Dual Mechanism for Inhibition of Calcium-Dependent Action Potentials by Acetylcholine in Avian Ventricular Muscle

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SUMMARY Acetylcholine (ACh) and carbamylcholine (Carb) inhibited Ca-dependent action potentials and contractions in ventricular muscle from the avian heart. The inhibition by cholinergic drugs was antagonized by atropine (muscarinic) and occurred by two pathways, "indirect" and "direct." Before hatching, ACh had no effect per se, but it inhibited Ca-dependent action potentials that had been augmented by isoproterenol (ISO). This "indirect" pathway for inhibition was detected as early as the 7th incubation day. Acetylcholine had no effect on basal cyclic AMP content, but it reduced cyclic AMP when the nucleotide had been increased by ISO. After hatching, ACh inhibited Ca-dependent action potentials and contractions not only "indirectly" but also "directly." "Direct" inhibition by ACh occurred in preparations treated with 6-hydroxydopamine (or reserpine) and propranolol, so that endogenous cardiac catecholamines probably were not involved. Further, basal levels of cyclic AMP increased after hatching and "direct" inhibition by ACh was associated with a reduction of cyclic AMP. These results are interpreted in a model in which cyclic AMP modulates the permeability of the membrane to Ca and in which GTP regulates the β-adrenergic receptor and adenylate cyclase. It is speculated that "indirect" muscarinic inhibition results from interference with GTP-dependent regulation of β-adrenergic receptor/adenylate cyclase interaction and "direct" muscarinic inhibition results from interference with GTP-dependent regulation of adenylate cyclase.


THE cardiac action potential is generated by two inward currents, a primary inward Na+ current that produces the rapidly rising phase and a secondary inward current (iCa) that is carried largely by Ca2+ and produces the plateau phase (McAllister et al., 1975; Beeler and Reuter, 1977). iCa is increased by sympathetic neurotransmitter in atrial and ventricular cardiac cells. This effect has been related to an increase in adenosine 3',5'-cyclic monophosphate (cyclic AMP) and accounts for the augmented strength of contractions (reviewed by Tsien, 1977; see also, Watanabe and Besch, 1974; Niedergerke and Page, 1977; Drummond and Severson, 1979).

In contrast to the general effect of sympathetic transmitter to increase iCa, the ability of the parasympathetic transmitter, acetylcholine (ACh), to reduce iCa and consequently to diminish contractions is not the same for all cardiac cells. In atrial muscle, ACh reduces contraction strength; the mechanism involves, in part, a reduction of iCa as shown by voltage clamp experiments in the frog (Giles and Noble, 1976) and in the mammal (Ten Eick et al., 1976). ACh also increases potassium conductance (gK), an effect which decreases contraction strength by reducing the duration of the action potential. Increased gK is the primary mechanism of action of ACh in the mammalian atrium (Ten Eick et al., 1976), whereas decreased gCa is the primary mechanism in the frog atrium (Giles and Noble, 1976). In ventricular muscle, the effectiveness of ACh is different in mammalian and nonmammalian species. ACh has little or no effect on electrical and mechanical activity in mammalian ventricular muscle (Hoffman and Cranefield, 1960). Presumably, ACh by itself lacks an inhibitory effect on iCa. However, several laboratories have reported that ACh does "indirectly" inhibit mammalian ventricles. That is, the inhibitory effect of ACh occurs only in the presence of substances which cause the accumulation of cyclic AMP. Excitation and contractions augmented by isoproterenol (ISO) (Bailey et al., 1979; Inui and Imamura, 1977; Watanabe and Besch, 1975), epinephrine (Meester and Hardman, 1967), norepinephrine (Dempsey and Cooper, 1969; Kissling et al., 1972), glucagon (Lucchesi, 1968), and phosphodiesterase inhibitors (Meester and Hardman, 1967) were diminished by a muscarinic effect of choline esters. These results raise the possibility that, in the mammalian ventricle, ACh inhibits only that portion of the contractile response which de-
depends on elevated levels of cyclic AMP. In the frog ventricle, muscarinic inhibition is demonstrable in the absence as well as in the presence of catecholamines (McAfee et al., 1978; Antoni and Rotmann, 1968). In the avian ventricle, as in the frog, ACh inhibits contractions "directly," that is, even in the presence of drugs that block either transmitter release from adrenergic nerves or adrenergic receptors (Bolton, 1967; Kissling et al., 1972).

