On the Ionic Mechanism Underlying Adrenergic-Cholinergic Antagonism in Ventricular Muscle

IRA JOSEPHSON and NICK SPERELAKIS

From the Department of Physiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

ABSTRACT In atrial muscle, acetylcholine (ACh) decreases the slow inward current (I\textsubscript{si}) and increases the time-independent outward K\textsuperscript{+} current. However, in ventricular muscle, ACh produces a marked negative inotropic effect only in the presence of positive inotropic agents that elevate cyclic adenosine monophosphate (AMP). A two-microelectrode voltage-clamp method was used on cultured reaggregates of cells from 16-20-d-old embryonic chick ventricles to determine the effects of ACh on I\textsubscript{si} and outward current during \beta-adrenergic stimulation. Only double penetrations displaying low-resistance coupling were voltage-clamped. Cultured reaggregates are advantageous because their small size (50-250 /m) permits better control of membrane potential and adequate space clamp. Tetrodotoxin (10\textsuperscript{-6} M) and a holding potential of -50 to -40 mV were used to eliminate the fast Na\textsuperscript{+} current. Depolarizing voltage steps above -40 mV caused a slow inward current to flow that was sensitive to changes in [Ca\textsubscript{o}] and was depressed by verapamil (10\textsuperscript{-6} M). Maximal I\textsubscript{si} was obtained at -10 mV and the reversal potential was about +25 mV. Isoproterenol (10\textsuperscript{-6} M) increased I\textsubscript{si} at all clamp potentials. Subsequent addition of ACh (10\textsuperscript{-6} M) rapidly reduced I\textsubscript{si} to control values (before isoproterenol) without a significant effect on the net outward current measured at 300 ms. The effects of ACh were reversed by muscarinic blockade with atropine (5 \times 10\textsuperscript{-6} M). We conclude that the anti-adrenergic effects of ACh in ventricular muscle are mediated by a reduction in Ca\textsuperscript{2+} influx during excitation.

INTRODUCTION

Avian myocardium contains a rich supply of cholinergic nerve terminals (Bolton, 1967), and muscarinic cholinergic receptors are present in both ventricular and atrial sarcolemmal membranes during embryonic development (Galper et al., 1977). However, in embryonic chick as well as in mammalian species, the parasympathetic transmitter acetylcholine (ACh) produces almost no effect on the contractile state of the ventricle in the
absence of adrenergic nerve stimulation (Hoffman and Cranefield, 1960; Biegon and Pappano, 1980). In contrast, in the presence of adrenergic tone, ACh exerts a negative inotropic effect on the ventricle, which has been termed accentuated antagonism (Levy, 1977).

The influx of Ca\(^{2+}\), through kinetically slow voltage-sensitive channels, is the trigger and modulator of the contractile force in myocardial cells (e.g., New and Trautwein, 1972). For studying this slow inward current \(I_{\text{si}}\), functional removal of the fast Na\(^{+}\) current (by blockade with tetrodotoxin, or by voltage inactivation using elevated external K\(^{+}\)) is often done. Under these conditions, a slowly rising action potential (AP) is produced and has a plateau component resembling that of the normal action potential. Because changes in the rate of rise and overshoot of these slow APs are related to changes in \(I_{\text{si}}\), these parameters can be used as a relative measure of the amount of Ca\(^{2+}\) entering the cell during excitation. A number of positive inotropic agents (such as catecholamines, histamine, and methylxanthines) act to restore or enhance slow APs (Shigenobu and Sperelakis, 1972; Schneider and Sperelakis, 1975; Josephson et al., 1976). These agents also raise intracellular cyclic AMP levels (McNeill and Muschek, 1972; Tsien, 1977). This suggests the intriguing possibility that slow channel conductance may be controlled by phosphorylation of a protein constituent of the slow channel via a cyclic AMP-dependent protein kinase (Sperelakis and Schneider, 1976; Reuter and Scholz, 1977).

One action of ACh, leading to negative inotropy in ventricular muscle, could be to inhibit the Ca\(^{2+}\) influx across the sarcolemma during excitation. Using the Ca\(^{2+}\)-dependent slow action potential, several groups have reported that ACh did reduce the rate of rise of slow APs that had been enhanced by catecholamines, histamine, or methylxanthines. However, ACh did not affect slow APs enhanced by increasing the driving force for Ca\(^{2+}\) (Inui and Imamura, 1977; Biegon and Pappano, 1980). These results are consistent with the hypothesis that the effects of ACh are related to the elevation of intracellular levels of cyclic AMP by these positive inotropes, and that ACh may somehow act to interfere with the production or the expression of this cyclic nucleotide (Biegon and Pappano, 1980) (see Discussion).

