Pacemaker activity in the insect (T. molitor) heart: role of the sarcoplasmic reticulum

Danielle F. Feliciano,1 Rosana A. Bassani,1 Pedro X. Oliveira,1,2 and José W. M. Bassani1,2

1Center for Biomedical Engineering, and 2Department of Biomedical Engineering, School of Electrical and Computer Engineering, University of Campinas, Campinas, Brazil

Submitted 22 February 2011; accepted in final form 11 September 2011

Feliciano DF, Bassani RA, Oliveira PX, Bassani JWM. Pacemaker activity in the insect (T. molitor) heart: role of the sarcoplasmic reticulum. Am J Physiol Regul Integr Comp Physiol 301: R1838–R1845, 2011. First published September 14, 2011; doi:10.1152/ajpregu.00089.2011.—The electrophysiological properties of the myogenic cardiac cells of insects have been analyzed, but the mechanisms that regulate the pacemaker activity have not been elucidated yet. In mammalian pacemaker cells, different types of membrane ion channels seem to be sequentially activated, perhaps in a cooperative fashion with the current generated by Ca2+ extrusion mediated by the electrogenic Na+/Ca2+ exchanger, which is sustained by the diastolic sarcoplasmic reticulum (SR) Ca2+ release. The objective of the present work was to investigate the role of the SR function on the beat rate (BR), and BR modulation by extracellular Ca2+ concentration ([Ca2+]o) and neurotransmitters in the in situ dorsal vessel (heart) of the mealworm beetle Tenebrio molitor. The main observations were as follows: 1) basal BR was reduced by 50% by inhibition of SR function, but not affected by perfusion with CsCl or ZD7288; 2) spontaneous activity was abolished by Cd2+; 3) a robust positive chronotropic response could be elicited by serotonin (5-HT), but not to norepinephrine or carbamylcholine; 4) SR inhibition abolished the sustained chronotropic stimulation by [Ca2+]o elevation and by 5-HT, while the latter was unaffected by CsCl. It is concluded that, in T. molitor heart, BR is markedly, but not exclusively, dependent on the SR function, and that BR control and modulation by both [Ca2+]o, and 5-HT requires a functional SR.

Heart pacemaker activity is a basic physiological process considered as one of the fundamental issues in cardiac electrophysiology. Despite the wide morphological diversity observed in insect circulatory organs (14), the heart is essentially the abdominal part of the dorsal vessel, which admits the hemolymph during diastole through valvulated openings called ostia and pumps it toward the head through the thoracic part of the vessel, the aorta, during systole. Hemolymph then flows from the head to the posterior/caudal regions of the body, bathing all organs, and finally returning to the heart during the next diastole (27). It is accepted that the spontaneous electrical activity normally originates at the posterior end of the heart tube, traveling in an undulatory pattern to the anterior region, as, for instance, described in the mealworm beetle, Tenebrio molitor (14, 23).

The insect heart is considered to be essentially myogenic, i.e., the electrical activity is generated by its striated muscle cells, rather than by a well-defined pacemaker structure, and subjected to chronotropic and inotropic modulation by neurotransmitters, hormones, and other cardioactive peptides (25, 27, 38). Although the influence of extracellular Ca2+ concentration ([Ca2+]o) on the action potential (AP) time course and cell spontaneous activity has been examined (8, 23, 24), the literature lacks information on the specific mechanisms responsible for the generation of the spontaneous activity in the insect heart.

In mammalian sinoatrial pacemaker, different sets of membrane ion channels are gradually activated in a complex, nonlinear dynamic interaction (19), which seems to include the diastolic extrusion of Ca2+ released from the sarcoplasmic reticulum (SR), coupled to the electrogenic influx of Na+ via the Na+/Ca2+ exchanger (NCX) (12, 21, 34). Whether the SR-dependent current or the so-called pacemaker current (Ip) plays a more central role in determining the spontaneous nature of the heartbeat is still a matter of debate (10, 16, 20, 22).

