorous tadpole adapts to a new feeding behaviour as a carnivorous frog. In the stomach, these changes include considerable growth of the muscular layers and remodelling of the gastric mucosa (Ishizuya-Oka and Ueda, 1996; Rovira et al., 1995; Schreiber et al., 2005; Sundqvist and Holmgren, 2004). Previously, we have shown that the expression of neurotrophin-like receptors is increased in the myenteric plexus during metamorphic climax (Sundqvist and Holmgren, 2004), suggesting that the enteric nervous system also undergoes modifications at this stage. Studies of the earliest developmental stages of the tadpole indicate that the control of gastrointestinal motility during these stages differs from that of adult gut (Sundqvist and Holmgren, 2006). Thus, changes in the regulation of gut motility are likely to take place during metamorphosis.

In adult *Xenopus* stomach, the transmitters pituitary adenylate cyclase-activating polypeptide, vasoactive intestinal peptide (VIP) and nitric oxide (NO) relax circular stomach muscle preparations (Olsson, 2002). Furthermore, the nitric oxide synthase (NOS) inhibitor L-NAME increases contractions in *Xenopus* stomach suggesting that NO exerts an endogenous inhibitory tonus in the preparations. In newly hatched tadpoles from *Xenopus* [stage 43 according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967)], VIP and NO decreased gastrointestinal motility; however, tonic nitrergic activity was absent (Sundqvist and Holmgren, 2006). Serotonin (5-hydroxytryptamine, 5-HT), another known modulator of gastric activity, has been shown to have a biphasic effect in adult *Xenopus* stomach (Johansson, 2003). Low concentrations elicit excitatory responses, while high concentrations elicit relaxation, probably indicating the presence of more than one 5-HT receptor in the tissue. The effect of 5-HT on gastric motor activity has not been studied in *Xenopus* tadpoles.

In the *Xenopus* intestine, changes in the regulation of motor activity by the purinergic system occur during metamorphosis (Sundqvist, 2007). The purinergic system affects motor function in the gut of many vertebrate species *via* purinoceptors (Burnstock, 1996; Burnstock, 2001). Responses to adenosine are mediated through metabotropic P1 receptors (A_1, $A_{2A\text{-}B}$, A_3). Receptors that are activated by ATP, ADP, UTP or UDP are called P2 receptors and consist of two subfamilies, the metabotropic P2Y [$P2Y_{1–2}$, $P2Y_4$, $P2Y_6$, $P2Y_{11–14}$; species specific: p2p3 (chicken), $p2y_8$ (*Xenopus*), tp2y (turkey)] receptors and the ionotropic P2X ($P2X_{1–7}$) receptors (Burnstock, 2006; von Kugelgen and Wetter, 2000). In *Xenopus*, the adenosine A_1 receptor (gene accession number: AJ249842) has been cloned along with a number of receptors for ATP including $P2Y_1$, $p2y_8$, $P2X_4$, $P2X_7$ (Bogdanov et al., 1997; Cheng et al., 2003; Juranka et al., 2001; Paukert et al., 2002) and $p2y_{11}$ (gene accession number: AM040941).

In a previous study performed in our laboratory (Sundqvist, 2007), adenosine relaxed intestinal smooth muscle strips from *Xenopus* both prior to and after metamorphosis, while ATP caused both relaxing and contracting responses. However, prior to metamorphosis, the ATP-evoked relaxing response appeared to be mediated by adenosine (from metabolized ATP) acting on A_1 receptors, while after metamorphosis the response to ATP was at least partly mediated by ATP *per se* acting directly on a $P2Y_{11}$-like receptor. Similarly, changes in the expression pattern or activity of the $P2Y_1$ receptor occur in the rodent stomach and intestine during late development. Interestingly, in longitudinal smooth muscle of rodents, $P2Y_1$ receptors switch from mediating contracting responses to mediating relaxing responses around the time of weaning (Brownhill et al., 1997; Furukawa and Nomoto, 1989; Giaroni et al., 2006; Hourani, 1999; Nicholls et al., 1990). This may be an adaptation to the altered content of nutrients in the food, when a more carbohydrate-containing food source replaces the lipid-rich diet of the young pups (Giaroni et al., 2006; Hourani, 1999).

Since our previous studies suggest changes in the control of intestinal motility during late development, the aim of this study was to investigate the changes, if any, in the control of gastric motor activity immediately around and during amphibian metamorphosis when the food intake of the animal changes from herbivorous to carnivorous and the gastrointestinal tract undergoes adaptive remodelling. We therefore studied the effect of a number of different neurotransmitters on gastric activity in stomach muscle strips prior to, during and after metamorphosis, with special emphasis on the purinergic system. The results show distinct changes in the control of gastric activity during metamorphosis including the development of a nitrergic tonus, a 5-HT receptor involved in muscle contraction and a switch in the smooth muscle response to UTP resulting in muscle contraction as well as the possible development of neural UTP receptors.

MATERIALS AND METHODS

Animals

Tadpoles from the African clawed frog, *Xenopus laevis* Daudin, were obtained according to a previously described procedure (Sundqvist, 2007). Briefly, adults (obtained from Nasco, Fort Atkinson, WI, USA) were injected with chorionic gonadotropin and allowed to breed. The fertilized eggs developed into tadpoles and were kept until metamorphosis in plastic aquariums. Tadpoles and froglets were fed 5 days a week on suitable *Xenopus* diets (Blades Biological Ltd, Edenbridge, UK). All experimental procedures were approved by the animal ethics committee of the city of Göteborg.

Drugs

Adenosine hemisulphate salt (adenosine), adenosine 5′-triphosphate (ATP), adenosine 5′-[γ-thio]-triphosphate tetralithium salt (ATPγS), carbamoylcholine chloride (carbachol), α-β-methyleneadenosine 5′-triphosphate lithium salt (α-β-meATP), *N*-nitro-L-arginine methyl ester hydrocloride (L-NAME), serotonin (5-hydroxytryptamine, 5-HT) and uridine 5′-triphosphate (UTP) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). 4-Amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-*d*]pyrimidine (ABT-702), N^6-cyclopentyladenosine (CPA), 6-*N*,*N*-diethyl-D-β,γ-dibromomethylene ATP trisodium salt (ARL67156), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and tetrodotoxin (TTX) were obtained from Tocris Cookson Ltd (Bristol, UK). Human neurokinin A (NKA) was obtained from Euro-Diagnostika (Malmö, Sweden) and methysergide maleate from Sandoz (Holzkirchen, Germany).

Stock solutions of all compounds except ABT-702 and DPCPX were created by dissolving the compound in Millipore water and then stored in aliquots at –20°C. ABT-702 and DPCPX were dissolved in DMSO to stock concentrations of 10 mmol l^{-1}. Further dilution of all compounds was made in McKenzie's amphibian Ringer solution.

Experimental procedure

The animals were anaesthetized and killed by immersion in a $NaHCO_3$-buffered solution of 0.05% MS 222 (3-aminobenzoic acid ethyl ester, Sigma) and the developmental stage was determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Prometamorphic tadpoles were taken at stages 56 and 57, while metamorphic tadpoles were taken at stages 61–63 and juveniles were at stage 66.

The stomach was dissected out and muscle strips from the cardiac stomach were prepared and mounted in organ baths as previously described for intestinal strips (Sundqvist, 2007). Briefly, ring-formed sections (~2–3 mm wide) were cut from the stomach and the circular muscle strips were then mounted in 10 ml organ baths containing 5 ml McKenzie's amphibian Ringer solution (NaCl 115 mmol l^{-1}, $NaHCO_3$ 20 mmol l^{-1}, Hepes 5.0 mmol l^{-1}, KCl 3.2 mmol l^{-1}, $MgSO_4$ 1.4 mmol l^{-1}, $CaCl_2$ 1.3 mmol l^{-1}, pH 7.8, 23°C) bubbled with gas (0.3% CO_2 in air). After preliminary testing, the prometamorphic and metamorphic muscle strips were stretched to a force of 2 mN (since higher forces caused them to tear) while juvenile muscle strips were stretched to 5 mN to account for the differences in muscle layer development and strength. The muscle strips were allowed to equilibrate for 1 h before the experiments were started. The Ringer solution was changed every 30–45 min during the experiment. The mean force developed from the muscle strips was measured using Grass FT03 force transducers (Astro-Med House, Slough, UK) and recorded by a Grass amplifier coupled to a computer running a custom-made program called General Acquisition (Labview version 6.01, National Instruments, Austin, TX, USA).

