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NOVEL OSCILLATORY MECHANISMS IN THE CHOLINERGIC CONTROL OF GUINEA PIG SINO-ATRIAL NODE DISCHARGE

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Abstract

Introduction—The role of the oscillatory after-potential V_{os} and prepotential ThV_{os} in cholinergic control of discharge was studied in sino-atrial node (SAN).

Methods and Results—A microelectrode technique was used in isolated guinea-pig SAN superfused *in vitro* in high $[K^+]_o$ to visualize V_{os} and ThV_{os} . The cholinergic agonist carbachol (CCh) decreased the amplitude and slope of V_{os} and ThV_{os} at a time when there was no increase in maximum diastolic potential. The slowing in SAN rate was due to slower and smaller ThV_{os} that missed intermittently the threshold and occurred gradually later in diastole, but not to a decrease in the intrinsic rate of ThV_{os} . Eventually, quiescence followed. Larger CCh concentrations quickly induced a hyperpolarization that altogether prevented the occurrence of oscillatory potentials. During CCh washout, ThV_{os} reappeared and consistently reinitiated discharge. Lower $[Ca^{2+}]_o$ also decreased slopes and amplitude of V_{os} and ThV_{os} , thereby slowing and stopping SAN discharge, as CCh did. Overdrive temporarily offset the negative chronotropic effects of CCh and of low $[Ca^{2+}]_o$. Cesium (a blocker of hyperpolarization-activated current I_f) did not abolish CCh inhibitory effects on oscillatory potentials.

Conclusions—The cholinergic agonist CCh: (1) slows SAN discharge by decreasing the amplitude of V_{os} and ThV_{os} , but not the rate of ThV_{os} ; (2) can cause hyperpolarization that altogether suppresses the oscillatory potentials; (3) is mimicked in its effects by low $[Ca^{2+}]_o$; (4) is antagonized by procedures that increase cellular calcium; and (5) modifies the oscillatory potentials independently of I_f .

Keywords

Sino-atrial node discharge; sino-atrial node cholinergic control; oscillatory after-potential V_{os} ; oscillatory pre-potential ThV_{os} ; calcium; sinus node; vagus; automaticity; autonomic nervous system

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INTRODUCTION

The mechanisms by which muscarinic receptor activation inhibits the discharge of the sino-atrial node (SAN) are not agreed upon.¹ Cholinergic agonists act on several voltage-dependent currents, which are purportedly involved in SAN dominant pacemaker mechanism. Thus, acetylcholine increases the muscarinic K^+ current $I_{K,ACh}$ ² and decreases the calcium current I_{CaL} .^{3, 4, 5} as well as the hyperpolarization-activated current I_f .⁶ However, on the one hand, the role of these currents on dominant pacemaker mechanism has not been established¹ and, on the other, different mechanisms may be involved in the action of different concentrations of cholinergic agonists.⁷

Recently, it has been shown that the after-potential V_{os} and the prepotential ThV_{os} are obligatory components of SAN dominant pacemaker mechanism. The presence and role of these oscillatory potentials in the SAN have been investigated by unmasking them by suitably increasing $[K^+]_o$. High $[K^+]_o$ visualizes the oscillatory potentials by decreasing their amplitude, so that they intermittently miss the threshold for the upstroke. In this fashion, the SAN dominant U-shaped diastolic depolarization becomes dissociated in its three components: DD proper, V_{os} and ThV_{os} .

V_{os} is obligatorily superimposed on the initial diastolic depolarization (DD_1). As the subsequent late DD (DD_2) enters a less negative voltage range ("oscillatory zone") ThV_{os} appear.⁸⁻¹² That high $[K^+]_o$ unmasks the oscillatory potentials by decreasing their amplitude and making them intermittently miss the threshold is shown by the fact that during recovery in Tyrode solution, the amplitude of the oscillatory potentials *increases* until they fuse again into the U-shaped DD. Furthermore, V_{os} and ThV_{os} appear also in Tyrode solution in SAN exposed to enhanced calcium load¹³ and in Purkinje fibers superfused in a suitably low $[K^+]_o$.¹⁴

Adrenergic agonists increase SAN discharge by increasing the oscillatory potentials¹² and this finding suggests that cholinergic agonists might act by having the opposite effect. Therefore, our *aim* was to test the hypothesis that cholinergic agonists decrease SAN rate also by decreasing the oscillatory potentials V_{os} and ThV_{os} . The results obtained provide evidence for a novel mechanism of cholinergic inhibition of SAN discharge, as CCh slowed SAN discharge by decreasing V_{os} and ThV_{os} and induced SAN standstill by altogether suppressing these oscillatory potentials through hyperpolarization. During the recovery from cholinergic inhibition, ThV_{os} had a unique role in reinitiating spontaneous discharge. Several results suggest that CCh decreases V_{os} and ThV_{os} by decreasing calcium load and not by decreasing I_f .

METHODS

The experiments were performed in accordance with national and institutional ethical guidelines. The protocols for the experiments were approved by the local Animal Care and Use Committee. Eighteen Hartley adult guinea pigs of either sex were euthanized with sodium pentobarbital (60 mg/kg, i.p.). Once the respiration had stopped, the heart was rapidly excised and placed in a Petri dish filled with oxygenated Tyrode solution.

The sino-atrial node was separated from the surrounding red-brown atrial tissue. The SAN was superfused in a narrow chamber at 37°C with oxygenated (95% O_2 and 5% CO_2) Tyrode solution of the following composition (mM): NaCl 129, KCl 4, $CaCl_2$ 2.7, $NaHCO_3$ 20, NaH_2PO_4 0.45, $MgCl_2$ 1.05 and glucose 5.5. Membrane potentials were recorded by means of glass microelectrodes filled with 3 M KCl and coupled to a Dagan probe and to a Dagan model 8500 operational amplifier. Contractile force was recorded by means of a force transducer (Grass Model FTO3C) connected to a Grass Model 7D Polygraph. The

traces were displayed on a Tektronix model 5111 storage oscilloscope and recorded on paper on a 3-channel chart recorder (Gould Brush 2400).