Heart cells of insects are known to present SR (35), which is less abundant (27) compared with the well-developed, T-tubule-associated organelle found in the insect skeletal muscle (13, 40) or in vertebrate cardiac cells, especially mammals (4). However, such morphological information is insufficient to allow the conclusion that SR Ca2+ handling is not important for the generation of myocyte spontaneous activity in insect heart.

In the present work, we studied the partially isolated heart of the mealworm beetle (Tenebrio molitor), aiming at investigating whether SR Ca2+ release contributes to the pacemaker activity and to modulation of beating rate (BR) by neurotransmitters and [Ca2+]o.

METHODS

T. molitor beetles were reared in colonies at the Center for Biomedical Engineering of the University of Campinas (CEB/UNICAMP) and fed on fruit, potatoes, bran, and low-protein bird food supplement. Animals were kept at room temperature (25°C) under photoperiod of 12:12 h (light-dark) and were not manipulated before the experiments, which were carried out with 10- to 15-day old beetles of either sex.

The animal was decapitated, and the wings and legs were removed. An incision was made along the abdomen midline. A window was then opened to the abdominal cavity by trimming out the two sides of the exoskeleton. The digestive organs, glands, and abdominal muscles were removed to expose the dorsal vessel (23, 38). The animal was then placed on the dorsal side down to the bottom of a Plexiglas chamber, which consists of a glass coverslip covered by a thin layer of petroleum jelly to keep the animal steady during superfusion. The preparation was composed of the heart (i.e., dorsal vessel), some alary muscles, internal body muscles, and the dorsal cuticle. The whole preparation was continuously superfused (1 ml/min, at 23°C) with the Tenebrio molitor solution (TMS) with the following composition:

HEART PACEMAKER ACTIVITY

Address for reprint requests and other correspondence: J. W. M. Bassani, Centro de Engenharia Biomédica, UNICAMP, R. Alexander Fleming 181, Cid. Universitária Zéferino Vaz, 13083-881 Campinas, SP, Brazil (e-mail: bassani@ceb.unicamp.br).
(mmol/l): 274 NaCl, 19 KCl, 4 CaCl₂, 5 glucose, 5 HEPES; pH adjusted to 7.0 with NaOH (38).

The heart holder with the heart preparation was mounted on the stage of a light microscope (mod. III, Carl Zeiss) equipped with a charge-coupled device camera. The image of the heart was captured with a Pinnacle Studio DeLuxe 2 capture card running at 25 frames/s under control of the software Pinnacle Studio Plus 9.3 (Avid). To record the contractile activity, a sensor based on a light-dependent resistor was developed, so as to measure the amount of light transmitted in the center of the dorsal vessel at the caudal one-third of the abdomen (segment 5, 6, or 7 of the heart tube). Thus contraction amplitude could be recorded as the black edges of the vessel moved toward the center of the cardiac tube during systole. Tracings were obtained by using a LabView virtual instrument, to which a data-acquisition board NI USB-6008 (National Instruments, Austin, TX) interface was connected. The output was exported to an Excel spreadsheet for further analysis of the amplitude and frequency of the heart contractions.

In some experiments, electromgrams were recorded simultaneously with the mechanical activity, using low-impedance glass micropipette suction electrodes (23). An Ag-AgCl electrode was used as the reference. The tip of the micropipette was driven by a micromanipulator to contact either one of the segments (4 to 6) of the heart, and a small negative pressure was applied to improve tissue-electrode contact and minimize leak currents. Electromgrams were amplified using a high-input impedance differential preamplifier (10× gain) connected to a differential amplifier with variable gain (1, 10, or 100×), developed at CEB/UNICAMP. The signal was then filtered (0.5-Hz high-pass, 50-Hz low-pass filter) and acquired at 1 kHz.

After 40- to 60-min stabilization, preparations were superfused with different test solutions, as detailed below. A video movie (20- to 40-min duration) was recorded in all experiments, for offline analysis and documentation.

