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The Action of Acetylcholine Upon Heart Rate Changes Markedly With Development in Bullfrogs

WARREN BURGGREN AND MICHAEL DOYLE
Department of Zoology, University of Massachusetts, Amherst, Massachusetts 01003-0027

ABSTRACT Dose-response curves for the action of acetylcholine (ACh) and its antagonist, atropine, upon heart rate in situ were made for three larval stages and adults of the bullfrog, *Rana catesbeiana*. The absolute magnitude of heart rate inhibition at any ACh concentration progressively increased throughout larval development. Unexpectedly, metamorphosis to the adult resulted in a sharp decrease in cholinergic cardiac sensitivity down to levels similar to the earliest larvae. Thus, cholinergic sensitivity of heart rate, reflecting properties of the cardiac pacemaker, is greatest immediately before metamorphosis and lowest in early larvae and adults.

It is generally perceived that amphibian development results in progressively increasing complexity, culminating in the adult. Yet, at least with respect to the respiratory and cardiovascular systems, amphibian larvae are physiologically and morphologically of equal or even far greater complexity than the adult (see Burggren, '84; Malvin, '85). As but one example, in the bullfrog *Rana catesbeiana* chronic cardiac vagal inhibition producing a low resting heart rate (f_H) appears quite early in larval development and persists in older larval stages (Burggren and Doyle, '86). However, vagal tone at rest disappears upon metamorphosis, such that resting f_H in adults simply reflects intrinsic f_H, as in the younger larvae (Burggren and Doyle, '86). Clearly, cardiovascular function of larval amphibians is not a simple, preliminary manifestation of that eventually found in the adult.

Do ontogenetic changes in cardiac regulation in amphibians occur only at the tissue level (e.g., progressive innervation of the atria), or are changes in the membrane properties of the cardiac pacemaker also involved? Certainly, the cholinergic sensitivity of the cardiac pacemaker cells will largely dictate f_H and thus overall cardiac performance, since cholinergically mediated vagal activity is a major modulator of f_H in amphibians (Heath, '80; Burggren and Doyle, '86).

This study assesses how modulation of f_H via pacemaker inhibition by acetylcholine is influenced by development in the bullfrog, *Rana catesbeiana*. Dose-response curves for the effects of acetylcholine and its antagonist, atropine, on f_H have been constructed for three larval stages and adults, and reveal unexpected changes associated with ontogeny.

MATERIALS AND METHODS

Experiments were performed at 20–22°C using 30 in situ preparations of larval and postmetamorphic bullfrogs. Frogs were maintained at 20–23°C on a 12:12 photoperiod for at least 1 week before experimentation. Larval groups used (with mean body mass) were VII–IX (12.0 g), X–XIV (19.1 g), and XVI–XIX (10.2 g), staged after Taylor and Kollros ('46). The adults had completed metamorphosis within 2 months (mean mass 11.9 g). The mean masses of the four groups were not significantly different (ANOVA, $P > .10$).

After delivery of a carefully directed blow to the cranium (larvae) or pithing (adults), animals were immobilized ventral surface up. The branchial chambers of the larvae were continuously ventilated with aerated water via a cannula in the mouth. In adults, the glottis was occlusively cannulated, and the lungs were deflated and inflated at regular 5–10-min intervals. After opening the animal's chest, a small slit was made in the

Michael Doyle is now at Department of Physiology, School of Medicine, University of New Mexico, Albuquerque, NM 87131.

pericardium, and its free edges were reflected back to expose the intact, beating heart lying within the fluid-filled "well" formed by the pericardial chamber. Such preparations usually remained viable for several hours, as evidenced by a strong, regular heart beat. Moreover, control f_H did not change significantly during the test period (see "Results").

Heart rate was recorded continuously during the entire experimental period. To record heart movement, a 50-mg weight suspended by a thread from an isometric force transducer was balanced on the ventricle. Ventricular contraction caused displacement of the weight, which was detected by the force transducer. The transducer output was displayed on a Narco MK-IV recording system, which provided signal conditioning and computation of instantaneous f_H.

Dose-response curves for f_H were constructed for both acetylcholine chloride (ACh) and the muscarnic antagonist, atropine sulfate (ATR). After initial stabilization of f_H following surgery (5–10 min), a standard 100-μl aliquot of Ringer's was pipetted directly onto the ventral surface of the heart, and any change in f_H (almost invariably none—see "Results") was recorded. Then, the Ringer's in the pericardial well was flushed with a 100-μl aliquot of 10^{-8} M ACh in Ringer's. This aliquot volume was many times greater than the total volume of the pericardial "well" and ensured total replacement of all fluid previously in the well. When the maximum effect on f_H had developed, the pericardial well was again flushed with one standard aliquot of Ringer's, whereupon f_H returned to control values. This cycle was repeated using, in order, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M ACh, in each case interspersed with a Ringer's treatment that returned f_H to control levels. Consequently, for every ACh concentration there was an immediately preceeding control period with which to compare the induced f_H response. The maximum effect at each ACh level was recorded as the difference between the immediately preceeding control f_H and the lowest transient f_H during ACh inhibition. The responses were presented as a percentage reduction from control f_H to facilitate both intra- and intergroup comparisons.

