Bramich, Narelle J., and Helen M. Cousins. Effects of sympathetic nerve stimulation on membrane potential, \([\text{Ca}^{2+}]_i\), 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 \(\text{Ca}^{2+}\) concentration ([\(\text{Ca}^{2+}\)]) 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 beat 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 [\(\text{Ca}^{2+}\)] with little change in peak [\(\text{Ca}^{2+}\)], measured during each action potential. Depletion of intracellular \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\)-ATPase inhibitor thapsigargin (30 \(\mu\)M), abolished irreversibly the chronotropic and inotropic responses evoked by sympathetic nerve stimulation. Ryanodine (10 \(\mu\)M) 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 \(\text{Ca}^{2+}\) from intracellular \(\text{Ca}^{2+}\) stores.

cardiac; intracellular calcium

STIMULATION OF THE sympathetic nerves innervating the pacemaker region of the mammalian and amphibian heart causes an increase in the rate and force of beat (13, 26). Superficially, these positive inotropic and chronotropic responses can be mimicked by the exogenous application of catecholamines to the heart (norepinephrine in mammals and epinephrine in amphibians). In both classes the exogenous application of catecholamines activates \(\beta\)-adrenoceptors, which, via a cAMP-dependent pathway, causes the phosphorylation and subsequent modulation of voltage-dependent channels involved in pacemaking: the hyperpolarization-activated inward current, the delayed rectifier current, and the voltage-dependent L-type \(\text{Ca}^{2+}\) current (5, 14, 23).

The cAMP-dependent modulation of these voltage-dependent ion channels evokes positive inotropic and chronotropic responses and causes marked changes in the shape of pacemaker action potentials: 1) an increased rate of diastolic depolarization, 2) an increased rate of action potential repolarization, and 3) an increased amplitude of pacemaker action potentials.

Recently, it has been shown that the mechanisms that underlie the positive chronotropic responses in the pacemaker region of the mammalian (8) and amphibian (3, 4) heart differ markedly, depending on whether transmitter is released from the nerve terminals or applied to the preparation exogenously. In the mammalian and amphibian heart, stimulation of sympathetic nerves and the subsequent release of catecholamines evoke an increase in the rate of generation of pacemaker action potentials that is associated with an increase in the rate of diastolic depolarization, but there is little other change in the shape of pacemaker action potentials (4, 8). Furthermore, in the mammalian and the amphibian the receptors activated by neuronally released catecholamines do not appear to activate a cAMP-dependent pathway (3, 8). These observed differences between responses evoked by sympathetic nerve stimulation and applied catecholamines have led to the suggestion that two different populations of receptor are activated by the two different sources of catecholamine in both classes (3, 4, 8). In the toad heart it has been shown that these receptors can be distinguished pharmacologically. The exogenous application of epinephrine most readily activates \(\beta\)-adrenoceptors (35). However, neuronally released epinephrine most readily activates a set of receptors that are neither \(\alpha\)-nor \(\beta\)-adrenoceptors (4, 22). These receptors are also activated by high concentrations of epinephrine and can be blocked by dihydroergotamine (3, 4). Activation of these non-\(\alpha\), non-\(\beta\)-adrenoceptors causes an increase in the rate and force of beat with no associated increase in the amplitude of pacemaker action potentials. This might suggest that the increased force of beat generated after activation of non-\(\alpha\), non-\(\beta\)-adrenoceptors does not result from an increased influx of \(\text{Ca}^{2+}\) through voltage-dependent \(\text{Ca}^{2+}\) channels.

We recently showed, in preparations of toad sinus venosus in which the contribution of voltage-dependent \(\text{Ca}^{2+}\) channels has been removed by “arresting” the tissue with voltage-dependent \(\text{Ca}^{2+}\) channel antagonists such as nifedipine, that sympathetic nerve stimulation triggers the release of \(\text{Ca}^{2+}\) from intracellular stores (10). We suggested that in arrested sinus venosus preparations the release of \(\text{Ca}^{2+}\) from intracellular stores triggers not only the oscillatory increase in force but also the membrane depolarization evoked after sympathetic nerve stimulation. A number of observations suggest that the mechanisms underlying responses evoked by sympathetic nerve stimulation in spontaneously beating preparations are the same as those underlying the responses recorded in arrested preparations: 1) Similar to the nerve-evoked responses recorded from arrested preparations, the positive inotropic and chronotropic responses evoked by sympathetic nerve stimulation result from the activation of non-\(\alpha\), non-\(\beta\)-adrenoceptors and can be blocked by dihydroergotamine (4). 2) All the responses evoked by sympa-
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$^{2+}$ concentration ([Ca$^{2+}$]) 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 venous (3). It therefore seems possible that sympathetic nerve stimulation may also trigger Ca$^{2+}$ store release in spontaneously beating preparations of toad sinus venous.

