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Tight coupling between the rate of rise of Ca^{2+} transient and peak twitch contraction in guinea-pig papillary muscle

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Abstract

We evaluated the relationship between cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and force in guinea-pig papillary muscles loaded with a fluorescent Ca^{2+} indicator, fura-PE3. In the absence of ryanodine, $[Ca^{2+}]_i$ transient and force were altered by changing extracellular Ca^{2+} concentration and stimulation frequency, and also by adding methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644) or ouabain. Under these conditions, the peak force correlated linearly with the maximal rate of rise of $[Ca^{2+}]_i$ ($\gamma = 0.948$) more than the peak $[Ca^{2+}]_i$ transient ($\gamma = 0.737$). Ryanodine inhibited the increase in the maximal rate of rise of $[Ca^{2+}]_i$ resulting in abolishment of the correlation between force and the maximal rate of rise of $[Ca^{2+}]_i$. These results suggest that the maximal rate of rise of $[Ca^{2+}]_i$ reflects Ca^{2+} release from the sarcoplasmic reticulum, and this fraction of $[Ca^{2+}]_i$ is crucial for determining the amplitude of twitch contractions when the sarcoplasmic reticulum is intact in guinea-pig papillary muscle.

Keywords: Cardiac muscle; Mechanical force; Ca^{2+} level, cytosolic; Fura-PE3; Ouabain; Bay K 8644

1. Introduction

In cardiac muscle, the action potential-induced increase in $[Ca^{2+}]_i$ is attributable to both Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release from the cardiac sarcoplasmic reticulum. Ca^{2+}-induced Ca^{2+} release has been shown to be the main mechanism that generates contraction in cardiac muscle (Fabiato, 1983), because Ca^{2+} influx is insufficient whereas Ca^{2+}-induced Ca^{2+} release provides sufficient $[Ca^{2+}]_i$ increase to generate twitch contraction. However, it has been suggested that the contribution of Ca^{2+}-induced Ca^{2+} release to the increase in $[Ca^{2+}]_i$ is variable in different animal species (Bers, 1985). For example, Ca^{2+} released from the sarcoplasmic reticulum is the major factor for the increase in $[Ca^{2+}]_i$ in rat ventricle whereas the contribution of Ca^{2+}-induced Ca^{2+} release to the $[Ca^{2+}]_i$ transient is smaller in guinea-pig ventricle (Terracciano and MacLeod, 1997). Therefore, it is unclear whether Ca^{2+}-induced Ca^{2+} release is the major mechanism to generate contraction in the species, such as guinea-pig in which the contribution of Ca^{2+}-induced Ca^{2+} release to $[Ca^{2+}]_i$ increase may not be great. In isolated cardiac myocytes loaded with fluorescent Ca^{2+} indicator, it has been suggested that the maximal rate of rise of the $[Ca^{2+}]_i$ transient indexes the Ca^{2+} release from the sarcoplasmic reticulum (Isenberg and Han, 1994; Terracciano et al., 1995). However, the relationship between the maximal rate of rise of $[Ca^{2+}]_i$ transient and the peak force is not clarified.

In cardiac muscle, a number of studies have been performed to analyze the relationships between $[Ca^{2+}]_i$ and isometric contractions using the bioluminescent protein, aequorin (Yue et al., 1986; Yue, 1987; Endoh and Blinks, 1988). An alternative method to measure $[Ca^{2+}]_i$ is to use the fluorescent indicators including fura-2 and indo-1 (Backx and Ter Keurs, 1993). Because of the different properties of aequorin and fluorescent dyes in stoichiometry and sensitivity for Ca^{2+}, qualitatively and quantitatively different Ca^{2+} signals have been obtained using these two types of Ca^{2+} indicators in vascular smooth muscle (Karaki, 1989; Rembold et al., 1995; Karaki et al.,

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1997). However, only few studies have so far been carried out to quantitatively evaluate the relationship between $[Ca^{2+}]_i$ and isometric twitch contraction in isolated cardiac muscle preparations using fluorescent indicators (Backx and Ter Keurs, 1993; Hotta et al., 1995), and this has been the purpose of the present study.

2. Materials and methods

2.1. Langendorff's method to load fura-PE3 into papillary muscle

Male guinea-pigs (350–500 g) were anesthetized with gaseous diethylether and the heart was rapidly removed and washed in ice-cold (4°C) normal physiological salt solution (PSS) (mM: NaCl 145.0, KCl 5.0, $CaCl_2$ 1.6, $MgCl_2$ 1.0, glucose 10.0 and HEPES 10.0). Hearts were perfused in the Langendorff's mode with modified PSS (containing 3 mM $CaCl_2$) warmed to 30°C and gassed with 100% O_2. The coronary flow was normalized to 8 ml min^{-1}. Changes in $[Ca^{2+}]_i$ were monitored with the fluorescent Ca^{2+} indicator, fura-PE3, an analog of fura-2, because the leakage of fura-PE3 is less than that of fura-2 and compartmentalization of fura-PE3 in intracellular organelles such as mitochondria is less than that of fura-2 (Vorndran et al., 1995). After a 20 min equilibration period, the perfusion was switched to recirculating PSS (20 ml) containing 4 μM acetoxymethyl ester of fura-PE3 (fura-PE3/AM) and 0.025% cremophor EL for 0.5–1.5 h. Upon completion of the loading, the perfusion was switched to fura-PE3-free PSS for 15 min to wash out the uncleaved fura-PE3/AM in the tissue. Papillary muscles with a diameter less than 1 mm were excised from the fura-PE3-loaded heart and one end of the muscle was fixed to the siliconized floor of a specialized perfusion chamber with stainless steel pins.