Spontaneous activity was recorded for 5–10 min after which drugs were added in a cumulative manner to construct a concentration–response curve. In some inhibitory concentration–response experiments, papaverine (100 μmol l^{-1}) was added to obtain total relaxation and facilitate calculation of EC_{50} values. In the antagonist and TTX experiments, the agonist (5-HT, ATP, adenosine or α-β-meATP) was added as a single concentration in the absence or presence of TTX or antagonist. After the agonist response had been recorded for a minimum of 5 min, or until a maximum response was obtained, the agonist was washed out and antagonist was administered. The antagonist was allowed to equilibrate for 20–30 min before another single concentration of the same agonist was added. Potassium chloride (80 mmol l^{-1}) was added at the end of experiments to check the viability of the muscle strips.

Data analysis and statistics

The mean force elicited by the muscle strips was measured over 200 s using a Labview-based analysis program. The mean force parameter was chosen since it reflected most changes seen in the preparations. However, in the experiments using UTP and TTX, the frequency was also analysed since this parameter was affected differently to the mean force parameter. To normalize the mean force

values, the resting tension value during the control period was subtracted from all values in an experiment. Further calculations and statistical analyses were performed in Excel and Graphpad Prism 4.0 (GraphPad Software, San Diego, CA, USA), respectively. EC_{50} values from each individual experiment were calculated using a sigmoidal dose–response model in GraphPad Prism 4.0, although when the plateau phase was not reached (see respective figures) with the available concentrations, the values should only be considered as tentative. This is indicated by using the sign for larger than or equal to ($\geqslant$) in the text. The figures show the curve from the combination of all experiments, which is why the calculated EC_{50} values sometimes deviate from the midpoint seen in the curve. Experiments were analysed using repeated measures one-way analysis of variance (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, depending on the experiment type. The statistical method used for each experiment is indicated in the figure legend. Results are presented as means ± s.e.m., and $P<0.05$ is considered statistically significant.

RESULTS

Spontaneous activity

All of the juvenile muscle strips developed spontaneous activity consisting of a basic tonus with superimposed phasic contractions shortly after mounting the preparation in the organ bath. A regular contraction frequency was more common in muscle strips from the distal stomach regions (56%) compared with strips taken from more proximal regions (14%). In contrast, 38% of prometamorphic and 16% of metamorphic stomach strips were sometimes inactive. The amplitude of the contractions seen in prometamorphic and metamorphic strips was smaller compared with the amplitude seen in the juvenile strips (0.02±0.004 mN prometamorphosis, 0.12±0.03 mN metamorphosis and 0.25±0.03 mN juvenile, $P<0.05$, $N=9$), although the contraction profile of individual muscle strips varied. On the whole, the mean force developed during basal conditions in stomach circular muscle strips increased significantly in juvenile froglets compared with prometamorphic and metamorphic tadpoles (Fig. 1A).

Carbachol

Administration of the cholinergic agonist carbachol (0.001–100 μmol l^{-1}) resulted in a concentration-dependent increase in mean force developed in smooth muscle from all three stages (Fig. 1B). The potency of carbachol was similar at all stages (EC_{50}: 0.5±0.2 μmol l^{-1} prometamorphosis, 1.4±0.3 μmol l^{-1} metamorphosis and 0.9±0.6 μmol l^{-1} juvenile).

NKA

The tachykinin NKA (0.0001–1 μmol l^{-1}) elicited a concentration-dependent increase in mean force at all stages (Fig. 1C). The potency of NKA was in the same range after metamorphosis as prior to or

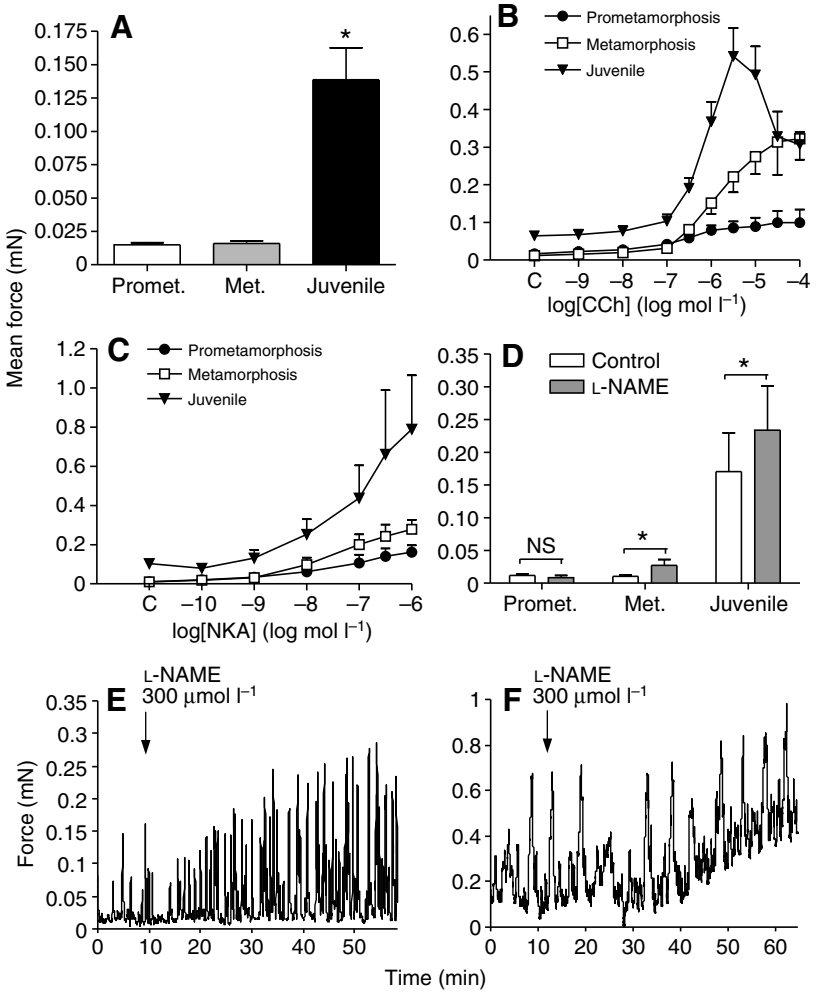

Fig. 1. (A) Mean force developed in gastric smooth muscle under basal conditions in prometamorphic (Promet.), metamorphic (Met.) and juvenile *Xenopus*. Statistical significance was calculated using a one-way ANOVA followed by Bonferroni's *post hoc* test ($N=28$). (B) Concentration-dependent increase in mean force in response to carbachol (CCh). Statistically significant increase was obtained at $\geqslant$1 μmol l^{-1} at all stages ($N=6$). (C) Concentration-dependent increase in mean force in response to neurokinin A (NKA). A statistically significant increase was obtained at $\geqslant$0.1 μmol l^{-1} for prometamorphosis ($N=7$) and metamorphosis ($N=8$), and at $\geqslant$1 μmol l^{-1} for juveniles ($N=6$). For concentration–response curves, statistical analysis was performed using repeated measures one-way ANOVA followed by Dunnett's *post hoc* test. Control value prior to administration of agonist is denoted C on the *x*-axis. (D) Effect of 300 μmol l^{-1} *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME) administered 20–30 min prior to measurements in prometamorphosis ($N=7$), metamorphosis ($N=8$) and juveniles ($N=9$). Asterisk indicates a statistically significant difference, $P<0.05$. (E,F) Representative traces of the response to L-NAME (300 μmol l^{-1}) in (E) metamorphic tissues and (F) juvenile tissues.

