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Different effects of isoproterenol and dihydroouabain on cardiac Ca^{2+} transients

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Abstract

Cytosolic fura-2 Ca^{2+} transient signals (T_{Ca}) and the left ventricular pressure or contraction of myocardium under the positive inotropic effects of the β-adrenoceptor agonist, isoproterenol, and the cardiac glycoside, dihydroouabain, were measured simultaneously and the results were compared. T_{Ca} was observed preceding the onset of force development and showed a steeper rise and slower decay than did the contraction curve of papillary muscle. Isoproterenol increased the steepness and the amplitude of T_{Ca}, reflecting the speed and peak force of contraction, and clearly biphasic T_{Ca} were observed with biphasic contractions developed at low frequency. Ryanodine reduced not only the early component of the contraction but also T_{Ca}, without affecting the diastolic Ca^{2+} level. These effects of isoproterenol were attributed to the enhanced uptake of Ca^{2+} by the sarcoplasmic reticulum. In contrast, dihydroouabain elevated the Ca^{2+} level at diastole without any change in the amplitude of T_{Ca}, suggesting that dihydroouabain inhibits the membrane Na pump thereby increasing the intracellular Ca^{2+} via Na^{+}-Ca^{2+} exchange. Furthermore, a comparison of the time course of the isometric twitch curve with that of T_{Ca} in rested state contraction indicated that there are distinct differences between the mechanisms of the positive inotropic effects of isoproterenol and of dihydroouabain.

Keywords: Fura-2; Ca^{2+}, transient signal; Heart, guinea-pig; Isoproterenol; Dihydroouabain

1. Introduction

Cardiac glycosides and β-adrenoceptor agonists are the most widely used agents for the treatment of cardiac failure. Free calcium ions in the myocardial cytosol $[Ca^{2+}]_i$ play a second-messenger role in exerting the positive inotropic effects of these two classes of cardiotonic agents by maintaining the resting level, or diastolic, intracellular Ca^{2+} transients (T_{Ca}) at a very low concentration.

Inhibition of the sarcolemmal Na^{+},K^{+} pump by cardiac glycosides is known to be an essential mechanism by which they exert their marked positive inotropic effects (Barry et al., 1985; Lee, 1985). The resulting increases in internal Na^{+} activity result in elevation of $[Ca^{2+}]_i$ by suppressing the Ca^{2+} extrusion mediated by Na^{+}-Ca^{2+} exchange. It is widely accepted that β-adrenoceptor agonists first stimulate adrenoceptors and then activate adenylate cyclase via Gs proteins increasing 3′,5′-cyclic monophosphate (c-AMP) levels which in turn stimulates c-AMP-dependent protein kinase. Activation of the latter enzyme increases Ca^{2+} influx by opening the Ca^{2+} channels, and increases the sequestration of Ca^{2+} by the sarcoplasmic reticulum (Tada and Katz, 1982; Callewaert et al., 1988).

There are as yet limited data available regarding the working heart muscle to clarify the mode of action of these two classes of cardiotonic agents on $[Ca^{2+}]_i$, especially under identical experimental conditions (Morgan and Blinks, 1982). Ca^{2+} transients have been measured using various agents and techniques such as Ca^{2+}-selective microelectrodes, metallochromic indica-

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tors, bioluminescent indicators and fluorescent indicators (Blinks, 1986; Cobbold and Rink, 1987). The fast Ca^{2+} transients in papillary muscle have been measured using the bioluminescent photoprotein, aequorin, but its sensitivity for Ca^{2+} is insufficient for measuring resting Ca^{2+} (Allen and Blinks, 1978; Morgan, 1985; Blinks, 1986; Endoh and Blinks, 1988; Cobbold and Rink, 1987).

Fluorescent Ca^{2+} probes such as fura-2 and indo-1 have recently become available to measure changes in cytosolic Ca^{2+} concentrations in various biological preparations (Tsien et al., 1985; Sato et al., 1988). Jackson et al. (1987) reported on the kinetics of binding constants of fura-2 and indo-1 with Ca^{2+}, using stopped-flow techniques and anticipated that these new indicators will be applicable for the measurement of the rapid changes in intracellular Ca^{2+} levels. This technique has been applied to isolated heart cells and tissue (Sheu et al., 1984; Lattanzio et al., 1986; Wier et al., 1987; Kim et al., 1987), and was shown to reach into the cells to measure changes in the resting or diastolic levels of $[Ca^{2+}]_i$ (Cobbold and Rink, 1987).

In the present study, intracellular fura-2 Ca^{2+} transient signals and mechanical performance were measured concomitantly, using both Langendorff heart and papillary muscle preparations treated with a cardiac glycoside with Na^+-dependent positive inotropic effects, dihydroouabain, and the β-adrenoceptor agonist, isoproterenol, which has a Ca^{2+}-dependent positive inotropic effect mediated by a slow inward Ca^{2+} current. The purpose of the present study was to assess both qualitatively and quantitatively the Ca^{2+}-dependent signals from fura-2-loaded beating heart muscle, and to examine the role of cytosolic Ca^{2+} in heart muscle contraction. Preliminary findings from this study have appeared elsewhere (Hotta et al., 1989).

2. Materials and methods

2.1. Measurement of fura-2 fluorescence in Langendorff heart and papillary muscle preparations

Cytosolic Ca^{2+} fluorometry was performed with a fluorometer (CAF-100, Japan Spectroscopic Co., Tokyo, Japan) which was specially designed to measure the surface fluorescence of living tissues. The excitation light was supplied by from a xenon high pressure lamp (75 W), equipped with a rotating wheel (48 Hz) containing filters for the light at 340 and 380 nm ($\pm$5.5 nm). The ratio of the strengths of fluorescence (R340/380) at 340 nm (F340) and 380 nm (F380) was