The aim of this study was to determine whether the positive inotropic and chronotropic responses recorded from the spontaneously beating toad sinus venous evoked by sympathetic nerve stimulation are mediated by the release of Ca$^{2+}$ from intracellular Ca$^{2+}$ 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 venous 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 venous 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 venous 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. Recorded force was digitized and stored on disk for later analysis.

In a separate series of experiments in which [Ca$^{2+}$], 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 venous and epicardium of the atria. The sinus venous 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 venous. 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$^{2+}$] were measured simultaneously from the pinned sinus venous preparations. Preparations were loaded with the fluorescent Ca$^{2+}$ indicator fura 2 by incubation in HEPES-buffered physiological saline with low Ca$^{2+}$ (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 esterification 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$_3$, 3.1 NaH$_2$PO$_4$, 1.8 CaCl$_2$, 1.4 MgCl$_2$, and 16.7 glucose, gassed with 95% O$_2$-5% CO$_2$) 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$^{2+}$]. 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$_{340}$/F$_{380}$) was taken as a qualitative indicator of [Ca$^{2+}$]. The ratio was not affected by nifedipine (10 µM) or norepinephrine (100 µM). The effects of the two drugs were compared to control levels by 10.220.32.246 on October 23, 2017 http://ajpheart.physiology.org/ Downloaded from by 10.220.32.246 on October 23, 2017 http://ajpheart.physiology.org/ Downloaded from
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 $10 \text{ min.}$

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 $31 \pm 1 \text{ beats/min}$, which increased to $48 \pm 2 \text{ beats/min}$ (n = 27). Contractions had a mean amplitude of $1.6 \pm 0.1 \text{ mN}$, which increased to $2.3 \pm 0.2 \text{ mN}$ 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 $14 \pm 1 \text{ beats/min}$ (mean baseline and peak rate $= 30 \pm 1$ and $44 \pm 1 \text{ beats/min}$, respectively, n = 28; Fig. 1B, trace b), a minimum latency of $2.5 \pm 0.1 \text{ s}$, a rise time of $2.0 \pm 0.1 \text{ s}$, and a half-width of $11.5 \pm 0.9 \text{ s}$ (n = 27; Fig. 1B, trace b). The secondary increase in rate reached a maximum of $2.0 \pm 0 \text{ beats/min}$ (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 $1.6 \pm 0.1 \text{ mN}$, 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/)
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
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 Ca2+ from intracellular stores. In contrast, the increases in force and rate after β-adrenoceptor stimulation are essentially independent of intracellularly stored Ca2+.

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 Ca2+ through voltage-dependent L-type Ca2+ 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

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**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.
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.

Fig. 5. Effect of ryanodine (10 µM) on membrane potential and force responses evoked by sympathetic nerve stimulation and caffeine (10 mM) in preparations of toad sinus venosus that have been arrested with nifedipine. Stimulation of sympathetic nerves (A) evoked a membrane depolarization (trace a) and increase in force (trace b), both of which were oscillatory in nature. In same preparation, rapid bath application of caffeine (B) also evoked oscillations in membrane potential (trace a) and force (trace b). C and D: effect of ryanodine on responses evoked by sympathetic nerve stimulation and caffeine, respectively. In all traces, membrane potential was ~36 mV. Nifedipine (10 µM) was present throughout. Voltage calibration bar refers to all a traces; force calibration bar refers to all b traces; time calibration bar refers to all traces.
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

