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Inotropic effects of OR-1896, an active metabolite of levosimendan, on canine ventricular myocardium

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Abstract

We performed experiments in dog ventricular trabeculae loaded with aequorin to elucidate the mechanism of positive inotropic effect of (*R*)-*N*-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydro-pyridazin-3-yl)-phenyl]-acetamide (OR-1896), an active metabolite of (*R*)-([4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]-hydrazono)-propanedinitrile (levosimendan). Concentration–response curve for OR-1896 was biphasic: positive inotropic effect of OR-1896 reached a plateau at 10^{-5} M (1st phase) and the concentration–response curve became steeper at 10^{-3} M and higher (2nd phase). Maximum response of the 1st phase was 29% of maximal response to isoproterenol and associated with an increase in Ca^{2+} transients of 13% of the maximal response to isoproterenol. For a given increase in force, the increase in Ca^{2+} transients by OR-1896 was lower than that induced by elevation of $[Ca^{2+}]_o$. The positive inotropic effect of OR-1896 was not associated with impairment of relaxation and it was abolished by carbachol. In conclusion, OR-1896 has a positive inotropic effect partly due to an increase in myofibrillar Ca^{2+} sensitivity that is exerted via cross-talk with signal transduction mediated by cAMP.

Keywords: Positive inotropic effect; Ventricular muscle, dog; Ca^{2+} transient; Myofibrillar Ca^{2+} sensitivity; cAMP; Levosimendan

1. Introduction

Development of cardiotonic agents that act via novel mechanism of action has been inevitable for the treatment of contractile dysfunction in congestive heart failure, because most of the classical cardiotonic agents, including digitalis and catecholamines, are known to have potential risk to elicit arrhythmia and myocardial cell injury due to Ca^{2+} overload (Farah et al., 1984). Meanwhile, several novel cardiotonic agents, such as amrinone, milrinone, olprinone and vesnarinone have been developed in last two decades. Although these agents administered intravenously improved the hemodynamic parameters and excise capacity and thereby raised quality of life of patients, they failed to prolong the life span or even aggravated the prognosis of patients with chronic congestive heart failure (Baim et al., 1983; DiBianco et al., 1989; Packer et al., 1991).

Therefore, current interests have been focused on the development of Ca^{2+} sensitizers that act through an increase in the myofibrillar Ca^{2+} sensitivity. Ca^{2+} sensitizers might be particularly useful as therapeutic agents of contractile dysfunction because: (1) they do not increase the activation energy that is required for handling intracellular Ca^{2+} ions; (2) they are not associated with induction of arrhythmia and cell injury due to Ca^{2+} overload in myocardial cells; and (3) they have potential to reverse the myocardial dysfunction that is produced under pathological conditions, such as acidosis and myocardial stunning (Endoh, 1995; Haikala and Linden, 1995; Végh et al., 1995).

(*R*)-([4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]-hydrazono)-propanedinitrile (levosimendan) was identified as a compound that binds selectively to cardiac troponin C during high performance liquid chromatography on a troponin C-coupled affinity column (Haikala et al., 1995a). It has been shown that levosimendan has a Ca^{2+} sensitizing action on mammalian cardiac preparations including guinea pig (Edes et al., 1995; Haikala et al., 1995c) and human (Hasenfuss et al., 1995, 1998). The characteristics of levosimendan action are: (1) the shift of pCa-tension relation to the left in skinned and intact myocardial cells; (2) the binding to the amino-terminal

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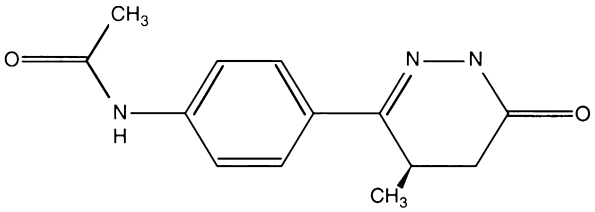

Fig. 1. Chemical structure of OR-1896.

region of troponin C (Pollesello et al., 1994); (3) the binding to troponin C in a Ca^{2+} dependent manner (Haikala et al., 1995a,c); and (4) the inhibitory action on phosphodiesterase III, which is comparable to that of milrinone (Boknik et al., 1997; Haikala et al., 1997; Raasmaja et al., 1991; Todaka et al., 1996). Whereas it has long been postulated that Ca^{2+} sensitizers might impair diastolic function as a result of an increased myofibrillar Ca^{2+} sensitivity (Böhm et al., 1991; Hajjar and Gwathmey, 1991; Hajjar et al., 1997; Kawabata and Endoh, 1993; Lues et al., 1993; Neumann et al., 1996; White et al., 1993), levosimendan neither impair diastolic relaxation nor prolong the relaxation time (Haikala et al., 1995c; Hasenfuss et al., 1995, 1998; Sato et al., 1998). This property of levosimendan is considered to contribute in part to the beneficial effects in clinical application of the compound because impaired diastolic function has risk to lead to elevated ventricular end-diastolic pressure and to aggravate both systemic and pulmonary congestion.

