Mechanisms underlying overdrive suppression and overdrive excitation in guinea pig sino-atrial node

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Summary

The hypothesis that the pause that follows overdrive of the sino-atrial node (SAN) might be the net result of overdrive excitation and overdrive suppression was tested by studying rate and force patterns induced by overdrive in isolated guinea pig SAN superfused *in vitro*. In Tyrode solution, the pause is short and changes but little with longer or faster drives. In high $[K^+]_o$ solution, longer overdrives increase force percent-wise more than in Tyrode solution, shorten the pause and are followed by greater rate and force. When the SAN (quiescent in high $[K^+]_o$) is driven at 6/min, faster overdrives are followed by stronger, slowly decreasing contractions. Alternating 10 s drives with 10 s pauses have little effect on force and rate in Tyrode solution, but progressively increase force and rate in high $[K^+]_o$. Cesium has effects similar to high $[K^+]_o$. High $[Ca^{2+}]_o$ increases force and in high $[K^+]_o$ increases the rate as well as it shortens the pause, whereas Ni^{2+} decreases force as well as rate and lengthens the pause. Barium dissociates the effects on force and rate. Lidocaine and tetrodotoxin decrease rate and force, and increase the pause duration. In overdrive excitation, the increase in rate is associated with an enhancement of diastolic voltage oscillations. It is concluded that in SAN the prevalence of Ca^{2+} load leads to overdrive excitation whereas the prevalence of Na^+ load leads to overdrive suppression. In Tyrode solution, the pause after drive appears to be the *net* result of these two different mechanisms.

Abbreviations: AP – action potential; MDP – maximum diastolic potential; DD – diastolic depolarization; R – control rate (prior to drive); F – control contractile force; DF_1 – force of first driven beat; DF_2 – force of last driven beat; pause – time interval between the last driven beat and the first spontaneous beat; PDF_1 – force of the first beat after the pause; PDR_1 – rate immediately after the pause; PDR_2 – late (10–15 s) post-drive rate; PDF_2 – late (10–15 s) post-drive force (see Figure 1); SR – sarcoplasmic reticulum; g_K – K^+ conductance; a_{Na}^i – intracellular Na^+ activity; TTX – tetrodotoxin; NCX – Na^+ – Ca^{2+} exchange current

Introduction

An increase in rate of sino-atrial node (SAN) often occurs under normal and abnormal conditions. It entails an enhanced ion exchange between extra- and intra-cellular environments, since the number

of action potentials increases in the unit of time. Indeed, a faster rate increases intracellular Na^+ [1, 2] and Ca^{2+} [e.g., 3–5] in cardiac tissues, including the SAN [6]. Overdrive increases contractile force as well [e.g., 5, 7].

Overdrive of cardiac pacemakers is followed by a temporary suppression of discharge which is much shorter in the sino-atrial node [8, 9] than in Purkinje fibers [10]. The reason for this difference

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has not been determined. It might be related to the fact that morphologically and functionally the SAN is not a homogenous structure, since slow responses are recorded in dominant pacemaker cells and fast responses in subsidiary pacemaker cells [11–13]. The upstroke of dominant action potentials is caused by Ca^{2+} entry through the slow Ca^{2+} channel [11] whereas that of subsidiary pacemakers is mostly caused by Na^+ entry through the fast Na^+ channel [12]. Therefore, an increase in rate is expected to predominantly increase Ca^{2+} load in dominant and Na^+ load in subsidiary pacemakers.

To maintain homeostasis, ions moving across the cell membrane during systole must be returned to their original environment during diastole. Ca^{2+} is extruded in exchange for extracellular Na^+ by the $\text{Na}^+-\text{Ca}^{2+}$ exchange current in the forward mode [14], whereas Na^+ is extruded in exchange with extracellular K^+ by the Na^+-K^+ pump [15]. Both the extrusion of Na^+ and of Ca^{2+} are electrogenic, although the pump current is outward [15] and the exchange current NCX at diastolic potentials is inward [14].

The loading of different ions during a faster discharge would be expected to have important physiological implications for SAN pacemaker activity. In dominant pacemaker cells, a prevalent extrusion of Ca^{2+} would steepen the slope of diastolic depolarization whereas in subsidiary pacemakers a prevalent extrusion of Na^+ would decrease it. In fact, overdrive of the cat SAN subsidiary pacemaker region surgically separated from the dominant region is followed by a prolonged inhibition [8], as in Purkinje fibers (“overdrive suppression”, [10]). Conversely, when in high $[\text{K}^+]_o$ SAN action potentials acquire the characteristics of dominant responses [16–20], overdrive may enhance or induce spontaneous discharge [18, 20] (“overdrive excitation”, [21]).

The *general aim* of the present experiments was to investigate the hypothesis that in the SAN overdrive can cause excitation under conditions that increase Ca^{2+} load and suppression under conditions that increase the Na^+ load; and that the short pause induced by overdrive in SAN discharge may be the *net* result of an electrotonic interplay between these opposite mechanisms. Since the electrical changes associated with overdrive have been reported [8, 18, 20], the main focus was on force behavior, as it gives essential insights

on both Ca^{2+} changes on a beat to beat basis and simultaneous rate changes.

The *specific aims* were to: (i) verify the effects of overdrives of different duration and rate on force and discharge patterns in Tyrode solution (both dominant and subsidiary pacemakers are present); (ii) study the effects of a prevalent Ca^{2+} loading by depolarizing the pacemaker cells in high $[\text{K}^+]_o$ (slow responses prevail); (iii) use different patterns of drive to determine if Ca^{2+} extrusion is impaired when the cells have a less negative diastolic potential; and (iv) use blockers of either Ca^{2+} or Na^+ channels to decrease the load of these ions; and (v) increase the entry of Ca^{2+} by using higher $[\text{Ca}^{2+}]_o$ or Ba^{2+} .

Materials and methods

The procedures concerning the animals used were in accordance with national and institutional guidelines. Hartley guinea pigs (259.0 ± 12.2 g) of either sex were euthanized with an overdose of sodium pentobarbital (60 mg/kg intraperitoneally). The heart was rapidly excised and placed in a Petri dish filled with oxygenated Tyrode solution. The SAN was identified as a translucent whitish area and was separated from surrounding red-brown atrial tissue. The SAN of guinea pig is thin (and therefore well superfused *in vitro*) and is made up of SAN cells throughout its thickness [22].

