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Quantifying the Altered Cardiac Response to Atropine Following Pyridostigmine in Rhesus Macaques[1]

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BIRNBAUM, S. G., B. C. RICHARDSON AND J. A. DELLINGER. *Quantifying the altered cardiac response to atropine following pyridostigmine in rhesus macaques.* PHARMACOL BIOCHEM BEHAV 31(2) 381–386, 1988.—An estimate of the amplitude of respiratory sinus arrhythmia ($\hat{V}$) has been proposed as a noninvasive measure of parasympathetic activity. This experiment monitored $\hat{V}$ in response to a subclinical dose of pyridostigmine bromide (PYR) and a pharmacological challenge of atropine sulfate (ATR). Twelve male rhesus macaques received 200 μg/kg of PYR 30 min prior to an injection of 0, 14, 44, or 140 μg/kg ATR. The decrease in $\hat{V}$ after both the 44 and 140 μg/kg ATR doses was similar to the response to ATR alone in a previous experiment. The 14 μg/kg dose of ATR did not significantly decrease $\hat{V}$ in this experiment, which is in contrast with the large decrease of $\hat{V}$ after ATR alone in a previous experiment. Neither drug affected respiration. The dose of ATR which would be effective in causing a 30% decrease of $\hat{V}$ in the presence of PYR was estimated to be 18.3 μg/kg of ATR. This is twice the dose of ATR calculated to have the same effect without PYR. The attenuated response of $\hat{V}$ after a pharmacological challenge of ATR may be used to quantify the latent muscarinic effects from exposure to anticholinesterase agents. The attenuated response to ATR may also be useful for evaluating the return of normal cholinergic function after disruption by cholinesterase inhibitors.

Respiratory sinus arrhythmia (RSA) Pyridostigmine Atropine Vagal tone monitor Cholinesterase Pharmacological challenge Anticholinesterase

ANTICHOLINESTERASE (anti-ChE) agents cause many behavioral alterations including changes in motor activity, learning, and memory (17) in addition to the clinical signs of their toxicity (excessive salivation, lacrimation, defecation, and urination). Atropine, a cholinergic antagonist used as a therapeutic agent for anti-ChE intoxication, also causes behavioral changes (14). Animals exposed to chronic or low doses of anti-ChE compounds may not exhibit clinical symptoms due to tolerance (29) and the compensatory capacity of the nervous system (32). Additional biological, physical, or chemical stresses on these animals may disclose their compromised nervous system. A partially impaired system may not be able to respond to the additional stress in the same manner as a fully functional nervous system. A pharmacological challenge can be used to demonstrate neurobehavioral impairment in animals which otherwise show no clinical symptoms (29,32).

Suspected anti-ChE exposures are generally confirmed by an assay for blood cholinesterase (ChE) activity, although the detection of anti-ChE exposures using blood ChE activity has several disadvantages. Normal ChE values can vary substantially between species (13,20), within a population, between sexes (25) and within one individual across time (26,27). These variabilities necessitate a recent baseline ChE activity level for each individual before a meaningful diagnosis can be obtained. In addition, the relationship between blood ChE levels and clinical symptoms is equivocal. Although inhibition of normal enzyme activity by approx-

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imately 60% in humans is generally correlated with the onset of cholinergic symptoms (30), cases have been reported where humans have suffered extreme symptoms with very little change in ChE activity (15). Blood ChE activity is only an indirect measurement of the status of the enzyme in vital tissues such as the diaphragm, heart, and central nervous system. Inhibition of enzyme activity in these tissues results in the adverse symptoms from exposure to anti-ChE agents.

A more direct method for assessing the effects of anti-ChE compounds would be to monitor changes in the activity of cholinergic neurons due to the inhibition of ChE and the concomitant build-up of the endogenous neurotransmitter acetylcholine. Although direct measurement of nerve activity and transmission across the synapse can be measured in experimental animals, no noninvasive method is available for measurements in awake or free-ranging animals. A method which quantifies the neural control of heart rate has been proposed as a means for assessing parasympathetic nervous system activity (10,18).