2.2. Measurements of cytosolic Ca^{2+} concentration and muscle force

Fluorescent signals were directed to and obtained from a portion of the papillary muscle through a single quartz fiber optic (800 μm diameter). Fura-PE3 fluorescence was measured with a dual excitation fluorometer (CAM230, Jasco, Tokyo, Japan). Excitation light with a wavelength of either 340 or 380 nm (alternated at 1000 Hz) through two monochrometers in front of the UV-light source (150 W Xenon-lamp), was introduced to the muscle through the fiber optic. The emitted light (fluorescent light of fura-PE3) was led to a photomultiplier through the fiber optic fitted with a 500 ± 10 nm bandpass filter. The ratio of the fluorescence (F340/F380) was calculated by a computer and used as an indicator of $[Ca^{2+}]_i$.

The muscles were maintained throughout the experiments by perfusing the chamber continuously (12 ml min^{-1}) with a pre-warmed, pre-oxygenated PSS solution. Temperature within the muscle chamber was maintained at 30°C. The force of contraction was recorded isometrically. One end of the muscle was connected to strain gauge transducer to monitor mechanical activity and the resting tension applied to each preparation was adjusted to give 90% of the maximum tension development on the ascending limits of the length-tension relation. During the equilibration period, the muscles were stimulated electrically by square pulses of 5 ms duration at 0.5 Hz with a voltage 1.2-fold greater than the threshold intensity, using an electronic stimulator (SEN-3201, Nihon Kohden, Tokyo, Japan) and a pair of platinum wires. $[Ca^{2+}]_i$ and contractile force were digitized at the sampling rate of 110 points s^{-1}.

Unless otherwise described, the amplitudes of changes in fura-PE3 signal and isometric contraction obtained at a stimulation frequency of 0.5 Hz in the presence of 1.6 mM Ca^{2+} were taken as control (100%). For the quantitative assessment of the signal, five successive twitch signals were averaged.

2.3. Evaluation of the method using fura-PE3/AM

Fig. 1A shows that electrical stimulation transiently increased the 500 nm fluorescence excited at 340 nm (F340), and decreased that excited at 380 nm (F380), and thus increased the ratio (F340/F380), an indicator of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ increased immediately after the electrical stimulation. Force development occurred 27.3 ± 3.7 ms ($n = 4$) after the onset of increase in $[Ca^{2+}]_i$ and reached a maximum 161.4 ± 55.2 ms ($n = 4$) after $[Ca^{2+}]_i$ reached the maximum at 0.5 Hz. The time courses of the changes in fluorescence ratio, such as the time-to-peak (136.4 ± 18.6 ms at 1 Hz) and the time to 50% relaxation of ratio (413.6 ± 19.8 ms at 1 Hz), were similar to those obtained by using fura-2 or indo-1 in guinea-pig ventricle stimulated at 1 Hz (Gambassi et al., 1993; Hotta et al., 1995). These findings suggest that the fura-PE3 fluorescence in the guinea-pig papillary muscle reflects the changes in $[Ca^{2+}]_i$ during twitch contractions.

There are several problems in the estimation of $[Ca^{2+}]_i$ using fluorescent indicators, especially in the experiments in which cardiac cells are loaded with AM ester. First, possible incorporation of the fluorescent Ca^{2+} indicator into intracellular organelles, such as mitochondria (Spurgeon et al., 1990), could be a source of error in the estimation of $[Ca^{2+}]_i$. To assess the extent of compartmentalization of fura-PE3 within intracellular organelles, we examined the effects of Mn^{2+} on fura-PE3 loaded muscles (Fig. 1B). Mn^{2+} has been shown to quench preferentially the cytosolic fluorescent indicator signal leaving intracellular organelle fluorescence intact (Miyata et al., 1991). Exposure to Mn^{2+} (100 μM) for 40 min attenuated the fluorescent transients, but had no apparent effect on the amplitude of twitch force (not shown), suggesting that