**BR dependence on [Ca²⁺]**. The preparations were exposed to TMS containing 0.1, 1, 2, 4, 8, or 16 mM CaCl₂, applied at a random fashion. Each different solution remained in contact with the preparation for at least 15 min. **Ca²⁺ transport inhibition.** SR Ca²⁺ uptake was blocked by the highly selective, irreversible inhibitor of the SR Ca²⁺-ATPase thapsigargin [TG, 100 μM (2, 33)], added to TMS immediately before use. Preliminary experiments showed that, at the concentration used, the vehicle (DMSO, <0.1%) did not significantly affect BR. The preparation was incubated with TG for 15 min in the dark, during which perfusion was interrupted. Measurements were performed after BR stabilization following TG washout. In some preparations, the response of the preparation to increase in [Ca²⁺], from 4 to 8 mM was investigated before and after TG treatment. In addition to TG, the selective, competitive SR Ca²⁺-ATPase inhibitor 2,5-tert-butyl-4,5-hydroquinone (BHQ, 30 μM) was used. BHQ was added to the superfusion solution and kept in contact with the preparation for at least 15 min.

The role of SR Ca²⁺ release was assessed by using the alkaloid rydandeine (Rya), which binds irreversibly to the SR Ca²⁺ release channels [Rya receptors (RyR)] with very high affinity and selectivity. At submicromolar concentrations, Rya is known to keep the SR release channels at a subconductance state. In this condition, Ca²⁺ leaks from the SR, which is progressively depleted of the ion. At high concentrations (0.3–2 mM), Rya is thought to block SR Ca²⁺ release (26, 32). We used a relatively high Rya concentration (100 μM), but still in the range that renders the SR leaky. Rya was washed out after a 30-min incubation period. Another procedure used to impair the SR function was the application of caffeine, added to the perfusion solution at the concentrations of 2 and 20 mM.

NCX-mediated Ca²⁺ extrusion was thermodynamically inhibited by perfusion with Na⁺-free TMS (equimolar replacement of NaCl with choline chloride).

**BR modulation by drugs.** The preparation was challenged with the muscarinic agonist carbachol, as well as with a relatively supramaximal concentration (1 μM) of norepinephrine or isoproterenol. Concentration-effect curves to serotonin (5-HT) were determined both in the presence (control) and in the absence of functional SR (after treatment with TG + Rya). Response to these drugs was recorded after 2- to 4-min exposure.

**Inhibition of ion channels.** CsCl (10 mM) and ZD7288 [4-(N-methyl-N-phenyl-amino)-1,2-dimethyl-6-(methylamino) pyrimidin chloride] (1 and 5 μM) were used to block K₄ (10).

As in vertebrate sinoatrial cells, the voltage-dependent, L-type Ca²⁺ current, rather than the voltage-dependent Na⁺ current, is accepted to be the main component of the cardiac AP upstroke in *T. molitor* (23). Tetracaine [20 μM; over 25-fold the Kₛ value at voltage-dependent Na⁺ channels (7)] was used to investigate the effect of voltage-dependent Na⁺ current blockade on BR. Sarcomial voltage-dependent Ca²⁺ channels were blocked by perfusion with TMS containing 100 μM CdCl₂ (15).

**Statistical analysis.** Data were expressed as means ± SE and compared by one-way analysis of variance, followed by Bonferroni’s test, or by Student’s t-test for paired or unpaired samples, as appropriate. Statistically significant difference was considered for *P* < 0.05.

**RESULTS**

Under steady-state conditions, BR was 66.0 ± 0.8 beats/min (*N* = 35) and remained stable at a regular rhythm for several hours under perfusion with TMS. Perfusion rates lower than 1 ml/min markedly reduced BR and led to rhythm irregularity, whereas little change was observed when flow rate was increased above 1 ml/min. Hearts in the present preparations were cylindrical with diastolic diameter of 64.4 ± 5.8 μm at the fifth to sixth abdominal segments. During systole, heart diameter decreased ~38% (40.0 ± 5.0 μm diameter). Figure 1 shows the dorsal vessel as prepared for the present study, in which the heart chambers and the ostia (valvulated openings through which hemolymph enters the heart tube) can be seen. Figure 1 (inset) shows the heart after further dissection, when only the dorsal abdominal cuticle is left, to emphasize the cylindrical shape of the heart tube.

