Regulation of Acetylcholinesterase in Avian Heart

Studies on Ontogeny and the Influence of Vagotony

Sangmee Ahn Jo, Dennis M. Higgins, and Harvey Alan Berman

This article examines the role of innervation in regulating expression of acetylcholinesterase (AchE), butyrylcholinesterase (BuchE), and the muscarinic acetylcholine receptor (mAchR) in avian heart. Two distinct approaches are taken. The first approach examines the relation between the onsets of parasympathetic and sympathetic innervation and the appearance of AchE and BuchE. All molecular forms of AchE and BuchE are present in early embryonic chick heart well before the onset of parasympathetic and sympathetic innervation. These molecular forms are characterized by sedimentation coefficients of 4.5S, 11S, 15S, and 19S. With further development, the amounts of AchE fall; the reductions in AchE parallel the onset of functional parasympathetic innervation. The amounts of BuchE increase progressively throughout embryonic development, independent of autonomic innervation, and in mature (56) predominate over the much less abundant amounts of AchE. The 15S and 19S forms of AchE in heart are lost during early embryogenesis but reappear in skeletal muscle during later embryogenesis. The second approach examines the influence of vagotomy and sympathetic denervation of 8-day-old chick myocardium on expression of the molecular forms of AchE, BuchE, mAchR, and β-adrenergic receptors. The amounts of AchE and BuchE molecular forms in avian heart are not measurably influenced by bilateral vagotomy for a duration of 4 days, unilateral vagotomy for a duration of 28 days, or sympathetic denervation. A measurable upregulation is observed in muscarinic receptors (35 to 46%) after vagotomy but not sympathectomy and in β-adrenergic receptors (29%) after sympathectomy but not vagotomy. In all cases, results in atria and ventricles are nearly identical. The present results indicate that expression of AchE in the myocardium is unique and different from that in skeletal muscle and not directly linked with autonomic innervation. (Circulation Research 1992;70:633–643)

Key Words: • acetylcholinesterase • butyrylcholinesterase • vagotomy • sympathetic denervation • muscarinic acetylcholine receptor • parasympathetic innervation • sympathetic innervation • ontogeny • avian heart

Innervation is well known to exert a profound influence on protein expression in skeletal muscle and has been studied extensively with reference to synthesis and degradation of acetylcholinesterase (AchE) and the nicotinic acetylcholine receptor. Although the influence of nerve on skeletal muscle can be attributed to the roles of both muscle activity and trophic factors, much less is known concerning neural regulation of the myocardium. Skeletal muscle is innervated by a single somatic motor nerve that serves to initiate contraction. Cardiac muscle, in contrast, is characterized by a high degree of automaticity and displays a more complex pattern of innervation by branches from the sympathetic and parasympathetic nervous system. Therefore, neural control of protein metabolism in cardiac muscle might not be expected to parallel that in skeletal muscle. Indeed, although protein turnover in intact and tissue-cultured heart is well studied, little is known concerning protein metabolism in the denervated heart. Since expression of AchE in skeletal muscle displays a critical dependence on innervation, it is of interest to know whether such neural regulation of AchE extends to other types of muscle.

This article describes two distinct experimental approaches used to ascertain the extent of neuronal regulation of AchE in the avian myocardium. The first approach investigates the influence of development and the sequential onsets of parasympathetic innervation and sympathetic innervation on the appearance of AchE in developing chick heart. The avian heart provides an ideal system for study, since there exists in this tissue a natural temporal separation of parasympathetic and sympathetic innervation, thereby permitting resolution of the different functional types of nerve–muscle interaction. Parasympathetic responsiveness, when monitored as a slowing of the heart, is observed as early as the 11th or 12th embryonic day, whereas sympathetic responsiveness, when monitored as an acceleration of heart rate, is seen much later in development, during the 16th embryonic day. In the present studies, AchE

From the Departments of Biochemical Pharmacology (S.A.J., H.A.B.) and Pharmacology and Therapeutics (D.M.H.), State University of New York at Buffalo.

Supported by grants from the National Institutes of Health (ES-03085) and the US Army Research Office, Research Triangle Park, N.C.

Address for reprints: Dr. Harvey Alan Berman, Department of Biochemical Pharmacology, State University of New York at Buffalo, Buffalo, NY 14260.

Received April 15, 1991; accepted November 18, 1991.
molecular forms in hearts from 4-day embryos to 5-week-old hatched chicks are examined. The presence of butryrycholinesterase (BuchE) is also monitored. Although the function of BuchE remains unknown, this enzyme is found to accompany AchE in a variety of tissues and displays the range of molecular forms similar to those for AchE.9-11 The relations derived from developing chick heart are compared with those found in developing skeletal muscle. The second approach examines the influence of vagotomy and sympathetic denervation on the expression of AchE and the muscarinic acetylcholine receptor (mAChR) in avian myocardium.