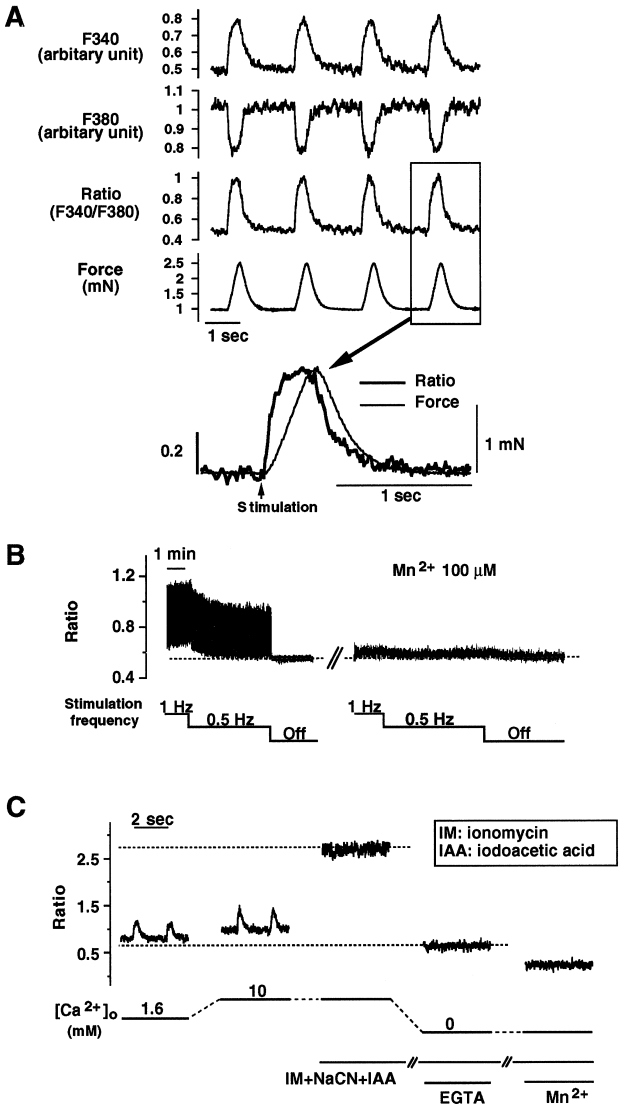

Fig. 1. (A) Simultaneous measurements of force of isometric contraction and the $[Ca^{2+}]_i$ transient in guinea-pig papillary muscle. Typical tracings of changes in F340, F380, ratio (F340/F380) and force of isometric contraction in a fura-PE3 loaded guinea-pig papillary muscle electrically stimulated at 0.5 Hz at 30°C. In lower panel, $[Ca^{2+}]_i$ transient and isometric contraction are superimposed on expanded time scale. (B) Effects of manganese on fura-PE3 fluorescence in the guinea-pig papillary muscle. Left and right panels represent the effect of changing stimulation frequency on fluorescence ratio (F340/F380) in the absence and presence of 100 μM Mn^{2+}, respectively. Muscle was superfused with Mn^{2+} for 40 min. Similar results were obtained in other two muscles. (C) In vivo calibration of fura-PE3 fluorescence. Initially, the muscle driven at 0.5 Hz was superfused with 1.6 mM Ca^{2+} solution and then switched to 10 mM Ca^{2+} solution. The muscle was superfused with 10 mM Ca^{2+} solution containing 25 μM ionomycin (IM), 4 mM NaCN and 2 mM iodoacetic acid (IAA) to obtain the maximum ratio. External medium was then changed to Ca^{2+}-free solution containing 10 mM EGTA, 25 μM ionomycin, 4 mM NaCN and 2 mM iodoacetic acid to obtain the minimum ratio. Finally, the Ca^{2+}-free solution containing 5 mM Mn^{2+}, 25 μM ionomycin, 4 mM NaCN and 2 mM iodoacetic acid was superfused to obtain the autofluorescence.

cytosolic fura-PE3 fluorescence was quenched by 100 μM Mn^{2+} without affecting $[Ca^{2+}]_i$. In the absence of Mn^{2+}, diastolic levels of fluorescent signals were decreased by decreasing stimulation frequency. In the presence of 100 μM Mn^{2+}, which quenched preferentially cytosolic fura-PE3, on the other hand, changing the stimulation frequency showed almost no change in fluorescent signals. These results suggest that, in our loading conditions, although the accurate evaluation of diastolic $[Ca^{2+}]_i$ is difficult, most of fura-PE3 may distribute in the cytoplasm.

Secondly, the K_d for Ca^{2+} of the fluorescent indicator in vivo may be different from that in vitro, which probably arises from differences in environment between in vivo and in vitro, such as viscosity and ionic strength (Roe et al., 1990). This may also result in the difficulty in calibration of fluorescent indicator. Therefore, we used the ratio of F340 to F380 as a relative indicator of $[Ca^{2+}]_i$ in this study. However, it may be important to evaluate the maximum and the minimum fluorescence of fura-PE3 in the intact tissue (in vivo calibration procedure) (Grynkiewicz et al., 1985). As shown in Fig. 1C, in the muscle metabolically inhibited by 4 mM NaCN and 2 mM iodoacetic acid, we applied ionomycin (25 μM) in the presence of 10 mM external Ca^{2+} concentration ($[Ca^{2+}]_o$) to determine the maximum fluorescence ratio value. This caused a further large increase in fluorescence ratio up to 3 times higher than the amplitude of $[Ca^{2+}]_i$ transients driven by electrical stimulation. Furthermore, removing external Ca^{2+} with 10 mM EGTA decreased fluorescence ratio to a level below diastolic level. Mn^{2+} (5 mM) in the absence of $[Ca^{2+}]_o$ further decreased the fluorescence ratio. These results showed that the fura-PE3 signals achieved during muscle contraction was far below the maximum level of fura-PE3 signal, and that the saturation of fura-PE3 molecules by Ca^{2+} should not be considered to be a limiting factor in this study.

The other problem with the use of fluorescent indicator is that it may act as a Ca^{2+} buffer (Spurgeon et al., 1990). We examined this possibility by comparing contractions of fura-PE3 loaded muscles with those of unloaded muscles. Results indicated that there were no significant differences in the time-to-peak, the time to 50% relaxation, duration of isometric contraction, maximal rate of rise and maximal rate of decrease of isometric contraction between loaded muscles ($n = 6$) and unloaded muscles ($n = 5$). Differences between mean values were compared by using Student's *t*-test for unpaired data. These results suggest that, in our experimental condition, fura-PE3 did not cause any significant buffering action on $[Ca^{2+}]_i$ involved in contractile activation.

2.4. Drugs

Chemicals used were acetoxymethyl ester of fura-PE3 (fura-PE3/AM; Texas Fluorescent Laboratories, Austin, USA), ryanodine (Sigma), cremophor EL, iodoacetic acid

and NaCN (Nacalai Tesque, Kyoto, Japan), ionomycin (Calbiochem, La Jolla, USA), methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644; Bayer Yakuhin, Osaka, Japan), ouabain (Wako, Osaka, Japan). Bay K 8644, fura-PE3/AM, ouabain and ryanodine were dissolved in dimethyl sulfoxide. Ionomycin was dissolved in ethanol. The final concentration of the solvent was less than 0.01% which alone had no effect on $[Ca^{2+}]_i$ or contractile force.

2.5. Statistics

The numerical data were expressed as mean ± standard error. Differences between mean values were evaluated by paired Student's *t*-test and, where appropriate, analysis of variance (ANOVA) followed by the Bonferroni test. Fisher's Z-transformation was used to evaluate significance of the correlation coefficients and also to compare the correlation coefficients. A probability of less than 0.05 was taken as a statistically significant difference.

3. Results

3.1. Effects of external Ca^{2+} concentration and stimulation frequency

With stepwise increases in external Ca^{2+} concentration ($[Ca^{2+}]_o$) from 0.5 mM to 10 mM, the amplitude of twitch contractions in response to electrical stimulation at 0.5 Hz increased from 35.9 ± 4.3% to 325.4 ± 54.1% ($n = 5$) in a concentration-dependent manner (Fig. 2B, left panel). The peak amplitude of fura-PE3 signal (peak $[Ca^{2+}]_i$ transient) and the maximal rate of rise of $[Ca^{2+}]_i$ also increased from 71.4 ± 1.9% to 140.4 ± 4.0% and from 56.1 ± 4.2% to 173.5 ± 11.1%, respectively (Fig. 2A and C, left panels). Increasing $[Ca^{2+}]_o$ also decreased the time-to-peak of both $[Ca^{2+}]_i$ transients and isometric contractions in a concentration-dependent manner (Fig. 2A and B). The durations of both the $[Ca^{2+}]_i$ transients and isometric contractions were little influenced by increasing $[Ca^{2+}]_o$.