Atropine dose-response curves were determined as follows. First, the control f_H response to a standard aliquot of 10^{-4} M ACh was determined. The pericardial well was then flushed with 500 μl of 10^{-8} M ATR. This both removed any ACh from the well and induced a muscarinic blockade proportional to the ATR dose. Preliminary experiments demonstrated that the effect of atropine at any concentration developed fully within 2 min. When f_H had stabilized for a 5-min period following flushing with ATR, another standard aliquot of 10^{-4} M ACh was delivered to the heart. Following the development of the maximum chronotropic response to ACh, the heart was flushed with 10^{-7} M ATR. After 5 min, the standard aliquot of 10^{-4} M ACh was again placed on the heart, and the maximum f_H response was measured. This protocol, using alternating treatments with 10^{-4} M ACh and progressively higher concentrations of ATR, was repeated for 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M ATR.

The influence of development for any given drug concentration was assessed with one-way analysis of variance (ANOVA). Where significant differences between means for developmental stages occurred, individual means subsequently were tested with the Tukey-Kramer procedure. A fiducial limit of 0.05 was adopted for all analyses.

RESULTS

While standard aliquots of Ringer's alone produced no significant change in f_H at any developmental stage, a 100-μl aliquot of 10^{-6}–10^{-3} M ACh produced a significant slowing of f_H within two to three heart beats (Fig. 1). In some preparations from all stages, ACh produced a transient slowing or cessation of heart beat, which then began to return to control levels before flushing with Ringer's (e.g., Fig. 1A). In other preparations, f_H briefly stabilized at the new post-treatment level for 1–3 min (Fig. 1B). In either situation, flushing of the pericardial "well" with Ringer's caused f_H to return to the pretreatment "control" levels within 1–3 min. There were no statistical differences ($P > .10$) between control f_H before any ACh treatment and after construction of the entire ACh dose-response curve.

Dose-response curves for the effect of ACh on f_H are shown for the four developmental groups in Figure 2. Significant differences (ANOVA, $P > .05$) between the responses of the four developmental groups occurred at the pharmacologic doses of 10^{-5}–10^{-3} M ACh. The magnitude of the f_H reduction by ACh clearly increased with larval development, with the response at 10^{-5}–10^{-3} M ACh being significantly larger ($P < .05$) in stages XVI–XIX than in stages VII–IX ($P < .05$)

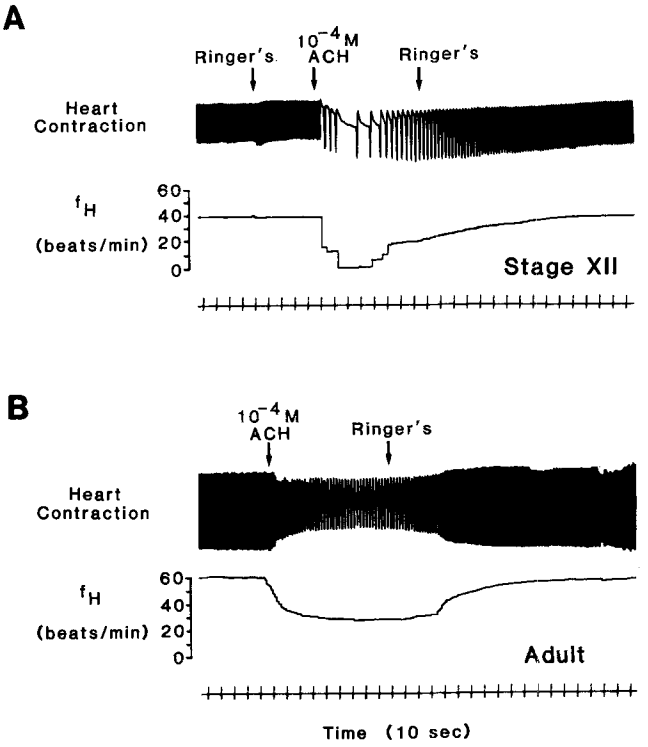

Fig. 1. Representative recordings of heart contraction and instantaneously computed heart rate in a 11.8-g stage XII larva (A) and a 12.76-g postmetamorphic adult (B) of the bullfrog *Rana catesbeiana*. Arrows show the application of Ringer's solution and 10^{-4} M acetylcholine (ACh), as indicated. Time marker in 10-sec intervals.