AChE in vertebrate tissues appears as distinct molecular forms, containing different numbers of globular catalytic subunits. The globular forms, denoted as G1, G2, and G4, contain one, two, and four catalytic subunits, respectively. The asymmetrical forms, denoted A4, A8, and A12, contain four, eight, or 12 globular catalytic units linked with an extended fibrillar collagen-like tail. These enzyme forms are readily detected by their molecular asymmetry as reflected in their sedimentation coefficients of 4–5S for G1, 6–7S for G2, 10–11S for G4, and 15–19S for A8 and A12. The asymmetrical forms have attracted interest as markers of neuromuscular function because they appear in skeletal muscle coincident with innervation and disappear after denervation.9,12 Although the molecular forms of AchE in all tissues are structurally distinct, the enzyme forms present in avian muscle and neurons appear to arise as products of a single gene, suggesting a linkage between innervation on the one hand and transcriptional and translational control of expression on the other.

**Materials and Methods**

**Materials**

Fertilized white leghorn chicken eggs were obtained locally from Enoch Farms or Delia-Smith, Clarence, N.Y. All chemicals and inhibitors were obtained from Sigma Chemical Co., St. Louis, Mo. Sucrose (ultrapure) for use in density gradient centrifugation was purchased from Schwarz-Mann, Cleveland, Ohio. (l)-[3H]Quinuclidinyl benzylate ([3H]QNB, 41.8 Ci/mmol) and (l)-[3H]dihydroalpranolol ([3H]DHA, 95.0 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Scintillation cocktail (Liquiscint) was purchased from National Diagnostics, Highland Park, N.J.

**Tissue Preparation**

Eggs were incubated in a self-turning incubator maintained at 90% humidity and 37°C. Hatched chicks were kept in a cage free to food and water at room temperature. Hearts were quickly removed from chicks and were cleared of any contaminating plasma activity by inserting a cannula in the aorta and perfusing with Krebs' solution (20–50 ml, 37°C) at a flow rate of 5 ml/min. Perfused hearts were placed on ice, and the great vessels were trimmed away. Hearts were rinsed with ice-cold phosphate-buffered saline, pH 7.4, and then dissected into ventricle and atrium as needed. Heart tissues were weighed, flash-frozen in liquid nitrogen, and stored at −80°C until use.

**Parasympathetic Deafferentation by Vagotomy**

Chicks (48–60 g, 8 or 9 days of age) underwent bilateral or unilateral (left side) vagotomy by removing a 1–2 cm length of vagus nerve under sodium pentobarbital anesthesia (20–30 mg/kg i.p.), as described by Cohen et al. Control animals underwent a sham operation whereby the vagus nerves were isolated but not sectioned. All animals were housed under identical conditions and given free access to food and water. At the indicated times after surgery, animals were killed by decapitation, and the ventricles and atria were obtained and stored as described above.

**Sympathetic Denervation**

Chicks (51–63 g, 8 days of age) received intraperitoneal injections of 6-hydroxydopamine (100 mg/kg) in a vehicle of saline containing 0.1% sodium ascorbate, adjusted to pH 7.4. For treatment of 6-day denervation, chicks were injected once a day for the first 5 days before death. For treatment of 10-day denervation, chicks were injected once a day for the first 9 days before death. Control chicks received injections of vehicle only.

**Extraction and Resolution of AchE and BuchE Molecular Forms on Sucrose Density Gradients**

The frozen tissue was pulverized in a metal mortar and pestle at liquid nitrogen temperature and homogenized in 5 vol per gram wet tissue in 0.01 M sodium phosphate buffer, pH 7.0, containing NaCl (1N), Triton X-100 (1%), EGTA (0.01 M), and a mixture of antiproteases, as described previously.9,16 The supernatant (360 μl), derived from a low-speed spin (23,400g for 30 minutes), was layered on a linear 5–20% sucrose density gradient. Sedimentation markers (3.3S, carbonic anhydrase; 11.4S, catalase; and 16S, β-galactosidase) were added with the sample to calibrate the gradient. After ultracentrifugation (SW41-Ti rotor, Beckman Instruments, Inc., Fullerton, Calif.) at 40,000 rpm for 21 hours, gradients were fractionated by upward extrusion and analyzed for AchE and BuchE in the presence of 5.5'-dithiobis-(2-nitrobenzoic acid) (3.3×10⁻⁵ M) and acetylthiocholine iodide (5×10⁻⁴ M) by the method of Ellman et al.17 Activity is presented as the change in absorbance per minute per gram wet weight of tissue. The proportion of AchE and BuchE in each pooled fraction was assayed in the presence of tetraisopropyl pyrophosphoramide (1×10⁻⁴ M), an inhibitor specific for BuchE, and 1.5-bis(allyldimethylammonium)pentan-3-one dibromide (5×10⁻³ M), an inhibitor specific for AchE.9,12

**Quantitation of mAChR and β-Adrenergic Receptors in Cardiac Membranes**

The frozen tissue was pulverized in a metal mortar and pestle at liquid nitrogen temperature and homogenized in ice-cold 50 mM Na⁺-K⁺ phosphate buffer, pH 7.4. The membrane fraction obtained after centrifugation of the homogenate (23,700g for 20 minutes at 4°C) was resuspended in 20–40 vol buffer (per original wet tissue weight) and homogenized again. To ensure the elimination of residual drug in the case of chicks treated with 6-hydroxydopamine, the membrane pellet was resuspended, washed, and centrifuged two additional times before the second homogenization. Membrane pellets from control chicks were obtained in a similar
Molecular Forms of AchE and BuchE and Their Relation With Development in Chick Heart

