Modulation of rate by autonomic agonists in SAN cells involves changes in diastolic depolarization and the pacemaker current

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Abstract

Two distinct intracellular mechanisms have been proposed to affect the firing rate of cardiac pacemaker cells: one involves modulation of the If current by the second messenger cAMP, and one relies upon disruption or alteration of SR Ca2+ transients during activity. Although both mechanisms are necessary for proper automaticity and autonomic rate control, the specific contribution of each to pacemaking is still debated. We investigated if the two processes can be separated based on potentially different effects on action potential characteristics during rate modulation. To identify specific If-mediated effects, we used the selective If blocker ivabradine and found that ivabradine (3 μM) slows rate (−16.2%) by selectively reducing (−31.9%) the steepness of early diastolic depolarization (EDD). On the other hand ryanodine (3 μM), used to evaluate the effects of abolishment of SR Ca2+ transients, slowed rate (−31.3%) by depolarizing the take-off potential (TOP, 18.1%) without affecting EDD. We therefore used these two parameters to identify If-based or SR Ca2+ transients-based processes and analyzed the effects on action potential’s characteristics of Rp-cAMPs (50 μM), a membrane permeable cAMP analogue directly activating f-channels; we found that Rp-cAMPs accelerates rate by increasing EDD (+42.3%) without modifying TOP. Finally, rate modulation was achieved by muscarinic (ACh 0.01 μM) or β-adrenergic (Iso 1 μM) stimulation; in both cases, rate changes were associated with modifications of EDD (ACh, −29.3% and Iso, +47.6%) and not of TOP. We conclude that rate-related changes in the EDD induced by autonomic agonists are mediated by If and not by processes involving SR Ca2+ transients.

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1. Introduction

A contribution of the funny (If) pacemaker current of sinoatrial node (SAN) cells to the generation and autonomic modulation of spontaneous activity in the heart has been proposed since the early description of this current [1,2] and is now well established [3–5], although debate continues on the extent of this contribution and participation of other mechanisms. Several reports have proposed a role for Ca2+ transients in rate regulation based on observations of Ca2+ release from the sarcoplasmic reticulum during activity [6–11]. These reports have highlighted a correlation between the amplitude of the Ca2+ transients and rate. For example, β-adrenoreceptor (βAR) stimulation causes parallel increases in Ca2+ transients and rate in SAN cells, and reduction or abolishment of Ca2+ transients leads to proportional changes in the frequency of spontaneous activity of pacemaker cells, as well as reduced responsiveness to βAR stimulation [11].

While these studies clearly illustrate the importance of normal Ca2+ homeostasis to the persistence of stable automaticity, the data have led to the further proposal that Ca2+ transients represent a main mechanism responsible for both normal SAN rate maintenance and β-adrenergic-induced positive chronotropism. However, experiments investigating the mode of action of Ca2+ transients inhibition on rate modulation showed that while abolishment of Ca2+ transients by ryanodine does inhibit βAR-induced rate acceleration, it does not abolish the rate acceleration caused by increasing intracellular cAMP levels [12]. This suggests that although normal Ca2+ homeostasis is required for maintenance of a proper activation cascade leading from βAR stimulation to effectors, including f-channel ac-
tivation, rate regulation also involves the contribution of a cAMP-mediated, \( I_f \)-dependent mechanism.

To further investigate the mechanisms controlling rate regulation, and specifically the control of rate by autonomic agonists, we asked the question whether different processes affecting rate can be classified based on their effects on action potential configuration. What is the "signature", for example, of changes of rate induced by \( I_f \) changes? Is this different from the signature of rate changes attributable to modifications of \( Ca^{2+} \) transients? Can any of these signatures be recognized in the way autonomic neurotransmitters affect rate? These questions were prompted by the preliminary observations that ryanodine slows rate both by altering the late diastolic depolarization and by shifting the action potential threshold in SAN cells to more positive values [12], while direct modulation of \( I_f \) by Rp-cAMPS increases rate by altering the early diastolic depolarization without an apparent effect on the action potential threshold (see for example [12], Fig. 4). We therefore proceeded to identify parameters describing action potential features such as action potential threshold and early diastolic depolarization rate and to study how different rate-modifying stimuli, and specifically neurotransmitters, affect these parameters.