To analyze the characteristics of V_{os} (also referred to in the literature as oscillatory after-potential or delayed after-depolarization) and of ThV_{os} (the oscillatory potential leading to the threshold for the upstroke¹⁴), $[K^+]_o$ was increased by mixing the Tyrode solution with a solution containing 40 mM KCl, but otherwise identical with Tyrode solution and gassed with the same mixture. The flow of Tyrode solution was kept constant (Gilson pump, model PP-4-A) at 1.71 ml/min and the flow of the K^+ -rich solution was changed by means of a peristaltic pump (LKB, 12000 Varioperpex) between 0 and 1.54 ml/min in different tests. The two solutions mixed before entering the thermostatic bath. The $[K^+]_o$ of the final admixture was calculated by means of equation (1):

$$K = \frac{F_T \cdot K_T + F_K \cdot K_K}{F_T + F_K} \quad (1)$$

where K is $[K^+]$ in the bath, F_T is the Tyrode flow, K_T is $[K^+]$ in Tyrode (4 mM), F_K is the flow of the K^+ -rich solution and K_K is $[K^+]$ in the K^+ -rich solution (40 mM). By suitably mixing the two solutions, $[K^+]_o$ in the bath solution could be chosen to a mM fraction to obtain the desired effect on SAN (e.g., dominant APs). The effects induced by high $[K^+]_o$ on SAN reached the desired new steady-state in less than 5 minutes.

The isolation of the guinea-pig SAN abolished the electrotonic interference from atrial muscle, since the SAN is made of sinus cells throughout its thickness.¹⁵ Exposure to suitably high $[K^+]_o$ made the SAN electrically more homogeneous, since the APs assumed a dominant configuration (smooth transition from DD into a slow upstroke and smaller APs). This allowed the study of CCh on dominant pacemaker potentials and the separation of U-shaped DD into its components V_{os} , ThV_{os} and DD. That the cells studied in high $[K^+]_o$ were pacemaker cells is shown by the persistence of their DD during recovery in Tyrode solution.

The abbreviations V_{os} and ThV_{os} are used for both singular and plural. The presence and amplitude of V_{os} was identified by its peak, which decayed back to the early diastolic depolarization DD_1 (see reference¹⁶). The late diastolic depolarization DD_2 entered a less negative potential range ("oscillatory zone") and led to the appearance of ThV_{os} . The maximum diastolic potential (MDP) and the resting potential are said to decrease when becoming less negative. When the SAN became quiescent during a procedure, the characteristics of AP, V_{os} and ThV_{os} were measured just before stoppage and ThV_{os} also during the "diamond pattern".

Carbamylcholine chloride (carbachol) and cesium chloride (Sigma) were added to the Tyrode solution and the final concentration in the bath was calculated by taking into account the flow of high $[K^+]_o$ solution. The results of the tests performed (n) are expressed as means $\pm$ standard error of the mean (SEM). Student's paired t-test was used and a P value <0.05 was considered significant (indicated in text and figures by an asterisk, *).

RESULTS

Cholinergic inhibition of diastolic oscillations

In Fig. 1A, in high $[K^+]_o$ the SAN was spontaneously active at 97 beats min^{-1} (panel *a*). The peak of V_{os} (indicated by the short bar) continued into the less steep slope of ThV_{os} , which is emphasized by the dashed line. The administration of CCh caused every other ThV_{os} to

miss the threshold (panel *b*), thereby slowing the rate of discharge. In panel *d*, the amplitude of V_{os} (short bar) and the slope of ThV_{os} (dashed line) were decreased.

In panel *e*, V_{os} was smaller yet and the following ThV_{os} (star) missed the threshold for the upstroke. Only the subsequent ThV_{os} initiated an AP. The black shaded area depicts the shape of V_{os} , which peaks and decays back to the underlying early diastolic depolarization (DD_1).^{10, 16} The gray shaded area outlines ThV_{os} that oscillates above and below DD (see below). In panels *c*, *d* and *e*, APs and MDP were hardly affected by CCh.

In Fig. 1*B*, panel *f*, the amplitude of V_{os} (short bars) and of ThV_{os} (vertical arrows) gradually decreased. During diastole, gradually more and larger ThV_{os} were needed to reach the threshold, resulting in a further gradual decrease in rate. In panel *g*, the increasing ThV_{os} (shaded area) initiated the last AP, which was followed by a small V_{os} and by gradually increasing ThV_{os} that reached a peak (without attaining the threshold) and then decayed into quiescence (“diamond pattern”).

In inset 1, the superimposed traces show that slowing of discharge was indeed due to simultaneous gradual decrease in the amplitude of V_{os} (downward arrow) and of ThV_{os} (rightward oblique arrow). During the recovery from CCh, V_{os} and ThV_{os} re-increased toward the control patterns (panel *h*).

As shown in inset 2, in $n = 24$, CCh decreased the rate, the depolarizing slope and magnitude of V_{os} , the depolarizing slope of ThV_{os} , the amplitude of diastolic depolarization and force, while having no effect amplitude and duration of the action potential. Thus, the slowing of discharge by CCh was due mostly to decrease in amplitude and slope of the oscillatory potentials.

The events responsible for the resumption of discharge during recovery from cholinergic inhibition were also studied. In Fig. 2*b*, CCh gradually slowed and then stopped SAN discharge by decreasing the oscillatory potentials and leading to the diamond pattern (star). In panel *c*, during recovery from CCh, the decay of hyperpolarization led to the re-appearance of ThV_{os} (first rightward oblique arrow) that initiated an AP. The AP suppressed ThV_{os} , since the membrane undershot the oscillatory zone by hyperpolarizing to MDP. Only when DD_2 again entered the oscillatory zone did ThV_{os} re-appear (second rightward oblique arrow) and elicit the subsequent AP. During recovery, V_{os} progressively re-increased (panel *d*) toward control. Recovery of discharge was consistently initiated by the re-appearance of ThV_{os} .

If the inhibitory effect of CCh is associated with decrease in $[Ca^{2+}]_i$, a period of fast drive (by re-increasing cellular calcium) may temporarily restore ThV_{os} and discharge. This is shown in inset 2 where SAN, quiescent in the presence of CCh, was driven at 60/min for 30 s. At the end of drive, ThV_{os} appeared during the late DD and elicited three APs followed by V_{os} and diamond pattern. Similar results were obtained in five tests, two of which in the presence of CCh and cesium.

Initial slowing versus late hyperpolarization by CCh

CCh could cause a late hyperpolarization (Fig. 2), which presumably was related to increasing concentration of CCh at the cell membrane as perfusion continued. If so, then increasing CCh concentrations would be expected to induce earlier and larger hyperpolarizations.