The SAN was superfused at 37 °C with gassed (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. To analyze the effect of a fast rate on predominantly dominant pacemaker activity, $[\text{K}^+]_o$ 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 at 1.71 ml/min and the flow of the K^+ -rich solution was changed by means of a peristaltic pump (LKB Broma, model 12000 Perpex) over the range 0–1.54 ml/min in different tests. The two solutions mixed before entering the thermostatic bath and tissue chamber. $[\text{K}^+]_o$ of the final admixture was calculated by means of the following equation:

$$K = \frac{F_T \cdot K_T + F_K \cdot K_K}{F_T + F_K}$$

where K is $[K^+]_o$ in the bath, F_T is the Tyrode flow, K_T is $[K^+]_o$ in Tyrode, F_K is the flow of the K^+ -rich solution and K_K is $[K^+]_o$ in the K^+ -rich solution. The effects of a higher K^+ were quickly established (< 5 min): once a new steady-state was attained, tests were carried out as warranted. The increase in $[K^+]_o$ was adjusted for each SAN to a value that resulted in slowing or in quiescence, as required by different procedures. $[Ca^{2+}]_o$ was increased by directly adding $CaCl_2$ to oxygenated freshly prepared Tyrode solution.

Spontaneously discharging SAN was held at one end by means of a double-tipped stainless steel electrode and at the other end by a short silk thread attached to the rigid rod of a force transducer (Grass Model FTO3C). The force transducer was coupled to a Grass Model 7D Polygraph. Membrane potentials were recorded by means of microelectrodes filled with 3 M KCl and coupled to a Dagan probe and to a Dagan model 8500 operational amplifier. The traces were displayed on a Tektronix model 5111 storage oscilloscope and recorded on paper on a three channel chart recorder (Gould Brush 2400S). The preparations were allowed to equilibrate for about 1 h in the tissue bath before beginning the experiment. The electrical stimuli were provided by a Grass 8800 Stimulator and were applied between the electrode holding the SAN and an electrode placed in the solution near the force transducer.

Tetrodotoxin, lidocaine, CsCl, $BaCl_2$ (Sigma Chemical Co.) and $NiCl_2$ (Fluka Chemika) were used. The results of the tests performed (n) are expressed as means $\pm$ standard error of the mean (SEM). Student's paired t test between two terms of comparison, ANOVA between data groups and the Page Test of ordered alternatives [23] to determine whether the mean values statistically changed in a monotonic manner were applied: a $p < 0.05$ was considered significant (marked in the text by an asterisk *, paragraph sign § and two crosses ‡, respectively).

Results

Effects of overdrives of different duration and rate in Tyrode solution

In Tyrode solution, both dominant and subsidiary pacemaker action potentials are present [8, 11–13].

Therefore, increasing the duration or rate of overdrive might have little effect on the pause if opposing mechanisms are both enhanced and electrotonically interact.

In Figure 1A, a 300/min overdrive was applied for 5, 10, 30 and 60 s. During overdrive, force initially decreased and then re-increased toward a new steady state value which was greater than the pre-drive value. The resting force also increased, but this was due to diastole being too short during overdrive to allow full relaxation of the twitch, as seen by the fact that the last driven beat relaxed to control resting tension during the longer diastole. After the 5 s drive, the pause was longer than control cycle length (+16.2%) and was followed by a more forceful twitch. After the pause, the rate and the markedly increased contraction subsided back to the control value. When the duration of drive was increased to 10, 30 and 60 s, the duration of the pause increased very little, whereas force increased more.

In Figure 1B, the average results ($n=19$) show that during 5 s overdrive force decreased at the beginning of drive (DF_1) below control and it increased by the end of drive (DF_2) above control, and more so the longer the drives. After the 5 s drive, the pause was longer than control cycle length (+23.2%) and changed little after longer drives. The first beat after the pause (PDF_1) became larger with longer drives and was followed by a slight slowing of the early rate (PDR_1). During late recovery, the rate (PDR_2) and force (PDF_2) were similar to control, suggesting a complete extrusion of extra Ca^{2+} entered during overdrive.

The results with faster drives are reported in Table 1. PDF_1 and PDF_2 increased more after the faster (375/min) drive and yet, the pause and rate immediately after pause were much less affected. Thus, in Tyrode solution, the pause was less affected than force by longer or faster overdrives.

Drive-dependent suppression and excitation in high $[K^+]_o$

$[K^+]_o$ was increased in order to decrease the diastolic potential, so that all SAN cells tend to assume the configuration of the slow response action potential [17–20].

In Figure 2A, the same SAN as in Figure 1 was superfused in high $[K^+]_o$ and the same drives were

Figure 1. Effects of overdrives of different duration in Tyrode solution. The traces are a record of contractile force. In A, rate and duration of overdrives are indicated above the traces. In B, the graph shows the average results. R = control rate in beats/min; F = control force in mg; DF₁ = force of the first driven beat in mg; DF₂ = force of the last driven beat in mg; pause = time interval between the last driven beat and the first spontaneous beat in s; PDF₁ = first contraction in mg after the pause; PDR₁ = rate in beats/min immediately after the pause; PDR₂ = late post-drive rate in beats/min; PDF₂ = late post-drive force in mg. In this and in the following figures, the number before the division sign indicates the rate of drive (e.g., 300 stimuli per minute) and number after the division sign indicate the duration of the drive (e.g., 5 s). The asterisk (*) indicate a statistical difference with the respective control. The pause duration was compared with the control cycle length.

carried out. In high $[K^+]_o$ (which decreases I_{Ca} [24]), the rate was slower and force smaller than in Tyrode solution. This may be reflected also in the fact that during the shorter drives (5 and 10 s) force did not increased above pre-drive value. The 5 s drive was followed by a marked suppression. After the pause, the twitch was large, but force

decreased markedly and quickly to control value. The 10 s drive showed similar characteristics, but the pause was somewhat shorter and PDF₁ greater.

By the end of the 30 and 60 s drives, force had increased above the pre-drive value and the pauses were much shorter than after the 5 and 10 s drives.

