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Cellular calcium fluctuations in mammalian heart: Direct evidence from noise analysis of aequorin signals in Purkinje fibers

(intracellular Ca^{2+} concentration/oscillations/cardiac physiology/contractile activation)

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ABSTRACT **Indirect evidence suggests that fluctuations, or oscillations, in the intracellular free calcium concentration ($[Ca^{2+}]_i$) can occur spontaneously in intact cardiac preparations, but such $[Ca^{2+}]_i$ fluctuations have never been demonstrated directly. We used the bioluminescent Ca^{2+}-sensitive protein aequorin to detect fluctuations in the $[Ca^{2+}]_i$ in canine cardiac Purkinje fibers. Noise analysis of the aequorin luminescence reveals prominent peaks of power density at frequencies of 1–4 Hz; these peaks become larger and shift to higher frequencies as the $[Ca^{2+}]_i$ increases. Caffeine and ryanodine abolish the $[Ca^{2+}]_i$ fluctuations, suggesting that Ca^{2+} release and uptake by the sarcoplasmic reticulum generate these events. When $[Ca^{2+}]_i$ fluctuations are present, less tension is produced at any given level of mean aequorin luminescence. Thus, $[Ca^{2+}]_i$ fluctuations will undermine attempts to relate $[Ca^{2+}]_i$ and force in intact myocardium.**

Changes in the intracellular free calcium concentration ($[Ca^{2+}]_i$) mediate cellular processes as varied as chemotaxis and secretion, in systems ranging from prokaryotes to mammalian endocrine cells (1). In excitable cells, such changes in the $[Ca^{2+}]_i$ are usually initiated by electrical stimuli, but $[Ca^{2+}]_i$ in cardiac muscle can oscillate even after the stimulated action potential is over and twitch tension has decayed. These $[Ca^{2+}]_i$ oscillations are manifested as oscillations in force and membrane potential, first described 40 years ago (2) and since witnessed frequently under conditions of increased $[Ca^{2+}]_i$ (see ref. 3 for review). Direct evidence for these afteroscillations in $[Ca^{2+}]_i$ has come from preliminary studies using the Ca^{2+}-sensitive bioluminiscent protein aequorin, showing a transient increase in luminescence associated with aftercontractions (4–6).

Oscillations in force, membrane potential, and related parameters can also occur *spontaneously*—i.e., not coupled to any prior electrical stimulation (see references in refs. 3, and 7–12). A large body of evidence also points to Ca_i^{2+} as the cause of these oscillations: spontaneous mechanical oscillations correlate closely with those in membrane potential (or current) (7); both mechanical and electrical fluctuations occur most readily under conditions of increased Ca^{2+} loading; conversely, both are attenuated by intracellular EGTA injection, presumably by chelation of excess Ca^{2+} (7, 8). While compelling, the evidence for spontaneous $[Ca^{2+}]_i$ fluctuations remains indirect, based on mechanical measurements that can be unreliable indicators of $[Ca^{2+}]_i$ (see, e.g., refs. 11–13). There is clearly a need for direct measurements to confirm the existence of spontaneous $[Ca^{2+}]_i$ fluctuations and to provide a basis for quantitative understanding of these oscillations. $[Ca^{2+}]_i$ fluctuations are likely to have profound implications for the relationship between the $[Ca^{2+}]_i$ and contractile force (11, 12), as well as for many other Ca^{2+}-dependent cellular processes. These relationships cannot be characterized adequately without direct $[Ca^{2+}]_i$ measurements. For this purpose, the rapid response time of aequorin represents a distinct advantage over Ca^{2+}-sensitive microelectrodes, which are not able to resolve fast Ca^{2+} transients (14). We used noise analysis of Ca^{2+}-activated aequorin luminescence to try to obtain direct evidence for spontaneous $[Ca^{2+}]_i$ oscillations in cardiac Purkinje fibers. A preliminary report of this work has appeared (15).