In Fig. 3*A*, the lowest concentration of CCh (39.7 nM) slowed the rate by decreasing ThV_{os} amplitude, so that during diastole ThV_{os} missed the threshold (cf. traces *a* and *b*,

superimposed in inset 1) and the subsequent ThV_{os} took longer to grow enough and initiate an AP (trace *b*). Eventually, ThV_{os} disappeared and quiescence followed with a small increase (< 2 mV) in resting potential. The two parallel gray lines above the lettering *a* and *b* show that CCh decreased DD amplitude, but it did not make the MDP more negative. During the recovery, the decay of hyperpolarization led to the reappearance of ThV_{os} and resumption of discharge (panel *c*). As usual, ThV_{os} appeared gradually sooner during a larger DD, thereby increasing the rate of discharge (cf. superimposed traces *c* and *d* in inset 2).

In Fig. 3*B*, doubling the concentration of CCh (79.5 nM) caused ThV_{os} to miss the threshold, as shown by the superimposed traces *f* and *g* in inset 3. CCh rapidly suppressed discharge as the diastolic potential became more negative, as emphasized by the dashed line in panel *h* and by the superimposed traces in inset 4. The hyperpolarization shifted the membrane potential negative to the oscillatory zone, as indicated by the small oscillations of the diamond pattern (filled star). The usual events (subsiding of hyperpolarization, reappearance of ThV_{os} , initiation of discharge, gradually larger DD and earlier ThV_{os}) characterized the recovery.

In Fig. 3*C*, the highest concentration of CCh (159 nM) slowed the rate of discharge by preventing ThV_{os} from attaining the threshold, as shown by traces *j* and *k*, superimposed in inset 5. ThV_{os} rapidly decreased in size and increased less during DD₂ as the diastolic potential shifted in a negative direction. V_{os} amplitude also decreased. ThV_{os} during the diamond pattern (empty star) were so small as to be barely visible. The MDP of the last AP increased and was followed by even a greater increase of diastolic potential. The recovery entailed the subsiding of hyperpolarization, until small ThV_{os} , labeled by rightward oblique arrows within and without inset 6, again initiated discharge.

DD slope was markedly decreased by the two larger concentrations, as emphasized in insets 4 and 5 and by the superimposition of control traces *e* and *i* on the recovery traces at the end of panels *B* and *C*, respectively. The lack of an inflection after exposure to the largest CCh concentration indicates the abolition of V_{os} and the unmasking of a reduced DD₁ (cf. oblique dashed lines at the end of panel *C*).

Therefore, all CCh concentrations decreased the rate by decreasing ThV_{os} (as shown in the insets 1, 3 and 5) and V_{os} (as shown by the short bars). However, higher concentrations shifted the diastolic potential negative to the oscillatory zone, thereby quickly abolishing the oscillatory potentials and inducing SAN standstill.

In the presence of CCh, ThV_{os} first missed the threshold at a time when no increase in MDP had occurred, but the size of DD had decreased (-24% , Table 1). In these same experiments, CCh induced its usual effects (Table 1). In $n = 3$ (CCh 371 ± 182 nM), ThV_{os} missed the threshold when the MDP had increased by 1.73 ± 0.54 mV.

In $n = 7$ (12.3 ± 0.6 mM $[\text{K}^+]_{\text{o}}$), in the presence of CCh (53.8 ± 7.6 nM) when SAN stopped, there was no measurable hyperpolarization of the resting potential. In $n = 11$ (13.6 ± 0.8 mM $[\text{K}^+]_{\text{o}}$, CCh 160.9 ± 65 nM), after the complete cessation of discharge, the resting potential increased by 5.28 ± 0.83 mV*. During the recovery, the hyperpolarization subsided and ThV_{os} consistently re-initiated a gradually faster discharge. In Tyrode solution, CCh decreased the rate, the AP amplitude and duration, the amplitude and slope of DD and force (Table 2).

Cholinergic inhibition of discharge and the rate of ThV_{os}

To verify whether the slowing of SAN also involves a slowing of the *rate* of ThV_{os}, this parameter was measured prior to the last AP as well as during the diamond pattern in the presence of CCh. The same measurements were carried out for other experiments in high [K⁺]_o alone, since suitably high [K⁺]_o leads to quiescence also by decreasing ThV_{os} and V_{os}.

In Table 3, CCh decreased AP rate to 21 min⁻¹ just before stoppage whereas the rate of ThV_{os} prior to the last AP was 73.7* min⁻¹ (250% faster than the AP rate). During the diamond patterns, ThV_{os} amplitude and rate were slightly greater than those prior to the last AP (+6% and +5.5%*, respectively). ThV_{os} depolarizing slope was less steep than the repolarizing slope (*, ratio 0.43)

In Table 3, high [K⁺]_o alone decreased the SAN rate to 19.6 min⁻¹ just before stoppage whereas the rate of ThV_{os} prior to the last AP was 63.5 min⁻¹* (223% faster than AP rate). During the diamond patterns, ThV_{os} amplitude and rate were greater than those prior to the last AP. ThV_{os} depolarizing slope was less steep than the repolarizing slope (*, ratio 0.41). There were no statistical differences between the data in CCh and those in high [K⁺]_o alone as compared with a non-paired t-test.

Thus, both in CCh and in high [K⁺]_o alone, SAN rate just prior to arrest was much smaller than the rate of ThV_{os} either immediately before the last AP or during the diamond patterns. Also, ThV_{os} amplitude and the ratio between its depolarizing and repolarizing slopes were similar in these two solutions.

Effects of low [Ca²⁺]_o on oscillatory potentials and SAN rate

If CCh slows the SAN by decreasing Ca²⁺ entry, then low [Ca²⁺]_o could slow the SAN through electrophysiological changes similar to those of CCh.

In Fig. 4*a*, on exposure to low [Ca²⁺]_o, the threshold was intermittently missed and ThV_{os} were unmasked (panel *b*). As ThV_{os} continued to decrease in size (dots in panel *c*), during diastole ThV_{os} took longer to increase sufficiently in size to attain the threshold. At the same time, V_{os} size also decreased (short bars) and the rate of discharge decreased gradually. The superimposition of control trace (star) on those labeled with an asterisk (panel *b*) and by square (last AP in panel *c* recorded at a greater time base) emphasizes the gradual decline in amplitude of both V_{os} and ThV_{os}. Quiescence followed shortly thereafter. As in the case of CCh, low [Ca²⁺]_o decreased SAN discharge by decreasing the oscillatory potentials and not by decreasing the rate of ThV_{os}. During the recovery in normal [Ca²⁺]_o (panel *d*), ThV_{os} reappeared and became larger (dots) until the control pattern were restored (cf. panel *e* with control panel *a*). Driving the SAN quiescent in low [Ca²⁺]_o at 120 for 60 seconds (and thereby increasing [Ca²⁺]_i) temporarily restored spontaneous discharge (not shown).