It is of interest to determine whether changes in $i_a$ and contractility necessarily are linked to alterations in cyclic AMP. The increased susceptibility of the avian as compared to the mammalian ventricle to direct inhibition by ACh prompted a study of this matter in the chick. The following questions were considered: (1) Does ACh inhibit calcium-dependent action potentials in the absence of catecholamines? (2) If so, when does this effect appear during ontogenesis? and (3) Is the inhibitory effect of ACh in the avian ventricle related to a change of cyclic AMP?

**Methods**

Fertilized eggs (White Leghorn from SPAFAS) were kept at 37.5°C in a humidified incubator until the embryos reached the desired stage of development. The incubation ages given in the text are comparable with those given by others because chicks hatched on the 21st incubation day (Hamberger-Hamilton Stage 46) as expected. The animals were decapitated and the hearts were rapidly excised. After the ventricles had been separated from the atria, the ventricles were pinned to the bottom of a tissue chamber (3.5-ml volume) with the endocardial surface up. For 3- to 7-day preparations, both presumptive ventricles were used. For preparations 9 days and older, only the right ventricular free wall was excised. The preparations were superfused at a rate of 2.3 ml/min with modified Tyrode's solution containing (mM): $K^+$, 5.4; $Na^+$, 149; $Ca^{2+}$, 1.8; $Mg^{2+}$, 1.0; $Cl^-$, 148; $HCO_3^-$, 11.9; $H_2PO_4^-$, 0.4; and dextrose, 5.5, equilibrated with 95% $O_2$-5% $CO_2$. The tissue chamber was kept at 35°C; temperature did not vary by more than 0.5°C during an experiment. Membrane potentials were recorded with glass microelectrodes filled with 3 M KCl and having resistances of 15–30 MΩ. The micro electrode was connected to an Ag-AgCl half-cell; the reference electrode was a chlorided Ag wire. A DC preamplifier (WPI, model M-701) with a high input impedance and negative capacitance for optimal recording of potential transients was used to record membrane potentials. Membrane potentials were displayed on an oscilloscope and differentiated by an operational amplifier (Tektronix, type O) with a response linear from 0 to 500 V/sec to provide a measurement of $V_{max}$ during phase 0 of the action potential. Preparations were electrically driven by rectangular pulses delivered through a glass insulated silver electrode with a tip diameter of 250 μm.

(In some experiments, constant current pulses were used.) The method used to inactivate the primary $Na^+$ conductance and permit examination of Ca-dependent action potentials has been described by this laboratory (Pappano, 1970) and has been applied to embryonic chick heart muscle (Shigenobu and Sperelakis, 1972). The 25 mM $K^+$ solution used to inactivate the primary $Na^+$ conductance was made by substitution of $K^+$ for $Na^+$ in the Tyrode solution: the sum of $K^+$ and $Na^+$ concentrations was constant at 154.4 mM.

All drug solutions were prepared at the time of experiments. Ascorbic acid (57 μM) was present in all solutions which contained catecholamines to retard oxidation. 6-Hydroxydopamine [dissolved in 25% (wt/wt) ascorbic acid made in 0.9% NaCl] was injected intravenously 24 hours before experiments, at 100 mg/kg body weight. Reserpine was dissolved in 25% (wt/wt) ascorbic acid in distilled water and injected intraperitoneally 24 hours before experiments, at 5 mg/kg body weight. Cyclic AMP was measured by radioimmunoassay (New England Nuclear) and the content expressed per milligram wet weight of tissue and per milligram protein. The values were corrected for percent recovery of cyclic AMP, which averaged 92 ± 3% before hatching and 91 ± 3% after hatching. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard. Tension was measured (in normal Tyrode’s solution) using a displacement force transducer. Measurements are given as the mean ± standard error of the mean (SEM). Student’s t-test was used to evaluate the difference between means.

**Results**

**Calcium-Dependent Action Potentials in Ventricles Depolarized by 25 mM [K+]o**

In all preparations studied from the 3rd incubation day to the 10th day after hatching, the resting potential averaged −40 mV when ventricular muscle was superfused with Tyrode’s solution containing 25 mM $K^+$. At this potential, the fast $Na^+$ conductance was completely inactivated (Iijima and Pappano, 1979). However, most preparations could be excited to produce action potentials which exhibited very low rates of rise ($V_{max} = 10$ V/sec) and were not affected by tetrodotoxin (TTX). Such action potentials are called “slow” because of the slow $V_{max}$ and because they represent the activation of the secondary inward conductance mechanism ($g_a$). Slow action potentials are graded in amplitude and have a long refractory period. Therefore, high intensity stimulation at low frequencies (typically 0.05 Hz) was used to maximize the action potential amplitude. Spontaneously occurring slow action potentials were observed frequently in ventricles from the 3rd to the 9th incubation day (22 out of 32
TABLE 1
Effects of 10^{-6} M ISO* on Ca-dependent Action Potentials

<table>
<thead>
<tr>
<th>Age (days after fertilization)</th>
<th>Peak potential (E_p) (mV)</th>
<th>Rate of rise (V_{max}) (V/sec)</th>
<th>Duration (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n)</td>
<td>ISO (n)</td>
<td>Control (n)</td>
</tr>
<tr>
<td>4</td>
<td>20 ± 3 (14)</td>
<td>41 ± 1 (7)</td>
<td>4 ± 1 (11)</td>
</tr>
<tr>
<td>6</td>
<td>16 ± 2 (18)</td>
<td>34 ± 2 (3)</td>
<td>5 ± 0 (3)</td>
</tr>
<tr>
<td>9</td>
<td>26 ± 3 (12)</td>
<td>41 ± 2 (8)</td>
<td>3 ± 0 (9)</td>
</tr>
<tr>
<td>12</td>
<td>13 ± 1 (26)</td>
<td>40 ± 1 (7)</td>
<td>1 ± 0 (23)</td>
</tr>
<tr>
<td>15</td>
<td>15 ± 4 (12)</td>
<td>41 ± 1 (8)</td>
<td>2 ± 0 (11)</td>
</tr>
<tr>
<td>18</td>
<td>16 ± 2 (46)</td>
<td>41 ± 1 (60)</td>
<td>2 ± 0 (39)</td>
</tr>
<tr>
<td>21</td>
<td>15 ± 3 (29)</td>
<td>41 ± 3 (7)</td>
<td>3 ± 0 (16)</td>
</tr>
<tr>
<td>28</td>
<td>36 ± 1 (37)</td>
<td>44 ± 2 (8)</td>
<td>7 ± 0 (34)</td>
</tr>
</tbody>
</table>

* Isoproterenol increased E_p, V_{max}, and duration significantly (P < 0.05) at all ages. Duration equals the time between the upstroke of the action potential and 80% repolarization.

Inhibitory Effects of Cholinergic Drugs before Hatching

In embryonic ventricles, ACh had no effect on calcium-dependent action potentials evoked in 25 mM [K^+]_o in the absence of isoproterenol. The ineffectiveness of ACh (at a concentration of 10^{-5} M) was observed in 20 ventricles (a total of 30 different cells) that ranged in age from the 4th through the 18th incubation days. However, in ventricles of this group, ACh (10^{-6} to 10^{-4} M) or carbamylcholine (Carb) (10^{-7} to 10^{-5} M) inhibited action potentials that had been augmented by ISO. As shown in Figure 2, ACh alone had no effect on the calcium-dependent action potential evoked in a ventricular cell from the 18th incubation day (Fig. 2, A–C). Addition of ISO increased the amplitude, duration, and V_{max} of the action potential (Fig. 2D). Under these conditions, addition of ACh partially inhibited the action potential; amplitude, duration, and V_{max} decreased toward values observed prior to addition of ISO (Fig. 2, E and F). The effects of
Figure 2  The effects of $10^{-5}$ M ACh on the Ca-dependent action potential (AP) in the embryonic chick ventricle. All AP's in this figure were recorded during one impalement in an 18-day ventricle. Calibration format as in Figure 1. A: Control in 25 mM [K$^+$]. B: 4 minutes after introduction of $10^{-5}$ M ACh. C: The superimposed traces in A and B. D: After removal of ACh and addition of ISO (10$^{-6}$ M), the AP is larger than in the control. (The $V_{max}$ signal is now obscured by the stimulus artifact.) E: In the presence of ISO, $10^{-5}$ M ACh diminished the amplitude, duration, and rate of rise of the AP. This was taken 5 minutes after ACh was introduced. F: Traces in D and E are superimposed.

