Decrease in Ca\(^{2+}\)-Sensitizing Effect of UD-CG 212 Cl, a Metabolite of Pimobendan, under Acidotic Condition in Canine Ventricular Myocardium

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ABSTRACT
We studied the influence of acidosis on the positive inotropic effect of UD-CG 212 Cl (4,5-dihydro-6-[2-(4-hydroxyphenyl)-1H-benzimidazole-5-yl]-5-methyl-3(2H)-pyridazinone), an active metabolite of pimobendan, in canine ventricular trabeculae loaded with aequorin. The positive inotropic effect of UD-CG 212 Cl was markedly suppressed under acidic conditions. The maximal contractile response to UD-CG 212 Cl was attained at 10\(^{-8}\) M in the control condition at pH 7.4, but was not achieved even at 10\(^{-4}\) M during acidosis. The maximal inotropic effect of UD-CG 212 Cl was 18\% of the maximal response to isoproterenol (ISO\(_{\text{max}}\)) in association with an increase in Ca\(^{2+}\) transients of 7\% of ISO\(_{\text{max}}\) in the control, while they are 8 and 6\% of ISO\(_{\text{max}}\) under acidosis, respectively. Acidosis abolished the increase in myofilament Ca\(^{2+}\) sensitivity induced by UD-CG 212 Cl, whereas the increase in Ca\(^{2+}\) transients induced by the compound was not affected by acidosis. In conclusion, UD-CG 212 Cl elicited a positive inotropic effect even under acidosis, however, UD-CG 212 Cl was much less effective as a cardiotonic agent under acidosis mainly due to a decrease in the Ca\(^{2+}\)-sensitizing effect under acidotic condition.

Cardiotonic agents are indispensable for improvement of contractile dysfunction in heart failure. Pimobendan (UD-CG 115 BS; 4,5-dihydro-6-[2-(p-methoxyphenyl)-5-benzimidazolyl]-5-methyl-3(2H)-pyridazinone) is a unique cardiotonic agent that has already been launched for treatment of patients with heart failure (Hagemeijer, 1993). It has an inhibitory action on phosphodiesterase III (Scholz and Meyer, 1986) and prolongs the action potential duration (Hörnger et al., 1984). A part of the increase in force of contraction has been shown to be due to the myofilament Ca\(^{2+}\) sensitization (Fujino et al., 1988; Scheld et al., 1989; Böhm et al., 1991). In addition, pimobendan is converted to the active metabolite UD-CG 212 Cl (4,5-dihydro-6-[2-(4-hydroxyphenyl)-1H-benzimidazole-5-yl]-5-methyl-3(2H)-pyridazinone) by hepatic demethylation (Hagemeijer et al., 1989), which may contribute to the favorable hemodynamic effects of the mother compound (Verdonw et al., 1987). UD-CG 212 Cl is 7.7 times more potent than pimobendan as a phosphodiesterase III inhibitor (Böhm et al., 1991). We have recently shown that the positive inotropic effect of UD-CG 212 Cl is partially due to myofilament Ca\(^{2+}\) sensitization in aequorin-loaded canine ventricular myocardium (Takahashi and Endoh, 2001).

Acidosis affects various processes of cardiac E-C coupling (Bountra and Vaughan-Jones, 1989; Orchard and Kentish, 1990). Acidosis decreases myofilament Ca\(^{2+}\) sensitivity (Allen and Orchard, 1983; Orchard and Kentish, 1990; Palmer and Kentish, 1994), which is partly due to a decrease in the affinity of troponin C for Ca\(^{2+}\) (Palmer and Kentish, 1994), and a direct depressant action on the crossbridge cycling (Hulme and Orchard, 1998). In canine ventricular myocardium, acidosis markedly suppressed the positive inotropic effect elicited by an increase in Ca\(^{2+}\) mobilization, whereas the Ca\(^{2+}\) sensitizer Org-30029 reversed effectively the acidosis-induced myofilament Ca\(^{2+}\) desensitization (Watanabe et al., 1996).

The present study was undertaken to examine the influence of acidosis on the positive inotropic effect of UD-CG 212 Cl. For this purpose, we carried out experiments in isolated canine right ventricular trabeculae loaded with aequorin. Our results indicate that the Ca\(^{2+}\)-sensitizing effect of UD-CG 212 Cl is abolished under acidic condition in the canine ventricular myocardium.

Materials and Methods
The study involving treatment of experimental animals conforms to the institutional standards. This study was conducted in

ABBREVIATIONS: [Ca\(^{2+}\)]\(_{i}\), intracellular Ca\(^{2+}\) concentration; ISO\(_{\text{max}}\), maximal response to isoproterenol; [Ca\(^{2+}\)]\(_{\text{ex}}\), extracellular Ca\(^{2+}\) concentration.
according with the Guidance for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. The approval for the animal experiments was obtained from the Committee of Animal Experimentation, Yamagata University School of Medicine, Yamagata, Japan, prior to the experiments and the study was carried out also in accordance with the Declaration of Helsinki.

