I\textsubscript{f}-dependent modulation of pacemaker rate mediated by cAMP in the presence of ryanodine in rabbit sino-atrial node cells

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Abstract

I\textsubscript{f} contributes to generation and autonomic control of spontaneous activity of cardiac pacemaker cells through a cAMP-dependent, Ca\textsuperscript{2+}-independent mechanism of rate regulation. However, disruption of Ca\textsuperscript{2+} release from sarcoplasmic reticulum (SR) by ryanodine (Ry) has been recently shown to slow spontaneous rate and inhibit \beta\textsubscript{AR}-induced rate acceleration, leading to the suggestion that the target of \beta\textsubscript{AR} modulation of pacemaking is the intracellular Ca\textsuperscript{2+}-regulatory process. We have investigated whether the Ry-induced decrease of \beta\textsubscript{AR} rate modulation alternatively involves disruption of the \beta\textsubscript{AR}-adenylate-cyclase-cAMP-I\textsubscript{f} mechanism. Prolonged exposure to Ry (3 \mu M, >2 min) slowed spontaneous rate of pacemaker cells by 29.8\% via a depolarizing shift of take-off potential (TOP) without significantly changing early diastolic depolarization rate. Ry depressed rate acceleration caused by isoproterenol (Iso) (1 \mu M, 23.6\% in control vs. 8.0\%), but did not modify that caused by two membrane-permeable cAMP analogs, CPT-cAMP (300 \mu M, 17.7\% vs. 17.3\%) and Rp-cAMPs (50 \mu M, 18.0\% vs. 20.6\%). Consistent with the rate effect, exposure to Ry decreased the shift induced by Iso, but not that induced by either cAMP analog on the I\textsubscript{f}-activation curve. We conclude that disruption of Ry receptor function and SR Ca\textsuperscript{2+} release depresses \beta\textsubscript{AR}-induced modulation of heart rate, but does not impair cAMP-dependent rate acceleration mediated by I\textsubscript{f}. However, abolishment of normal Ca\textsuperscript{2+} homeostasis may result in the failure of \beta\textsubscript{AR} agonists to sufficiently elevate cAMP near f-channels. The molecular basis for Ca\textsuperscript{2+}-dependent interference in \beta\textsubscript{adrenergic} signaling remains to be determined.

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1. Introduction

Cardiac pacemaking is a complex event that requires the combined contribution of several ion channel and ion transport mechanisms. Typical of cardiac pacemaker cells is a slow (diastolic or pacemaker) depolarization that at the end of an action potential drives membrane voltage towards threshold for a new action potential, thus ensuring spontaneous, repetitive activity. The diastolic depolarization of pacemaker cells, as well as determining spontaneous activation, allows for atrial chamber refilling and ensures an appropriate chronotropic function perfectly tailored to cardiac metabolic requirements. It is precisely because of this function as a chronotropic spacer that most of the regulatory mechanisms of cardiac rate are targeted to modify the currents that determine the slow diastolic depolarization. Among these, a prominent role is played by the I\textsubscript{f} current [1,2].

While I\textsubscript{f}-mediated autonomic modulation of rate has long been established and characterized [3-5], a newer mechanism able to affect pacemaking rate, and based on the contribution of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, has been recently proposed by several groups [6,7]. According to this mechanism, subsarcolemmal Ca\textsuperscript{2+} released transiently from the sarcoplasmic reticulum (SR) activates the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger that, by being electrogenic, carries an inward current and thus contributes to the last fraction of diastolic depolarization of pacemaker cells [10]. The same cascade of events has also been implicated in the \beta\textsubscript{adrenergic} receptor (\beta\textsubscript{AR})-induced modulation of pacemaker rate, based on evidence that \beta\textsubscript{AR} stimulation increases both SR Ca\textsuperscript{2+} transients and rate, and...
that disruption of the function of ryanodine receptors (RyRs) strongly slows spontaneous activity and abolishes both SR Ca\(^{2+}\) transients and βAR-mediated rate acceleration [6,11,12].

