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# Time-Dependent Changes in the Chronotropic Response to Vagal Stimulation in the Newborn Canine

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ABSTRACT. **We characterized changes in the vagal chronotropic response during 3-min trains of vagal stimulation at 3, 5, and 8 Hz in anesthetized, chemically sympathectomized (6-hydroxydopamine) newborn canines (<15 d of age). In response to vagal stimulation, the sinus cycle length gradually increased (within 30 s) to a maximum value that was dependent upon the stimulation frequency ($p < 0.001$). The chronotropic response then attenuated over the remainder of the vagal train. However, unlike in adult dogs, the degree of attenuation of the vagal chronotropic response (fade) was also highly dependent upon the frequency of vagal stimulation in the range 3–8 Hz ($p < 0.002$). We then compared the maximum change in sinus cycle length and fade in a group of neonates while stimulating the vagus at 3 Hz before and after the administration of physostigmine (0.2 mg/kg i.v.). Physostigmine resulted in a significant increase not only in the maximum percent change in sinus cycle length but in the magnitude of attenuation of the vagal response as well. Therefore, unlike the adult, in the newborn the magnitude of both the vagal response and fade are dependent upon concentrations of acetylcholine released in response to modest levels of vagal stimulation (≤8 Hz). This may be the result of differences between the newborn and the adult in the prestreceptor mechanisms of fade or in the susceptibility of the muscarinic receptor to desensitization by the neurotransmitter acetylcholine. (*Pediatr Res* 34: 139–143, 1993)**

### Abbreviations

ACh, acetylcholine
VST, vagosympathetic trunk
ANOVA, analysis of variance

It is well known that the chronotropic response to constant vagal nerve stimulation or exogenous ACh infusion reaches a maximum value that then gradually fades over the rest of the period of stimulation or ACh infusion. This fade phenomenon has been mainly attributed to a desensitization of the postsynaptic muscarinic receptor, although an inhibition of ACh release via presynaptic muscarinic receptors has also been postulated (1–7). Importantly, in *in vivo* and *in vitro* studies of the adult canine heart, the vagal chronotropic response increases in magnitude as vagal stimulation frequency is increased. However, while the vagal chronotropic response increases, the fade ratio (a measure of the attenuation of the vagal chronotropic response over time) remains remarkably constant over the range of vagal stimulation frequencies of 1–10 Hz (1, 5). In this study we examined the time-dependent changes in sinus cycle length observed in response to tonic vagal stimulation in the intact newborn dog. We report that in the newborn, unlike the adult, the degree of fade of the vagal chronotropic response is highly dependent upon the intensity of vagal stimulation over the range of stimulation frequencies of 3–8 Hz. It is suggested that this stimulation frequency dependency of the fade ratio in the newborn may reflect muscarinic receptor differences that result in an alteration of the relationship between the amount of released ACh and pre- and postsynaptic receptor desensitization. This may represent yet another important manifestation of immaturity of parasympathetic nervous system function in the newborn.

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## MATERIALS AND METHODS

This study was performed in a total of 15 neonatal mongrel canines, aged 8.7 ± 3.5 d (mean ± SD). All experiments were approved by the Tulane University School of Medicine Advisory Committee for Animal Resources and were conducted in accordance with federal guidelines for the care and use of laboratory animals. All neonates were chemically sympathectomized with 6-hydroxydopamine (6-hydroxydopamine hydrobromide, dissolved in 0.9% NaCl plus 0.1% ascorbic acid) given intraperitoneally (50 mg/kg/d for 3 d) before the day of study. Chemical sympathectomy was performed because we have previously demonstrated that in the newborn canine an attenuation of the vagal chronotropic response can be observed that is rapidly acquired, long lasting, and likely caused by the release of the sympathetic cotransmitter neuropeptide Y from sympathetic nerves (8). The efficacy of sympathectomy was verified by assessing the changes in sinus cycle length and systolic blood pressure in response to a test dose of tyramine (100 μg/kg i.v.) as previously described (8). All animals were anesthetized at the time of autonomic testing with pentobarbital 30 mg/kg given intraperitoneally. Pentobarbital was chosen as an anesthetic because of the stable plane of anesthesia and reproducible vagal responses achievable in this newborn model (9). Resting heart rates under pentobarbital anesthesia in this study were not different from resting heart rates reported in normal unanesthetized neonatal mongrel canines (10). All animals were mechanically ventilated via a Harvard model 613 ventilator (Harvard Apparatus, South Natick, MA). A left femoral artery catheter was placed for monitoring of blood pressure and arterial blood gases. The left femoral vein was cannulated for the administration of pharmacologic agents. Via the right femoral vein, a no. 4 bipolar electrode catheter was placed in the vicinity of the sinus node for the purpose of

recording the high right atrial electrogram as a measure of sinus cycle length. The arterial blood pressure, high right atrial electrogram (filtered at 300–1000 Hz), and surface ECG lead II were amplified, displayed, and recorded at a paper speed of 50–100 mm/s with a Gould TA-2000 thermal chart recorder (Gould Inc., Cleveland, OH).

