"Fade" of Hyperpolarizing Responses to Vagal Stimulation at the Sinoatrial and Atrioventricular Nodes of the Rabbit Heart

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SUMMARY. Previous studies have suggested that maintained vagal stimulation or acetylcholine infusion results in a fade of responses in the sinoatrial node but not in the atrioventricular node, implying different muscarinic receptor subtypes in the two regions. We investigated this hypothesis in 23 isolated rabbit atrial preparations made quiescent by continuous superfusion with verapamil (1 µg/ml). Transmembrane potentials were recorded simultaneously from cells in the sinoatrial pacemaker region and from the "N" region of the atrioventricular node. Postganglionic vagal stimulation was achieved by the application of trains of pulses (50-150 µsec; 10-20 V; 200 Hz). Simultaneous application of long-lasting (1-10 sec) vagal trains produced hyperpolarizations which were nearly identical for both nodal regions. Maximal hyperpolarizations (~ 24 mV for sinoatrial node; 26 mV for atrioventricular node) were reached about 500 msec after initiation of the vagal train. Thereafter, hyperpolarizations faded, following a biphasic time course, and thus displaying two different time constants, one fast (τ fast = 580 msec for sinoatrial node; 550 msec for atrioventricular node), and one slow (τ slow = 9.2 sec for both sinoatrial and atrioventricular nodes). Hyperpolarizations during brief (200-msec) but repetitive vagal trains also faded biphasically, but approached a steady state much more rapidly than responses to long-lasting trains. Recovery from hyperpolarization decay occurred rather slowly and was linear. Our results demonstrate that the membrane potential responses to vagal stimulation in the atrioventricular node are indistinguishable from those in the sinoatrial node, and suggest that similar muscarinic receptors are operative in both regions. These phenomena may play an important role in the response of the cardiac conducting system to direct or reflexly mediated vagal input. (Circ Res 56: 718-727, 1985)

A NUMBER of reports have noted a gradual decay or "fade" in the magnitude of cardiac responses to either sustained or brief repetitive cholinergic interventions. Sinoatrial (SA) rate and atrial contractile responses consistently have been shown to fade after reaching a maximum despite continued vagal stimulation (Chess and Caralesu, 1974; Jalife et al., 1980; Loeb et al., 1981; 1982; Martin et al., 1982; Martin, 1983). In vivo studies of atrioventricular (AV) conduction responses to maintained vagal stimulation have yielded variable results. Loeb et al. (1981) reported that, unlike heart period, AV conduction remained prolonged to the same extent throughout maintained vagal stimulation. They hypothesized that such effects might indicate a difference in muscarinic receptor subtypes between the SA and AV nodes.

In contrast, Martin (1983) found that "secondary" dromotropic responses in the AV node were dependent upon the "initial" magnitude of the response. When the initial response was large, there was a secondary, relatively rapid fading. At intermediate initial levels, there was no appreciable fade. If the initial dromotropic response was small, there was actually a secondary increase in conduction time. He suggested that some aspect of the mechanism responsible for secondary changes in chronotropic and inotropic responses was considerably different from that of the AV conduction response, but questioned the hypothesis invoking different receptor subtypes.

Previous in vitro studies from this laboratory (Jalife et al., 1980) demonstrated that hyperpolarizations of quiescent SA node preparations evoked by vagal stimulation or by exogenous acetylcholine (ACh) or carbamylcholine (CCh) decay with time despite maintained cholinergic influence. Similar findings were reported from responses in bullfrog sinus (Satow, 1968), bullfrog atrium (Tokimasa et al., 1980, 1981), guinea pig atrium (Baumgarten et al., 1984), and rabbit Purkinje fibers (Mubagwa and Carmeliet, 1983).