However, studies using the slow AP are not able to rule out an effect of ACh on K\(^{+}\) conductances. Although ACh has little or no effect on the duration of the normal ventricular AP, it is well known to produce profound shortening of the atrial AP. This effect has been explained as an increase in the K\(^{+}\) conductance of the membrane (Hutter and Trautwein, 1956), and \(^{42}\)K flux experiments support this conclusion (Harris and Hutter, 1956). Examinations of atrial tissue using voltage-clamp techniques have revealed that in addition to an increase in K\(^{+}\) conductance, \(I_{\text{si}}\) is inhibited by ACh. In frog atrium, a decrease in \(I_{\text{si}}\) is the dominant mechanism for the negative inotropic effects of ACh (Giles and Noble, 1976), whereas an increase in K\(^{+}\) conductance appears to be the more important mechanism in mammalian atrium (Ten Eick et al., 1976).

In the present study, we wished to ascertain whether the primary action of ACh on adrenergically stimulated ventricular myocardial cells was to reduce \(I_{\text{si}}\) or to increase \(I_{\text{K}}\). For these studies, we have used small (50–250 \(\mu\)m) cultured
reaggregates of embryonic chick ventricular myocytes that can be voltage-clamped using a standard two-microelectrode technique for analysis of the membrane currents (see DeHaan and Fozzard, 1975; Nathan and DeHaan, 1979; and Ebihara et al., 1980).

**METHODS**

**Culture Preparation**

Reaggregate cell cultures were prepared with cells isolated from 16–20-d-old embryonic chick hearts (ventricles) using methods previously described (Josephson et al., 1976). In brief, 12–24 hearts were isolated under sterile conditions, and the atria were dissected free and discarded. The ventricles were washed free of blood, minced, and collected in cold (4°C) culture medium (medium 199 containing 10% fetal calf serum). A suspension of single cells was obtained by 8–10 successive dissociations (each lasting 5 min) in a Ca2+- and Mg2+-free Ringer solution containing trypsin (0.05%); the dissociation solution was gently stirred using a magnetic stirring bar. Since the first two dissociation periods yielded only relatively few viable ventricular cells, they were usually discarded. The cells obtained from the remaining dissociations were pooled, pelleted, and then washed several times to remove any remaining trypsin not inactivated by the serum. The cells were suspended in culture medium and plated at a density of about 10⁶ cells/ml into plastic culture dishes to which the cells did not adhere. Reaggregates, ranging from 50–250 μm, formed during incubation of the cell suspensions (at 37°C in a moist atmosphere gassed with 95% air and 5% CO2) for 24–48 h. The cultures were maintained for periods of up to 3 wk by frequent changes of the culture medium.

**Electrophysiology**

For electrophysiological experiments, reaggregates were transferred by pasteur pipette to a heated (34 ± 1°C) bath (volume 0.5 ml) and were superfused with oxygenated (95% O2, 5% CO2) Tyrode solution (pH 7.4 ± 0.2) at a rate of 1 ml/min. The composition of the Tyrode solution was: 136.9 mM NaCl; 2.68 mM KCl; 1.84 mM CaCl₂; 1.03 mM MgCl₂; 11.91 mM NaHCO₃; 0.38 mM NaH₂PO₄·H₂O; 5.5 mM dextrose.