The same procedures were applied to the preparations driven at 0.2 Hz (Fig. 2, right panels). Increasing $[Ca^{2+}]_o$ also increased the peak $[Ca^{2+}]_i$ transient, the maximal rate of rise of $[Ca^{2+}]_i$ and peak force (from 67.2 ± 1.0% to 130.7 ± 3.1%, from 46.0 ± 2.7% to 130.0 ± 8.1% and from 24.8 ± 2.4% to 199.1 ± 27.7%, respectively, $n = 5$). As compared with the muscle driven at 0.5 Hz, the time-to-peak of both $[Ca^{2+}]_i$ transients and isometric contractions were longer, and the increments of peak $[Ca^{2+}]_i$ transient and peak force were smaller at all $[Ca^{2+}]_o$ levels.

Fig. 3 shows the influence of stimulation frequency on the $[Ca^{2+}]_i$ transient and force at 1.6 mM $[Ca^{2+}]_o$. Decreasing the frequency of stimulation from 0.5 Hz to 0.2 Hz slightly decreased the peak $[Ca^{2+}]_i$ transient (to 90.8 ± 3.4%, $P > 0.05$). The force, on the other hand, was significantly decreased to 41.1 ± 4.6% ($n = 5$, $P < 0.05$). Increasing the frequency of stimulation from 0.5 Hz to 1 Hz slightly increased the peak $[Ca^{2+}]_i$ transient (to 115.1 ± 6.2%, $P > 0.05$). The force, on the other hand, was significantly increased to 221.3 ± 22.4%($n = 5$, $P < 0.05$). The peak $[Ca^{2+}]_i$ transient and the peak force at 1 Hz were significantly larger than those at 0.2 Hz. Changing stimulation frequency caused frequency-dependent changes in the maximal rate of rise of $[Ca^{2+}]_i$ (68.0 ± 5.6%, $P < 0.05$, at 0.2 Hz, and 154.8 ± 16.4%, $P < 0.01$, at 1 Hz). The maximal rate of rise of $[Ca^{2+}]_i$ at 1 Hz was significantly larger than those at 0.2 Hz. Results obtained at 0.125 Hz were not statistically different from that at 0.2 Hz. As shown in Fig. 3, increasing the frequency of stimulation also decreased the time-to-peak and duration of both

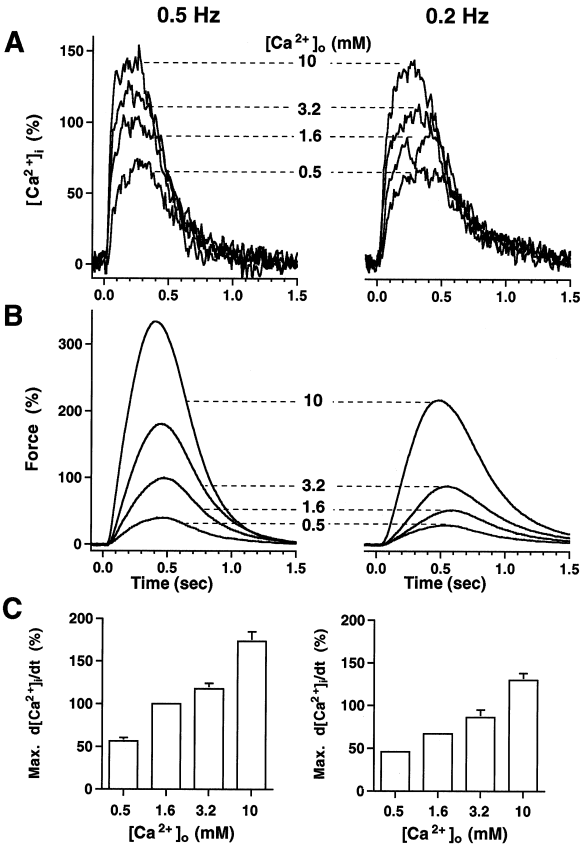

Fig. 2. Influence of changing external Ca^{2+} concentration on the $[Ca^{2+}]_i$ transient and force of isometric contraction at 0.2 Hz and 0.5 Hz stimulation. External Ca^{2+} concentration was changed from 0.5 to 1.6, 3.2, and 10 mM. Left and right panels show the effects of extracellular Ca^{2+} concentration at 0.5 Hz and at 0.2 Hz respectively. Averaged tracings of 5 successive $[Ca^{2+}]_i$ transients (A) and isometric contractions (B) are shown. (C) Summarized effects of changing external Ca^{2+} concentration on the maximal rate of rise of $[Ca^{2+}]_i$ (Max. $d[Ca^{2+}]_i/dt$) at different frequencies of stimulation. Values obtained in normal PSS at 0.5 Hz were taken as 100%. The time when the muscle was electrically driven was taken as time zero.