While it has been known that levosimendan produces an active metabolite, OR-1896 [(*R*)-*N*-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydro-pyridazin-3-yl)-phenyl]-acetamide] (see Fig. 1 for chemical structure) that may partially contribute to the positive inotropic effect of levosimendan in vivo, the pharmacological characteristics of the metabolite have not yet been reported. The present study was undertaken to elucidate the mechanism of the positive inotropic effect of OR-1896 in mammalian cardiac muscle. For this purpose, we carried out the experiments in isolated dog right ventricular muscles loaded with aequorin to investigate whether the compound has an effect on myofibrillar Ca^{2+} sensitivity in intact ventricular myocardium. We studied the concentration-dependent effects of OR-1896 on the relationship between contractility and Ca^{2+} transients. OR-1896 may elicit a positive inotropic effect by increasing myofibrillar Ca^{2+} sensitivity. In addition, the phosphodiesterase III inhibitory action of the compound may contribute to the absence of the negative lusitropic effect on canine ventricular myocardium.

2. Methods

The study involving experimental animals conforms to the institutional standards. This study was conducted in accordance with the Guidance for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The approval for the animal experiments was obtained from the Animal Research Committee, Yamagata University School of Medicine, prior to the experiments, and the study was carried out also in accordance with the Declaration of Helsinki.

2.1. Isolated canine trabeculae of the right ventricle

Mongrel dogs of either sex (8–12 kg) were used in the present study. Animals were anesthetized by intravenous administration of pentobarbital sodium (30 mg/kg). Hearts were rapidly excised from mongrel dogs, and free-running trabeculae (<1 mm diameter) were dissected from the wall of the right ventricle. Muscles were mounted in 20-ml organ baths containing Krebs–Henseleit solution. The composition of the solution was as follows (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, and glucose 11.1 (with 0.057 mM ascorbic acid and 0.027 mM EDTA added to prevent autoxidation of the compounds examined). The solution was bubbled with 95% O_2–5% CO_2 and maintained pH 7.4.

The preparations were stimulated electrically by square-wave pulses of 5-ms duration at a voltage about 20% above the threshold, at a frequency of 0.5 Hz, through bipolar platinum electrodes. The force of contraction was recorded continuously on a thermal pen-writing oscillograph (Recti-Horiz-8K; NEC Sanei Instruments, Tokyo, Japan) by means of force-displacement transducers (Shinkoh UL 10 GR; Minebea, Tokyo, Japan). In most preparations, after an equilibration period of 1 h, the resting and contractile force of the muscle was stable during the course of experiments for several hours. During the equilibration period, the muscles were stretched initially at a resting tension of 5 mN, and the length was later adjusted to give 90% of the maximal contractile force (Lmax). Preparations in which the resting tension increased progressively during the equilibration period were discarded.

At the beginning of each experiment, the responsiveness and stability of individual preparations were checked by successive administration of isoproterenol at a submaximally effective concentration (10^{-7} M). Only those preparations that produced consistent and reproducible increase in contractile force in response to the successive administration of isoproterenol were used for the experiments. At the end of each experiment with OR-1896, the maximal response to isoproterenol was determined in each muscle by cumulative administration of isoproterenol.

2.2. Aequorin-loaded trabeculae

For simultaneous detection of contractile force and intracellular Ca^{2+} transients, the Ca^{2+}-sensitive bioluminescent protein aequorin was loaded by the modified

macro-injection technique, as described elsewhere in detail (Kihara and Morgan, 1989; Sato et al., 1998; Sawada and Endoh, 1999; Watanabe et al., 1996).

The muscle preparation of right ventricular free wall was mounted horizontally in a 12-ml organ bath that contained the nominally Ca^{2+} free modified Krebs–Henseleit solution at 4°C. Aequorin was dissolved at concentrations of approximately 1.0 μg/ml in a solution of 150 mM KCl and 0.1 mM EDTA-2Na. After immersion of the preparations in nominally Ca^{2+} free solution for 5 min, 3–4 μl of the solution of aequorin were gently injected just beneath the endocardium through a fine-tipped glass micropipette. Then the aequorin-loaded preparation was transferred to a 50-ml organ bath that contained modified Krebs–Henseleit solution. The concentration of Ca^{2+} ions was gradually raised stepwise to 0.025, 0.25, 1.25, and finally to 2.5 mM at intervals of 15 min. Simultaneously, the temperature was gradually raised to 37°C.

The 50-ml organ bath specially designed for the simultaneous high-efficacy detection of light from aequorin and minimization of motion artifacts due to isometric contractions was used for the experiments (Blinks and Endoh, 1986). Aequorin light signals were detected with a photomultiplier (9789A; Thorn EMI Electron Tubes, Ruislip, UK) and light signals were smoothed by a low-pass filter. The isometric contractile force was recorded simultaneously with the transducer mentioned above. Both signals were recorded on digital audio tape (PC-108M; Sony Magnescale, Tokyo, Japan) for subsequent analysis.

The muscle was electrically stimulated by square-wave pulses of 5-ms duration at a voltage about 20% above the threshold, at a frequency of 0.5 Hz, through bipolar platinum electrodes. The aequorin-loaded preparation was equilibrated for about 120 min, meanwhile the bioluminescence declined to a steady low level. During the equilibration period, the length of the muscle was adjusted to Lmax. Only the preparations with a baseline contractile force of > 4 mN/mm^2 and with stable bioluminescence signals and contraction amplitudes during the course of experiments were used for the analysis of drug action in the present study.