The molecular forms of AchE and BuchE, their amounts, and relative proportions underwent substantial changes during embryogenesis and adult development. The sedimentation profiles typical of hearts derived from 4-, 5-, 6-, and 12-day embryos and from 1- and 36-day-old hatched chicks are presented in Figures 1 and 2; the proportions of AchE and BuchE in each of the indicated forms are presented in the insets. In heart from the fourth embryonic day, at least four distinct molecular forms of AchE and BuchE having sedimentation coefficients of 4.5S, 11S, 19S, and 19S were resolved. During the fourth to sixth embryonic day, amounts of the 1S and 19S forms were reduced, and resolution of these forms was obscured, while the amounts of the 4.5S and 11S forms increased slightly. The combined amounts of 4.5S, 11S, and 19S AchE in heart on the sixth embryonic day accounted for ~57% of the total enzyme activity; BuchE accounted for 43% (Figure 1C, inset). At and beyond the 12th embryonic day, three principal molecular forms of AchE and BuchE, characterized by sedimentation coefficients of 4.5S, 11S, and 19S, were resolved. In the 36-day-old hatched chicks, AchE was present principally as an 11S species, accounting for only 20% of total enzyme activity, whereas BuchE was present as 4.5S and 11S forms, accounting for nearly 80% of total activity (Figure 2C, inset). In 36-day-old heart, the combined 11S forms of AchE and BuchE, representing <5% of total activity of the mature myocardium, were not readily detectable; therefore, the relative proportions of these enzymes are not reported.

Figure 3 shows the amounts of AchE and BuchE molecular forms present in hearts from the fourth embryonic day through the 35th day after hatching. The 4.5S, 11S, and 19S forms of AchE underwent graded reductions between the fourth and 12th embryonic day. The amounts of these forms, measured in terms of specific activity, decreased sharply, reached their lowest amounts on the 12th embryonic day, and remained essentially constant thereafter. After this time these enzymes were present in only small amounts and underwent no discernible change with further embryogenesis. The 11S species represented the most prominent molecular form of AchE in mature myocardium, whereas the 4.5S and 19S forms were present in relatively small amounts. Although the specific activities of the 11S and 19S forms of BuchE were essentially constant through the entire period of observation, the amounts of 4.5S BuchE increased in a monotonic fash-
Molecular Forms of Acetylcholinesterase and Butryrylcholinesterase in Chick Heart and Avian Skeletal Muscle

Hearts were homogenized in 5 vol of the extraction phosphate buffer per gram wet weight of tissue, and then analyzed for cholinesterase as described in “Materials and Methods.” Three principal molecular forms of cholinesterase appear as 4.5S (peak I), 11S (peak II), and 19S (peak III). The arrows indicate the position of the marker proteins (from left to right: 3.3S, carbonic anhydrase; 11.4S, catalase; 16S, β-galactosidase). Activity is expressed as changes in absorbance per minute per gram wet weight of tissue. Insets show the proportion of enzyme activity attributable to the presence of acetylcholinesterase (open bars) and butryrylcholinesterase (hatched bars). Specific activity is presented as the change in absorbance per minute per microgram protein.

In 16-day embryos, the specific activities of AchE and BuchE in the atria exceeded those in the ventricles by only twofold to threefold (Table 1). Similar results were observed from atria and ventricles of mature chick myocardium. Moreover, the relative proportions of AchE and BuchE in the atria were essentially identical with those in the ventricles. The findings in whole heart were therefore not biased by differences in the regional distribution of AchE and BuchE.

Molecular Forms of AchE and BuchE and Their Relation With Development in Chick Skeletal Muscle

As a frame of reference, the molecular forms of AchE and BuchE were examined during embryonic days 10–20 in chick pectoral muscle. As shown in Figure 4, the principal molecular forms of AchE and BuchE present in 10-day embryonic skeletal muscle were 4.5S, 7S, 11S, and 19S. AchE represented >90% and BuchE <10% of total enzyme activity. This ratio was invariant with development. The 19S form of AchE was substantially more abundant in 20-day embryonic muscle than in 10-day embryonic muscle. It was noteworthy also that 15S AchE, a form that was not evident in early embryonic skeletal muscle, was present clearly in muscle from 20-day embryos.

Influence of Vagotomy on AchE and BuchE Molecular Forms in Avian Heart

The influence of parasympathetic deafferentation on expression of AchE in avian myocardium was examined in 8-day-old chicks that underwent bilateral severance of the vagus nerves. Hearts obtained from animals killed between 0.5 and 5 days after surgery were weighed and assayed for AchE, BuchE, mAchR, and the β-adrenergic receptor. Results from denervated chicks were compared with those obtained from sham-operated chicks and with age-matched untreated chicks. Heart weight from sham-operated animals (0.54±0.03 g, n=14) showed no signifi-
by guest on June 23, 2017 http://circres.ahajournals.org/ Downloaded from

ventricles and forms, after atrophy animals. AchE sham-operated bilateral vagotomy of control and chick atria molecular forms of cholinesterase; AchE, acetylcholinesterase; BuchE, butyrylcholinesterase. The indicated regions of avian heart were dissected, weighed, and flash-frozen in liquid N₂. The tissues were homogenized in 5 vol of buffer per gram wet weight of tissue, and the supernatants from a low-speed spin (23,400g for 30 minutes) were placed over linear sucrose density gradients and analyzed as described in "Materials and Methods."