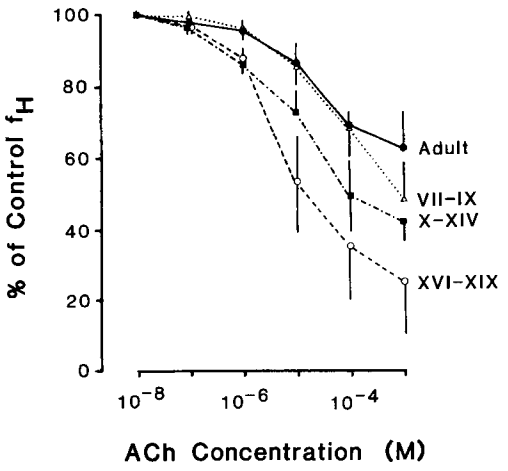

Fig. 2. Dose-response curves for the effect of acetylcholine upon control heart rate in three larval groups and adult bullfrogs. Data are presented as mean ± 1 standard error. Numbers of preparations used were 7,9,6, and 8, in ascending developmental order.

(Fig. 2). Unexpectedly, the effect of ACh on f_H in adults was quite muted when compared with intermediate and late larvae. The mean response of f_H in adults was significantly smaller than in stage XVI–XIX and X–XII larvae at 10^{-4} M and 10^{-3} M ACh, and significantly smaller than stage XVI–XIX larvae at 10^{-5} M ACh.

In the presence of 10^{-8} M atropine (ATR), the reduction in f_H by 10^{-4} M ACh was not significantly different ($P > .10$) from that produced before ATR treatment (Fig. 3), and closely reflected the distinctive cholinergic sensitivities of the various developmental groups evident in Figure 2. As [ATR] increased, the reduction in f_H by ACh was progressively diminished, and 10^{-5} M ATR almost completely abolished the ACh effect in all developmental groups. No differences in the sensitivity to ATR were observed between developmental groups.

DISCUSSION

The heart of developing bullfrog larvae becomes increasingly sensitive to the inhibi-

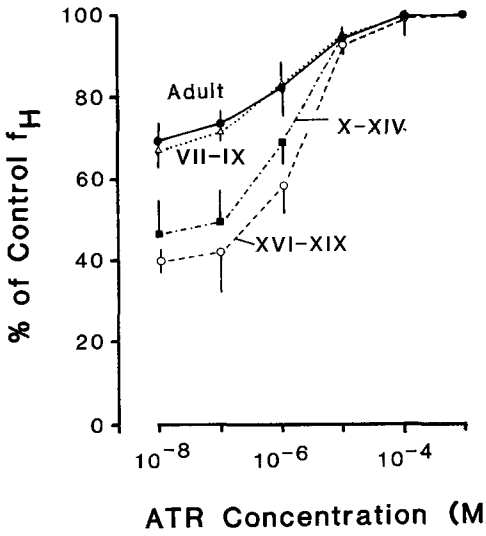

Fig. 3. Dose-response curves for the inhibitory effect of atropine on the heart rate reduction produced by 10^{-4} M ACh in three larval groups and adult bullfrogs. Data are presented as mean ± 1 standard error. Numbers of preparations used were 7,9,6, and 8, in ascending developmental order.

tory action of acetylcholine (Fig. 2). The cholinergic sensitivity of the heart of the chicken and several mammals also generally increases during prenatal development (see reviews by Pappano, '77; Sperelakis and Pappano, '83), with such changes often being associated with increased density of muscarinic binding sites (Nedoma et al., '86). The mechanism underlying the developmental changes in cardiac cholinergic sensitivity in bullfrog larvae remains to be determined, and could involve changes in ACh receptor numbers and/or affinity and acetylcholinesterase concentration.

In intact bullfrog larvae, a chronic vagal tone maintains resting f_H well below the intrinsic rate of the larval heart (Burggren and Doyle, '86). This could reflect the appearance and continued elaboration of cardiac vagal innervation. However, in light of the present findings, the onset of a chronic vagal cardioinhibition in developing bullfrog larvae could also be accounted for by the increasing sensitivity of the pacemaker cells to a constant level of transmitter being released from functional vagal neurons already morphologically in place.

Changes in cholinergic sensitivity of the atria of mammals sometimes continue after birth. Although the direction of change varies between species, the magnitude of the effect is generally small (see Pappano, '77). Against this background of varied findings in mammals, a change in cholinergic sensitivity of the heart following metamorphosis from larva to adult in bullfrogs was not entirely unexpected. However, the magnitude of the reduction in ACh sensitivity of the heart immediately upon metamorphosis was very large, and indicated a major reversal of the ontogenetic trend apparent earlier during larval development. Such marked development "regression" in transmitter sensitivity during cardiac ontogeny has not been reported for any lower vertebrate to our knowledge. This finding emphasizes that the development of cardiovascular function in amphibians should not be assumed as progressive or linear.

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