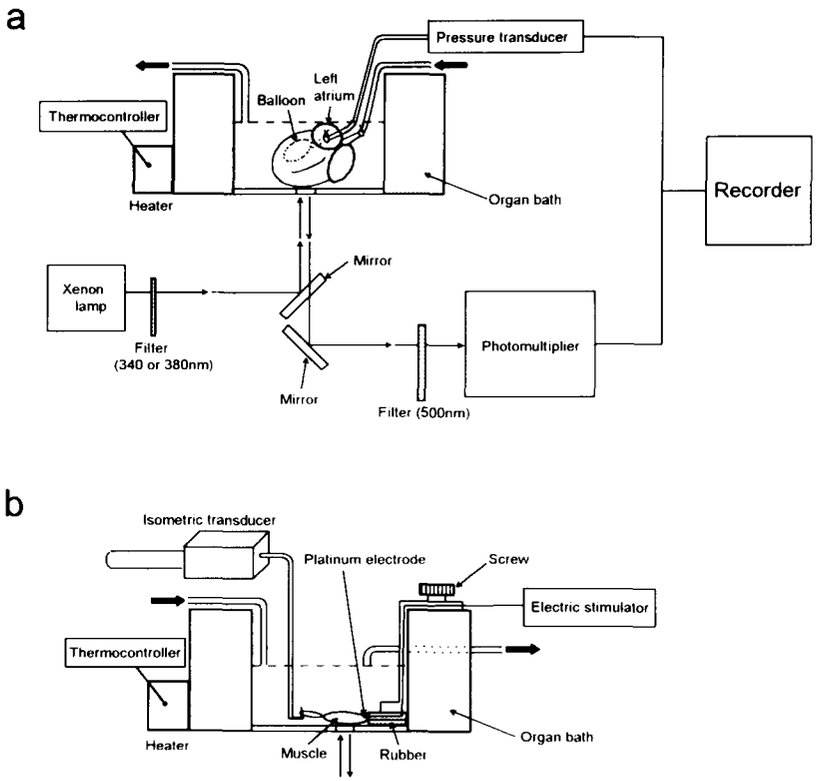

Fig. 1. Block diagram of the experimental set-up for Ca^{2+} measurement using a fluorescence spectrometer specially designed for the Langendorff hearts (a) and papillary muscles (b).

calculated in real time by a microcomputer. Whole hearts or papillary muscle were held on the apparatus in a temperature-controlled organ bath at 30°C (Fig. 1a and b), and illuminated alternatively at excitation light (at 340 and 380 nm). The light emitted from the muscle was collected by a photomultiplier through a bandpass filter at 500 nm (±10.0 nm).

Hartley strain guinea-pigs of either sex, weighing between 250–300 g, were anesthetized with diethyl ether. The hearts were rapidly excised and the aortae were cannulated. The Langendorff heart preparations were perfused with Krebs-Henseleit solution (K-H solution, pH 7.4, at 30°C) containing 115 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4 and 10 mM glucose. The solution was saturated with a gas mixture containing 95% O_2 and 5% CO_2, and the hearts were perfused at a flow rate of 7 ml/min using a peristaltic pump. A latex balloon was inserted into the left ventricle and inflated sufficiently to measure the left ventricular developed pressure at an end-diastolic pressure of 5–10 mm Hg.

The pharmacodynamic influence of acetoxymethylester of fura-2 (fura-2AM) was first observed in preparations of isolated guinea-pig atria with spontaneous pacemaker activity. Neither chrono- nor inotropic effects of 10 μM fura-2AM containing cremophore EL were detected between the test and control over the 60-min observation period (data not shown). To facilitate loading of fura-2 into the cells, K-H solution containing 2 μM fura-2AM and 0.025% cremophore EL was recirculated into the heart, and fura-2AM solution was sonicated prior to use to avoid precipitation. After perfusion for fura-2 loading, the heart was perfused with normal K-H solution in the non-recirculating mode for 10 min to remove excess fura-2AM in the extracellular space. After fura-2 loading for a period of 30 min, F340 from the hearts increased 5–7-fold compared with that before fura-2 loading. As the F340 did not change after fura-2 loading over 60 min, loading was performed in subsequent experiments by 30 min of recirculation mode perfusion.

The fura-2-loaded papillary muscles were obtained from the Langendorff hearts loaded with 2 μM fura-2AM for 30 min. The papillary muscles with a diameter less than 1 mm were excised from the hearts and mounted horizontally under a fluorescent spot with a diameter of about 1 mm in an organ bath containing K-H solution, saturated with a gas mixture containing 95% O_2 and 5% CO_2 (pH 7.4) at 30°C. The force of contraction of papillary muscle at a driving rate of 1.0 Hz was measured with a force-displacement transducer (Nihon kohden, SB-1TH) connected to an amplifier and was recorded with a pen recorder on oscillographic

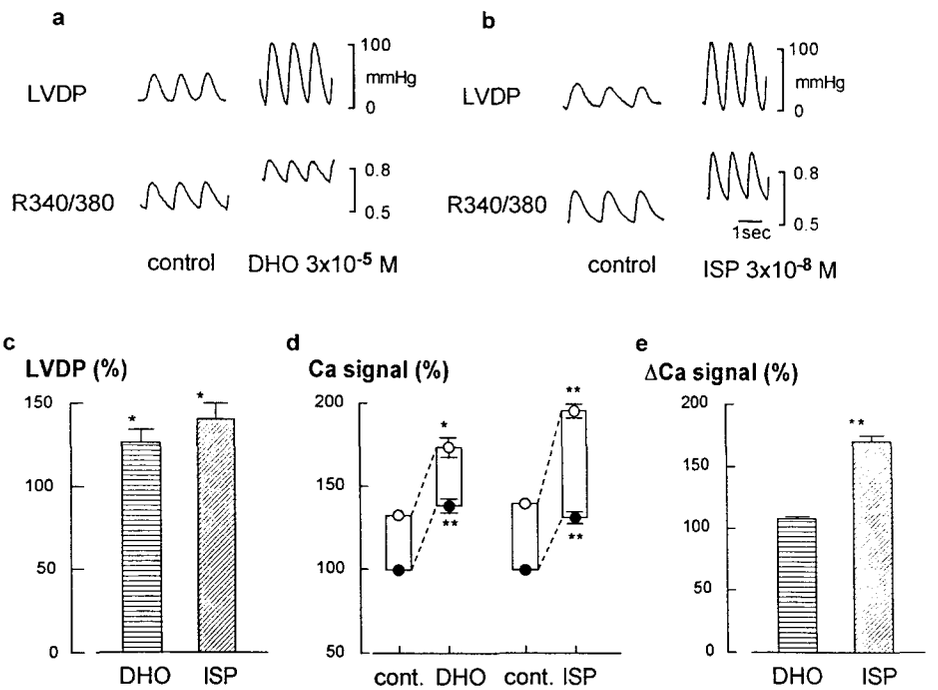

Fig. 2. Effects of dihydroouabain (DHO) at 3×10^{-5} M (a) or isoproterenol (ISP) at 3×10^{-8} M (b) on Ca^{2+} transient signals (R340/380) and left ventricular developed pressure of Langendorff hearts. Summarized effects of DHO and ISP on the maximum ratio (R340/380) during the systolic phase (S_{Ca}), the minimum ratio during the diastolic phase (D_{Ca}) and the amplitude of the Ca^{2+} transient signal (T_{Ca}) in Langendorff hearts. (c) Left ventricular developed pressure (LVDP), (d) S_{Ca} (○) and D_{Ca} (●), (e) ΔCa signal of T_{Ca}. Each point and bar represents the mean ±S.E. ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the control (drug-free) value as 100%.

paper. The muscle was field-stimulated using a pair of platinum plate electrodes.