As expected, contraction of the insect heart followed the electrical activity wave (Fig. 2A). To confirm that the electrical signal recorded was not due to or largely contaminated by motion artifacts, mechanical activity was selectively suppressed by perfusion with the electromechanical uncoupling agent, 2,3-butanedione monoxime [BDM, 5 mM (30)]. As observed in Fig. 2A, BDM progressively attenuated the con-
tractions, but not the electrographic signal, which indicates that the latter does not depend on mechanical activity. Even at a lower rate, the preparation remained electrically active after exposure to BDM for over 10 min, whereas contractile activity was nearly abolished. Both spontaneous electrical activity and contractions were reversibly abolished by perfusion with 100 μM Cd²⁺ (Fig. 2B) in all tested preparations (N = 6). On the other hand, BR increased exponentially with [Ca²⁺]₀ elevation (P < 0.05), reaching saturation at ~8 mM (Fig. 3).

Inhibition of the SR function markedly affected BR and time course of heart contractions (Fig. 4A). We used two compounds (TG and Rya), which, by different mechanisms, are expected to deplete the SR of Ca²⁺ (i.e., by inhibiting SR Ca²⁺ uptake and by increasing SR Ca²⁺ leak, respectively). Figure 4B shows pooled data for the effect on BR of these two classical maneuvers. BR was reduced significantly and by a similar extent by treatment with 100 μM Rya (from 61.2 ± 2.9 to 33.0 ± 3.7 beats/min, N = 6; P < 0.05) and with 100 μM TG (from 67.0 ± 3.8 to 36.0 ± 3.4 beats/min, N = 7; P < 0.05; t-test for paired samples). The combined treatment of the heart with TG and Rya did not produce further decrease in BR (from 67.0 ± 3.8 to 31.7 ± 3.5 beats/min, N = 7; P < 0.05). Since TG and Rya are both irreversible, a competitive inhibitor of the SR Ca²⁺-ATPase was also tested. Incubation with 30 μM BHQ produced an effect comparable to that produced by TG and/or Rya on BR (from 61.2 ± 2.9 to 33.3 ± 3.3 beats/min, N = 6; P < 0.05; Fig. 4B).

Assuming that inhibition of the SR function resulted in lower BR due to decrease in the electrogenic NCX-dependent current driven by Ca²⁺ release (I_NCX), it would be expected...
that stimulation of diastolic SR Ca\(^{2+}\) release should enhance the depolarizing current resulting from Ca\(^{2+}\) extrusion. To test this possibility, we used a relatively low concentration of caffeine, which increases RyR activity (37) and enhances diastolic SR Ca\(^{2+}\) leak in multicellular preparations (11). Figure 5A shows the biphasic time-dependent effect of 2 mM caffeine on BR. From a precaffeine value of 67.2 ± 2.3 beats/min (N = 5), BR peaked within ~45 s after caffeine application (76.4 ± 2.2 beats/min; \(P < 0.01\)), and then subsided to a steady-state level significantly lower than the control value (47.2 ± 2.0 beats/min; \(P < 0.01\); analysis of variance for paired samples, Bonferroni test). Figure 5B shows pooled data for a 10-fold higher (20 mM) caffeine concentration. In this case, BR was reduced significantly from 59.0 ± 11.6 to 32.0 ± 1.8 beats/min 20 min after caffeine application (\(P < 0.05, N = 6\)). This effect was completely reversible after washout, as seen in Fig. 6. Interestingly, a negative chronotropic effect was also observed in the presence of BDM (Fig. 2A), which, in addition to other effects [e.g., on myofilaments (30)], was shown to cause SR Ca\(^{2+}\) depletion (31).