We found that rate changes due to \( I_f \) modification are associated with specific changes of EDD, while those due to SR \( Ca^{2+} \) modifications are associated with specific changes of TOP. We further found that rate changes elicited by autonomic agonists involve changes of EDD, but not of TOP. These data confirm that \( I_f \) modulation is a major mechanism responsible for rate control.

2. Materials and methods

2.1. Cell isolation

Animal protocols conformed to the guidelines of the care and use of laboratory animals established by Italian (DL. 116/1992), European (86/609/CEE) and US (National Institutes of Health publication No 85-23) directives.

Young white albino rabbits (0.8–1.2 kg) were deeply anesthetized by intramuscular injection of xilazine (4.6 mg/kg) (Sigma-Aldrich Co) and euthanized by intramuscular injection of xilazine (4.6 mg/kg) (Sigma-Aldrich Co) and euthanized by cervical dislocation, exsanguination and cardiectomy. After quick removal of the heart, the SAN tissue was dissected out and strips from cervical dislocation, exsanguination and cardiectomy. After quick removal of the heart, the SAN tissue was dissected out and strips were placed in 30 mm plastic petri dish placed on the stage of an inverted microscope and superfused with a Tyrode solution containing (mM): 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl2, 5 HEPES-NaOH, 10 d-glucose, pH 7.4. Patch-clamp analysis was performed in the whole-cell configuration. The pipette solution contained (mM): 130 K-Aspartate, 10 NaCl, 2 CaCl2, (pCa = 7), 2 MgCl2, 10 HEPES, 5 EGTA, 2 ATP(Na2), 0.1 GTP, 5 Creatine Phosphate, pH 7.2. Spontaneous activity was recorded from small aggregates of 2–6 cells beating uniformly. In addition to providing stable seals for longer times, recording from cell clusters, rather than single cells, was preferred because it prevented cell dialysis and interference with normal intracellular \( Ca^{2+} \) homeostasis. Test solutions were delivered on top of the cell under study by a fast perfusion device allowing solution changes near the cell in less than 1 s. The temperature was 34±0.5 °C.

Isoproterenol (Iso) and acetylcholine (ACh) were purchased from Sigma-Aldrich Co, and ryanodine and Rp-cAMPS were obtained from Calbiochem. Ixabradine (Iva) was provided by the Institut de Recherches Internationales Servier, France.

2.2. Data analysis

All data were acquired with pClamp software and an Axopatch 200B amplifier (Axon Instruments). Action potentials were recorded from small uniformly beating aggregates as continuous traces for several hundred seconds at a sampling rate of 2 kHz and filtered at 1 kHz with pClamp software. Only recordings where the control frequency was stable over a period of several tens of seconds were used. Raw action potential records were digitally smoothed by a 10-point adjacent averaging smoothing procedure and the time derivative calculated according to a second polynomial, 8-point smoothing differentiating routine (Origin 7, Origin Lab, Northampton, MA).

Data thus obtained were then processed with customized software. For each action potential cycle, we obtained the following parameters (Fig. 1):

(a) maximum diastolic potential (MDP, mV), defined as the most negative potential reached during action potential repolarization;

(b) \( f \)-dependent mechanism.
(b) take-off potential (TOP, mV), defined as the voltage measured at the time when the voltage derivative (dV/dt) overtakes a given threshold, set to 0.5 mV/ms for all records; this value was chosen since it normally represents a level across which, regardless of rate, the voltage derivative dV/dt changes abruptly during the transition from phase 4 to phase 0 of the action potential (see Fig. 1B); since dV/dt is proportional to the whole-cell current density (I_{wc}) according to the equation \(-dV/dt = I_{wc}/C\), with C being the cell capacity, the sudden change of dV/dt apparent in Fig. 1B corresponds to the sudden change of current associated with activation of the Ca^{2+} current; the TOP value can therefore also be viewed as the voltage at which the Ca^{2+} current responsible for action potential depolarization takes off;

c) slope of early diastolic depolarization (EDD, mV/ms); for each cycle, the diastolic depolarization (DD) was defined as the phase of the action potential in the interval between MDP and TOP (\(\Delta t = \text{time of TOP} - \text{time of MDP}\), and EDD calculated as the mean slope in the early fraction of the DD interval (defined as 0.1 \(\Delta t\) to 0.5 \(\Delta t\)); the mean duration of the above interval in a long record (about 20 s) in control conditions was used for calculation throughout analysis of the same cell. We did not measure late diastolic depolarization since, as seen in Fig. 1B, this phase is not well approximated by a linear function (see also the Results section below for further discussion about the use of late diastolic depolarization);

d) action potential duration (APD, ms), defined as the time interval between TOP and following MDP;

e) maximal rate of upstroke depolarization (dV/dt max, mV/ms);

(f) rate (Hz), calculated as the reciprocal of the cycle length (in seconds) from peak-to-peak dV/dt traces (Fig. 1B).