In Fig. 4, inset *I*, (*n* = 12), low [Ca²⁺]_o decreased the rate, the depolarizing slope and magnitude of V_{os}, the depolarizing (as well as the repolarizing) slope of ThV_{os}, the amplitude of ThV_{os} as well as of DD and force, as CCh did. However, low [Ca²⁺]_o decreased also the amplitude of the AP. In two additional tests, ThV_{os} decreased as usual, but during diastole they re-increased markedly: eventually, the threshold was missed and quiescence followed. In no instance, did low [Ca²⁺]_o decrease the rate of ThV_{os}.

In *n* = 5, overdrive applied during quiescence in low [Ca²⁺]_o caused a transient appearance of oscillatory potentials, spontaneous APs and/or the diamond pattern. In one experiment in Tyrode solution, in a SAN stopped by CCh, 120 min⁻¹ 10 s overdrive in 6.7 mM [Ca²⁺]_o was followed by a steady spontaneous discharge at 48 min⁻¹.

Cesium block of I_f and SAN slowing induced by carbamylcholine

If CCh decreased V_{os} and ThV_{os} by decreasing I_f , CCh should fail to affect the oscillatory potentials and rate of discharge in the presence of Cs^+ -induced block of I_f .

In Fig. 5A, panel *a*, the SAN was active in the presence of Cs^+ . When CCh was added, the amplitude of V_{os} (short bars) and the depolarizing slope of ThV_{os} (dashed lines) gradually decreased. In Fig. 5B, ThV_{os} missed the threshold more often, until the diamond pattern appeared, followed by quiescence. The trace above the gray line is amplified in inset 1. Inset 2 shows the progressive decrease in amplitude of V_{os} (from *a* to *e*, vertical downward arrow) and ThV_{os} (oblique rightward arrow). The progressively less steep slope of ThV_{os} is emphasized by the superimposed traces labeled by squares within and without inset 2. Also in the presence of Cs^+ , CCh slowed the rate of APs, but not that of ThV_{os} .

In Fig. 5C, during the recovery in Cs^+ solution (no CCh), the resting potential decreased slightly (see horizontal dashed line) and ThV_{os} appeared (rightward oblique arrows) that reinitiated spontaneous discharge. In panel *g*, the patterns seen prior to the exposure to CCh reappeared (cf. panel *a*). Thus, CCh decreased oscillatory potentials and rate in the same way whether or not I_f was blocked by Cs^+ . In $n = 4$, in two tests CCh was administered first and Cs^+ was then added, and in two other tests Cs^+ was administered first and CCh was added. Cs^+ had the usual effect in the presence of CCh, and CCh had the usual effect in the presence of Cs^+ .

DISCUSSION

We conclude from these results that the inhibitory action of CCh on SAN dominant pacemakers involves two mechanisms. The *first inhibitory mechanism* was brought about by smaller concentrations or during the early phase of exposure to higher concentrations and it involved the decrease in amplitude of V_{os} , ThV_{os} and DD without changes in AP or in MDP. This mechanism appears to be related to a decrease in Ca^{2+} loading by CCh, since contractile force also decreased. Indeed, low concentrations of ACh decrease I_{CaL3-5} and contractile force.³ Furthermore, in SAN arrested by CCh, overdrive or high $[Ca^{2+}]_o$ could temporarily reinstate V_{os} and ThV_{os} . Conversely, low $[Ca^{2+}]_o$ inhibited SAN discharge as CCh did, namely, by reducing V_{os} and ThV_{os} slope and amplitude and not by decreasing the rate of ThV_{os} .

The *second inhibitory mechanism* was brought about by longer exposures or larger concentrations of CCh, which (through hyperpolarization) shifted the diastolic potential negative to the oscillatory zone. This hyperpolarization is consistent with the increase in K^+ conductance by cholinergic agonists.^{17,18} This mechanism appears to involve a stronger inhibition, since the hyperpolarization altogether prevented ThV_{os} by shifting the membrane potential negative to the oscillatory zone and thereby quickly leading to SAN arrest.

During recovery from CCh, ThV_{os} played an exclusive role in the resumption of SAN activity and in slow spontaneous discharge, since it bridged the voltage gap between DD and threshold for the upstroke. The recovery of V_{os} contributed to reincrease in rate by speeding up the attainment of the oscillatory zone. The hyperpolarization-activated current I_f had no role in the oscillatory potentials and in their regulation by cholinergic agonists in dominant pacemakers, since the I_f -blocker Cs^+ did not modify the effects of CCh on the oscillatory potentials and on SAN discharge.

The reduction of V_{os} , ThV_{os} and force preceded the hyperpolarization not only when CCh increases at the cell membrane as a function of perfusion time, but also with higher concentrations of CCh.

Role of calcium in the negative chronotropic effect of cholinergic agonists

In SAN dominant DD, the pacemaker mechanism includes the pacemaker current I_K , $20 V_{os}$ and ThV_{os} .^{10, 12, 19} Our results show that the negative chronotropic effect of CCh involves all those three components in a way that is mimicked by a low $[Ca^{2+}]_o$. The question then becomes how cholinergic activation diminishes V_{os} , ThV_{os} and DD.

