Action Potentials in Chick Atria

Ontogenetic Changes in the Dependence of Tetrodotoxin-Resistant Action Potentials on Calcium, Strontium, and Barium

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SUMMARY Action potentials were recorded from chick embryo atrial muscle cells bathed in Tyrode's solution. Tetrodotoxin (TTX), 3.1 μM, was added to block the early, transient, Na+-dependent conductance system. Rectangular stimuli were used to evoke action potentials the peak amplitude (E_p) of which depend on the external concentration of divalent cations, [Me₁⁺]. The relationship between potentials the peak amplitude (E_p) and [Me₁⁺] shifted to the right with increasing age. For example, the slope of E_p was 33 ± 2, 22 ± 1 and 11 ± 3 mV per 10-fold change in [Ca²⁺] on the 9th, 12th, and 18th incubation days, respectively. Solutions with reduced [Ca²⁺], E_p increased when Ba²⁺ or Sr²⁺ was added to the bath. The potency of Me₁⁺ in generating action potentials was Ba²⁺ > Sr²⁺ > Ca²⁺ and this sequence did not change during development. Action potential amplitude, which was reduced in 18-day preparations, was increased by isoproterenol (increased Ca²⁺ conductance, g_Ca) and by tetraethylammonium (TEA) ion (decreased K⁺ conductance, g_K). The results show that (1) Me₁⁺-dependent action potentials support membrane excitation in chick atrial cells treated with TTX, and (2) the ability of Me₁⁺ to support action potentials decreases during ontogenesis. We conclude from these experiments that the ontogenetically related decrease in Me₁⁺-induced action potentials is the result of a reduction in g_Me₁⁺/g_K during stimulation.

Methods

Fertilized eggs (white Leghorn) were obtained from a commercial supplier and were kept at 37.5°C in a humidified incubator. The incubation ages given in the text agree with those given by others, since chicks hatched on the 21st incubation day as expected. The chicks were decapitated and the hearts were removed and pinned to the bottom of a tissue chamber that contained about 10 ml of a modified Tyrode's solution containing (mM): K⁺, 5.4; Na⁺, 149; Ca²⁺, 1.8; Mg²⁺, 1.0; Cl⁻, 148; HCO₃⁻, 11.9; H₂PO₄⁻, 0.4; and glucose, 5.5. The atrial endocardial surface was exposed by cuts made along the atrioventricular groove and the lateral margins of the atria. The preparations were equilibrated in Tyrode's solution for 30 minutes. The solution was equilibrated with 95% O₂-5% CO₂ and had a pH of 7.3. Bath temperature was maintained at 30 ± 1°C for all experiments and did not vary by more than 0.5°C in individual experiments.

Membrane potentials were recorded with glass microelectrodes having tip diameters <1 μm and resistances of 15-30 megohms. Signals were fed through a high input impedance preamplifier: capacitance neutralization was used to obtain optimal recording of membrane potential transients. The signals were displayed on an oscilloscope. In some experiments, the maximum rate of rise of the action potential was obtained with an operational amplifier (Tektronix, type 0) the response of which was linear from 0 to 500 V/sec. The output of the differentiator was displayed on the second beam of the oscilloscope. Rectangular pulses of constant voltage were applied to the right atrium-right sinoatrial valve region through platinum-iridium wires insulated to the tip. Additional details of the experimental changes on the resistance of action potentials to blockade by TTX.
The Ca$^{2+}$ and Mg$^{2+}$ analyses were made with an atomic absorption spectrophotometer (Perkin-Elmer 290B). Drugs used in this study include isopropylnaterenol hydrochloride, tetraethylammonium chloride, and TTX.