Statistical differences were determined at the \(P<0.05\) level by one-sample, or paired, Student’s \(t\)-tests. Data points were plotted as mean±SEM values.

3. Results

3.1. Inhibition of \(I_f\) and inhibition of Ca^{2+} transients slow rate by affecting different action potential parameters

To identify which of the action potential parameters are affected when spontaneous rate is modified by specific changes of \(I_f\), we chose a pharmacological dissection approach and used ivabradine, a drug known to interact selectively with f-channels [14,15] and cause heart rate reduction without affecting other currents or contractility [16].

In Fig. 2 representative data from a 300 s recording from one small aggregate of SAN myocytes are shown. Perfusion with 3 \(\mu\)M ivabradine, a concentration known to block about 66% of \(I_f\) [15], led to slowing of rate that stabilized within about 100 s. Neither the MDP nor the TOP were modified significantly by ivabradine, as seen in both the superimposed samples of action potential traces (Fig. 2A) and the plotted time course (Fig. 2B). It also is apparent from Fig 2B that the EDD decreased with a time course similar to that of the spontaneous rate. This indicates that the slowing action associated with the partial and selective inhibition of \(I_f\) results from a reduced steepness of diastolic depolarization, already measurable in its early fraction. In \(n=6\) recordings, ivabradine 3 \(\mu\)M slowed spontaneous rate by 16.2±1.5% of its control value (2.9±0.1 Hz), and this was
associated with an EDD decrease of 31.9±4.5% of its control value (0.101±0.009 mV/ms; significant), while the TOP did not vary (1.4±1.7% more negative than its control value of −40.6±1.8 mV, not significant). Ivabradine did not affect either the mean MDP (−0.07% of its control value of −60.5±1.0 mV) or dV/dt max (1.7% of its control value of 3.53±0.11 mV/ms), while it slightly increased mean APD (9.4% of its control value of 212.3±9.3 ms, significant, n=6) in agreement with previous reports [17].

We next used ryanodine as a tool to inhibit SR Ca2+ transients [7,18] and investigated the effects on action potential parameters. A representative set of data is shown in Fig. 3. As previously reported [12], perfusion with 3 μM ryanodine led to a rate decrease that became stable in about 70 s.

In contrast to the effect of ivabradine, the rate decrease induced by ryanodine was not accompanied by a decrease of EDD, but rather by a marked increase of the TOP. This is apparent in both the sample superimposed tracings of Fig. 3A and the time course plots of Fig. 3B. The MDP was not modified. Ryanodine 3 μM decreased rate by 31.3±4.0% of its control value of 3.4±0.3 Hz in n=6 cells and depolarized the TOP by 18.1±2.3% of its control value (−42.2 ±2.2 mV; significant), while EDD was not modified relative to its control value (0.117± 0.016 mV/ms; −1.1±1.9%, not significant). No significant changes were induced by ryanodine on mean MDP (−1.6% of its control value of −60.3±2.9 mV), dV/dt max (−6.4% of its control value of 3.29±0.14 mV/ms) or APD (0.65% of its control value of 172.9±11.9 ms, n=6). In the light of the results of Fig. 2 above, lack of action of ryanodine on EDD is also in agreement with published evidence that this drug does not affect If at the concentrations used here [19,20]. Thus, the slowing arising from inhibition of Ca2+ transients by ryanodine appears to involve processes quite different from those associated with If inhibition, i.e. processes which modify the action potential threshold and leave unaltered the early part of diastolic depolarization. To further highlight the different processes underlying rate slowing due to either If inhibition or the abolishment of Ca2+ transients, we plotted in Fig. 4 the TOP (upper panels) and the EDD (lower panels) against rate for the two sample experiments shown in Figs. 2 and 3 (A and B, respectively); these plots represent the cycle-by-cycle relationships between TOP or EDD and rate in each cell, and the time dependence of the rate changes is therefore not apparent in these plots.