Fifty to hundred-fifty signals of Ca^{2+} transients and isometric contractions were averaged to improve the signal-to-noise ratio by means of data analysis software (Visual Designer; Intelligent Instrumentation, Tucson, AZ, USA) in IBM PC/AT personal computer (FMV-Deskpower S13; Fujitsu, Tokyo, Japan). The 2.5th root of the peak amplitude of aequorin signals was calculated as an indicator of the peak $[Ca^{2+}]_i$, because the strength of the bioluminescence of aequorin varies approximately in proportion to the 2.5th power of the concentration of Ca^{2+} ions within a range of physiological value of $[Ca^{2+}]_i$ (Blinks et al., 1982). At the end of each experiment, the maximal response of both signals in response to isoproterenol was determined. The effects of drugs and elevation of $[Ca^{2+}]_o$ on the peak amplitudes of both signals were expressed as the percentage of the maximal response to isoproterenol.

In the experiment to determine the concentration–response curve for OR-1896, the drug was administrated in a cumulative manner in steps of 0.5 log units. When a steady contractile force had been achieved, OR-1896 was added to the next higher concentration. In some experiments, the muscarinic cholinergic receptor agonist carbachol was allowed to act for 30 min or longer before the administration of OR-1896, and it was present in the bathing solution throughout the period of administration of OR-1896. All experiments with aequorin-loaded preparations were carried out in the presence of 3×10^{-7} M (±)-bupranolol to avoid the modulation of the drug action by β-adrenoceptor stimulation induced by norepinephrine released if any by electrical stimulation or by the drug itself.

Solutions of OR-1896 were faint yellowish. During spectrophotometry, however, the absorbance of OR-1896 in solution at 469 nm, the peak wavelength of the aequorin light signal, was negligibly low even at the highest concen-

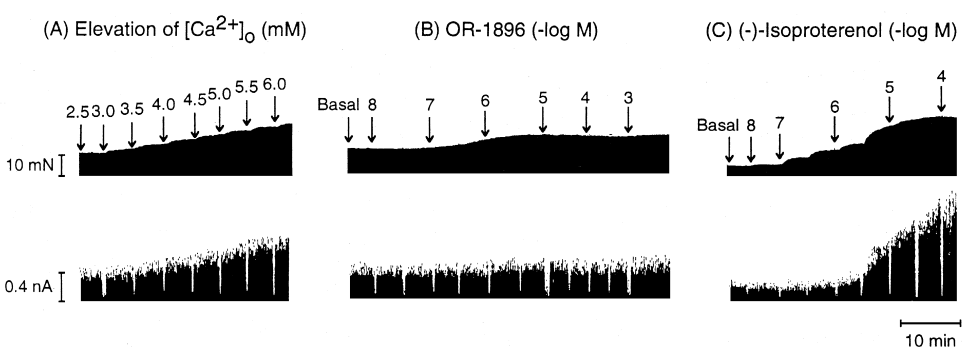

Fig. 2. Continuous recordings of the concentration-dependent effects of elevation of $[Ca^{2+}]_o$ (A), OR-1896 (B) and (−)-isoproterenol (C) on isometric contractions and Ca^{2+} transients in the same dog ventricular trabecula electrically driven at 0.5 Hz at 37°C. Upper panel: isometric contractions; lower panel: aequorin light signals. The experiments with elevation of $[Ca^{2+}]_o$ and OR-1896 were performed in the presence of 3×10^{-7} M (±)-bupranolol, the effects of (−)-isoproterenol were determined after washout of (±)-bupranolol for 2 h.

tration used in this study. To determine whether OR-1896 had any direct effect on aequorin bioluminescence, we performed a cuvette test in vitro according to the procedures originally developed by Blinks et al. (1978). The fractional luminescence of aequorin light signals at various concentrations of Ca^{2+} ions in the presence of 10^{-2} M OR-1896 fell on the curve obtained without OR-1896. Thus, light emission from aequorin was not influenced by OR-1896 itself.

OR-1896 was dissolved in dimethylsulfoxide. In preliminary experiments, dimethylsulfoxide up to 0.4% in the bathing solution scarcely influenced the baseline contractile force or the aequorin light signals. The highest concentration of dimethylsulfoxide employed in the present study was 0.27%.

In each preparation, the maximal response to isoproterenol was determined at the end of experimental protocol. It has been shown in dog ventricular trabeculae that the maximal response to isoproterenol was not affected by (±)-bupranolol up to 3×10^{-6} M (Ishihata et al., 1988) because (±)-bupranolol is a competitive β-adrenoceptor antagonist. Nonetheless, to minimize the influence of all drugs examined the maximal response was determined after washout of (±)-bupranolol and OR-1896 for more than 2 h. The positive inotropic effect and the increase in the amplitude of Ca^{2+} transients induced by OR-1896 or elevation of $[Ca^{2+}]_o$ were expressed as a percentage of the maximal response to isoproterenol.

2.3. Chemicals

The drugs used were as follows: OR-1896 (Orion-Farmos, Espoo, Finland); (−)-isoproterenol hydrochloride, carbamylcholine chloride (carbachol), (Sigma, St. Louis, MO, USA); (±)-bupranolol hydrochloride (Kaken Pharmaceutical, Tokyo); and pentobarbital sodium (Tokyo Kasei Kogyo, Tokyo). Aequorin was purchased from Friday Harbor Photoproteins, Friday Harbor, WA, USA.