Mean heart rate and rhythmic fluctuations in the cardiac beat-to-beat interval (heart period) are controlled by input from both the sympathetic (3, 11, 23) and the parasympathetic nervous system (22,28). A spectral density plot of the variations in heart period reveals three principle peaks (3). Although some of the heart period variability is sensitive to alterations in sympathetic activity (1), the amplitude of the peak which corresponds to the variations in heart rate occurring at the same frequency as respiration (respiratory sinus arrhythmia, RSA) is influenced primarily by the parasympathetic nervous system (3, 18, 19). Reduction of vagal efferent activity to the heart through pharmacological and surgical manipulations decreases RSA (1, 3, 12, 18). Conversely, increases in parasympathetic activity by elevating blood pressure leads to an increased RSA (18). Changes in sympathetic activity do not alter RSA (3, 10, 12). This direct relationship between vagal efferent activity and RSA suggests that measurement of RSA may be used as a representation of the status of the parasympathetic nervous system.

The Vagal Tone Monitor (VTM; Delta Biometrics, Bethesda, MD) estimates RSA by digitizing ECG signals and filtering out the portion of heart period variability due to sympathetic activity and nonneural factors. The portion of heart period variance which occurs within the same frequency range as respiration is then quantified and termed $\hat{V}$ (8). Pharmacological and surgical manipulations have shown that this estimate of RSA is sensitive to alterations in parasympathetic activity, but is relatively insensitive to sympathetic manipulations (21,31).

During anticholinergic therapy, atropine is currently administered until symptoms of atropinization, such as tachycardia and a dry mouth, occur. Previous work in our laboratory documented the decrease in $\hat{V}$ in the rhesus macaque after exposure to atropine (16). That experiment also demonstrated the potential of $\hat{V}$ for quantifying the effects of atropine rather than relying on subjective symptoms. In this experiment, a subclinical dose of reversible anti-ChE agent, pyridostigmine bromide, was administered to the monkeys preceding the atropine challenge. Pyridostigmine has been shown to have no effect on $\hat{V}$ in the rhesus monkey (4). Dichlorvos, an irreversible ChE inhibitor, has also been shown to have no direct effect on $\hat{V}$ in dogs (6). When the dichlorvos-treated dogs were subsequently given a pharmacological challenge of atropine and pralidoxime chloride, the response of $\hat{V}$ was diminished compared to the control animals.

The objective of this experiment was to use pharmacological challenges of atropine to discern the latent muscarinic effects of pyridostigmine. The pyridostigmine dose utilized had previously been found to cause a significant inhibition of ChE without resulting in overt symptoms of rhesus monkeys (4). The atropine doses were chosen to allow for a direct comparison between the results in this experiment and those obtained from a previous study which investigated the effects of atropine alone in rhesus macaques (16).

METHOD

Experimental Animals

Twelve young adult (5–10 years; 6.14–9.43 kg) captive-born male rhesus monkeys (*Macaca mulatta*), received six months prior to the onset of this study, were housed individually and given food twice daily. Water was available ad lib. Following a period of acclimation to the housing facility, the monkeys were trained to sit in a primate restraining chair for a total of 3.5 hr. A pole and collar minimized the handling necessary to remove the monkeys from their home cage and place them into the restraining chairs. All monkeys had previously been used in two experiments with similar experimental designs. They were allowed a six week recovery period before the onset of this experiment.

Experimental Design

Atropine sulfate (ATR; Med-Tech Inc., Elwood, KS) was prepared in isotonic saline and individual doses were drawn weekly. Pyridostigmine bromide (PYR; Hoffman-La Roche Inc., Nutley, NJ) was prepared in isotonic saline and individual doses were drawn daily from this preparation. All doses were administered with a final volume of 0.1 cc/kg body weight.

A Latin-square design, with experimental week as columns and dose sequence groups as rows, was used to assign ATR doses to all 12 monkeys. The monkeys were randomly assigned to groups and the investigator was blind to the randomized treatment sequence. Each animal received an injection (IM) of 200 μg PYR/kg body weight followed by either 0, 14, 44, or 140 μg of ATR/kg body weight. All injections were administered into the lateral aspect of the right calf. Fourteen measurements of $\hat{V}$ were obtained from each experimental session. Experimental sessions were separated by a week for each animal.

Data Collection

During each session, two monkeys were individually seated in primate restraining chairs and a 2 cc baseline venous blood sample was drawn from each monkey. The animals were then placed in separate sound-attenuating chambers with low levels of light and white noise. Cardiac parameters were collected using a standard lead II ECG connection.