Table 1. Compositions of solutions used

Name	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	Dextrose
Control	161	2.7	2	2.7	149.1	24	5.6
0K	161	0	2	2.7	146.4	24	5.6
0K24Na*	24	0	2	2.7	9.4	24	5.6

All solutions were gassed with 95% O_2/5% CO_2 and adjusted to pH 7.4.
*NaCl removed was replaced by equimolar sucrose.

MATERIALS AND METHODS

Experimental Techniques. Hearts were removed from mongrel dogs anesthetized with T-61 euthanasia solution (0.5 mg/kg; Hoechst, Hounslow, Middlesex, United Kingdom). Free-running Purkinje fibers 200- to 400-μm in external diameter were chosen and mounted on fine stainless steel hooks, one end fixed to a pedestal and the other attached to a force transducer (Akers 801 element), in a bath chamber perfused with control solution (Table 1). Flow was controlled by a pump and was kept constant during any single experiment to ensure comparable solution changes. Temperature was maintained at 35°C–36°C by a Peltier device. The fibers were field stimulated at frequencies of 1–3 Hz during recovery from mounting. Those fibers that exhibited a normalized twitch force of $\approx$2.5 g/mm^2 of total cross-sectional area with a stimulus duration of 1 msec were then pressure injected with aequorin as described (16, 17). Typically, 10–20 cells in each fiber were injected. The chamber was then shielded from external light, and light from the preparation was collected through a Lucite light guide leading to a photomultiplier tube (EMI 9893 B/350). Light was recorded continuously as an analog signal on magnetic tape (1 V/2×10^3 cps) and was also collected in digital form as photon

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Abbreviations: $[Ca^{2+}]_i$ and $[Na^+]_i$, intracellular free calcium and sodium concentration, respectively; L/L_{max}, fractional luminescence; SR, sarcoplasmic reticulum.

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counts (using discriminator control unit model 1121A and signal averager model 4203, Princeton Applied Research, Princeton, NJ). Tension and membrane potential (when measured) were also recorded continuously on magnetic tape and on a chart recorder.

The experimental protocol used was patterned after that of Corabouef *et al.* (18). Fibers were bathed in 0K solution (Table 1) for a "priming" interval [to inhibit the Na^+ pump and allow the intracellular free sodium concentration ($[Na^+]_i$) and $[Ca^{2+}]_i$ to rise] and then exposed to 0K24Na solution (Table 1) for a sufficiently long time for tension to plateau. The low-Na^+ solution increases the $[Ca^{2+}]_i$ even further, presumably via the Na^+/Ca^{2+} exchange mechanism (19, 20). Control solution was then restored, the fiber was allowed to recover to within 90% of the original control twitch tension, and the above protocol was repeated. In some experiments, caffeine (4–10 mM) or ryanodine (1 μM) was added at various points in the protocol. Fibers were not stimulated during the collection of data for fluctuation analysis (see below).

Data Analysis. The light records stored on magnetic tape were low-pass filtered at 15 Hz and digitized at 40 Hz on a Nicolet digital oscilloscope. Data segments of 40- to 500-sec duration were analyzed using a fast Fourier transform program (modified from Ralston and Wilf, ref. 21) to generate power spectra (e.g., Fig. 1*C*). Prior studies of oscillatory phenomena in heart cells have found power density to be concentrated in the range below 10 Hz (3, 8); we confirmed this in our own experiments by examining several power spectra generated from unfiltered data.

Great care was taken to exclude from analysis data segments showing any twitch activity, which might be due to electrically triggered rather than spontaneous $[Ca^{2+}]_i$ oscillations. We did not measure membrane potential routinely, but in two experiments, we confirmed the findings of Corabouef *et al.* (18): action potentials or low-voltage oscillations (22) could be reliably excluded when there was no sign of twitch activity.