As also shown by the linear regression analysis of the plots, ivabradine-induced rate changes did not involve a significant modification of the TOP (correlation coefficient r=0.15), but were rather associated with changes of EDD (r=0.95); in contrast, ryanodine modified rate by acting on the TOP (r=−0.95), while the EDD was hardly modified (r=−0.06). Mean correlation coefficients for TOP and EDD were r=0.15±0.16 and 0.81±0.07 (n=6) with ivabradine and r=−0.86±0.05 and 0.23±0.05 (n=6) with ryanodine.

The data presented above thus indicate that changes in the action potential parameters EDD and TOP reflect essentially selective contributions of If and SR Ca2+ transients to rate modulation. A more complex situation arises when the later portion of the diastolic depolarization is considered since during late DD both mechanisms have been shown to be functionally involved. This is shown for example by evidence that both Cs+ [6] and ryanodine [12] affect this phase. In the light of the selective dependence of EDD and TOP upon separate mechanisms, we adopted these two parameters as a means to evaluate If-dependent and/or SR Ca2+-dependent contributions to rate modulation by autonomic transmitters.
3.2. cAMP accelerates rate by increasing the steepness of early diastolic depolarization

The $I_f$ current mediates the autonomic control of heart rate through its sensitivity to cAMP, which is increased and decreased by β-adrenergic and muscarinic stimulation, respectively [3,21].

Increases of internal cAMP levels, if acting solely on $I_f$ activation, should therefore lead to rate changes with properties symmetrical to those observed during ivabradine-induced $I_f$ inhibition in Fig. 2 above. However, cAMP is involved in several different biochemical pathways, including those dependent upon protein kinase A (PKA)-induced phosphorylation [22]. In order to limit possible phosphorylation-dependent effects, we superfused cells with Rp-adenosine cyclic 3',5'-phosphorothioate (Rp-cAMPs), a diastereomeric phosphorothioate derivative of cAMP known to act as an antagonist of the cAMP-dependent activation of PKA [23] but also capable of modulating $I_f$ by direct interaction with the channel [24].

Fig. 5 shows the effects on rate and other action potential parameters of Rp-cAMPs 50 μM, a concentration yielding near-saturating activation of $I_f$ [24]. Rp-cAMPs accelerated rate by increasing the EDD, while the TOP remained unchanged (Figs. 5A, B). Shown in Fig. 5C are time-independent plots of EDD and TOP versus rate, also illustrating that the predominant effect of Rp-cAMPs was on EDD. According to linear regression analysis, rate changes were correlated with changes of EDD ($r=0.90$), but not of TOP ($r=0.25$).

This was confirmed by the mean data. In $n=5$ cells, Rp-cAMPs increased rate by 20.6±0.8% of its control value (2.9±0.2 Hz); this was associated with a 42.3±11.1% increase in EDD relative to its control value (0.083±0.015 mV/ms; significant) with no appreciable change in TOP (−3.4±1.9% of its control value of −38.1±2.5 mV, not significant).

Mean correlation coefficients were $r=−0.19±0.20$ and 0.64±0.07 ($n=5$) for TOP and EDD, respectively. Rp-cAMPs did not modify significantly mean MDP (−2.0% of its control value of −55.4±1.8 mV), $dV/dt_{max}$ (−0.3% of its control value of 3.34±0.37 mV/ms) or APD (−5.1% of its control value of 183.6±12.4 mV, $n=5$). The experiments with Rp-cAMPs are consistent with those involving ivabradine and thus confirm that agents able to modulate the $I_f$ current in either direction induce changes of EDD but not of TOP.

3.3. Autonomic agonists alter rate mostly by modifying early diastolic depolarization

The indication of the existence of distinct mechanisms for modifying spontaneous pacemaker activity raises the obvious question whether autonomic modulation of rate is based specifically on either of these two mechanisms. Alternatively, if both mechanisms contribute to autonomic modulation of rate, which, if any, is the most significant? To answer these questions we analyzed the action of Iso (1 μM) and ACh (0.01 μM) on action potential parameters (Figs. 6 and 7). These concentrations were chosen to achieve accelerating/slowing rate changes in a limited range (approximately between 15 and 25% [12,25], comparable to the range of frequency changes observed with the protocols shown above in Figs. 2–5 (16 to 31%)).

As is apparent from Fig. 6, Iso-induced rate acceleration was accompanied by an increased EDD, whereas the TOP remained approximately constant (see also sample action potentials in panel B). Plotting TOP and EDD values against rate in time-
independent, cycle-to-cycle plots (Fig. 6C) and performing linear regression analysis showed that, as with rate changes caused by ivabradine or Rp-cAMPs, the βAR-induced acceleration was much more strongly correlated to changes of the early fraction of diastolic depolarization ($r=0.90$) than of the TOP ($r=-0.18$).