2.2. Measurement of cytosolic Ca^{2+}

To calculate the absolute amount of cytosolic Ca^{2+}, it is necessary to determine the dissociation constant of fura-2 for Ca^{2+} under a given set of conditions: this value was reported to be 224 nM (Grynkiewicz et al., 1985) in solution at 37°C. However, Konishi et al. (1988) found that the dissociation constant in the tissues is affected by binding with various proteins in living cells. Due to the difficulty of determining the real dissociation constant in myocardial cells, in the present study we used only the ratio of F340 to F380 (R340/380) as an indicator of cytosolic Ca^{2+}, as in the study reported previously by Ozaki et al. (1988).

2.3. Chemicals

Agents used in these experiments were fura-2AM (Dojindo Laboratories), cremophore EL (Sigma Chemical), isoproterenol (Nikken Co.), ryanodine (Sigma Chemical) and verapamil hydrochloride (Sigma Chemical). Dihydroouabain was prepared by semi-synthesis from ouabain (Merck Co.). The purity was confirmed by ^{1}H- and ^{13}C-NMR spectra.

2.4. Statistical analysis

The results of the experiments are expressed as means ± S.E. Student's *t*-test was used for statistical analysis of the results.

3. Results

3.1. Fura-2 Ca^{2+} transient signals and the force of contractions

Simultaneous recordings of fluorescence from fura-2-loaded preparations and the left ventricular developed pressure in Langendorff hearts or contraction in papillary muscles (driven at 1.0 Hz) are shown in Figs. 2 and 3, respectively. The fluorescence changes due to intracellular Ca^{2+} (increase in F340, decrease in F380 and increase in R340/380) were in accordance with the changes in left ventricular developed pressure and in contraction development. The resting auto-fluorescence (F340, F380, R340/380) of the fura-2-unloaded hearts did not change after addition of 3×10^{-5} M dihydroouabain or 3×10^{-8} M isoproterenol.

An intracellular Ca^{2+} transient signal (T_{Ca}) developed consistently 10–15 ms faster than the start of contraction, and rose more steeply and decayed more

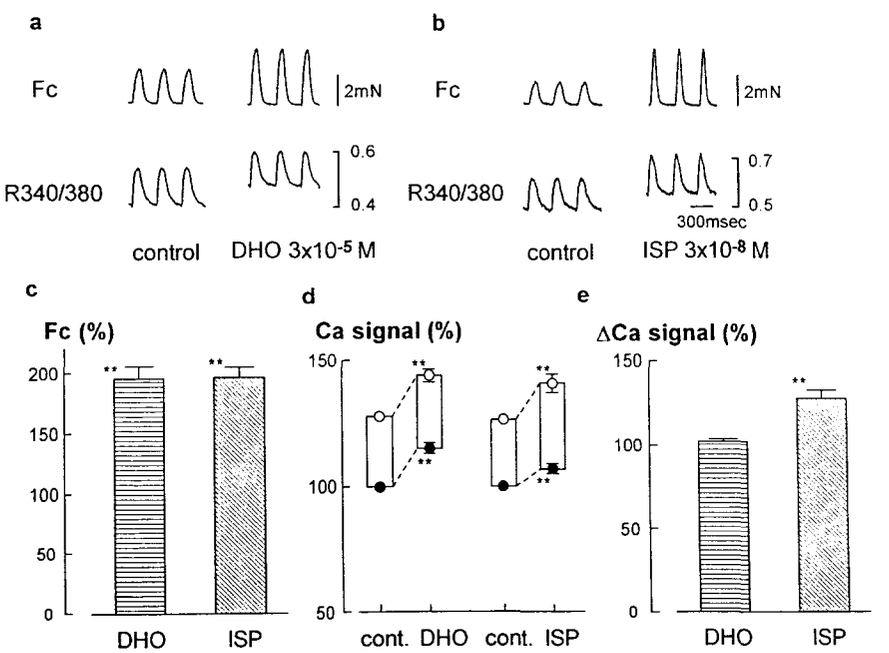

Fig. 3. Effects of dihydroouabain (DHO) at 3×10^{-5} M (a) or isoproterenol (ISP) at 3×10^{-8} M (b) on Ca^{2+} transient signals (R340/380) and contraction (Fc) of guinea-pig papillary muscle driven at 1.0 Hz. Summarized effects of DHO and ISP on the maximum ratio (R340/380) during the systolic phase (S_{Ca}), the minimum ratio during the diastolic phase (D_{Ca}) and the amplitude of Ca^{2+} transient signal (T_{Ca}) in papillary muscle, (c) contraction (Fc), (d) S_{Ca} (○) and D_{Ca} (●), (e) ΔCa signal of T_{Ca}. Each point and bar represents the mean ± S.E. ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from the control value as 100%. Mean values of the time to peak and the half-decay time of contraction curves obtained from the 5 muscles mentioned in Table 1.

slowly than did the contraction curve of papillary muscles ($n = 4$). The time to peak T_{Ca} (167.3 ± 13.9 ms) from a stimulation was shorter by about 60 ms than that of the contraction curve (226.2 ± 15.9 ms), while, conversely, the half-decay time of T_{Ca} (121.3 ± 12.7 ms) was longer by about 60 ms than that of contraction (180.3 ± 14.5 ms).