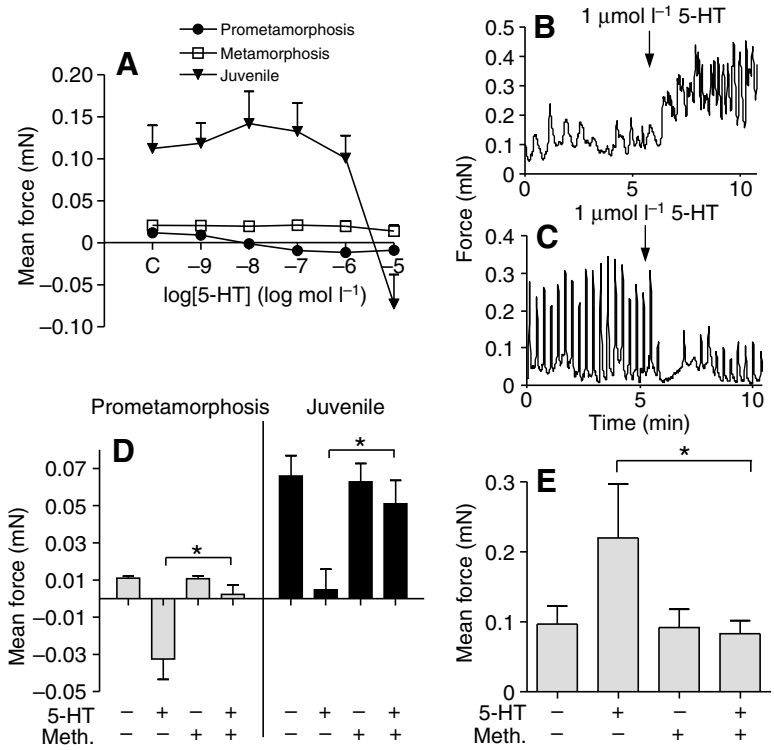

Fig. 2. Responses to 5-hydroxytryptamine (5-HT, serotonin) in stomach muscle. (A) Concentration-dependent effect on mean force in response to 5-HT. A statistically significant decrease was obtained at ⩾0.01 μmol l^{-1} in prometamorphosis (N=10) and at ⩾10 μmol l^{-1} in juveniles (N=8). There was no effect during metamorphosis (N=7). For concentration–response curves, statistical analysis was performed using repeated measures one-way ANOVA followed by Dunnett's *post hoc* test. (B) Representative trace of the response to an excitatory concentration of 5-HT (1 μmol l^{-1}) in juveniles. (C) Representative trace of the response to an inhibitory concentration of 5-HT (1 μmol l^{-1}) in juveniles. (D) Relaxing effect of 5-HT (1 μmol l^{-1}) in the presence and absence of the 5-HT receptor antagonist methysergide (Meth., 10 μmol l^{-1}) in prometamorphic (N=4) and juvenile (N=5) stomach muscle strips. (E) Contracting effect of 5-HT (0.1–1 μmol l^{-1}) in the presence and absence of the 5-HT receptor antagonist methysergide (10 μmol l^{-1}) in juvenile (N=12) stomach muscle strips. Statistical analysis was performed using repeated measures one-way ANOVA followed by Bonferroni's *post hoc* test. Asterisk indicates a statistically significant difference, P<0.05.

during metamorphosis (EC_{50}: 0.09±0.06 μmol l^{-1} prometamorphosis, 0.08±0.04 μmol l^{-1} metamorphosis and 0.4±0.2 μmol l^{-1} juvenile).

L-NAME

Inhibition of the NO-producing enzyme NOS using L-NAME (300 μmol l^{-1}) did not affect mean force prior to metamorphosis. In contrast, in both metamorphosing and juvenile animals an increase in mean force in stomach strips was recorded 20–30 min after L-NAME administration (Fig. 1D–F).

5-HT

Cumulative administration of 5-HT (0.001–10 μmol l^{-1}, Fig. 2A) elicited a concentration-dependent decrease in mean force in prometamorphic tadpoles (EC_{50} 0.31±0.26 μmol l^{-1}). Tissues taken from tadpoles during metamorphosis did not respond to 5-HT. Although not evident from Fig. 2A, which includes mean data from all experiments, tissues from juvenile froglets sometimes responded with an increased mean force at low concentrations of 5-HT followed by relaxation at higher concentrations, while in other juvenile preparations only relaxation at high concentrations could be demonstrated. The potency of 5-HT at inducing relaxations in the juvenile stomach strips was 10-fold lower than prior to metamorphosis (EC_{50} ⩾4.6±2.1 μmol l^{-1}).

The excitatory and inhibitory phases of the biphasic response of juvenile stomach strips were studied separately in another set of experiments. To determine a concentration giving only contraction for each muscle strip, low concentrations of 5-HT (between 0.01 and 1 μmol l^{-1}, Fig. 2B,E) were administered cumulatively until an increased mean force was seen, which occurred in eight out of 14 stomach strips. Contraction evoked by 5-HT was blocked by methysergide (Fig. 2E, P<0.05). The remaining six muscle strips relaxed in response to 5-HT, an effect also blocked by methysergide (Fig. 2C,D, P<0.05). A high concentration of 5-HT (10 μmol l^{-1}) always elicited relaxation.

Responses to ATP

ATP

Cumulative administration of ATP (0.01–1000 μmol l^{-1}) produced a biphasic response in muscle strips from prometamorphic tadpoles (Fig. 3A,B), with a significant decrease in mean muscle force occurring between 3 and 30 μmol l^{-1} (EC_{50} 5.4±4.1 μmol l^{-1}) and contraction occurring at the highest concentrations (300–1000 μmol l^{-1}). A single high concentration of ATP (1000 μmol l^{-1}) at this stage elicited contraction in the stomach strips, demonstrating that the contraction phase of the concentration–response curve is in fact a contraction and is not dependent on a possible desensitization or autoinhibition of the preceding inhibitory response. ATP did not affect muscle strips from tadpoles in metamorphic climax in a consistent manner, although a weak relaxation at low concentrations and a weak contraction at high concentrations could be seen in some preparations (Fig. 3A, P>0.05). In juvenile froglets, a decrease in mean force occurred at 300–1000 μmol l^{-1} ATP (EC_{50} ⩾233±99 μmol l^{-1}, Fig. 3A,C). The potency of ATP with regard to its relaxing effect was at least 40 times lower in juvenile animals compared with prometamorphic animals. A single high concentration of ATP (1000 μmol l^{-1}) in some cases (7 of 12 strips) elicited a dual response in juvenile stomach strips with a transient contraction peak preceding the relaxing response (Fig. 3D). Administration of TTX (1 μmol l^{-1}) did not affect the inhibitory effects of ATP in either prometamorphic (3 μmol l^{-1} ATP, N=7) or juvenile animals (1000 μmol l^{-1} ATP, N=7, data not shown).