The present study was undertaken to determine whether the in vitro muscarinic responses of the AV node are the same as those of the SA node. Vagally induced membrane hyperpolarizations were used as a physiological indicator of muscarinic receptor activity. A preliminary report of these findings has appeared elsewhere in abstract form (Salata et al., 1983).
Methods

Isolated SA node-AV node preparations were obtained from 23 albino rabbits (1.5-3.0 kg), which were anesthetized with sodium pentobarbital (35 mg/kg, iv). Hearts were excised and quickly placed in oxygenated Tyrode’s solution at 37°C. Dissection was performed in a manner similar to that described by Hoffman et al. (1959). The right atrium, together with both venae cavae, the interatrial septum, the upper portions of both the right ventricular free wall and interventricular septum were cut out. An incision was made starting at the inferior end of the right ventricular free wall, extending through the atrioventricular groove along the anterior border of the right atrium, and terminating with a longitudinal cut through the anterior wall of the superior vena cava. A second incision was made along the lateral border of the crista terminalis, extending inferiorly and then medially along the junction of the right atrial free wall and septum. Preparations then were pinned to a wax bottom Plexiglas tissue bath with the endocardial surface up. Equilibration was allowed for at least 1 hour while the preparation was superfused at a rate of 20 ml/min with a modified Tyrode’s solution that was saturated with 95% O₂ and 5% CO₂ and maintained at 37°C. The composition of the solution was (miu): NaCl, 130; KCl, 4; NaH₂PO₄, 1.2; NaHCO₃, 24; CaCl₂, 1.8; MgCl₂, 1.0; and dextrose, 5.6. Propranolol (4 HM) was added to eliminate any β-adrenergic effects due to release of norepinephrine.

A schematic diagram of the preparation is shown in Figure 1. Transmembrane potentials were recorded differentially from the primary pacemaker region (ME1) of the SA node and N-region (ME2) of the AV node using microelectrodes (20-30 MΩ) filled with 2.7 M KCl and connected to high input impedance amplifiers (WPI models 750 and KS-700). The electrical signals were displayed on a Tektronix 565 oscilloscope and photographed with a Grass C4 kymographic camera. The primary pacemaker region of the SA node and N-region of the AV node were identified by their characteristic action potential configurations and responses to vagal stimuli (cf. Cranefield et al., 1959; West and Toda, 1967). Postganglionic vagal nerve stimulation was performed by a technique modified from Vincenzi and West, (1963). Stimuli were applied through two pairs of Teflon-coated silver electrodes exposed only at the tips which were separated by 3-5 mm. The stimulating electrodes were positioned directly on the endocardial surface of the preparation, one pair near the cephalic portion of the SA node (VS1) and the other pair near the coronary sinus anterior to the AV node (VS2; Fig. 1). Final electrode positioning was determined by optimal responses to vagal stimulation at any given intensity (cf. West and Toda, 1967). Stimuli were generated by two P61 Frederick Haer units and consisted of trains of biphasic pulses, 50-150 μsec in duration, 10-20 V in amplitude, and at a frequency of 200 Hz. These pulses were subthreshold for cardiac cells but were of sufficient magnitude to stimulate postganglionic vagal nerve terminals (Jalife et al., 1983; Slentner et al., 1984).

Test vagal stimuli were applied while preparations were discharging spontaneously to assess the effects on SA nodal rate and on AV conduction or junctional pacemaker rate in order to (1) confirm that recordings were from the primary pacemaker region of the SA node and N-region of the AV node, (2) assure that satisfactory vagal effects were achieved, and (3) aid in positioning of the stimulating electrodes and optimize the stimulation parameters. Once the optimal vagal stimulation parameters were achieved, only the number of pulses and, thus, the duration of the vagal trains were altered to vary the intensity of the vagal stimulus.

After obtaining sufficient information from the spontaneously beating preparations, they were superfused with Tyrode’s solution containing 2.2 μM verapamil for the remainder of the experiments. Most preparations became quiescent within 60 minutes after superfusion was initiated, and vagally induced hyperpolarizations could be studied without interference from spontaneous action potentials. In some preparations, it was necessary to cut away bordering myocardial tissue exhibiting subsidiary pacemaker activity which was refractory or less sensitive to verapamil. In 11 of 23 preparations, the AV node was carefully dissected free from surrounding tissue and was studied in isolation. The other 12 preparations were studied with both SA and AV nodes intact.