**Results**

There was no significant change in the resting potential (E$_r$) and action potential peak (E$_{ap}$) of atrial cells between the 9th and 18th days of incubation (Table 1). The maximum rate of rise (V$_{max}$) increased significantly between the 9th and 12th days (P < 0.01); no statistically significant changes in V$_{max}$ occurred after the 12th day of incubation. These data confirm and extend those published previously; no statistically significant differences were measured systematically for 36 impalements of atrial cells in 10 preparations from the 18th day of incubation.

The spread of contraction from the sinoatrial pacemaker (sinus venous-sinoatrial valve region) to the atria ceased in preparations from chicks of all ages when TTX (3.1 x 10$^{-4}$ M) was added to the bathing solution. In addition, the incidence of spontaneous contractions in the sinoatrial region of preparations treated with TTX decreased progressively during development. Spontaneous contractions in the sinoatrial region occurred in four of nine preparations isolated on the 9th day of incubation, two of seven on the 12th day of incubation, one of four on the 15th day, and in one of 10 preparations on the 18th day. Spontaneous contractions of the sinoatrial region from all preparations stopped when the external [Ca$^{2+}$]$_0$ was reduced to one-half normal.

Stimuli were applied through electrodes placed adjacent to the sinoatrial valves in order to evoke action potentials in the presence of TTX. The recording microelectrode was placed in left atrial cells. In the presence of TTX, rectangu-
Ca-DEPENDENT POTENTIALS IN CHICK ATRIA/Pappano

Table 2: Membrane Potentials of Chick Atrial Cells in Tetrodotoxin (3.1 μM)

<table>
<thead>
<tr>
<th>Incubation age (days)</th>
<th>Resting E_m (mV)</th>
<th>E_p (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 (48)</td>
<td>−59 ± 1</td>
<td>+9 ± 1</td>
</tr>
<tr>
<td>12 (38)</td>
<td>−58 ± 1</td>
<td>+1 ± 3</td>
</tr>
<tr>
<td>15 (18)</td>
<td>−56 ± 1</td>
<td>−25 ± 7</td>
</tr>
<tr>
<td>18 (65)</td>
<td>−58 ± 1</td>
<td>−34 ± 4</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE.

Resting E_m = resting membrane potential; E_p = membrane potential as peak of action potential. Numbers in parentheses denote the number of cells studied.

The peak of the action potential was positive (that is, there was an overshoot) in preparations from the 9th and 12th incubation days, but not in those from the 15th and 18th incubation days. The values of E_p given in Table 2 for the 15th and 18th incubation days included results from all cells tested. If the values of E_p were taken only from cells that displayed regenerative potential changes, E_p averaged −5 ± 5 mV and −10 ± 4 mV on the 15th and 18th incubation days, respectively. These values, like those given in Table 2, are significantly lower than those obtained from atrial preparations on the 9th and 12th incubation days.

EFFECT OF CALCIUM ON MEMBRANE POTENTIALS IN THE PRESENCE OF TTX

The effects of variations in [Ca^{2+}]_o (0.5 to 3 times normal) on the membrane potentials of chick embryo atrial cells are shown in Figure 2. The resting potentials obtained in the presence of normal [Ca^{2+}]_o did not differ significantly from those obtained in solutions containing ½ normal [Ca^{2+}]_o. Elevation of [Ca^{2+}]_o above normal was associated with a 4-mV decrease (P < 0.05) in the resting potential of 9-day preparations and a 5-mV (P < 0.02) increase in the resting potential of 18-day preparations. Regression analysis showed that the resting potential tended to decrease as [Ca^{2+}]_o increased (−8 ± 1 mV per 10-fold change in [Ca^{2+}]_o) in cells from the 9th incubation day. Similar results (−8 ± 1 mV per 10-fold change in [Ca^{2+}]_o) were obtained from analysis of resting potential data from preparations on the 6th incubation day in which [Ca^{2+}]_o ranged from 0.5 to 3.6 mM. The slope of this relationship decreased for atrial cells from the 12th incubation day (−6 ± 0.1 mV per 10-fold change in [Ca^{2+}]_o) and was reversed in cells from the 18th incubation day (8 ± 1 mV per decade change in [Ca^{2+}]_o).