ARL67156 and ATP

Administration of the ectoATPase inhibitor ARL67156 (100 μmol l^{-1}) 20 min prior to generating a cumulative concentration–response curve to ATP completely blocked the ATP-induced decrease in mean force seen in prometamorphic tadpoles (Fig. 4A). Instead, ATP had a potent stimulatory effect in the

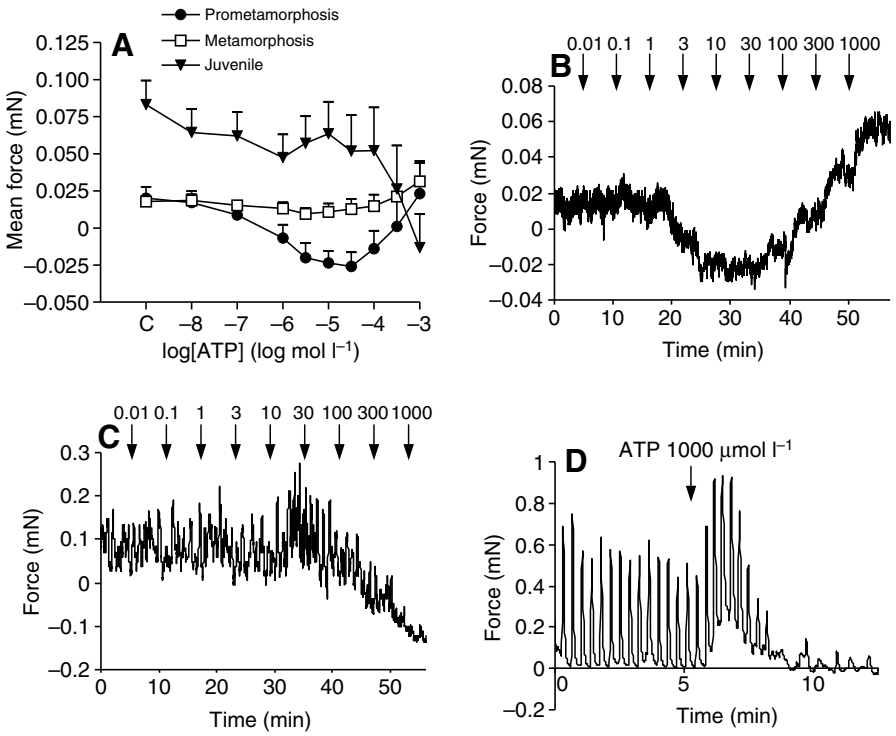

Fig. 3. Responses to ATP in stomach muscle preparations. (A) Concentration-dependent mean force response to ATP. A statistically significant decrease was obtained at $\geqslant$3 μmol l^{-1} in prometamorphosis (N=10) and at $\geqslant$300 μmol l^{-1} in juveniles (N=10). There was no effect in metamorphic tissues (N=8). Control value prior to administration of agonist is denoted C. Statistical analysis was performed using repeated measures one-way ANOVA followed by Dunnett's *post hoc* test. (B,C) Representative traces of the response to ATP in (B) prometamorphic tissues and (C) juvenile tissues (concentrations are in μmol l^{-1}). (D) Representative trace of the response to a single high concentration of ATP (1000 μmol l^{-1}).

presence of ARL67156 at this stage, resulting in a concentration–dependent increase in mean force (Fig. 4A). In juvenile stomach strips the decrease in mean force induced by ATP was only partly blocked by ARL67156; however, the response was shifted to higher concentrations (Fig. 4B).

DPCPX and ATP

To further elucidate whether the ATP-elicited relaxing response was mediated by metabolites acting on P1 receptors, the selective adenosine A_1 receptor antagonist DPCPX (1 μmol l^{-1}) was administered 20 min prior to ATP in prometamorphic and juvenile *Xenopus*. In prometamorphic animals, DPCPX abolished the relaxing response to ATP (Fig. 4C). In juvenile froglets, 1 μmol l^{-1} DPCPX did not affect ATP-evoked relaxation (data not shown). At 3 μmol l^{-1}, DPCPX *per se* appeared to attenuate contractions (possibly because of the 0.03% DMSO vehicle); however, it was not able to inhibit the ATP-induced relaxation (Fig. 4D).

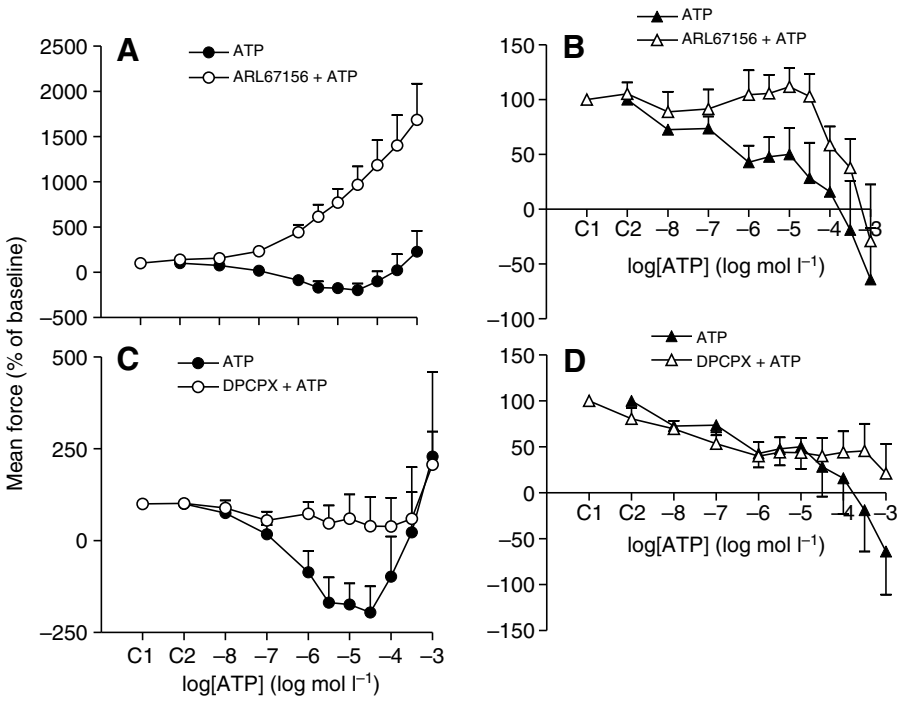

Fig. 4. (A,B) Responses to adenosine 5′-triphosphate (ATP) in the absence and presence of the ectonucleotidase inhibitor 6-*N*,*N*-diethyl-D-β,γ-dibromomethylene ATP (ARL67156, 100 μmol l^{-1}) in (A) prometamorphic tissues (N=8) and in (B) juveniles (N=9). A statistically significant difference between responses was obtained at $\geqslant$0.01 μmol l^{-1} in prometamorphosis and between 1 and 10 μmol l^{-1} in juveniles. (C,D) Responses to ATP in the absence and presence of the A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) in (C) prometamorphic tissues (1 μmol l^{-1}, N=9) and in (D) juveniles (3 μmol l^{-1}, N=8). A statistically significant difference between responses was obtained between 3 and 30 μmol l^{-1} in prometamorphic tissues but not at all in juvenile tissues. Control value prior to administration of antagonist or enzyme blocker is denoted C1 and that prior to ATP administration as C2 on the x-axis (A–D). Statistical significance for ARL67156/DPCPX effects was calculated using Student's unpaired t-test for each concentration.