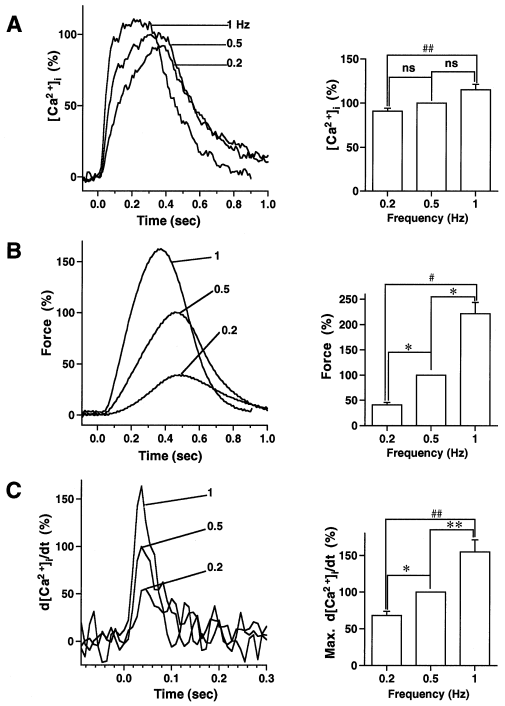

Fig. 3. Influence of stimulation frequency on the $[Ca^{2+}]_i$ transient and isometric contraction. Left panels, averaged tracings of 5 successive $[Ca^{2+}]_i$ transients (A), isometric contractions (B) and the rate of rise of $[Ca^{2+}]_i$ (C). $d[Ca^{2+}]_i/dt$: rate of rise of $[Ca^{2+}]_i$. Numbers represent stimulation frequency (Hz). Right panels, summarized data of the effects of stimulation frequency on the peak $[Ca^{2+}]_i$ transient, the peak force and the maximal rate of rise of $[Ca^{2+}]_i$ (Max. $d[Ca^{2+}]_i/dt$). Values obtained in normal PSS at 0.5 Hz were taken as 100%. Each column represents the mean and S.E.M. ($n = 5$). Differences between mean values were compared by using analysis of variance (ANOVA). ns: not significantly different from the control. $^*P < 0.05$ ** $P < 0.01$ vs. the control. # and ##: Differences between at 0.2 Hz and 1 Hz are significant with $P < 0.05$ and 0.01 respectively.

$[Ca^{2+}]_i$ transients and isometric contractions in a frequency-dependent manner.

3.2. Effects of Bay K 8644

Fig. 4A indicates that, in muscles stimulated at 0.5 Hz, 1 μM Bay K 8644 increased the peak force to 272. 3 $\pm$31.2% ($n = 5$, $P < 0.01$), with a relatively small effect on the peak $[Ca^{2+}]_i$ transient (to 114.8 $\pm$ 5.2%, $P < 0.05$). Bay K 8644 also increased the maximal rate of rise of $[Ca^{2+}]_i$ to 145.1 $\pm$ 11.8% (Fig. 4A, $P < 0.05$) Although Bay K 8644 did not affect the time-to-peak of both $[Ca^{2+}]_i$ transients and isometric contractions, it greatly prolonged plateau levels of both $[Ca^{2+}]_i$ and force.

3.3. Effects of ouabain

Fig. 4B indicates that, in the muscle stimulated at 0.5 Hz, 1 μM ouabain markedly increased the maximal rate of rise of $[Ca^{2+}]_i$ to 159.1 $\pm$ 10.4% ($P < 0.05$) and the peak force to 390.6 $\pm$ 57.0% ($P < 0.05$). However, the increase in the peak $[Ca^{2+}]_i$ transient was relatively small (to 115.5 $\pm$ 3.9%, $P < 0.05$) ($n = 4$). Ouabain significantly decreased the time-to-peak and the duration of both the $[Ca^{2+}]_i$ transients and isometric contractions.

3.4. Effects of ryanodine

After the muscle was incubated with ryanodine (300 nM) for 25 min, the amplitude of $[Ca^{2+}]_i$ transients at 0.5 Hz decreased to 69.9 $\pm$ 7.4% ($n = 5$, $P < 0.05$) (Fig. 5A). Ryanodine increased the peak force in two out of five muscle preparations, (to 130.5% and to 115.5%), while it

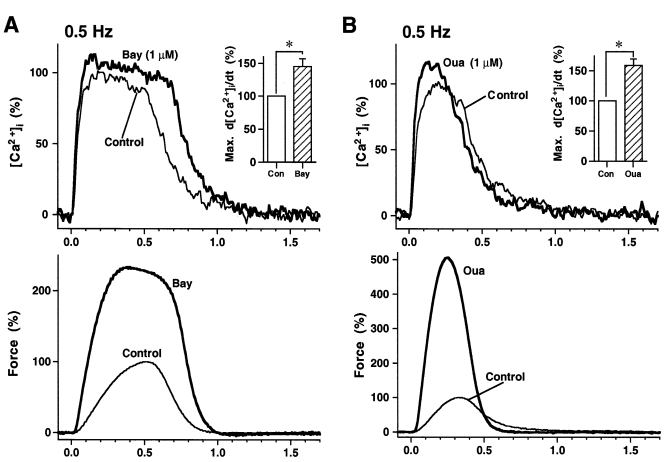

Fig. 4. Effects of Bay K 8644 and ouabain on the $[Ca^{2+}]_i$ transient and isometric contraction. Averaged tracings of 5 successive $[Ca^{2+}]_i$ transients and isometric contractions are shown. Upper and lower panels represent the $[Ca^{2+}]_i$ transient and isometric contraction, respectively. Muscles were stimulated at 0.5 Hz. (A) Effects of 1 μM Bay K 8644 (Bay). (B) Effects of 1 μM ouabain (Oua). Changes in the maximal rate of rise of $[Ca^{2+}]_i$ (Max. $d[Ca^{2+}]_i/dt$) are shown in the inset in the upper panels. Open and hatched columns represent results obtained in the absence (Con) and presence of Bay K 8644 (Bay) or ouabain (Oua), respectively. Values obtained in normal PSS at 0.5 Hz were taken as 100%. Each column represents the mean $\pm$ S.E.M. ($n = 5$). $^*P < 0.05$ vs. the control.