Some of these studies rely on the use of ryanodine (Ry), which at low concentrations (<10 µM), forces RyRs in the open state and depletes SR Ca\(^{2+}\) stores, resulting in the abolishment of SR Ca\(^{2+}\) release and Ca\(^{2+}\) transients[6,9,12]. However, SR Ca\(^{2+}\) depletion and the subsequent disruption of normal SR Ca\(^{2+}\) homeostasis may alter the normal mechanism of βAR-dependent rate regulation if any elements of the signaling cascade are Ca\(^{2+}\)-sensitive in sino-atrial node (SAN). We, therefore, used Ry (3 µM) to address a two-fold question:

1. How does impairment of Ca\(^{2+}\) release by Ry decrease spontaneous rate? This question originated from the observation that there is no agreement in the literature as to whether Ry decreases the slope of the full length of diastolic depolarization [11] or only the late fraction [13].

2. Is the \(I_{f}\)-dependent rate-regulation mechanism still functional after inhibition of the SR Ca\(^{2+}\) transients? Since \(I_{f}\) channels are not directly regulated by Ca\(^{2+}\) ions [14], abolition of any \(I_{f}\)-dependent mechanism by inhibition of SR Ca\(^{2+}\) release requires that at least one of the steps of the cascade βAR-adenylate-cylase-cAMP-f-channels is impaired by disruption of normal subsarcolemmal Ca\(^{2+}\) homeostasis [15].

Our results show that while βAR-mediated modulation of rate and \(I_{f}\) are impaired following Ry, the \(I_{f}\)-dependent modulation of rate of SAN cells mediated by cAMP elevation is functional even after prolonged exposure to Ry, and is quantitatively similar to that in control conditions.

2. Materials and methods

Animal protocols conformed to the guidelines of the care and use of laboratory animals established by Italian (DL 116/1992) and European Directives (86/609/CEE). Rabbits (weighing 0.8-1.2 kg) were anesthetized by intramuscular injection of a mixture of xilazine (4.6 mg kg\(^{-1}\)) and ketamine (60 mg kg\(^{-1}\)) (Sigma-Aldrich Co) and euthanized by cervical dislocation and exsanguination. The heart was quickly removed and the SAN tissue dissociated into single cells and small clusters of cells using a two-step (enzymatic and mechanical) procedure described previously [16]. During experiments, cells were placed in a chamber on an inverted microscope and superfused at 34 ± 0.5 °C via a fast perfusion device that permitted exchange of the solution near the cell in <1 s. External Tyrode solution contained (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 5 Hepes-NaOH, 10 D-glucose, pH 7.4. The pipette solution contained (in mM): 130 K-aspartate, 10 NaCl, 2 CaCl\(_2\), (pCa = 7), 2 MgCl\(_2\), 10 Hepes, 5 EGTA, 2 ATP(Na\(_2\)), 0.1 GTP, 5 creatine phosphate, pH 7.2. The whole-cell configuration of the patch clamp was employed in all experiments. In current-clamp studies of spontaneous rate, small clusters consisting of two to six cells were often employed since these more consistently maintained a constant spontaneous beating frequency than normally did single cells. All voltage-clamp studies of \(I_{f}\) were conducted on single cells, with 1 mM BaCl\(_2\) and 2 mM MnCl\(_2\) added to the external solution.

In studying the effect of Ry on the ability of isoproteorenol (Iso) or cAMP analogs to modulate rate, we employed a protocol involving two successive exposures to agonist in the same cell, before and during ryanodine superfusion. In the presence of Ry (3 µM), exposure to the test substance (Iso or cAMP analogs) was performed after a suitable time, when the spontaneous rate had stabilized following addition of Ry (2–3 min).

All data were acquired with pClamp 7 software and an Axopatch 200B amplifier (Axon Instruments). For current-clamp studies, data were acquired at 2 kHz and filtered at 1 kHz, then processed with custom software to measure the interval between successive action potential upstrokes and convert this to a continuous beat-to-beat measure of frequency. Rate effects were measured as the peak change during the period of drug superfusion. Voltage-clamp data were acquired at 0.66 kHz.

Shifts of the \(I_{f}\)-activation curve induced by Iso and cAMP analogs (see Fig. 6) were obtained by a method which did not require measurement of the conductance-voltage relation. Trains of hyperpolarizing steps (1/6 Hz) were applied to near the mid-activation voltage; upon perfusion with cAMP analogs, the holding potential (~35 mV in the control solution) was adjusted until the cAMP-induced current increase during the hyperpolarizing step was compensated and the control current size fully restored. The measurement of the displacement of the holding potential (in mV) allowed the estimation of the shift of the activation curve [17,18].