The ECG signals were continuously passed into an oscilloscope and then into the Vagal Tone Monitor (VTM). An adult human respiratory range of 0.12 to 0.40 Hz or a human neonatal respiratory range of 0.3 to 1.3 Hz was determined for each monkey the week prior to the onset of this experiment. The VTM was set to the predetermined range for each monkey. The cardiac parameters output every 30 sec from the VTM were stored directly on a computer disc for later analysis. Respiration was monitored using a bellows/pressure transducer apparatus. The respiratory data were

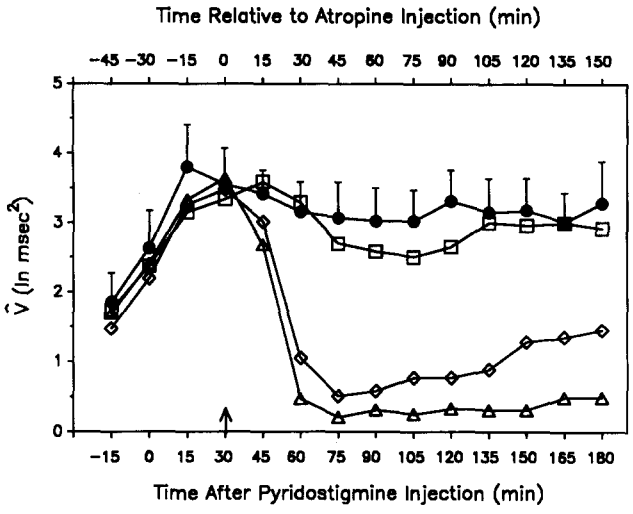

FIG. 1. Effects of atropine on the estimate of the amplitude of respiratory sinus arrhythmia ($\hat{V}$) in the presence of pyridostigmine. All animals received a 200 μg/kg pyridostigmine injection at Time 0. The arrow at Time 30 indicates the atropine injection of ● 0 μg/kg ±S.E.M.; □ 14 μg/kg; ◇ 44 μg/kg; △ 140 μg/kg. Data presented are means from 12 monkeys.

transmitted to a physiograph and recorded on thermal sensitive paper every 15 min for measurement of respiratory rates.

After a 30 min baseline period in the sound-attenuating chambers, each monkey received 200 μg of PYR/kg body weight. Thirty min after the PYR injection another 2 cc blood venous sample was drawn, immediately followed by the atropine sulfate injection. A final venous blood sample was drawn 180 min after the first (PYR) injection.

Cholinesterase Determination

All blood samples were collected by cephalic venipuncture into EDTA tubes. Each sample of whole blood was analyzed for both erythrocyte and plasma cholinesterase activity using modifications of a colorimetric method developed by Ellman *et al.* (9).

The whole blood was separated by centrifugation at 1200 × g for 10 min. Unlike Ellman's technique, the erythrocytes (0.1 ml) were hemolyzed with 1.9 ml of a 5% Triton-X solution (octyl phenoxy polyethoxyethanol). The lysed erythrocytes (0.5 ml) were then diluted to 25 ml with 0.1 M phosphate buffer, pH 8.0. The erythrocyte sample (1 ml) was further diluted with the phosphate buffer (2 ml) in order to obtain a linear rate of color change (final dilution 1:3000). The plasma (0.01 ml) was diluted to 10 ml with phosphate buffer (0.1 M, pH 8.0; final dilution 1:1000).

A 3 ml volume of each buffered sample was assayed for ChE activity. Dithiobisnitrobenzoic acid (0.01 M; 0.05 ml) was added to both a reference and the sample cuvette. The substrate, acetylthiocholine iodide (ATCI 0.075 M; 0.02 ml) was added to the sample cuvette only. The absorbance changes at 412 nm were measured against the reference cuvette for 3 min using a Beckman DU-5 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). One technician performed all of the blood analyses which were completed within one hour from the time of the blood draw. A human serum standard (SeraChem Normal Clinical Chemistry Control Serum, Fisher Scientific Co., Pittsburgh, PA) was also analyzed daily. All reagents were prepared weekly. The data were reported as mM of ATCI hydrolyzed/l/min.