Table 1. Effects of faster drive on rate and force patterns.

n = 5	DF ₁	DF ₂	Pause	PDF ₁	PDR ₁	PDR ₂	PDF ₂
300/min	-39.5	+56.2	+25	+177	-6.2	+2.8	+4.2
375/min	-55.7*#	+54.7	+32.9*#	+369.0*#	-8	+4.3	+7.1*#

n = number of tests. DF₁, DF₂, pause, PDF₁, PDR₁, PDR₂, PDF₂ = as explained in legend of Figure 1. 300/min and 375/min = rates of 10 s overdrives. * Statistically different from their own control values; # Statistically different from values in the top row.

The first contraction after the 60 s drive was larger than that after the 30 s drive (at the end of two drives, force was 6.3 and 4.8 mg, respectively). As emphasized in the boxed insets *a* and *b*, after the drives force and rate increased above control (overdrive excitation). When $[K^+]_o$ was increased to 11.0 mM, the SAN became quiescent (not shown): overdrive (after a brief pause) actually induced fast discharge (146.3/min) which continued for several tens of seconds.

In Figure 2B ($n=13$, $[K^+]_o = 13.6 \pm 1.2$ mM), longer drives (270.0 ± 8.3 /min for 5, 10, 30 and 60 s) gradually more increased force (DF_2 , §‡; PDF_1 , §‡) above control (and more so than in Tyrode solution), markedly diminished the pause (§‡), caused a subsequent acceleration (PDR_1 and PDR_2 , §‡) and markedly slowed the force decline

after the drives (PDF_2 , §‡). Thus, in high $[K^+]_o$, faster drives induce overdrive excitation as they increase force and rate, and shorten the pause.

Changes in force at constant rate after overdrives

In high $[K^+]_o$, the greater increase in force during and the slower decline of force and rate after longer drives suggest the possibility that a decreased Ca^{2+} extrusion might contribute to the enhanced force and rate. If so, in non-spontaneous SAN driven at 6/min and overdriven for 2 min at progressively faster rates, a decreased Ca^{2+} extrusion should be reflected in the magnitude and decay of force during recovery from overdrive, *the rate being constant at 6/min*.

Figure 2. Effects of overdrives of different duration in high $[K^+]_o$. In A, the SAN was spontaneously active in 10.4 mM $[K^+]_o$ and was overdriven at the rate and for the durations indicated. The same symbols identify the same traces within and without the boxed inset *a* and *b*. In B, the average values are shown. Other explanation as in legend of Figure 1.

Figure 3. Effect of drives of increasing frequency and fixed duration on the subsequent behavior of force in high $[K^+]_o$. In A, the rates of the 2 min overdrives are indicated above the respective traces. After the overdrives, 6/min drive was resumed, except for the bottom trace where overdrive induced excitation. In the boxed inset, the first beat after the overdrive indicated by numbers is shown. In B, the average values are shown for force of the first beat of overdrive (DF₁), of the last beat of overdrive (DF₂), of the first post-drive beat (PDF₁), and of a late post-drive beat (PDF₂) in mg, as well as recovery intervals in seconds

In Figure 3A, during the 6/min drive force was constant (top trace). At the beginning of 30, 60, 120 and 240/min drives force decreased with respect to that during the preceding 6/min drive (not shown). At the end of 15, 30, 60, 120 and 240/min drives, force had increased by 0, +6.2, +66.6, +281.8 and +900%, respectively, with respect to the first beat of the overdrives. After the 15, 30, 60 and 120/min drives, the first beat at 6/min drive was +117.3, +235.2, +305.0 and +447.0%, respectively, above the last beat of the previous drive (see boxed inset). After an initial brief pause, the 240/min drive induced overdrive excitation. After the overdrives, recovery of force took longer the faster the previous drive.

In Figure 3B ($n = 4$), force became gradually smaller at the beginning of 15, 30, 60, 120/min

drives (DF₁, ‡) and gradually larger by the end of the same drives (DF₂, §‡) compared to DF₁. After overdrives, at 6/min PDF₁ was larger than DF₂ and more so with longer drives (§, ‡ $p = 0.079$) and then decreased to a similar value (PDF₂ ~ 2.5 mg) after gradually longer periods (Recovery time). In $n = 2$, 240 and 300/min drives were followed by overdrive excitation with a strong initial contraction. Thus, during a steady slow post-overdrive rhythm, the greater force takes a gradually longer time to decrease to the original value.

Effects of intermittent drives on force in quiescent SAN

If force increases with overdrives also because of a slower Ca^{2+} extrusion (as suggested by the above

results), then in quiescent SAN successive drives at the *same* rate separated by periods of quiescence of the same duration as the drives may lead to a progressive increase in force. This approach also allows to test how long the quiescence needs to last for force to return to control patterns.

In Figure 4A, in SAN quiescent in high $[K^+]_o$, 10 s drives were separated by 10 s periods of quiescence. During successive drives, force gradually increased, but from a gradually greater initial value. Thus, in the top B trace (last drive of the series), the first (F_1) and second (F_2) beat after quiescence, and the last beat (F_3) of drive increased by +88.8, +1528.5 and +222.2%, respectively, in comparison to F_1 , F_2 and F_3 of the first drive (top A trace). Eventually, force stabilized during the drives as a new steady state was attained.

To verify how long quiescence needs to be in order to restore control force patterns (top A trace), in Figure 4B the quiescent period preceding the 10 s drive was gradually increased. When drives were separated by three successive pauses of 30 s each, the increase in force began to subside, but only after additional pauses of 60 and 120 s did force return to the initial patterns.

An average of 11.8 ± 1.8 drives of 10 s duration and $274.2 \pm 12.1/\text{min}$ rate were separated by 10 s periods of quiescence. In Table 2, force during the

last drive was greater than during the first drive, with the greatest increment in F_2 . In two additional experiments, the intermittent drives induced spontaneous discharge.

Intermittent drives of active SAN in Tyrode solution and in high $[K^+]_o$

In quiescent SAN, the lack of activity allowed to study the effects on force of repeated drives separated by equal periods of quiescence. However, since overdrive appears to affect not only force but also the rate, the effects of intermittent drives on force and rate were also studied in spontaneously active SAN.

In Figure 5A, in Tyrode solution, the first, second, fourth and seventh drives were followed by similarly short pauses and by an increase in force that subsided in less than 5 s after the pause. That less than 5 s were needed for the subsiding of the increase in force was verified by reducing the non-driven interval to 5 s for five times. The 12th drive was followed by the fifth shorter interval and still force fell to a steady state by the end of 5 s. The behavior of force was similar after the last (15th) drive.