3.2. Effects of dihydroouabain and isoproterenol on Ca^{2+} transient signals and left ventricular developed pressure of Langendorff hearts

Dihydroouabain at 3×10^{-5} M and isoproterenol at 3×10^{-8} M increased the systolic left ventricular developed pressure to the same degree without any change in diastolic pressure. Dihydroouabain elevated the minimum ratio (Ca^{2+} level) during free diastolic phase (D_{Ca}) in parallel to the increase in left ventricular developed pressure without any change in the amplitude of T_{Ca} (Fig. 2a). On the other hand, isoproterenol increased the amplitude of T_{Ca} concurrently with increases in left ventricular developed pressure and heart rate (Fig. 2b). The level of D_{Ca} was increased by addition of either dihydroouabain or isoproterenol.

D_{Ca}, the maximum ratio during the systolic phase (S_{Ca}), and T_{Ca} changes in response to dihydroouabain and isoproterenol are summarized in Fig. 2c, d and e from Langendorff heart data shown in Fig. 2a and b. The increased changes (%) in D_{Ca} and S_{Ca} by dihydroouabain or isoproterenol in the Langendorff hearts were shown as relative values, taking the diastolic level of the control as 100%. Dihydroouabain did not significantly change T_{Ca}, while it increased D_{Ca}. In contrast, isoproterenol significantly increased T_{Ca} in combination with increases in both D_{Ca} and S_{Ca}.

3.3. Effects of dihydroouabain and isoproterenol on Ca^{2+} transients and isometric contraction of papillary muscle

Isolated papillary muscles were used to investigate the relationship between the time courses of isometric contraction and intracellular Ca^{2+} signal in detail. Dihydroouabain or isoproterenol added cumulatively into the bath produced positive inotropic effects but the response patterns of T_{Ca} to both agents were strikingly different, although dihydroouabain at 3×10^{-5} M and isoproterenol at 3×10^{-8} M increased the force of contraction by about the same extent as shown in Fig. 3a and b. D_{Ca}, S_{Ca} and T_{Ca} values in papillary muscle are summarized with the force of contraction in Fig. 3c, d and e. Force of contraction with dihydroouabain and isoproterenol increased to the same extent (Fig. 3a and c). However, fura-2 Ca^{2+} transient signals also appeared to have different effects on T_{Ca} (Fig. 3d and e) essentially similar to those in Langendorff hearts (Fig. 2d and e).

The changes in the time to peak and the half-decay time of Ca^{2+} transient signals and contraction curves with dihydroouabain and isoproterenol are summarized in Table 1. The time to peak and the half-decay time of T_{Ca} after addition of isoproterenol were significantly shortened by 37% (61 ms) and by 17% (33 ms) ($P < 0.01$), respectively, relative to control values accompanied with a shortening of the time to peak and the half-decay time in the contraction curve by 52% (156 ms) and by 31% (34 ms) of the relative control values ($P < 0.01$).

On the other hand, the addition of dihydroouabain did not significantly alter the time to peak or the half-decay time (reductions of 7%, i.e. 11 ms, and 0%, 0 ms, respectively). The time to peak and the half-decay time of the contraction curve were also not significantly different, changing by only 18% (56 ms) and 0% (0 ms), respectively.

3.4. Influence of dihydroouabain and isoproterenol on rested state contraction and Ca^{2+} transient signals

To obtain further information with regard to the cellular mechanisms of the different effects of dihydroouabain and isoproterenol on T_{Ca}, we compared their effects on T_{Ca} during steady and rested state contractions (RSC) in isolated guinea-pig papillary muscles by observing the time course of the force of contractions and fura-2 Ca^{2+} transient signals.

Table 1
Influence of isoproterenol and dihydroouabain on the time to peak and the half-decay time of contraction curves and Ca^{2+} transients recorded from guinea-pig papillary muscle

	Time to peak (ms)		Half-decay time (ms)	
	Control	Isoproterenol	Control	Isoproterenol
Contraction (Fc)	300.0 ± 24.2	144.4 ± 10.5 [a]	112.0 ± 14.3	77.8 ± 8.4 [b]
Ca^{2+} signal (R340/380)	166.6 ± 14.2	105.6 ± 9.2 [b]	200.0 ± 20.3	166.7 ± 13.7 [b]
	Control	Dihydroouabain	Control	Dihydroouabain
Contraction (Fc)	311.1 ± 27.3	255.6 ± 22.4	111.0 ± 12.3	111.1 ± 13.5
Ca^{2+} signal (R340/380)	177.8 ± 15.4	166.7 ± 15.2	200.0 ± 21.9	200.0 ± 23.4

Data are presented as means ± S.E. for each of 5 muscles in the absence (control) and presence of isoproterenol (10^{-7} M) or dihydroouabain (3×10^{-4} M) as shown in Fig. 3a and b. [a] $P < 0.01$, [b] $P < 0.05$, significantly different from control.

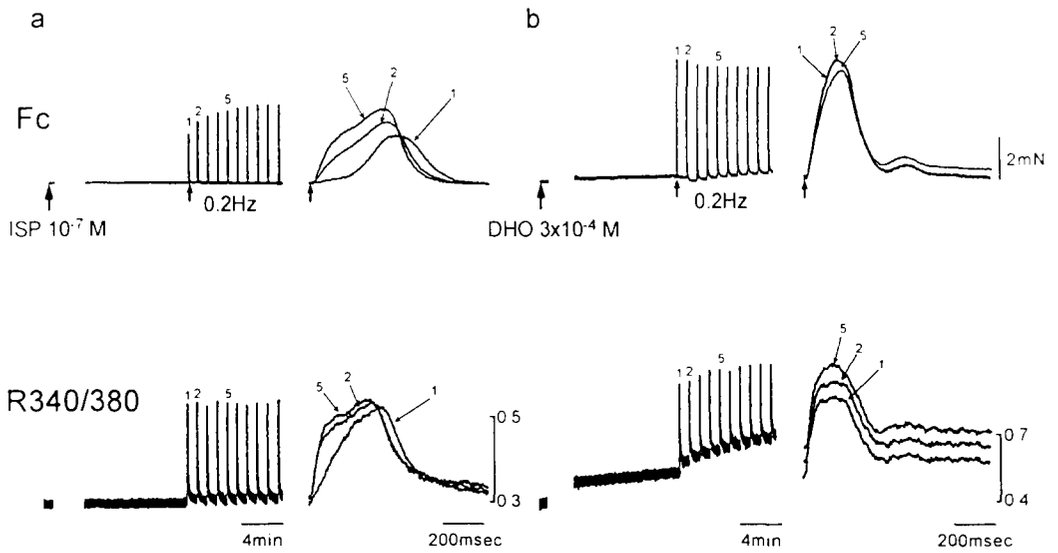

Fig. 4. Influence of isoproterenol (ISP) at 10^{-7} M (a) and dihydroouabain (DHO) at 3×10^{-4} M (b) on contraction and Ca^{2+} transient signal at 0.2 Hz after a rest period of 15 min. The muscle was stimulated at 0.2 Hz. Each drug was added 10 min prior to stimulation. The numbers on both the recordings and the superimposed curves denote the sequence of contractions and Ca^{2+} transient signals. The stimulus is marked by an arrow.

The first T_{Ca} stimulated at a low rate of 0.2 Hz after a 15-min rest period in the presence of 10^{-7} M isoproterenol developed latency then reached a peak at more than 360 ms from the start of the stimulus (Fig. 4a). The time to peak of T_{Ca} at 360 ms developed more quickly than the time to peak force of RSC at 440 ms. The early compartment of T_{Ca} and contraction at 0.2 Hz steady state was clearly visible on the second beat and reached its approximate maximum on the fifth beat. The level of D_{Ca} during the addition of isoproterenol did not increase between the rest period and with stimulation at 0.2 Hz.

On the other hand, the first T_{Ca} signal for RSC of papillary muscle treated with dihydroouabain at 3×10^{-4} M showed a steep rise early after stimulus and reached the maximum peak within 147 ms accompanied by an early component (Fig. 4b). The time to peak of T_{Ca} after the stimulus developed 20 ms earlier than the time to peak force of contraction of 168 ms. The shapes of T_{Ca} for the first stimulation after a 15-min rest period and at a 0.2 Hz steady state were less clear, as shown in Fig. 4b. However, the level of D_{Ca} during the rest period was increased by administration of 3×10^{-4} M dihydroouabain and was further increased rapidly by stimulation at 0.2 Hz without any change in the amplitude of T_{Ca}.

To evaluate the steepness (S_1) of the isometric contraction (S_1, see Fig. 5) and that of T_{Ca} (FS_1, see Fig. 5) under the influence of dihydroouabain and isoproterenol, the mean gradient of this component was measured in a group of 5 muscles stimulated at 0.2 Hz following rested state contraction. As shown in Fig. 5, dihydroouabain produced a maximum value from the first beat in T_{Ca} and contraction, but isoproterenol caused an increase in steepness of the early component at a 0.2 Hz steady state.

3.5. Effects of ryanodine and verapamil on biphasic Ca^{2+} transient signals

The sources of activator Ca^{2+} participating in biphasic contractions at 0.2 Hz in the presence of isoproterenol at 10^{-7} M were examined using ryan-

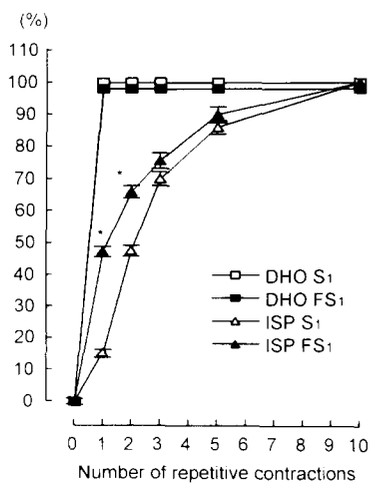

Fig. 5. Development of both the early component of contraction and Ca^{2+} transient signal during stimulation resumed at 0.2 Hz after a rest period. Ordinate: steepness (S_1) of the isometric contraction curve (□, dihydroouabain (DHO); △, isoproterenol (ISP)) and that (FS_1) of Ca^{2+} transient signal (■, DHO; ▲, ISP) determined from the 10th segment after the stimulus. Abscissa: number of contractions and Ca^{2+} transient signals during the rest period. Mean values obtained from 6 guinea-pig muscles. $^{*}P < 0.05$, $^{**}P < 0.01$, significant difference between S_1 and FS_1 under the influence of each drug.

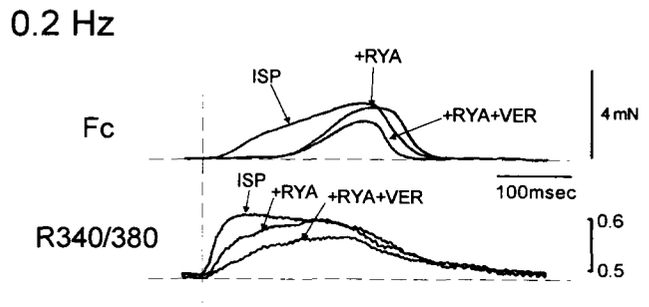

Fig. 6. Influence of ryanodine (RYA) at 3×10^{-7} M and verapamil (VER) at 10^{-6} M on the positive inotropic effects of isoproterenol (ISP) 10^{-7} M. RYA was added to the bathing solution of guinea-pig muscle after establishing the steady state positive inotropy at a contraction frequency of 0.2 Hz, and further VER was added. Contraction curves and Ca^{2+} transient signals 20 min after the addition of each drug are illustrated.

odine, a modulator of the function of the sarcoplasmic reticulum, and verapamil, a Ca^{2+} channel blocker. Both the early component of biphasic contraction and the Ca^{2+} transient signal were decreased by treatment of the muscle with ryanodine at 3×10^{-7} M, as shown in Fig. 6. Subsequently, verapamil at 10^{-6} M depressed both the late component of the biphasic contraction and the Ca^{2+} transient signals.

4. Discussion

In the present study, we measured the changes in intracellular Ca^{2+} transient signal (T_{Ca}) and the force of contraction (the left ventricular developed pressure and contraction) simultaneously using a fura-2 Ca^{2+} fluorescent probe in working preparations of Langendorff hearts and in papillary muscles isolated from guinea pigs. T_{Ca} in myocardial cells have strikingly characteristic features: (1) T_{Ca} were consistently observed preceding the onset of force development; (2) the time to the peak of T_{Ca} from the start of the stimulus was shorter than that of contraction; (3) T_{Ca} persisted much longer even after termination of contraction (Figs. 2 and 3). These changes in T_{Ca} in the present study were in essential agreement with results for fura-2 Ca^{2+} transient signals in single rat myocytes or cultured chick heart cells (Cannell et al., 1987; Kim et al., 1987), and also in rabbit heart containing another Ca^{2+} fluorescence indicator, indo-1 (Lee et al., 1988; Auffermann et al., 1989).

Cytosolic T_{Ca} using a photoprotein, aequorin, measured firstly to monitor cytosolic free Ca^{2+}, rapidly increased and returned to a resting Ca^{2+} signal level in the time taken to reach the peak of a contraction (Allen and Blinks, 1978; Allen and Orchard, 1984; Morgan and Morgan, 1984). As the luminescence of aequorin is proportional to the cube of the concentration of intracellular Ca^{2+}, a steeper increase in bioluminescent signals of aequorin was observed. In contrast, as fura-2 binds in amounts equimolar with free Ca^{2+}, and the amplitude of Ca^{2+} signal is proportional to the degree of binding of fura-2 and free Ca^{2+}, fura-2 Ca^{2+} signals persist apparently with the unchanged amplification of Ca^{2+} signals. For this reason, the shapes of T_{Ca} are altered in different modes using each probe to measure Ca^{2+} concentration. The development of fura-2 Ca^{2+} transient signals was not more rapid than for those of aequorin. Further study of Ca^{2+} transients will be required using a lower affinity fluorescent indicator such as furaptra (Mg-fura-2) which has a faster off rate constant than both fura-2 and aequorin and thus should have a more reliable time course (Konishi et al., 1991).

Fura-2 Ca^{2+} fluorescence in test solutions containing free acid type fura-2 at 1 μM, 115 mM KCl, 25 mM NaCl, 1 mM $CaCl_2$, 10 mM Hepes (pH 7.4, 30°C) could detect Ca^{2+} at concentrations from 10^{-9} to 10^{-5} M. This spectrum was not influenced by Na^+ (0, 25, 50 mM), K^+ (0, 50, 100, 150 mM), Mg^{2+} (5, 10, 20 mM), pH (6.8, 7.4, 8.2) or temperature (25, 30, 35°C) (Nishiyama, 1991). Although fura-2 Ca^{2+} signals may include artifacts originating from cardiac muscle motion, these signals appeared not to be affected by contraction, based on the following three main observations: (1) no change in fluorescence was observed in myocardium not loaded with fura-2 accompanying contraction; (2) the fluorescence ratio (R340/380) consistently preceded force development; (3) the fluorescence of F340 and that of F380 were mirror images (Hotta et al., 1989).

The major disadvantage of the fura-2 method for Ca^{2+} determination is that it cannot quantify intracellular Ca^{2+} concentration in living tissues. Although the absolute value of the cellular Ca^{2+} concentration has been estimated from calibration curves of fura-2 and Ca^{2+} concentrations in test tubes, accurate quantification from fura-2 Ca^{2+} signals is almost impossible and is presented only as a relative change for the following reasons: (1) fura-2-unloaded myocardial muscles show a little self-fluorescence (Cobbold and Rink, 1987); (2) the properties of fura-2 Ca^{2+} fluorescence change with the interference of Ca^{2+}-sensitive cellular proteins, so that the dissociation constant between fura-2 and free Ca^{2+} in myocardial cells cannot be measured exactly (Konishi et al., 1988); (3) photobleaching of fura-2 dye occurs on exposure to excitation light for long periods (Ozaki et al., 1988); (4) the intermediate fura-2AM generated by hydrolysis has the same fluorescence as fura-2 (Highsmith et al., 1986). However, Jensen et al. (1993) estimated the intracellular dissociation constant (K_d) of fura-2-Ca^{2+} complex in rat mesenteric resistance arteries, and thus in papillary muscle prepara-

tions; it should be possible to estimate K_d for fura-2 and also to calibrate the signals.

In contrast to other methods used for determination of intracellular Ca^{2+} content, assay techniques using fluorescent indicators such as fura-2 or indo-1 are easier with regard to Ca^{2+}-probe loading into the cells and allow convenient measurement of cellular T_{Ca} accompanying each contraction of living myocardium. Simultaneous measurement of cytosolic fura-2 Ca^{2+} transient signals and force of contraction (either the left ventricular developed pressure or contraction of myocardium) under the positive inotropic effect of isoproterenol and dihydroouabain was performed in this study using fura-2 Ca^{2+} fluorometry. As shown in Figs. 2 and 3, dihydroouabain (with Na^+-dependent positive inotropic effect) elevated the intracellular Ca^{2+} level at diastole without any change in the amplitude of T_{Ca}, while isoproterenol (with Ca^{2+}-dependent positive inotropic effect increased by the slow inward Ca^{2+} current) increased T_{Ca} in comparison with dihydroouabain. Thus, the shapes of T_{Ca} with different modes of action of these two cardiotonic agents were strikingly different. The positive inotropic effect of dihydroouabain is the result of its inhibition of the membrane Na^+,K^+-pump, thereby increasing firstly intracellular Na^+ concentration (Hotta et al., 1994) and then intracellular Ca^{2+} by way of Na^+-Ca^{2+} exchange. It is highly probable that the elevation of the diastolic intracellular Ca^{2+} concentration reflects this mechanism. Further, neither an increase in Ca^{2+} uptake by the sarcoplasmic reticulum nor Ca^{2+} influx (I_{Ca}) modulation via Ca^{2+} channels takes place following dihydroouabain treatment since the amplitude of T_{Ca} did not change.

On the other hand, isoproterenol stimulates β-adrenoceptors and increases the intracellular c-AMP level by activating adenylate cyclase, which in turn stimulates c-AMP-dependent protein kinase. Activation of the latter enzyme increases I_{Ca} by phosphorylation of Ca^{2+} channels and promotes sequestration of Ca^{2+} by the sarcoplasmic reticulum (Reuter and Scholz, 1977; Tada and Katz, 1982; Callewaert et al., 1988). The observed increases in the amplitude of T_{Ca} by isoproterenol can be interpreted as increases in Ca^{2+} influx and the release of Ca^{2+} from the sarcoplasmic reticulum.

Comparison of the time course of the isomeric twitch curve with that of fura-2 Ca^{2+} signals in the rested state contraction of the papillary muscle (Fig. 4) further supported the view that there is a distinct difference between the mechanisms of the positive inotropic effects of isoproterenol and of dihydroouabain. The development of the early component of Ca^{2+} signals produced by isoproterenol was more rapid than that of the contraction curve, but dihydroouabain produced almost maximum contraction in the first beat (Fig. 5). The early component of the biphasic Ca^{2+} signals produced by isoproterenol was depressed after exposure to ryanodine which selectively abolishes the release of Ca^{2+} from the sarcoplasmic reticulum, and the late component was further decreased by treatment with the Ca^{2+} channel blocker, verapamil (Barcenas-Raiz and Wier, 1987), as shown in Fig. 6. These results regarding T_{Ca} from the heart muscle in the rested and steady state contractions support the view that the early component of biphasic contraction depends on Ca^{2+} stores in the sarcoplasmic reticulum and the late component depends on the Ca^{2+} influx during the plateau phase of cardiac action potential as suggested previously (Seibel and Takeya et al., 1978; Honoré et al., 1987; Reiter et al., 1984, 1988).

Lee et al. (1988) showed that most of the Ca^{2+}-specific fluorescence due to the free acid type of fluorescent dye was located in the soluble cytosolic fraction: cytosolic fraction 72.2%, mitochondrial fraction 3.6%, microsomal fraction 3.4% and nuclear fraction 20.8%. No evidence has been seen of any toxicity, because very little free acid was present in the mitochondria. Although the binding constant between cellular free Ca^{2+} and fura-2 has not yet been determined exactly, our results with Ca^{2+}-dependent fura-2 fluorescence transients demonstrated a rapid upstroke and slow decay during twitch. The increase in fura-2 Ca^{2+} fluorescence ratio (R340/380) during contraction appeared to reflect binding with an increased amount of intracellular free Ca^{2+} originating from the sarcoplasmic reticulum, mitochondria, and Ca^{2+} influx, while in the relaxed state, the fura-2 Ca^{2+} ratio will decrease by reuptake of Ca^{2+} by cellular organelles (sarcoplasmic reticulum, mitochondria, etc.) and binding with ubiquitous Ca^{2+}-binding proteins such as calmodulin (K_d = 3–4 μM). It will be necessary to investigate carefully the respective and total relationships between fura-2, Ca^{2+} and various proteins on account of the regulation of cytosolic Ca^{2+}.

Similar observations were reported previously with regard to the effects of isoproterenol and dihydroouabain on the Ca^{2+} transients and contraction in cultured rat ventricular cells by Tatsukawa (1993). As shown by the physiological performance of fura-2-loaded, beating hearts, the shape of the contraction curve (the left ventricular developed pressure and contraction) of Langendorff hearts and papillary muscles was established more exactly than that (shortening) of myocytes, and the shape of fura-2 Ca^{2+} transients was essentially identical to that of the corresponding contraction curve. The global ischemia in the Langendorff heart preparations can also make induction easier by stopping the perfusate, than with myocyte and papillary muscle preparations. Cytosolic free Ca^{2+} in ischemic myocardium can be estimated by another technique using ^{19}F-NMR and the Ca^{2+}-selective indicator,

1,2-bis(2-amino-5-difluorophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (Marban et al., 1990). We observed an increase in the amplitude of fura-2 Ca^{2+} transient signals and diastolic Ca^{2+} level accompanied by the disappearance of the left ventricular developed pressure during global ischemia in guinea-pig Langendorff hearts (Takeya and Hotta, 1994). The changes in fura-2 Ca^{2+} ratio in guinea-pig hearts could be recorded sufficiently accurately during ischemia and reperfusion.

In the present study, we measured simultaneously the intracellular Ca^{2+} transient changes in response to dihydroouabain and isoproterenol using the dual wavelength excitation method to detect fura-2 Ca^{2+} transients, and mechanical activity in intact guinea-pig hearts. We also observed that, in the presence of the Ca^{2+}-sensitizing agent, pimobendan, the shapes of fura-2 Ca^{2+} transients of papillary muscles were distinctly different from those of these two drugs (unpublished data). These results indicate that the shape of the fura-2 Ca^{2+} transient signal is a useful indicator for estimating the cytosolic Ca^{2+} level corresponding to the contraction curve in the working heart.

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References

Allen, D.G. and J.R. Blinks, 1978, Calcium transients in aequorin-injected frog cardiac muscle, Nature 273, 509.

Allen, D.G. and C.H. Orchard, 1984, Measurements of intracellular calcium concentration in heart muscle: the effect of inotropic interventions and hypoxia, J. Mol. Cell. Cardiol. 16, 117.

Auffermann, W., T. Stefenelli, S.T. Wu, W.W. Parmley, J. Wikman-Coffelt and D.T. Mason, 1989, Influence of positive inotropic agents on intracellular calcium transients. Part I. Normal rat heart, Am. Heart J. 118, 1219.

Barcenas-Ruiz, L. and W.G. Wier, 1987, Voltage dependence of intracellular $[Ca^{2+}]_i$ transients in guinea pig ventricular myocytes, Circ. Res. 61, 148.

Barry, W.H., Y. Hasin and T.W. Smith, 1985, Sodium pump inhibition, enhanced calcium influx via sodium-calcium exchange, and positive inotropic response in cultured heart cell, Circ. Res. 56, 231.

Blinks, J.R., 1986, Intracellular (Ca^{2+}) Measurements. The Heart and Cardiovascular System (Raven Press, New York) p. 671.

Callewaert, G., L. Cleemann and M. Morad, 1988, Epinephrine enhances Ca^{2+} current-regulated Ca^{2+} release and Ca^{2+} reuptake in rat ventricular myocytes, Proc. Natl. Acad. Sci. USA 85, 2009.

Cannell, M.B., J.R. Berlin and W.J. Lederer, 1987, Effect of membrane potential changes on the calcium transient in single rat cardiac muscle cells, Science 238, 1419.

Cobbold, P.H. and T.J. Rink, 1987, Fluorescence and bioluminescence measurement of cytoplasmic free calcium, Biochem. J. 248, 313.

Endoh, M. and J.R. Blinks, 1988, Actions of symphathomimetic amines on Ca^{2+} transients and contractions of rabbit myocardium: reciprocal changes in myofibrillar responsiveness to Ca^{2+} mediated through α- and β-adrenoceptors, Circ. Res. 8, 247.

Grynkiewicz, Z., M. Poenie and R.Y. Tsien, 1985, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260, 3440.

Highsmith, S., P. Bloebaum and K.W. Snowdowne, 1986, Sarcoplasmic reticulum interacts with the Ca(2+) indicator precursor fura-2-AM, Biochem. Biophys. Res. Commun. 138, 1153.

Honoré, E., M. Adamantidis and B. Dupuis, 1987, Calcium channels and excitation-contraction coupling in cardiac cells. I. Two components of contraction in guinea-pig papillary muscle, Can. J. Physiol. Pharmacol. 65, 1822.

Hotta, Y., M. Nishiyama, H. Ando, M. Kato and K. Takeya, 1989, Different modes of the effects of dihydroouabain and isoproterenol on the fura-2 calcium transients and contractile response of the isolated guinea-pig heart, J. Aichi Med. Univ. Assoc. 17, 911.

Hotta, Y., H. Ando, K. Takeya and J. Sakakibara, 1994, Direct measurement of increased myocardial cellular ^{23}Na NMR signals in perfused guinea-pig heart induced by dihydroouabain and grayanotoxin-I, Mol. Cell. Biochem. 139, 59.

Jackson, A.P., M.P. Timmerman, and C.R. Bagshaw, 1987, The kinetics of calcium binding to fura-2 and indo-1, FEBS Lett. 216, 35.

Jensen, P.E., M.J. Mulvany, C. Aalkjer, H. Nilsson and H. Yamaguchi, 1993, Free cytosolic Ca^{2+} measured with Ca^{2+}-selective electrodes and fura 2 in rat mesenteric resistance arteries, Am. J. Physiol. 265, H741.

Kim, D., A. Okada and T.W. Smith, 1987, Control of cytosolic calcium activity during low sodium exposure in cultured chick heart cells, Circ. Res. 61, 29.

Konishi, M., A. Olson, S. Hollingworth and S.M. Baylor, 1988, Mycoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements, Biophys J. 54, 1089.

Konishi, M., S. Hollingworth, A.B. Harkins and S.M. Baylor, 1991, Myoplasmic calcium transients intact frog skeletal muscle fibers monitored with the fluorescent indicator furaptra, J. Gen. Physiol. 97, 271.

Lattanzio, F.A. and B.C. Pressman, 1986, Alterations in intracellular calcium activity and contractility of isolated perfused rabbit hearts by ionophores and adrenergic agents, Biochem. Biophys. Res. Commun. 139, 816.

Lee, C.O., 1985, 200 years of digitalis: the emerging central role of the sodium ion in the control of cardiac force, Am. J. Physiol. 249, C367.

Lee, H.-C., R. Mohabir and N. Smith, 1988, Effect of ischemia on calcium-dependent fluorescence transients in rabbit heart containing indo-1, Circulation 78, 1047.

Marban, E., M. Kitakaze, Y. Koretune, D.T. Yue, V.P. Chacko and M.M. Pike, 1990, Quantification of $[Ca^{2+}]_i$ in perfused hearts: critical evaluation of 5F-BAPTA and nuclear magnetic resonance method as applied to study of ischemic and reperfusion, Circ. Res. 66, 1255.

Morgan, J.P., 1985, The effects of digitalis on intracellular calcium transients in mammalian working myocardium as detected with aequorin, J. Mol. Cell. Cardiol. 17, 1065.

Morgan, J.P. and J.R. Blinks, 1982, Intracellular Ca^{2+} transients in the cat papillary muscle, Can. J. Physiol. Pharmacol. 60, 524.

Morgan, J.P. and K.G. Morgan, 1984, Calcium and cardiovascular function: intracellular calcium levels during contraction and relaxation of mammalian cardiac and vascular smooth muscle as detected with aequorin, Am. Med. 5, 33.

Nishiyama, M., 1991, The change in intracellular calcium of working fura-2 loaded myocardium in normal and hypoxic conditions, J. Aichi Med. Univ. Assoc. 19, 139.

Ozaki, H., T. Satoh, H. Karaki and Y. Ishida, 1988, Regulation of metabolism and contraction by cytoplasmic calcium in the intestinal smooth muscle, J. Biol. Chem. 263, 14074.

Reiter, M., 1988, Calcium mobilization and cardiac inotropic mechanisms, Pharmacol. Rev. 40, 189.

Reiter, M., V. Vierling and K. Seibel, 1984, Where is the origin of the activator calcium in cardiac ventricular contraction? Basic Res. Cardiol. 79, 1.

Reuter, H. and H. Scholz, 1977, The regulation of the calcium conductance of cardiac muscle by adrenaline, J. Physiol. 264, 49.

Sato, K., H. Ozaki and H. Karaki, 1988, Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura 2, J. Pharmacol. Exp. Ther. 246, 294.

Seibel, K., E. Karema, K. Takeya and M. Reiter, 1978, Effect of noradrenaline on an early and late component of the myocardial contraction, Naunyn-Schmied. Arch. Pharmacol. 305, 65.

Sheu, S., V.K. Sharma and S.P. Banerjee, 1984, Measurement of cytosolic free calcium concentration in isolated rat ventricular myocytes with quin 2, Circ. Res. 55, 830.

Tada, M. and A.M. Katz, 1982, Phosphorylation of the sarcoplasmic reticulum and sarcolemma, Annu. Rev. Physiol. 44, 423.

Takeya, K. and Y. Hotta, 1994, A new approach to evaluation of cardioprotective agents against myocardial ischemia. Symposia II. How to prevent cell damage in ischemic heart considering electrolyte and contractility alterations, Jpn. J. Pharmacol. 64 (Suppl. 1), 40p.

Tatsukawa, Y., M. Arita, T. Kiyosue, Y. Mikuriya and M. Nasu, 1993, A comparative study effects of isoproterenol and dihydroouabain on calcium transients and contraction in cultured rat ventricular cells, J. Mol. Cell. Cardiol. 25, 707.

Tsien, R.Y., T.J. Rink and M. Poenie, 1985, Measurement of cytosolic free Ca^{2+} in individual small cells using fluorescence microscopy with dual excitation wavelengths, Cell Calcium 6, 145.

Wier, W.G., M.B. Cannell and J.R. Berlin, 1987, Cellular and subcellular heterogeneity of (Ca^{2+}) in single heart cells revealed by fura-2, Science 235, 325.