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**Fig. 6.** Effect of thapsigargin (30 µM) and caffeine (3 mM) on force and rate responses evoked by sympathetic nerve stimulation recorded from spontaneously beating toad sinus venosus. Sympathetic nerve stimulation (●) evoked positive inotropic (trace a) and chronotropic (trace b) responses when recorded from toad sinus venosus in presence of propranolol (1 µM; A). Addition of thapsigargin decreased, but did not abolish, inotropic and chronotropic responses evoked by sympathetic nerve stimulation (B). Arrow, secondary increase in force in presence of thapsigargin. Subsequent addition of caffeine to physiological saline, in presence of propranolol and thapsigargin, abolished increase in force and increase in beat rate evoked by stimulation of sympathetic nerves (C). Responses evoked by sympathetic nerve stimulation were not restored after washout of caffeine in continued presence of propranolol and thapsigargin (D). Time calibration bar refers to all traces.
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+]i 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 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+]i, compared with the diastolic [Ca2+]i recorded before sympathetic nerve stimulation: the diastolic [Ca2+]i 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+]i, there was little change in the peak [Ca2+]i achieved during each [Ca2+]i oscillation. Thus the mean amplitude of the transient increase in [Ca2+]i associ-
ated with pacemaker action potentials after sympathetic nerve stimulation was decreased to $0.10 \pm 0.02$ F$_{340/380}$ compared with the control value $0.17 \pm 0.03$ 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$^{2+}$] 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$^{2+}$]. The time course of the positive chronotropic response and the time course of the increase in the diastolic [Ca$^{2+}$] 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$^{2+}$]. The positive chronotropic response produced by β-adrenoceptor activation is associated with an increase in the amplitude of pacemaker action potentials via a cAMP-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$^{2+}$] 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$^{2+}$] (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$^{2+}$] 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$^{2+}$] oscillations associated with each action potential (Fig. 7B). Clearly, during the positive chronotropic response produced by isoprenaline, the basal levels of [Ca$^{2+}$] did not increase (Fig. 8B). This suggests that an increase in beat rate alone cannot account for the changed basal levels of [Ca$^{2+}$], 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$^{2+}$] and force production after sympathetic nerve stimulation might be intracellular.

Effect of caffeine on sympathetically evoked changes in membrane potential and [Ca$^{2+}$]. To determine whether the sympathetically evoked changes in [Ca$^{2+}$] 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$^{2+}$] was examined. In a series of five experiments, sympathetic nerve stimulation increased the
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 [Ca\(^{2+}\)], by 0.06 ± 0.01 F\(_{340/380}\) and decreased the amplitude of the [Ca\(^{2+}\)], oscillations (mean amplitude of [Ca\(^{2+}\)], oscillation before and after sympathetic nerve stimulation = 0.20 ± 0.04 and 0.13 ± 0.03 F\(_{340/380}\), 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 [Ca\(^{2+}\)], 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 [Ca\(^{2+}\)], (change in diastolic [Ca\(^{2+}\)], after sympathetic nerve stimulation = 0.01 ± 0.01 F\(_{340/380}\), amplitude of [Ca\(^{2+}\)], oscillations before and after sympathetic nerve stimulation = 0.18 ± 0.04 and 0.17 ± 0.04 F\(_{340/380}\), 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 [Ca\(^{2+}\)], oscillation before and after sympathetic nerve stimulation = 0.26 ± 0.07 and 0.16 ± 0.07 F\(_{340/380}\), respectively; Fig. 9C).

These results indicate that the positive chronotropy and the changes in [Ca\(^{2+}\)], evoked by sympathetic nerve stimulation result from the release of Ca\(^{2+}\) from intracellular stores.

**DISCUSSION**

This study has examined the mechanisms by which sympathetic nerve stimulation and the subsequent activation of non-\(\alpha\), non-\(\beta\)-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 \(\beta\)-adrenoceptor stimulation are quite different.

In heart muscle, responses to the exogenous application of catecholamines result from the activation of \(\beta\)-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 [Ca\(^{2+}\)], associated with each contraction (12). Similar observations after \(\beta\)-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 [Ca\(^{2+}\)], that accompanied each action potential. These effects of isoprenaline were not changed by caffeine. Therefore, \(\beta\)-adrenoceptor activation apparently initiates an increase in force production by the increased influx of extracellular Ca\(^{2+}\). As a consequence of the increased influx of Ca\(^{2+}\), [Ca\(^{2+}\)], may then be further amplified by the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from intracellular Ca\(^{2+}\) stores.