In Figure 5B, in high $[K^+]_o$, the first drive (top B trace) was followed by a relatively long pause, a larger beat and a subsequent acceleration

Figure 4. Effects of intermittent drives on force development in high $[K^+]_o$. In A, repeated 240/min 10 s drives separated by 10 s pauses were applied to a SAN quiescent in 13.8 mM $[K^+]_o$. In B, the duration of the pauses is indicated by the numbers (in seconds) next to the traces. F_1 indicates the force of the first beat after the pause, F_2 the force of the second beat and F_3 the force of the last beat of the drives.

Table 2. Effects of intermittent drives on force in quiescent SAN.

$n=7$, $[K^+]_o$ 14.5 ± 1.1 mM	F_1	F_2	F_3
First drive	9.9 ± 3.2	0.15 ± 0.06	1.5 ± 0.66
Last drive	$24.1 \pm 4.7^*$	$3.9 \pm 1.5^*$	$7.5 \pm 2.4^*$
% Change	+141.5%	+2464.0%	+379.2%

First drive = results obtained with the first drive. Last drive = results obtained with the last drive. F_1 , F_2 , F_3 = force in mg of the first, the second and the last beat of drive, respectively. % Change = percent increase in F_1 , F_2 and F_3 during the last drive compared to the first. * Statistically different from values in top row.

(+108.5% of the pre-drive rate). After the successive drives, the pause gradually decreased, and the faster spontaneous discharge started earlier and was

associated with a greater force. At the end of the last (11th) drive, force was +240%, the pause -56.5% and the spontaneous rate +59.1% with respect to the first drive. During the recovery from the 11th drive, force and rate decreased but slowly.

In Figure 5C, in Tyrode solution, the changes in rate and force for the last drive with respect to the first are small and quickly decaying. In Figure 5D, in high $[K^+]_o$, the changes with the last drive with respect to the first show that repeated drives increase *both* force and rate, and decrease the pause.

Effects of cesium on overdrive patterns in Tyrode solution

High Cs^+ depolarizes the SAN and reduces its spontaneous discharge [16], as high $[K^+]_o$ does.

Figure 5. Effects of intermittent drives on force and rate in active SAN in Tyrode solution and in high $[K^+]_o$. In A, repeated 300/min 10 s drives separated by 10 s pauses were applied to a SAN perfused in Tyrode solution and in B to an active SAN in 11.9 mM $[K^+]_o$. Successive traces are identified by the numbers next to them. In A, the five pauses preceding the 12th drive were reduced to 5 s. The average values for the first and last drive in Tyrode solution are shown in C and those in high $[K^+]_o$ in B.

If high $[K^+]_o$ impairs Ca^{2+} extrusion by decreasing the diastolic potential, then high Cs^+ may lead to effects similar to those by high $[K^+]_o$.

In Figure 6A, in Tyrode solution, overdrive induced a pause followed by a slight acceleration of discharge (see boxed inset *a*, where the same symbols identify the same traces within and without the inset). In Figure 6B, in 15 mM Cs^+ , the subsidiary action potentials recorded in Tyrode solution (TYR.) underwent a considerable depolarization (emphasized by the dashed line) and the development of a diastolic tail (see grey area) [16, 25, 26] while rate and force decreased. In Cs^+ , overdrive increased force more slowly, was followed by a longer pause (+203.5% of the pre-drive cycle length), a larger first beat and a substantial acceleration of discharge (see boxed inset *b*). The increase in rate and force subsided slowly during recovery from overdrive, as also seen with high $[K^+]_o$.

In Figure 6C ($n=5$), with respect to Tyrode control, in 15 mM Cs^+ all parameters (except the pause) decreased. Nevertheless, in the presence of Cs^+ , overdrive increased percentage-wise rate and force more than in Tyrode, as it does in high $[K^+]_o$.

Effects of high $[Ca^{2+}]_o$ on overdrive patterns

If the changes induced by drives are related to a progressive increase in $[Ca^{2+}]_i$, then increasing Ca^{2+} load by increasing $[Ca^{2+}]_o$ should enhance the excitatory effects of drive on force and rate, but not necessarily to the same extent in Tyrode and high $[K^+]_o$ solutions.

In Figure 7A, in Tyrode solution, high $[Ca^{2+}]_o$ increased the rate slightly and force markedly (note the different force calibrations). During overdrive, force reached a steady state sooner (cf. dashed lines and gray areas). During drive, the first beat decreased less than in control and there was a small decrease in the steady state force. After drive, the pause was similar to control and the absolute value of the first beat after the pause was greater in high $[Ca^{2+}]_o$ (79.2 mg) than in control (49.8 mg). Early post-drive rate was slightly slower than pre-drive rate.

In Figure 7B, in high $[K^+]_o$, the smaller force increased more slowly but substantially during drive, as usual. Rate and force were greater after drive than before (upper traces in boxed inset). In the bottom B trace, high $[Ca^{2+}]_o$ increased rate and force (note the different force calibrations), as also shown by a comparison of the left traces in

Figure 6. Effects of high cesium on rate and force overdrive patterns. In A and B, the same symbols identify the same traces within and without the boxed insets *a* and *b*. The action potentials and twitches were recorded in Tyrode solution and in 15 mM cesium, as indicated. The dashed line extrapolates the maximum diastolic potential in Tyrode solution and the grey area the Cs^+ -induced tail. In C, the average values in Tyrode solution and in the presence of Cs^+ are shown.

Figure 7. Effects of high $[Ca^{2+}]_o$ on rate and force overdrive patterns. In A, the top trace was recorded in Tyrode solution and bottom trace in the presence of 5.4 mM $[Ca^{2+}]_o$. The grey areas and dashed lines emphasize the more rapid increase in force during drive in high $[Ca^{2+}]_o$. In B, drive was repeated in 16.0 mM $[K^+]_o$ (top trace) and in high $[K^+]_o$ plus 3.6 mM $[Ca^{2+}]_o$ (bottom trace). The same symbols identify the same trace within and without the boxed inset. The action potentials were recorded in high $[K^+]_o$. The average values of Ca^{2+} effects are shown in C for Tyrode solution and in D for high $[K^+]_o$.

the boxed inset. By the end of drive, force had increased above the pre-drive value. After drive, while the pause was little altered in high $[Ca^{2+}]_o$, both the late post-drive rate and force were greater than prior to overdrive (cf. bottom traces in boxed inset), and decreased very slowly.