A DAGAN intracellular preamp-clamp (model 8500; DAGAN Corp., Minneapolis, Minn.) was used for intracellular recording and for voltage-clamp experiments. Glass microelectrodes, filled with 3 M KCl (resistance 10–30 MΩ), were connected via Ag:AgCl half-cells to the differential inputs of the preamplifier. The microelectrode connected to the inverting input was positioned outside the reaggregate in the bath and served as the voltage reference. Intracellular current pulses could be applied, when desired, through the microelectrode via a bridge circuit housed in the preamplifier section. A third microelectrode was used to pass the feedback current from the voltage-clamp circuit to the preparation. The voltage-clamp amplifier has a maximum gain of 25,000 and was capable of switching ± 90 V in 30 μs. In practice, however, the speed of the clamp was limited by the access resistance, which includes the microelectrode resistance and a component of axial resistance between the current and voltage electrodes. The current microelectrode could also be switched to voltage recording when desired. A virtual ground circuit was used to measure the membrane current during voltage-clamp experiments. Another microelectrode, connected to a WPI voltage follower (WP Instruments, New Haven, Conn.), and positioned using a de Fonbrune pneumatic micromanipulator, was used for experiments testing the spatial uniformity of the voltage clamp.
Extracellular field stimulation, used to excite action potentials in quiescent cells, was delivered to the preparation by platinum plate electrodes placed in the bath. The first time derivative of the rising phase of the action potential ($+V_{\text{max}}$) was obtained with an operational amplifier that was linear over the range of 0–500 V/s. Voltage and current signals were displayed on the Tektronix 565 oscilloscope (Tektronix, Inc., Beaverton, Oreg.) and photographed using a Grass Kymograph camera (Grass Instrument Co., Quincy, Mass.). The reaggregates were observed through a Zeiss dissecting microscope ($25–100 \times$ magnification) (Carl Zeiss, Inc., New York). Distance measurements were made using an ocular graticule to an accuracy of ±10 µm. The voltage and current microelectrodes were held and positioned by a Zeiss sliding micromanipulator (Carl Zeiss, Inc.). Pharmacological agents to be tested were added to the solution reservoir from concentrated stock solutions to give the desired final concentration. All drug solutions were prepared at the time of the experiment. Ascorbic acid (10 µM) was added to solutions containing isoproterenol to prevent oxidation.

Reaggregates displaying automaticity (i.e., spontaneous action potentials and contractions) showed a high degree of electrical coupling (see Results) and were selected for voltage-clamp experiments. The highly differentiated reaggregates (i.e., having large, stable resting potentials) showed a much reduced amount of electrical coupling, as recently described by McLean and Sperelakis (1980).

**RESULTS**

*Effects of ACh on Ventricular Slow Action Potentials*

Intracellular penetrations in spontaneously contracting reaggregates gave action potentials with the following characteristics: maximal diastolic potential, $-76 \pm 3.7$ mV; peak overshoot potential, $+26 \pm 2.4$ mV; maximum rate of rise, $140 \pm 8$ V/s; and duration (at 50% repolarization), $185 \pm 12$ ms ($n = 40$) (see Fig. 1). The addition of tetrodotoxin (TTX) ($10^{-6}$ M) to the normal Tyrode solutions bathing ventricular reaggregate cultures resulted in a marked reduction in the maximal rate of rise of the normal action potential to $\sim 5$ V/s (Fig. 2). In four experiments, the addition of ACh ($10^{-6}$ M) had no effect on these slow action potentials persisting in TTX (not shown). Isoproterenol ($10^{-6}$ M) increased the maximal rate of rise of the persisting slow action potential to $\sim 10$ V/s, and increased the duration. Both enhancements produced by isoproterenol were rapidly abolished by the addition of ACh ($10^{-6}$ M) (five experiments) as shown in Fig. 2. Subsequent exposure to atropine ($5 \times 10^{-6}$ M) antagonized the effects of ACh and restored slow action potentials to the previous (isoproterenol-enhanced) values (not shown).

*Intercellular Coupling*

Impalement of a reaggregate with a second low-resistance electrode (10–20 MΩ) usually resulted in an initial depolarization (5–20 mV) with loss of spontaneous action potential generation. After both microelectrodes had "sealed in" and the resting potential had recovered, cell-to-cell electrotonic coupling was tested. Square current pulses (0.1–10 nA) of variable duration (50–500 ms) were delivered through one voltage electrode via a bridge circuit, and the resulting voltage change was recorded in both impaled cells. The ratio of the voltage change in the distance cell, compared with the voltage change
in the cell into which current was injected ($\Delta V_2/\Delta V_1$), gives a measure of the spread of current (in steady-state conditions) from a point source through this three-dimensional structure.