The amplitude of the action potentials was directly related to [Ca^{2+}]_o (Fig. 2). The rate of change of E_p with re-

Figure 2: Effect of [Ca^{2+}]_o on membrane potentials of developing chick atrial cells exposed to tetrodotoxin. Ordinate: membrane potential in millivolts. Abscissa: concentration of Ca^{2+} in bathing fluid in millimoles per liter (logarithmic). The nonfilled symbols give the resting potential (E_r) and the filled symbols give the peak of the action potential (E_p) for cells from the 9th (O, ∆), 12th (Δ, △), 15th (O, ★), and 18th (O, ♦) incubation days. The number of measurements at each [Ca^{2+}]_o is given in parentheses. The symbols for resting potential do not show a standard error for this is included in the dimensions of the symbol.

Figure 3: Effect of Ba^{2+} on membrane potentials of atrial cells on the 12th and 18th incubation days. Ordinate: membrane potential in millivolts. Abscissa: [Ba^{2+}]_o in millimoles per liter. The nonfilled and filled symbols refer to the resting potential (E_r) and peak potential (E_p), respectively, obtained from cells on the 12th (Δ, △) and 18th (O, ♦) incubation days. See Figure 2 legend for additional details.
The results obtained with Ba\textsuperscript{2+} in preparations from the 12th and 18th incubation days are shown in Figure 3. The values for resting potential and peak potential obtained in TTX and \(\frac{1}{2}\) the normal \([Ca^{2+}]_0\) (no Sr\textsuperscript{2+} added) are shown on the far left for the 12th incubation day. Addition of 0.5 mM Sr\textsuperscript{2+} was associated with an increase in the peak of the action potential and an overshoot (8 ± 3 mV) was observed when \([Sr^{2+}]_0\) was 1 mM in preparations from the 9th incubation day. No consistent change in the resting potential occurred as \([Sr^{2+}]_0\) was changed. The relationship between \(E_p\) and \([Sr^{2+}]_0\) shifted to the right for cells from the 18th incubation day. Thus, an overshoot of 10 mV was obtained at 1.4 mM and 5 mM \([Sr^{2+}]_0\) on the 9th and 18th incubation days, respectively. The effects of added Sr\textsuperscript{2+} on the ability of atrial cells to generate action potentials, shown in Figure 6, are taken from a preparation on the 12th incubation day. In the presence of TTX and \(\frac{1}{2}\) the normal \([Ca^{2+}]_0\), 1 mM Sr\textsuperscript{2+} allowed action potentials (Fig. 6A). Increasing the \([Sr^{2+}]_0\) to 3 mM augmented the amplitude of the action potential (Fig. 6B).

It was estimated that the \([Ba^{2+}]_0\) at an overshoot of 10 mV was 0.16, 0.10, and 1.5 mM on the 9th, 12th, and 18th incubation days, respectively. An overshoot of 10 mV occurred at \([Ca^{2+}]_0\) of 1.8 and 3.0 mM on the 9th and 12th incubation days, respectively. Therefore, the order of potency of Me\textsuperscript{2+} in generating evoked action potentials was Ba\textsuperscript{2+} > Sr\textsuperscript{2+} > Ca\textsuperscript{2+} at any of the ages examined during development. As shown in Table 3, the ability of Me\textsuperscript{2+} to induce spontaneously occurring action potentials followed the same pattern.

**FIGURE 4** Restoration of action potentials by Ba\textsuperscript{2+} in atrial cell (18th incubation day). Records taken from a single impalement, calibration for voltage (50 mV) applies to both records. Horizontal calibration for time equals 100 msec in panel A and 400 msec in panel B. Zero potential is shown by upper horizontal line in panel B. (A) Control showing no action potential with intense stimulation (80 mA, 10 msec) in the presence of tetrodotoxin (TTX) and \(\frac{1}{2}\) normal Ca\textsuperscript{2+}. (B) Spontaneously occurring action potential at 1 minute after addition of 1 mM Ba\textsuperscript{2+}.