In Figure 7C ($n=7$) in Tyrode solution, 5.4 mM $[Ca^{2+}]_o$ changed little the rate, but more than doubled force. In high $[Ca^{2+}]_o$, 30 s 300/min overdrive substantially increased force parameters, but had little effect on the rate. Shorter drives (5 and 10 s) showed trends similar (not shown).

In Figure 7D ($n=5$) in 13.9 ± 1.8 mM $[K^+]_o$, 3.9 mM Ca^{2+} markedly increased both rate and force and enhanced the effects of overdrive, including a shortening of the pause. Therefore, in Tyrode, high $[Ca^{2+}]_o$ increases force more than the (already high) rate and influences but little the pause. In high $[K^+]_o$, high $[Ca^{2+}]_o$ increases force and rate before, during and after drive, and shortens the pause.

Nickel enhances overdrive suppression

If overdrive excitation and overdrive suppression interact in determining the pause, then decreasing Ca^{2+} load by means of a Ca^{2+} channel blocker would be expected to enhance overdrive suppression.

In Figure 8A, in Tyrode solution, overdrive caused the usual changes in force and rate patterns. As shown in the trace underneath, Ni^{2+} reduced both force and rate. During overdrive, force increased more slowly than in the absence of Ni^{2+} (cf. grey areas), but by the end of drive force had increased substantially. The cessation of overdrive was followed by a longer pause and a larger first contraction than in control. The longer pause apparently contributed to subsequent stronger first beat, since force declined relatively quick to the low steady state. In the boxed inset, with respect to the pre-drive cycle length (marked by the joined gray lines), drive prolonged the pause much more in the presence of Ni^{2+} .

Figure 8. Effects of nickel on rate and force overdrive patterns. In A, the top trace was recorded in Tyrode solution and the bottom trace after adding 0.5 mM nickel. The slower growth of force during drive is emphasized by the grey areas. In the boxed inset, the joined vertical grey lines label the cycle length in control and in the presence of Ni^{2+} . In B, the average values in the Tyrode and in Ni^{2+} solutions are shown.

In the presence of Ni^{2+} , the ratio overdrive/spontaneous rate was greater than in control since the basal rate was less. Still, prolonging the drive from 30 to 60 and to 120 s (bottom two A traces) led to much longer pauses, which were dependent on drive duration and not on the overdrive/spontaneous rate unchanged ratio.

In Figure 8B, in Tyrode solution ($n=8$) 0.5 mM Ni^{2+} decreased rate and force prior, during and after 30 s overdrive and increased the pause. In high $[\text{K}^+]_o$ ($n=4$), 0.35 mM Ni^{2+} also decreased the rate (R -15%, PDR₁ -8.9%, and PDR₂ -1.1%), decreased force (F -25.7%*, DF₂ -28.7%, PDF₁ -44.3%*, PDF₂ -25.1%*), and increased the pause (+36.0%). Therefore, Ni^{2+} has similar effects in high $[\text{K}^+]_o$. Overall, Ni^{2+} enhances overdrive suppression while decreasing rate and force.

Effects of barium in Tyrode solution and high $[\text{K}^+]_o$

Both diastolic depolarization and diastolic voltage oscillations play a role in SAN discharge [20]. Ba^{2+} increases I_{Ca} , but decreases the delayed rectifier current I_{K} [27], which is the SAN dominant pacemaker current [28]. Therefore, Ba^{2+} may provide information as to the relative importance of Ca^{2+} load versus that of diastolic depolarization on drive-related rate and force patterns in Tyrode solution and high $[\text{K}^+]_o$.

In Figure 9A, in Tyrode solution Ba^{2+} increased force prior, during and after overdrive whereas it decreased the rate and increased the pause (see boxed inset). In Figure 9B ($n=11$), in Tyrode solution, 0.64 ± 0.1 mM Ba^{2+} decreased the rate and increased the pause as well as force (R -30.7%,

Figure 9. Effects of barium on rate and force patterns of overdrive. In A, overdrive was carried out in Tyrode solution (top trace) and after adding 0.2 mM Ba^{2+} (bottom trace). In B, the average values in Tyrode and in Ba^{2+} solutions are shown.

F + 54.3%, DF_2 + 8.5%, pause + 12.2%, PDF_1 + 15.4%, PDR_1 - 15.2%, PDR_2 - 14.1% and PDF_2 + 88.7%). In high $[\text{K}^+]_o$ ($n=4$), 0.15 ± 0.01 mM Ba^{2+} had little effect on rate, increased force and shortened the pause (R + 3.3%, F + 23.9%, DF_1 - 28.5%, DF_2 + 32.4%*, pause - 9.5%, PDF_1 + 27.0%, PDR_1 + 30.0%, PDR_2 - 0.3%, and PDF_2 + 45.4%*). Thus, while generally force and rate change in the same direction, the change can be dissociated by Ba^{2+} .

Electrical and mechanical events associated with overdrive and Na^+ channel blockers

In high $[\text{K}^+]_o$, overdrive increases the diastolic oscillatory potentials [20]. The relation between changes in diastolic potentials and in force with overdrive is shown in Figure 10 in high $[\text{K}^+]_o$.

Before drive (Contr.), the distinct inflection during diastolic depolarization is typically due to a superimposed V_{os} (diamond in A and arrow in C). In Figure 10A, after the 5 s overdrive, several changes are apparent. During the pause, diastolic depolarization no longer shows V_{os} , (see top traces in boxed inset a). After the pause, both force and V_{os} (cf. the grey areas in the traces labeled "Contr." and "5 s" in boxed inset b) increased above control. The steeper and larger V_{os} was responsible for increase in the late post-drive rate above control rate. The subsequent return of V_{os}

toward control patterns was associated with a gradual subsiding of the rate toward the pre-drive value.