In principle, changes in muscle position could affect the efficiency of light collection, leading to spurious fluctuations in luminescence. This is unlikely to be important in our experiments because tension fluctuations are very small even when large luminescence fluctuations are recorded (see Figs. 1–4). Nevertheless, we tried to minimize this possibility by using a light guide with a diameter (6.5 mm) that is large relative to the injected area of the preparation and by mounting fibers so that little or no rotation was evident visually.

During some of the experimental interventions, the $[Ca^{2+}]_i$ rose to high levels for a long enough period of time that appreciable utilization of aequorin occurred. We corrected some of the luminescence records for this problem by using the following method. After the experiment, we integrated the luminescence throughout the entire experiment, so that we knew at any given moment how much aequorin remained in the preparation. Luminescence records could be corrected from that point onward by using the known relationship between the fractional luminescence (L/L_{max}) and the rate of aequorin consumption (ref. 14; see also the legend to Fig. 1).

RESULTS

The results from a fiber during exposure to low-Na^+ solution and at rest are shown in Fig. 1 (*A*, tension; *B*, light). The increase in tension during low-Na^+ exposure is associated with a marked rise in aequorin luminescence, which decays rapidly along with the relaxation on return to control solution. The power spectra of aequorin luminescence during 0K24Na exposure (1) and from the fiber at rest (2) are shown in Fig. 1*C*. The power

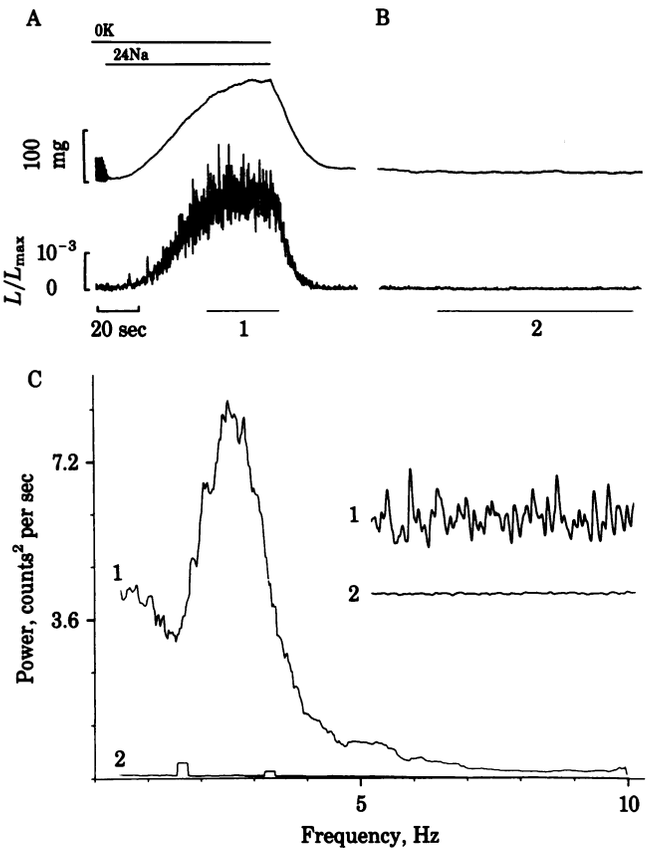

FIG. 1. Tension and aequorin luminescence during K^+-free low-Na^+ solution exposure and at rest. (*A* and *B*) Tension (upper records) and luminescence (lower records) during exposure to the solutions indicated by the solid lines above the tension records (Table 1). The absence of lines above the records in this and subsequent figures indicates superfusion with control solution. The calibration bar for tension is arbitrarily positioned with respect to zero. L/L_{max} was determined by using the method of Allen and Blinks (16). The numbered lines below the luminescence records indicate the segments of the light records used for the spectral analysis shown in *C*; each power spectrum in *C* is labeled with the number of the corresponding data segment. The vertical axis in *C* is in units of counts² per sec, corresponding to (count rate)² × sec and obtained from V^2-sec by calibration of our photomultiplier ratemeter output. (*Inset*) Part of data segments 1 and 2 are displayed at the same vertical gain as in *A* and *B* but at a 10 times faster time base. The luminescence records in this figure were not corrected for aequorin consumption. Preparation 2-22 was used.