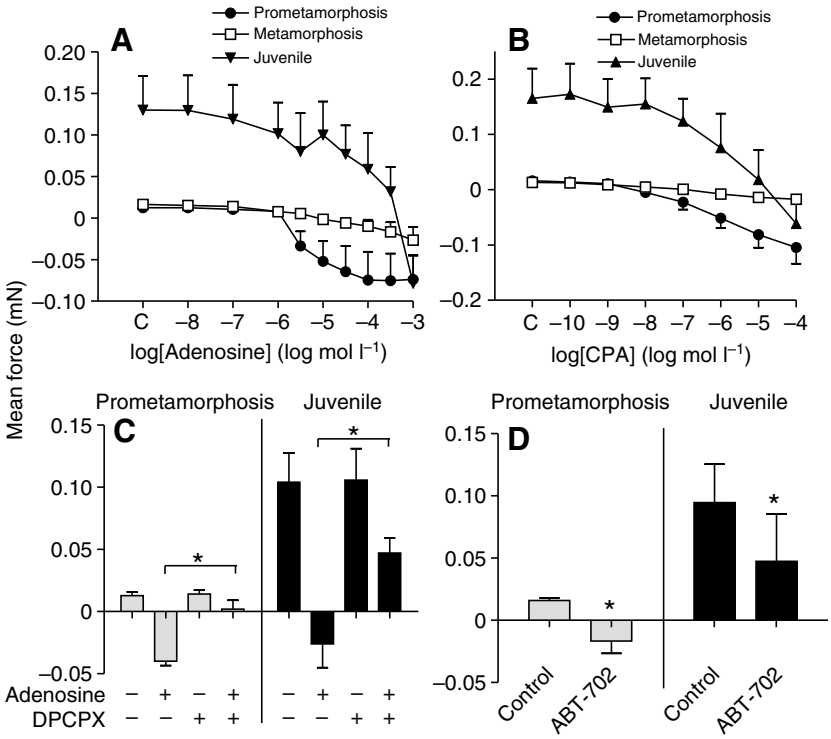

Fig. 5. (A) Concentration-dependent decrease in mean force in response to adenosine. A statistically significant decrease was obtained at ⩾10 μmol l^{-1} in prometamorphosis (N=12), at ⩾100 μmol l^{-1} in metamorphosis (N=6) and at ⩾300 μmol l^{-1} in juveniles (N=12). (B) Concentration-dependent decrease in mean force in response to N^6-cyclopentyladenosine (CPA) in prometamorphosis (N=8), metamorphosis (N=11) and juveniles (N=10). Statistically significant decrease obtained at ⩾1 μmol l^{-1} for all stages. Control value prior to administration of agonist is denoted C on the x-axis. Statistical analysis was performed using repeated measures one-way ANOVA followed by Dunnett's *post hoc* test (A,B). (C) Effect of adenosine in the presence and absence of the A_1 receptor antagonist DPCPX (1 μmol l^{-1}) in prometamorphic (100 μmol l^{-1} adenosine, N=4) and juvenile (1000 μmol l^{-1} adenosine, N=9) stomach muscle strips. Statistical analysis was performed using repeated measures one-way ANOVA followed by Bonferroni's *post hoc* test. Asterisk indicates P<0.05. (D) Effects of the adenosine kinase inhibitor 4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-*d*]pyrimidine (ABT-702, 1 μmol l^{-1}) on mean force developed under control conditions in prometamorphic (N=7) and juvenile (N=10) tissues. Statistical significance was calculated using Student's t-test for paired observations. Asterisk indicates P<0.05.

P1 receptor-mediated responses

Adenosine

Adenosine (0.01–1000 μmol l^{-1}) decreased the mean force developed in muscle strips from prometamorphic and metamorphic tadpoles in a concentration-dependent manner (EC_{50} 25±17 and 39±18 μmol l^{-1}, respectively, Fig. 5A). Adenosine also decreased mean force in stomach strips from juvenile froglets, although the potency was almost 10 times lower (EC_{50} ⩾188±76 μmol l^{-1}). Administration of TTX (1 μmol l^{-1}) did not block adenosine-dependent relaxation (1000 μmol l^{-1} adenosine) in either prometamorphic or juvenile animals (N=6 and N=7, respectively, data not shown).

CPA

The selective adenosine A_1 receptor agonist CPA (0.0001–100 μmol l^{-1}) produced a dose-dependent decrease in mean force developed in stomach strips from all stages (Fig. 5B). The potency of CPA was slightly higher before (EC_{50} ⩾0.6±0.3 μmol l^{-1}) than during and after metamorphosis (EC_{50} ⩾2.1±0.9 and ⩾3.2±2.0 μmol l^{-1}, respectively).

DPCPX and adenosine

The selective A_1 receptor antagonist DPCPX (1 μmol l^{-1}) blocked adenosine-induced relaxation in muscle strips from prometamorphic tadpoles (Fig. 5C), and attenuated the adenosine-induced relaxation in juvenile stomach strips by 50% (P<0.05). The vehicle (0.01% DMSO) had no effect on the muscle strip preparations.

ABT-702

Administration of the adenosine kinase inhibitor ABT-702 (1 μmol l^{-1}) caused a significant decrease in mean force in both prometamorphic and juvenile *Xenopus* (Fig. 5D). The vehicle (0.01% DMSO) had no effect on the muscle strip preparations.

P2 receptor-mediated responses

UTP

Administration of the P2Y receptor agonist UTP [most potent at $P2Y_2$, $P2Y_4$ and $P2Y_6$ (Abbracchio et al., 2006; Brunschweiger and Muller, 2006)] elicited a concentration-dependent decrease in mean force in muscle strips from prometamorphic tadpoles (EC_{50} 7.9±3.1 μmol l^{-1}, Fig. 6A,B). In contrast, muscle strips from metamorphic tadpoles responded to UTP with an increase in mean force (EC_{50} 30±13 μmol l^{-1}, Fig. 6A,C). In juveniles, UTP elicited a decrease in phasic contractions (Fig. 6D). However, this was often accompanied by an increase in tension resulting in a non-significant change in mean force (Fig. 6A).

Similarly, a single dose of UTP (100 μmol l^{-1}) elicited relaxation in prometamorphic stomach (Fig. 7A) and contraction in most metamorphic stomach strips (Fig. 7B). The response seen in juvenile stomach was clearer in single dose administrations than in the concentration–response curve and consisted of an increase in the basal tension level concurrent with abolished contractions (Fig. 7C). Administration of TTX (1 μmol l^{-1}) blocked the UTP-induced decrease in phasic contractions (Fig. 7C,D, P<0.05) but had no effect on the UTP-induced increase in basal tension (Fig. 7C,E) in juveniles. TTX *per se* had no significant effect on the frequency or mean force of contractions although there was a slight increase in the amplitude of contractions after TTX administration. TTX did not affect the UTP-induced effects in prometamorphic or metamorphic stomach preparations (Fig. 7A,B). Furthermore, L-NAME (300 μmol l^{-1}) also blocked the UTP-induced decrease in phasic contractions in juvenile stomach (Fig. 7F). L-NAME was not tested on UTP effects in prometamorphic or metamorphic stomach.

ATPγS

The stable ATP analogue ATPγS is particularly potent at $P2Y_2$, $P2Y_{11}$ and $P2X_5$ purinoceptors but is also an agonist at $P2Y_1$, $P2X_{1–4}$, $P2X_6$, $P2X_{1/5}$ and $P2X_{2/3}$ purinoceptors (Abbracchio et al., 2006;