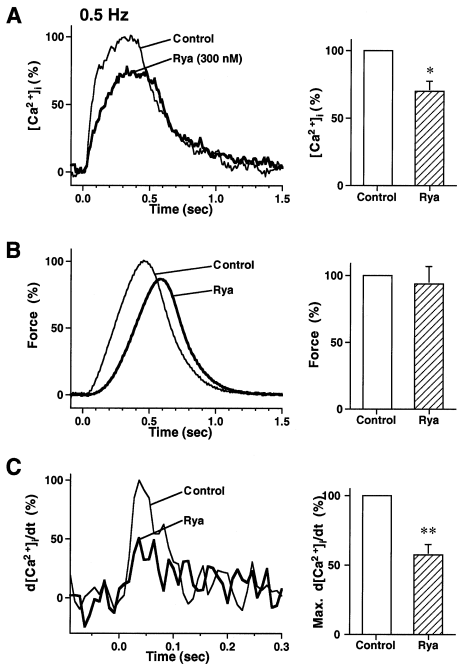

Fig. 5. Effects of ryanodine on the $[Ca^{2+}]_i$ transient and isometric contraction. Muscles were treated with ryanodine (300 nM) for 25 min. Effects of ryanodine (Rya) on the $[Ca^{2+}]_i$ transient (A), isometric contraction (B) and rate of rise of $[Ca^{2+}]_i$ (C) at 1.6 mM $[Ca^{2+}]_o$ in the muscles stimulated at 0.5 Hz are shown. In left panels, averaged tracings of 5 successive signals are shown. In right panels, summarized data of the effects of ryanodine on the peak $[Ca^{2+}]_i$ transient, the peak force and the maximal rate of rise of $[Ca^{2+}]_i$ (Max. $d[Ca^{2+}]_i/dt$) are shown. Open and hatched columns represent the results obtained in the absence and presence of ryanodine, respectively. Values obtained in normal PSS at 0.5 Hz were taken as 100%. Each column represents the mean ± S.E.M. ($n = 5$). $^*P < 0.05$, $^{**}P < 0.01$ vs. the control.

decreased the peak force (to 87.9%, 76.9% and 58.0%) in three other muscle preparations, which altogether resulted in the averaged peak force of 93.8 ± 13.1% (Fig. 5B). Variable effects of ryanodine on the peak force have also been reported by Sutko and Kenyon (1983) in the same preparations. On the other hand, ryanodine consistently decreased the maximal rate of rise of $[Ca^{2+}]_i$ to 57.3 ± 7.4% (Fig. 5C, $P < 0.01$). Ryanodine-treatment also prolonged the time-to-peak of both $[Ca^{2+}]_i$ transients and isometric contractions by 60.0 ± 22.3% and by 23.0 ± 3.9% ($n = 5$), respectively. At different stimulation frequencies (0.125, 0.2 and 1 Hz), ryanodine also decreased the peak $[Ca^{2+}]_i$ transient and the maximal rate of rise of $[Ca^{2+}]_i$ with a relatively small decrease in the peak force (Fig. 7).

To evaluate the contribution of Ca^{2+} release from the sarcoplasmic reticulum to the effects of Bay K 8644 or ouabain, we examined the effects of ryanodine (Fig. 6). As shown in Fig. 6, values obtained in Bay K 8644 or ouabain at 0.5 Hz were taken as 100%. Muscles pretreated with Bay K 8644 (1 μM) were incubated with ryanodine (300 nM) for 25 min (Fig. 6A). Ryanodine gradually decreased the peak $[Ca^{2+}]_i$ transient to 88.6 ± 4.0% ($P < 0.05$) and the maximal rate of rise of $[Ca^{2+}]_i$ to 52.9 ± 3.9% ($P < 0.01$), whereas the peak force was enhanced to 123.2 ± 7.2% ($n = 5$. $P < 0.05$). Ryanodine did not inhibit the prolonged plateau phase of both $[Ca^{2+}]_i$ and force, resulting in prolonged time-to-peak of both $[Ca^{2+}]_i$ transients and isometric contractions (Fig. 6A).

Application of ryanodine to the ouabain-treated preparations driven at 0.5 Hz for 25 min. Ryanodine gradually inhibited the $[Ca^{2+}]_i$ transient and force, and finally decreased the peak $[Ca^{2+}]_i$ transient and the maximal rate of rise of $[Ca^{2+}]_i$ to 72.5 ± 2.6% ($P < 0.01$), and 61.6 ± 3.8% ($P < 0.01$), respectively (Fig. 6B, $n = 5$). Ryanodine also decreased the peak force to 73.2 ± 7.9% ($P < 0.05$).

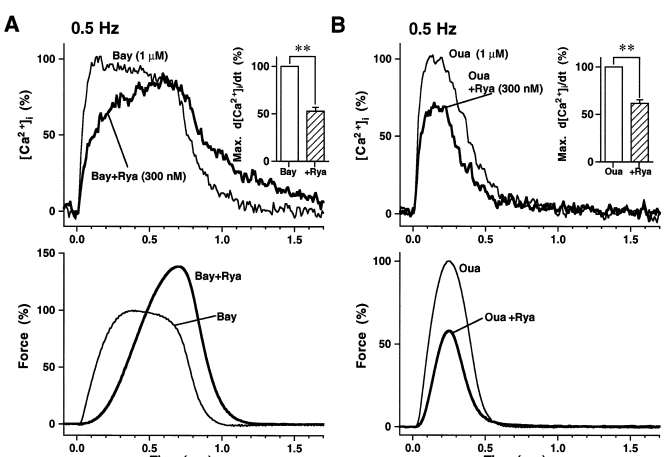

Fig. 6. Effects of ryanodine in the presence of Bay K 8644 or ouabain. Averaged tracings of 5 successive signals are shown. Upper and lower panels represent the $[Ca^{2+}]_i$ transient and isometric contraction, respectively. Muscles treated with Bay K 8644 or ouabain were subsequently treated with ryanodine (300 nM) for 25 min at 0.5 Hz. (A) Effects of ryanodine (Rya) in the presence of 1 μM Bay K 8644 (Bay). (B) Effects of ryanodine in the presence of 1 μM ouabain (Oua). Changes in the maximal rate of rise of $[Ca^{2+}]_i$ (Max. $d[Ca^{2+}]_i/dt$) are shown in the inset in the upper panels. Open and hatched columns represent results obtained in the absence and presence of ryanodine. Values obtained in Bay K 8644 or ouabain at 0.5 Hz were taken as 100%. Each column represents the mean ± S.E.M. ($n = 5$). $^{**}P < 0.01$ vs. Bay K 8644 or ouabain.