Resolution of AchE and BuchE molecular forms in sham-operated control and vagotomized 12-day-old chick atria and ventricles is shown in Figure 5. Three major molecular forms of cholinesterase having sedimentation coefficients of 4.5S, 11S, and 19S were resolved from both atria and ventricles of heart from sham-operated chicks. As indicated in the insets, BuchE accounted for the major proportion of the 4.5S form, whereas AchE accounted for the larger proportion of the 11S and 19S forms. The density gradient profiles of cholinesterase in atria and ventricles were similar with respect to the relative proportions of AchE and BuchE (Figure 5, insets), whereas atria contained twofold greater amounts of 11S and 19S forms than the ventricles.

Bilateral vagotomy of chick heart for 4 days caused only a slight reduction of cholinesterase activity in the 4.5S form and no appreciable alteration in the 11S and 19S forms (Figures 5B and 5D). Overall, the influence of bilateral vagotomy was indistinguishable in atria and ventricles and was invariant over 4 days of observation. It was noteworthy that no changes in the 11S and 19S forms were evident in bilaterally denervated cardiac muscle. Since 4.5S AchE represented only a small percentage (<10%) of the total cholinesterase activity in control heart, the small reductions observed in the 4.5S form after denervation were attributable to the loss of BuchE activity. It appeared that the relative proportions of AchE and BuchE in atrium and ventricle were unchanged after denervation (Figures 5B and 5D).

All forms of AchE were essentially unchanged after bilateral vagotomy. However, bilateral vagotomy allowed examination of denervation for only short periods of time, up to 4 days, because of the high mortality observed under these conditions. The mortality rate after 4 days of denervation exceeded 60% (n=53), and the mortality rate after 5 days approached 80% (n=12). Bilaterally denervated animals displayed severe difficulties in breathing and intake of food, and these symptoms appeared soon after surgery. The main causes for death were likely due to pulmonary and esophageal dysfunction as reported also for rat, guinea pig, and dog.19,20

The period of observation was extended to 25 days by using unilateral vagotomy (left side). Under these conditions all chicks remained viable and continued to feed normally throughout the period of examination. After 25 days of denervation, there was observed a slight reduction in heart weight (20%, n=4) compared with control animals; however, there were no changes in either the molecular forms or the relative proportion of AchE and BuchE (Table 2).

### Effects of Vagotomy on Cardiac Muscarinic Receptors

The regional distribution of mAChR in atria and ventricles of sham-operated and 4-day-denervated chicks was determined using the radiolabeled muscarinic antagonist [3H]QNB. Association of [3H]QNB with crude cardiac membranes derived from sham-operated and denervated chicks was saturable and characterized by a dissociation constant of ~30 pM. Although the ligand affinity was identical for membranes from ventricles and atria from sham-operated heart, the maximum number of binding sites in ventricles (636±24 fmol/mg protein) was ~30% lower than in atria (857±82 fmol/mg protein). In contrast, the muscarinic receptor densities in chick atria and ventricles on the fourth day after vagotomy were 1,251±159 and 861±69 fmol/mg protein, respectively, representing a measurable increase in the number of [3H]QNB binding sites in atrium (46%) and ventricle (35%) (Figure 6, Table 3).

After 1 day of denervation, atria and ventricles showed respective increases of 19% and 23% in the number of [3H]QNB binding sites, suggestive of a dependence on duration of denervation. Overall, the increase in the specific binding of [3H]QNB after bilateral vagotomy was not remarkably different in atrium and ventricle.

### Effects of Sympathetic Denervation on AchE and BuchE Molecular Forms and Cardiac Muscarinic Receptors

The influence of sympathetic denervation on AchE and BuchE molecular forms and mAChR was examined
in chicks that underwent treatment with 6-hydroxydopamine for 6 and 10 days. As reported in Table 2, treatment with 6-hydroxydopamine caused no significant alterations in the presence of AchE and BuchE molecular forms and their relative proportions in avian heart. The results after 6 and 10 days of denervation were identical.