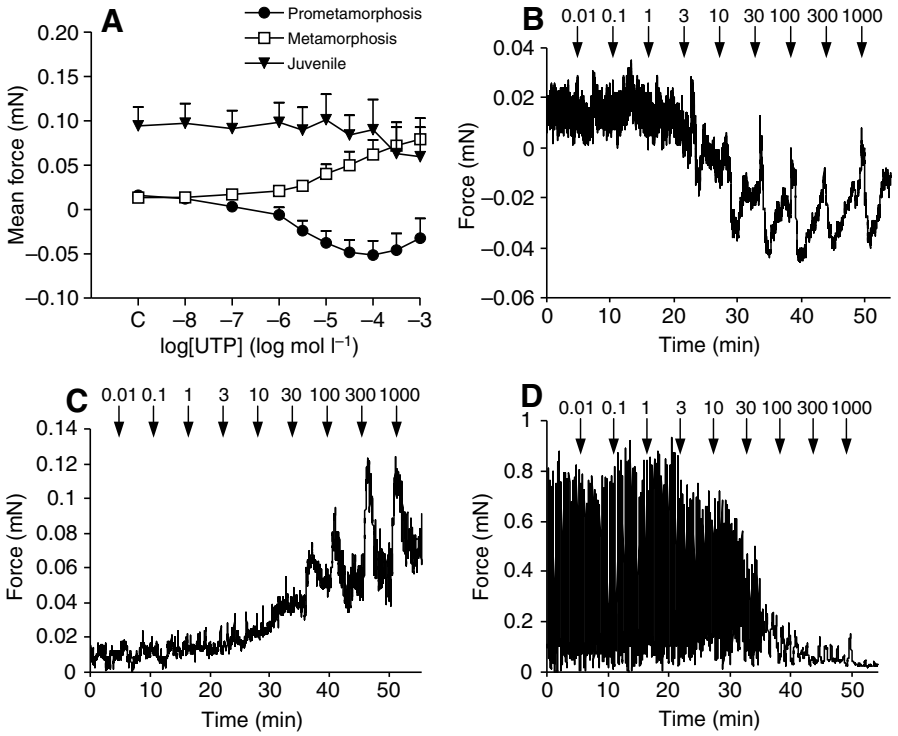

Fig. 6. Responses to uridine 5′-triphosphate (UTP) in stomach muscle preparations. (A) Concentration-dependent response of mean force to UTP. A statistically significant decrease was obtained at ⩾10 μmol l^{-1} in prometamorphosis (*N*=9) and in juvenile tissues at 1000 μmol l^{-1} (*N*=12). In metamorphic tissues a significant increase was obtained at ⩾30 μmol l^{-1} (*N*=6). Control value prior to administration of agonist is denoted C on the *x*-axis. Statistical analysis was performed using repeated measures one-way ANOVA followed by Dunnett's *post hoc* test. (B–D) Representative traces of the response to UTP in (B) prometamorphic tissues, (C) metamorphic tissues and (D) juvenile tissues (concentrations in μmol l^{-1}).

Lambrecht, 2000). Administration of ATPγS (0.1–300 μmol l^{-1}) resulted in a concentration-dependent increase in mean force developed in stomach strips from prometamorphic tadpoles (EC_{50} ⩾102±45 μmol l^{-1}, Fig. 8A) and tadpoles in metamorphic climax (EC_{50} ⩾618±232 μmol l^{-1}). Muscle strips from juvenile froglets only responded with a weak increase in mean force at the highest concentration (300 μmol l^{-1}) resulting in EC_{50} values too uncertain to be reported.

α-β-MeATP

Administration of the purinergic $P2X_1/P2X_3$-selective agonist α-β-MeATP (0.01–100 μmol l^{-1}) elicited a concentration-dependent increase in mean force at all three stages (EC_{50}: ⩾35±15 μmol l^{-1} prometamorphosis, ⩾69±27 μmol l^{-1} metamorphosis and ⩾198±69 μmol l^{-1} juveniles, Fig. 8B). TTX (1 μmol l^{-1}) failed to block the effect of α-β-MeATP in either prometamorphic tadpoles (10 μmol l^{-1} α-β-MeATP, *N*=6) or juvenile froglets (30 μmol l^{-1} α-β-MeATP, *N*=7, data not shown). Metamorphic tadpoles were not tested.

PPADS and α-β-MeATP

The P2 receptor antagonist PPADS (100 μmol l^{-1}), effective at homomeric $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$ receptors, heteromeric $P2X_{2/3}$ and $P2X_{1/5}$ receptors and $P2Y_1$, $P2Y_6$ and $P2Y_{13}$ receptors (Abbracchio et al., 2006; Jacobson and Knutsen, 2001), inhibited α-β-MeATP-induced contractions (Fig. 8C) in both prometamorphic tadpoles (10 μmol l^{-1} α-β-MeATP, $P<0.05$) and juvenile stomach strips (30 μmol l^{-1} α-β-MeATP, $P<0.05$). PPADS had no effect on ATP-induced relaxation at any stage tested (*N*=5, data not shown).

DISCUSSION

In this study, the control of gastric activity around the period of metamorphosis was investigated in the amphibian *Xenopus laevis*. Considerable changes in the response to L-NAME, 5-HT and purinergic agents during development were detected, as well as changes in spontaneous gastric motor activity. An increase in spontaneous activity of the stomach after metamorphosis resulting in a larger mean force measured in juvenile stomach muscle strips is most probably due to the increase in circular muscle layer thickness that occurs during this time period (Kordylewski, 1983; Sundqvist and Holmgren, 2004).

Carbachol and NKA

Carbachol, a cholinesterase-resistant analogue of the classical neurotransmitter acetylcholine, had a similar potency all through metamorphosis. The larger force elicited during the juvenile stage may again reflect an increase in smooth muscle layer thickness. The bell-shaped concentration–response curve obtained with carbachol after metamorphosis suggests a degree of receptor internalization or desensitization at higher concentrations that is not seen in the earlier stages. Alternatively, inhibitory muscarinic (M) receptors (e.g. of the M2-type) may be expressed during this stage. The consistently excitatory effect of the tachykinin NKA through metamorphosis, with possibly only a slightly lower potency after metamorphosis, supports previous studies of the *Xenopus* gut, both in newly hatched tadpoles (Sundqvist and Holmgren, 2006) and in adult *Xenopus* (Johansson et al., 2002). In the present study, mammalian NKA was used, since the study by Johansson et al. (Johansson et al., 2002) showed that there was no difference in potency or maximal effect between endogenous *Xenopus* NKA and mammalian NKA.

Nitrergic mechanisms

The NO donor sodium nitroprusside (SNP) inhibits contractions in newly hatched tadpoles (Sundqvist and Holmgren, 2006) and relaxes stomach muscle from adult frogs (Olsson, 2002). Neurons containing the NO-synthesizing enzyme NOS were detected in *Xenopus* gut from stage 46 (Holmberg et al., 2001). Following this stage, VIP-induced relaxation of the intestine was partially blocked

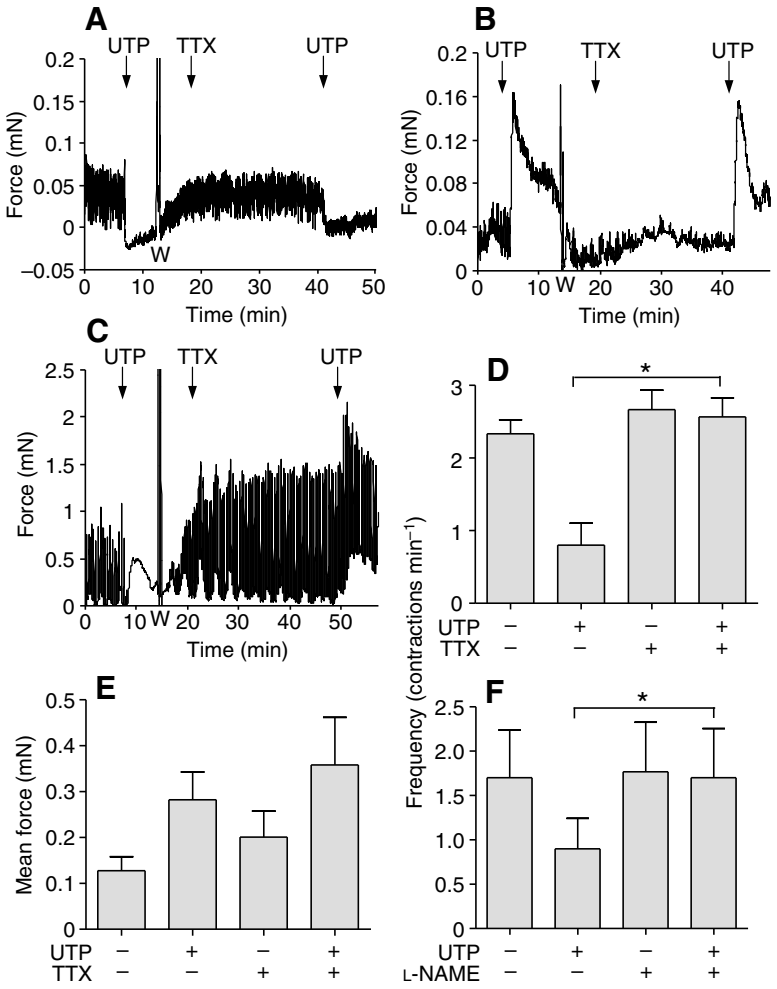

Fig. 7. (A,B) Representative trace of the tetrodotoxin (TTX)-insensitive responses to UTP (100 μmol l^{-1}) in (A) prometamorphic stomach and (B) metamorphic stomach. (C) Representative trace of the partially TTX-sensitive response to UTP (100 μmol l^{-1}) in juvenile stomach. (D,E) TTX (1 μmol l^{-1}) blocks the UTP-induced decrease in frequency (D, *N*=9) but not the increase in mean force (E, *N*=6) in juvenile stomach. (F) L-NAME (300 μmol l^{-1}) blocks the UTP-induced decrease in frequency (*N*=5) in juvenile stomach. Statistical analysis was performed using repeated measures one-way ANOVA followed by Bonferroni's *post hoc* test. Asterisk indicates $P<0.05$. W, wash-out.