Fig. 5. Modification of action potential parameters by Rp-cAMPs 50 μM. (A) Dot-plots of time course of action potential parameters during application of Rp-cAMPs (bar). (B) Superimposition of sample traces recorded in control and following perfusion with Rp-cAMPs, as indicated (arrows in the rate panel in A); corresponding TOP values also plotted (filled squares, control; open squares, Rp-cAMPs). (C) Cycle-by-cycle TOP vs rate plot (upper) and EDD vs rate plot (lower) as from corresponding panels in A. Correlation coefficients from linear regression analysis (lines) were $r=0.25$ and $r=0.90$ for TOP vs rate and EDD vs rate plots, respectively.

Fig. 6. Modification of action potential parameters by isoprenaline 1 μM. (A) Dot-plots of time course of action potential parameters during application of isoprenaline (Iso, bar). (B) Superimposition of sample traces recorded in control and following perfusion with isoprenaline, as indicated (arrows in the rate panel in A); corresponding TOP values also plotted (filled squares, control; open squares, isoprenaline). (C) Cycle-by-cycle TOP vs rate plot (upper) and EDD vs rate plot (lower) as from corresponding panels in A. Correlation coefficients from linear regression analysis (lines) were $r=-0.18$ and $r=0.90$ for TOP vs rate and EDD vs rate plots, respectively.
In n=7 cells, Iso increased rate by 26.3±5.4% of its control value of 3.2±0.3 Hz and increased EDD by 47.6±7.8% of its control value (0.089±0.008 mV/ms, significant) without affecting the TOP (−2.9±1.2% of its control value of −41.6±2.0 mV, not significant). Mean correlation coefficients were r=−0.29±0.08 and 0.86±0.04 (n=7) for TOP and EDD, respectively.

It should be noticed that the same correlation between EDD and rate is observed here and in Figs. 4 and 5 above regardless of whether the rate is increasing (as with Iso and Rp-cAMPs) or decreasing (as with ivabradine) since these plots are independent from the direction of chronotropic change. Iso (1 μM) did not change mean MDP (−0.9% of its control value of −59.2±1.5 mV) or dV/dt max (−1.0% of its control value of 3.17±0.21 mV/ms), while it decreased slightly the APD (−11.3% of its control value of 176.7±13.5 mV, significant, n=7).

Similar observations were made when ACh 0.01 μM was applied to slow spontaneous rate (Fig. 7). Again, according to linear regression analysis, the rate change due to ACh was much more strongly correlated to changes of EDD (r=0.92) than of TOP (r=−0.24; Fig. 7C).

In n=9 cells, application of ACh 0.01 μM resulted in a 20.8±3.2% decrease of spontaneous rate (control rate 3.3±0.3 Hz). At the same time, it decreased EDD by 29.3±4.4% of its control value (0.116±0.017 mV/ms, significant) but did not alter the TOP (decrease of 0.2±1.7% relative to its control value of −37.4±1.7 mV, not significant). Mean correlation coefficients were r=−0.12±0.17 and 0.89±0.03 (n=9) for TOP and EDD, respectively. This concentration of ACh did not affect significantly MDP (2.8% of its control value of −57.9±2.1 mV) or APD (3.8% of its control value of 183.2±11.4 mV), while it increased slightly dV/dt max (5.0% of its control value of 2.82±0.16 mV/ms, significant, n=7).

We also tested higher concentrations of Iso and ACh. A higher concentration of Iso (10 μM) caused a larger increase of EDD (114.0% of its control value of 0.084±0.004 mV/ms, significant), but also altered significantly the TOP (9.0% of its control value of −42.9±1.4 mV, significant), while leaving the MDP unaltered (−0.3% of its control value of −62.1±0.6 mV, n=3). A higher concentration of ACh (0.03 μM), on the other hand, decreased EDD more strongly (by 76.6% of its control value of −62.1±0.6 mV, n=3). A higher concentration of ACh (0.03 μM), on the other hand, decreased EDD more strongly (by 76.6% of its control value of −62.1±0.6 mV, n=3). A higher concentration of ACh (0.03 μM), on the other hand, decreased EDD more strongly (by 76.6% of its control value of −62.1±0.6 mV, n=3). A higher concentration of ACh (0.03 μM), on the other hand, decreased EDD more strongly (by 76.6% of its control value of −62.1±0.6 mV, n=3). A higher concentration of ACh (0.03 μM), on the other hand, decreased EDD more strongly (by 76.6% of its control value of −62.1±0.6 mV, n=3). A higher concentration of ACh (0.03 μM), on the other hand, decreased EDD more strongly (by 76.6% of its control value of −62.1±0.6 mV, n=3). A higher concentration of ACh (0.03 μM), on the other hand, decreased EDD more strongly (by 76.6% of its control value of −62.1±0.6 mV, n=3).