Iso and CPT-cAMP were obtained from Sigma-Aldrich Co, and Ry and Rp-cAMPS were obtained from Calbiochem. Statistical differences were determined at the \(P < 0.05\) level by Student’s \(t\)-test. Data points were plotted as mean ± SEM values.

3. Results

3.1. Ryanodine slows spontaneous rate

Cells were initially perfused with a control Tyrode solution and spontaneous activity was recorded from either single cells or small clusters. As shown in [Fig. 1a] addition of Ry (3 µM) to the bath solution typically yielded a biphasic effect with an initial transient increase in firing activity, followed by a slower decrease [Fig. 1b]. The transient increase in rate was not further studied. The rate of decay of the decrease, when fitted by a single exponential, had a time constant of 61.6 ± 15.1 s (\(n = 6\). As illustrated in [Fig. 1b] when traces were superimposed, the rate of diastolic depolarization in control did not differ substantially from that of the first fraction of diastolic depolarization after Ry. The action potential dura-
The modifications we observed are in agreement with previous observations [12,13] although other data in the literature do not distinguish between early and late phases of diastolic depolarization and report a decrease of the slope of diastolic depolarization by ryanodine [11]. Our data suggest that the major changes induced on action potential parameters by a reduction in the SR Ca2+ transients concern the late fraction of diastolic depolarization and the TOP.

### 3.2. Isoproterenol-induced rate acceleration is depressed by ryanodine

To verify whether a long exposure to Ry affected the frequency response to Iso, we applied 1 µM Iso (a concentration known to cause nearly maximal rate responses [11,19]) before and during perfusion with 3 µM Ry, when rate reduction had approached steady state (Fig. 2).

As apparent by comparing the time course of frequency during Iso exposure [Fig. 2a] and the Iso-induced modification of spontaneous action potentials before and after treatment with Ry [Fig. 2b], Ry depressed but did not fully abolish the response to Iso. In n = 6 cells, Iso increased rate by 23.6 ± 2.4% in control conditions, and by 8.0 ± 2.7% after Ry (P < 0.05). The mean rate increase we recorded under control conditions was similar to data reported previously under similar conditions in isolated SAN cells [11] although higher rate increases have been reported in intact SAN tissue [20].

In separate control experiments (n = 4), where Ry was not employed, there was no significant difference between the rate increase during the first (22.5 ± 3.7%) and second exposure to Iso, applied about 150 s later (29.6 ± 5.8%, P > 0.05; inset to Fig. 2a). The effect of Ry on the Iso-induced frequency modulation can be interpreted to indicate that the Ca2+ transients associated with subsarcolemmal RyR activity represent the main mechanism in the β-adrenergic modulation of rate [11]. However, these results could also reflect a Ca2+ dependence of the previously established βAR-adenylate-cyclase-cAMP-I f mechanism for modulation of heart rate [15].

### 3.3. Ryanodine does not impair rate acceleration by CPT-cAMP

As a way to verify if cAMP-dependent processes affecting rate are still operating after treatment with Ry, we analyzed
the action on rate of a membrane-permeable cAMP analog, CPT-cAMP, before and after Ry (Fig. 3). If SR Ca\(^{2+}\) regulation is the ultimate target by which \(\beta\)AR activation accelerates rate, than CPT-cAMP should be ineffective in the presence of Ry. Alternatively, if Ry exposure results in a lesion elsewhere in the adenylate-cyclase cascade such that Iso fails to elevate cAMP and/or modulate \(I_f\), then CPT-cAMP should bypass that lesion and restore acceleration of rate.

CPT-cAMP (300 µM) was first tested in control conditions, where it elicited a reversible increase in spontaneous rate by 17.0% (from 3.29 to 3.85 Hz). When CPT-cAMP was re-applied after Ry had reached a steady-state effect, it was still able to accelerate rate by 14.7% (from 2.59 to 2.97 Hz). In \(n = 6\) cells, CPT-cAMP accelerated rate by 17.7 ± 2.0% in control conditions, and by 17.3 ± 1.6 after exposure to 3 µM Ry (not significantly different, \(P > 0.05\)). Note that the response to Iso is depressed after Ry. The rate increased by 30.3% in control and by 8.1% during Ry perfusion.