**FIGURE 5** Effects of \([Sr^{2+}]_0\) on membrane potentials of tetrodotoxin-treated preparations from the 9th and 18th incubation days. Ordinate: membrane potentials in millivolts. Abscissa: \([Sr^{2+}]_0\) in millimoles per liter. See Figure 2 legend for additional details.
The amplitude of the TTX-resistant component of the action potential was directly related to $[Ca^{2+}]_0$, $[Ba^{2+}]_0$, and $[Sr^{2+}]_0$, all divalent metal ions ($Me^{2+}$) that display current-carrying ability in the adult myocardium. Barium restored action potentials to atrial cells bathed in Na+-free (Tris+ substitution), TTX-containing saline solution; this finding supports the conclusion that $Me^{2+}$ is a current carrier in these cells (Pappano, unpublished observations). Pharmacological evidence also supported this conclusion. The ability of isoproterenol to increase the amplitude of action potentials in the presence of TTX is attributed to an increased membrane conductance to $Ca^{2+}$ ($g_{Ca^{2+}}$). This conclusion has been reached by others who studied the effects of catecholamines on cardiac tissues from adult18 and embryonic9 animals. In addition, the results of experiments with TEA+ demonstrated that the increased amplitude depended upon [TEA+] and increased $[Ca^{2+}]_0$. Calcium-dependent action potentials were evoked in mammalian Purkinje fibers when 146 mM TEA+ replaced Na+ in the bathing solution;17 the effects of TEA+ may have been due to inhibition of outward K+ current as well as to osmotic replacement of Na+. However, the lower concentration of TEA+ used for the present study would not be in keeping with the alkylammonium ion acting as an Na+ replacement but could allow observation of the effects of the inward $Ca^{2+}$ current by inhibiting $g_K$.

The current carried by $Me^{2+}$ ($i_{Me^{2+}}$) across the atrial membrane is the product of the ionic conductance ($g_{Me^{2+}}$) and the electrical driving force for $Me^{2+}$, that is, $i_{Me^{2+}} = g_{Me^{2+}} (E_{Me^{2+}} - E_m)$, where $E_m$ is the resting potential and $E_{Me^{2+}}$ is the equilibrium potential of a particular divalent cation. It is assumed that the driving force of $Me^{2+}$ did not decrease over the developmental time span examined. The resting potentials in TTX were not different and it can be inferred that $E_{Me^{2+}}$ did not decrease. The latter is supported by the finding that the maximum amplitude of $Ba^{2+}$-dependent and $Sr^{2+}$-dependent action potentials was not diminished in atrial cells of day 18 chicks as compared to those from days 9 and 12. It is reasonable to assume that the peak potential is determined by the simultaneous flow of an outward current and an inward current when the peak of the action potential is considerably less than $E_{Me^{2+}}$. Therefore, it is concluded that the ability of $Me^{2+}$ to support action potentials declined during ontogenesis because of a reduction in $g_{Me^{2+}}/g_K$ during excitation where $g_{Me^{2+}}$ represents membrane conductance to inward $Me^{2+}$ current and $g_K$ represents membrane conductance to outward K+ current.

Consistent with the suggestion that $g_{Me^{2+}}$ during excita-

### Table 3: Concentrations of $Me^{2+}$ that Allowed Spontaneous Action Potentials*

<table>
<thead>
<tr>
<th>Incubation age (days)</th>
<th>$[Ba^{2+}]_0$ (mM)</th>
<th>$[Sr^{2+}]_0$ (mM)</th>
<th>$[Ca^{2+}]_0$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.2-0.5</td>
<td>0.5-2</td>
<td>1.6-4.8</td>
</tr>
<tr>
<td>12</td>
<td>0.2-1</td>
<td>2-5</td>
<td>1.4-3.7</td>
</tr>
<tr>
<td>18</td>
<td>0.5-1.2</td>
<td>Not observed</td>
<td>Not observed at 5 mM</td>
</tr>
</tbody>
</table>

* Spontaneous action potentials observed when $Me^{2+}$ was added to bathing solution containing tetrodotoxin ($3.1 \times 10^{-6}M$) and 8 normal $Ca^{2+}$. 