To inhibit \(I_{\text{NCX}}\), Na\(^{+}\) was removed from the perfusate. After the perfusate was switched to Na\(^{+}\)-free TMS (Fig. 7), BR was transiently increased (from 62.6 ± 4.8 to 85.3 ± 7.0 beats/min, \(P < 0.05, N = 4\)) and then declined to a lower steady-state level within ~20 min (22.0 ± 2.0 beats/min; \(P < 0.01\) vs. basal BR; analysis of variance for paired samples and Bonferroni test). During perfusion with Na\(^{+}\)-free TMS, the rhythm of the contractions became irregular. On the other hand, tetracaine (20 \(\mu\)M), a well-known blocker of voltage-dependent Na\(^{+}\) channels (7), did not affect BR significantly (Fig. 7, inset), which indicates that the late negative chronotropic effect of the Na\(^{+}\)-free solution cannot be attributed to suppression of the current through these channels.

Neurotransmitters that typically control heart rate in vertebrates had no chronotropic effect on this insect preparation. Figure 8 shows that, at a high concentration (1 \(\mu\)M), norepinephrine, isoproterenol, or carbamylcholine did not significantly modify BR in the T. molitor heart (\(P > 0.05\)). Under the same conditions, CsCl and ZD7288, well-known inhibitors of \(I_{\text{f}}\), which is modulated by the \(\beta\)-adrenergic and muscarinic

---

**Fig. 5** Effects of caffeine (Caff) on the BR of the T. molitor heart. A: biphasic effect of 2 mM Caff, which caused an early, transient chronotropic stimulation followed by BR depression. B: steady-state negative chronotropic effect of 20 mM Caff, which was completely reversible after washout (CTR, before Caff treatment). Values are means ± SE. *Significant difference, \(P < 0.05\).

**Fig. 6** Reversible negative chronotropic effect of prolonged (15 min) exposure to 20 mM Caff on T. molitor heart. Top trace: extracellular electrogram. Bottom trace: contractile activity (CTR, before Caff treatment).

**Fig. 7** Biphasic effect of removal of extracellular Na\(^{+}\) on the BR of the T. molitor heart. The arrow indicates perfusate switching to the Na\(^{+}\)-free solution (0Na). Inset: lack of significant chronotropic effect of tetracaine (TTC; 20 \(\mu\)M). Values are means ± SE.
cascades in vertebrate pacemaker, did not significantly affect BR ($P_{H11022} 0.05$).

On the other hand, 5-HT exerted marked positive chronotropic and inotropic effects on the *T. molitor* heart (Fig. 9A). From the concentration-response curve determined for the chronotropic effect of 5-HT, the maximum response was $28.4 \pm 2.1$ beats/min, and the $pD_2$ value was $8.07 \pm 0.31$ ($N = 8$). It is interesting to observe, however, that the chronotropic response to 5-HT was completely abolished after impairment of the SR function with TG + Rya (Fig. 9B). In contrast, inclusion of 10 mM CsCl in the perfusate did not prevent the increase in BR by exposure to 0.1 $\mu$M 5-HT (from $74.0 \pm 3.9$ to $89.3 \pm 4.4$ beats/min; $N = 6$; $P < 0.05$). Additional demonstration of the importance of the SR in the control of BR is presented in Fig. 10, which shows the time course of the chronotropic response to $[Ca^{2+}]_o$ increase from 4 to 8 mM. Under control conditions, raising $[Ca^{2+}]_o$ produced a sustained positive chronotropic response, whereas, in the absence of a functional SR (after TG treatment), only a small, transient increase in BR was observed.