In many cases, the true value of $\Delta V_1$ was difficult to assess because of bridge imbalance that developed during the impalement. In most cases, the bridge electrode was found to be off-balance after removal from the cell. Correspondingly, an additional voltage drop was seen in the voltage traces during the impalement due to the incomplete nulling of the microelectrode tip resistance.
This additional voltage component could be reduced, nulled, or even reversed in sign by the adjustment of the bridge balance. This indicates that this voltage change, seen in the measurements using the bridge, is an artifact of the bridge imbalance, which is caused by an additional voltage drop due to the microelectrode resistance. Therefore, the magnitude of the fast component was subtracted from the total voltage measured, to give a corrected value for $\Delta V_1$. In addition, since the amount of applied current was known and $\Delta V_2$ could be measured reliably, the polarization resistance ($r_{pol}$) could be determined. The polarization resistance serves as a relative measure of the resistance to electrotonic spread of current within the reaggregate.

An example of a coupling experiment is shown in Fig. 3. In this example, a spontaneously contracting reaggregate (150 μm in diameter) was impaled with two microelectrodes having an interelectrode distance of 60 μm. An intracellular hyperpolarizing current pulse of 4 nA injected into cell 1 produced a 15.8-mV voltage change in cell 1 and a 14.7-mV voltage change in cell 2. The $r_{pol}$ was 3.7 MΩ, also indicating a large degree of current spread between the impaled cells. Upon cessation of the current pulse, anodal-break excitation produced action potentials in both cells. Such reaggregates displaying substantial amounts of electrotonic coupling (>90% interaction) were selected for voltage-clamp experiments. Representative data from the coupling experiments are given in Table I.

**Voltage-Clamp Experiments**

Since we wanted to examine the slow inward current, TTX ($10^{-6}$ M) was routinely used to block the fast Na$^+$ current and, as an extra precaution, a
holding potential of -50 or -40 mV was used to voltage-inactivate the fast 
Na⁺ conductance. Depolarizing voltage steps of 300 ms duration were deliv-
ered at a frequency of 0.3/s, and the resulting membrane currents were 
recorded. In four experiments, the spatial homogeneity of the voltage clamp 
was monitored using an independent voltage electrode (V₂), as shown in Fig. 
4. As can be seen, there was a close agreement between V₂ and the membrane 
potential in the controlled cell (V₁).

To simulate the conditions of sympathetic nerve stimulation, the β-adren-
ergic agonist isoproterenol (10⁻⁶ M) was used. It has been suggested by Reuter 
and Scholz (1977) that such an augmentation of the slow inward 
current by adrenaline reflects an increase in the number of functional con-
ductance channels, rather than a change in channel kinetics or the conduct-
ance per channel. Representative current traces from several voltage-clamp 
steps are shown in Fig. 5. After a brief outward capacitive current (not visible 
in some records), there is a transient slow inward current, followed by a small 
slowly increasing outward current. Upon repolarization of the clamp step, an 
inward capacitive current flowed, followed by a small, slowly decaying inward 
tail current. The peak amplitude of the slow inward current, during voltage 
steps from -50 mV (holding potential) to +20 mV, is plotted in Fig. 6 (closed 
circles). The voltage threshold for the slow inward current is around -35 mV, 
and the peak slow current occurs at around -10 mV. The apparent reversal 
potential for the slow inward current is around +26 mV; contributing factors 
for this low value include a small outward leak conductance that has not been 
subtracted. The open circles in Fig. 6 represent the magnitude of the net 
outward current developed at the end of the clamp pulse (measured at 300 
ms). This current, which increases at potentials positive to -30 mV, is the 
sum of the delayed K⁺ current, the leakage current, and a slowly inactivating 
or maintained component of Iₛₐ.

The data from the measured current-voltage relationship for Iₛₐ are graphed

| Reaggregate diameter | Inter electrode distance | Iₖ | ΔV₁ | ΔV₂ | Percent interaction | Rₛₑₚ |
|---------------------|-------------------------|----|-----|-----|---------------------|------|
| µm                  | µm                      | nA | mV  | mV  |                     | mΩ   |
| 150                 | 60                      | -4 | -13.8 | -14.7 | 93                | 3.7  |
| 150                 | 60                      | -4 | -16.8 | -13.8 | 94                | 3.9  |
| 150                 | 60                      | -1 | -4.2  | -3.3  | 79                | 3.3  |
| 180                 | 70                      | -8 | -26.3 | -23.3 | 96                | 3.2  |
| 160                 | 80                      | -3 | -13.7 | -10.5 | 77                | 2.1  |
| 170                 | 50                      | -5 | -21.0 | -17.4 | 83                | 3.5  |
| 250                 | 100                     | -10| -15.2 | -5.0  | 32                | 0.5  |
| 200                 | 70                      | -5 | -10.6 | -3.2  | 30                | 0.6  |

* Values have been corrected for bridge imbalance.
Percent interaction = (ΔV₂/ΔV₁) x 100%.
Rₛₑₚ = ΔV₂/Iₖ and is independent of bridge balance.
as conductance (semi-log) versus potential in Fig. 7. The apparent slow conductance is obtained from the relation:

$$I_{si} = g_{si} \cdot (E_{clamp} - E_{rev})$$

where $E_{rev}$ includes a component of outward current. It can be seen that the slow conductance rises exponentially with increasing depolarization to a maximum sustained value above 0 mV.