density is maximum at ≈3 Hz in the record obtained during exposure to 0K24Na solution. By comparison, there is little power in the resting spectrum. The raw signals that correspond to the power spectrum are also shown on an expanded time base (Fig. 1*C Inset*): note that in record 1 the light exhibits large fluctuations at ≈3 Hz; the record obtained at rest in control solution shows much smaller fluctuations (record 2).

As shown by the oscillations in aequorin luminescence in Fig. 1, the $[Ca^{2+}]_i$ does indeed oscillate spontaneously in cardiac cells. The figure also suggests that these $[Ca^{2+}]_i$ fluctuations become more prominent as the degree of Ca^{2+} loading increases. This possibility was tested by examining power spectra from progressive stages of Ca^{2+} loading during Na^+ pump inhibition (Fig. 2). The fiber was exposed to 0K solution for 13 min, then bathed with 0K24Na solution (Fig. 2*A*). The power spectrum of light during the first 4 min in 0K solution (Fig. 2*B*, record 1) reveals very little power density. In the following 4 min (Fig. 2*A*, record 2), as tension and light are gradually rising, there is more power concentrated in the frequency range below 2 Hz (Fig.

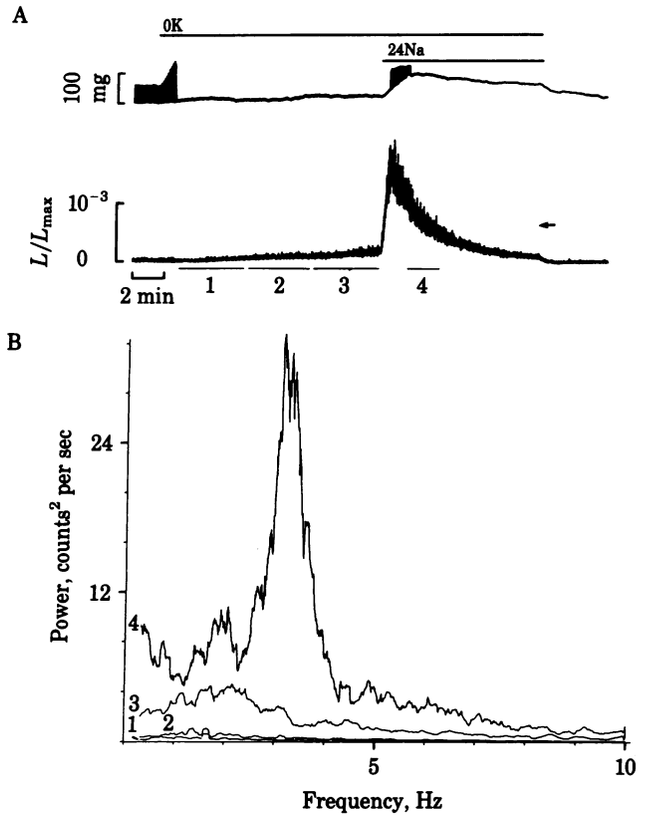

FIG. 2. Effects of progressive Ca^{2+} loading. (*A*) Tension (upper record) and aequorin luminescence (lower record) during solution exposures as indicated above the tension record, with the data segments used for analyses 1–4 below the luminescence records (same conventions as Fig. 1). Twitches are present before and just after washing in 0K solution, as well as in the first 2 min of 0K24Na exposure. Data from these periods were not chosen for spectral analysis. The luminescence record drops precipitously during 0K24Na treatment, perhaps disproportionately as compared with the decrease in tension over the same period. This decrease in luminescence may reflect a true decrease in $[Ca^{2+}]_i$, or it may be due in part to aequorin consumption. The records shown here have not been corrected for aequorin consumption continuously, but when such a correction is made, the aequorin luminescence at the end of 0K24Na treatment decreases only to the level indicated by the arrow. Therefore, ≈40% of the decrease in luminescence here is attributable to consumption. This reflects much more consumption than is usually encountered in our experiments because of the relatively prolonged exposure to 0K and 0K24Na solutions. (*B*) Spectral analysis of the luminescence record, corresponding to the data segments as numbered in *A*. Preparation 1-18 was used.