by L-NAME, suggesting that endogenous NOS activity can be triggered by transmitters such as VIP (Sundqvist and Holmgren, 2006), although a constant NO tonus under basal conditions did not appear to be present. The current experiments in the stomach indicate the development of a nitrergic tone during metamorphosis, which could be important in modulating the increasingly complex, adult-like spontaneous activity that develops during metamorphosis. In the urodele amphibian the axolotl (*Ambystoma mexicanum*), nitrergic neurons develop late, during the first juvenile stages (Badawy and Reinecke, 2003).

5-Hydroxytryptaminergic mechanisms

The responses to 5-HT, with a relaxation prior to metamorphosis changing to a biphasic effect in juvenile stomach, indicate a change in 5-HT receptor populations during metamorphosis. Developmental changes have previously been found in 5-HT innervation and receptor ($5HT_{1A}$) expression during the early postnatal period in rat motor neurons (Talley et al., 1997) and in respiratory motor control in the amphibian *Rana catesbeiana* during metamorphosis (Kinkead et al., 2002). As in *Rana* respiratory control and in adult *Xenopus* gut (Johansson, 2003), the biphasic effect of 5-HT in the juvenile stomach is manifested as contraction at low 5-HT concentrations followed by relaxation at higher concentrations. The contraction could counteract a relaxation at low concentrations, explaining the apparent decrease in potency for 5-HT-induced relaxation in juveniles.

In mammals, several receptors are involved in gastric relaxation and contraction in response to 5-HT (Komada and Yano, 2007; Tamura et al., 1996; Xue et al., 2006). In the present study, the 5-HT_{1-2} and 5-HT_{5-7} receptor antagonist methysergide (Komada and Yano, 2007; Prins et al., 2001) inhibited all 5-HT-induced changes in tonus. Even though both types of response (after metamorphosis) were blocked, the relaxing and contracting effects could still be mediated by different receptors given the nonselective nature of methysergide. A previous study in our laboratory using TTX on *Xenopus* gut indicated that 5-HT receptors are situated mainly on smooth muscle (Johansson, 2003). The gastric mucosa of amphibians, both prior to and after metamorphosis, contains numerous enterochromaffin (EC) cells containing 5-HT (Sundqvist and Holmgren, 2004; Villaro et al., 2001), while 5-HT-containing neurons are reported to be absent in the amphibian (*Bufo marinus*) stomach (Anderson and Campbell, 1989). Surprisingly, muscle strips responded very weakly to 5-HT during metamorphosis, possibly due to receptor desensitization or internalization during a time when the EC cells, containing the major source of 5-HT in the tissue, are being reorganized.

P1 receptor-mediated purinergic mechanisms

The response to ATP elicited in prometamorphic *Xenopus* stomach was similar to responses previously described in the intestine (Sundqvist, 2007). The biphasic response indicates the existence of at least two receptors in the tissue. The relaxation is probably induced by an ATP-derived metabolite, since ATP can be rapidly metabolized by ectonucleotidases present on cell surfaces, and blockade of ectonucleotidases using ARL67156 completely abolished the response. That the more stable ATP analogue ATPγS only produced contraction at this stage lends further support to this hypothesis, along with the fact that the relaxing response to ATP could be blocked by the adenosine A_1 receptor antagonist DPCPX.

Blockade of ATP breakdown revealed an ATP-induced increase in mean force, which was not seen in the ATP + DPCPX experiments. We conclude that intact ATP may cause contraction in the stomach, while a metabolite such as adenosine is responsible for relaxation prior to metamorphosis.

The blockade of adenosine-induced relaxation by an A_1 receptor antagonist, while a selective A_1 receptor agonist (CPA) potently reduced mean force suggests that the receptor mediating the adenosine-induced relaxation is A_1-like prior to metamorphosis, as was previously shown in the intestine (Sundqvist, 2007).

The overall inconsistent response to ATP *per se* in the metamorphosing tadpole could be due to a rapid change in sensitivity during this stage. Some muscle strips taken in the beginning of the transition (stage 61) tended to respond similarly to prometamorphic tissues while some taken at the end (stage 63) tended to respond similarly to juvenile tissues, thereby resulting in an unchanged mean response during metamorphosis. As mentioned above, tissues collected from tadpoles during metamorphosis also responded inconsistently to 5-HT. Thus, metamorphosis appears to reflect a transition stage during the control of gastric motility.

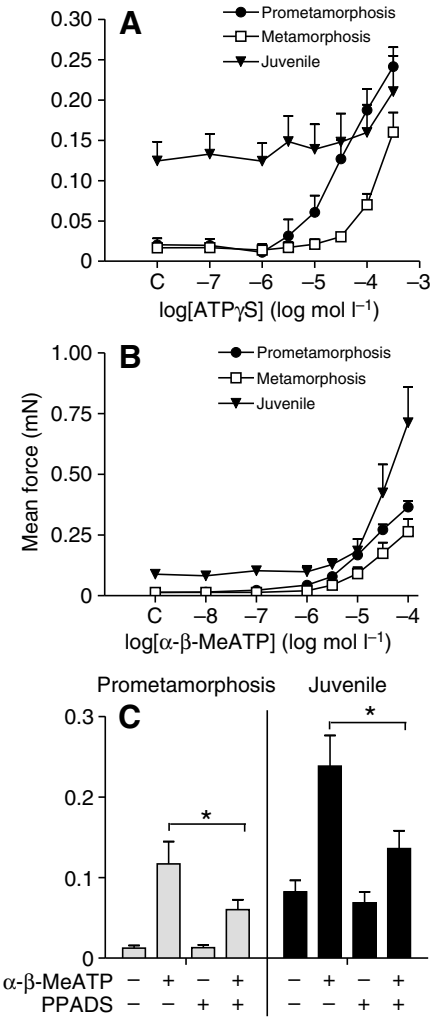

Fig. 8. Responses to adenosine 5′-[γ-thio]-triphosphate (ATPγS) and α-β-methyleneadenosine 5′-triphosphate (α-β-MeATP) in stomach muscle preparations. (A) Concentration-dependent increase in mean force in response to ATPγS. A statistically significant increase was obtained at ⩾30 μmol l^{-1} in prometamorphic (*N*=7), at ⩾100 μmol l^{-1} (*N*=9) in metamorphic and at 300 μmol l^{-1} in juvenile tissues (*N*=15). (B) Concentration-dependent increase in mean force in response to α-β-MeATP. Statistically significant increase obtained at ⩾10 μmol l^{-1} in prometamorphic (*N*=8) and at ⩾30 μmol l^{-1} (*N*=6) in metamorphic and juvenile tissues (*N*=10). Control value prior to administration of agonist is denoted C on the *x*-axis. Statistical analysis was performed using repeated measures one-way ANOVA followed by Dunnett's *post hoc* test. (C) Effect of α-β-MeATP in the presence and absence of the P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 100 μmol l^{-1}) in prometamorphic (10 μmol l^{-1} α-β-MeATP, *N*=7) and juvenile (30 μmol l^{-1} α-β-MeATP, *N*=8) stomach strips. Statistical analysis was performed using repeated measures one-way ANOVA followed by Bonferroni's *post hoc* test. Asterisk indicates $P<0.05$.