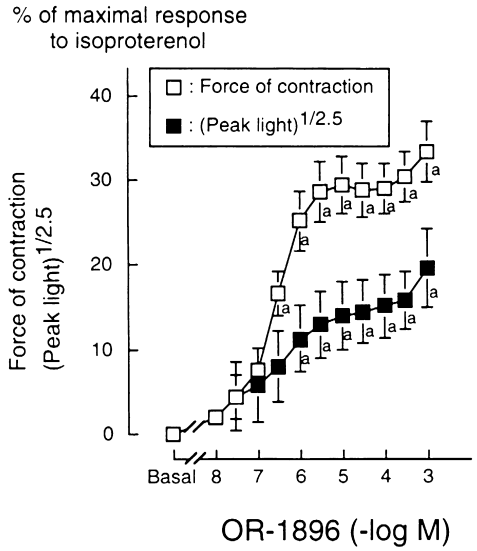

Fig. 4. Concentration–response curves for increases in force of contraction (open squares) and aequorin light signals (closed squares) induced by OR-1896 administered in a cumulative manner in the presence of 3×10^{-7} M (±)-bupranolol in isolated canine right ventricular trabeculae electrically driven at 0.5 Hz at 37°C. The 2.5th root of the amplitude of aequorin light signals has been used as an indicator of the amplitude of the Ca^{2+} transients because over a large range of $[Ca^{2+}]_i$ in vitro, the light emission from aequorin varies approximately in proportion to the 2.5th power of $[Ca^{2+}]_i$ (Blinks et al., 1978). Ordinate: the positive inotropic effect and the increase in Ca^{2+} transients induced by OR-1896 expressed as a percentage of the maximal response to isoproterenol in each preparation; abscissa: the concentration of OR-1896 expressed as −log M. Symbols with vertical bars represent mean ± S.E.M. Alphabet a indicate significant difference from the baseline values in respective parameters induced by OR-1896 (a: $P < 0.05$). Actual values of control contractile force and peak light transients: 6.57 ± 2.74 mN/mm^2 and 0.37 ± 0.12 nA in control ($n = 7$, each); and the maximal response to isoproterenol: 47.6 ± 11.9 mN/mm^2 and 3.33 ± 1.71 nA at 3×10^{-5} M OR-1896 ($n = 7$, each), respectively.

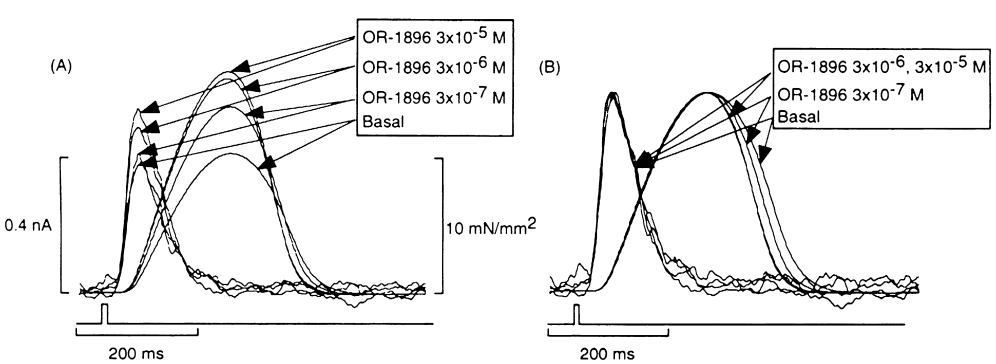

Fig. 3. Concentration-dependent effects of OR-1896 on aequorin light transients (noisy recordings) and isometric contractions in the presence of 3×10^{-7} M (±)-bupranolol in canine right ventricular trabecula electrically driven at 0.5 Hz at 37°C. (A) Superimposed tracings of aequorin light transients (noisy recordings) and isometric contractions recorded during cumulative administration of OR-1896. (B) Amplitudes of peak light transients and isometric contractions were normalized to facilitate the comparison of the time course of both signals induced by OR-1896. Actual values of contractile force and peak light transients: 10.2 mN/mm^2 and 0.44 nA in control; and 17.2 mN/mm^2 and 0.66 nA at 3×10^{-5} M OR-1896. Each tracing represents signal-averaged recordings of 50 successive signals. The line below each tracing represents the recording of stimulus pulse.