When overdrive duration was increased to 10 s (Figure 10B) and 30 s (Figure 10C), the pause became shorter (as emphasized by the grey vertical line in boxed inset a). The slope and size of V_{os} increased further (see "10 s" trace, boxed inset b) and then V_{os} became fused with $\text{Th}V_{os}$ in a U-shaped DD ("30 s" trace, boxed inset b). The growth of V_{os} is shown by the superimposed traces in the same inset (arrow). Thus, the pause involves a less steep DD and the acceleration involves an increase in slope and amplitude of V_{os} associated with an increase in force. The increase in V_{os} and force was greater with the longer drives.

Since norepinephrine increases Ca^{2+} load, the increase in V_{os} could be due to the release of NE by the electrical stimuli. To test this possibility, overdrive was carried out also in the presence of blockers of the fast Na^+ channels (lidocaine [29] and tetrodotoxin [30]). In Figure 11A, in high $[\text{K}^+]_o$, V_{os} was superimposed on DD (dot) which was absent during the pause (square, see superimposed control traces and grey area in boxed inset a). After the pause, slope and size of V_{os} increased until a U-shaped DD developed and V_{os} was no longer visible (see traces labeled with an asterisk and superimposed in the boxed inset b under Cont.). At the same time, both rate and force were

Figure 10. Effects of overdrives of different durations on diastolic potentials and force. The duration of the 240 drives was 5 s in A, 10 s in B and 30 s in C. In the boxed inset *a*, diastolic depolarization in control (Con.) has been superimposed on that during the pause (the vertical grey line emphasizes the shortening of the pause). In the boxed inset *b*, the grey areas emphasize the growth of the slope and magnitude of V_{0s} after the longer drives: the same symbols identify the same trace within and without the boxed inset. The arrow points to the successive changes in DD.

greater than prior to drive. The last A panel shows the reappearance of V_{0s} , as rate and force returned toward pre-drive values.

In Figure 11B, lidocaine had decreased force to the baseline and abolished V_{0s} (triangle). Overdrive did not increase force above the baseline and induced a small pause (bottom traces in boxed inset *a*). After the pause, slope and size of DD increased with the appearance of a faint V_{0s} (see middle trace in the boxed *b* inset under Lido.) and this was associated with an increase in rate.

Tetrodotoxin was also tested since it blocks nerve action potentials in the nanomolar range [30], decreases intracellular Na^+ activity as well as force (see [31, 32]), and decreases V_{0s} and ThV_{0s} [33]. In the boxed inset *c*, in a SAN superfused in high $[K^+]_o$, the bottom part of the last two driven action potentials and the subsequent pause are shown. Prior to overdrive, V_{0s} was not visible either in control or in the presence of TTX as apparently it was fused with DD. But during the pause, in the presence of TTX small V_{0s} appeared (dots) as they missed the threshold.

Thus, the pause appears associated with a temporary reduction in the slope of DD which is later followed by an enhanced V_{0s} and an increase in rate and force. In the presence of lidocaine and TTX, the less steep DD during the pause also

tends to re-increase after the drive, but less than in control as the diastolic potentials are smaller.

In Figure 11C, in Tyrode solution ($n=9$), lidocaine ($100\mu M$) decreased rate and force and prolonged the pause (+184.2%). In high $[K^+]_o$ (11.5 ± 1.2 mM, $n=4$), lidocaine slowed the SAN in two experiments and stopped it in the other two. In $n=2$, overdrive for 60 s increased the force by 993.3% and induced overdrive excitation. In Figure 11D, in Tyrode solution ($n=7$), TTX ($2.08\mu M$) also decreased rate and force, and prolonged the pause, as lidocaine did.

Discussion

Our results lead us to conclude that: (1) the excitatory effects of overdrive (increase in force and rate, shorter pause or induction of spontaneous discharge) are enhanced when Ca^{2+} loading prevails at less negative potentials; (2) less negative diastolic potentials appear to decrease the rate of Ca^{2+} extrusion, since force increases more during overdrive and the greater force and rate subside more slowly during recovery; (3) at less negative diastolic potentials, overdrive-induced increase in Ca^{2+} load leads to simultaneous increase in force and in the size and slope of diastolic potential (and therefore in rate of

Figure 11. Effects of lidocaine and TTX on rate and force overdrive patterns. The action potentials and force were recorded in 16.2 mM $[K^+]_o$ in the absence (panel A) and the presence (panel B) of 65.9 μ M lidocaine. In boxed inset *a*, the diastolic potential before overdrive has been superimposed on that during the pause in control (Contr.) and in the presence of lidocaine (Lido.); the same symbols identify the same trace within and without the boxed inset. The diastolic potentials marked by asterisks have been superimposed in boxed inset *b* in control (Contr.) and in the presence of lidocaine (Lido.). In boxed inset *c*, the lower part of the last two driven APs and of the first two spontaneous APs after drive are shown. The dots emphasize subthreshold diastolic oscillations. The average results of overdrive on rate and force patterns are shown in C for lidocaine and in D for tetrodotoxin.

discharge); (4) conversely, the inhibitory effects of overdrive (decrease in force and rate, longer pause) are enhanced when the Ca^{2+} load is decreased by Ni^{2+} , lidocaine and TTX; (5) the effects of Ca^{2+} loading on force can be separated from those on rate by Ba^{2+} which increase I_{Ca} but decreases the pacemaker current; and (6) the unmasking of overdrive excitation and overdrive suppression under suitable conditions suggests that in Tyrode solution the pause is little affected by longer or faster drive, because of the interaction between the simultaneous increase in the excitatory (dominant pacemakers) and inhibitory (subsidiary pacemakers) effects of overdrive.

Overdrive, increased calcium load and force

It is generally agreed that force development is a function of Ca^{2+} loading [see 5, 34]: indeed, an increase in rate increases cellular Ca^{2+} in all cardiac tissues including the SAN (e.g., [3–6]). Also, overdrive-induced force patterns in SAN are similar to those in other cardiac tissues (e.g., [2, 7]). During longer or faster overdrives, contraction increases as a function of the number of driven APs, since during the AP Ca^{2+} enters the cells through the more often activated I_{Ca} and the reverse NCX. Furthermore, during overdrive a shorter diastole decreases the time available for Ca^{2+} extrusion from the cell by the Na–Ca exchange and this too

would contribute to the gradual increase in force. Thus, force (DF_2) increases as a function of the duration of overdrive (Figure 1B) and even more so in high $[K^+]_o$ (Figure 2B).