These results suggest that cAMP is able to modulate sinus rhythm in the absence of a functionally active SR Ca\(^{2+}\)-handling mechanism. A direct activation of \(f\)-channels by CPT-cAMP is likely to have a role in the accelerating effect on rate in Fig. 3. However, CPT-cAMP could also affect the activity of channels contributing to the diastolic depolarization through phosphorylation by cAMP-dependent kinases (PKA); these include \(f\)-channels, L-type Ca\(^{2+}\) channels and delayed K\(^+\) channels.[22–28]

3.4. Ryanodine does not impair rate acceleration by Rp-cAMPS, a cAMP analog ineffective on PKA

To further restrict our investigation to phosphorylation-independent mechanisms involved in the modulation of SAN rate by cAMP, we used the cAMP analog Rp-cAMPS. Rp-cAMPS lacks the ability to activate cAMP-dependent kinases, although it activates \(f\)-channels directly by binding to the channel cAMP-binding site.[18] The effect of Rp-cAMPS (50 µM, a concentration known to nearly maximally activate \(f\)-channels[18]) on spontaneous activity was evaluated before and after treatment with Ry.

As apparent in the example of Fig. 4, Ry did not decrease the effectiveness of Rp-cAMPS in speeding rate. In the cell
shown, Rp-cAMPs (50 µM) accelerated rate by 22.3% (from 3.36 to 4.11 Hz) in control conditions and by 30.0% (from 2.77 to 3.60 Hz) following Ry. In n = 4 cells, Rp-cAMPs increased spontaneous frequency by 18.0 ± 1.6% in control conditions, and by 20.6 ± 3.2% after Ry (not different, P > 0.05). As with CPT-cAMP, Rp-cAMPs appeared to affect rate by acceleration of the diastolic depolarization with no substantial modification of the action potential duration (Fig. 4b). Also, Rp-cAMPs modified, like CPT-cAMP, the DD1 rate: in n = 4 cells, this was 104.1 ± 17.4 mV/s in control and 130.2 ± 20.6 mV/s in the presence of Rp-cAMPs (significantly different, P < 0.05).

The results obtained on spontaneous rate with Iso, CPT-cAMP and Rp-cAMPs (Figs. 2–4) are summarized in the bar graphs of Fig. 5. The data clearly indicate that the Ry treatment, while strongly inhibiting the Iso-induced rate modulation, is ineffective on rate changes induced by membrane-permeable cAMP analogs. The accelerating actions of CPT-cAMP and Rp-cAMPs are comparable, suggesting that cAMP-dependent phosphorylation processes do not play a major role in the rate-controlling action of cAMP. In the absence of Ry, the mean Iso-accelerating effect is larger than that of the cAMP analogs, although the difference does not reach the significance level (P > 0.05).

3.5. Ryanodine impairs modulation of I_f current by Iso, but not by cAMP analogs

The experimental data collected so far agree with previous evidence pointing to I_f as a major effector of the cascade responsible for cAMP-mediated rate regulation by β-adrenergic [1,16] and muscarinic stimulation [5,29]. Therefore, if the effects of cAMP analogs on rate are due to stimulation of I_f, an increase in I_f should be measured independently of Ry treatment upon perfusion of these analogs. On the contrary, I_f modulation by Iso should be inhibited by long exposure of cells to Ry. This was investigated in Fig. 6.