Fig. 8 compares the mean effects of each of the agents used, as fractions of control values, for rate, EDD and TOP. In each of the bar graphs, empty/gray bars represent mean amplitudes (normalized to control) which are/are not significantly different from 1, respectively. The graphs clearly illustrate that, at the concentrations used, the autonomic agonists Iso and ACh modulate EDD but not TOP, performing like agents that selectively modulate If, but unlike agents that selectively modulate Ca2+ transients.

4. Discussion

The aim of this work was to select between different processes mediating rate modulation by autonomic stimuli. Our results indicate the existence of at least two separate mechanisms able to modify spontaneous rate of SAN myocytes, one due to If changes and responsible for modulation of early dias-
tolic depolarization rate, and one associated with changes of SR Ca\(^{2+}\) transients and acting on the action potential activation threshold. While the functional contributions of the two mechanisms to EDD and TOP are clearly separable, evidence that changes of \(I_f\) [6] and of SR Ca\(^{2+}\) transients [12,19,20] both modify late DD confirms the combined involvement of the two mechanisms during this phase and rules against its use as a discriminating parameter. Although we did not analyze late DD, all our data consistently showed modification of late DD following interventions altering either of the two mechanisms investigated (see for example Figs. 2 and 3).

The mode of rate modulation achieved by autonomic agonists most closely resembles that of substances whose action is mediated specifically by \(f\)-channels. In particular, ACh and ivabradine, at the concentrations tested, both reduced EDD without affecting TOP. Similarly, Iso and Rp-cAMPs both increased EDD without altering TOP. It should be noted that Rp-cAMPs is not a specific activator of \(f\)-channels. It is a permeable cAMP analog that can interact with the cyclic nucleotide binding domain of pacemaker channels, but also can act as a PKA inhibitor. Any PKA inhibiting action of Rp-cAMPs in the present study would be expected to reduce rate if anything, via actions on Ca\(^{2+}\) handling and/or Ca\(^{2+}\) current. Thus, at worst our observed effect of Rp-cAMPs on rate is an underestimate of the actual contribution of \(I_f\) to autonomic rate modulation. In fact, it has been reported that inhibition of PKA reduces basal rate in SAN cells by a reduction in local Ca\(^{2+}\) release [26]. We did not observe this effect with Rp-cAMPs, presumably because the direct effect of this agent to enhance \(I_f\) and rate overwhelmed any effect due to inhibition of PKA.

Although the evidence that different agents modify action potential shape similarly does not prove that they have equivalent modes of action, the indication that two rate-modulating processes such as \(I_f\) and Ca\(^{2+}\) transients act in distinctly separable ways allows agents to be classified according to their effects on action potential shape. Since different “signatures” are incompatible with an equivalent underlying mechanism, our data rule against the view that autonomic stimuli and Ca\(^{2+}\) transients act through common pathways in rate modulation. Indeed, at the concentration used, \(\beta\)-adrenergic and muscarinic agonists modify rate predominantly by altering early diastolic depolarization slope, consistent with these agents acting primarily through modulation of the pacemaker current, \(I_f\). This does not exclude the possibility that at higher concentrations autonomic agonists can also affect SR Ca\(^{2+}\) release. The observation that autonomic agonists primarily modify early diastolic depolarization slope is consistent with the proposal that they act by targeting pacemaker channels. This agrees with our previous studies on the dose dependence of ACh and Iso actions on channels involved in pacemaker activity and on rate. We found that ACh inhibited \(I_f\) at lower concentrations than those required to modify the ACh activated K\(^+\) current or the L-type Ca\(^{2+}\) current, and that rate also was modified at these low concentrations [25], although the exact threshold of ACh concentration at which the \(I_{K, ACh}\) current is activated is a matter of debate [27]. We also found that while Iso modified \(I_f\) and L-type Ca\(^{2+}\) current at comparable concentrations, the enhancement of L-type Ca\(^{2+}\) current largely affected late diastolic potential [28]. Thus, modulation of \(I_f\) appears to be the main mechanism by which moderate concentrations of autonomic agonists modify spontaneous rate in isolated SAN myocytes. Of course, at higher concentrations, where more marked rate effects are seen, other mechanisms can contribute. For example, at sufficient concentrations the ACh effect on K\(^+\) current is apparent in a
more negative MDP [25]. Furthermore, our data do not rule out that mechanisms other than \( I_f \) also affect EDD during autonomic agonist modulation.