Fig. 9. Effects of caffeine (3 mM) on changes in membrane potential and [Ca\(^{2+}\)], evoked by sympathetic nerve stimulation recorded from spontaneously beating pacemaker region of toad heart. In control physiological saline, sympathetic nerve stimulation (\(\bullet\); A) evoked an increase in rate of action potential generation recorded from sinus venosus (trace a). Associated with each action potential is an oscillation of [Ca\(^{2+}\)] (A, trace b). After sympathetic nerve stimulation, there was an increase in basal level of [Ca\(^{2+}\)] (A, trace b). Caffeine (B) abolished increase in beat rate (trace a) and increase in basal [Ca\(^{2+}\)] (trace b) produced by sympathetic nerve stimulation. Responses were fully restored after washout of caffeine (C). Time calibration bar refers to all traces.

In contrast, the positive inotropic and chronotropic responses evoked by sympathetic nerve stimulation, which result from the activation of non-\(\alpha\), non-\(\beta\)-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 [Ca\(^{2+}\)]. Rather, after sympathetic nerve stimulation, there was an apparent decrease in the magnitude of the transient increases in [Ca\(^{2+}\)], associated with each pacemaker action potential. This was a consequence of a marked increase in the basal level of [Ca\(^{2+}\)], measured during diastole with no apparent increase in peak
[Ca$^{2+}$], attained during each action potential. The increase in the basal level of [Ca$^{2+}$] 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$^{2+}$] would also accompany the increase in the rate of action potential generation after β-adrenoceptor stimulation with isoprenaline, which was not apparent (Fig. 8B). Therefore, it seems likely that basal [Ca$^{2+}$] 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$^{2+}$], associated with each action potential does not reflect an inability of fura 2 to measure higher concentrations of [Ca$^{2+}$]. That this is the case is illustrated by the observation that β-adrenoceptor activation with isoprenaline did evoke an increase in the peak levels of [Ca$^{2+}$], associated with each action potential. However, it is conceivable that an increase in the peak [Ca$^{2+}$], 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$^{2+}$ complex fluorescence emission spectrum for different [Ca$^{2+}$]. Even so, our results demonstrate that the increase in the peak [Ca$^{2+}$] evoked by β-adrenoceptor stimulation was larger than that evoked by sympathetic nerve stimulation. Alternatively, the increase in basal [Ca$^{2+}$] during diastole could have led to the partial inactivation of voltage-dependent L-type Ca$^{2+}$ channels and resulted in a reduction of voltage-dependent L-type Ca$^{2+}$ 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$^{2+}$] after sympathetic nerve stimulation differ greatly from those produced by β-adrenoceptor activation.

It is possible that all the changes evoked by sympathetic nerve stimulation result from the release of intracellularly stored Ca$^{2+}$. In preparations of toad sinus venosus that have been arrested with a voltage-dependent Ca$^{2+}$ 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$^{2+}$] and force production, even though Ca$^{2+}$ entry through voltage-dependent L-type Ca$^{2+}$ channels has been inhibited (10). All these responses are abolished after the depletion of Ca$^{2+}$ 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$^{2+}$ 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$^{2+}$ 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$^{2+}$ 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$^{2+}$-ATPase inhibitor thapsigargin, suggests that caffeine acted to deplete intracellular Ca$^{2+}$ stores. This suggests that the release of intracellularly stored Ca$^{2+}$ 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 β-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$^{2+}$ stores. In rat hepatocytes it has been suggested that caffeine acts as a low-affinity antagonist of the inositol 1,4,5-trisphosphate (IP$_3$) receptor (21). There is a plethora of evidence that IP$_3$ is the second messenger that mediates intracellular Ca$^{2+}$ store release in smooth muscle preparations and nonexcitable cells (2). In mammalian cardiac muscle, positive inotropic responses produced by α$_1$-adrenoceptor stimulation are associated with increases in the levels of IP$_3$ (25, 31). IP$_3$ has also been shown to cause directly the release of Ca$^{2+}$ in cardiac skinned fibers (24). Therefore, IP$_3$-induced Ca$^{2+}$ 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-α-, non-β-
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\(^{2+}\)], 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 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). Although 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 Na\(^{+}\) 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 ß-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-ß-, non-ß-adrenoceptors is a consequence of the release of intracellularly stored Ca\(^{2+}\).

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