In Fig. 6a, I_f was recorded during trains of hyperpolarizing steps from a holding potential of -35 to -80 mV and Iso applied before (left) and after perfusion with Ry (474 s, right). Exposure to Ry decreased the response to Iso (see legend to Fig. 6). In n = 5 cells, Iso shifted the I_f-activation curve by 7.3 ± 0.5 mV in control conditions, and by 3.7 ± 0.7 mV after Ry (significantly different; see Fig. 6d). We next measured the effect of Ry treatment on activation curve shifts caused by cAMP analogs (sample records in Fig. 6bc). As shown by bar graphs in Fig. 6d, CTP-cAMP shifted the I_f-activation curve by an average 7.8 ± 0.7 mV in control conditions, and by 7.3 ± 0.6 mV after Ry (n = 4); similarly, the shift induced by Rp-cAMPs was 7.7 ± 1.3 mV in control conditions, and 7.9 ± 1.2 mV after Ry (n = 4); for both analogs the shifts before and after Ry were not significantly different (P > 0.05).
The increase in local Ca$^{2+}$ can be detected at times preceding the fluorescence methods indicates that Ca$^{2+}$ sparks and an inflow to–outflow of Ca$^{2+}$ from the sarcoplasmic reticulum (SR) may be coupled to a mechanism aimed at ensuring a safer kick of the systolic depolarization phase and rate slowing$^{[6,9,10]}$. The picture that emerges by these and other results is the following$^{[10,30]}$: (1) during the last third of pacemaker depolarization, Ca$^{2+}$ sparks localized in subsarcolemmal spaces are elicited by Ca$^{2+}$ entering through T-type Ca$^{2+}$ channels; (2) local elevation of Ca$^{2+}$ activates an inward current, identified as the Na$^^{+}$–Ca$^{2+}$ exchange current$^{[31]}$; (3) the depolarizing effect of the inward current further promotes voltage-dependent activation of T-type Ca$^{2+}$ entry and Ca$^{2+}$ sparks, causing a positive feedback mechanism, which “boosts” depolarization up to action potential threshold.

An intrinsic consequence of the set of events described above is that any process affecting the subsarcolemmal Ca$^{2+}$ transients during activity will affect rate. Since β-adrenergic stimulation typically increases the Ca$^{2+}$ transients$^{[6,11]}$, this has led to the proposal that the release of SR Ca$^{2+}$ is a main mechanism for pacemaker rate modulation by β-adrenergic input$^{[6,11]}$. Based on data from embryonic stem (ES) cells, where RyR had been functionally knocked out, this view has recently been extended to the more general hypothesis that RyRs act as modulators of spontaneous rate by regulation of the slope of diastolic depolarization$^{[32]}$. Although the contribution to automaticity of localized RyR-mediated Ca$^{2+}$ release is apparent, the proposal that this process is a main mechanism controlling pacemaker rate and its autonomic modulation$^{[11,32]}$ in a physiological setting contrasts with several observations.

A first observation is that RyR-mediated Ca$^{2+}$ concentration changes only affect the last fraction of diastolic depolarization. This appears by using voltage steps or voltage ramps to analyze the timing of changes induced by alterations of the Ca$^{2+}$ release process$^{[7,10]}$. As seen by fluorescence measurements indicating that subsarcolemmal Ca$^{2+}$ sparks due to RyR activity just precede action potential firing$^{[7,9,35]}$, the contribution to automaticity of localized RyR-mediated Ca$^{2+}$ release is apparent, the proposal that this process is a main mechanism controlling pacemaker rate and its autonomic modulation$^{[11,32]}$ in a physiological setting contrasts with several observations.

A second observation concerns the existence of known rate-controlling processes that do not directly depend upon Ca$^{2+}$ release from the SR. In particular, the adenylate-cyclase-cAMP–I$i$ process$^{[5,29]}$ operates by the direct action of cAMP on f-channels$^{[21]}$ whose stimulation/inhibition contributes to acceleration/slowing of the diastolic depolarization$^{[1,2]}$. Modification of rate by the I$i$ mechanism involves changes of early as well as late portions of the diastolic depolarization, in agreement with a key role of I$i$ in generating this phase$^{[1]}$. Although intracellular Ca$^{2+}$ has

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**Fig. 6.** The effect of Iso and of cAMP analogs on $I_f$ in control cells and in cells exposed to Ry. (a) Sample traces showing the action of Iso (1 µM) on $I_f$ recorded during trains (1/6 s) of hyperpolarizing steps from –35 to –80 mV in control conditions (left) and after exposure to Ry 3 µM (474 s, right). (b), (c) Similar protocols showing the action of CPT-cAMP (b, 300 µM), and Rp-cAMPs (c, 50 µM) on $I_f$ recorded during hyperpolarizing steps from –35 to –80 mV in control (left) and after Ry 3 µM (right, 222 and 252 s for CPT-cAMP and Rp-cAMPs, respectively). (d) Bar graphs of shifts of current-activation curve, measured as explained in the methods, were (mV): 7.4, 1.9 (a); 6.6, 6.2 (b); 11.3, 10.4 (c) in control and after Ry, respectively. (d) Bar graphs of shift of $I_f$-activation curve (in mV) by Iso (n = 5), CPT-cAMP (n = 4) and Rp-cAMPs (n = 4) in control and after Ry (see Section 2 for details); * indicates significant difference between +Ry and corresponding –Ry data.