The observation that autonomic agonists typically modulate rate via an \( I_f \)-dependent mechanism does not mean that \( Ca^{2+} \) dependent mechanisms do not contribute to SAN automaticity. Indeed, the evidence that \( Ca^{2+} \) sparks or transients can impact basal SAN automaticity is compelling [7–11,26]. In addition, the T- and L-type currents are important determinants of action potential shape and duration as any reduction of the net inward current flow during diastolic depolarization is expected to slow spontaneous rate. Furthermore, these currents are essential in replenishing the source of \( Ca^{2+} \) transients.

In recent work, rhythmic \( Ca^{2+} \) transients with a frequency close to that of spontaneous electrical activity have been demonstrated in SAN cells even during voltage clamp, leading to the suggestion that they represent an independent mechanism able to drive, via regulation of the \( Na^- \)–\( Ca^{2+} \) exchange current, the pacemaker function of these cells [29]. However, \( Ca^{2+} \) transients have been reported to affect the latest part of the diastolic depolarization [12,19,20] in agreement with the view that this mechanism impacts more on events following the EDD than on events determining the slope of EDD itself.

While disruption of \( Ca^{2+} \) dependent mechanisms clearly negatively impacts basal automaticity, these mechanisms are not necessarily the primary contributors to the fine beat-to-beat modulation of rate. The beat-to-beat modulation of rate appears to largely involve modification of \( I_f \). This is not surprising in that this current is ideally suited for this role. First, it flows almost exclusively during diastole [30], so that any modification will preferentially affect rate and not action potential duration or other portions of the action potential. Second, because it binds cAMP directly [21] it can adjust rapidly to demands for a change in rate and is less susceptible to disruption of this function than targets that also involve kinases and phosphatases in their pathway. It is interesting to note that our data fully agree with evidence that HCN4 knock-out mice have lost cAMP-dependent modulation of heart rate [31].

5. Conclusions

Our study has compared two mechanisms proposed to contribute to rate modulation and has shown that the mechanism relying on \( I_f \) can explain changes of action potential parameters induced by autonomic transmitters. A possible limitation of this study is that all data refer to rabbit myocytes; since species differences are known to exist with regard to \( Ca^{2+} \) handling [32] a different degree of complexity may exist in other species. A further limitation is that the evidence that \( I_f \) modulation and autonomic transmitters cause similar changes in action potential configuration is not sufficient, by itself, to prove equivalent mechanisms of action. Clearly however, lack of changes of action potential threshold rules against a strong contribution of \( Ca^{2+} \) transients to autonomic rate regulation. While other mechanisms can play a role, the \( I_f \)-based mechanism appears the simplest and most direct contributor to rate modulation by autonomic transmitters under our experimental conditions.

In summary, our data are consistent with the idea that the diastolic depolarization is contributed to during its full development by two major mechanisms. The first one is activation of \( I_f \) and represents the initiation of the diastolic depolarization. \( I_f \) controls the early part of diastolic depolarization and governs the autonomic regulation of rate via changes in the slope of this fraction of diastolic potential. The second mechanism involves \( SR Ca^{2+} \) transients and intervenes in late diastole and determination of action potential threshold. This mechanism has been investigated in detail by experiments analyzing the effects on rate of changes in \( SR Ca^{2+} \) transients [29]. Interpretation of this mechanism involves the entry of \( Ca^{2+} \) through T-type \( Ca^{2+} \) channels in late diastole, activation of \( Ca^{2+} \) sparks and subsequent activation of \( Na^- \)–\( Ca^{2+} \) exchange current according to a regenerative process whose final aim is to increase the probability of action potential firing. The relevance of this mechanism to development of the last part of the diastolic depolarization may explain a depolarizing shift of AP threshold upon inhibition of \( SR Ca^{2+} \) transients.

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