*Vagal stimulation.* Both cervical VST were isolated and divided, and the distal end of the right VST was prepared for electrical stimulation with a pair of platinum-iridium electrodes. The nerve was kept constantly moistened with oxygenated physiologic saline to prevent nerve damage or drying. Vagal stimuli were delivered to the right VST at constant current and at a pulse duration of 2 ms via a Bloom DTU-210 programmable stimulator (Bloom Associates, Reading, PA). Stimulation intensity in mA was selected by stimulating the right VST at a frequency of 8 Hz while gradually increasing the current delivered. The stimulation current was set at that current output at which a further increase in current produced no further appreciable slowing of the sinus cycle length. As defined, the current used for stimulation averaged 1.3 ± 0.4 mA (mean ± SD).

In the first experimental protocol, tonic stimulation of the right VST was performed in six neonates (aged 7.8 ± 4.4 d) for 3 min at three different stimulation frequencies in random order: 3, 5, and 8 Hz. Changes in the sinus cycle length were measured with a precision of ±5 ms from electrograms recorded from the high right atrial electrical catheter. Sinus cycle length was measured at 2, 4, 6, 8, 10, 12, 15, 20, 30, 40, and 60 s, and at 30-s intervals thereafter for the duration of the 3-min train. Vagal chronotropic responses were expressed as the percent change in the sinus cycle length from control. To characterize the change in the vagal chronotropic response during the course of the vagal train, a fade ratio was calculated. The fade ratio was calculated as the difference between the maximum percent change in sinus cycle length and the percent change in sinus cycle length at the end of the 3-min vagal train, divided by the maximum percent change (Fig. 1).

To demonstrate recovery of the vagal chronotropic response from fade, a 5-s vagal train was delivered to the right VST 2 min after the end of each of the 3-min stimulation trains. The change in sinus cycle length during both the primary 3-min train and the 5-s test train were measured, for consistency, 4 s into each vagal train as previously described (8). Recovery of the vagal chronotropic response was then assessed by comparing the percent change in sinus cycle length during the primary and test vagal trains. Recovery from fade was defined as complete if the percent change in sinus cycle length in response to the test train was equal to or greater than 90% of the primary train response.

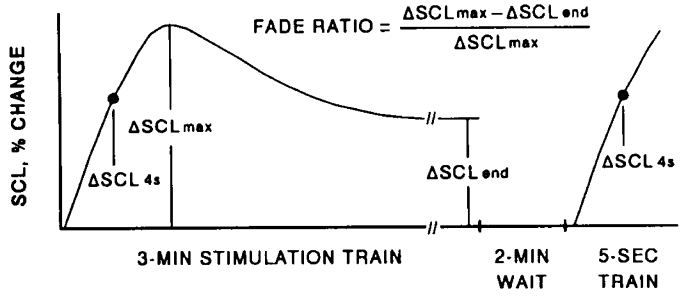


Fig. 1. Diagram illustrating the method for determining the maximum vagal chronotropic response and calculating the fade ratio during a 3-min vagal stimulation train. The change in sinus cycle length ($\Delta SCL$), expressed as percent change from control, is plotted as a function of time. The fade ratio is the ratio of the percent change in SCL measured at the end of the 3-min vagal train ($\Delta SCL_{end}$) to the maximum value ($\Delta SCL_{max}$). To demonstrate recovery of the vagal chronotropic response, the ΔSCL was determined 4 s after the onset of the vagal train ($\Delta SCL_{4s}$). Two min after the end of the vagal train, a 5-s test train was delivered. Recovery was considered complete when the vagal chronotropic response, measured 4 s into the second vagal train, was within 90% of the original value.