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**4. Discussion**

Previous work from several laboratories has indicated that the Ca$^{2+}$ transients due to rhythmic release from the SR are involved in the regulation of the rate of spontaneous activity in pacemaker cells. This conclusion is based on several observations both in amphibian and mammalian pacemaker cells. Measurement of subsarcolemmal Ca$^{2+}$ transients by fluorescence methods indicates that Ca$^{2+}$ sparks and an increase in local Ca$^{2+}$ can be detected at times preceding the large Ca$^{2+}$ peak due to Ca$^{2+}$ entry through L-type channels, which is responsible for the action potential upstroke$^{[7,9]}$. As shown in the transitional SAN cells, these early Ca$^{2+}$ transients are probably associated with activity of T-type Ca$^{2+}$ channels, since they are activated by low-voltage step or ramp depolarizations, and are inhibited by nicardipine$^{[10]}$. Inhibition of the Ca$^{2+}$ transients (by nicardipine or Ry) leads to a prolongation of the diastolic depolarization phase and rate slowing$^{[6,9,10]}$. The picture that emerges by these and other results is the following$^{[10,30]}$: (1) during the last third of pacemaker depolarization, Ca$^{2+}$ sparks localized in subsarcolemmal spaces are elicited by Ca$^{2+}$ entering through T-type Ca$^{2+}$ channels; (2) local elevation of Ca$^{2+}$ activates an inward current, identified as the Na$^^{+}$–Ca$^{2+}$ exchange current$^{[31]}$; (3) the depolarizing effect of the inward current further promotes voltage-dependent activation of T-type Ca$^{2+}$ entry and Ca$^{2+}$ sparks, causing a positive feedback mechanism, which “boosts” depolarization up to action potential threshold.

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been reported to affect \( I_{f} \) [34]; this is likely to involve slow, phosphorylation-dependent regulation of channel turnover [24,27] since a fast, direct action of \( Ca^{2+} \) on \( f \)-channels able to affect their activity in a beat-to-beat fashion has not been observed [14].

A third, related observation concerns the action of \( \beta \)-adrenergic stimulation. Data in the literature indicate that disruption of the RyR function and of SR \( Ca^{2+} \) release inhibits \( \beta \)AR modulation of pacemaker rate [6,11,12]. This indicates that normal SR \( Ca^{2+} \) release/uptake turnover is essential for a correct functioning of the \( \beta \)-adrenergic cascade, but does not demonstrate that the localized \( Ca^{2+} \) increase is a direct step in the chain of events leading from \( \beta \)AR stimulation to acceleration of diastolic depolarization. For example, if any of the links in this chain of events depends upon normal \( Ca^{2+} \) homeostasis, its disruption is bound to affect the entire cascade [15]. Failure of \( \beta \)AR stimulation to modify pacemaker rate in the presence of Ry does not, therefore, necessarily imply that the \( \beta \)AR modulation of \( I_{f} \) has no role in the \( \beta \)AR modulation of rate, but may more simply indicate that at least one of the steps involved is inhibited by disruption of normal \( Ca^{2+} \) homeostasis.

Here we have investigated the integrity of the cAMP-\( I_{f} \) mechanism in the regulation of pacemaker rate. Our data show that this mechanism is still functional, and is able to speed the rate of spontaneous activity of SAN pacemaker cells, following prolonged exposure to Ry. We found that Ry inhibits the accelerating action of Iso, but not of cAMP analogs. In this action, CPT-cAMP and Rp-cAMPSs are equivalent, indicating that phosphorylation processes do not play a significant role. This argues against cAMP analogs acting by increasing Na–Ca exchange via elevation of intracellular \( Ca^{2+} \) mediated by L-type \( Ca^{2+} \) current.