2*B*, record 2). As $[Ca^{2+}]_i$ continues to rise late during the 0K exposure (Fig. 2*A*, record 3), there is a further increase in power density and a shift to higher frequencies (Fig. 2*B*, record 3). During the subsequent exposure to 0K24Na solution (record 4), which causes a substantial further increase in the $[Ca^{2+}]_i$, the power density increases further still, and a pronounced peak occurs at 3–4 Hz. This result implies that the amplitude and frequency of the $[Ca^{2+}]_i$ oscillations are graded with the degree of Ca^{2+} loading.

What cellular mechanisms might be responsible for the $[Ca^{2+}]_i$ oscillations? Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) has been invoked to explain force fluctuations in mechanically skinned (23) or hyperpermeable (24) mammalian heart cells. The frequency characteristics of these force fluctuations and their dependence on the extent of Ca^{2+} loading are similar to the results in Fig. 2. Further hints suggesting an important role for the SR come from interspecies comparisons of Ca^{2+}-dependent scattered light-intensity fluctuations (10, 25), which occur as a result of spontaneous asynchronous microscopic cellular motion (12) and which do not occur in species with a sparse SR, such as the frog. We tested the involvement of the SR pharmacologically with caffeine and ryanodine. Caffeine is believed to inhibit Ca^{2+} uptake by the SR (26, 27), whereas ryanodine primarily inhibits Ca^{2+} release from the SR (28, 29). Caffeine attenuates fluctuations in force and membrane current (30, 31), and both agents abolish scattered light-intensity fluctuations (32).

The results from a fiber that was exposed to 0K and 0K24Na solutions and then to 10 mM caffeine in the continuing presence of 0K24Na are shown in Fig. 3*A*, record 1. Both the light signal (middle trace) and a derived plot of the apparent mean free Ca^{2+} concentration (bottom trace) are shown. The power spectrum of light without drug (Fig. 3*C*, record 1) resembles that in Fig. 1 in showing a prominent peak, here greatest at 0.5–1.5 Hz. When caffeine is added, there is a sudden increase in light intensity, most likely representing Ca^{2+} release from the SR, but the coarse oscillations that were present prior to