In other experiments, we examined the possible influence of circulating (*i.e.* adrenal) catecholamines on the vagal chronotropic responses. These experiments were performed in a group of chemically sympathectomized newborns ($n$ = 7, 8.3 ± 2.7 d old). In these experiments, determinations of the maximum percent change in sinus cycle length and the fade ratio (as defined above) were made during 3-min trains of vagal stimulation at 5 Hz. Vagal trains were then delivered after the administration of propranolol and phentolamine (1 mg/kg i.v.), given to block both β- and α-adrenergic receptors. The vagal chronotropic responses and fade ratios in the unblocked and adrenergically blocked states were analyzed (see below). The differences were taken as evidence for a possible effect of circulating catecholamines on the vagal responses.

Finally, the effects of acetylcholinesterase inhibition on the vagal chronotropic response and fade ratio were examined in five chemically sympathectomized neonates, aged 10.0 ± 2.9 d. In these experiments, a 1-min train of vagal stimulation was delivered at the lowest stimulation frequency (3 Hz). The train was then repeated after the administration of 0.2 mg/kg i.v. of physostigmine. Comparisons of the maximum percent change in sinus cycle length and the fade ratio were made before and after acetylcholinesterase inhibition to evaluate the effect of increasing junctional ACh concentration by a mechanism other than an increase in vagal stimulation intensity.

*Statistical analysis.* To assess the influence of vagal stimulation frequency on the vagal chronotropic response and on the fade ratio, one-way ANOVA for repeated measurements was performed. A $p$ value <0.05 was taken to indicate a significant ANOVA. Bonferroni multiple comparison tests were performed to determine at which stimulation frequencies differences became significant (11). Recovery of the vagal response at each stimulation frequency was assessed using a two-way analysis of variance. For paired observations (*i.e.* before and after adrenergic blockade or physostigmine) two-tailed $t$ tests were applied, again with $p < 0.05$ used to indicate a significant difference. All values are expressed as the mean ± SD.

## RESULTS

*Verification of chemical sympathectomy.* Before the start of vagal stimulation, a dose of tyramine (100 μg/kg i.v.) was administered and changes in sinus cycle length and arterial blood pressure were recorded. In the 15 sympathectomized neonates (aged 8.7 ± 3.5 d) the sinus cycle length remained essentially unchanged (control sinus cycle length, 323 ± 35 ms; posttyramine, 319 ± 37 ms) and systolic blood pressure rose by only 10 ± 6% (from 76 ± 9 to 84 ± 11 mm Hg). We have previously shown in our laboratory that in nonsympathectomized neonates ($n$ = 7) the same dose of tyramine results in a shortening of the sinus cycle length of 24 ± 6% and an increase in the systolic blood pressure of 88 ± 19% (8). Therefore, in the present study group, the lack of change in sinus cycle length and very small increase in systolic blood pressure after tyramine provides evidence for an adequate (if not total) chemical sympathectomy.

*Characterization of chronotropic response during 3-min vagal stimulation trains at 3, 5, and 8 Hz (Table 1).* The mean percent changes in sinus cycle length over the course of 3-min vagal stimulation trains in six neonatal canines (aged 7.8 ± 4.4 d) are illustrated in Figure 2. Recovery of the vagal chronotropic response (see Materials and Methods) measured 107 ± 19% at 3 Hz, 94 ± 7% at 5 Hz, and 109 ± 29% at 8 Hz. Thus, as reflected by the recovery values, no significant difference in the magnitude of the vagal chronotropic response was observed after a 2-min rest period (ANOVA $p$ = 0.91).

At each stimulation frequency the (percent) change in sinus cycle length increased to a maximum value. This maximum value increased as a function of stimulation frequency (26 ± 9%

Table 1. *Vagal chronotropic responses**

| | 3 Hz | 5 Hz | 8 Hz | ANOVA |
|---|---|---|---|---|
| $\Delta SCL_{max}$ | 26 ± 9 | 52 ± 13 | 109 ± 34† | $p < 0.001$ |
| $\Delta SCL_{end}$ | 25 ± 7 | 44 ± 10 | 53 ± 21 | |
| Fade ratio | 3 ± 5 | 14 ± 12 | 48 ± 26‡ | $p < 0.002$ |

* Values are the means ± SD in percent; $n = 6$. $\Delta SCL_{max}$, maximum percent change in sinus cycle length; $\Delta SCL_{end}$, percent change in sinus cycle length at end of vagal stimulation train; Fade ratio, percent attenuation of vagal response.