In accordance with the data on spontaneous rate, we also found that Ry inhibits the activating action of Iso on \( I_{f} \), represented by a depolarizing shift of the current-activation curve [35], but does not affect the action of cAMP analogs [Fig. 6]. These results imply that:

1. the cAMP-\( I_{f} \) mechanism is still functional;
2. \( I_{f} \) stimulation can modulate rate independently of SR \( Ca^{2+} \) transients;
3. normal \( Ca^{2+} \) homeostasis is essential for the normal function of \( \beta \)AR-adenylate-cyclase-signaling cascade.

The previous observation that Iso increases L-type \( Ca^{2+} \) current in the presence of Ry [11] does not contradict this conclusion that the adenylate-cyclase cascade is disrupted, since there are potential cAMP-independent mechanisms by which Iso could increase \( I_{Ca,L} \) [36,37].

Our data also suggest that the major mechanism by which activity of the RyR, and the associated SR \( Ca^{2+} \) transients, affect rate is by modification of the TOP, while the slope of early diastolic depolarization is little affected. Entry of \( Ca^{2+} \) through T-type \( Ca^{2+} \) channels in the late fraction of diastolic depolarization is known to favor \( Na^{+}–Ca^{2+} \) exchange-dependent depolarization through stimulation of SR \( Ca^{2+} \) release [10] thus, a displacement of TOP to more positive levels is expected to result from inhibition of SR \( Ca^{2+} \) transients.

According to this view, SR \( Ca^{2+} \) release may represent a “safety” mechanism, whose main aim is to ensure the attainment of action potential threshold, and thus contribute to rate regulation in connection with other mechanisms involved more specifically in regulation of the early phase of diastolic depolarization.

Our data highlight the presence of two pathways involved in \( \beta \)AR-mediated rate regulation:

1. the \( \beta \)AR-adenylate-cyclase-cAMP-\( I_{f} \) mechanism, which operates to affect rate by modulation of diastolic depolarization and
2. SR \( Ca^{2+} \) cycling, whose function is necessary for the above mechanism to work properly and which affects rate by alteration of threshold.

The evidence that Iso fails to fully activate \( I_{f} \) and modify rate in the presence of Ry, whereas CPT-cAMP continues to be effective, shows that abolishment of SR \( Ca^{2+} \) cycling impairs one of the steps leading from \( \beta \)-receptor stimulation to elevation of cAMP in the vicinity of \( f \)-channels.

An intriguing question arising from our results is why \( \beta \)AR stimulation fails to effectively activate the adenylate-cyclase-cAMP-\( I_{f} \) cascade after Ry. It is interesting to note that a substantial response to \( \beta \)-stimulation is observed in intact SAN, leading to the suggestion that regional differences may exist in the action of Ry [20].

Since under our conditions cAMP analogs are still able to speed rate, and this action involves direct cAMP interaction with \( f \)-channels independent of any role of PKA and phosphorylation, the action of Ry is likely to be at the level of impairing \( \beta \)AR-induced elevation of cAMP, either globally or in a sub-cellular compartment associated with \( f \)-channels. Although no information is available concerning compartmentation of \( f \)-channels, there are examples in the literature of sub-cellular localization of other ionic channels [38–40] as well as elements of the \( \beta \)AR-adenylate cyclase or muscarinic-adenylate-cyclase-signaling cascades [41–43].

Disruption of SR \( Ca^{2+} \) transients could lead to redistribution or modification of \( f \)-channels such that they no longer are associated with \( \beta \)AR-dependent elevations in cAMP, or to perturbation of a \( Ca^{2+} \)-dependent element of the cAMP-signaling cascade. For example, while the dominant cardiac adenylate-cyclase isoforms (Types V and VI) are inhibited by \( Ca^{2+} \) [43–45], other isoforms are stimulated by \( Ca^{2+} \) (e.g., Types I, III and VIII [43–45]). Message for both Types III [46,47] and VIII [48] adenylate cyclase have been reported in heart, and if either isoform is localized to the SAN then depletion of SR \( Ca^{2+} \) by Ry could result in inhibition of adenylate-cyclase function and disruption of \( \beta \)AR signaling.

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