Statistical Analysis

Fifteen min averages of the estimate of respiratory sinus arrhythmia amplitude ($\hat{V}$) were used to study the major changes in $\hat{V}$ and to standardize the data with previous experiments in our laboratory. These means were used to test the main effects (animal, group, week, time, and dose) and the interactions of the main effects with a repeated measures multivariate analysis of variance (MANOVA). Wilk's criterion was used to calculate the F-statistic. Individual variability was corrected for in the overall statistical model by partitioning the appropriate error terms. Differences within the main effects were further analyzed using Tukey's Studentized Range Tests. Interactions were investigated using multiple *t*-tests with Bonferroni adjusted significance levels. Plasma and erythrocyte cholinesterase activity were analyzed using a similar model. An overall alpha level of $p<0.05$ was used for all MANOVA tests and subsequent multiple comparisons.

The dose of ATR effective in causing a 30% decrease from baseline levels in $\hat{V}$ for 50% of the animals (ED_{50}) was calculated using Probit Analysis. Chi-Square test ($p>0.10$) was used to assess the fit of the probit line to the data. The number of animals which responded at each dose level were used to calculate the ED_{50}. This ED_{50} for $\hat{V}$ was compared to the ED_{50} previously calculated in an experiment which involved only ATR (16).

RESULTS

The respiratory rate of six monkeys was within the adult human frequency range of 0.12 to 0.40 Hz (7.2 to 24 breaths/min) and six within the human neonate frequency range of 0.3 to 1.3 Hz (18 to 78 breaths/min). These rates were maintained within the original ranges after exposure to both of the drugs.

Although examination of the ECG traces revealed that several of the monkeys occasionally exhibited minor cardiac arrhythmias, none of these could be attributed to the administration of either drug (Dr. David Smetzer, personal communication). One monkey exhibited a very small R-wave and a large S-wave. The VTM, which responds to a large positive deflection, required us to reverse the polarity of the electrodes for this animal each week.

The decrease of $\hat{V}$ in response to both PYR and ATR, as shown in Fig. 1, was very similar to the decrease of $\hat{V}$ in response to ATR alone (16). The MANOVA indicated a significant dose effect, $F(39,24.44)=2.76$, $p<0.0049$, time effect, $F(12,9)=23.96$, $p<0.0001$, and dose-time interaction, $F(36,27.3)=2.88$, $p<0.0027$. Tukey tests indicated that the high (44 and 140 μg/kg) doses of ATR were significantly different from the low (0 and 14 μg/kg) doses. Further investigation revealed that the high doses first differed from the control at 30 min after ATR and they remained separate for the duration of the experiment. The maximum depression of $\hat{V}$ occurred at 45 min after the ATR injection. The response of $\hat{V}$ to the 14 μg/kg dose of ATR did not differ from control at any time during the experiment. Also, $\hat{V}$ did not differ between the 44 and 144 μg/kg ATR doses.

The analysis of the data from this experiment combined with the experiment involving only ATR revealed a strong drug-dose-time interaction, $F(30,50.57)=1.69$, $p<0.0494$.

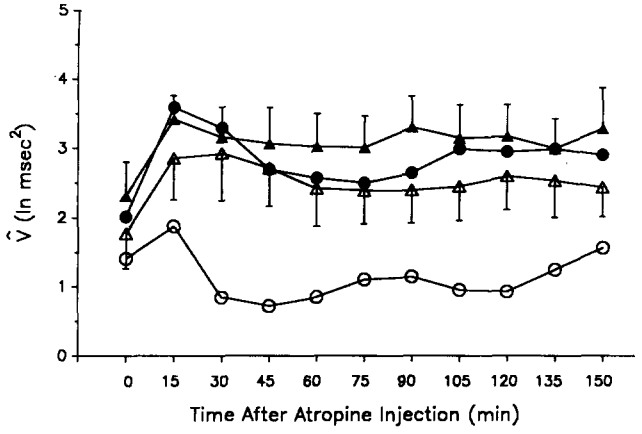

FIG. 2. Comparison of the effect on $\hat{V}$ of 14 μg/kg of atropine in the presence and absence of pyridostigmine. ▲ 200 μg/kg pyridostigmine and 0 μg/kg atropine ±S.E.M.; ● 200 μg/kg pyridostigmine and 14 μg/kg atropine; △ 0 μg/kg pyridostigmine and 0 μg/kg atropine ±S.E.M.; ○ 0 μg/kg pyridostigmine and 14 μg/kg atropine. The pyridostigmine pretreatment was administered 30 min prior to the atropine injection. Data presented are means from 12 monkeys.