Discussion

Atrial cells of the chick embryo heart have two components of inward current during the action potential.3 The early current is kinetically rapid, Na+-dependent, and sensitive to blockade by TTX; the late current is kinetically slow, Ca2+-dependent, and insensitive to blockade by TTX. It generally is agreed that the fast and slow components of inward current produce the rapidly rising phase and the plateau phase, respectively, of the cardiac action potential.6

The present results confirm and extend the conclusion regarding the calcium-dependence of the slow component of
the amplitude of Me\textsuperscript{2+}-dependent action potentials decreased at specified [Me\textsuperscript{2+}]_o and that the relationship between the action potential peak and [Me\textsuperscript{2+}]_o shifted to the right with increasing age. Also, stimulus durations required to evoke action potentials in atrial cells increased with increasing age. The fact that the ability of Me\textsuperscript{2+} to allow atrial action potentials followed the same sequence (Ba\textsuperscript{2+} > Sr\textsuperscript{2+} > Ca\textsuperscript{2+}) during the period studied suggested that the selectivity of the conductance mechanism was not appreciably changed. Experiments with Ba\textsuperscript{2+} and Sr\textsuperscript{2+} were not conducted in the absence of Ca\textsuperscript{2+} because the membrane depolarized. This depolarization (to about -30 mV) is greater than that reported for 3-day chick embryo ventricular cells bathed in Ca\textsuperscript{2+}-free saline. It may be that atrial membranes are more sensitive than ventricular membranes to the stabilizing effect of Ca\textsuperscript{2+}. Alternatively, the stabilizing effect of Ca\textsuperscript{2+} increases with age and the function of Ca\textsuperscript{2+} shifts during development from current carrier to membrane stabilizer. An example of this shift toward increased stabilization can be found in the effects of Ca\textsuperscript{2+} on the resting potential. The resting potential increased as [Ca\textsuperscript{2+}]_o increased in cells from the 18th but not from the 6th, 9th, and 12th incubation days. It is of interest that the amplitude of potentials in preparations from the 18th incubation day, particularly when [Ca\textsuperscript{2+}]_o was increased. Tetraethylammonium ion, which inhibited g_k in nerve and muscle membranes of adult animals, prolonged the action potential in embryonic rat ventricular muscle. This action of TEA\textsuperscript{+}, which is due to inhibition of g_k, increased with age. The actions of Ba\textsuperscript{2+} can also be related to inhibition of g_k. Barium is the most potent of the divalent cations (Ba\textsuperscript{2+} > Sr\textsuperscript{2+} > Ca\textsuperscript{2+}) in supporting spontaneous and evoked atrial action potentials. The same sequence was reported for Me\textsuperscript{2+}-dependent action potentials in cultured chick ventricular cells.17 Atrial membranes on the 18th incubation day were more sensitive than those on the 9th and 12th incubation days to the depolarizing action of Ba\textsuperscript{2+}. This change is attributed to greater inhibition of g_k in membranes from older preparations and agrees with the conclusion that Ba\textsuperscript{2+} depolarized heart muscle by reducing outwardly directed K\textsuperscript{+} current.18 Barium is the most potent of these cations (Ba\textsuperscript{2+} > Sr\textsuperscript{2+} > Ca\textsuperscript{2+}) with respect to its ability to inhibit g_k as well as with respect to its ability to carry current in barnacle muscle fibers.19 The relationship between peak potential and [Ba\textsuperscript{2+}]_o, which can have a slope of >60 mV per 10-fold change in [Ba\textsuperscript{2+}]_o, has been attributed to an inward Me\textsuperscript{2+} current carried by Ba\textsuperscript{2+} and to a simultaneous inhibition of outward K\textsuperscript{+} current by Ba\textsuperscript{2+} in voltage-clamped barnacle muscle fibers.19 It seems probable that this explanation applies to chick embryo atrial cells. The decreased ability of Me\textsuperscript{2+} to generate action potentials as shown in the present study is paralleled by an increased contribution of Na\textsuperscript{+} to the early component of the action potential.2 It has not been possible to verify the proposal that conversion from "slow" to "fast" channels accompanies the increased susceptibility to TTX during development.25 The mechanism responsible for rapid transition in membrane excitation properties of heart cell aggregates also remains unresolved. The change from TTX-sensitive, fast channel action potentials to verapamil-sensitive, slow channel action potentials occurred within 4 hours, that is, considerably more rapidly than the reverse transition observed during ontogenesis. However, it cannot be decided whether the transition depended on changes in existing fast channels or in the appearance of newly synthesized slow channels in ventricular membranes. The transition in susceptibility of action potentials to blockade by TTX and in cation-dependence of excitation in