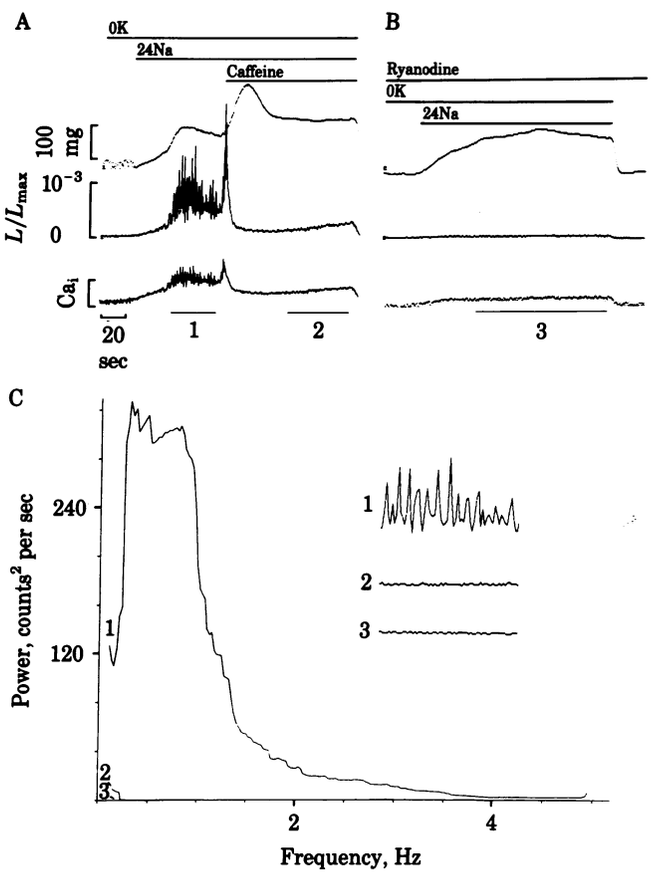

FIG. 3. Caffeine (10 mM) and ryanodine (1 μM) effects on fluctuations in aequorin luminescence. (*A* and *B*) Tension (top records), luminescence (middle records), and the corresponding apparent values of $[Ca^{2+}]_i$ (bottom records, labeled Ca_i and ranging from 0 to 4 μM). Apparent values of $[Ca^{2+}]_i$ were derived on a point-by-point basis from an aequorin calibration curve obtained at 35°C under the following conditions: 150 mM KCl/3 mM Mg^{2+}, pH 7.2 (see ref. 14 for a general review of aequorin calibration methods). The luminescence records (and the derived $[Ca^{2+}]_i$) were corrected continuously for aequorin consumption. Solutions and data segments used for analysis are as described in Figs. 1 and 2. Drugs were added to each solution without osmotic correction. (*C*) Spectral analysis of the aequorin luminescence data segments indicated in *A* and *B*. (*Inset*) Parts of those data segments at a 6 times faster time base than in *A* and *B*. Preparation 3-22 was used.

caffeine treatment (Fig. 3*C Inset*, record 1) are abolished (record 2). The power spectrum in the presence of caffeine is correspondingly flat (Fig. 3*C*, record 2). Tension is greater in the presence of caffeine, despite the fact that light intensity decreases substantially after the initial release. This may reflect an increase in myofilament sensitivity with caffeine treatment (27, 33), or it may simply result from attenuation of the oscillations and a reduction in spatial inhomogeneity of the $[Ca^{2+}]_i$ (see *Discussion*). The fiber was allowed to recover in control solution, then 1 μM ryanodine was added. This concentration abolishes the twitch, but not the slow tension changes, in voltage-clamped calf Purkinje fibers (29). In our canine fibers, the twitch was abolished within 10 min. 0K and 0K24Na solutions were then reapplied in the continued presence of ryanodine (Fig. 3*B*). The effect of ryanodine on the fluctuations is similar to that of caffeine, as judged by its effects on the power spectrum of light (Fig. 3*C*, record 1), on the raw light signal (*Inset*), and on the mean level aequorin luminescence (Fig. 3*B*). Unlike caffeine, ryanodine does not alter the affinity of the myofilaments for Ca^{2+} (A. Fabiato, personal communication). The results in Fig. 3 provide strong evidence for involvement of the SR in the generation of the spontaneous $[Ca^{2+}]_i$ oscillations.

We did not attempt to reverse the effect of ryanodine, which is generally believed to act irreversibly (28). The effect of caffeine, on the other hand, was fully reversible, as shown in Fig. 4. This preparation was bathed in 4 mM caffeine until twitch tension reached steady state, then exposed to 0K and 0K24Na solutions while still in the presence of caffeine. There is no initial burst of light (the fiber had already been exposed to caffeine for 10 min, so that any initial release of Ca^{2+} stores would have already occurred), but otherwise the results resemble those of Fig. 3: the light level is relatively low, and the oscillations are attenuated (Fig. 4*C Inset*, record 1). The fiber was allowed to recover, then exposed to 0K and 0K24Na solutions in the absence of drug (Fig. 4*B*). The oscillations in light intensity are once again prominent and, in addition, the mean level of light intensity is markedly increased compared with that in the presence of caffeine (Fig. 4*C*, record 2).