As far as Ca^{2+} is concerned, even small concentrations of cholinergic agonists reduce I_{CaL} , $3\text{--}5$ the calcium transient²¹ and contractile force.²² In fact, a low concentration of ACh decreased I_{CaL} , but did not shift the holding current and nor made the MDP more negative, indicating that it did not increase K^+ conductance. In addition, there was no I_f in the SAN dominant pacemaker range (-40 to -65 mV), but ACh also decreased I_f at more negative potentials.⁵ At concentrations in the range $0.05\text{--}10$ μM , ACh decreased the basal I_{CaL} by decreasing the high basal levels of adenylate cyclase activity, of cAMP and of cAMP-dependent protein kinase activity with a consequent dephosphorylation of calcium channels.⁴

The decreased calcium load decreases the amplitude and slope of both V_{os} and ThV_{os} , since these oscillatory potential depend on cellular calcium.^{8–12} Indeed, the decrease in amplitude and slope of V_{os} and ThV_{os} by CCh was mimicked by lowering $[Ca^{2+}]_o$. Furthermore, the inhibitory action of CCh was antagonized by an increase in calcium load by means of overdrive. High $[Ca^{2+}]_o$ fostered SAN discharge by enhancing the oscillatory potentials^{9,10} and here we show that it opposed the inhibitory action of CCh. Also, adrenergic agonists increase SAN discharge by increasing V_{os} , ThV_{os} and DD,¹² consistent with the fact that they increase calcium current and contractile force. As for the Ca^{2+} channel involved in ThV_{os} , $Ca_v1.3$ (α_{1d}) L-type channels may be responsible for the depolarizing slope of ThV_{os} , as they activate at more negative potentials than the $Ca_v1.2$ (α_{1c}) L-type channels.^{23, 24} The latter are responsible for the upstroke of the AP (see1).

As for the *mechanisms underlying the oscillatory potentials*, V_{os} is related to diastolic release of calcium by a calcium-overloaded sarcoplasmic reticulum (SR), consistent with its suppression by ryanodine¹⁰ as well as with its association with an aftercontraction (see19) and with $[Ca^{2+}]_i$ elevation in late diastole.⁷ Diastolic Ca^{2+} release stimulates an extrusion of calcium by the Na^+-Ca^{2+} exchange (see19). Since at diastolic potentials Ca^{2+} extrusion by the Na^+-Ca^{2+} exchange creates an inward current, its enhancement causes a depolarizing event (V_{os}) superimposed on DD_1 . Several other lines of evidence link V_{os} to a Ca^{2+} overloaded SR and to a diastolic Ca^{2+} extrusion by the Na^+-Ca^{2+} exchange (see19).

As for ThV_{os} , its depolarizing phase could be caused by calcium entry, as suggested by the activation of an inward calcium component toward the end of diastolic depolarization.⁷ The depolarizing phase of ThV_{os} would be expected to activate I_K , thereby causing the ensuing hyperpolarization. This under-swing (by increasing calcium channel availability and deactivating I_K) would increase the size of the subsequent depolarization phase of ThV_{os} , which, in turn, would cause a larger hyperpolarization. Indeed, in voltage-clamped single SAN cells, small hyperpolarizing pulses were followed by an inward current component when applied at -40 mV but not at -80 mV (see Fig. 6 in25). Furthermore, in single SAN cell, a small (3 mV) depolarizing voltage-clamp step from -40 mV caused an inward transient, which was followed by a decaying tail (H. Zhang and M. Vassalle, unpublished experiments).

Therefore, a decrease in I_{CaL} by CCh would have direct and indirect effects on the oscillatory potentials. Decreased calcium entry reduces the calcium overload of the SR and therefore V_{os} . Indeed, I_{os} (the current underlying V_{os}) was markedly decreased by low $[Ca^{2+}]_o$ or impairment of SR function by caffeine.²⁶ As for ThV_{os} , a decrease in calcium

entry through $\text{Ca}_v1.3$ channels could directly decrease their magnitude and slope. In turn, the smaller depolarizing phase of ThV_{os} would decrease the activation of I_K . The decrease in amplitude of V_{os} and ThV_{os} by CCh would also decrease the amplitude of DD upon which V_{os} and ThV_{os} are superimposed.

The maintained rate of ThV_{os} rate prior to and during the diamond patterns indicates that CCh did not change the kinetics of the currents involved in ThV_{os} ; indeed, ACh reduces the calcium current without affecting its kinetics.²⁷ Therefore, ThV_{os} seems to act as link between DD and the threshold for the AP upstroke, and not primarily by changing SAN discharge through changes in its rate (as a “clock” would). This is also shown by the temporary suppression of ThV_{os} by the AP through its hyperpolarization to the MDP. Yet, when DD enters the oscillatory zone, it is the restoration of ThV_{os} that leads to the attainment of the threshold for the upstroke. Changes in SAN rate are determined by changes in slope and magnitude of DD, V_{os} and ThV_{os} , whether or not fused in the U-shaped DD.

Role of increased K^+ conductance in the negative chronotropic effect of cholinergic agonists

With higher concentrations of CCh, the more negative MDP and the hyperpolarization of diastolic potential are consistent with the fact that suitable concentrations of cholinergic agonists increase K^+ conductance,²⁸ radioactive K^+ fluxes¹⁷ and I_{KACH} .¹⁸ This inhibitory mechanism abolished ThV_{os} altogether (rather than just decrease their amplitude) by shifting the membrane potential negative to the oscillatory zone. The standstill caused by the suppression of ThV_{os} also abolished V_{os} , since there were no APs (V_{os} obligatorily follows the action potential; present results and⁹⁻¹²).

Negative shift of the hyperpolarization-activated current I_f

ACh shifted the activation curve of hyperpolarization-activated I_f in a negative direction by 9.9 mV when measured at -100 mV.⁷ However, -100 mV is negative not only to the SAN dominant, but even to the subsidiary pacemaker range. More importantly, cholinergic activation decreased the amplitude and slope of V_{os} and ThV_{os} independently of I_f , since CCh had the same negative chronotropic effects after the block of I_f by Cs^+ (cf. inset 2 in Fig. 5 with inset 1 in Fig. 1). This is consistent with the finding that I_f blockade by Cs^+ did not affect the negative chronotropic effect of any concentration of CCh.²⁹ Furthermore, our findings are in agreement with the fact that lower CCh concentrations decreased the Ca^{2+} current and higher concentrations also increased I_{KACH} .²⁹

It is generally agreed that I_f is not activated at -40 mV, whereas ThV_{os} occur at that resting potential.¹⁰ Indeed, I_f begins to slowly activate negative to -60 mV (e.g.,²⁵⁻²⁹), namely, negative to the dominant pacemaker range. Instead, as shown by the changes in subsidiary APs in Tyrode solution, the negative shift of I_f could decrease SAN subsidiary DD, thereby hindering the emergence of subsidiary pacemakers when the dominant ones are inhibited by cholinergic agonists.