**DISCUSSION**

It is still a matter of debate whether insect and vertebrate hearts have evolved independently (5), but they have at least one feature in common: automaticity. The mechanisms by which heart automaticity is generated and controlled are not completely understood yet. However, mostly from studies with small mammals (e.g., mouse, rat, rabbit), it is clear that several membrane currents (e.g., the “funny” current $I_{if}$, T- and L-type $Ca^{2+}$ currents), activated at different times, are responsible for the diastolic depolarization toward the threshold for AP triggering. In addition, a $Ca^{2+}$-dependent, $I_{if}$-independent current seems to contribute to the late phase of the diastolic depolarization and is attributable to the electrogenic extrusion, via NCX, of the $Ca^{2+}$ locally released from the SR during diastole and accumulated in the subsarcolemmal space (43). More recently, both $I_{if}$ and $I_{NCX}$ were demonstrated to contribute to the pacemaker activity also in canines (12).

In mammalian myocardium, the higher the SR $Ca^{2+}$ content, the greater the rate of spontaneous SR release of $Ca^{2+}$ during diastole (3, 36), which has been considered as one of the basic mechanisms involved in heart rate control in different species (12, 21). A consistent finding in the present study was that maneuvers that impair the ability of the SR to retain $Ca^{2+}$ for subsequent release, by either inhibiting SR $Ca^{2+}$ uptake (TG and BHQ) or by enhancing SR $Ca^{2+}$ leakage (Rya and caffeine) produced marked chronotropic depression in the *T.
The molitor heart, reducing BR by ~50%. The absence of additive effects of TG and Rya indicates that either kind of interference seems to have been equally effective at inducing SR Ca\(^{2+}\) depletion. Another finding that reinforces the proposal of an important role of diastolic SR Ca\(^{2+}\) release in the insect cardiac automatisms is the biphasic effect of caffeine, which promotes Ca\(^{2+}\) release (11, 37). Given sufficient time, this should result in SR Ca\(^{2+}\) depletion, as it occurs with Rya. However, at the early phase of exposure to caffeine, the SR would be expected to still contain a reasonable amount of Ca\(^{2+}\). Accordingly, we observed transient chronotropic stimulation by 2 mM caffeine within the first minute of exposure, followed by a progressive decrease in BR to subnormal values. Increasing caffeine concentration to 20 mM depressed BR further, to a value that was similar to those observed after treatment with Rya and/or TG. In summary, the marked depression of BR observed after treatment with different inhibitors of SR function can be taken as a strong indication that diastolic SR Ca\(^{2+}\) release, most likely via RyRs, plays a significant role in the determination of BR in T. molitor heart tube.

It is well known that BR in insects is Ca\(^{2+}\)-dependent (23, 24, 27). Changes in [Ca\(^{2+}\)]\(_o\), are expected to directly affect the transmembrane electrochemical gradient of the ion and its influx via voltage-dependent Ca\(^{2+}\) channels, therefore changing the AP upstroke and the amount of Ca\(^{2+}\) available for contraction. It has been suggested that Ca\(^{2+}\)-influx is important for the AP upstroke in insects (23), although the diastolic depolarization phase is still present when [Ca\(^{2+}\)]\(_o\) is reduced (23, 24). Accordingly, we observed total suppression of mechanical and electrical activity during exposure to Ca\(^{2+}\), a nonselective blocker of voltage-dependent Ca\(^{2+}\) channels (28), at a concentration in which NCX inhibition is very small (15).

This indicates that Ca\(^{2+}\) influx through these channels is essential for generation and/or propagation of the pacemaker activity of T. molitor heart, as observed in mammalian sinoatrial node cells (4). BR was clearly sensitive to increase in [Ca\(^{2+}\)]\(_o\), and this is suggestive that Ca\(^{2+}\) influx is also able to modulate the basal chronotropic activity. A possibility to explain the observed chronotropic stimulation would be that the greater Ca\(^{2+}\) influx at higher [Ca\(^{2+}\)]\(_o\) would increase availability of the ion for SR uptake, thus augmenting the SR Ca\(^{2+}\) content, and, consequently, the rate of diastolic Ca\(^{2+}\) release of the ion and the magnitude of the depolarizing I\(_{\text{NCX}}\). Increase in Ca\(^{2+}\) influx alone via sarcosomal Ca\(^{2+}\) channels might stimulate chronotropism, but the requirement of a functional SR for the sustained positive response, as observed in this study, argues in favor of a prominent participation of Ca\(^{2+}\) release from intracellular stores. This is in line with our laboratory’s previous demonstration that diastolic SR Ca\(^{2+}\) release is crucial for the generation of automatism in mammalian cardiomyocytes under Ca\(^{2+}\)-overload and adrenergic stimulation (3, 6). Such a mechanism might also explain the negative chronotropic effect of reduced [Ca\(^{2+}\)]\(_o\), on mammalian sinoatrial node, which may be partially overcome by procedures that increase the cell Ca\(^{2+}\) load (41).