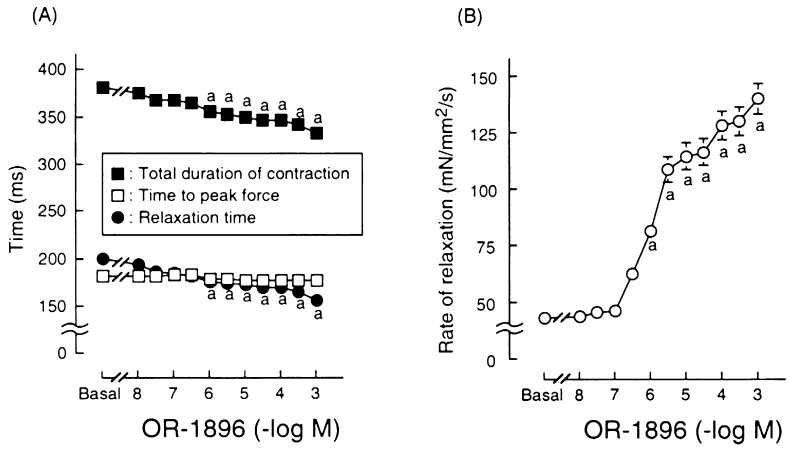

Fig. 5. Time course of contractions induced by OR-1896 in the presence of 3×10^{-7} M (±)-bupranolol in isolated canine right ventricular trabeculae electrically driven at 0.5 Hz at 37°C. (A) Concentration–response curves for time courses of isometric contractions induced by OR-1896 administered in a cumulative manner ($n = 7$). Ordinate: total duration of contraction (closed squares), time to peak force (open squares) and relaxation time (closed circles) expressed as ms; abscissa: the concentration of OR-1896 expressed as $-\log$ M. (B) Influence of OR-1896 on the rate of relaxation of twitch contraction of canine right ventricular trabeculae ($n = 7$). Ordinate: rate of relaxation (open circles) expressed as mN/mm^2/s; abscissa: the concentration of OR-1896 expressed as $-\log$ M. Alphabet a indicate significant difference from the baseline values in respective parameters induced by OR-1896 (a: $P < 0.05$). Symbols with vertical bars represent mean ± S.E.M.

2.4. Statistical analysis

Data are expressed as means ± S.E.M. For analysis of multiple measurements obtained from a single preparation, we used one-way analysis of variance (ANOVA) for repeated measures with Bonferroni's test or Dunnett's test. The mean values between two groups were compared by Student's *t*-test. A P value smaller than 0.05 was considered to indicate statistically significant difference.

3. Results

3.1. Positive inotropic effect of OR-1896 on isolated canine right-ventricular trabeculae loaded with aequorin

OR-1896 had a concentration-dependent positive inotropic effect in association with a small increase in the amplitude of aequorin light transients in the presence of 3×10^{-7} M bupranolol in right-ventricular trabeculae isolated from the dog. Fig. 2 shows actual continuous record-

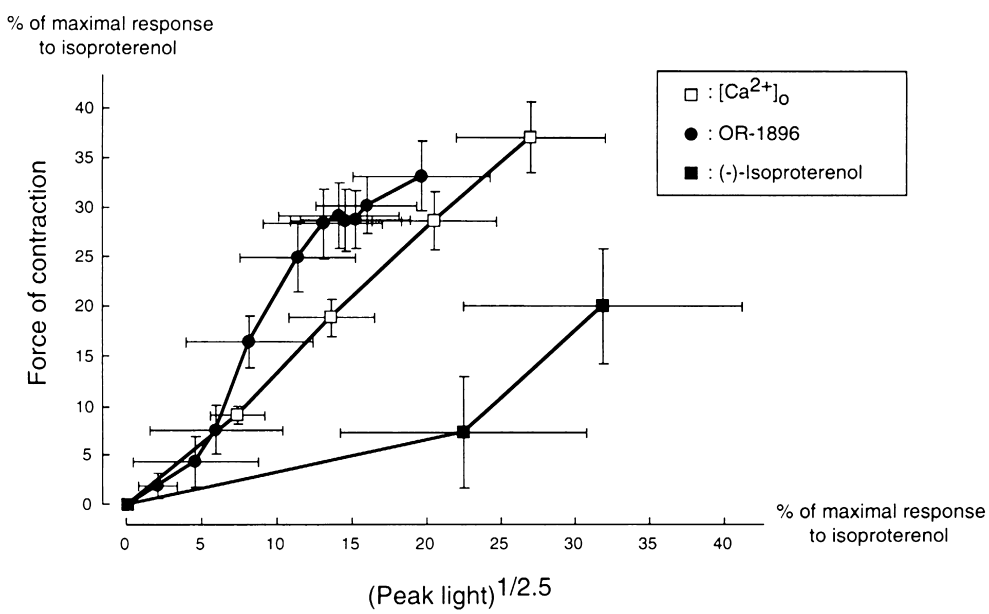

Fig. 6. The relationship between the peak Ca^{2+} transients and force of contraction during application of different inotropic interventions including elevation of $[Ca^{2+}]_o$, OR-1896 and (−)-isoproterenol in isolated canine right ventricular trabeculae. Three interventions were applied in each muscle preparation successively as shown in Fig. 1 ($n = 7$). Symbols with vertical and horizontal bars represent mean ± S.E.M.

ings of the effects of elevation of $[Ca^{2+}]_o$, OR-1896 and isoproterenol in the same dog ventricular trabecular muscle. In continuous recordings, it is evident that the extent of the positive inotropic effect of OR-1896 (Fig. 2B) is small and associated with little change in the amplitude of aequorin light transients compared with those of elevation of $[Ca^{2+}]_o$ (Fig. 2A) and isoproterenol (Fig. 2C). The elevation of $[Ca^{2+}]_o$ and isoproterenol increased definitely both the peaks of aequorin light transients and the peak force of contraction in a concentration-dependent manner. It is visible that isoproterenol, especially at higher concentrations, is more effective than the elevation of $[Ca^{2+}]_o$ to increase the peak Ca^{2+} transients.