ACh in hearts studied before hatching are summarized in Table 2.

An indirect inhibitory effect of ACh was observed as early as the 7th incubation day. In Figure 3, records from a single cell in a 7-day ventricle are shown. ACh alone had no effect on this cell (not illustrated). After ISO had caused an increase in the action potential, however (Fig. 3B), ACh had an inhibitory effect (Fig. 3C). In 3- and 4-day embryos, an inhibitory effect of ACh did not occur even in the presence of ISO (three ventricles). ACh did not reduce the frequency of spontaneous calcium-dependent action potentials in 3- to 6-day ventricles in the presence of 10$^{-6}$ M ISO. In 5- and 6-day embryos, the results with evoked action potentials were inconclusive.

Inhibition of calcium-dependent action potentials by ACh was observed when the action potentials had been augmented by histamine (10$^{-5}$ M) as well as by ISO. The inhibitory effect of ACh or Carb was antagonized by 3 x 10$^{-7}$ M atropine. The muscarinic nature of the cholinergic inhibitory effect is illustrated in the records of Figure 4, taken from a single cell in a 15-day ventricle. The inhibitory effect of ACh (Fig. 4B) is reversed either by removal of ACh (Fig. 4C) or by the addition of atropine in the presence of ACh (Fig. 4F).

Experiments were done to determine if a direct inhibitory effect of ACh could be demonstrated in the embryonic ventricle by increasing the driving force for Ca$^{2+}$. In two 18-day ventricles, action potential amplitude was increased by raising the Ca$^{2+}$ in the bathing fluid from 1.8 to 3.6 mM. Under these conditions, however, ACh did not inhibit the action potentials (see example in Fig. 8, A and B). We considered the possibility that the relatively low resting potential in elevated potassium somehow interfered with the direct inhibitory effect of ACh. In three experiments (all in 12-day ventricles), the fast Na$^+$ conductance was inhibited by addition of TTX (3 x 10$^{-7}$ or 3 x 10$^{-6}$ M) in normal Tyrode's solution (5.4 mM K$^+$), and calcium-dependent action potentials were evoked from resting potentials greater than -70 mV. Under these conditions, ACh had no effect in the absence of ISO while continuing to exhibit its inhibitory effect in the presence of ISO. One of these experiments is illustrated in Figure 5.

### Table 2  Effects of ACh (10$^{-5}$ M) on Ca-dependent Action Potentials before Hatching: Differences ($\Delta n$) from Control Values Caused by ACh Alone and by ACh in the Presence of ISO (10$^{-6}$ M)*

<table>
<thead>
<tr>
<th>Age (days after fertilization)</th>
<th>Peak potential ($E_p$) (mV)</th>
<th>Rate of rise ($V_{max}$) (V/sec)</th>
<th>Duration (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACh alone $\Delta n$ (n)</td>
<td>ACh + ISO $\Delta n$ (n)</td>
<td>ACh alone $\Delta n$ (n)</td>
</tr>
<tr>
<td>7</td>
<td>2 ± 1 (4)</td>
<td>6 ± 3 (4)</td>
<td>0 ± 0 (2)</td>
</tr>
<tr>
<td>12</td>
<td>-2 ± 3 (4)</td>
<td>-10 ± 2 (3)</td>
<td>0 ± 0 (4)</td>
</tr>
<tr>
<td>15</td>
<td>-1 ± 2 (3)</td>
<td>-9 ± 1 (5)</td>
<td>-1 ± 0 (3)</td>
</tr>
<tr>
<td>18</td>
<td>-1 ± 1 (17)</td>
<td>-11 ± 2 (6)</td>
<td>0 ± 0 (13)</td>
</tr>
</tbody>
</table>

* At every age, the mean reduction in $E_p$, $V_{max}$, and duration caused by ACh (at 3-7 minutes after introduction of 10$^{-5}$ M ACh) was greater in the presence of ISO (10$^{-6}$ M) than in the absence of ISO. Each difference value (each n) was obtained during an impalement in one cell. Duration is defined in Table 1.
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Inhibitory Effects of Cholinergic Drugs after Hatching

By 5 days after hatching, muscarinic inhibition of calcium-dependent action potentials occurred in all preparations, even in the absence of catecholamines. A "direct" effect of Ach was seen only occasionally in ventricles taken from chicks on the day of hatching (day 21). Because endogenous catecholamines can be released from adrenergic nerves in the innervated ventricle (Higgins and Pappano, 1978), the effect of Ach was tested after 6-hydroxydopamine or reserpine pretreatment (see Methods) and/or in the presence of 3 × 10⁻⁷ M propranolol. A direct inhibitory effect of muscarinic drugs occurred in the presence of these agents (14 experiments). The results of such an experiment on a ventricle from the 7th day after hatching are shown in Figure 6, A-C. The animal had been treated with 6-hydroxydopamine 24 hours previously, and propranolol had been added to the 25 mM [K⁺]o solution. The calcium-dependent action potential, present in the control period (Fig. 6A), was inhibited by 10⁻⁵ M Ach (Fig. 6, B-C).

After hatching, ventricular cells continued to display "indirect" inhibition by Ach. Ach inhibited "directly" over the same range of concentrations (10⁻⁷ to 10⁻⁴ M) needed to inhibit "indirectly." However, the presence of ISO [or 3-isobutyl-1-methylxanthine (IBMX) or histamine] limited the inhibitory effect of any particular concentration of Ach. This is illustrated in Figure 6, D-F. Addition of IBMX (10⁻⁵ M) increased the amplitude, duration, and V_max of the action potential (Fig. 6E). Addition of 10⁻⁵ M Ach inhibited the calcium-dependent action potential (Fig. 6F), although the inhibition did not proceed to the same point as in the absence of IBMX (Fig. 6, F compared to C). The "direct"
TABLE 3  Effects of ACh (10⁻⁵ M) on Ca-dependent Action Potentials at and after Hatching: Differences (Δs) from Control Values Caused by ACh Alone and by ACh in the Presence of ISO (10⁻⁶ M)*

<table>
<thead>
<tr>
<th>Age (days after fertilization)</th>
<th>Peak potential (Eₚ) (mV)</th>
<th>Rate of rise (Vₘₚ) (V/sec)</th>
<th>Duration (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACh alone Δ(Δ)</td>
<td>ACh + ISO Δ(Δ)</td>
<td>ACh alone Δ(Δ)</td>
</tr>
<tr>
<td></td>
<td>A(n)</td>
<td>A(n)</td>
<td>A(n)</td>
</tr>
<tr>
<td>21 (hatching)</td>
<td>-2 ± 2 (6)</td>
<td>-15 ± 4 (3)</td>
<td>-1 ± 1 (3)</td>
</tr>
<tr>
<td></td>
<td>-7 ± 4 (3)</td>
<td>-7 ± 1 (5)</td>
<td>-6 ± 1 (5)</td>
</tr>
<tr>
<td>28 (propranolol)</td>
<td>-23 ± 7 (12)</td>
<td>-3 ± 1 (10)</td>
<td>-105 ± 11 (12)</td>
</tr>
<tr>
<td></td>
<td>-15 ± 7 (6)</td>
<td>-15 ± 7 (6)</td>
<td>-118 ± 8 (3)</td>
</tr>
<tr>
<td></td>
<td>-5 ± 9 (3)</td>
<td>-6 ± 9 (5)</td>
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</table>

* On day 21, but not on day 28, the mean reduction in Eₚ, Vₘₚ, and duration caused by ACh (10⁻⁵ M) was greater in the presence of ISO (10⁻⁶ M) than in its absence (P < 0.05). In addition, the effect of ACh alone was greater on the 28th day (with or without propranolol) than on the 21st day (P < 0.05); n is explained in Table 2.

(a) Absolute values of Ca⁺ are not understood in this Table.

In some experiments in posthatched ventricles, concentrations of ACh greater than 10⁻⁵ M were required to inhibit maximally the calcium-dependent action potential via the "direct" as well as the "indirect" mode. An illustration of the concentration dependence of the "direct" effect of ACh is given in Figure 7 for a cell in a ventricle 9 days after hatching. During cumulative addition of ACh (10⁻⁷ to 10⁻⁴ M), the action potential amplitude, the rate of rise, and the duration were increasingly diminished. In higher concentrations of ACh, the rate of repolarization of the action potential increased.

The direct effect of ACh appears at an age in which the control calcium-dependent action potential (that is, the action potential in drug-free 25 mM [K⁺]o Tyrode's solution) has increased significantly in amplitude, duration, and Vₘₚ (Table 1). This observation prompted experiments to test whether reduction of the action potential amplitude in the hatched ventricle would limit the direct effect of ACh. In two experiments, [Ca⁺]o was reduced from 1.8 to 0.9 or 0.45 mM. This modified the calcium-dependent action potential, as expected. However, 10⁻⁵ M ACh further reduced the action potential amplitude and duration. This result is illustrated in Figure 8 (C and D), where it is contrasted to the previously mentioned finding that augmentation of the action potential (by doubling [Ca⁺]o) did not allow observation of a direct effect of ACh in an 18-day ventricle (Fig. 8, A and B).

The age-dependence of the inhibitory effects of ACh in cells depolarized by K⁺ was paralleled by similar effects in normal Tyrode's solution. In seven out of eight ventricles, taken either from 18-day embryos or from chicks on the day of hatching, 10⁻⁵ or 10⁻⁴ M ACh had no inhibitory effect on action potentials (in one 21-day ventricle, action potential duration was reduced). In five ventricles from chicks 5–11 days after hatching, ACh reversibly reduced action potential duration. The resting potential was not affected by ACh. In embryonic ventricles, twitch tension was not reduced by ACh.
Effects of ACh on Basal Cyclic AMP and the Accumulation of Cyclic AMP Caused by ISO

<table>
<thead>
<tr>
<th></th>
<th>Cyclic AMP (pmol/mg wet weight)</th>
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<tbody>
<tr>
<td></td>
<td>18 days embryonic</td>
</tr>
<tr>
<td>Basal</td>
<td>0.4 ± 0.04 (18)</td>
</tr>
<tr>
<td>ACh (10^{-6} M)</td>
<td>0.4 ± 0.05 (4)</td>
</tr>
<tr>
<td>ISO (10^{-6} M)</td>
<td>0.7 ± 0.08 (5)</td>
</tr>
<tr>
<td>ISO + ACh</td>
<td>0.5 ± 0.08 (4)</td>
</tr>
</tbody>
</table>

Number of experiments given in parenthesis.

* ACh exposure for 3 minutes by itself or during last 3 minutes of a 6-minute exposure to ISO.

† Propranolol (3 × 10^{-7} m) present to antagonize endogenous catecholamines, had no significant effect per se on cyclic AMP.

‡ P < 0.05 when compared to basal values for the same age.

Effects of ACh on Cardiac Cyclic AMP—Relationship to "Indirect" and "Direct" Muscarinic Inhibition

The effects of ACh on basal cyclic AMP and the accumulation of cyclic AMP caused by ISO were examined before and after hatching. The basal cyclic AMP content of ventricular muscle increased significantly (P < 0.05) between the 18th incubation day (3 days before hatching) and 1 week after hatching (Table 4). When cyclic AMP content was expressed relative to protein, there was no significant difference (0.5 > P > 0.4) between the 18th incubation day (4.2 ± 0.41 pmol/mg protein) and 1 week after hatching (4.8 ± 0.15 pmol/mg protein). However, because of hypertrophy, the amount of protein per chick ventricle doubles by 7 days after hatching when compared to the value observed on the 18th incubation day. By contrast, the DNA content per ventricle changes slightly (~10%) and therefore the number of cells per milligram wet weight changes very little during this time (Doyle et al., 1974). In our experiments, the ratio of protein to wet weight increased significantly (P < 0.01) from 0.10 ± 0.003 on the 18th incubation day to 0.14 ± 0.003 at 1 week after hatching. Therefore, it is appropriate to express cyclic AMP activity relative to tissue weight. It may be concluded that the cyclic AMP content per cell is increased. Acetylcholine by itself had no effect on cyclic AMP in ventricles from the 18th incubation day, but it reduced the accumulation of cyclic AMP caused by ISO. After hatching, ACh reduced cyclic AMP in the absence and presence of ISO. Under conditions in which ACh reduced cyclic AMP, there was a concomitant reduction of developed tension. Acetylcholine had no effect on tension when it had no effect on cyclic AMP content.

Discussion

Inactivation of the fast Na⁺ conductance by K⁺-induced depolarization (Pappano, 1970) allows the study, in the embryonic avian heart, of the properties of the secondary inward current (i si) carried by Ca²⁺ (Shigenobu and Sperelakis, 1972). The present experiments confirm the results obtained by others insofar as catecholamines augmented calcium-dependent action potentials, presumably by increasing gCa (Niedergerke and Page, 1977; Reuter and Scholz, 1977; Tsien, 1977). The results extend those of Shigenobu and Sperelakis (1972) in that catecholamines augmented action potentials as early as the 3rd incubation day. Further, the results are consistent with the finding that β-adrenergic receptors and a positive inotropic effect of catecholamines are present in the chick on the 4th incubation day (Poison et al., 1977).

Others have concluded that intact embryonic chick ventricle is insensitive to muscarinic agents (Shigenobu and Sperelakis, 1972; Nakashishi and Takeda, 1969). (A different view is held by Hermseymeyer and Robinson, 1977). Our results agree with the conclusion that ACh alone had no effect on the calcium-dependent action potential in embryonic chick ventricle (Shigenobu and Sperelakis, 1972). However, the muscarinic receptor is clearly present on embryonic chick ventricle at least by the 7th day in ovo, since inhibition was observed in the presence of ISO. This result differs from that of Shigenobu and Sperelakis (1972). It may be noted that muscarinic receptors have been detected biochemically on embryonic chick ventricle as early as the 3rd day in ovo (Galper et al., 1977).

Cholinergic inhibition of calcium-dependent action potentials and contractions is of interest because of the apparent differences in mechanism of action of ACh on the atrium vs. the ventricle and in mammalian and nonmammalian vertebrates. The present report shows that muscarinic inhibition is the avian ventricle before hatching has properties much like those observed in the adult mammalian ventricle (reviewed by Levy, 1977). First, the inhibition is initiated by activation of muscarinic cholinergic receptors. Second, it occurs only in the presence of substances that increase gCa by increasing cyclic AMP; such substances include catecholamines, histamine, phosphodiesterase inhibitors, and glucagon (cf., Introduction). Finally, muscarinic inhibition of calcium-dependent action potentials is not observed when the electrochemical gradient for Ca²⁺ is increased, either by elevation of Ca²⁺ or by impulse initiation at more negative membrane potentials.

After hatching, muscarinic inhibition of calcium-dependent action potentials in the chick ventricle occurs "directly," that is, without the addition of substances that increase cyclic AMP. ACh and Carb retained their direct effects when animals were pretreated with 6-hydroxydopamine and reserpine, and in the presence of propranolol. Therefore, the "direct" inhibitory effect is not dependent on release of neuronal stores of catecholamines. The presence of "direct" in addition to "indirect" inh-
bition by ACh distinguishes the avian ventricle after hatching from the mammalian ventricle. The results indicate that, in the avian heart, there is a transition from a single to a dual mode for cholinergic inhibition during the 1st week after hatching.

Although several mechanisms can be offered to explain the two inhibitory actions of ACh in the hatched chick ventricle, we have considered the one shown in Figure 9. We considered a model in which both direct and indirect effects occur through a reduction in cyclic AMP synthesis, because ACh reduces cyclic AMP in parallel with its inhibitory effects on the action potential and contractions.

Experimental evidence in support of the general features of this model are given in Figure 9. With regard to the embryonic chick heart, it is pertinent that: (1) catecholamines interact with the β-adrenergic receptor, activate adenylate cyclase, and increase cyclic AMP as early as the 4th incubation day (Polson et al., 1977; Renaud et al., 1978); (2) the accumulation of cyclic AMP by catecholamines is associated with increased membrane permeability to calcium (Shigenobu and Sperelakis, 1972; present report) and increased contractility as early as the 4th incubation day (Polson et al., 1977); (3) the calcium-dependent action potential of the chick heart is increased by f-5-guanylimidodiphosphate [Gpp(NH)p], presumably by direct activation of adenylate cyclase (Josephson and Sperelakis, 1978); and (4) cholera toxin stimulates adenylate cyclase and increases the force of contraction as early as the 4th incubation day (Hedtke et al., 1976).

Our results are consistent with the possibility that, by reducing the accumulation of cyclic AMP, muscarinic agents interfere with activation of gsi and thereby inhibit calcium-dependent action potentials and contractions. Several laboratories have reported that catecholamine-induced accumulation of cyclic AMP is diminished by muscarinic agonists (Murad et al., 1962; Lee et al., 1971; McAfee et al., 1978), although there are exceptions to this pattern (Watanabe and Besch, 1975). It has been suggested that muscarinic drugs reduce activation of adenylate cyclase by modifying the GTP-dependent regulation of the β-receptor/adenylate cyclase system (Watanabe et al., 1978). Alternatively, diminished accumulation of cyclic AMP could be the result of more rapid degradation by phosphodiesterase. LaRaia and Sonnenblick (1971) have excluded the possibility that the diminished accumulation of cyclic AMP resulted from the stimulation of phosphodiesterase by ACh. Cholinergic inhibition of cyclic AMP accumulation in the thyroid gland has been attributed to a cyclic GMP-dependent activation of phosphodiesterase (Erneux et al., 1977). However, this mechanism may be unusual for cholinergic inhibition (Butcher, 1978), and there is considerable evidence for an inhibitory effect of muscarinic drugs on cardiac adenylate cyclase (Murad et al., 1962; Lee et al., 1971; Watanabe et al., 1978).

It is proposed that direct cholinergic inhibition seen after hatching is related to a reduction in basal cyclic AMP content and thereby to a reduction in the availability of gsi channels needed to generate the calcium-dependent action potential. Measurements of basal cyclic AMP content showed that the levels were higher after hatching than on the 18th incubation day and that ACh reduced cyclic AMP content only when it also inhibited the calcium-dependent action potential and contractions. It may be suggested that it is the rise of basal cyclic AMP which allows "direct" cholinergic inhibition after
hatching. Other data suggest that it may not be the rise in basal cyclic AMP per se which allows "direct" muscarinic inhibition. "Direct" inhibition by ACh could not be demonstrated at any age prior to hatching, yet preliminary experiments showed that basal cyclic AMP was greater in the 12-day embryonic ventricle (0.52 ± 0.03 pmol/mg wet weight, n = 6) than in the 18-day ventricle. Others have reported that basal cyclic AMP levels are much greater in young (4- to 7-day) than in old embryonic hearts (Renaud et al., 1978). A rise in basal cyclic AMP can explain the inhibition only if this increase of cyclic AMP occurs in an ACh-sensitive pool. Perhaps the rise of cyclic AMP in this pool may be related to an ontogenetic change in the GTP-dependent binding protein that allows direct stimulation of adenylate cyclase by GTP (mechanism II in Fig. 9). Whereas this proposal is admittedly speculative, it is noteworthy that Watanabe et al. (1978) reported that GTP increased basal adenylate cyclase activity and revealed muscarinic inhibition of the enzyme, even in the absence of β-receptor activation. In addition, these authors confirmed previous reports that the regulation of β-receptor and adenylate cyclase by GTP are mechanistically separable. We propose, as a working hypothesis, that these two aspects of GTP-dependent regulation may appear at different times during development.

Although the hypothesis that the inhibitory effects of ACh are caused by reductions of cyclic AMP is consistent with our results, alternative explanations should be considered. First, ACh may interfere with some effect of cyclic AMP, that is, with step(s) subsequent to cyclic AMP production (Watanabe and Besch, 1975). If this were the case, it might be predicted that the inhibition caused by ACh would not be overcome by providing excess cyclic AMP (in the form of exogenous dibutyryl-cyclic AMP, for example). This has not been tested. A second alternative is that the observed changes in cyclic AMP could be coincidental with an action of ACh on another cyclic nucleotide, namely, 3',5'-cyclic guanosine monophosphate (cyclic GMP). Some have suggested that the effects of ACh are mediated by increased production of cyclic GMP (reviewed in Goldberg et al., 1973). For example, Kohlhardt and Haap (1978) reported that 8-bromocyclic GMP mimicked the inhibitory effect of ACh on another cyclic nucleotide, namely, 3',5'-cyclic guanosine monophosphate (cyclic GMP). However, it has not been shown that cyclic GMP represents a trophic effect of cholinergic nerves on cardiac muscle cells. Whereas a systematic investigation is needed to test this proposal, we have observed inhibitory cholinergic innervation as early as the 18th embryonic day in the chick ventricle (Biegon and Pappano, unpublished observations).

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