Limitations of the approach used

In the present experiments, we used high $[\text{K}^+]_o$ and this raises the question as the relation of the findings obtained in high $[\text{K}^+]_o$ to the events in Tyrode solution. If high $[\text{K}^+]_o$ induced the oscillatory potentials, their relevance to cholinergic control of SAN in Tyrode solution would be rather uncertain. Instead, high $[\text{K}^+]_o$ decreases the oscillatory potentials and this implies their presence in Tyrode solution, albeit fused in the U-shaped DD. By decreasing them, high $[\text{K}^+]_o$ prevents the oscillatory potentials from attaining the threshold for the upstroke and therefore unmask them. In fact (once unmasked), a further gradual increase in

$[K^+]_o$ gradually decreases the magnitude and slope of the oscillatory potentials.¹⁰ As it would be expected, the reverse occurs during recovery in Tyrode solution: the magnitude and slope of the oscillatory potentials *increased* until they fused in the U-shaped DD.^{10, 12}

Similarly, if Purkinje fibers are quiescent in Tyrode solution, *lowering* $[K^+]_o$ makes both V_{os} and ThV_{os} appear and initiate spontaneous discharge.¹⁴ Furthermore, in Tyrode solution the oscillatory potentials appear under suitable conditions in both SAN and Purkinje fibers (e.g., 13, 14, 16, 19, 26, 30). Indeed, it was the separation of the components of the pacemaker mechanism by high $[K^+]_o$ that made it possible to determine that the inhibitory mechanisms of dominant pacemaker discharge by cholinergic activation include the decrease or suppression of V_{os} and ThV_{os} .

Still, since both carbachol and high $[K^+]_o$ decrease the oscillatory potentials, cholinergic agents might unmask the oscillatory potentials in Tyrode solution. This would require that in *dominant* pacemakers, the concentrations of cholinergic agonists, on the one hand, should not merely decrease the slope of U-shaped DD and, on the other, should not hyperpolarize the diastolic potential negative to the oscillatory zone (thereby suppressing ThV_{os}). Indeed (in agreement with our findings with ThV_{os}), the hyperpolarization caused by stimulation of vagal fibers in small pieces of SAN quiescent in Tyrode solution at ~ -40 mV was followed by oscillatory potentials that could reach the threshold and initiate dominant pacemaker discharge.³¹

CONCLUSIONS

The present results provide evidence for novel mechanisms of cholinergic inhibition of SAN discharge. *In vivo*, with *graded* vagal activation, SAN bradycardia may be due to the decrease in oscillatory potentials, whereas with *strong* vagal activation the sudden SAN arrest may result from the hyperpolarizing mechanism. While V_{os} steepens DD, ThV_{os} acts as a calcium dependent link between DD and the threshold in the initiation, maintenance and autonomic control of dominant pacemakers. Although the oscillatory potentials are similar in SAN and Purkinje fibers, ThV_{os} may have different ionic substrates.^{32, 33}

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REFERENCES

1. Mangoni ME, Nargeot J. Genesis and regulation of the heart automaticity. *Physiol Rev.* 2008; 88:919–982. [PubMed: 18626064]
2. Sakmann B, Noma A, Trautwein W. Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. *Nature.* 1983; 303:250–253. [PubMed: 6302520]
3. Giles WR, Noble SJ. Changes in membrane currents in bullfrog atrium produced by acetylcholine. *J Physiol (London).* 1976; 61:103–123. [PubMed: 1086900]
4. Petit-Jacques J, Bois P, Bescond J, Lenfant J. Mechanism of muscarinic control of the high-threshold calcium current in rabbit sino-atrial node myocytes. *Pflügers Arch.* 1993; 423:21–27.
5. Brown HF, Denyer JC. Low dose of acetylcholine reduces calcium current in isolated sino-atrial node cell of rabbit. (Abs) *J Physiol.* 1989; 410:65.
6. DiFrancesco D. The onset and autonomic regulation of cardiac pacemaker activity: relevance of the f current. *Cardiovasc Res.* 1995; 29:449–456. [PubMed: 7540954]
7. Zaza A, Robinson RB, DiFrancesco D. Basal responses of the L-type Ca^{2+} and hyperpolarization-activated currents to autonomic agonists in the rabbit sino-atrial node. *J Physiol (London).* 1996; 491:347–355. [PubMed: 8866859]