It is known that 5-HT is a neurotransmitter that stimulates chronotropism in the insect heart (17). A surprising observation was that BR modulation by 5-HT was completely dependent on the functional integrity of the SR. When the SR function was inhibited by TG plus Rya, not only was basal BR decreased, but also the preparations became completely insensitive to 5-HT. In the light of our present results, it is plausible to suppose that the transduction cascade coupled to 5-HT receptors leads to increased availability of Ca\(^{2+}\) for the SR, by stimulation of Ca\(^{2+}\)-influx and/or SR Ca\(^{2+}\) uptake, with direct or indirect augmentation of diastolic release. Consequently, depolarizing I\(_{\text{NCX}}\) would be enhanced, so that a shorter diastolic interval would be required for the AP-triggering threshold to be reached. More investigation is required to characterize the type/subtype of 5-HT receptor involved in this effect and the signaling pathway coupled to it. It should be recognized, however, that, although by different receptors and cascades, diastolic SR Ca\(^{2+}\) release has been considered as an important mechanism of neural regulation in vertebrate pacemaker (18, 21, 43).

Despite some striking similarity of cardiac pacemaking function in T. molitor and vertebrates, such as the apparent requirement of voltage-dependent Ca\(^{2+}\) current and the modulatory influence of the SR function on BR, other observations point out important differences. Neither ZD7288 (at 0.5 μM) nor CsCl, commonly used inhibitors of the hyperpolarization-activated, cyclic nucleotide-gated cation channels that mediate I\(_{f}\), produced significant effect on basal BR or on the responsiveness to 5-HT. Moreover, in contrast with vertebrates and Drosophila (17), BR in T. molitor was not sensitive to adrenergic and muscarinic cholinerigic stimulation. In vertebrate pacemaker, the signaling pathways coupled to these receptors converge to the regulation of intracellular cAMP levels, which directly affects hyperpolarization-activated, cyclic nucleotide-gated channel properties, consequently affecting the rate of diastolic depolarization and spontaneous AP rate (1). Our present results suggest that this mechanism of BR regulation appears to be absent in T. molitor, and that I\(_{f}\) does not seem to play a significant role in the determination of the basal BR, which is in agreement with the apparent lack of involvement of cAMP in chronotropic regulation in the Drosophila heart (17).

A nominally Na\(^+-\)free perfusion solution is expected to shift the NCX reversal potential (E\(_{\text{NCX}}\)) to very negative values (less than ~200 mV), which should virtually abolish Ca\(^{2+}\) extrusion by the exchanger. On the other hand, the driving force for Ca\(^{2+}\)-influx (in exchange for Na\(^+\)-efflux) would be greater, so that Ca\(^{2+}\)-overload should develop as extracellular Na\(^+\) concentration ([Na\(^+\)]\(_o\)) gradually decreases during perfusion with Na\(^+-\)free solution. As a result, the SR Ca\(^{2+}\) content (and the diastolic release rate) would be expected to increase and partially offset the negative shift of E\(_{\text{NCX}}\), which might cause some transient stimulation of Ca\(^{2+}\)-efflux. As [Na\(^+\)]\(_o\), decreases further and SR Ca\(^{2+}\)-load reaches saturation, E\(_{\text{NCX}}\) would become very negative, and net Ca\(^{2+}\) efflux would virtually cease. Accordingly, we observed a small, transient increase in BR during the first minute of perfusion with Na\(^+-\)-free TMS. However, BR subsequently dropped and maintained a value remarkably close to that observed after SR inhibition. This similarity, although indirectly, argues in favor of a [Na\(^+\)]\(_o\)-dependent depolarizing mechanism, closely coupled to diastolic SR Ca\(^{2+}\) release in the regulation of the insect cardiac BR, namely I\(_{\text{NCX}}\).