The time-course for the activation of $I_{si}$ displayed a pronounced potential dependence. A plot of the time from the beginning of the clamp step to the peak slow inward current as a function of potential is given in Fig. 8. In three representative experiments (represented by triangles, squares, circles), the time to peak $I_{si}$ is longest near threshold potential (43–50-ms range), and becomes more rapid at more positive potentials (i.e., <10 ms at +10 mV).

The steady-state voltage dependence of slow inward current inactivation ($f_{\infty}$) was studied by measuring the effect of various conditioning clamps on the peak slow inward current elicited at a fixed test potential. Fig. 9 presents
FIGURE 5. Membrane currents obtained during voltage clamp of a reaggregate cell culture. TTX (10^-6 M) and isoproterenol (10^-6 M) were present in the superfusing solution. The holding potential was -50 mV. Depolarizing clamp steps of 300 ms duration were applied at a frequency of 0.3/s. Capacity currents are not visible in some cases.

FIGURE 6. Current-voltage curve for the peak slow inward (closed circles) and steady-state outward current (open circles) obtained in Tyrode solution containing TTX (10^-6 M) (circles) and 5 min after the addition of isoproterenol (10^-6 M) (triangles).
FIGURE 7. A graph of conductance vs. potential constructed from slow inward current data. Points were obtained from the relation: \( I_{si} = g_{si} (E_{clamp} - E_{rev}) \). Isoproterenol (10^{-6} M) was present.

the results from three experiments using a 1-s conditioning clamp and a test pulse of 0 mV. It can be seen that the full complement of slow channels that are available at -40 mV decreases in a sigmoid fashion and is completely inactivated at 0 mV.
Calcium

To determine the Ca\(^{2+}\) dependence of \(I_{\text{s}i}\), voltage-clamp experiments were performed in normal Tyrode (1.8 mM \([\text{Ca}]_o\)), and then repeated after elevating \([\text{Ca}]_o\) to 3.6 mM. The current-voltage curves in Fig. 10 show data obtained from such an experiment. It can be seen that \(I_{\text{s}i}\) was increased at all clamp potentials by elevation of external Ca\(^{2+}\). In addition, the observed shift of the reversal potential upon doubling the \([\text{Ca}]_o\) (8.5 mV) was very close to the predicted shift of \(E_{\text{Ca}}\), using the Nernst relation for Ca\(^{2+}\) (9 mV).

Verapamil

A number of agents are known to inhibit the slow Ca\(^{2+}\) current in cardiac muscle. The effects of one such Ca\(^{2+}\) antagonist, verapamil, on \(I_{\text{s}i}\) are shown...
in Fig. 11. Verapamil (10⁻⁶ M) greatly depressed the slow inward current at all clamp potentials. A small reduction in the amount of net outward current was also seen (see Kass and Tsien, 1975). Similar effects on $I_\text{si}$ were observed with Mn²⁺ (1 mM) (not shown).

**Acetylcholine**

The effect of ACh on membrane current was tested in the presence of isoproterenol to simulate the conditions under which accentuated antagonism occurs in the ventricle. Fig. 12 shows the membrane currents resulting from clamp steps from -40 mV (holding potential) to -20 mV in the presence of isoproterenol (top traces), after the addition of ACh (10⁻⁶ M) (middle traces) and after antagonism of ACh by atropine (5 × 10⁻⁶ M) (lower traces). The addition of ACh (10⁻⁶ M) rapidly produced a reduction in the magnitude of isoproterenol-enhanced slow inward current in four experiments. Relatively small (<10%) increases in the net outward current over control values were also seen in the presence of ACh (Fig. 13).

To test whether ACh affects the amount of outward K⁺ current present near the normal resting potential, a more extended range of clamp potentials was examined. These results, shown in Fig. 14, demonstrate that over the range of potentials from -85 to -10 (HP = -40 mV), ACh produces only small or negligible increases in the late currents. Again, the slow inward current was markedly reduced by ACh. It should be noted that the decrease in net inward current seen in these experiments can be attributed to a decrease
FIGURE 12. Effects of ACh (10^{-6} M) on the isoproterenol-enhanced $I_{in}$. Holding potential, -40 mV; voltage-clamp steps to -20 mV. Top traces: isoproterenol (10^{-6} M); middle traces: isoproterenol and ACh (10^{-6} M); lower traces: isoproterenol, ACh, and atropine (5 \times 10^{-6} M). TTX (10^{-6} M) was present throughout the experiment.

FIGURE 13. Effects of ACh on membrane currents in the presence of isoproterenol. Solid circles represent $I_{in}$ obtained in the presence of TTX (10^{-6} M) and isoproterenol (10^{-6} M); outward currents (at 300 ms) are shown as open circles. Solid triangles are inward currents recorded after 3 min exposure to ACh (10^{-6} M); outward currents are plotted as open triangles.
in $I_{si}$ because any shift in the $I-V$ relation caused by the small increases in outward current could not account for the pronounced reduction of the net inward current.

Values for the peak $I_{si}$ from the pharmacological experiments described above are summarized in Table II.

![Figure 14](image-url)

**Figure 14.** Extended $I-V$ relations showing lack of effect of ACh (open triangles) on steady-state currents (at 300 ms) over the potential range -85 to -10 mV. Slow inward currents before (solid circles) and after ACh (solid triangles) are also shown. Isoproterenol ($10^{-6}$ M) and TTX ($10^{-6}$ M) were present throughout the experiment.

**Table II**

**SUMMARY OF THE EFFECTS OF AGENTS ON SLOW INWARD CURRENT ($I_{si}$)**

| A | B | C | Ratio |
|---|---|---|-------|
| Control | Ca$^{2+}$/ISO | Verap/ACh | B/A or C/B |
| 1.25 (2) | 2.3 (2) | | 1.84 |
| 1.2±0.4 (5) | 3.1±0.8 (5) | | 2.58 |
| ISO: 3.5±1.1 (3) | Verap: 0.8±0.3 (3) | | 0.23 |
| ISO: 3.0±1.0 (4) | ACh: 0.8±0.3 (4) | | 0.28 |

Values for $I_{si}$ are means ±SE.  
Number of experiments given in parentheses.  
TTX ($10^{-6}$ M) was present in all experiments, including the controls.  
Agents were added to the Tyrode solution to achieve the following concentrations: isoproterenol ($10^{-6}$ M), Ca$^{2+}$ (3.6 mM), verapamil ($10^{-6}$ M), ACh ($10^{-6}$ M).
DISCUSSION

The findings presented in this paper demonstrate that during β-adrenergic stimulation, the primary effect of ACh on membrane currents in chick ventricular cells is to markedly reduce the slow inward current. Such a reduction in $I_{si}$ (carried by Ca$^{2+}$ and Na$^+$) can account for the negative inotropic effect of ACh found under these conditions. In addition, no significant effects of ACh were observed on the net membrane currents measured at 300 ms. It can be concluded, therefore, that the ACh-mediated reduction in $I_{si}$ is responsible for the decrease in the maximal rate of rise and duration of the slow action potential in ventricular muscle observed in the presence of catecholamines. In contrast, Hino and Ochi (1980), using a single sucrose gap voltage-clamp technique on guinea pig papillary muscle, found that ACh inhibited basal $I_{si}$ and the delayed outward K$^+$ current without affecting the time-independent K$^+$ current. That is, ACh inhibited $I_{si}$ in the absence of catecholamine stimulation.

Reaggregate cultures were chosen for these voltage-clamp experiments because they offer several advantages over other cardiac preparations. First, their small size allows a rapid charging of the membrane capacity and permits a more uniform polarization of the preparation during voltage-clamp steps. A second reason for the use of cultured reaggregates is that they do not contain nerve terminals, which are present in other intact cardiac preparations used for voltage-clamp studies. Therefore, agents can be tested for their direct effects on the myocardial cells that comprise the reaggregates.

During the voltage clamp of a multicellular preparation, the degree of spatial uniformity of potential depends on the presence of a low intercellular resistance as compared with the surface membrane resistance. In the present experiments, only double impalements in a given reaggregate displaying a high degree of electrotonic coupling were selected for voltage clamp. In addition, a direct test of the spatial uniformity of the preparation showed that the potential was controlled during the flow of $I_{si}$ in a distant cell; i.e., large voltage gradients did not exist. Adequate control of potential during the flow of $I_{si}$ was also suggested by the gradual slope of the negative resistance region of the current-voltage curve to peak $I_{si}$.

In the present experiments, the net membrane current recorded at 300 ms probably reflects the summation of several currents. In addition to a leak current and the delayed outward K$^+$ current, there may have been a contribution from a Ca$^{2+}$-mediated K$^+$ current, as has been described in invertebrate neurons, as well as for several cardiac preparations. These outward currents may have been of sufficient magnitude to have masked the presence of a steady-state slow inward current. A significant steady-state $I_{si}$ "window" current is predicted by the overlap of the $G_{si}$ and steady-state inactivation curves (see Figs. 7 and 9). Therefore, a decrease of the steady-state $I_{si}$ by ACh or verapamil would shift the net current at the end of the clamp step in the outward direction. However, this shift might have been offset by a corresponding reduction in a Ca$^{2+}$-mediated K$^+$ current. Since only small changes in the net outward current were observed with acetylcholine or verapamil (at clamp
potentials below the activation of the delayed current), this suggests that the opposing effects of these currents would tend to balance each other. Further experimentation, using more specific blockers for $I_{\text{sl}}$, will be necessary to answer this question definitively.

Alternatively, it is possible that the predicted steady-state $I_{\text{sl}}$ was gradually reduced with time, so that by 300 ms it was of small magnitude. Such a reduction could have resulted from a current-dependent inactivation of $I_{\text{sl}}$.

Although the mechanism through which ACh exerts its anti-adrenergic actions on the slow inward current in ventricular muscle is not yet known, several hypotheses have been proposed. One possibility is that muscarinic agents act to reduce the accumulation of cyclic AMP produced by catecholamines, i.e., that ACh inhibits adenylate cyclase activity. This reduction in the cyclic AMP level may lead to a diminished availability of slow channel conductance (Sperelakis and Schneider, 1976; Reuter and Scholz, 1977; Biegon and Pappano, 1980). Such a depression of catecholamine-stimulated levels of cyclic AMP by ACh has been reported for embryonic chick ventricle (Biegon and Pappano, 1980), as well as for the myocardium of other species (Murad et al., 1962; Lee et al., 1971; McAfee et al., 1978). In this regard, experiments using cardiac membrane preparations suggest that a diminished adenylate cyclase activity produced by ACh results from a muscarinic receptor-mediated action on a GTP-dependent regulatory component of $\beta$-receptor/adenylate cyclase system (Watanabe et al., 1978).

Thus, the fact that ACh did not affect the slow action potential in the absence of catecholamines may reflect the possibility that there are two different populations of slow channels in the ventricle. One population (type I), which conducts the basal slow current, may not be required to be phosphorylated for voltage activation, whereas the second population (type II), which is regulated by autonomic agents, does require phosphorylation for activity. In all other respects, these two types of slow channels would have identical properties (i.e., voltage dependence, kinetics, and blockade by Ca\textsuperscript{2+} antagonists). Therefore, ACh addition, in the absence of catecholamine stimulation of cyclic AMP and resultant phosphorylation, should have no effect on $I_{\text{sl}}$.

Alternatively, the effects of ACh on the slow channel conductance may be mediated by cyclic GMP (George et al., 1970; Lee et al., 1972). However, since there sometimes is a clear dissociation between the elevation of cyclic GMP level and the decrease in contractility, it is unlikely that this cyclic nucleotide mediates all of the anti-adrenergic effects of ACh (Nawrath, 1977; Linden and Brooker, 1979).

In conclusion, these experiments demonstrate that reaggregate cell cultures of embryonic chick hearts are a useful preparation for studying the slow inward Ca\textsuperscript{2+} current in myocardial cells and its regulation by autonomic agents. Using this preparation, we found that ACh exerts its anti-adrenergic effect on the ventricular myocardial cells by inhibiting the slow inward current. Thus, the autonomic transmitters acetylcholine and norepinephrine may exert a dual control over the availability of ventricular slow channels,
and thereby govern the amount of Ca$^{2+}$ entering the myocardial cell during excitation.

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