**Table 4** Effect of Tetraethylammonium (TEA) on Membrane Potentials in 18-Day Chick Embryo Atria

<table>
<thead>
<tr>
<th>Tetrodotoxin, 3.1 ( \mu \text{M} ) (14 impalements)</th>
<th>Plus TEA, 20 mm, (15 impalements)</th>
<th>Plus TEA, 20 mm, and Ca, 3.9 mM (10 impalements)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting ( E_m ) (mV)</td>
<td>(-57 \pm 1)</td>
<td>(-57 \pm 2)</td>
</tr>
<tr>
<td>( E_p ) (mV)</td>
<td>(-37 \pm 8)</td>
<td>(-11 \pm 9)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE.

Resting \( E_m \) = resting membrane potential; \( E_p \) = membrane potential at peak of action potential.

* \( P < 0.05 \) when compared to control.

**Figure 7** Isoproterenol-induced increase of action potential amplitude in atrial cell from the 18th incubation day. Vertical (50 mV) and horizontal (50 msec) calibrations in panel B apply to both records. Zero line is shown by uppermost horizontal line in panel B. (A) control action potential evoked by intense stimulus (150 V, 50 msec) in the presence of tetrodotoxin (TTX) 3.1 \( \times 10^{-4} \) M. (B) superimposed traces showing control action potential (record 1) and action potential recorded 3 minutes after addition of isoproterenol, 5 \( \times 10^{-4} \) M (record 2).
chick embryo atria may be a specific example of a general phenomenon occurring in excitable cells during ontogenesis. Action potentials in the tunicate were transformed from a dependence on Na⁺ and Ca⁺⁺ in the unfertilized egg to a dependence on Ca⁺⁺ in the differentiated skeletal muscle cell. A Ca-dependent component of the action potential was observed in cultured rat myotubes (Bade-dependent action potentials also occurred) in which TTX does not block the action potential. The action potential of fetal rat skeletal muscle is resistant to the inhibitory action of TTX, whereas that evoked in muscles from rats 20 days after birth is blocked by TTX. The membrane of adult skeletal muscle can display resistance to TTX similar to that of the fetus, if the nerve is cut and allowed to degenerate. Transmission between postganglionic cholinergic nerves and sinoatrial pacemaker cells can be detected on the 12th incubation day. The time course for development of functional innervation and for the transition in membrane excitation properties does not support the possibility of a causal relationship between the two phenomena in cardiac muscle. In addition, experiments with heart cell cultures show that the change in membrane excitation properties (loss or retention, or both, of TTX reactivity) can be independent of functional innervation.

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