Developmental Changes in the Acetylcholine Influence on Heart Muscle of *Rana catesbeiana*: In Situ and In Vitro Effects

BERND PELSTER, WARREN W. BURGGREN, STEVEN PETROU, AND INGER WAHLQVIST

*Institut für Physiologie, Ruhr-Universität Bochum, Universitätsstr. 150, 44780 Bochum, Germany (B.P.); Department of Biological Sciences, University of Nevada, Las Vegas, Nevada 89154-4004 (W.W.B.); Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01655 (S.P.); and Astra Hässle, Kärragatan 5, S-43183 Mölndal, Sweden (I.W.)*

**ABSTRACT**

The influence of acetylcholine (ACh) on cardiac performance of larval (Taylor Kollros [TK] stages II–XVIII) and postmetamorphic (3–609 g) *Rana catesbeiana* was analyzed in situ (circulatory system intact) and in vitro (isolated heart or ventricular strip preparations).

Topical application of ACh to the heart in situ resulted in a dose-dependent decrease in heart rate and in a slight decrease in systolic ventricular pressure in all developmental stages. Injection of acetylcholine into the ventricle lumen in situ caused a dose-dependent transient decrease in systolic ventricular pressure, with little heart rate effect. Intraventricular ACh injection also changed the hemodynamic coupling between ventricle and conus arteriosus, generating a biphasic pressure profile in the conus due to sequential contractions of the ventricle and of the conus. In situ the sensitivity of the ventricle to ACh decreased during larval development, with the lowest sensitivity in small postmetamorphic adults.

ACh applied in vitro to cardiac muscle strips or small hearts produced a negative inotropic effect. The ACh dose necessary to induce a 50% reduction in muscle strip contraction force in vitro decreased substantially during larval development, indicating an increase in ACh sensitivity with development.

The effects of ACh both in vitro and in situ were diminished or eliminated by topical application or injection of atropine, suggesting the presence of muscarinic cholinergic receptors. After preincubation with the acetylcholinesterase blocker eserine, injection of ACh into the conus arteriosus decreased systolic ventricular pressure with a delay of 4–10 seconds, probably representing the minimum blood circulation time.

The observed inotropic and chronotropic responses result from the action of ACh on cardiac muscle, primarily affecting systolic ventricular pressure, and on the cardiac pacemaker, mainly influencing heart rate. These responses occur as early as TK, stage II, indicating a well-developed set of mechanisms to regulate cardiovascular performance early in development.

Heart rate and cardiac contractility in vertebrates is determined via a complex system of neuronal and humoral control mechanisms (Nilsson, '83). These control mechanisms have long been known for the adult amphibian heart (Loewi, '21; Nilsson, '83; Axelsson and Nilsson, '85). For example, vagal cholinergic fibers and acetylcholine represent a powerful inhibitory control mechanism for cardiac activity in frogs and most other vertebrates by exerting a negative chronotropic effect. Usually acetylcholine also has a negative inotropic effect (Nilsson, '83). Far less is known about cardiac control mechanisms in amphibian larvae, although an understanding of some developmental changes is emerging. For example, the cardiac pacemaker of anuran amphibians is vagally innervated early in larval development (Burggren and Doyle, '86a; Kimmel, '90; Burggren and Pinder, '91; Hou, '92; Protas and Leontieva, '92) and adjustments in vagal tone play an important role in heart rate adjustment associated with changes in gill and lung ventilation or activity (Burggren and Doyle, '86a). In situ studies of the intact circulation show that the negative chronotropic effect of ACh increases during larval development, but then greatly decreases at meta-

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morphic climax (Burggren and Doyle, '86b). With respect to inotropic responses, in vitro studies of spontaneously contracting atrial/sinus venous preparations of larval and adult *Rana catesbeiana* (Kimmel, '90) and of paced ventricular preparations of *Rana temporaria* (Protas and Leontieva, '92) have shown that the sensitivity of atrial and ventricular muscle to ACh decreases with development. Although the changes in inotropic and chronotropic sensitivity of the cardiac system thus appear to be opposite during development, the adult cardiac system generally shows the lowest responsiveness to ACh.

In spite of these studies on larval amphibian heart, the inotropic cholinergic responses of the larval ventricle—the main cardiac pump—remain unexplored in terms of their in vivo role in generating arterial blood pressure and cardiac output. Consequently, experiments were undertaken to establish the ontogeny of the inotropic response to acetylcholine in ventricular muscle in the bullfrog *Rana catesbeiana* by recording central arterial blood pressure in larvae and in adults in situ. Because blood pressure changes reflect changes in both central and peripheral elements of the cardiovascular system, in vitro preparations of ventricular muscle strips were also examined.