Association of \[^{[3}H\]QNB with atria and ventricles from 6-hydroxydopamine-treated chicks was saturable and compatible with a single class of sites characterized by a dissociation constant of \(\approx 30\) pM, a value essentially identical with that characteristic of control. After sympathetic denervation for 10 days, the number of muscarinic binding sites in atria (\(889\pm 70\) fmol/mg protein) and ventricles (\(615\pm 31\) fmol/mg protein) was not remarkably different from the number of sites found in atria (\(794\pm 38\) fmol/mg protein) and ventricles (\(642\pm 22\) fmol/mg protein) derived from sham-operated chicks. This result was identical with that obtained after 6 days of treatment. These findings were consonant with those seen in rat hearts \(^{21,22}\) (but also see Reference 23) and in chick hearts.\(^{24}\)

**Effects of Vagotomy and Sympathetic Denervation on Cardiac \(\beta\)-Adrenergic Receptors**

As a frame of reference, the influence of parasympathetic and sympathetic denervation on the amounts of \(\beta\)-adrenergic receptors in ventricles was determined using \[^{[3}H\]DHA. In all cases \[^{[3}H\]DHA association with membranes from ventricles was saturable and compatible with binding at a single class of binding sites characterized by a dissociation constant of \(\approx 0.87\pm 0.03\) nM. As seen in Table 3, the dissociation constants for \[^{[3}H\]DHA after bilateral vagotomy and after treatment with 6-hydroxydopamine were not significantly different from control values. However, the maximum number of \[^{[3}H\]DHA binding sites after vagotomy was unchanged from the control value, whereas that determined after treatment of chicks with 6-hydroxydopamine (\(84.0\pm 2.9\) fmol/mg protein) was increased \(\approx 29\)\% relative to the control value (\(65.1\pm 0.5\) fmol/mg protein). The increase in the maximum number of \[^{[3}H\]DHA binding sites without change in affinity was compatible with that seen in rat hearts after similar treatment with 6-hydroxydopamine.\(^{25}\)

**Discussion**

**Ontogeny of AchE and BuchE in Avian Myocardium**

These studies reveal that the ontogeny of AchE molecular forms in avian heart is opposite and distinct from that in skeletal muscle. As early as the fourth embryonic day, well before the onset of either anatomic or functional parasympathetic innervation, embryonic avian heart expresses a diversity of AchE molecular forms, comprising 4.5S, 11S, 15S, and 19S AchE. Before the 12th embryonic day, these species undergo graded reductions with a time course that parallels the onset of functional parasympathetic innervation. After the 12th embryonic day, the amounts of AchE remain unchanged. In all cases, results in atria resemble those in ventricles.\(^{26,27}\) These results in cardiac muscle contrast with the case for skeletal muscle: whereas the 7S and 11S forms undergo no marked reductions between the 10th and 20th embryonic days, the presence in skeletal muscle of 19S AchE increases dramatically. Therefore, these studies reveal a tissue-specific pattern for emergence of molecular forms of AchE in cardiac and skeletal muscle.

Avian heart displays a temporal separation of parasympathetic and sympathetic innervation. Anatomic parasympathetic innervation of chick heart, assessed through histochemical detection of vagal in-branches on the myocardium, begins at the interatrial septum on the fifth embryonic day, whereas functional parasympathetic innervation, assessed through atropine-sensitive responsiveness of the myocardium, occurs on the 12th embryonic day.\(^{7}\) Anatomic sympathetic innervation reaches the heart on the 10th embryonic day, whereas functional sympathetic innervation, detected with re-

**FIGURE 4.** Graphs showing molecular forms of acetylcholinesterase and butyrylcholinesterase present in embryonic chick pectoral muscle. Pectoral muscles from 10-day (panel A), 15-day (panel B), and 20-day (panel C) embryos were homogenized in 5 vol of the extraction phosphate buffer per gram wet weight of tissue and then analyzed for cholinesterase molecular forms as described in “Materials and Methods.” In 10-day embryonic muscle, three principal molecular forms of cholinesterase appear: 6.5S (peak I), 11S (peak II), and 19S (peak III). A 3S form migrates with the 6.5S form. A 13S form of acetylcholinesterase, absent from 10- and 15-day embryonic muscle, was evident in the 20-day embryonic muscle. The arrows indicate the position of the marker proteins (from left to right: 3.3S, carbonic anhydrase; 11.4S, catalase; 16S, \(\beta\)-galactosidase). Activity is expressed as the change in absorbance per minute per gram wet weight of tissue. Insets show the enzyme activity attributable to the presence of acetylcholinesterase (open bars); the amount of butyrylcholinesterase (hatched bars) was negligible. Specific activity is presented as the change in absorbance per minute per microgram protein.
spect to increased twitch tension on stimulation of intramural nerves, occurs on the 16th embryonic day.\textsuperscript{28,29}

The presence of AchE in embryonic chick heart before functional autonomic innervation correlates with findings that the muscarinic receptor,\textsuperscript{30,31} GTP-binding proteins,\textsuperscript{32} \(\beta\)-adrenergic receptor,\textsuperscript{33} slow Ca\(^{2+}\) channel,\textsuperscript{34} Na\(^+\) channel,\textsuperscript{35} Na\(^+\),K\(^+\)-ATPase,\textsuperscript{36} and choline acetyltransferase\textsuperscript{37} are present before the onset of parasympathetic innervation. The muscarinic receptor and Na\(^+\) and Ca\(^{2+}\) channels, although detectable in the aneural heart through their capacity to associate antagonist ligands, are functionally silent. These membrane-associated species become functional coincident with the onset of parasympathetic innervation. AchE, in common with the enzymes Na\(^+\),K\(^+\)-ATPase and choline acetyltransferase, is present in muscle in a catalytically competent form well before the onset of functional innervation. The expression of the asymmetric 15S and 19S AchE in aneural embryonic avian heart provides another indication, complementing that seen for skeletal muscle,\textsuperscript{12,38,39} that innervation is not required for expression of the asymmetric forms of AchE.

**TABLE 2.** Representation of Acetylcholinesterase and Butyrylcholinesterase in Avian Heart After Unilateral Vagotomy and Sympathetic Denervation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific activity ((\Delta A \cdot \text{min}^{-1} \cdot \text{g wet wt tissue} \times 10^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.5S</td>
</tr>
<tr>
<td>Unilateral vagotomy*</td>
<td></td>
</tr>
<tr>
<td>Control ((n=3))</td>
<td>1.30±0.27</td>
</tr>
<tr>
<td>Vagotomized ((n=3))</td>
<td>0.93±0.23</td>
</tr>
<tr>
<td>Sympathectomy†</td>
<td></td>
</tr>
<tr>
<td>Control ((n=4))</td>
<td>0.96±0.14</td>
</tr>
<tr>
<td>6-OHDA ((n=8))</td>
<td>1.09±0.13</td>
</tr>
</tbody>
</table>

Values are mean±SEM averaged over the number of independent determinations indicated within parentheses. A, absorbance; 4.5S, 11S, and 19S, three principal molecular forms of cholinesterase; AchE, acetylcholinesterase; BuchE, butyrylcholinesterase; 6-OHDA, 6-hydroxydopamine.

*Chicks of 8–9 days of age underwent unilateral vagotomy and were killed 25 days after surgery.
†Sympathetic denervation of 8-day-old chick was accomplished by daily injection of 6-OHDA (100 mg/kg i.p.). Control chicks received injections of vehicle only. Chicks were killed after 6–10 days of treatment.
AchE molecular forms in chick neurons and skeletal muscle appear to arise as allelic variants of a single gene.13,14 If this relation holds in avian heart, then these different forms can reflect either alternative splicing of a single primary transcript or differential promotion in which multiple transcripts are processed. Embryonic heart contains measurable amounts of all AchE forms present in skeletal muscle. The 15S and 19S forms of AchE expressed in chick myocardium display a collagenase sensitivity (Z. Luo, S.A. Jo, and H.A. Berman, unpublished observations, 1990) similar to that found for asymmetric forms in rat heart25,40 and therefore possess structural domains similar to the more extensively studied asymmetrical forms found in skeletal muscle.1 In this regard, it is noteworthy that the collagenase-sensitive 15S and 19S AchE species, although they are present in embryonic heart, undergo reduction before the onset of parasympathetic innervation and eventual elimination subsequent to innervation. In early embryonic skeletal muscle, in contrast, 15S AchE is either absent or undetectable and, together with 19S AchE, becomes abundant just before hatching. This reciprocal relation between appearance of the asymmetrical forms in cardiac and skeletal muscles is reminiscent of the developmental changes seen in these tissues for actin and myosin isoforms. α-Cardiac actin, the form present in adult cardiac muscle, accounts for 90% of the sarcomeric actin transcript found in embryonic skeletal muscle.14,41 Similarly, the myosin alkali light chain expressed in mammalian embryonic skeletal muscle is identical to an isoform expressed in adult cardiac muscle.43 The loss of 15S and 19S forms from embryonic cardiac muscle and the subsequent appearance of these forms in mature skeletal muscle represents the first indication of this phenomenon for proteins associated with cholinergic neurotransmission.

**Influence of Vagotomy and Sympathetic Denervation on Appearance of AchE and BuchE**

Preganglionic parasympathetic nerves innervate cardiac ganglia, which, in turn, project postganglionic neurons that terminate within the heart. Vagotomy severs the preganglionic neurons and thereby abolishes effrent cholinergic influences, while postganglionic fibers remain intact. In this condition, the heart is essentially parasympathetically deafferented. Preganglionic sympathetic nerve fibers run to paravertebral sympathetic ganglia, which, in turn, send projections through the heart wall. Treatment with 6-hydroxydopamine selectively destroys the adrenergic nerve terminal.44 Innervation exerts subtle but precise effects on proteins associated with parasympathetic and sympathetic neuroeffector junctions. As shown through direct comparison of parasympathetically deafferented and innervated heart, there is a measurable upregulation of muscarinic receptors after vagotomy but not sympathetic receptors and β-adrenergic receptors after sympathectomy but not vagotomy. Neither vagotomy nor sympathetic denervation exerts any measurable effects on the appearance or disappearance of AchE in the avian myocardium. The results of sympathetic denervation in avian heart are not incompatible with the marginal reductions seen under similar treatment of rat heart.45 Results obtained after bilateral vagotomy, monitored for 4 days, are similar to those obtained after unilateral vagotomy, monitored for 25 days. Hence, the relatively short 4-day duration of denervation was not a likely explanation for the lack of any discernible alteration in AchE. The influences of vagotomy and chemical sympathetic denervation are exerted almost equally in atria and ventricles, consistent with the rather uniform distribution of parasympathetic nerve endings in avian heart.7,8,46

**Expression of BuchE in Avian Heart**

The appearance in heart of BuchE contrasts with that for AchE. Whereas all forms of AchE undergo reduction before stabilization at the 12th embryonic day, the overall amounts of BuchE increase throughout embryogenesis. Since 11S and 19S BuchE remain essentially constant, the increase in BuchE is directly attributable to increasing amounts of the 4.5S species. Overall, it is striking that the mature myocardium contains measurable amounts of BuchE that greatly exceed the very much smaller amounts of AchE. The relations between amounts of AchE and BuchE in atria and ventricles are identical. All aspects of BuchE appearance in avian heart are opposite those seen in skeletal muscle. Of significance is that during cardiac myogenesis the amounts of AchE decrease and those of BuchE increase (Figure 3), whereas during myogenesis of skeletal muscle the amounts of AchE increase and those of BuchE decrease (Figure 4). Although the function of BuchE remains unknown, expression of this enzyme in mammalian skeletal muscle is unaltered by denervation,9 in marked contrast to the case for AchE.12 The absence in avian heart of any effect of the onset of parasympathetic or sympathetic innervation on ontogeny of BuchE, taken with the failure of bilateral vagotomy to alter the amounts of BuchE, suggests that expression of BuchE in heart also is unrelated to cholinergic function.


**TABLE 3. Dissociation Constants and Maximum Number of Binding Sites for (l)-[3H]Quinuclidinyl Benzilate and (l)-[3H]Dihydroalprenolol Binding to Cardiac Membranes From Chick Heart After Bilateral Vagotomy and 6-Hydroxydopamine Treatment**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Kd (pM)</th>
<th>Bmax (fmol/mg)</th>
<th>n</th>
<th>Kd (nM)</th>
<th>Bmax (fmol/mg)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parasympathectomy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Ventricle</td>
<td>27.8±1.5</td>
<td>636±24</td>
<td>8</td>
<td>0.56±0.09</td>
<td>62.5±2.9</td>
<td>4</td>
</tr>
<tr>
<td>Atrium</td>
<td>29.8±2.8</td>
<td>857±82*</td>
<td>4</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>1-Day vagotomy</td>
<td>29.9±1.8</td>
<td>787±49</td>
<td>3</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Ventricle</td>
<td>27.4±5.4</td>
<td>992±87</td>
<td>3</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Atrium</td>
<td>36.4±2.2</td>
<td>861±69†</td>
<td>8</td>
<td>0.98±0.15</td>
<td>65.6±6.3</td>
<td>4</td>
</tr>
<tr>
<td>4-Day vagotomy</td>
<td>36.3±4.9</td>
<td>1,251±159‡</td>
<td>4</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td><strong>Sympathectomy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Ventricle</td>
<td>28.4±1.4</td>
<td>642±22</td>
<td>8</td>
<td>0.87±0.03</td>
<td>65.1±0.5</td>
<td>3</td>
</tr>
<tr>
<td>Atrium</td>
<td>31.8±4.1</td>
<td>794±38</td>
<td>3</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>6-Day 6-OHDA</td>
<td>28.0±4.2</td>
<td>678±30</td>
<td>3</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Ventricle</td>
<td>28.8±1.1</td>
<td>615±31</td>
<td>5</td>
<td>0.91±0.07</td>
<td>84.0±2.9‡</td>
<td>3</td>
</tr>
<tr>
<td>Atrium</td>
<td>30.6±2.0</td>
<td>889±70</td>
<td>3</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Values are mean±SEM. [3H]QNB, (l)-[3H]quiniclidinyl benzilate; [3H]DHA, (l)-[3H]dihydroalprenolol; Kd, dissociation constant; Bmax, maximum number of binding sites; 6-OHDA, 6-hydroxydopamine. Parasympathectomy was accomplished by bilateral severance of the vagus nerve for the designated duration; control indicates sham-operated chicks that were killed 4 days after surgery. Sympathectomy was achieved by daily treatment with 6-OHDA (100 mg/kg i.p.) for the indicated duration; control chicks received vehicle of saline containing 0.1% sodium ascorbate.

*p=0.0009 compared with corresponding value in ventricle by unpaired two-tailed t test.

‡p=0.0084 compared with corresponding control value in ventricle.

§p=0.0031 compared with corresponding control value in atrium.

**Influence of Parasympathetic and Sympathetic Denervation on Cardiac Muscarinic and β-Adrenergic Receptors**

Although no change in the amounts of AchE or BuchE occurs after bilateral vagotomy, an increase of ~1.5-fold is observed in the number of muscarinic receptors (Figure 6). The increase in muscarinic receptor density after 4-day bilateral vagotomy is comparable in ventricles and atria and occurs after parasympathetic deafferentation but not sympathetic denervation. The upregulation is not a consequence of loss of protein, since denervation causes no changes in the amounts of AchE, BuchE, and β-adrenergic receptors. A case analogous to the relation between upregulation of nicotinic receptors and supersensitivity of tissues to acetylcholine in skeletal muscle4,47 can be considered for cardiac muscle, since, as seen by Smith et al,48 an increased responsiveness of canine heart to acetylcholine is observed after total extrinsic cardiac denervation. In this condition the heart is denervated pregangionically with respect to the parasympathetic nerves and postganglionically with respect to the sympathetic nerves. The increased sensitivity to acetylcholine may therefore reflect the upregulation of muscarinic receptors after denervation.

**Neural Control of AchE in Avian Heart**

The effects of vagotomy and chemical sympathectomy on avian myocardium contrast dramatically with the pronounced influence of motoneuron denervation on AchE expression in skeletal muscle. Denervation of rat skeletal muscle results in rapid and large decreases in total muscle AchE activity,9,12,49 whereas denervation of chicken and rabbit skeletal muscle causes an increase in AchE activity.50,51 Also, the asymmetrical forms of AchE are lost rapidly immediately after denervation of skeletal muscle. Indeed, in chicken, 19S AchE is eliminated within 6 days;51 in rat, 16S AchE disappears within 3 days.12,49,52 Yet, in cardiac muscle no discernible loss of 19S AchE is observed after 4 days of denervation. Since parasympathetic deafferentation by severance of the vagus leaves the cardiac ganglia and postsynaptic axons to the myocardium intact, the myocardium remains only partially innervated. Although the vagotomized myocardium is not therefore directly analogous to denervated skeletal muscle, it is noteworthy that loss of the vagal component of innervation is sufficient to produce an increase in the density of muscarinic receptors. Thus, the factors regulating appearance and disappearance of AchE in cardiac muscle appear to be unique and different from those in skeletal muscle and provide a clear distinction with respect to a neural influence on AchE expression.

These distinctions between skeletal muscle and cardiac muscle are revealing when considered with respect to functional and morphological characteristics of these two types of striated muscle. The fast action of acetyl-
choline at the clearly defined end plate in skeletal muscle is due in part to punctate release of the neurotransmitter and to a comparably rapid mechanism for termination of transmitter action. It is significant in skeletal muscle that diffusion of acetylcholine is subordinate in importance to enzymatic termination of acetylcholine and that synaptic AchE therefore plays an essential role in determining the temporal response of the neuromuscular synapse.53,54

Cardiac muscle is diffusely innervated and contains no clearly defined end plates. Instead, the sites of transmitter release along terminal axons are usually separated from the nearest target by a distance of several micrometers or more.55 By virtue of this morphology, acetylcholine is, in effect, as described by Hartzell,56,57 “bath applied” to the cardiac muscle, in contrast to the punctate release characteristic of the skeletal neuromuscular junction. Rat heart10,27 and chick heart (Z. Luo, S.A. Jo, and H.A. Berman, unpublished observations, 1990) contain collagenase-sensitive asymmetrical forms of AchE. The concentrations of these asymmetrical forms in heart, however, are far smaller than in skeletal muscle.40 For heart, therefore, the small amounts of collagen-tailed AchE in the basal lamina may facilitate a longer residence time and greater diffusion of acetylcholine throughout the myocardium, thereby allowing the neurotransmitter to encounter multiple environments. In this sense, diffusion of acetylcholine may play a role of greater importance in heart than in skeletal muscle.

Finally, although there exists some evidence for reduced cardiac performance after partial cardiac denervation,55 it is intriguing that the transplanted heart, a fully denervated organ, continues to maintain contractile function when compared with respect to chronotropy and inotropy of the resting innervated heart.56-60 This behavior provides a further contrast with the case for skeletal muscle, which, after denervation, is non-functional and undergoes marked atrophy and rapid loss of AchE. Indeed, whereas it is clear that skeletal muscle expression of AchE is under strict neural regulation, it is also clear that expression of AchE in the myocardium is not directly linked with autonomic innervation. These differences between cardiac and skeletal muscle imply, in turn, that membrane electrical activity, contractile activity, and trophic factors acting on the target cell, all of which play roles of profound importance in maintenance of skeletal muscle, bear no strict parallel in the case of cardiac muscle.

Acknowledgment

We wish to thank Shaheen Nakeeb for his advice and assistance with the techniques of cardiac denervation.

References

42. Paterson BM, Eldridge JD: α-Cardiac actin is the major sarcomeric isoform expressed in avian embryonic skeletal muscle. Science 1984;224:1346–1348
47. Miledi R: The acetylcholine sensitivity of frog muscle fibers after complete or partial denervation. J Physiol (Lond) 1960;151:1–12
53. Katz B, Miledi R: The binding of acetylcholine to receptors and its removal from the synaptic cleft. J Physiol (Lond) 1973;231:549–574
54. Hartzell HC, Kuffer SW, Yoshikami D: Post-synaptic potentiation: Interaction between quanta of acetylcholine at the skeletal muscle neuromuscular synapse. J Physiol (Lond) 1975;251:427–463
Regulation of acetylcholinesterase in avian heart. Studies on ontogeny and the influence of vagotomy.
S A Jo, D M Higgins and H A Berman

Circ Res. 1992;70:633-643
doi: 10.1161/01.RES.70.4.633

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/70/4/633