Fig. 3A shows superimposed tracings of Ca^{2+} transients and isometric contractions recorded during cumulative administration of OR-1896. The positive inotropic effect of OR-1896 that was exerted in a concentration-dependent manner was associated with small but definite increases in the amplitude of aequorin light transients, and with acceleration of relaxation. Acceleration of relaxation was accompanied by little alteration of time course of Ca^{2+} transients (Fig. 3B).

The concentration–response curve for increases in contractile force and Ca^{2+} transients are summarized in Fig. 4. The concentration–response curves for the positive inotropic effect and the increase in Ca^{2+} transients induced by OR-1896 were biphasic: the positive inotropic effect of OR-1896 reached a plateau level at 10^{-5} M (1st phase); and at 10^{-3} M and higher the concentration–response curve became steeper again (2nd phase). The maximum response of the 1st phase that was achieved at 10^{-5} M was $29.3 \pm 3.41\%$ of the maximal response to isoproterenol, which was associated with an increase in Ca^{2+} transients of $14.0 \pm 3.98\%$ of the maximal response to isoproterenol ($n = 7$, each). The EC_{50} value for the positive inotropic effect of the 1st phase of OR-1896 was 2.53×10^{-7} M, and the value for the increase in the amplitude of Ca^{2+} transients was 2.82×10^{-7} M. The 2nd phase positive inotropic effect of OR-1896 was also associated with an increase in the amplitude of Ca^{2+} transients (Fig. 4).

Alterations of time courses of isometric contractions are shown in Fig. 5A. Relaxation time was significantly abbreviated by OR-1896 at concentrations of 10^{-6} M and higher (Fig. 5A), which was reflected to the decrease in the total duration of contraction induced by OR-1896 during induction of the positive inotropic effect. Abbreviation of relaxation time caused by OR-1896 was accompanied by a concentration-dependent increase in the rate of relaxation induced by OR-1896 at concentrations of 10^{-6} M and higher (Fig. 5B). While it was difficult to calculate the change in the duration of aequorin light transients induced by OR-1896 because of noise associated with the signal, it was essentially unaffected by OR-1896 as shown in a representative preparation in Fig. 3B.

Table 1
The influence of 3×10^{-6} M carbachol on the positive inotropic effect and the increase in aequorin light transients induced by OR-1896 in the presence of 3×10^{-7} M (±)-bupranolol in isolated canine right-ventricular trabeculae

	Force of contraction (% of basal)	Aequorin light transients (% of basal)
Basal	100	100
Carbachol 3×10^{-6} M	97.1 ± 2.75	104.9 ± 4.47
+ OR-1896 10^{-7} M	94.0 ± 2.09	89.3 ± 1.41
10^{-6} M	96.8 ± 2.69	84.1 ± 1.89
10^{-5} M	99.5 ± 2.93	86.7 ± 3.16
10^{-4} M	98.9 ± 2.91	87.7 ± 4.08

Values presented are means ± S.E.M. ($n = 4$, each).

Fig. 6 shows the relationship between the peak Ca^{2+} transients and force of contraction during application of different inotropic interventions including elevation of $[Ca^{2+}]_o$, OR-1896 and (−)-isoproterenol. While the relationship was shifted by (−)-isoproterenol at 10^{-8} and 10^{-7} M to the right, that with OR-1896 was shifted to the left of the relation with elevation of $[Ca^{2+}]_o$. Thus, for a given increase in contractile force, OR-1896 elevated the peak of Ca^{2+} transients to a level that was lower than that induced by $[Ca^{2+}]_o$: while OR-1896 3×10^{-6} M increased the contractile force to a level equivalent to that induced by a $[Ca^{2+}]_o$ of 4.0 mM, the increase in the peak amplitude of Ca^{2+} transients induced by OR-1896 was significantly lower than that induced by elevation of $[Ca^{2+}]_o$ ($P < 0.05$). These observations indicate that OR-1896 may elicit an increase in the myofibrillar sensitivity to Ca^{2+} ions.

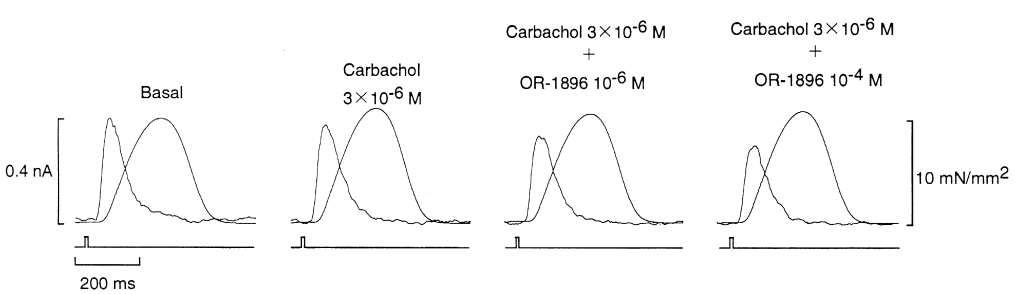

Fig. 7. Influence of 3×10^{-6} M carbachol on the changes in aequorin light transients and isometric contractions induced by OR-1896 in the presence of 3×10^{-7} M (±)-bupranolol in the isolated canine right-ventricular trabecula (0.5 Hz at 37°C). Each tracing represents signal-averaged recordings of 50 successive signals. The basal force of this preparation was 10.3 mN/mm^2 and the baseline peak amplitude of aequorin light transients was 0.41 nA.