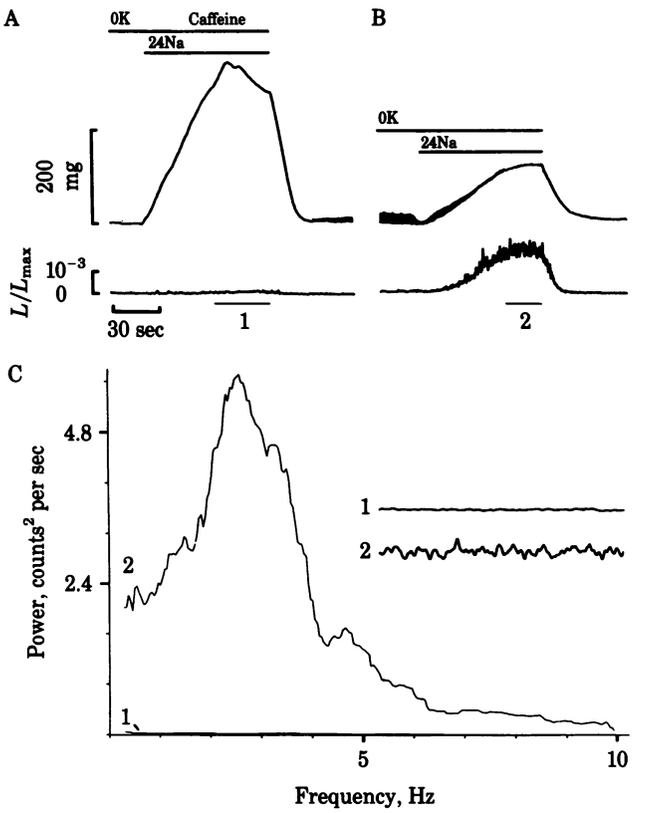

FIG. 4. Reversibility of caffeine-induced atttenuation of fluctuations. (*A* and *B*) Tension (upper records) and aequorin luminescence (lower records) during exposure to 4 mM caffeine (*A*) and after ≈1 hr in drug-free solution (*B*). The increased thickness of the tension traces late in *A* and early in *B* is due to twitch activity, which is absent during the periods chosen for analysis (indicated by 1 and 2). (*C*) Spectral analysis of the data from periods 1 and 2. (*Inset*) Luminescence data as in *A* and *B* but displayed at a 10 times faster time base. Preparation 2-22 was used; these records were obtained after those illustrated in Fig. 1.

DISCUSSION

Our results provide direct evidence that $[Ca^{2+}]_i$ fluctuations can occur spontaneously in cardiac muscle. Ions other than Ca^{2+}, such as Na^+, H^+, K^+, and Mg^{2+}, can affect aequorin luminescence (14), but implausibly large and rapid changes in the concentration of these ions would be required to produce the results we observed. Since $[Ca^{2+}]_i$ is capable of activating myofilaments as well as nonspecific surface membrane ion channels (34, 35), spontaneous $[Ca^{2+}]_i$ oscillations probably suffice to explain the previously described fluctuations in tension, surface membrane conductance, and scattered light intensity (7, 12). While our data are limited to Purkinje fibers, mechanical and electrical fluctuations have been reported in mammalian ventricular and atrial muscle (see, e.g., refs. 25 and 31), making it likely that $[Ca^{2+}]_i$ oscillations also occur in these preparations.