A sudden change in duration of diastole is a variable that appears to influence force of the next beat. One likely mechanism is that during diastole cytoplasmic Ca^{2+} is taken up by the sarcotubular network and transferred to the subsarcolemmal cisternae [35]. Therefore, after a sudden shortening of diastole, less Ca^{2+} is collected in and can be released from the SR cisternae by the next AP: the consequent contraction is weaker, as it happens with the first beat of drive (DF_1 , Figures 1B, 2B). Conversely, a sudden lengthening of diastole (e.g., during the pause) allows more time for Ca^{2+} re-accumulation into the subsarcolemmal cisternae [35] and this would contribute to the stronger contraction of the first beat after the pause (PDF_1 , Figures 1B, 2B). However, the predominant role of Ca^{2+} loading by overdrive on force development is shown by the fact that force increases during overdrive in the presence of a diastole of constant duration and PDF_1 increases progressively with longer drives even if the pause either changes very little (Tyrode solution) or actually shortens (high $[K^+]_o$) (Figures 1B, 2B).

The relationship between Ca^{2+} loading and force is emphasized by the fact that high $[Ca^{2+}]_o$ (Figure 7) and Ba^{2+} (Figure 9) increase force whereas Ni^{2+} decreases it (Figure 8). Also, high $[K^+]_o$ decreases I_{Ca} [24] and decreases force (compare control force in Tyrode solution in Figure 1B with that in high $[K^+]_o$ in Figure 2B). An increase or decrease of Ca^{2+} entry acts in so far it changes intracellular Ca^{2+} . Thus, lidocaine (Figure 11C) and TTX (Figure 11D) reduce force even more than the rate. In this respect, TTX and lidocaine act like high $[K^+]_o$ does, probably through a similar mechanism (a decrease in a_{Na}^i and therefore in $[Ca^{2+}]_i$). Thus, in Purkinje fibers, TTX decreases a_{Na}^i as well as force [31, 32]. Also, TTX markedly decreases V_{os} in SAN perfused in high $[K^+]_o$ [33] and, in the present experiments, reduced force and DD slope by decreasing the size of diastolic oscillations.

The findings in high $[K^+]_o$ show that an additional mechanism (peculiar to the slow responses) contributes to increase intracellular Ca^{2+} by overdrive. In high $[K^+]_o$, longer overdrives percentage-wise increase force more and the great-

er force returns more slowly toward pre-drive values (Figures 2–6) compared to Tyrode solution (Figures 1, 5). That a reduced rate of extrusion of Ca^{2+} contributes to the increase in force in high $[K^+]_o$ is indicated by the fact: (a) during recovery from overdrive, when the duration of diastole was kept constant and long enough for the full recovery of I_{Ca} , the greater force took longer to recede, as expected from diminished rate of Ca^{2+} extrusion (Figure 3); (b) a 10 s pause was not sufficient to allow the extrusion of all the Ca^{2+} that entered during the previous 10 s drive, since the force kept on increasing with successive drives; and (c) only after rather longer and repeated pauses did the overdrive force patterns revert to the original control value (Figure 4). The cumulative increment in force with intermittent overdrives and its slower subsiding in high $[K^+]_o$ (Figure 5B) are in sharp contrast with the findings in Tyrode solution (Figure 5A), since in Tyrode solution force changes little with successive drives and during recovery it subsided in ≤ 5 s.

As for the mechanism by which Ca^{2+} extrusion might be less efficient in high $[K^+]_o$ with respect to Tyrode solution, one major difference is that in high $[K^+]_o$ the diastolic potential is less negative than in Tyrode solution [17–20]. Since the driving force for Ca^{2+} extrusion is the Na^+ electrochemical gradient [14], a reduction of such a gradient due to the less negative diastolic potential would be expected to reduce Ca^{2+} extrusion in the unit of time. This conclusion is supported by the fact that high Cs^+ (which also decreases the SAN diastolic potential [16]) affected overdrive force patterns similarly to high $[K^+]_o$.

Thus, the present findings indicate that with overdrive force development in the SAN is a function of Ca^{2+} load (more numerous APs in the unit of time during overdrive), the sudden changes in the duration of diastole (transfer of Ca^{2+} to the SR subsarcolemmal cisternae) and Ca^{2+} extrusion (reduced by a shorter diastole during overdrive and by a less negative diastolic potential). As for the hyperpolarization-activated I_f being increased by high $[K^+]_o$, it should be noted that high $[K^+]_o$ decreases the rate and (by decreasing the diastolic potential) would diminish the I_f activation. Overdrive would act similarly, since the I_f activation during the shorter diastole would be less. Further, I_f is not the dominant pacemaker current in the SAN [28].

Calcium load and dominant discharge

The results indicate that overdrive excitation predominates when Ca^{2+} load is greater either absolutely (overdrive, high $[\text{Ca}^{2+}]_o$, Ba^{2+} , reduced extrusion of cellular Ca^{2+}) or relatively (lower Na^+ load due to depolarization and inactivation of fast Na^+ channels). Overdrive excitation is thus characterized by a simultaneous increase in force and rate. This raises the question as to whether and how an increase in force might be linked to an increased in rate.

In this connection, a major finding is that in the SAN perfused in suitably high $[\text{K}^+]_o$ the depolarization changes the subsidiary fast APs into slow responses [16–20, 36]. This on the one hand decreases the Na^+ load due to the inactivation of the fast Na^+ channel (slow responses instead of fast APs) and on the other enhances the Ca^{2+} load by overdrive (more slow responses and decreased rate of Ca^{2+} extrusion). If the behavior of the slow responses in high $[\text{K}^+]_o$ reflects that of dominant pacemakers (see below), the findings provide substantial insights in the operation of the SAN dominant pacemakers. This has to do with the presence of oscillatory potentials in the SAN [17–20].

In Tyrode solution, the U-shaped DD is usually ascribed to the pacemaker current (for the ionic species responsible for the dominant pacemaker current see [28]). However, when $[\text{K}^+]_o$ is gradually increased, DD misses the threshold and this unmasks two different diastolic oscillations [17–20, 33]. One is the oscillatory after-potential V_{os} , which is obligatorily superimposed on early DD (DD_1) since it requires a previous AP to be generated (see [37]). The other is the oscillatory pre-potential $\text{Th}V_{os}$ (the oscillatory potential near the threshold for the upstroke), which can occur at any time during diastole. Decreasing $[\text{K}^+]_o$ back to normal allows $\text{Th}V_{os}$ to increase in size, initiate sooner during diastole and fuse with V_{os} ; force at the same time increases. Eventually DD, V_{os} and $\text{Th}V_{os}$ become indistinguishable in the U-shaped DD [20].