Table 2
Effects of OR-1896 on the concentration–response curve for the positive inotropic effect of isoproterenol in isolated trabeculae of the dog

	Force of contraction (mN/nm^2)		EC_{50} values for ISO ($\times 10^{-7}$ M)
	Basal force	Maximal force	
Control	13.5 ± 6.98	44.2 ± 6.62	1.09 ± 0.35
OR-1896 10^{-7} M	5.01 ± 2.86	42.9 ± 8.70	0.87 ± 0.22
Control	8.27 ± 3.04	29.4 ± 4.66	1.03 ± 0.19
OR-1896 10^{-6} M	2.48 ± 0.62	28.7 ± 4.74	0.52 ± 0.07[a]

Values presented are means ± S.E.M. ($n = 4$, each); ISO: isoproterenol.
[a] $P < 0.05$ vs. the respective control values.

3.2. Influence of carbachol on the effect of OR-1896

We examined whether carbachol affects the positive inotropic effect of OR-1896 in dog ventricular trabeculae. The positive inotropic effect and an increase in Ca^{2+} transients induced by OR-1896 up to 10^{-5} M (1st phase) were abolished in the presence of 3×10^{-6} M carbachol (Fig. 7, Table 1): the contractile force was $-3.30 \pm 0.76\%$ and the amplitude of Ca^{2+} transients was $-14.8 \pm 3.51\%$ of the respective values in the presence of carbachol alone ($n = 4$).

It is noteworthy that positive inotropic effect of the 2nd phase induced by OR-1896 was less susceptible to carbachol: OR-1896 at 3×10^{-3} M increased the contractile force to $48.7 \pm 4.83\%$ in the absence and to $30.5 \pm 5.42\%$ of the maximal response to isoproterenol in the presence of 3×10^{-6} M carbachol in a series of aequorin-unloaded preparations ($n = 6$).

3.3. Interaction of OR-1896 with isoproterenol

It was examined whether OR-1896 enhances the positive inotropic effect of isoproterenol. The concentration–response curve for positive inotropic effect of isoproterenol was unaffected by OR-1896 at 10^{-7} M, but at 10^{-6} M it shifted the concentration–response curve for isoproterenol to the left without affecting the maximal response to isoproterenol (Table 2), an indication that OR-1896 may enhance the positive inotropic effect of isoproterenol by a phosphodiesterase III inhibitory action at concentrations of 10^{-6} M and higher in dog ventricular trabeculae.

4. Discussion

4.1. Mechanism of action of OR-1896

While the mechanism of action of levosimendan has been documented quite well (see Section 1), the effects of OR-1896, its active metabolite, have not yet been analyzed up to the current study. We investigated therefore the concentration-dependence of the positive inotropic effect of the compound in canine ventricular trabeculae. The relationship between the amplitude of Ca^{2+} transients and force of contraction after administration of OR-1896 over a wide range of concentrations (the 1st phase) was shifted to the left and upward compared with that produced by isoproterenol or by elevation of $[Ca^{2+}]_o$, an indication that the myofibrillar Ca^{2+} sensitivity may be increased by the compound.

OR-1896 did not impair the relaxation of contraction and actually, it accelerated relaxation, in spite of the shift of $[Ca^{2+}]_i$-force relationship to the left as did levosimendan in canine ventricular myocardium (Sato et al., 1998). Such an acceleration of relaxation induced by OR-1896 could be due to at least two potential subcellular mechanisms. First, the accumulation of cAMP resulting from a weak inhibition of phosphodiesterase III might lead to acceleration of relaxation by facilitation of sarcoplasmic reticulum Ca^{2+} pump induced by phospholamban phosphorylation and phosphorylation of troponin I. In the present study, OR-1896 at a concentrations of 10^{-6} M shifted the concentration–response curve for the positive inotropic effect of isoproterenol to the left and the positive inotropic effect and the increase in aequorin light transients induced by OR-1896 was abolished by carbachol. These results support the view that the cAMP-mediated process may partly contribute to the positive inotropic effect of OR-1896 and that the range of concentration of OR-1896, over which it induces an increase in myofibrillar Ca^{2+} sensitivity and increase of Ca^{2+} transients by phosphodiesterase inhibition, may be overlapping. Second, it has been proposed that the binding of levosimendan to troponin C is dependent on the concentration of Ca^{2+} ions and therefore, at diastolic levels of Ca^{2+} ions there is no sensitization to lead to impairment of diastolic relaxation (Boknik et al., 1997; Edes et al., 1995; Haikala et al., 1995b, 1997; Müller-Beckmann et al., 1988; Raasmaja et al., 1991). Although such effects have been shown biochemically only with levosimendan, it is highly likely that OR-1896 that is structurally closely related to levosimendan may act via similar mechanisms in intact myocardial cells. While the positive inotropic effect of both OR-1896 and levosimendan was abolished by carbachol, a notable difference between the two compounds is that after washout Ca^{2+} sensitizing effect of levosimendan reappeared (Sato et al., 1998) but such a phenomenon may not be observed after washout of OR-1896, which implies that the characteristics of action of both compounds on myofibrillar Ca^{2+} sensitivity may not be the same.