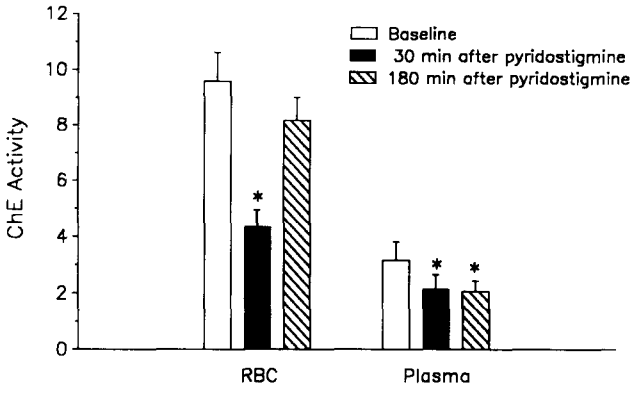

FIG. 3. The effects of 200 μg/kg pyridostigmine on erythrocyte and plasma cholinesterase activity. Data presented are means±standard deviation for 12 monkeys. *Denotes the mean differs significantly from baseline ($p<0.05$).

Bonferroni adjusted t-test comparisons of $\hat{V}$ for each time and ATR dose were used to determine the effect of PYR. There were no significant differences in $\hat{V}$ at any time after the 0 μg/kg doses. $\hat{V}$ was significantly less at all times after the 14 μg/kg dose of ATR alone compared with the ATR and PYR combination. The only significant difference in $\hat{V}$ was at 15 min after the 44 and 140 μg/kg ATR doses. Figure 2 shows the effect on $\hat{V}$ of the 0 and 14 μg/kg doses of ATR both in the presence and absence of PYR. The 14 μg/kg dose of ATR in the presence of PYR was not significantly different from the 0 μg/kg dose of ATR in the previous experiment in which only saline was administered. This is in sharp contrast with the reduction of $\hat{V}$ after the 14 μg/kg dose of ATR alone.

The dose of ATR which would cause a 30% decrease of $\hat{V}$ in the presence of PYR was estimated to be 18.3 μg/kg ($\chi^2=2.4$, $p>0.1219$). The ED_{50} for ATR alone was estimated in a previously reported experiment to be 9.0 μg/kg ($\chi^2=0.1$, $p>0.7458$).

The changes in erythrocyte and plasma ChE following the PYR injection are presented in Fig. 3. The baseline ChE activities were 9.59±1.030 mM/l/min (mean±standard deviation) and 3.17±0.661 mM/l/min for erythrocyte and plasma samples respectively. A significant time effect for both erythrocyte, $F(2,22)=970.44$, $p<0.0001$, and plasma, $F(2,22)=492.0$, $p<0.0001$, indicates the effect of the 200 μg/kg dose of PYR. Thirty min after the PYR dosing, erythrocyte ChE activity was 4.36±0.589 mM/l/min, an inhibition of 55% from baseline levels. The erythrocyte ChE activity had begun to recover towards the baseline level but was still 15% inhibited 180 min after the PYR dosing. Plasma ChE activity was 2.16±0.490 mM/l/min, an inhibition of 32%, at 30 min after the administration of PYR. Unlike the erythrocyte ChE, plasma ChE activity had not recovered even at 180 min following the PYR injection. There was no significant ATR dose-time effect in either erythrocyte, $F(6,44)=0.89$, $p<0.5105$, or plasma, $F(6,44)=1.74$, $p<0.1346$, ChE activity. The significant week effect in erythrocyte, $F(9,51.3)=2.13$, $p<0.0436$, and plasma, $F(9,51.3)=5.04$, $p<0.0001$, ChE activity was due to the low values during week 1 compared to the values measured during weeks 2, 3 and 4. There was not a consistent trend towards an increased activity level across the four experimental weeks. Analysis of the human serum standards also indicated a significant week effect, $F(3,36)=3.5$, $p<0.0245$, similar to the one seen for both plasma and erythrocyte ChE.