4. Discussion

Increments of external Ca^{2+} concentration ($[Ca^{2+}]_o$) from 0.5 mM to 10 mM increased the peak amplitude of fura-PE3 signal (peak $[Ca^{2+}]_i$ transient), the maximal rate of rise of $[Ca^{2+}]_i$ and peak force, both at 0.5 and 0.2 Hz (Fig. 2). Increasing the stimulation frequency from 0.2 Hz to 1 Hz in 1.6 mM $[Ca^{2+}]_o$ also increased the maximal rate of rise of $[Ca^{2+}]_i$ and peak force. However, changes in stimulation frequency induced a relatively small influence on the peak $[Ca^{2+}]_i$ transient (Fig. 3). These results indicate that the peak force correlates better with the maximal rate of rise of $[Ca^{2+}]_i$ rather than with the peak $[Ca^{2+}]_i$ transient. In addition, the area under the $[Ca^{2+}]_i$ transient curve does not correlate with the change in the peak force, because elevation of stimulation frequency did not increase but rather decreased the former due to the decrease in the duration of $[Ca^{2+}]_i$ transients (Fig. 3A). The stimulation frequency-dependent shortening of the $[Ca^{2+}]_i$ transients was also obtained in the presence of ryanodine (data not shown). The stimulation frequency-dependent shortening of the $[Ca^{2+}]_i$ transients is likely to be due to shortening of action potential duration, which may be the result of an increase in outward Na^+–Ca^{2+} exchange current in response to a rise in the intracellular Na^+ activity (Janvier and Boyett, 1996). Bay K 8644 (1 μM), a facilitator of the L-type Ca^{2+} channel (Thomas et al., 1985), and ouabain (1 μM), a cardiac glycoside, showed positive inotropic effects with significant increases in the maximal rate of rise of $[Ca^{2+}]_i$, and relatively small increases in the peak $[Ca^{2+}]_i$ transient (Fig. 4). These results support our suggestion that peak force development is more closely related to the maximal rate of rise of $[Ca^{2+}]_i$ than the peak $[Ca^{2+}]_i$ transient. Bay K 8644 markedly prolonged the plateau level of $[Ca^{2+}]_i$ transients and isometric contractions (Fig. 4A). Prolonged plateau levels of $[Ca^{2+}]_i$ transients and isometric contractions may be due to prolongation of the action potential plateau by Bay K 8644 (Thomas et al., 1985) accompanied by a prolonged Ca^{2+} influx-dependent slow phase. In contrast, ouabain decreased the time-to-peak and the duration of $[Ca^{2+}]_i$ transient and force, which might be due to an increase in Ca^{2+} release from the sarcoplasmic reticulum and outward Na^+–Ca^{2+} exchange current resulting from an increase in intracellular Na^+ activity, respectively (Levi, 1993).

To evaluate the contribution of Ca^{2+} release from the sarcoplasmic reticulum to $[Ca^{2+}]_i$ transients and isometric contractions, we used ryanodine which is believed to lock the sarcoplasmic reticulum Ca^{2+} release channel in a subconductance open state level (Rousseau et al., 1987) and to have no direct action on L-type Ca^{2+} current (Balke and Wier, 1991). In normal PSS and at 0.5 Hz stimulation frequency, 300 nM ryanodine decreased the peak $[Ca^{2+}]_i$ transient to about 70% (Fig. 5). The relatively small inhibition of $[Ca^{2+}]_i$ by ryanodine has been also reported in guinea-pig single myocytes (Lewartowski et al., 1994; Janiak et al., 1996) and it has been suggested that the contribution of Ca^{2+}-induced Ca^{2+} release to $[Ca^{2+}]_i$ increase is smaller in guinea-pig ventricle than the ventricle of other species, such as rat (Kapelko et al., 1994; Terracciano and MacLeod, 1997) and mouse (our unpublished observation). In contrast to the peak $[Ca^{2+}]_i$ transient, maximal rate of rise of $[Ca^{2+}]_i$ was greatly decreased by ryanodine, resulting in a slow increase in $[Ca^{2+}]_i$. The slow increase in $[Ca^{2+}]_i$ observed in the presence of ryanodine may be ascribed to Ca^{2+} influx via both L-type Ca^{2+} channels and Na^+–Ca^{2+} exchange (Wier and Beuckelmann, 1989). Similarly, in the presence of ryanodine, slow increase in force was observed and the peak force was slightly decreased. Thus, our results support the suggestion that $[Ca^{2+}]_i$ transients and isometric contractions may be composed of two phases; the early phase due to Ca^{2+} release from the sarcoplasmic reticulum and the late phase due to Ca^{2+} influx in the guinea-pig ventricular muscle (Reiter, 1988).

In the muscle stimulated at 0.5 Hz in the presence of Bay K 8644 (1 μM), ryanodine also decreased the maximal rate of rise of $[Ca^{2+}]_i$, resulting in the slow increases in $[Ca^{2+}]_i$ transients and isometric contractions. Ryanodine slightly decreased the peak $[Ca^{2+}]_i$ transient but rather enhanced the peak force at 0.5 Hz (Fig. 6A). As a result, ryanodine abolished the correlation between the peak force and the maximal rate of rise of $[Ca^{2+}]_i$ and also the correlation between the peak force and the peak $[Ca^{2+}]_i$ transient in the muscles stimulated by Bay K 8644. In the muscles stimulated at 0.5 Hz in the presence of ouabain, ryanodine decreased the peak $[Ca^{2+}]_i$ transient, the maximal rate of rise of $[Ca^{2+}]_i$ and the peak force, suggesting an important contribution of Ca^{2+}-induced Ca^{2+} release to positive inotropic effect of ouabain.