V_{os} and $\text{Th}V_{os}$ are Ca^{2+} -dependent, in that both are augmented by interventions that increase cellular calcium (high $[\text{Ca}^{2+}]_o$, fast drive [18, 20], norepinephrine [26], slower Ca^{2+} extrusion) and decreased by interventions that decrease cellular calcium (low $[\text{Ca}^{2+}]_o$, Ni^{2+} [20] and TTX [33]).

The role of a less negative diastolic potential in reducing Ca^{2+} extrusion in high $[\text{K}^+]_o$ would apply also to dominant pacemakers in Tyrode solution, since their diastolic potential is 20–30 mV less negative than that of SAN subsidiary pacemakers [19, 20, 26]. That the extrusion of Ca^{2+} is more effective in Tyrode solution is suggested by the fact that during intermittent overdrives force decreased toward pre-drive value within 5 s, thereby preventing a cumulative effect of repeated drives on force and rate. An overall more negative diastolic potential in Tyrode solution may play a role in the differences with high $[\text{K}^+]_o$.

In the present context, the evidence indicates that overdrive increases force and rate through the generally agreed [3–6] increase in cellular calcium. Furthermore, in high $[\text{K}^+]_o$ overdrive is shown to simultaneously increase the twitch and the size as well as slope of diastolic potential which then subside together during recovery (Figure 10). One mechanism linking the increase in Ca^{2+} load with a larger diastolic potential might be that in SAN cells the SR is sparse [38] and therefore easily Ca^{2+} overloaded. The obligatory role of Ca^{2+} load in overdrive excitation in high $[\text{K}^+]_o$ is indicated by the fact that short drives do not increase force above the pre-drive value and are followed by a longer (not a shorter) pause with respect to Tyrode solution (high $[\text{K}^+]_o$ decreases I_{Ca} [24] and force, Figure 2). Only longer drives increase force above control and are followed by a shorter pause as well as greater force and rate.

The implication of the present findings is that the SAN dominant pacemakers are not inhibited by an increase in rate (intrinsic or extrinsic) in virtue of the APs being slow responses (prevalent Ca^{2+} loading). The increased Ca^{2+} load and slower Ca^{2+} extrusion insures that the Ca^{2+} overloaded SR releases more Ca^{2+} in diastole. The consequent enhancement of the diastolic potentials leads to overdrive excitation. Instead, overdrive suppresses the discharge of cat surgically separated SAN subsidiary pacemakers (see [8]), since the latter are fast responses (enhancement of the electrogenic Na^+ extrusion by overdrive) and their DD is too negative for $\text{Th}V_{os}$ to be present [20]. In intact SAN, the reduced slope of DD of subsidiary pacemakers by Na^+ extrusion would electrotonically antagonize overdrive excitation of dominant pacemakers.

It should be added that dominant pacemaker slow responses in Tyrode solution and those in suitably high $[K^+]_o$ have the same configuration and behave similarly. Thus, the slow responses of dominant pacemakers and those in high $[K^+]_o$ are suppressed by acetylcholine [39], enhanced by norepinephrine [26], increased by high and decreased by low $[Ca^{2+}]_o$ [26], and are abolished by high Ni^{2+} [20].

Overdrive excitation and diastolic potential

The present results indicate that the prevalent Ca^{2+} loading and the less negative diastolic potential in the slow responses are essential for dominant pacemaker discharge and for overdrive excitation. These characteristics result in a contribution of Ca^{2+} -dependent diastolic oscillations to the U-shaped DD (and therefore to fast discharge) and to the lack of frequency-dependent self-suppression. In fact, the induction of excitation by overdrive in dominant pacemakers might be viewed as a protective mechanism against the simultaneous enhancement of overdrive suppression in SAN subsidiary pacemakers.

This functional organization of *dominant* pacemakers appears to center on the lack of expression of I_{K1} channels (see [40]). The consequently reduced background K^+ current would result in: (1) a less negative diastolic potential; (2) the inactivation of Na^+ channels even if expressed; and (3) a Ca^{2+} -dependent slow response. The less negative diastolic potential would permit DD to enter the oscillatory zone (induction of ThV_{os}) and a less efficient Ca^{2+} extrusion (Ca^{2+} overload of the SR and induction of V_{os}). The lack of I_{K1} acts only insofar as it depolarizes the diastolic potential, since high $[K^+]_o$ (which increases I_{K1}) also induces the slow responses through a depolarization (less negative E_K).

Clinical implications

Under normal conditions, SAN dominant pacemakers are not overdriven, but the mechanisms related to overdrive are operating all the time, since an increase in rate loads dominant pacemaker cells with Ca^{2+} . Thus, discharge of dominant pacemaker cells tend to sustain their spontaneous activity, instead of being self-inhibitory like for fast-response subsidiary pacemakers.

Arrhythmias (such atrial tachycardias, flutter and fibrillation) could overdrive the SAN at rates as fast as those tested here. The peculiar organization of the ionic exchange in dominant pacemaker cells tends to reduce overdrive suppression that would follow the stoppage of these tachycardias. This means that the dominant SAN pacemakers on the one hand inhibit subsidiary pacemakers in virtue of their fast rate and on the other they are protected from frequency-dependent suppression by the relation between Ca^{2+} load and dominant discharge (enhancement of oscillatory potentials).

Also, the results provide essential insights on the effects of hyperkalemia and on the therapeutic action of Ca^{2+} administration in patients. The SAN is particularly resistant to an increased K^+ plasma level and this is due in part to sympathetic innervation [41] and in part to the fact that (while high $[K^+]_o$ increases g_{K1}) it has little effects on the dominant pacemaker current I_K [24]. The present results indicate that the therapeutic effect of Ca^{2+} administration in hyperkalemia might be related to the fact that an increased Ca^{2+} loading of SAN depolarized in high $[K^+]$ fosters spontaneous discharge (Fig. 7B).

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