Unfortunately, the lack of selective NCX blockers that effectively inhibit the transporter in the Ca\(^{2+}\)-efflux mode prevents further confirmation of the role of NCX in pacemaker activity. A relevant question is whether diastolic SR Ca\(^{2+}\)
release could support the generation of a NCX-mediated inward current of magnitude sufficient to significantly contribute to diastolic depolarization up to the excitation threshold. Assuming that such a depolarization amounts to 10 mV (23, 24) at a cycle length of 1 s, and that cells are of similar geometry, but slightly smaller than a mammalian ventricular myocyte (100 μm long, 30 μm wide, and 10 μm thick), it is estimated that it would be necessary for the inward transport of ~8.5 amol Na⁺ to drive the membrane potential to the threshold. Considering 1) the usual NCX stoichiometry (i.e., the net influx of 1 Na⁺ ion for each Ca²⁺ ion extruded); 2) the diastolic SR Ca²⁺ release rate as 1 μmol Ca²⁺⁻¹ nonmitochondrial cell water⁻¹ s⁻¹, a value intermediate between those described by Bassani and Bers (2) and Shannon et al. (36) in mammalian myocytes; and 3) that the mitochondrial fraction of cell volume is 40% lower than in mammalians (0.2 vs. 0.35), then it is possible to calculate that NCX would mediate the influx of ~6 amol Na⁺. This corresponds to 70% of the charge transfer required for 10-mV depolarization during diastole. Although most of the assumptions for this estimation are not verifiable, yet, in this specific preparation, our preliminary calculations suggest that NCX-mediated extrusion of Ca²⁺ released by the SR during diastole could support, at least partially, the diastolic depolarization at a rate compatible with the basal BR in the present preparation. Alternatively, but not exclusively, SR Ca²⁺ release might modulate cycle length by affecting AP duration, for instance via a Ca²⁺-activated K⁺ current, as proposed by Markou and Theophilidis (23).

In conclusion, the present results indicate an important contribution of a SR-dependent current (possibly mediated by NCX) in the determination of the basal BR in T. molitor heart, although it is apparent that other mechanisms play a role in the genesis of pacemaker activity. In addition, our observations implicate this mechanism as the main effector of the 5-HT signaling pathway, based on the absolute requirement of SR function for manifestation of the positive chronotropic effect of 5-HT. This is, to our knowledge, the first time that evidence is provided for the involvement of this mechanism in the generation and modulation of cardiac automaticity in the insects.

Perspectives and Significance

The indirect modulation of cell excitability by SR Ca²⁺ release, via the activation of Ca²⁺⁻dependent membrane currents, seems to be a ubiquitous mechanism involved in the generation of cyclic electrical activity and in its regulation by signaling processes, as it has been identified in gut pacemaker and smooth muscle cells of a variety of organs in mammals (e.g., Refs. 9, 29, 39, 44). Interestingly, this mechanism was shown to be essential for the generation of spontaneous activity by mammalian heart cells during early cardiomyogenesis (42), while the present report provides evidence of its importance in the insect heart tube. All of these observations are suggestive that this mechanism seems to be a universal, successful solution for generation and regulation of pacemaker activity in muscle cells, in which Ca²⁺ signaling is used for both excitation and contraction.

ACKNOWLEDGMENTS

We are grateful to Elizângela S. Oliveira, Carlos A. L. Silva, and Renato S. Moura for the technical support.

GRANTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (Grant 300632/2005-3).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES