Effects of sympathetic nerve stimulation on membrane potential, \([Ca^{2+}]_i\), and force in the toad sinus venosus

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Bramich, Narelle J., and Helen M. Cousins. Effects of sympathetic nerve stimulation on membrane potential, \([Ca^{2+}]_i\), and force in the toad sinus venosus. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H115–H128, 1999.—The effects of sympathetic nerve stimulation on beat rate, force, intracellular \(Ca^{2+}\) concentration (\([Ca^{2+}]_i\)) measured using fura 2, and membrane potential were recorded from the spontaneously beating toad sinus venosus. Short trains of stimuli evoked an increase in the be rate and force. During this tachycardia the amplitude of pacemaker action potentials was not changed, but there was an increase in the basal level of \([Ca^{2+}]_i\) with little change in peak \([Ca^{2+}]_i\), measured during each action potential. Depletion of intracellular \(Ca^{2+}\) stores with caffeine (3 mM) abolished all responses to sympathetic nerve stimulation. The effects of caffeine were fully reversible. Caffeine (3 mM), in the presence of the \(Ca^{2+}\)-ATPase inhibitor thapsigargin (30 µM), abolished irreversibly the chronotropic and inotropic responses evoked by sympathetic nerve stimulation. Ryanodine (10 µM) attenuated, but did not abolish, these responses. These results suggest that, in the toad sinus venosus, increases in force and beat rate evoked by sympathetic nerve stimulation result from the release of \(Ca^{2+}\) from intracellular \(Ca^{2+}\) stores.

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thetic nerve stimulation in beating and arrested preparations exhibited a similar time course. That is, the membrane depolarization and the accompanying transient increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) and force recorded from arrested preparations have a latency and duration similar to the positive inotropic and chronotropic responses recorded from spontaneously beating preparations of toad sinus venosus (3). It therefore seems possible that sympathetic nerve stimulation may also trigger Ca\textsuperscript{2+} stores release in spontaneously beating preparations of toad sinus venosus.

The aim of this study was to determine whether the positive inotropic and chronotropic responses recorded from the spontaneously beating toad sinus venosus evoked by sympathetic nerve stimulation are mediated by the release of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores.

**METHODS**

The procedures were approved by the Animal Experimentation Ethics Committee at the University of Melbourne. Toads (Bufo marinus) were anesthetized by immersion in a solution of 0.5% tricaine methanesulfonate (Thomson and J. Joseph, Norwich, UK) in tap water. Preparations consisted of the sinus venosus and two atria, with the ventricle and truncus arteriosus removed. The left and right vagosympathetic trunks were dissected free back to the sympathetic chains. The force of beat from sinus venosus preparations was measured isometrically using a force transducer (model 52-9545, Harvard Apparatus). Preparations with the sympathetic nerves attached were set up in a 50-ml organ bath. A tie was placed around the posterior vena cava and anchored to a tissue holder. Preparations were then attached to the force transducer via a fine stainless steel hook placed through the sinus venosus close to the sinoatrial junction. Preparations were set up with a resting force of 2 mN. The sympathetic nerves were stimulated via a pair of platinum ring electrodes by using a stimulator (model S8800, Grass). Responses were digitized and stored on disk for later analysis.

In a separate series of experiments in which [Ca\textsuperscript{2+}]i and membrane potential were measured simultaneously, the preparations were pinned out in a shallow recording chamber, the base of which consisted of a microscope coverslip coated with silicone resin (Sylgard, Dow Corning, Midland, MI) (4). Fine pins, cut from 50-µm tungsten wire (Goodfellow, Cambridge Science Park, Cambridge, UK), were placed through the connective tissue surrounding the sinus venosus and epicardium of the atria. The sinus venosus was exposed by placing a ring of pins through the sinoatrial aperture. Care was taken not to damage the atrial septum and not to apply excessive stretch to the partly immobilized, pinned out region of sinus muscle. In experiments in which membrane potential recordings were made from preparations that had been arrested with nifedipine (10 µM), force generated by the tissue was measured by placing a fine stainless steel hook, attached to a tension transducer, through the sinus venosus. Sympathetic nerve fibers were stimulated by drawing the two sympathetic chains through a pair of platinum ring electrodes.

Membrane potential changes and changes in [Ca\textsuperscript{2+}]i were measured simultaneously from the pinned sinus venosus preparations. Preparations were loaded with the fluorescent Ca\textsuperscript{2+} indicator fura 2 by incubation in HEPES-buffered physiological saline with low Ca\textsuperscript{2+} (0.1 mM; pH adjusted to 7.39 with 1 M NaOH), containing 10 µM fura 2-AM and 0.01% Pluronic F-127 to aid dispersal of the fura 2-AM, for 2.5 h at 22°C. This concentration of fura 2-AM was used, inasmuch as it resulted in an optimal signal-to-noise ratio. Preparations were then warmed to 32°C for 30 min to assist efferentation of the fura 2-AM complex before they were washed in fura 2-AM-free physiological saline (in mM: 115 NaCl, 3.2 KCl, 20 NaHCO\textsubscript{3}, 3.1 NaH\textsubscript{2}PO\textsubscript{4}, 1.8 CaCl\textsubscript{2}, 1.4 MgCl\textsubscript{2}, and 16.7 glucose, gassed with 95% O\textsubscript{2}-5% CO\textsubscript{2}) for 40 min. There was no difference in control action potentials or responses evoked by sympathetic nerve stimulation after incubation in the fura 2 loading medium. It was therefore apparent that the loading medium itself had no effect on the behavior of preparations and that fura 2 was not buffering [Ca\textsuperscript{2+}]i. Tissue fluorescence at 510 nm was continuously monitored with a photomultiplier tube during alternate excitation, 50 Hz, with light of 340- and 380-nm wavelength. This was achieved by passing the incident light from a xenon arc lamp through a beam splitter to produce two beams of incident light: one beam of light was passed through a 340-nm filter and the other through a 380-nm filter. The resultant two beams of excitation light, 340 and 380 nm, were then alternately passed to the preparation using a chopper wheel. The ratio of the fluorescence at 340-nm excitation to that at 380-nm excitation (F\textsuperscript{340/380}) was taken as a qualitative indicator of [Ca\textsuperscript{2+}]i. There was no difference between relative changes in [Ca\textsuperscript{2+}]i measured before and after signals were corrected for background fluorescence (n = 3). Therefore, in all experiments, there was no correction for this condition. However, although it was apparent that the fura 2 was not saturated by Ca\textsuperscript{2+} within the cells (see Fig. 8, it is unlikely that the changes in F\textsuperscript{340/380} are linearly related to the changes in [Ca\textsuperscript{2+}]i. All records of F\textsuperscript{340/380} were passed through a moving-average filter (10 points, 5 passes) and stored on disk for later analysis. After such filtering, the amplitudes of [Ca\textsuperscript{2+}]i transients associated with each action potential were reduced by <5%, and there was little change in their time course.

Intracellular recordings were made using conventional techniques with fine glass microelectrodes (resistance 120-240 MΩ) filled with 0.5 M KCl. All membrane potential records were low-pass filtered (cutoff frequency 1 kHz), digitized, and stored on disk for later analysis. To eliminate any possible effect of stimulus spread to vagal nerve fibers, preparations were continuously superfused with physiological saline containing the muscarinic receptor antagonist hyoscine (1 µM) at a rate of 6 ml/min (bath volume 1.5 ml). Experiments were carried out at room temperature (22-25°C). Drugs were added to the preparation by changing the inflow line from the control solution to one containing the appropriate concentration of drug.

In all experiments the sympathetic nerves were stimulated with a train of 10 impulses delivered at 10 Hz (stimulation current = 10-60 mA, pulse width = 1.0 ms). After acquisition, beat-to-beat rate plots were calculated from the membrane potential and force recordings. Diastolic and systolic values of F\textsuperscript{340/380} were taken as the minimum and maximum changes in fluorescence, respectively, before and after sympathetic nerve stimulation. Latency measurements represent the time between the start of the stimulation train and the time to reach 10% of the peak amplitude. Rise time is the time between 10 and 90% of the peak amplitude. Half-width is measured as the time between 50% of peak amplitude on rising and falling phases. Values are means ± SE; each n represents the mean result from a different animal. Where indicated, the statistical significance of the difference between two means was determined using a Student's t-test.

Drugs used in this study were fura 2-AM (Molecular Probes, Eugene, OR) and ryanodine (Calbiochem, Alexandria,
NSW, Australia), epinephrine bitartrate, caffeine, dihydroergotamine tartrate, hyoscine hydrochloride, isoprenaline hydrochloride, nifedipine hydrochloride, propranolol hydrochloride, and thapsigargin (Sigma Chemical, St. Louis, MO). All drugs were dissolved in distilled water, except nifedipine and ryanodine, which were dissolved in absolute alcohol, and fura 2-AM, which was dissolved in DMSO (ICN Biomedicals, Aurora, OH). Neither solvent had any effect on the sinus venosus tissue itself or on responses evoked by sympathetic nerve stimulation (n = 3). In all experiments involving nifedipine, solutions were made daily and protected from the light with aluminum foil wrapping. Applied drugs were allowed to equilibrate with the tissue for ≥10 min.

RESULTS

General observations: inotropic and chronotropic responses. Bilateral sympathetic nerve stimulation caused an increase in the rate of contraction that displayed two distinct components (4) (Fig. 1A). Preparations had a basal beat rate of 31 ± 1 beats/min, which increased to 48 ± 2 beats/min (n = 27). Contractions had a mean amplitude of 1.6 ± 0.1 mN, which increased to 2.3 ± 0.2 mN after sympathetic nerve stimulation (n = 27). In the presence of the β-adrenoceptor antagonist propranolol (1 µM), the initial increase in rate had an amplitude of 14 ± 1 beats/min (mean baseline and peak rate = 30 ± 1 and 44 ± 1 beats/min, respectively, n = 28; Fig. 1B, trace b), a minimum latency of 2.5 ± 0.1 s, a rise time of 2.0 ± 0.1 s, and a half-width of 11.5 ± 0.9 s (n = 27; Fig. 1B, trace b). The secondary increase in rate reached a maximum of 2.0 ± 0 beats/min (n = 25) ~2 min after sympathetic nerve stimulation. This increase in beat rate was not further examined. The sympathetically evoked increase in the force of contraction had a time course similar to the initial positive chronotropy.

In the presence of propranolol (1 µM), control contractions had a mean amplitude of 1.6 ± 0.1 mN, which

![Fig. 1. Effect of propranolol (1 µM) and dihydroergotamine (20 µM) on inotropic and chronotropic responses evoked by sympathetic nerve stimulation recorded from toad sinus venosus. In control physiological saline (A), sympathetic nerve stimulation (●) evoked an increase in force (traces a) and beat rate (traces b) recorded from sinus venosus. In B, addition of propranolol to bathing solution decreased time course, but not amplitude, of inotropic and chronotropic responses evoked by sympathetic nerve stimulation. With addition of dihydroergotamine to bathing solution in C, increases in force and rate evoked by sympathetic nerve stimulation were markedly attenuated. Time calibration bar refers to all traces.](http://ajpheart.physiology.org/doi/10.2308/ajpheart-03-0078.s1)
increased to 2.3 ± 0.1 mN after sympathetic nerve stimulation (n = 27; Fig. 1B). Although the peak increases in beat rate and force production evoked by sympathetic nerve stimulation were not greatly attenuated by β-adrenoceptor blockade with propranolol (1 µM), the time course of both responses was decreased (Fig. 1, A and B; n = 27) (22).

To determine whether the increase in force evoked by sympathetic nerve stimulation results from the activation of non-α,- non-β-adrenoceptors, similar to the increase in beat rate (4), the effects of dihydroergotamine (20 µM) on the positive inotropic and chronotropic responses were examined. In a series of five experiments, sympathetic nerve stimulation evoked an increase in force of 0.6 ± 0.1 mN and an increase in rate of 8 ± 2 beats/min. After the addition of dihydroergotamine to the physiological saline, the increases in force and rate evoked by sympathetic nerve stimulation were reduced to 0.4 ± 0.1 mN and 3 ± 1 beats/min, respectively (Fig. 1, B and C). These effects of dihydroergotamine were significant for force (P = 0.009) and rate (P = 0.026). These results suggest that, similar to the increases in beat rate, the sympathetically evoked increases in the force of beat result from the activation of a set of non-α,- non-β-adrenoceptors.

Effect of ryanodine or caffeine on force responses evoked by sympathetic nerve stimulation or isoprenaline. To investigate the possibility that intracellularly stored Ca^{2+} is released by sympathetic nerve stimulation to generate the inotropic and chronotropic responses, the effects of drugs that interfere with such stores were examined.

In eight preparations of toad sinus venosus, ryanodine (10 µM) abolished the increase in beat rate evoked by sympathetic nerve stimulation (Fig. 2, A, trace b and B, trace b). Sympathetic nerve stimulation in the presence of propranolol (1 µM) evoked an increase in beat rate of 18 ± 2 beats/min (basal and peak rate = 28 ± 2 and 47 ± 2 beats/min, respectively; Fig. 2A, trace b). In the presence of ryanodine the basal rate was 36 ± 2 beats/min, and the rate after the stimulus train was 35 ± 2 beats/min (Fig. 2B, trace b). Ryanodine attenuated, but did not abolish, the nerve-evoked increase in force. Sympathetic nerve stimulation, in the presence of propranolol, evoked an increase in force of 0.8 ± 0.1 mN (basal and peak force = 1.6 ± 0.1 and 2.4 ± 0.2 mN, respectively; Fig. 2A, trace a). The increase in peak force in the presence of ryanodine (10 µM) was reduced to 60 ± 7% of control (basal and peak force = 0.8 ± 0.1 and 1.3 ± 0.1 mN, respectively, force increase = 0.5 ± 0.0 mN, P = 0.004; Fig. 2B, trace a). However, the ratio of peak force to basal force did not decrease in the presence of ryanodine (control = 1.5, ryanodine = 1.6), which may indicate that the decrease in the positive inotropic response reflects a decrease in the ability of the muscle to contract.

Figure 3 shows the effect of caffeine (3 mM) on the positive inotropic and chronotropic responses evoked by sympathetic nerve stimulation. Control responses are illustrated in Fig. 3A. Addition of caffeine to the physiological saline elicited an increase in basal force production and basal beat rate (control and caffeine-treated basal force = 1.5 ± 0.3 and 2.2 ± 0.3 mN, respectively, P = 0.002; control and caffeine-treated

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Fig. 2. Effect of ryanodine (10 µM) on increases in force and rate evoked by stimulation of sympathetic nerves recorded from spontaneously beating toad sinus venosus. A: control force and rate responses, after sympathetic nerve stimulation (●), recorded in presence of propranolol (1 µM). In B, addition of ryanodine to physiological saline markedly attenuated inotropic and chronotropic responses evoked by stimulation of sympathetic nerves. Time calibration bar refers to all traces.
basal beat rate = 26 ± 3 and 38 ± 3 beats/min, respectively, P < 0.0005; n = 7). These effects are presumably due to the inhibition of phosphodiesterases by caffeine to cause an accumulation of cAMP (6). However, the elevated basal levels of force production and beat rate in the presence of caffeine did not exceed the peak values evoked by sympathetic nerve stimulation in control solution (cf. Fig. 3, A and B). In control physiological saline, sympathetic nerve stimulation evoked an increase in the force of contraction by 0.6 ± 0.1 mN and an increase in beat rate of 16 ± 3 beats/min (n = 7). The increases in force and rate of contraction evoked by sympathetic nerve stimulation were reduced to 0.1 ± 0.0 mN and 1.0 ± 0.0 beats/min, respectively, after the addition of caffeine to the physiological saline. The effects of caffeine on basal force and rate production and also on the increases in force and rate evoked by sympathetic nerve stimulation were completely reversible: after removal of caffeine from the physiological saline, the inotropic and chronotropic responses evoked by sympathetic nerve stimulation were restored to control values (force increase = 0.9 ± 0.1 mN, P = 0.244; rate increase = 21 ± 3 beats/min, P = 0.031; Fig. 3C). To test the possibility that caffeine might act presynaptically to inhibit transmitter release, the effect of caffeine on the decrease in beat rate evoked by vagal nerve stimulation was examined. The decrease in beat rate was unaltered by the addition of 3 mM caffeine (n = 5), suggesting that caffeine at this concentration has no presynaptic action.

The blocking action of caffeine on the inotropic and chronotropic responses evoked by sympathetic nerve stimulation suggests that these responses are mediated by the release of intracellularly stored Ca^{2+}. In contrast, β-adrenoceptor activation is thought to mediate positive inotropic and chronotropic responses via the increased influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. To determine the selectivity of caffeine, the effects of intracellular Ca^{2+} store depletion with caffeine on isoprenaline-evoked responses were investigated. Figure 4 illustrates the effects of caffeine (3 mM) on force and rate responses evoked by the application of isoprenaline (10 µM) to the spontaneously beating toad sinus venosus. This high concentration of isoprenaline...
line was chosen, inasmuch as it produced an increase in beat rate similar to that evoked by sympathetic nerve stimulation. In four experiments, isoprenaline caused an increase in the force of beat (basal and peak force = 1.4 ± 0.4 and 2.1 ± 0.4 mN, respectively, force increase = 0.6 ± 0.1 mN; Fig. 4A, trace a) and an increase in beat rate (basal and peak beat rate = 35 ± 2 and 49 ± 1 beats/min, respectively, rate increase = 14 ± 3 beats/min; Fig. 4A, trace b). Again, the application of caffeine (3 mM) to the bathing saline caused an increase in the basal force production (1.9 ± 0.5 mN, P = 0.025) and basal beat rate (41 ± 1 beats/min, P = 0.031; cf. Fig. 4, A and B). However, caffeine had no significant effect on the peak force or rate evoked by isoprenaline (peak force = 2.1 ± 0.4 mN, P = 0.360; peak rate = 47 ± 1 beats/min, P = 0.162).

Taken together, these results suggest that in the toad sinus venosus the activation of non-α-, non-β-adrenoceptors after sympathetic nerve stimulation leads to increases in the force and rate of contraction, both of which are mediated by the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores. In contrast, the increases in force and rate after β-adrenoceptor stimulation are essentially independent of intracellularly stored Ca\(^{2+}\).

Effect of caffeine on membrane potential and force production recorded from arrested preparations of sinus venosus. To confirm that the actions of caffeine and ryanodine were to deplete intracellular stores, a series of experiments were performed on preparations of toad sinus venosus in which the influx of Ca\(^{2+}\) through voltage-dependent L-type Ca\(^{2+}\) channels had been prevented with nifedipine. 1) The effects of caffeine on membrane potential and force generation of the sinus venosus were examined. 2) The effects of ryanodine on responses evoked by caffeine were examined.

Caffeine (10 mM, 30 s) superfused onto arrested preparations evoked an initial membrane depolarization of 4–10 mV in amplitude (6.1 ± 1.6 mV, n = 5). This was followed by large oscillations in membrane potential that had a maximum amplitude of 12–42 mV (21.4 ± 6.1 mV, n = 5; Fig. 5B, trace a). These membrane potential oscillations were similar in time

![Fig. 4. Effect of caffeine (3 mM) on force and rate responses evoked by isoprenaline applied to spontaneously beating sinus venosus of toad. Addition of isoprenaline (10 µM, horizontal bar) to physiological saline bathing pacemaker preparation (A) increased force (trace a) and rate (trace b) of contraction recorded from sinus venosus. Addition of caffeine to physiological saline (B) also increased force of contraction and basal beat rate but did not significantly attenuate peak increase in force (trace a) or peak increase in rate (trace b) evoked by isoprenaline. Time calibration bar refers to all traces.](http://ajpheart.physiology.org/)

Control

- 3.5 mN
- 2.0 mN
- 0.5 mN
- 0.0 mN

Beats per min

- 30
- 20
- 10
- 0

Isoprenaline (10 µM)

Caffeine (3 mM)

- 3.0 mN
- 2.0 mN
- 1.0 mN
- 0.0 mN

Beats per min

- 60 s
- 30

Isoprenaline (10 µM)
course and frequency to those evoked by sympathetic nerve stimulation in arrested preparations (Fig. 5A, trace a) (10). The initial membrane depolarization and subsequent oscillations were accompanied by an increase in force produced by the sinus venosus (Fig. 5B, trace b). The initial increase in force evoked by caffeine had an amplitude of 17.3 ± 5.5 µN (n = 5). Such responses could be reproduced if ≥30 min were allowed between consecutive caffeine applications. However, if caffeine was reapplied after 60 s, the evoked membrane depolarizations and contractions were reduced in amplitude by ~50%. Subsequent applications of caffeine at 60-s intervals resulted in further decreases in the amplitudes of the membrane depolarization and increase in force. The observation that caffeine could evoke an increase in force in the absence of Ca2+ entry through voltage-dependent L-type Ca2+ channels suggested that caffeine was acting to deplete Ca2+ from intracellular stores. Presumably, these stores were being gradually depleted and not completely refilled after the consecutive applications of caffeine.

To ensure that caffeine and ryanodine had similar actions on intracellular Ca2+ stores, the effects of ryanodine on the membrane depolarization and contraction evoked by caffeine in the arrested toad sinus venosus were examined. We previously showed that ryanodine reduces the membrane depolarization and increase in force evoked by sympathetic nerve stimulation in arrested preparations of toad sinus venosus (10). Figure 5A shows a control depolarization (trace a) and increase in force (trace b) evoked by sympathetic nerve stimulation. The addition of ryanodine (10 µM) to the physiological saline reduced both responses evoked by sympathetic nerve stimulation (Fig. 5C; n = 3) (10). The same concentration of ryanodine abolished the membrane depolarization and increase in force evoked by the rapid bath application of caffeine (10 mM; n = 5; Fig. 5, B and D). The ability of ryanodine to abolish responses evoked by caffeine suggests that caffeine and ryanodine in this tissue were acting in the same way, that is, to deplete intracellular Ca2+ stores.

Effect of caffeine in the presence of thapsigargin on changes in force and rate evoked by sympathetic nerve stimulation. Additional experiments were performed in which the reuptake of Ca2+ into intracellular stores was prevented using the Ca2+-ATPase inhibitor thapsigargin (36). In the presence of propranolol (1 µM), sympathetic nerve stimulation caused an increase in force production (amplitude of contraction before and after sympathetic nerve stimulation = 1.4 ± 0.2 and 2.1 ± 0.3 mN, respectively, force increase = 0.7 ± 0.2 mN) and an increase in the rate of spontaneous contractions of the sinus venosus (basal and peak rate = 32 ± 2 and 45 ± 2 beats/min, respectively, rate increase = 13 ± 3 beats/min, n = 5; Fig. 6A). Addition of thapsigargin (30 µM) did not significantly alter the amplitude of contractions before sympathetic nerve stimulation (1.3 ± 0.1 mN, P = 0.106) or basal beat rate (32 ± 2 beats/min, P = 0.477). Furthermore, thapsigargin did not affect the inotropic response evoked by sympathetic nerve stimulation (36).
nerve stimulation, although its effect varied between preparations (amplitude of contraction before and after sympathetic nerve stimulation = 1.3 ± 0.1 and 1.7 ± 0.2 mN, respectively, force increase = 0.4 ± 0.1 mN, P = 0.122, n = 5). Thapsigargin did, however, decrease the chronotropic response produced by sympathetic nerve stimulation (basal and peak beat rate = 32 ± 2 and 38 ± 3 beats/min, respectively, rate increase = 6 ± 1 beats/min, P = 0.035; Fig. 6B, trace b). After the addition of thapsigargin to the physiological saline, a secondary increase in force production was apparent subsequent to sympathetic nerve stimulation (Fig. 6B, trace a, arrow). We have no explanation for this action of thapsigargin, and it was not further investigated.

The observation that thapsigargin had no effect on the basal contraction or beat rate and did not abolish the increase in force and rate evoked by sympathetic nerve stimulation suggests that inhibition of the Ca\(^{2+}\)-ATPase alone is not enough to deplete intracellular Ca\(^{2+}\) stores. In rabbit ventricular muscle it has been shown that there is a slow and incomplete depletion of intracellular stores after the addition of thapsigargin (1). We therefore investigated the effect of thapsigargin on nerve-evoked responses after stores had been depleted using caffeine. In the continued presence of thapsigargin (30 µM), the addition of caffeine (3 mM) abolished the positive inotropic response (amplitude of contraction before and after sympathetic nerve stimulation = 2.2 ± 0.3 and 2.2 ± 0.3 mN, respectively, force increase = 0.0 ± 0.0 mN) and chronotropic response (basal and peak rate = 36 ± 2 and 38 ± 2 beats/min, respectively, rate increase = 2 ± 1 beats/min) evoked by sympathetic nerve stimulation (Fig. 6C). However, after washout of caffeine, but in the continued presence of thapsigargin, only a secondary increase in force production was observed.

![Fig. 6](http://ajpheart.physiology.org/)
of thapsigargin, neither the inotropic (amplitude of contraction before and after nerve stimulation = 1.6 ± 0.2 and 1.8 ± 0.3 mN, respectively, force increase = 0.1 ± 0.1 mN) nor the chronotropic response (basal and peak rate = 32 ± 4 and 33 ± 4 beats/min, respectively, rate increase = 1 ± 0 beats/min) could be restored (Fig. 6D).

The observations with use of caffeine and thapsigargin provide further evidence to support the idea that sympathetic nerve stimulation evokes the release of intracellularly stored Ca2+. This therefore suggests that the action of ryanodine to decrease the positive inotropic response was not simply a consequence of its action on basal force production. Taken together, the results suggest that the increases in force and beat rate evoked by sympathetic nerve stimulation are the consequence of the release of Ca2+ from intracellular stores.

General observations: membrane potential and [Ca2+]. When intracellular recordings were made from sinus venosus cells, the rhythmic discharges of action potentials were detected. The frequency of action potential discharge was 38–52 beats/min (46 ± 2 beats/min, n = 10). Recordings of action potentials were assumed to have been from pacemaker cells if the diastolic depolarization led smoothly into the upstroke of the action potential. Pacemaker action potentials were similar to those described previously for this preparation (4). After a slow diastolic depolarization, action potentials were initiated at a threshold potential of about −50 mV; when measured from the maximum diastolic potential, action potentials had peak amplitudes of 78–109 mV (92.9 ± 3.1 mV, n = 9). Each pacemaker action potential was associated with an oscillation in [Ca2+], that had a mean amplitude of 0.17 ± 0.03 F340/380 (n = 10). Most recordings were made from such pacemaker cells. In some instances, recordings were made from cells in which the upstroke of the action potential rose more sharply from the diastolic depolarization. Although the action potential configuration differed slightly in these “driven” cells, there was no quantitative difference in the responses observed to sympathetic nerve stimulation in these cells compared with pacemaker cells.

Bilateral sympathetic nerve stimulation with trains of stimuli increased the rate of pacemaker action potential discharge, which again was composed of two distinct components (4). The initial increase in rate had an amplitude of 12 ± 2 beats/min (baseline and peak rate = 46 ± 2 and 58 ± 2 beats/min, respectively, n = 10). The initial positive chronotropic response had a latency of 1.7 ± 0.1 s, a rise time of 1.7 ± 0.2 s, and a half-width of 8.5 ± 1.4 s (Fig. 7B, trace c). The secondary increase in rate reached a maximum of 2.0 ± 0.2 beats/min (n = 10) − 2 min after sympathetic nerve stimulation. Associated with the initial positive chronotropy, there was no significant change in the amplitude of pacemaker action potentials (mean amplitude = 92.0 ± 3.4 mV, n = 8, P = 0.069; Fig. 7B, trace a). However, on some occasions, during this initial increase in rate, there was a small, 1- to 2-mV decrease in the peak diastolic potential and overshoot potential (4).

Even though the action potential amplitude remained unaltered, it occurred at a time when there was an increase in force production recorded from the sinus venosus (cf. Fig. 7, A, trace a and B, trace a) (3). During the initial positive chronotropy there was an increase in the diastolic [Ca2+] compared with the diastolic [Ca2+] recorded before sympathetic nerve stimulation: the diastolic [Ca2+] increased by 0.09 ± 0.02 F340/380 after sympathetic nerve stimulation, and this increase coincided in time with the peak increase in beat rate (Fig. 7B, trace b). However, even though sympathetic nerve stimulation evoked an increase in the diastolic [Ca2+], there was little change in the peak [Ca2+] achieved during each [Ca2+] oscillation. Thus the mean amplitude of the transient increase in [Ca2+] associ-
ated with pacemaker action potentials after sympathetic nerve stimulation was decreased to 0.10 ± 0.02 F_340/380 compared with the control value 0.17 ± 0.03 F_340/380 (P = 0.001; Fig. 7B, trace b). This might be a direct consequence of the increased frequency of action potential generation. That is, [Ca^{2+}]_i may not have had sufficient time to return to baseline levels during the positive chronotropic response. Alternatively, sympathetic nerve stimulation may cause an increase in the basal level of [Ca^{2+}]_i. The time course of the positive chronotropic response and the time course of the increase in the diastolic [Ca^{2+}]_i are similar (Fig. 7B, trace b and B, trace c). Both begin ~2 s after sympathetic nerve stimulation and have a total duration of ~14 s.

Effect of isoprenaline on membrane potential and [Ca^{2+}]_i. The positive chronotropic response produced by β-adrenoceptor activation is associated with an increase in the amplitude of pacemaker action potentials via a Ca^{2+}-dependent pathway (5, 15). This is due in part to the increased influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. This suggests that the change in [Ca^{2+}]_i associated with each pacemaker action potential would also increase after β-adrenoceptor activation. Figure 8 shows the effect of isoprenaline (10 µM) on membrane potential (A, trace a) and [Ca^{2+}]_i (B, trace a) recorded from separate preparations of toad sinus venosus. In six preparations, isoprenaline increased basal beat rate from 42 ± 2 to 59 ± 2 beats/min (P = 0.001). This positive chronotropy was associated with an increase in the amplitude of pacemaker action potentials from 74.2 ± 7.8 to 94.0 ± 4.2 mV (P = 0.003; Fig. 8A, trace a) and an increase in the amplitude of [Ca^{2+}]_i oscillations associated with each action potential from 0.35 ± 0.03 to 0.44 ± 0.04 F_340/380 (P = 0.003, n = 14; Fig. 8B, trace a). This is in marked contrast to the activation of non-α-, non-β-adrenoceptors after sympathetic nerve stimulation, which is characterized by no change in the amplitude of pacemaker action potentials and a decrease in the amplitude of [Ca^{2+}]_i oscillations associated with each action potential (Fig. 7B). Clearly, during the positive chronotropic response produced by isoprenaline, the basal levels of [Ca^{2+}]_i did not increase (Fig. 8B). This suggests that an increase in beat rate alone cannot account for the changed basal levels of [Ca^{2+}]_i, measured after sympathetic nerve stimulation.

Together, these observations suggest that the increase in force evoked by sympathetic nerve stimulation does not result from the increased influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. This raises the possibility that the Ca^{2+} responsible for changes in [Ca^{2+}]_i and force production after sympathetic nerve stimulation might be intracellular.

Effect of caffeine on sympathetically evoked changes in membrane potential and [Ca^{2+}]_i. To determine whether the sympathetically evoked changes in [Ca^{2+}]_i that accompany the positive chronotropy are due to the release of Ca^{2+} from intracellular stores, the effect of store depletion with caffeine on membrane potential and [Ca^{2+}]_i was examined. In a series of five experiments, sympathetic nerve stimulation increased the

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Fig. 8. Effects of isoprenaline (10 µM) on membrane potential, [Ca^{2+}]_i, and beat rate recorded from separate preparations of spontaneously beating preparations of toad sinus venosus. Bath application of isoprenaline (30 s; horizontal bar) increased rate of generation of pacemaker action potentials (A, trace b). In addition to increase in beat rate, isoprenaline increased peak diastolic potential and overshoot potential of pacemaker action potentials to produce an increase in action potential amplitude (A, trace a). In a separate preparation, bath application of isoprenaline (B, trace b) also increased beat rate and amplitude of [Ca^{2+}]_i oscillations associated with each beat compared with [Ca^{2+}]_i oscillations recorded during control (B, trace a). Time calibration bar refers to all traces.
rate of generation of pacemaker action potentials by 10 ± 2 beats/min (initial positive chronotropy; mean baseline and peak rate = 43 ± 2 and 54 ± 3 beats/min, respectively). In addition, sympathetic nerve stimulation increased the diastolic [Ca²⁺], by 0.06 ± 0.01 F₃₄₀/₃₈₀ and decreased the amplitude of the [Ca²⁺] oscillations (mean amplitude of [Ca²⁺], oscillation before and after sympathetic nerve stimulation = 0.20 ± 0.04 and 0.13 ± 0.03 F₃₄₀/₃₈₀, respectively, P = 0.008). After the addition of caffeine (3 mM) to the physiological saline, both phases of the positive chronotropy and also the increase in the diastolic [Ca²⁺], produced by sympathetic nerve stimulation (Fig. 9) were abolished. In the presence of caffeine, sympathetic nerve stimulation failed to evoke a positive chronotropic response (mean baseline and peak rate = 45 ± 2 and 45 ± 2 beats/min, respectively) or an increase in the diastolic [Ca²⁺], (change in diastolic [Ca²⁺], after sympathetic nerve stimulation = 0.01 ± 0.01 F₃₄₀/₃₈₀, amplitude of [Ca²⁺], oscillations before and after sympathetic nerve stimulation = 0.18 ± 0.04 and 0.17 ± 0.04 F₃₄₀/₃₈₀, respectively). These responses were fully restored after the washout of caffeine (baseline and peak rate = 40 ± 1 and 53 ± 3 beats/min, respectively, beat rate increase = 14 ± 3 beats/min, amplitude of [Ca²⁺], oscillation before and after sympathetic nerve stimulation = 0.26 ± 0.07 and 0.16 ± 0.07 F₃₄₀/₃₈₀, respectively; Fig. 9C).

These results indicate that the positive chronotropy and the changes in [Ca²⁺], evoked by sympathetic nerve stimulation result from the release of Ca²⁺ from intracellular stores.

DISCUSSION

This study has examined the mechanisms by which sympathetic nerve stimulation and the subsequent activation of non-α-, non-β-adrenoceptors cause positive chronotropic and inotropic responses in the toad sinus venosus. The results suggest that the mechanisms activated after sympathetic nerve stimulation and those activated after β-adrenoceptor stimulation are quite different.

In heart muscle, responses to the exogenous application of catecholamines result from the activation of β-adrenoceptors and the subsequent elevation of intracellular cAMP (32). Consequently, the amplitude of pacemaker action potentials is increased (4, 5), as is the magnitude of the transient increase in [Ca²⁺], associated with each contraction (12). Similar observations after β-adrenoceptor activation were made in this study (Fig. 8). Isoprenaline evoked an increase in the amplitude of pacemaker action potentials and a concurrent increase in the magnitude of the transient elevation of [Ca²⁺], that accompanied each action potential. These effects of isoprenaline were not changed by caffeine. Therefore, β-adrenoceptor activation apparently initiates an increase in force production by the increased influx of extracellular Ca²⁺. As a consequence of the increased influx of Ca²⁺, [Ca²⁺], may then be further amplified by the process of Ca²⁺-induced Ca²⁺ release (CICR) from intracellular Ca²⁺ stores.

In contrast, the positive inotropic and chronotropic responses evoked by sympathetic nerve stimulation, which result from the activation of non-α-, non-β-adrenoceptors (4), were not associated with an increase in the amplitude of the pacemaker action potentials or the magnitude of the concomitant transient increases in [Ca²⁺]. Rather, after sympathetic nerve stimulation, there was an apparent decrease in the magnitude of the transient increases in [Ca²⁺], associated with each pacemaker action potential. This was a consequence of a marked increase in the basal level of [Ca²⁺], measured during diastole with no apparent increase in peak
[Ca\textsuperscript{2+}]\textsubscript{i}, attained during each action potential. The increase in the basal level of [Ca\textsuperscript{2+}]\textsubscript{i} is unlikely to be a direct consequence of the increase in rate of generation of pacemaker action potentials. If this were the case, it might be expected that an increase in basal [Ca\textsuperscript{2+}]\textsubscript{i} would also accompany the increase in the rate of action potential generation after \(\beta\)-adrenoceptor stimulation with isoprenaline, which was not apparent (Fig. 8B). Therefore, it seems likely that basal [Ca\textsuperscript{2+}]\textsubscript{i} is actively increased by sympathetic nerve stimulation. It is clear that the failure of sympathetic nerve stimulation to evoke an increase in the peak levels of [Ca\textsuperscript{2+}]\textsubscript{i}, associated with each action potential does not reflect an inability of fura 2 to measure higher concentrations of [Ca\textsuperscript{2+}]\textsubscript{i}. That this is the case is illustrated by the observation that \(\beta\)-adrenoceptor activation with isoprenaline did evoke an increase in the peak levels of [Ca\textsuperscript{2+}]\textsubscript{i} associated with each action potential. However, it is conceivable that an increase in the peak [Ca\textsuperscript{2+}]\textsubscript{i} evoked by sympathetic nerve stimulation may have been too small to have been detected. This might be a consequence of the nonlinearity of the fura 2-Ca\textsuperscript{2+} complex fluorescence emission spectrum for different [Ca\textsuperscript{2+}]\textsubscript{i}. Even so, our results demonstrate that the increase in the peak [Ca\textsuperscript{2+}]\textsubscript{i} evoked by \(\beta\)-adrenoceptor stimulation was larger than that evoked by sympathetic nerve stimulation. Alternatively, the increase in basal [Ca\textsuperscript{2+}]\textsubscript{i} during diastole could have led to the partial inactivation of voltage-dependent L-type Ca\textsuperscript{2+} channels and resulted in a reduction of voltage-dependent L-type Ca\textsuperscript{2+} current (20). This might then account for the decrease in action potential amplitude seen in some preparations during the initial tachycardia evoked by sympathetic nerve stimulation. It is very clear that the changes in [Ca\textsuperscript{2+}]\textsubscript{i} after sympathetic nerve stimulation differ greatly from those produced by \(\beta\)-adrenoceptor activation.

It is possible that all the changes evoked by sympathetic nerve stimulation result from the release of intracellularly stored Ca\textsuperscript{2+}. In preparations of toad sinus venosus that have been arrested with a voltage-dependent Ca\textsuperscript{2+} channel antagonist such as nifedipine, stimulation of the sympathetic nerves evokes an oscillatory membrane depolarization (10). Such membrane depolarizations are associated with an oscillatory increase in [Ca\textsuperscript{2+}]\textsubscript{i} and force production, even though Ca\textsuperscript{2+} entry through voltage-dependent L-type Ca\textsuperscript{2+} channels has been inhibited (10). All these responses are abolished after the depletion of Ca\textsuperscript{2+} from intracellular stores (10). In the present experiments, caffeine increased the basal levels of beat rate and force of contraction but abolished responses to sympathetic nerve stimulation. These two effects of caffeine are likely to be due to two different modes of action. Together with its ability to deplete intracellular Ca\textsuperscript{2+} stores by lowering the threshold for CICR (29), caffeine is also a phosphodiesterase inhibitor (6). It has previously been shown that increasing levels of cAMP through the inhibition of phosphodiesterases increases beat rate and action potential amplitude (3). However, the application of phosphodiesterase inhibitors has no effect on responses evoked by sympathetic nerve stimulation (3). It therefore seems likely that the effects of caffeine to abolish nerve-evoked responses were due to its ability to deplete intracellular Ca\textsuperscript{2+} stores. This action of caffeine seems most likely for a number of reasons. 1) High concentrations of caffeine applied to the sinus venosus after Ca\textsuperscript{2+} entry had been abolished with nifedipine caused oscillatory changes in force. 2) Caffeine applied at short intervals resulted in progressively smaller contractions and depolarizations, presumably reflecting the gradual depletion of intracellular stores. 3) After the addition of ryanodine, caffeine failed to evoke a membrane depolarization or a change in force. This, together with the observation that the responses evoked by sympathetic nerve stimulation could not be restored after the washout of caffeine in the presence of the Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin, suggests that caffeine acted to deplete intracellular Ca\textsuperscript{2+} stores. This suggests that the release of intracellularly stored Ca\textsuperscript{2+} is essential to evoke the positive inotropic and chronotropic responses produced by sympathetic nerve stimulation in the toad sinus venosus. This is in contrast to the activation of \(\beta\)-adrenoceptors.

It is noteworthy, however, that ryanodine attenuated, but did not abolish, the positive inotropic responses evoked by sympathetic nerve stimulation. It is unclear why the effects of ryanodine and caffeine on sympathetically evoked responses in the sinus venosus were different. Ryanodine has been reported to be use dependent (17), such that prior activation of the CICR channel is required before ryanodine can render the sarcoplasmic reticulum release channel permanently open (30). This use-dependent action of ryanodine, and not caffeine, may explain the observed differences between these two drugs on the responses evoked by sympathetic nerve stimulation. That this is the case is suggested by the observation that ryanodine and caffeine, when applied in combination, irreversibly abolished the membrane depolarization evoked by sympathetic nerve stimulation in arrested preparations of toad sinus venosus (10). In the absence of ryanodine, the effects of caffeine are fully reversible. Another possibility that may explain the differences between the actions of caffeine and ryanodine is that caffeine, as well as modulating the CICR channel, may have an additional action on intracellular Ca\textsuperscript{2+} stores. In rat hepatocytes it has been suggested that caffeine acts as a low-affinity antagonist of the inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptor (21). There is a plethora of evidence that IP\textsubscript{3} is the second messenger that mediates intracellular store release in smooth muscle preparations and nonexcitable cells (2). In mammalian cardiac muscle, positive inotropic responses produced by \(\alpha\)-adrenoceptor stimulation are associated with increases in the levels of IP\textsubscript{3} (25, 31). IP\textsubscript{3} has also been shown to cause directly the release of Ca\textsuperscript{2+} in cardiac skinned fibers (24). Therefore, IP\textsubscript{3}-induced Ca\textsuperscript{2+} release and CICR may play a role in sympathetically evoked responses. Irrespective of the identity of the second messenger involved, it is clear that the increase in the force of beat evoked after activation of non-\(\alpha\)-, non-\(\beta\)-
adrenoceptors is the consequence of the activation of a biochemical pathway that involves the release of Ca\(^{2+}\) from intracellular stores.

The observation that the period of elevated [Ca\(^{2+}\)] was evoked by sympathetic nerve stimulation coincided with the time course of the first component of the positive chronotropy might suggest that the two events are related. The mechanism by which the release of intracellularly stored Ca\(^{2+}\) might cause an increase in beat rate after sympathetic nerve stimulation is unclear. In preparations of toad sinus venosus that have been arrested with nifedipine, sympathetic nerve stimulation evokes a membrane depolarization and increase in [Ca\(^{2+}\)]. (3, 4, 10). Given that both responses were abolished after the depletion of intracellular Ca\(^{2+}\) stores (10), it is possible that the membrane depolarization results from the activation of a Ca\(^{2+}\)-dependent conductance. Although there is evidence to suggest that Cl\(^{-}\) channels are activated by an elevation of [Ca\(^{2+}\)] in cardiac myocytes (9), it is unlikely that a Cl\(^{-}\) conductance is responsible for the membrane depolarization evoked in arrested preparations of toad sinus venosus (3). However, this does not rule out the possibility of the involvement of a Ca\(^{2+}\)-activated cation conductance in the positive chronotropic response evoked by sympathetic nerve stimulation. A Ca\(^{2+}\)-activated cation channel has been described in isolated guinea pig ventricular myocytes (11). However, no evidence of its contribution to transient inward currents during oscillations in [Ca\(^{2+}\)] was found in the same tissue (34).

Alternatively, the positive chronotropic response evoked by sympathetic nerve stimulation might result from the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores and the subsequent activation of the Na\(^{+}\)/Ca\(^{2+}\) exchanger. In cardiac tissue the Na\(^{+}\)/Ca\(^{2+}\) exchanger is one of the major mechanisms responsible for the removal of elevated Ca\(^{2+}\) from the cell after cardiac action potentials (28). In frog atrial myocytes, increases in Ca\(^{2+}\) have been shown to evoke inward currents that have been attributed to an electrogenic Na\(^{+}\)/Ca\(^{2+}\) exchanger (7, 16). Similarly, the activation of the Na\(^{+}\)/Ca\(^{2+}\) exchanger has also been proposed to account for transient inward currents observed during changes in [Ca\(^{2+}\)] in mammalian sinoatrial, atrial, and ventricular myocytes (19, 33, 37). Being electrogenic, the Na\(^{+}\)/Ca\(^{2+}\) exchanger is thought to exchange one Ca\(^{2+}\) for three Na\(^{+}\) (27), with the direction and amplitude of the current being dependent on the membrane potential and the ion gradients for Ca\(^{2+}\) and Na\(^{+}\) (18). By sympathetic nerve stimulation, it might be expected that elevated [Ca\(^{2+}\)] would be extruded from the cell via the Na\(^{+}\)/Ca\(^{2+}\) exchanger, resulting in a net inward current. Therefore, such an inward current could account for the increased rate of diastolic depolarization to produce the positive chronotropy after sympathetic nerve stimulation and could also account for the membrane depolarization evoked in arrested preparations of toad sinus venosus (10). This idea could not be tested further. Even though N\(^{1+}\) and a number of the amiloride derivatives, which are known to block the Na\(^{+}\)/Ca\(^{2+}\) exchanger (33), abolished the chronotropic response evoked by sympathetic nerve stimulation in the sinus venosus, they were also found to interfere with transmitter release in this tissue (unpublished observations).

In summary, the results of this study suggest that catecholamine released from sympathetic nerve terminals evokes positive inotropic and chronotropic responses via a mechanism that is distinctly different from that involved with the activation of β-adrenoceptors in cardiac tissue. It appears that the increase in force and beat rate evoked by sympathetic nerve stimulation and the subsequent activation of non-α-, non-β-adrenoceptors is a consequence of the release of intracellularly stored Ca\(^{2+}\).

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