Among newly developed positive inotropic agents that generate cAMP by selective inhibition of phosphodiesterase III, several agents, such as sulmazole, pimobendan, 6-[(4-4′-pyridylamino)phenyl]-4,5-dihydro-3(2*H*)-pyridazinone hydrochloride trihydrate (MCI 154), 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2*H*-1,3,4-thiadiazin-2-one (EMD

53998) and levosimendan, have been shown to have action to increase the myofibrillar sensitivity to Ca^{2+} ions and OR-1896 belongs to this class of agents. Previous studies suggest that carbachol inhibited selectively the positive inotropic effect mediated by generation of cAMP (Endoh, 1999), because it scarcely affected the positive inotropic effect of inotropic interventions that do not involve cAMP, such as α-adrenoceptor agonists, cardiac glycosides, elevation of $[Ca^{2+}]_o$ and dibutyryl cAMP (Endoh, 1979; Endoh and Blinks, 1988; Endoh and Motomura, 1979; Endoh et al., 1982, 1986; Müller-Beckmann et al., 1988). Whereas the increase in myofibrillar Ca^{2+} sensitivity induced by sulmazole and Org 30029 was insensitive to the inhibitory action of carbachol (Kawabata and Endoh, 1993), carbachol abolished both the positive inotropic effect and the increase in the Ca^{2+} transients induced by levosimendan (Sato et al., 1998) and OR-1896 (the 1st phase in the present study) that elicited the shift of the $[Ca^{2+}]_i$-tension relationship to the left, an indication that there may exist Ca^{2+} sensitizing mechanism that is susceptible to carbachol. The observation that the 2nd phase of the positive inotropic effect of OR-1896 is relatively resistant to carbachol indicates the similarity of the compound to the previous agents such as pimobendan, though the 2nd phase may have less clinical relevance from respects of the concentration.

Since it has generally been accepted that cAMP decreases the myofibrillar Ca^{2+} sensitivity in intact cardiac muscle (Endoh and Blinks, 1988; Okazaki et al., 1990), it is difficult to reconcile the observations that Ca^{2+} sensitizing action of OR-1896 and levosimendan was abolished by carbachol with current concept of muscarinic regulation of cAMP-mediated signal transduction. The findings with OR-1896 and levosimendan imply, however, that there exists Ca^{2+} sensitizing mechanism that may require a small extent of cAMP accumulation, which is inhibitable with the muscarinic receptor agonist, though there is no definite evidence at the level of molecular mechanism of such regulation of contractile proteins. Supporting the postulate described above, we recently found that the induction of positive inotropic effect of endothelin-1 that is partly mediated by an increase in Ca^{2+} sensitivity of contractile proteins requires activation of protein kinase A in addition to that of protein kinase C in canine ventricular muscle (Chu and Endoh, 2000).

4.2. Aequorin-loaded multicellular cardiac preparations: advantages and limitations

Simultaneous recordings of aequorin light transients and force of isometric contractions induced by OR-1896 were obtained from intact canine right ventricular muscle. Aequorin has been widely used as an indicator of $[Ca^{2+}]_i$ in a variety of cardiac preparations including single myocytes, isolated papillary muscles, and perfused heart (Endoh, 1995). While aequorin is relatively insensitive to the diastolic level of $[Ca^{2+}]_i$ compared with fluorescent dye, such as indo-1 and fura-2, it is an excellent $[Ca^{2+}]_i$ indicator which is available for analysis of systolic $[Ca^{2+}]_i$ facilitated by cardiotonic agents in intact myocardium (Allen and Kurihara, 1980; Blinks et al., 1982). Namely, since the strength of $[Ca^{2+}]_i$-dependent bioluminescence is altered in proportion to the 2.5th power of $[Ca^{2+}]_i$ changes, sensitivity of aequorin to detect alterations in systolic $[Ca^{2+}]_i$ is extremely high. In addition, the muscle contracts at the length close to Lmax, which is more appropriate for detecting contractility than isolated myocytes that contract at near slack length.

The method of analysis of the $[Ca^{2+}]_i$-force relationship during twitch contraction in cardiac muscle could lead to over or underestimation of alteration of Ca^{2+} sensitivity when the duration of the Ca^{2+} transient is prolonged or abbreviated, because the equilibrium between Ca^{2+} transient and force of contraction is not achieved during twitch contraction (Yue, 1987). Nevertheless, when the time course of Ca^{2+} transients is unchanged or when the duration of both parameters changes in opposite directions, the examination of the relationship does qualitatively reflect well the change in myofibrillar Ca^{2+} sensitivity in intact cardiac muscle (Blinks and Endoh, 1986; Kawabata and Endoh, 1993; Watanabe et al., 1996). Since neither OR-1896 nor $[Ca^{2+}]_o$ had a significant effect on the time course of Ca^{2+} transients, the leftward and upward shift of the relationship induced by OR-1896 might reflect an increase in myofibrillar Ca^{2+} sensitivity caused by the compound as observed with levosimendan in canine ventricular muscle (Sato et al., 1998).

In conclusion, OR-1896 has a positive inotropic effect that may be ascribed to combination of an increase in myofibrillar Ca^{2+} sensitization and a small extent of cAMP accumulation by weak inhibition of phosphodiesterase III, in association with a positive lusitropic effect in the canine ventricular myocardium. As shown in Fig. 5, at high concentrations the compound accelerates relaxation. The Ca^{2+} sensitizing effect of OR-1896 is susceptible to the inhibitory action of the muscarinic receptor agonist carbachol.

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