**MATERIALS AND METHODS**

**Animals**

Experiments were performed on larvae and adults of the bullfrog, *Rana catesbeiana*. All experiments were performed at 20–23°C from July to November. Larvae were staged after Taylor and Kollros (1946). Following the protocol of previous studies larval stages were grouped according to major developmental landmarks like limb development or onset of air breathing. Adults were grouped as small post metamorphic animals (body mass 3.2–11.4 g) and full-grown adults (body mass 349–609 g). Adults and some larvae were obtained from commercial suppliers, while the remainder of the larvae were collected locally in Hampshire County, Massachusetts. There were no apparent physiological or pharmacological differences between commercially acquired and captured animals. Animals were kept in an aquarium at 20–23°C on a 12:12 h light-dark photoperiod for at least one week before experimentation. Larvae were feed spinach while adults were fed grubs.

**In vitro studies of ventricular force**

Larvae were killed by a carefully directed blow to the head while adult animals were killed by cranial and spinal pithing. In each case the ventral body wall was immediately opened and the entire heart removed and placed in a Hepes-buffered saline solution (pH 7.4) with the following composition (in mmol·l⁻¹): Hepes 10.0, NaCl 115, KCl 3.2, MgSO₄ 1.4, glucose 16.7. From a stock solution CaCl₂ was added to a final concentration of 1.3 mmol·l⁻¹. In larger adults longitudinal ventricular strips were cut from the heart and fine thread was tied to each end of the strip. Due to the small size of the heart in larvae and smaller adults, a whole ventricle preparation was used, with a fine thread tied to the apex and base of the ventricle. A longitudinal cut in the ventricle exposed the inner surface of the heart to the bathing solution. Ventricular preparations were transferred to a 50 ml organ bath containing the buffered saline solution which was continuously aerated with O₂ and thermostatted to 22°C. One thread was anchored to the organ bath, while the other was attached to a Narco F-60 isometric force transducer. The transducer output was recorded on a Narco MKIV rectilinear chart recorder (Narco Bio-System, Houston, TX).

Contractions of whole ventricles (larvae) or ventricular strips (adults) were electrically paced at 0.7 cycles·sec⁻¹ (10 volts, 2 ms duration), using a Grass S-8 stimulator. Muscle preparations were carefully stretched in a stepwise manner until maximum force production was obtained. Cumulative concentration-action (dose-response) curves were produced for acetylcholine chloride (ACh), purchased from Sigma Chemicals (St. Louis, MO).

**In situ measurement of arterial blood pressure and flow**

Animals were either quickly decerebrated and spinaly pithed with a fine needle or were anaesthetized in 0.15 g neutralized MS 222. They were then placed ventral side up in a small experimental chamber containing approximately 1 cm of aerated water. The preparation of the central circulatory system and the measurement of blood pressure were performed essentially as described by Pelster and Burggren (1991). Briefly, the heart and conus arteriosus were exposed by a ventral incision in the body wall. The pericardium was opened to allow topical application of drugs directly to the heart (see below). The opened body cavity was flushed periodically with saline to prevent desiccation. Arterial blood pressure was recorded in the conus arteriosus using a glass micropipette attached to a servo-null micropressure system (model 900, World Precision Instruments, New Haven, CT). In full grown adults a catheter (PE 50) was inserted
into the conus arteriosus and blood pressure was recorded with a conventional pressure transducer (Gould Statham BD 231D).

Blood velocity was also recorded in some preparations using a pulsed Doppler flowmeter (Bioengineering, Iowa City, IA). A bare 1 mm crystal held in a micromanipulator was positioned close to the conus beside the micropressure electrode. The blood velocity signal was not calibrated (see Pelster and Burggren, 1991). All pressure and flow signals were continuously recorded on a Narco MKIV rectilinear chart recorder (Narco Bio-System, Houston, TX).

To test the effect of ACh on heart rate \( (f_h) \) and central arterial blood pressure, the drug was either added to the saline (external application) or injected into the lumen of the ventricle using a second glass micropipette held in a micromanipulator. The micropipette was connected to a Hamilton syringe, allowing injections of \( 1-10 \mu l \) of an ACh stock solution \( (10^{-5} \text{ or } 10^{-6} \text{M}) \) dissolved in saline. Assuming the total blood volume of a larva amounting to 5% of body mass, the injection volume was < 5% of the blood volume, and assuming full equilibration of the animal the final concentration of ACh was in the range of \( 10^{-5} \text{ to } 10^{-6} \text{ M} \). Sham injections using only saline had no affect on \( f_h \) or arterial blood pressure.

In some animals a single injection of atropine (final concentration \( 10^{-6} \text{ M} \)) was administered prior to ACh injections to verify the cholinergic action of ACh. In the in situ preparations the activity of acetylcholine esterase rapidly degrades the applied ACh, perhaps even before the drug reaches the receptors that mediate a response. This is of particular concern in assessing the frequency response, as the drug was injected into the ventricle or central arterial circulation and had to pass through the circulatory system before it could reach the pacemaker that is located in the sinus venosus. Therefore, \( 10^{-6} \text{ M} \) eserine (a cholinesterase blocker) was injected into the ventricle in a series of eight larvae of various stages and two small postmetamorphic adults. In these animals blood pressure was recorded in the ventricle, while ACh was injected into the conus arteriosus or the truncus arteriosus 30 min after application of eserine. From the delay between injection into the conus and the first response of the ventricle, an estimation of circulation time could be made.

Data analysis

Values of \( \text{pD}_2 \) ( \(- \log 50\% \text{ maximum response} \)) for in vitro effects of ACh were obtained for each curve by a probit analysis. Developmental effects on the influence of ACh on in vitro ventricular force were assessed using ANOVA. Data are expressed as mean ± 1 standard error.

RESULTS

In vitro ventricular preparations

Application of ACh to larval and adult in vitro ventricular preparations caused a dose-dependent decrease in force production. The cholinergic inotropic dose-response curves for in vitro ventricular preparations, expressed as % maximum response, are presented in Figure 1, while Figure 2 presents a comparison of the \( \text{pD}_2 \) values for the inotropic response of each of the following groups: V–VII, XI–XIV, XVI–XVII, and adults. ANOVA of the \( \text{pD}_2 \) data indicated that there was a highly significant \( (P<0.01) \) effect of development on the inotropic ACh response. Essentially, the ACh dose required to produce 50% of the maximal negative inotropic response showed that the youngest larvae were significantly less sensitive to ACh than middle or late larval stages, which in turn were significantly less sensitive than adults. The relationship between development and the cholinergic, inotropic sensitivity of the ventricle in vitro can be summarized as V–VII < XI–XIV = XVI–XVII < adults.

In situ effects of ACh on blood pressure measurements

Intraventricular injection of ACh (up to 10 ng·g\(^{-1}\) body mass) transiently decreased systolic arterial blood pressure and also changed the pressure

![Graph](https://via.placeholder.com/150)  
**Fig. 1.** Cumulative concentration- \( \text{pD}_2 \) (dose-response) curves for the inotropic effect of acetylcholine chloride (ACh) on ventricular muscle tension in three larval stage groups (n = 7, 9, 7) and adults (n = 13) of the bullfrog *Rana catesbeiana*.**
Fig. 2. The relationship between developmental stage and the pD₂ (− log 50% response dose) for the cholinergic, inotropic response of the ventricle of larvae and adults. The development effect was significant (P<0.01, ANOVA)—see text for further details.

profile recorded in the conus (Fig. 3). Systolic pressure declined sharply, and although there was only one peak before the injection, often two distinct pressure peaks rather than the typical single peak could be resolved during systole after ACh injection. Injection of higher concentrations of ACh (using a more concentrated stock solution) stopped cardiac activity in all larval stages.

The effects of internal and external application of ACh on conal blood pressure and flow are compared in Figure 4. Injection of ACh (top panel) resulted in a dose-dependent decrease in systolic blood pressure and in blood velocity (and presumably flow), but f_H showed little or no change. External application of ACh to the heart of the same larva, (middle, bottom panels) however, mainly reduced f_H, with only a small decrease in systolic arterial pressure, which occurred primarily in the first few heart beats. In the instance illustrated higher doses of ACh actually stopped the heart.

Figure 5 summarizes the reduction of systolic blood pressure in relation to the amount of ACh injected for animals grouped as stages VI–VII, X–XI and XII–XIV, postmetamorphic juveniles and full grown adults. The decrease in systolic blood pressure provoked by injection of a weight specific dose of ACh becomes smaller and smaller as development proceeds, indicating a decrease in inotropic sensitivity of the ventricle with development. The lowest sensitivity by far was observed in postmetamorphic small juveniles, where compared to all other animals an almost 10 times higher dose was necessary to induce any reduction in systolic blood pressure.