DISCUSSION

Respiratory and cardiac changes were recorded in the rhesus macaque following exposure to pyridostigmine bromide and atropine sulfate. A decrease in $\hat{V}$, an estimate of RSA, was observed after the ATR doses. A decrease in $\hat{V}$ could occur for several reasons. A direct interaction of the drug with the parasympathetic nerves controlling HR could effect $\hat{V}$. Atropine is known to be a muscarinic antagonist. Binding of atropine to receptors at the synapses could decrease the neural signals which cause changes in HR. This estimate of RSA is calculated by quantifying the variability in HR which occurs within a specific respiratory frequency band. If respiration shifts away from the preset frequency band, then the variability which is actually due to respiration would be removed since it would no longer occur within the frequency band. Angelone and Coulter (2) have also shown that changes in breathing frequency can cause both a direct change in the amplitude of the fluctuations in HR and a phase shift between the frequency of respiration and the frequency of the HR variability. Both of these effects would also cause a change in $\hat{V}$ using the methods utilized by the VTM.

Respiration in this experiment did not shift out of the frequency range which we had set. Thus the decrease in $\hat{V}$ after ATR is probably due to a direct effect at the cholinergic synapses. Evidence from subsequent experiments (Waldrop, unpublished observations) suggests that the effects of atropine on $\hat{V}$ are a result of an interaction with cholinergic receptors in the peripheral nervous system rather than in the central nervous system. An acidic solution, injected into the CSF surrounding the brainstem of cats, resulted in both respiratory and cardiac responses, while an ATR injection into this region had no effect on either. An IV injection of ATR

had no effect on blood pressure but resulted in an immediate decrease of $\hat{V}$.

After the 200 μg/kg PYR injection, there was no significant change in $\hat{V}$. The initial increase of $\hat{V}$ at 15 min appears to be the result of a continued acclimation of the animals to restraint. This increase might be avoided if the animals are allowed a longer period of acclimation prior to the first injection.

The attenuated response of $\hat{V}$ to ATR in the presence of an anti-ChE agent was evident from the ED_{50} results. The ED_{50} for ATR following the PYR predosing in the present experiment was 18.3 μg/kg, twice the previously reported ED_{50} for ATR alone (9.0 μg/kg). The attenuated effect of ATR was also evident from the comparison of the responses of $\hat{V}$ after the low dose of ATR in the presence and absence of PYR. The 14 μg/kg dose of ATR after 200 μg/kg of PYR did not reduce $\hat{V}$ in this experiment, but it substantially reduced $\hat{V}$ when presented alone. In both experiments, the 44 and 140 μg/kg doses reduced $\hat{V}$ to almost 0.0 ln $msec^2$. The lack of an attenuation at these doses may have been due to either a saturation of the receptors by atropine or a saturation of the response as measured by the VTM.

No clinical symptoms were observed in any of the monkeys after the 200 μg/kg dose of PYR. Clinical symptoms, generally observed after ChE inhibitions of 60% or greater (30), were not expected since the erythrocyte ChE was inhibited only 54% from baseline levels.

Although both erythrocyte and plasma ChE activity levels increased significantly after week 1, this was not a consistent trend across the four experimental weeks. The standard human serum samples exhibited a similar trend during these four weeks. It is unlikely that the animals compensated for the PYR by increased ChE concentrations or activity as a result of the dosing schedule used in this experiment. It is more likely that the change observed after the first week was due to either a difference in the temperature or the pH of the solutions. Both of these factors are known to significantly effect the relative activity of the ChE enzyme (24,27).

Cholinesterase values have been shown to be unreliable due to variability in vivo and to variability within the assays. Detection of exposures to low doses of anti-ChE agents can be difficult to interpret due to these variabilities. Although clinical symptoms may not be evident at these low doses, the normal functioning of the animal may be compromised following subsequent exposures to anti-ChE agents or under either physical or chemical stresses. Estimates of parasympathetic activity may prove to be a more reliable method for detecting and assessing exposure to anti-ChE compounds. Significant decreases of $\hat{V}$ have consistently been shown in humans (7) and several other species (5, 6, 16) after a low dose of atropine. The modification of this response after exposure to a subclinical dose of an anti-ChE has also been demonstrated (6). Thus, $\hat{V}$ appears to be useful as a noninvasive method for quantifying the latent muscarinic effects of anti-ChE agents in awake animals. This estimate of RSA may also be a useful tool for quantifying the therapeutic dose of ATR necessary to return to normal cholinergic function after exposure to ChE inhibiting compounds.

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