To quantitatively evaluate the relationship between $[Ca^{2+}]_i$ transients and force development, peak force was plotted against the peak $[Ca^{2+}]_i$ transient (Fig. 7A) and against the maximal rate of rise of $[Ca^{2+}]_i$ (Fig. 7B), and effects of ryanodine on the relations were examined. Data were obtained by changing extracellular Ca^{2+} concentration from 0.5 mM to 10 mM, stimulation frequency from 0.125 Hz to 1 Hz, and adding 1 μM Bay K 8644 or 1 μM ouabain at 0.2 Hz and 0.5 Hz. The linear regression of the data obtained in the absence of ryanodine showed that there were positive correlations ($P < 0.01$) between the peak force and both the peak $[Ca^{2+}]_i$ transient and the maximal rate of rise of $[Ca^{2+}]_i$. The correlation coefficient between the peak force and the maximal rate of rise of $[Ca^{2+}]_i$ ($\gamma = 0.948$) was significantly greater ($P < 0.05$) than that between the peak force and the peak $[Ca^{2+}]_i$ ($\gamma = 0.737$). Ryanodine almost inhibited the increases in the maximal rate of rise of $[Ca^{2+}]_i$ elicited by any interventions employed in this study (Fig. 7B), supporting the suggestion that the maximal rate of rise of $[Ca^{2+}]_i$ reflects Ca^{2+} release from the sarcoplasmic reticulum (Isenberg and Han, 1994; Terracciano et al., 1995). In the presence

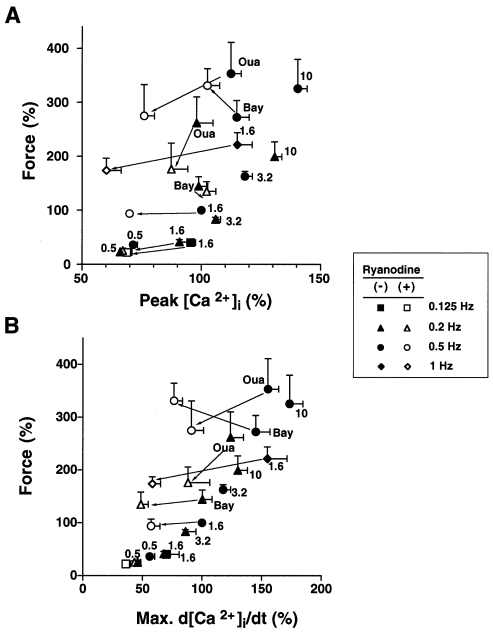

Fig. 7. Relationship between $[Ca^{2+}]_i$ and force of contraction. (A) Peak force was plotted against the peak $[Ca^{2+}]_i$ transient. (B) Peak force was plotted against the maximal rate of rise of $[Ca^{2+}]_i$. Open and closed symbols represent the results obtained in the presence and absence of ryanodine, respectively. The change in the relationship between $[Ca^{2+}]_i$ and force by the treatment with ryanodine are indicated by the arrows attached to individual symbols. Each point represents the mean ± S.E.M. ($n = 4–5$). Numbers represent external Ca^{2+} concentration (mM). Oua, ouabain (1 μM); Bay, Bay K 8644 (1 μM); ■, 0.125 Hz; ▲, 0.2 Hz; ●, 0.5 Hz; ◆, 1 Hz. Values obtained in normal PSS at 0.5 Hz were taken as 100%.

of ryanodine, the correlation between the maximal rate of rise of $[Ca^{2+}]_i$ and peak force was abolished (Fig. 7B). These results suggest that, when the sarcoplasmic reticulum is intact, despite a relatively small contribution of Ca^{2+}-induced Ca^{2+} release to the formation of the peak $[Ca^{2+}]_i$ in this preparation as compared with other species (Terracciano and MacLeod, 1997), fraction of Ca^{2+} released from the sarcoplasmic reticulum may directly coupled to cardiac contractile elements. In the presence of ryanodine, the correlation between the peak $[Ca^{2+}]_i$ transient and peak force was abolished ($\gamma = 0.526$, $P > 0.05$) (Fig. 7A) by decreasing the peak $[Ca^{2+}]_i$ transient with little effect on the peak force in some, but not all the interventions. Thus, the peak $[Ca^{2+}]_i$ transient correlates well with peak force only when the Ca^{2+} handling by the sarcoplasmic reticulum is intact.

It has been suggested that a portion of Ca^{2+} entering cell is rapidly buffered by the sarcoplasmic reticulum during twitch contraction in guinea-pig ventricle (Janczewski and Lakatta, 1993), and this might explain the relatively lower correlation between the peak $[Ca^{2+}]_i$ and peak force when the sarcoplasmic reticulum function is intact (in the absence of ryanodine). In contrast, when the sarcoplasmic reticulum function is impaired by ryanodine, Ca^{2+} derived from extracellular space could diffuse more easily to the contractile element and this fraction of Ca^{2+} may more effectively activate the contractile element of cardiac tissue during the slow time-course of the $[Ca^{2+}]_i$ transient. This might explain the relatively large force observed at given $[Ca^{2+}]_i$ in the presence of ryanodine. In aequorin loaded ferret papillary muscle, Yue et al. (1986) also observed that a slow increase in $[Ca^{2+}]_i$ induces greater amplitude of contraction.

In summary, we found that the peak force of contraction showed a better correlation with the maximal rate of rise of $[Ca^{2+}]_i$ than with the peak $[Ca^{2+}]_i$ transient in the isolated guinea-pig papillary muscle. Furthermore, ryanodine decreased the maximal rate or rise of $[Ca^{2+}]_i$, resulting in the disappearance of the positive correlation with the peak force. These results imply that the maximal rate of rise of $[Ca^{2+}]_i$ represents the Ca^{2+} release from the sarcoplasmic reticulum and that under normal circumstances this fraction of $[Ca^{2+}]_i$ plays an important role in determining the peak force of twitch contraction in guinea-pig ventricular myocardium, nevertheless the increase in $[Ca^{2+}]_i$ is only partly due to Ca^{2+}-induced Ca^{2+} release.

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