The approximately linear dose-response curves on a log-scale (Fig. 5) appear to indicate a saturation of the ACh effect. It should be noted, however,

Fig. 3. Influence of ACh injection into the ventricle on blood pressure recorded in the conus arteriosus of various larval stages and a postmetamorphic juvenile. The ACh concentration given in brackets has been calculated assuming full equilibration of the animal.
that an approximately 10-fold increase of the ACh dose at constant injection volume (using a higher concentrated stock solution) usually stopped the heart immediately in all stages analyzed.

Atropine (calculated final concentration in the animal $10^{-5}$M) injected prior to the application of ACh completely suppressed or greatly diminished the effect of ACh in all larval stages tested. Injection of the acetylcholinesterase blocker eserine resulted in a transient reduction of ventricular activity. Five to 10 min after eserine injection, however, $f_H$ and systolic arterial blood pressure returned to control values. Subsequent injection of ACh into the conus arteriosus of these animals resulted in a decrease of ventricular systolic pressure with a delay of 4–10 sec (Fig. 6), indicating that a complete transit through some circuit of the systemic circulation occurs within 4–10 sec. Systolic arterial blood pressure slowly recovered to control levels, indicating that complete acetylcholinesterase inhibition had not been achieved. About 30–50 sec after the injection a second reduction of ventricular activity was observed which, along with the reduction in systolic arterial pressure, included a reduction in $f_H$.

### DISCUSSION

**Comparison of topically applied ACh and ACh injected in situ**

The present study demonstrates in situ the presence of functional ACh receptors regulating not only $f_H$, but also force of ventricular contraction in larval *Rana catesbeiana* as early as stage II. This find-
ing extends the studies of Burggren and Doyle (‘86a,b), who observed a negative chronotropic effect of ACh to larvae as young as stage IV. The receptors are muscarinic, as suggested by the inhibitory influence of atropine. Histological evidence demonstrates the presence of cholinesterase containing fibers no earlier than larval stages IV–V in Rana temporaria, but in vitro even in preparations of late embryonic stages and larval stages I–III inotropic and chronotropic effects of acetylcholine are observed (Protas and Leontieva, ’92). In their study the cholinergic effects were also mediated exclusively via muscarinic receptors. Thus, like in chicken embryos (Pappano, ’77), amphibian heart muscle is able to respond to acetylcholine before the autonomic innervation of the muscle cells is complete.

The comparison of results obtained with internal and external application of ACh in situ consistently revealed a striking difference in the cardiac responses they evoke. External application mainly provoked a negative chronotropic response with only slight changes in blood pressure, while injection of ACh produced little chronotropic response but had a dramatic negative inotropic effect. ACh injected into the ventricle had an immediate response, apparently activating receptors located on the cardiac muscle of the ventricle. The receptors regulating heart frequency (pacemaker) are located in the sinus venosus, and thus ACh injected into the ventricle has to pass completely through the circulatory system before reaching the receptors modulating fH. Acetylcholinesterase in the blood, therefore may have degraded the ACh before it could affect the sinus venosus, and the results obtained after preincubation with the esterase blocker eserine demonstrate that injected ACh indeed does influence receptors modulating fH, if it remains long enough in the blood. But even with eserine inhibited esterase the frequency response is observed later than the pressure response, which could indicate a higher threshold of these receptors. Another explanation would be that the receptors mediating the inotropic response in the thick walled ventricle are more easily accessible from the blood, while the receptors which mediate the chronotropic response, located in the thin walled sinus venosus, are easily accessible for topically applied ACh.

**Comparison of in situ and in vitro responses**

Paradoxically, the pattern of change with development in the ACh inotropic sensitivity of the ventricular muscle is in distinct contrast in in situ preparations (where ACh sensitivity decreased with development) compared with in vitro muscle preparations (where ACh sensitivity increased with development). The reason for this qualitative difference is not immediately apparent; but it may relate to the fact that the in situ response to injected ACh, opposite to the in vitro response of muscle strips, actually represents a collage of responses by ventricular muscle, conal muscle, and smooth muscle in the vasoactive blood vessels.