† $p < 0.05$ compared with 3 Hz (Bonferroni multiple comparison test).

‡ $p < 0.01$ compared with 3 Hz.

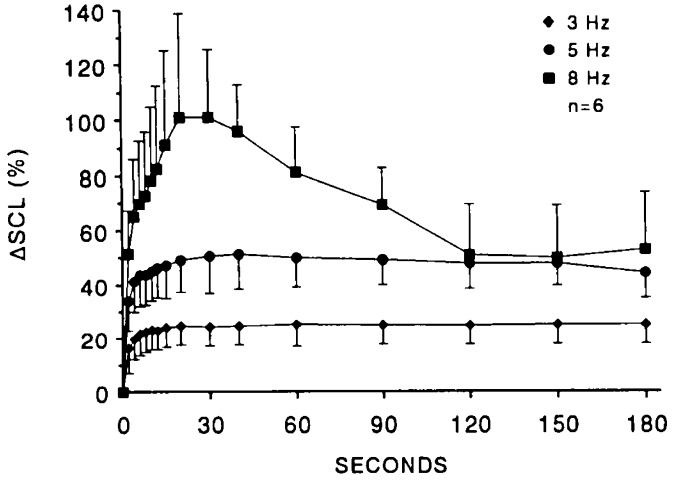


Fig. 2. Plot of the percent change in sinus cycle length (*SCL*) in response to 3-min trains of vagal stimulation delivered at 3, 5, and 8 Hz. See text for further discussion.

at 3 Hz, 52 ± 13% at 5 Hz, and 109 ± 34% at 8 Hz, ANOVA $p < 0.001$). This is consistent with our previous report of the effects of tonic vagal stimulation in the neonatal canine (9). During the remainder of the 3-min vagal stimulation train very little change in sinus cycle length was noted when stimulating at 3 Hz (percent change in sinus cycle length at end of stimulation train = 25 ± 7%). At 8 Hz, however, there was a very noticeable attenuation or fade of the vagal chronotropic response over the remainder of the 3-min train (percent change in sinus cycle length at end of train = 53 ± 21%). Fade ratios increased as stimulation frequency increased (ANOVA $p < 0.002$) and were 3 ± 5% at 3 Hz, 14 ± 12% at 5 Hz, and 48 ± 26% at 8 Hz. The fade ratio at 8 Hz was significantly larger than at 3 Hz ($p < 0.05$). This is notably different than the constant fade ratio reported over the same range of stimulation frequencies in the adult dog (5).

*Effect of α- and β-adrenergic blockade on fade ratio.* In a group of seven sympathectomized neonates (aged 8.3 ± 2.7 d), 3-min trains of vagal stimulation were delivered at 5 Hz before (control response) and after the administration of both propranolol and phentolamine (1 mg/kg i.v.). The maximum percent change in sinus cycle length of the control response was 54 ± 10%, and after adrenergic blockade the response was unchanged (54 ± 12%, $p = 0.89$). Therefore, circulating catecholamines did not alter the maximum response to vagal stimulation. The fade ratio was actually slightly greater after adrenergic blockade (fade ratio 9 ± 12% control, 14 ± 15% postadrenergic blockade, $p < 0.03$). This suggests that circulating catecholamines did not likely account for the observed attenuation of the vagal chronotropic response (*i.e.* via a positive chronotropic effect) during the 3-min stimulation trains.

*Effect of acetylcholinesterase inhibition on vagal chronotropic response and fade ratio.* In five sympathectomized neonates (aged 10 ± 2.9 d), we compared the chronotropic response to vagal stimulation for 1 min at the lowest stimulation frequency (3 Hz) before and after the administration of the acetylcholinesterase inhibitor physostigmine (0.2 mg/kg i.v.). Physostigmine was administered as a second method of increasing ACh concentration in the synaptic cleft. Before the administration of the acetylcholinesterase inhibitor, the sinus cycle length gradually increased during vagal stimulation, reaching a maximum percent change of 34 ± 8%, and remained essentially constant over the duration of the vagal train (percent change at end of train = 32 ± 7%). This response is essentially identical to that observed during the 3-min trains at 3 Hz depicted in Figure 2. After physostigmine, the same stimulation train resulted in a much larger maximum percent change in sinus cycle length (105 ± 47%, $p < 0.01$ compared with before physostigmine) with the mean maximum response occurring within 8 s after the onset of vagal stimulation (Fig. 3). Importantly, in addition to a larger maximum percent change, the fade ratio also increased significantly after physostigmine, from 6 ± 4% to 50 ± 14%, $p < 0.004$. This value is comparable to that observed during vagal stimulation for 3 min at 8 Hz (Fig. 2). Some differences were observed, however, in the rate of development of fade after physostigmine. Although the fade ratio after physotigmine was comparable to that observed during vagal stimulation at 8 Hz, the rate of development of fade appeared to be much more rapid when stimulating at 3 Hz in the presence of physostigmine. During vagal stimulation at 8 Hz, fade developed over a period of ~120 s (Fig. 2), whereas after physostigmine and vagal stimulation at 3 Hz, fade was nearly complete by 20 s. Thus, in these neonates, the increased concentration of ACh caused by acetylcholinesterase inhibition (and low level vagal stimulation intensity) resulted not only in a larger chronotropic response, but also caused a significant increase in the fade ratio and the rate of development of fade as well.

## DISCUSSION

In this study, we have shown that in the neonatal canine not only does the magnitude of the vagal chronotropic response increase as a function of vagal stimulation intensity but the attenuation of the vagal response (*i.e.* the fade ratio) increases as well. These results are quite different from those reported *in vivo* in adult dogs, in which the fade ratio remains constant during vagal stimulation at 0.5–8 Hz (5). Similar results are also noted in *in vitro* studies of the chronotropic response of the adult atrium to intramural parasympathetic stimulation. In these studies, it is only at very high stimulation intensities (30 Hz) that changes in the fade ratio have been reported (1). Although the chronotropic responses observed during vagal stimulation *in vitro* and *in vivo* may not be directly comparable, in the adult the fade ratio is essentially constant over stimulation intensities of <10 Hz in both *in vitro* and *in vivo* studies. In the neonate, unlike

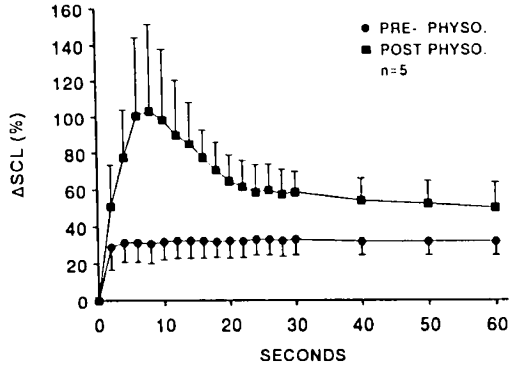


Fig. 3. Percent change in sinus cycle length (*SCL*) in response to a 1-min vagal stimulation train delivered at 3 Hz. The chronotropic response is plotted before (*PRE- PHYSO.*) and after (*POST PHYSO.*) the administration of the acetylcholinesterase inhibitor physostigmine. See text for further discussion.

the adult, the fade ratio is not constant but rather increases as stimulation intensity is increased from 3 to 8 Hz.

Attenuation, or fade, of the vagal chronotropic response is thought to represent an action of ACh itself at pre- and postsynaptic sites. Although the precise mechanism responsible for fade is not known, three principle mechanisms have been considered. The first involves a progressive decrease in the amount of ACh released during a continuous vagal stimulation train. This progressive decrease may occur as the result of a negative feedback mechanism mediated via presynaptic muscarinic receptors (12, 13). Previous studies have suggested that this negative feedback process is, itself, dependent upon vagal stimulation intensity and is only important at very high vagal stimulation frequencies (20 Hz) (12, 14). The second postulated mechanism for fade involves a decrease in the binding affinity of the muscarinic receptor for ACh, as the receptor is exposed to the agonist (3). This process, referred to as receptor desensitization, is well described for other receptor-agonist systems such as the nicotinic receptor of skeletal muscle (15). Finally, fade may be the result of changes in the intracellular response to receptor activation that result in a diminished effector response (16). These changes may include such processes as receptor uncoupling or changes in intracellular G protein activation and inactivation that alter the magnitude of the effector response (17). One or a combination of these mechanisms is likely involved in the attenuation of the vagal response observed in previous vagal stimulation studies and in our own study.

Our results clearly show that, in the newborn, as the concentration of ACh in the synaptic cleft is increased, either by increasing vagal stimulation intensity or by inhibiting hydrolysis with physostigmine, the fade ratio increases. Why the fade ratio is a direct function of the concentration of ACh in the neonate, but not in the adult, is not known and not readily determined by the methods used in our study. However, developmental differences in the neonate in pre- or postsynaptic muscarinic receptor concentration, or perhaps the response of the receptor/postreceptor pathway to ACh, may account for our findings. Important developmental differences in ACh receptor density and cholinergic signaling have been reported. In both rat and sheep myocardium, muscarinic receptor density has been found to be significantly higher in fetal and neonatal animals compared with adult animals (18, 19). In addition to receptor number, the linkage of the muscarinic receptor to postreceptor pathways, as measured in one study by the production of inositol polyphosphates, is also much greater in the fetus than in the adult (18). This finding is of potential importance when considering mechanisms that might contribute to the observed differences in receptor desensitization in the neonate. Inositol polyphosphate hydrolysis is believed to result in, among other things, the generation of diacylglycerol. Diacylglycerol, in turn, results in activation of protein kinase C. Protein kinase C, which has many substrates, is believed by some to play a pivotal role in the desensitization process. The presumed mechanism is the phosphorylation of the $\alpha$-subunit of the G inhibitory protein complex. This, in turn, renders the G protein inactive and thus unable to mediate the opening of ACh-responsive potassium channels (*i.e.* attenuation of agonist response) (17, 19). Finally, although fetal and adult myocardium express the muscarinic subtype M2, it is not really known to what extent other muscarinic subtypes might be present in the immature myocardium or how these subtypes might modulate the effector response and the subsequent attenuation, or fade, of that response. Thus, significant differences in muscarinic receptor number (and perhaps receptor subtype), as well as in cholinergic signaling, have been demonstrated in the immature myocardium. These receptor differences likely account, in some way, for the different relationship between ACh concentration and receptor desensitization demonstrated in the newborn in our study. Additional research would be required to delineate the specific mechanisms of receptor desensitization in the newborn.

We believe that the attenuation, or fade, of the vagal chronotropic responses observed in our study was in fact most likely mediated by ACh, rather than by any other mechanism. By chemically sympathectomizing the newborns, we have theoretically eliminated direct sympathetic activity or parasympathetic-sympathetic interactions from modifying the vagal chronotropic response. Although the use of 6-hydroxydopamine has been associated with some degree of hyperactivity of the adrenal medulla (20, 21), the administration of both $\alpha$- and $\beta$-adrenergic blocking agents in our model resulted in no change in the maximum vagal chronotropic response. The fade ratio, if anything, seemed to be slightly greater after adrenergic blockade. Thus, any positive chronotropic effect of circulating catecholamines did not appear to account for the fade observed during vagal stimulation.

The use of 6-hydroxydopamine also makes attenuation of the vagal response secondary to the release of the sympathetic cotransmitter neuropeptide Y unlikely. Furthermore, the time course of recovery of the vagal chronotropic response in these experiments (less than 2 min) is not consistent with a neuropeptide Y mechanism. It is notably shorter than the more prolonged time course of recovery of the vagal response noted in the newborn canine after exogenous neuropeptide Y administration or (presumed) endogenous release (8). We also do not believe that the attenuation of the vagal response was mediated by the release of the putative autonomic cotransmitter, vasoactive intestinal peptide. Although a positive chronotropic response to vasoactive intestinal peptide has been noted in the adult animal (22), Mas *et al.* (23) reported that vasoactive intestinal peptide does not increase automaticity in the neonatal dog. In fact, vasoactive intestinal peptide exerts a mild negative chronotropic effect in the newborn. For all of the reasons cited above, we believe the fade of the vagal chronotropic response observed in our experiments is most likely the phenomenon of receptor desensitization that others have attributed to an effect of ACh (1–7).

In summary, our data demonstrate that in the newborn both the vagal chronotropic response and the fade ratio increase as stimulation frequencies are increased from 3 to 8 Hz. This is different from the adult, in which the fade ratio remains constant over the same range of stimulation intensities. That this increase in the fade ratio is the direct result of ACh is demonstrated by the response to acetylcholinesterase inhibition. An increase in the vagal chronotropic response, the fade ratio and the rate of development of fade occurs when the vagus nerve is stimulated at the lowest stimulation intensity after acetylcholinesterase inhibition. We speculate that developmental differences in muscarinic receptor concentration and/or intracellular signaling may account for these findings. A greater attenuation of closely coupled, strong vagal stimuli compared with weak stimuli, as predicted by our study, could render repetitive, strong bursts of vagal activity somewhat less effective in modulating parasympathetic tone in the newborn compared with the adult.

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