8. Kim EM, Choy Y, Vassalle M. Mechanisms of suppression and initiation of pacemaker activity in guinea-pig sino-atrial node superfused in high $[K^+]_o$. *J Mol Cell Cardiol.* 1997; 29:1433–1445. [PubMed: 9201628]
9. Choy Y, Kim EM, Vassalle M. Overdrive excitation in the guinea pig sino-atrial node superfused in high $[K^+]_o$. *J Biomed Sci.* 1997; 4:179–191. [PubMed: 11725151]
10. Nett MP, Vassalle M. Obligatory role of diastolic voltage oscillations in sino-atrial node discharge. *J Mol Cell Cardiol.* 2003; 35:1257–1276. [PubMed: 14519436]
11. Catanzaro JN, Nett MP, Rota M, Vassalle M. On the mechanisms underlying diastolic voltage oscillations in the sino-atrial node. *J Electrocardiol.* 2006; 39 e1-342-e14.
12. Vassalle M, Catanzaro JN, Nett MP, Rota M. Essential role of diastolic oscillatory potentials in adrenergic control of guinea pig sino-atrial node discharge. *J. Biomed Sci.* 2009; 16:101. [PubMed: 19922640]
13. Gonzalez MD, Vassalle M. Role of oscillatory potential and pacemaker shifts in digitalis intoxication of the sino-atrial node. *Circulation.* 1993; 87:1705–1714. [PubMed: 8387899]
14. Spiegler P, Vassalle M. Role of voltage oscillations in the automaticity of sheep cardiac Purkinje fibers. *Can J Physiol Pharmacol.* 1995; 73:1165–1180. [PubMed: 8564885]
15. Anderson RH. The disposition, morphology and innervation of the cardiac specialized tissue in the guinea-pig. *J Anat.* 1972; 111:453–468. [PubMed: 4560935]
16. Vassalle M; Musso, E. On the mechanisms underlying digitalis toxicity in cardiac Purkinje fibers. In: Roy, P-E.; Dhalla, NS., editors. *Recent Advances in Studies on Cardiac Structure and Metabolism.* Vol. 9. University Park Press; Baltimore: 1976. p. 355-376. The Sarcolemma
17. Lipsius SL, Vassalle M. Effects of acetylcholine on potassium movements in the guinea pig sinus node. *J Pharmacol Exptl Therap.* 1977; 201:669–677. [PubMed: 864603]
18. Sakmann B, Noma A, Trautwein W. Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. *Nature.* 1983; 303:250–253. [PubMed: 6302520]
19. Vassalle M, Lin C-I. Calcium overload and cardiac function. *J Biomed Sci.* 2004; 11:542–565. [PubMed: 15316129]
20. Brown HF, Giles WR, Noble SJ. Membrane currents underlying activity in frog sinus venosus. *J Physiol.* 1977; 271:783–816. [PubMed: 303699]
21. van Borren MM, Verkerk AO, Wilders R, Hajji N, Zegers JG, Bourier J, Tan HL, Verheijck EE, Peters SL, Alewijnse AE, Ravesloot JH. Effects of muscarinic receptor stimulation on Ca^{2+} transient, cAMP production and pacemaker frequency of rabbit sinoatrial node cells. *Basic Res Cardiol.* 2010; 105:73–87. [PubMed: 19639379]
22. Vassalle M, Zhang H. On the mechanisms of cholinergic control of the sino-atrial node discharge. *J Cardiovasc Pharmacol.* 2001; 37:173–186. [PubMed: 11210000]
23. Zhang Z, Xu Y, Song H, Rodriguez J, Tuteja D, Namkung Y, Shin HS, Chiamvimonvat N. Functional Roles of $Ca_v1.3$ (α_1D) calcium channel in sinoatrial nodes: insight gained using gene-targeted null mutant mice. *Circ Res.* 2002; 90:981–987. [PubMed: 12016264]
24. Mangoni ME, Couette B, Bourinet E, Platzer J, Reimer D, Striessnig J, Nargeot J. Functional role of L-type $Ca_v1.3$ Ca^{2+} channels in cardiac pacemaker activity. *Proc Natl Acad Sci U S A.* 2003; 100:5543–5548. [PubMed: 12700358]
25. Zhang H, Vassalle M. Role of I_K and I_f in the pacemaker mechanisms of sino-atrial node myocytes. *Canadian J Physiol Pharmacol.* 2001; 79:963–976.
26. Vassalle M, Di Gennaro M. Caffeine actions on currents induced by calcium overload in Purkinje fibers. *Europ J Pharmacol.* 1984; 106:121–131.
27. Ikemoto Y, Goto M. Effects of ACh on slow inward current and tension components of the bullfrog atrium. *J Mol Cell Cardiol.* 1977; 9:313–323. [PubMed: 301193]
28. Hutter OF, Trautwein W. Vagal and sympathetic effects on the pacemaker fibres in the sinus venosus of the heart. *J Gen Physiol.* 1956; 39:715–733. [PubMed: 13319658]
29. Lyashkov AE, Vinogradova TM, Zahanich I, Li Y, Younes A, Nuss HB, Spurgeon HA, Maltsev VA, Lakatta EG. Cholinergic receptor signaling modulates spontaneous firing of sinoatrial nodal cells via integrated effects on PKA-dependent Ca^{2+} cycling and I_{KACH} . *Am J Physiol Heart Circ Physiol.* 2009; 297:H949–H959. [PubMed: 19542482]

30. Irisawa, H.; Noma, A. Miniature fluctuations of the membrane potential in the rabbit sinoatrial node cell; Proc Intern 27th Congr Physiol; Sci, Paris: 1977. p. 345
31. Goto J, Toyama J, Yamada K. Effects of timing of vagal stimulation on the sinoatrial nodal cell discharge. J Electrocardiol. 1983; 16:45–52. [PubMed: 6833923]
32. Rota M, Vassalle M. Patch-clamp analysis in canine cardiac Purkinje cells of a novel sodium component in the pacemaker range. J Physiol (London). 2003; 548:147–165. [PubMed: 12588904]
33. Vassalle M. The vicissitudes of the pacemaker current I_{Kdd} of cardiac Purkinje fibers. J Biomed Sci. 2007; 14:699–716. [PubMed: 17564816]

Fig.1.

CCh decreases SAN discharge through smaller V_{os} and ThV_{os} . SAN was superfused in 11.9 mM $[K^+]_o$. CCh was administered between the two downward arrows. In panel *e*, the star identifies the ThV_{os} that missed the threshold, the black area emphasizes V_{os} and the gray area ThV_{os} . In panel *f*, the arrows point to the decreasing ThV_{os} . In panel *g*, the shaded area emphasizes the growth of ThV_{os} toward the threshold for the upstroke. In inset *I*, the vertical downward arrow points to the CCh-induced decrease of V_{os} and the rightward oblique arrow the decrease in ThV_{os} . The slope of the depolarizing phase of ThV_{os} is emphasized by dashed lines and the peak of V_{os} by short horizontal bars. The graph illustrates mean data and standard error in control (13.2 ± 0.49 mM $[K^+]_o$, empty columns) and in the presence of 118.8 ± 37.2 CCh (filled columns) of the various parameters indicated in the abscissa: rate in min^{-1} , action potential amplitude in mV (APa), action potential duration in ms (APd), depolarizing slope of V_{os} (V_{osds}) and of ThV_{os} (ThV_{osds}) in mV s^{-1} , amplitude of V_{os} in mV (V_{osa}), diastolic depolarization amplitude in mV (DDa), and force in mg. The percent changes induced by CCh are indicated below the respective parameter. Asterisks (*) indicate statistical difference with respect to control ($P < 0.05$).

Fig. 2.

Recovery of discharge from CCh-induced inhibition. The control is shown in panel *a*. CCh was administered between the downward arrows. In panel *b*, the star labels the diamond pattern and in panel *c*, the arrows point to ThV_{os} that re-initiated discharge. In insert 1, the labels identify V_{os}, DD₁, DD₂, ThV_{os} and the MDP. In inset 2, the SAN, quiescent in CCh, was driven at 60 min⁻¹ for 30 s between the arrows.

Fig. 3.

Larger concentrations of CCh induce marked hyperpolarization. The *a* and *b* traces have been superimposed in inset 1; traces *c* and *d* in inset 2; traces *f* and *g* in inset 3; traces *e* and *h* in inset 4; and traces *f*, *k* and *i* in inset 5. In inset 6, the arrow points to ThV_{os} . In panels *B* and *C*, the dashed lines emphasize the CCh-induced hyperpolarization. In the traces at the end of panels *B* and *C*, the control traces *e* and *i*, have been superimposed on the respective CCh traces.