A recent study measuring the development of tension of ventricular preparations of Rana temporaria reported a decrease in ACh sensitivity with proceeding development (Protas and Leontieva, ’92), thus paralleling the changes observed in our in situ preparations. The decrease in the chronotropic sensitivity of the cardiac pacemaker with larval development in their study, however, is in distinct contrast to the results of Burggren and Doyle (1986b), who reported an increase in sensitivity for Rana catesbeiana larvae. Although Protas and Leontieva (’92) suggest that these differences may be related to the comparison of in vitro and in situ results, which appears to be supported by our results on the inotropic responses (see above), it can not yet be excluded that species differences are involved.

Compared to the more or less continuously decreasing responsiveness of cardiac muscle to ACh with development the sensitivity of postmetamorphic juvenile cardiac muscle in situ is especially low. A dramatic decrease in the responsiveness of the cardiac pacemaker in juveniles has been reported by Burggren and Doyle (’86b). These findings may in-
dicate that metamorphosis coincides with transient changes in regulation of the central cardiac system.

**The role of the conus arteriosus**

The inotropic effect of ACh not only reduced the force of ventricular contraction, but also changed the pressure profile recorded in the conus such that sometimes a double peak pressure signal emerged indicating separate contraction of the ventricle and conus arteriosus. In adult anurans the conus arteriosus is a contractile chamber, beating with a time delay of about 0.3 sec with respect to the ventricle (Langille and Jones, '77). Electrocardiographic analysis shows a B wave following the depolarization of the ventricle, indicating a transmission of the electrical signal to the muscle cells of the conus (Mullen, '74). The onset of "conal contraction" or the point of maximum force of conal contraction usually is difficult to define from the arterial pressure trace in adults frogs (Shelton and Jones, 1968; Langille and Jones, 1977), although in larval *Rana catesbeiana* during the winter months (Pelster and Burggren, '91) and in skates (Johansen et al., '66) a distinct conus wave in the pressure profile has been observed.

Injection of ACh depresses ventricular contractility at all larval stages examined. The weakened ventricular activity permits a distinct conal pressure wave to become detectable, proving the capability of the conus for muscular contraction irrespective of the developmental stage.

**Evaluation of the circulation time**

After inhibition of acetylcholinesterase with eserine, injection of ACh into the conus arteriosus or into the truncus arteriosus resulted in a biphasic response in arterial pressure. The initial response was observed after a delay of 4–10 seconds. Regurgitation of blood appears to be very unlikely due to the pylargal valve between ventricle and conus and of the syngangial valve between conus and truncus in larval *Rana catesbeiana* (Pelster and Burggren, '91) and adults (Shelton and Jones, '65; Johansen and Burggren, '80), and the pressure traces we recorded present indication that these values were intact. The drug must have passed into the arterial tree, through the remainder of the circulatory system, and then reached the ventricle from the venous side. Therefore, it can be assumed that the shortest pathway from the truncus to the ventricle requires 4–10 seconds. ACh decreased cardiac activity and certainly prolonged the circulation time. The second cardiac response, occurring with a delay of 30–50 seconds, indicates that the injected bo-

lus of ACh passed through the heart a second time, before ACh was completely degraded by the remaining esterase.

**Comparison of the "developmental trajectories" of chronotropic and inotropic cardiac responses**

Although qualitatively speaking ACh in situ exerts similar chronotropic and inotropic responses in all developmental stages of *Rana catesbeiana*, profound quantitative differences in the responsiveness of the cardiac system occur as a function of development. Cholinergic sensitivity of the cardiac pacemaker increases as development progresses, but decreases again sharply with metamorphosis (Burggren and Doyle, '86b). Using spontaneously active atrial/sinus venous muscle preparations exposed to different concentrations of ACh, Kimmel ('90) also noted the decreased sensitivity of adult compared to larval bullfrogs, but observed no differences between different larval stages. The in situ component of the present study, performed under experimental conditions similar to those of Burggren and Doyle ('86b), again reveals a higher inotropic cardiac responsiveness in the larvae compared to juveniles and full grown adults. However, in distinct contrast to the chronotropic effect, the inotropic sensitivity in situ continuously decreases during larval development.

Generally, then, the adult bullfrog heart is much less sensitive to ACh than in the larvae. The influence of ACh on force of contraction of the cardiac system appears to decrease with development and in the adult the effect was almost saturated at a decrease in systolic pressure of only 10–20%. On the other hand, ACh can completely block the activity of the cardiac pacemaker irrespective of the developmental stage. It thus seems possible that in the heart of young larvae both heart frequency and force of contraction (and thus stroke volume) are under cholinergic control while in adults ACh mainly exhibits a chronotropic effect. Further experiments are required to test this hypothesis as it applies to in vivo functions.

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**LITERATURE CITED**