Ca^{2+}-induced Ca^{2+} release and Ca^{2+} uptake by the SR together constitute a probable mechanism for generating the $[Ca^{2+}]_i$ oscillations. Our results with progressive Ca^{2+} loading are analogous to observations in isolated cells (23, 24) and in intact fibers (12) in which the frequency and amplitude of the mechanical oscillations varied with the level of Ca^{2+} within the preparation. Caffeine and ryanodine attenuate $[Ca^{2+}]_i$ fluctuations and decrease the apparent mean free $[Ca^{2+}]_i$. The attenuation of the fluctuations by caffeine and ryanodine is most likely a reflection of their inhibition of Ca^{2+} uptake and release by the SR. It is important to note that spatiotemporal inhomogeneity of $[Ca^{2+}]_i$, which is likely to occur in association with the fluctuations we measure, would render the estimate of spatial average $[Ca^{2+}]_i$ from aequorin luminescence erroneously high, given the nonlinearity of the relationship of aequorin luminescence to Ca^{2+} (14). Thus, the reduction in apparent spatial average $[Ca^{2+}]_i$ by caffeine and ryanodine could result simply from a reduction in spatial nonuniformity of $[Ca^{2+}]_i$.

Fluctuations in $[Ca^{2+}]_i$ such as we have observed could have major implications for the investigation of cardiac function. Several studies of excitation–contraction coupling have assumed that $[Ca^{2+}]_i$ reaches steady levels, particularly under conditions giving rise to "tonic" tension. As an example, attempts to determine the coupling ratio of Na^+–Ca^{2+} exchange have assumed that $[Ca^{2+}]_i$ reaches steady state, if not thermodynamic equilibrium, during tonic tension (36–38). The presence of $[Ca^{2+}]_i$ oscillations would render any such assumptions strictly incorrect.

We have undertaken mathematical modeling to try to predict the effects of $[Ca^{2+}]_i$ fluctuations on force generation, functionally the most important Ca^{2+}-dependent process in heart. Our model, which is an extension of one previously described (11, 12), represents a multicellular preparation as a matrix composed of individual contractile elements, each of which can either be relaxed or contracted. While an extensive discussion of the model is beyond the scope of this paper, the salient results can be summarized as follows: $[Ca^{2+}]_i$ fluctuations that are asynchronous in different regions of a cell or within different re-

gions of a muscle will give rise to a subset of contracted areas that pull on their relaxed neighbors. Thus, much of the Ca^{2+}-activated force will be dissipated. For any given level of mean $[Ca^{2+}]_i$, less force will be produced at the ends of the preparation. This is equivalent to an apparent "shift to the right" of the Ca^{2+}–tension relation. This rightward shift will be greater as the frequency and the amplitude of the $[Ca^{2+}]_i$ oscillations increases—i.e., with increasing $[Ca^{2+}]_i$. Interventions that attenuate the $[Ca^{2+}]_i$ oscillations will tend to make the Ca^{2+}–tension relation steeper and shift it to the left.

These considerations demonstrate the errors that could undermine any attempt to infer $[Ca^{2+}]_i$ from tension in preparations in which spontaneous $[Ca^{2+}]_i$ fluctuations occur. Such deductions are tempting because of the relative ease of measuring tension in conjunction with other factors that influence $[Ca^{2+}]_i$, such as membrane potential or $[Na^+]_i$ (37–39). Tonic tension in particular should be interpreted cautiously because it is usually measured under conditions of heavy Ca^{2+} loading (e.g., prolonged 0K exposure as in ref. 37) and at a time when asynchronous activation is expected to be greatest. For these reasons, tonic tension alone will not provide reliable information about $[Ca^{2+}]_i$ and Ca^{2+}-dependent processes in mammalian preparations.

Ryanodine may prove to be a useful tool because it clearly attenuates the $[Ca^{2+}]_i$ fluctuations but does not influence myofilament Ca^{2+} sensitivity. With ryanodine present to attenuate fluctuations, force will not be subject to the errors discussed above. Furthermore, the estimate of $[Ca^{2+}]_i$ from aequorin luminescence is straightforward in the absence of spatiotemporal $[Ca^{2+}]_i$ fluctuations. Thus, experiments such as the one shown in Fig. 4*B* might be used to obtain reliable measurements of the Ca^{2+}–tension relation in intact heart tissue.

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