Fig 4.

Low $[Ca^{2+}]_o$ suppresses discharge by decreasing the oscillatory potentials. In panels *a* and *b*, star and asterisk label the ThV_{os} that have been superimposed on the trace (square) at the end of panel *c*. In panel *c*, the dots and the short bars emphasize the gradual decrease in amplitude of ThV_{os} and of V_{os} , respectively, by low $[Ca^{2+}]_o$. In panel *d*, during recovery, the dots emphasize the progressive increase of ThV_{os} . In panel *f*, the mean data in control (K^+)_o 13.5 ± 0.02 mM) and in low $[Ca^{2+}]_o$ (1.2 ± 0.07 mM) on the parameters indicated on the abscissa are shown. The percent changes induced by low $[Ca^{2+}]_o$ are shown below the respective parameter. Other explanations are as in the legend of Fig. 1.

Fig. 5.

CCh inhibits SAN discharge in the presence of Cs⁺-induced block of I_f. Cs⁺ was administered throughout and CCh between the two downward arrows. The diamond pattern labeled with a gray line is shown at greater gain in inset 1. In inset 2, traces *a*, *b*, *c*, *d* and *e* have been superimposed and the vertical downward arrow points to the decrease in V_{os} amplitude by CCh. The long rightward oblique arrow points to the decrease in ThV_{os} by CCh. The depolarizing phases of ThV_{os} labeled by squares in *a*, *c*, *d*, and *e* have also been superimposed in inset 2 (square with small rightward oblique arrow). In panel C, during the recovery from CCh in the presence of Cs⁺, the dashed line emphasizes the subsiding of a small hyperpolarization and the rightward oblique arrows point to ThV_{os} initiating APs.

Changes induced by CCh on several parameters

TABLE 1

<i>n</i> = 15	[K ⁺] _o , mM	CCh, mM	DDa, mV	AP rate, min ⁻¹	APa, mV	APd, ms	V _{osds} , mV s ⁻¹	V _{osa} , mV	ThV _{osds} , mV s ⁻¹
Control	10.2 ± 0.5		9.3 ± 0.8	86.55 ± 9.67	77 ± 1.71	261.3 ± 6.61	31.77 ± 3.02	6.01 ± 0.62	10.72 ± 2.73
CCh		82.5 ± 22.5	7.3 ± 0.7 *	15.98 ± 9.20 *	76.7 ± 1.67	254.67 ± 6.61 *	20.57 ± 2.77 *	4.69 ± 0.54 *	5.97 ± 0.88 (P=0.05)
change			-24%	-81.5%	0.03%	0.03%	-36%	-21.9%	-44.3%

n: number of tests carried out in control and in the presence of CCh. [K⁺]_o mM: K⁺ concentration in mM. CCh, mM: concentration of CCh in mM. DDa: amplitude of diastolic depolarization mV. AP rate, min⁻¹: rate of the APs per minute in control and just prior to stoppage in CCh. APa, mV: amplitude of the action potential in mV. APd, ms: duration of the action potential in ms. V_{osds}, mV s⁻¹: depolarizing slope of V_{os} in mV per second. V_{osa}, mV: amplitude of V_{os} in mV. ThV_{osds}: depolarizing slope of ThV_{os} in mV per second. Change: percent changes induced by CCh.

* The asterisk indicates a statistical difference between CCh values and respective controls.

TABLE 2

Effects of CCh on subsidiary pacemakers in Tyrode solution

Tyrode <i>n</i> = 3	CCh, nM	AP rate, min ⁻¹	APa, mV	APd, ms	DDs, mVs ⁻¹	DDa., mV	Force, mg
Control		154.3 ± 0.8	119.17 ± 3.63	226.67 ± 13.33	28.17 ± 1.17	3.20 ± 0.65	5 ± 1.53
	400 ± 300	91.3 ± 45.65	106.67 ± 9.82	176 ± 38.16	11.67 ± 5.9	2.23 ± 1.13	2.33 ± 1.20
change		-40%	-10.4%	-22.3%	-58.4%	30.3%	53.4%

Tyrode: results obtained in SAN superfused in Tyrode solution. DDs, mVs⁻¹; slope of diastolic depolarization in mV per second. Force, mg: contractile force in mg. Other explanations as in the legend of Table 1.

TABLE 3

CCh and high $[K^+]_o$ decrease SAN rate, but not ThV_{OS} rate

	$[K^+]_o$, mM	AP rate, min ⁻¹	ThV _{OS} rate, min ⁻¹	ThV _{OSa} , mV	V _{OSa} , mV	V _{OSd} , mVs ⁻¹	D- ThV _{OSa} , mV	D- ThV _{OSds} , mVs ⁻¹	D-Rate min ⁻¹
CCh <i>n</i> = 17	13.1 ± 0.6	21.0 ± 3.1	73.7 ± 3.4	3.2 ± 0.4	4.8 ± 0.5	22.2 ± 2.6	3.4 ± 0.5	7.7 ± 1.2	77.8 ± 3.2
High $[K^+]_o$ <i>n</i> = 16	14.5 ± 0.4	19.6 ± 3.7	63.5 ± 3.2	2.7 ± 0.4	5.5 ± 0.4	24.4 ± 2.2	3.9 ± 0.5	8.4 ± 1.7	68.0 ± 3.9

n: number of tests carried out in the presence of 120.0 ± 45 nM CCh (top row) and of high $[K^+]_o$ (bottom row). AP rate, min⁻¹: rate of the APs per minute just prior to stoppage. ThV_{OS} rate, min⁻¹: rate of ThV_{OS} per minute prior to the last AP. ThV_{OSa}, mV: amplitude of ThV_{OS} in mV just prior to the last AP. V_{OSa}, mV: amplitude of V_{OS} in mV just prior to stoppage. V_{OSds}, mVs⁻¹: depolarizing slope of V_{OS} in mV per second just prior to stoppage. D-ThV_{OSa}, mV: maximal amplitude of ThV_{OS} during diamond pattern. D-ThV_{OSds}, mVs⁻¹: depolarizing slope of ThV_{OS} during the diamond pattern in mV per second. D-Rate min⁻¹: rate of ThV_{OS} per minute during the diamond pattern