In the juvenile stomach, ATP-induced relaxation was only seen at the highest concentrations. Adenosine is probably not the only relaxing mediator, since the relaxation was only partly blocked by ectonucleotidase inhibition. The existence of relaxing P2 receptors may be suggested, but the results could also be explained by an increase in ectonucleotidase activity over-ruling the effect of the enzyme blocker. Similarly, the A_1 receptor antagonist (1 μmol l^{-1}) failed to block the ATP-induced relaxation. However, the same concentration only blocked 50% of adenosine-induced relaxation too, possibly indicating that higher concentrations are required or that another type of receptor is present in this tissue as well, for example A_3 receptors.

The decrease in tonus in both prometamorphic and juvenile animals after inhibition of adenosine kinase using ABT-702 indicates that the endogenous levels of adenosine are sufficient to elicit relaxation if inactivation of adenosine by phosphorylation is inhibited.

P2 receptor-mediated purinergic mechanisms

UTP is a potent agonist at $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, although its metabolite UDP is the most potent agonist known at $P2Y_6$ (Abbracchio et al., 2006). $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors have all been found in the stomach of different mammalian species (Chang et al., 1995; Communi et al., 1996; Giaroni et al., 2002; Moore et al., 2001; Suarez-Huerta et al., 2001; Van Nassauw et al., 2006). UTP has also been reported to be an agonist at $P2Y_{11}$ receptors (White et al., 2003). The *Xenopus*-specific receptor p2y8 (Bogdanov et al., 1997) also binds UTP and may be a $P2Y_4$ receptor orthologue (Abbracchio et al., 2006), but its presence in *Xenopus* stomach has not been investigated.

UTP had complex effects in *Xenopus* stomach. The response could conceivably be mediated by UDP but, in contrast to ATP, the instantaneous effect and the rapid decline of the response rather suggest that UTP itself is the effector. It should be noted that ATPγS is a potent agonist at $P2Y_2$ receptors but did not elicit comparable responses to UTP in this study, especially not prior to metamorphosis. Further studies using selective pharmacological tools are required to determine the receptor subtype(s) involved in the responses to UTP.

Interestingly, a submaximal concentration of UTP induced a transient increase in basal tension in juvenile stomach. The increase in tension was not seen prior to metamorphosis, suggesting that this effect of UTP develops at metamorphosis. The immediate decline in tension after the initial peak indicates desensitization or rapid metabolism of UTP. The insensitivity to TTX of the tension increase suggests that it is non-neuronal, due to direct stimulation of smooth muscle cells, in contrast to the simultaneous TTX-sensitive decrease in frequency of phasic contractions.

That TTX was only effective in juveniles suggests that the neuronal receptors responding to UTP develop after metamorphosis. It is possible that UTP stimulates inhibitory motor- or interneurons resulting in the release of inhibitory transmitters that block phasic contractions in the muscle. Indeed, this hypothesis was supported by the fact that the UTP-induced decrease in phasic contractions was blocked by NOS inhibition. Although a tonic NOS activity is present in these preparations, this activity seems to be relatively small. Conceivably, the removal of this tonus (by L-NAME or TTX) would not on its own result in the dramatic reversal of the UTP-induced inhibition of phasic contractions.

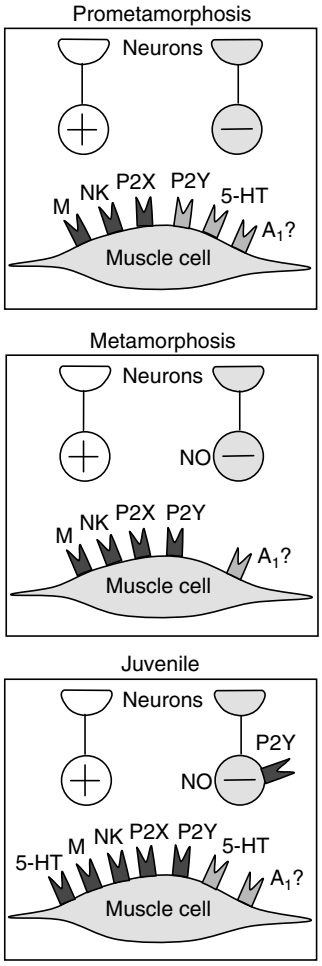

Fig. 9. A schematic overview of the different receptors indicated in this study to be present in the gut at prometamorphosis, metamorphosis and juvenile stages. Most receptors seem to be directly situated on the smooth muscle. Plus sign indicates excitatory neuron, whereas minus sign indicates inhibitory neuron. Black receptors indicate excitatory effects while grey receptors indicate inhibitory effects. For possible receptor subtypes see discussion. M, muscarinic receptor; NK, neurokinin receptor. Other abbreviations as previously used in the text.

The UTP-induced relaxation in prometamorphic tadpoles is either non-neuronal, or mediated by TTX-insensitive nerves that have been shown to exist in *Xenopus* (Buchanan et al., 1996). The existence of a receptor for the UTP metabolite uridine mediating inhibitory actions has been claimed, although this receptor has yet to be identified (Connolly and Duley, 1999). The effects of ATP, adenosine or α-β-MeATP were unaffected by TTX in this study, similarly indicating that the purinergic receptors in *Xenopus* gut responding to these agents are situated directly on the smooth muscle or on TTX-insensitive nerves.

The contractions elicited by ATP and the P2X receptor agonist α-β-MeATP at different stages, taken together with the blockade of the α-β-MeATP-induced contractions by the nonselective P2 antagonist PPADS, indicates the existence of a $P2X_1$ and/or $P2X_3$ receptor in the stomach of *Xenopus*, as has previously been shown in *Xenopus* intestine (Sundqvist, 2007). Since the response to α-β-MeATP did not desensitize at any stage, the possibility of a heteromeric receptor consisting of $P2X_2$ and $P2X_3$ subunits could also be indicated, although the presence of such a receptor remains to be established in *Xenopus*.

The present study of the stomach shows larger changes in the regulation of motility during metamorphosis than the subtle adjustments found in the previous study of the intestine (Sundqvist, 2007). Both the nutritional content, from carbohydrate to protein, and the consistency of the food, from liquid to solid, ingested by the *Xenopus* frog change during metamorphosis. But as the food is processed down the gut the differences in mechanical properties between the food eaten at different stages grows less pronounced, which could suggest that larger changes in the regulation of motility could be expected in the stomach than in the intestine. Further, one could speculate that the need to mix the food with pepsin [an enzyme not present in tadpoles (Rovira et al., 1995)] to start protein degradation also requires a more complex motility and motility regulation after metamorphosis than before.

To conclude, this study indicates the development of an excitatory 5-HT receptor, a nitrergic tonus, excitatory smooth muscle UTP receptors and possibly inhibitory neural UTP receptors during or after metamorphosis in *Xenopus* (Fig. 9). These changes could conceivably be related to the change from liquid to solid and from herbivorous to carnivorous food intake during this period, and indicate that the extensive remodelling affecting the connective and muscular tissues of the amphibian stomach during metamorphosis also affects the enteric nervous system. The study also shows the presence of an A_1-like adenosine receptor mediating relaxation in the stomach both prior to and after metamorphosis, as well as a P2X-like purinergic receptor mediating contraction, similar to previous findings in *Xenopus* intestine (Sundqvist, 2007).

LIST OF ABBREVIATIONS

ABT-702	4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl) pyrido[2,3-*d*]pyrimidine
ARL67156	6-*N*,*N*-diethyl-D-β,γ-dibromomethylene ATP
ATPγS	adenosine 5′-[γ-thio]-triphosphate
CCh	carbachol
CPA	N^6-cyclopentyladenosine
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
L-NAME	*N*-nitro-L-arginine methyl ester
NKA	neurokinin A
NO	nitric oxide
NOS	nitric oxide synthase
PPADS	pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid
TTX	tetrodotoxin
VIP	vasoactive intestinal peptide
α-β-meATP	α-β-methyleneadenosine 5′-triphosphate

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