For analysis of the data, the following definitions were employed: latency is defined as the time interval between the onset of vagal stimulation and the onset of the induced hyperpolarization. During prolonged (200 msec or longer) vagal stimulation, the maximal membrane hyperpolarization (nadir) is defined as Emax, and the time interval from the onset of vagal stimulation to Emax is termed Tp. Thus, Tp corresponds to the point in time at which Emax is achieved (see inset, Fig. 4).

### Table 1

<table>
<thead>
<tr>
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<th>RMP (mV)</th>
<th>Emax (mV)</th>
<th>Tp (msec)</th>
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<tbody>
<tr>
<td>SAN (n = 7)</td>
<td>-43.2 ± 1.1</td>
<td>24.4 ± 3.5</td>
<td>479 ± 46</td>
</tr>
<tr>
<td>AVN (n = 14)</td>
<td>-48.3 ± 3.1</td>
<td>26.0 ± 2.3</td>
<td>512 ± 51</td>
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</table>

Data expressed as mean ± SEM; vagal train durations 2-5 seconds. SAN: sinoatrial node; AVN: atrioventricular node; RMP: resting membrane potential; Emax: maximal membrane hyperpolarization; Tp: time from onset of vagal stimulation to Emax. Analysis by Dunnett’s method revealed no significant differences between SAN and AVN.
Table 2
Regression Analysis of Recovery Process during Twin Vagal Pulses

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<thead>
<tr>
<th></th>
<th>Slope</th>
<th>Intercept</th>
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<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Range</td>
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<tr>
<td>SAN (n = 12)</td>
<td>7.4 ± 0.7</td>
<td>2.6–11.8</td>
</tr>
<tr>
<td>AVN (n = 10)</td>
<td>7.7 ± 1.9</td>
<td>2.7–14.9</td>
</tr>
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No significant difference between the slopes of the regression lines for the SAN and AVN (Student's t-test). Abbreviations defined in footnote to Table 1.

Analysis of variance was used for statistical analysis of vagally induced changes in membrane potential (Table 1) and Dunnott's t-test (Steel and Torrie, 1960) was used for comparisons. Recovery from fade was studied via regression analysis (Table 2), and was compared in SA vs. AV nodes via Student's t-test.

The time constants for the fade of hyperpolarization were determined by curve peeling (Zierler 1981). The varying amplitude (Ec) of the hyperpolarizations was measured at time (t) after E(max), was expressed as a fraction of E(max), and plotted on semilogarithmic coordinates as a function of time (Fig. 4). Straight lines were drawn by eye through the linear portions of the curve. The fast component was determined by subtracting the slow component from the total curve, and the time constants (τ) were determined from the resulting lines.

**Results**

**Hyperpolarization in SA and AV Nodes**

Previous studies have shown that vagal stimulation produces membrane hyperpolarization in quiescent SA nodal cells (Jalife and Moe, 1979; Jalife et al., 1980). Such hyperpolarization increases in amplitude and duration as a direct function of the vagal stimulus train duration.

In this study, we found that graded hyperpolarizations mediated by postganglionic vagal stimulation are also characteristic of cells within the AV node region. This is illustrated in Figure 2, which shows transmembrane potentials recorded from an N-cell in a quiescent AV node preparation with a resting potential of −50 mV. Six superimposed traces demonstrate the responses induced by postganglionic vagal trains consisting of 5, 10, 15, 25, and 30 pulses, from top to bottom, respectively. Hyperpolarizations began after a latency of about 80 msec following initiation of the vagal trains (arrow), and varied in amplitude between 4 and 21 mV, depending on vagal train duration. Longer vagal trains (not shown) produced increasingly greater hyperpolarizations, which, in this case, reached an E(max) of 25 mV at an interval of 490 msec (T,o) after the onset of vagal stimulation. Similar results were observed in seven isolated AV node and seven combined SA-AV node preparations (Table 1). These results in the rabbit AV node were analogous to previous results in cat SA node-vagus nerve preparations (Jalife et al., 1980) and bullfrog sinus node preparations (Saitow, 1968).

Study of vagally induced hyperpolarizations of the SA and AV nodes simultaneously in the same preparation substantiated the similarities of the re-

![Figure 2. Transmembrane potential recordings from an N-cell of an isolated AV node preparation. Six superimposed traces show progressively greater hyperpolarizations induced by submaximal vagal stimulus trains of 5, 10, 15, 20, 25, and 30 pulses (top to bottom). Arrow indicates onset of vagal trains. Calibrations are 400 msec and 10 mV. (Experiment of 6-17-82.)](image)

![Figure 3. Simultaneous transmembrane potential recordings from the SA node and AV node during vagal stimulation (top bars). Panel A: submaximal (100-msec) vagal train results in very similar responses in both the SA and AV nodes. Panel B: supramaximal (1-sec) vagal train produces maximal amplitude hyperpolarizations in both regions, which decay with time despite maintained vagal stimulation. Longer vagal trains (2 sec) in panels C and D further demonstrate the decay phenomenon. Note slower sweep speed in panel D. Scale: vertical: 20 mV; horizontal: A, B, C = 400 msec, D = 1 sec. (Experiment of 5-5-83.)](image)
responses in the two regions. Indeed, as shown by the example in Figure 3, the hyperpolarizations were found to be nearly identical. In panel A, a 100-msec vagal train (top horizontal bar) produced submaximal hyperpolarizations of 15 and 18 mV in the SA and AV nodes, respectively. A 1.0-second vagal train in panel B produced maximal responses with an $E_{\text{max}}$ of 18 mV in the SA node and 22 mV in the AV node. $T_e$ values were 320 and 260 msec, respectively. Most importantly, despite maintained vagal stimulation beyond attainment of $E_{\text{max}}$, the amplitude of the hyperpolarization in both regions was not maintained, but decayed gradually in a very similar biphasic manner. Application of a longer vagal train (2.0 sec) in panels C and D, further demonstrated this time-dependent decay process. Note that the time scale in panel D was reduced to illustrate better the biphasic nature of the decay.

In the example of Figure 3, the $E_{\text{max}}$, $T_e$, and time course of decay of the SA nodal cell are slightly different from those in the AV node cell. Such minor variations were often seen within a given preparation. Yet, statistical analysis yielded no significant differences in any of the parameters recorded from the two regions (see Table 1 and Fig. 4).

**Long Vagal Trains and Time Course of the Fade**

Upon quantitative analysis (see Methods), the decay phenomenon in both SA and AV nodes was nearly identical. Figure 4 shows semilogarithmic plots of the mean membrane potential changes with time ($E_t$), expressed as a function of $E_{\text{max}}$. The top panel shows results from the SA node and the bottom panel those from the AV node. In either case, the data best fit a double exponential, and thus displayed two different time constants, one "fast" and one "slow." The fast time constants for the SA and AV nodes were nearly equal (580 vs. 550 msec), whereas the slow time constants were exactly the same (9.2 sec). Clearly, quantitative comparison of SA and AV node hyperpolarizing responses to prolonged vagal stimulation demonstrates that these responses are practically indistinguishable from each other.

The slow time constant has been estimated at 9.2 seconds using vagal trains of less than 5 seconds. Ideally, a time constant should be determined over several multiples of the suggested value. We attempted to analyze quantitatively the decay during long vagal trains (>5 sec) in these studies, but were unable to obtain satisfactory results in all but a few experiments. Typically, when the vagal train duration exceeded 5 seconds, the membrane potential of one or both regions (SA or AV node) became unstable. Subthreshold oscillations were common, and spontaneous action potentials appeared occasionally during and, often, after termination of such vagal trains (cf. Goto et al., 1983). In five experiments, the decay of the membrane potential followed a predictable time course during long vagal trains. One such AV node experiment is shown in Figure 5. The high vertical sensitivity resulted in a noisy trace due to the vagal stimulus throughout the recording. A 50-second vagal train produced a typical biphasic fade of the membrane potential response, which did not reach a steady state. Similar results were obtained in two other AV node and two SA node experiments. The estimated individual time con-
FIGURE 5. Hyperpolarization of an AV node cell during a long vagal train (50 sec), which terminated just beyond the oscilloscope trace. Dotted line indicates resting membrane potential level of –50 mV. (Experiment of 9-26-84.)

stants from these experiments ranged from 8–15 msec.

Brief but Repetitive Vagal Trains

Whereas the responses to a continuous vagal train of prolonged duration exhibit essentially the same time curve or decay in both the SA and AV nodes, such continuous vagal activity would not be expected to occur under physiological conditions. Rather, efferent vagal activity has been shown to occur in brief bursts as a result of baroreflex activation during the systolic pressure wave (Jewett, 1964). Therefore, we examined the hyperpolarizing responses produced by brief repetitive vagal stimulation. Figure 6 shows two examples of simultaneous responses of the SA and AV nodes to brief repetitive postganglionic vagal trains (200-msec duration) delivered at 1400- and 850-msec intervals (A and B, respectively). Hyperpolarization amplitude decreased in a biphasic manner that was similar to that demonstrated during continuous vagal stimulation. In the example of Figure 6, the return toward the resting membrane potential for each hyperpolarization was slower for the SA node than for the AV node and, as a result, each successive hyperpolarization was initiated from a more negative membrane potential (i.e., closer to the equilibrium potential for K). This might account in part for the smaller amplitude of the SA node hyperpolarizations in this experiment, and for the apparently more pronounced decay of successive responses in the SA node. As noted previously, such variations between the responses of the two nodal systems were not uncommon. However, no quantitative distinctions between the two regions could be made (see below and Fig. 7).

FIGURE 6. Simultaneous responses of SA and AV nodes to repetitive supramaximal vagal stimuli at cycle lengths of 1400 and 850 msec. Representative traces show that decay occurs in a manner similar to that with continuous vagal stimulation. (Experiment of 5-6-83.)

FIGURE 7. Analysis of responses to repetitive supramaximal vagal stimuli at various cycle lengths. Panel A: the peak amplitude of each successive hyperpolarization, expressed as a percent of the initial hyperpolarization amplitude (E_max), is plotted on the ordinate as a function of time. Thus, the "envelope" of the responses is outlined for various vagal stimulus cycle lengths (VSCL). Panel B: the "steady state" amplitude of repetitive responses for the SA node (left ordinate; filled circles) and AV node (right ordinate; unfilled squares) is plotted as a function of the VSCL. See text for details. (Experiment of 5-6-83.)
The results of Figure 6 suggest that, during repetitive application of brief vagal trains, the rates of fade in both SA and the AV nodes are dependent on the frequency of the trains. This suggestion is confirmed by the data presented in Figure 7, which were taken from the same experiment as those in Figure 6. Figure 7A shows the time course of the "envelope" for the responses in the AV node. Brief vagal trains of constant duration (200 msec) were applied repetitively at various cycle lengths (VSCL). In this experiment, the fade of the responses was more rapid at shorter VSCL. In addition, the amplitude of subsequent responses to repetitive vagal trains appeared to approach a steady state value much more rapidly than the responses to continuous vagal stimulation (cf. Fig. 5).

The "steady state" amplitude of hyperpolarization, measured after 30 seconds of repetitive vagal stimulation and expressed as a percentage of the initial amplitude ($E_{\text{max}}$), is plotted in Figure 7B, as a function of the VSCL. This steady state amplitude increased with increasing VSCL in a linear manner. Furthermore, as evident from Figure 7B, the slopes of the lines for the SA and AV nodes were indistinguishable. Similar data were obtained in all experiments tested in this manner. Regression analysis yielded lines with slopes ($\% E_{\text{max}}$/sec) of 11.3 ± 1.9 (mean ± SEM; $n = 6$) and 9.0 ± 2.2 ($n = 5$) for the SA and AV nodes, respectively. These slopes were not significantly different from each other (Student's $t$-test).

Recovery from the Fade

The observation that the rate of fade during repetitive vagal stimulation displayed a frequency dependence suggests that, at lower frequencies of vagal stimulation, there was more time for recovery from the process(es) responsible for the decay phenomenon. We studied the recovery process by applying twin vagal trains at either increasing or decreasing coupling intervals. Each vagal train was of sufficient duration to produce a hyperpolarization of maximal amplitude ($E_{\text{max}}$). Examples of separate experiments in the SA node (panel A) and AV node (panel B) are shown in Figure 8. Superimposed traces in each panel demonstrate the responses to twin vagal trains at increasing coupling intervals. Identical results were obtained using decreasing coupling intervals; therefore, no hysteresis was apparent. These results demonstrate that recovery of the muscarinic response is rather slow, and best fit by a linear time course upon regression analysis (Table 2). The slopes of the regression lines for the SA node and AV node are not significantly different.

Discussion

Membrane Hyperpolarization, Fading, and Recovery

We studied membrane potential responses simultaneously in the SA and AV node during postganglionic vagal stimulation as a physiological indicator of muscarinic receptor activation. The interaction of ACh with cardiac muscarinic receptors causes an increase in the membrane conductance to potassium ions (Harris and Hutter, 1956), resulting in hyperpolarization toward the potassium equilibrium potential. Hyperpolarizations of AV node cells increased in amplitude with increasing vagal train duration (Fig. 1), similar to SA nodal and atrial cell membrane responses in previous publications (Toda and West, 1967; Glitsch and Pott, 1978; Jalife and Moe, 1979; Jalife et al., 1980).

In this study, simultaneous application of long vagal trains of supramaximal intensity to both nodal regions, produced hyperpolarizations that reached a maximum and then decayed in a biphasic manner. In all cases, a maximal hyperpolarization was achieved in both nodes, leading to the assumption that a saturating concentration of ACh was present throughout the period of vagal stimulation. In this manner, it was possible to standardize the vagal influence on both regions and to demonstrate that their hyperpolarizing responses and time courses of decay were qualitatively and quantitatively the same (see Table 1 and Figs. 3 and 4). Indeed, maximal hyperpolarizations of about 24 and 26 mV were demonstrated for SA and AV nodes, respectively.
and, in both cases, the decay phenomenon was described by a biexponential process yielding two time constants which were nearly identical for the two nodes.

Decay of membrane responses to prolonged muscarinic receptor activation has been described in a number of other cardiac tissue preparations from several species. Hyperpolarization in quiescent bullfrog sinus returned gradually toward the original resting potential, despite continued vagal stimulation (Satow, 1968). Similar results were obtained in kitten sinus node preparations during vagal stimulation or iontophoretic application of ACh (Jalife et al., 1980). Also, in bullfrog atrial muscle, effects on membrane potential, membrane conductance, and action potential amplitude and duration all were found to diminish gradually during prolonged exposure to ACh or CCh (Tokimasa et al., 1980). Moreover, holding clamp current in small SA node preparations has been shown to decline progressively with time in the continuous presence of ACh (Noma et al., 1979). More recent experiments have shown that prolonged exposure to ACh or CCh can result in a concentration-dependent biphasic decay of membrane hyperpolarization or action potential shortening in rabbit Purkinje fibers (Mubagwa and Carmeliet, 1983).

Some investigators have found a lack of decay of muscarinic response (Glitsch and Pott, 1978; Hartzell, 1979; Boyden et al., 1983). Glitsch and Pott (1978) reported that, for the most part, ACh-induced hyperpolarization in guinea pig atria did not dissipate with time. However, more recent experiments in the same preparation (Baumgarten et al., 1984) have indeed demonstrated a significant time-dependent decline in the hyperpolarization, particularly in the presence of high ACh concentrations (see Baumgarten et al., 1984; their Fig. 5).

In a recent study in anesthetized dogs (Martin, 1983), high frequencies of vagal stimulation produced large initial dromotropic responses that gradually decayed toward control. The time course of decay of the dromotropic responses was similar to that of the chronotropic responses found in earlier studies (Loeb et al., 1981; Martin et al., 1982), but was substantially slower than the lowest decay observed in our study (τ = 33 sec vs. 9.2 sec, respectively). This discrepancy may be related to different kinetic parameters being responsible for the fade in each experimental situation (see below), or to differences in the parameters measured, i.e., SA rate and AV conduction time vs. membrane hyperpolarizations.

During application of twin vagal trains at variable coupling intervals (Fig. 8), we found that recovery from hyperpolarization decay in both the SA and AV nodes occurs rather slowly and follows a linear time course. Furthermore, the mean slope of the linear recovery was essentially the same for both regions. A similar type of recovery for AV node conduction has been observed in vivo (Martin, 1976). In atrially paced dogs, two identical brief stimulus bursts were applied to either vagus separated by increasing time intervals. The change in AV nodal conduction time in response to the second stimulus burst was reduced maximally at a coupling interval of about 5 seconds, and it took about 50 seconds to recover completely. This time to recovery is about 10 times longer than that demonstrated in our experiments for the hyperpolarizing response (see Fig. 8). This suggests that, in addition to hyperpolarization, ACh-receptor interactions at the AV nodal cell membrane may trigger changes in other parameters [e.g., inhibition of slow inward current (Ten Eick et al., 1976)] which have slower kinetics and contribute to conduction delay.

**Duration- and Frequency-Dependence of the Fade**

Our results indicate that the magnitude and rate of decay of vagally induced membrane potential responses in both nodal structures were dependent on the duration and frequency of vagal stimulation (see Figs. 2 and 5). Other authors have demonstrated similar duration and/or frequency dependent effects of vagal stimulation on membrane hyperpolarization, chronotropic responses of the SA node and dromotropic responses of the AV node (Vincenzi and West, 1965; West and Toda, 1967; Chess and Calaresu, 1974; Jalife et al., 1980; Martin et al., 1982).

Although quantitatively different, our results are in general qualitative agreement with those of Martin et al. (1982) in that the pattern of the fade depends on the frequency of vagal stimulation. These results are also consistent with previous data in the isolated kitten sinus node (Jalife et al., 1980). In those experiments, hyperpolarization and negative chronotropic response faded gradually at vagal stimulus frequencies of 25 Hz or more. However, at lower frequencies, maximal hyperpolarization and chronotropic response were either achieved rapidly and maintained (12.5 Hz), or approached more gradually, never reaching a steady state (3.1 and 6.2 Hz). Likewise, in the present experiments, when low frequency vagal stimuli were used to stimulate post-ganglionic vagal terminals in the AV node region (not shown), the initial hyperpolarization was small and increased continuously for as long as the vagal train was applied.

The hyperpolarizing responses to brief repetitive vagal trains also fade following a biphasic time course (Fig. 6). However, in contrast to the responses to long-lasting stimulation (Fig. 5), the amplitude of brief hyperpolarizations reaches a steady state (Fig. 7) after 10–20 seconds of repetitive stimulation. This observation, together with the demonstration that both the rate of fade and the amplitude of the responses are functions of the interval between repetitive inputs (Figs. 7A and B), explains the reason for the recently demonstrated difference in the am-
A number of factors may contribute to the decay of responses observed in this study, including (1) a gradual reduction in the release of ACh from synaptic terminals, (2) a decrease in ACh concentration induced by an increase in the activity of cholinesterase, (3) a decrease in the effector response to a constant (saturated) level of receptor occupancy, (4) accumulation of potassium ions in the extracellular space secondary to an increase in potassium conductance, (5) a vagally induced time-dependent increase in inward current, which would tend to depolarize the membrane and oppose the hyperpolarizing response, and (6) desensitization of the post-junctional muscarinic receptors. Our results do not permit an extensive discussion in this regard. However, some of the proposed mechanisms appear to be unlikely, in the light of previous results from this and other laboratories. Indeed, the first two possibilities can be ruled out by the experiments of Jalife et al. (1980) in the kitten SA node in which iontophoretic application of exogenous ACh or CCh also demonstrated fade in the hyperpolarizing response. Similar results were obtained by Tokimasa et al. (1980, 1981) in the bullfrog atrium, and by Mubagwa and Carmeliet (1983) in Purkinje fibers.

The third possibility is also doubtful. A fade in the hyperpolarizing response has been observed even when brief vagal trains or ACh pulses are used to test the responsiveness after conditioning inputs of submaximal intensity (Jalife et al., 1980). In addition, it has been demonstrated that full response to ACh can take as long as 15 minutes to recover, whereas potential and conductance changes reach control values soon after removal of the drug (Tokimasa et al., 1981). With respect to the fourth possibility, potassium accumulation has been proposed to explain the fade in the muscarinic effects on membrane potential in guinea pig atrium and the transient depolarization beyond the resting potential upon washout (Glitsch and Pott, 1978; Baumgarten et al., 1984). Although potassium accumulation might contribute partly to the decay in the hyperpolarizing response in our preparations, recent experiments in Purkinje fibers (Mubagwa and Carmeliet, 1983) argue against this being a major factor in the development of the fade.

The data presented here are consistent with the last two postjunctional mechanisms listed above, namely the activation of a hyperpolarization-induced inward current, possibly $i_{h}$ (Yanagihara et al., 1980), and the development of desensitization (Katz and Thesleff, 1957; Rang and Ritter, 1970; Jalife et al., 1980) of the muscarinic receptor at the SA and AV node membranes. Whether one or both of these mechanisms are responsible for the fade under our experimental conditions, remains to be determined.

**Different Receptor Subtypes?**

Loeb et al. (1981) reported that during prolonged vagal stimulation in anesthetized dogs, chronotropic responses of the SA node decayed secondarily with time, but the negative dromotropic effect on AV nodal conduction remained nearly constant. They proposed a difference in the ability of muscarinic receptors at the SA and AV node to respond to prolonged activation by ACh, and suggested that different muscarinic receptor subtypes may exist between the SA and AV nodes. Our results and those of Martin (1983) indicate that the lack of decay of AV node responses might be due to frequency or ACh concentration dependence. Loeb et al. (1981) stimulated at only one vagal frequency, which may have been insufficient to demonstrate the fade in the AV node response.

Our findings show that the hyperpolarizing responses of the SA and AV nodes to vagal stimulation are both qualitatively and quantitatively identical. As such, these data do not support the contention that there are different muscarinic receptor subtypes in the two regions. However, muscarinic receptor subtypes have been classified, based on differences in binding affinities or kinetics (Barlow et al., 1976; Galper and Smith, 1978; Hulme et al., 1981), rather than physiological responses. It thus remains possible that different subtypes of muscarinic receptors exist in different cardiac regions or co-exist within a given region.

One final point in this regard deserves consideration. Equating changes in SA node pacemaker rate with those of AV node conduction time as quantitative indicators of muscarinic receptor activation (Loeb et al., 1981) may not be entirely valid. One must assume that these two different responses are modulated equally by the same effector mechanism, or at least by mechanisms having equal gain. Yet, AV node conduction has been found to be less sensitive to vagal influences than is sinus pacemaker activity (Spear and Moore, 1973) and the two systems exhibit different characteristic responses to vagal stimulation (de la Fuente et al., 1969, Jalife et al., 1982). Indeed, when conduction was monitored as a common parameter, i.e., during constant atrial drive, the response of the SA node to cholinergic stimulation was analogous to that of the AV node (West and Toda, 1967). However, since AV node conduction is dependent on the slow inward current, which was blocked in our study, we cannot exclude the possibility that the effect of ACh on this current does not fade.
Yet, it seems reasonable to speculate that the biphasic time course of the fade in our SA and AV node preparations might have resulted from at least two different kinetic constants of desensitization (see Clapham and Neher, 1984), and that these constants are identical in the two systems. It is also possible that the complex time course of the decay is the result of a combination of two completely different mechanisms. Thus, the fast initial decay might have resulted from rapid depolarization induced by activation of $I_h$ (Yanagihara et al., 1980), whereas the slower decay could have been associated with muscarinic receptor desensitization.

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