Preparation of Aequorin-Loaded Canine Right Ventricular Trabeculae. Mongrel dogs of either sex (8–12 kg) were anesthetized by intravenous administration of pentobarbital sodium (30 mg/kg). Hearts were rapidly excised and free-running trabeculae (<1 mm in diameter) were dissected from the free wall of the right ventricle. The muscle preparations had an average dimension of 14.8 ± 0.66 mm (range 13–17 mm) in length and 1.08 ± 0.11 mm² (range 0.85–1.38 mm²) in cross-sectional area (n = 12).

For simultaneous detection of contractile force and intracellular Ca²⁺ transients, the Ca²⁺-sensitive bioluminescent protein aequorin was loaded by the modified macroinjection technique, as described elsewhere in detail (Sawada and Endoh, 1999; Takanashi and Endoh, 2001). The muscle was electrically stimulated by square wave pulses of 5-ms duration at a voltage about 20% above the threshold at 0.5 Hz in modified Krebs-Henseleit solution at 37°C. The composition of the solution was as follows: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 24.9 mM NaHCO₃, 1.2 mM KH₂PO₄, and 11.1 mM glucose (with 0.057 mM ascorbic acid and 0.027 mM EDTA). The solution was bubbled with 95% O₂, 5% CO₂ and maintained at pH 7.4 in the control (5 mM ascorbic acid and 0.027 mM EDTA). The solution was bubbled with 95% O₂, 5% CO₂ and maintained at pH 7.4 in the control (5 mM ascorbic acid and 0.027 mM EDTA).

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 (cut-off frequency of 100 Hz; 9789A; Thorn EMI Electron Tubes, Ruislip, UK) and light signals were smoothed by a low-pass filter (cut-off frequency of 100 Hz; 9789A; Thorn EMI Electron Tubes, Ruislip, UK) and light signals were smoothed by a low-pass filter (cut-off frequency of 100 Hz; 9789A; Thorn EMI Electron Tubes, Ruislip, UK).

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 for repeated measures with Bonferroni’s test. A P value smaller than 0.05 was considered to indicate statistically significant difference.

Results

Influence of Acidosis on Effects of Elevation of [Ca²⁺]o. Figure 1 shows the representative actual tracings (A, pH 7.4; B, pH 6.6) and summarized data (C, pH 7.4; D, pH 6.6) on the influence of acidosis on the increase in aequorin light transients and isometric contractions induced by elevation of [Ca²⁺]o. At 2.5 mM [Ca²⁺]o, acidosis produced a pronounced depression of contractile force (by 64.9 ± 7.50% of the baseline level in the control; P < 0.01) and a significant increase in the amplitude of aequorin light transients (by 9.65 ± 5.11% of the baseline level in the control; P < 0.01) in association with a prolongation of aequorin light transients (Figs. 1, A and B, and 3A). Increase of [Ca²⁺]o increased the force of contraction even under acidosis but to a lesser extent compared with the control at pH 7.4 (Fig. 1, C and D; P < 0.01 versus the increase in contractile force induced by elevation of [Ca²⁺]o at the corresponding concentrations at pH 7.4). For example, at pH 7.4 the increase in contractile force at 4.0 mM [Ca²⁺]o was 26.2 ± 3.32% of ISOmax and it was associated with an increase in Ca²⁺ transients by 28.8 ± 1.98% of ISOmax (n = 5). During acidosis the increase in force at 4.0 mM [Ca²⁺]o was 14.8 ± 3.27% of ISOmax, which was approximately half of the control (P < 0.01) and it was associated with an increase in aequorin light transients by 24.0 ± 6.00% of ISOmax (n = 5), which was not significantly different from the control at pH 7.4 (P > 0.05).

Influence of Acidosis on Effects of UD-CG 212 Cl. Figure 2 shows the representative actual tracings (A, pH 6.6) and summarized data (B, pH 7.4; C, pH 6.6) on the influence of acidosis on the increase in Ca²⁺ transients and isometric contractions induced by UD-CG 212 Cl. Figure 2A shows actual tracings with application of UD-CG 212 Cl, which indicates that the compound is able to induce a positive inotropic effect in association with a moderate increase in aequorin light transients even under acidosis. Figure 2B and C, show the concentration-response curve for increases in Ca²⁺ transients and contractile force induced by UD-CG 212 Cl in the control (Fig. 2B) and acidotic (Fig. 2C) conditions. In the control condition the concentration-response curve for UD-CG 212 Cl was bell-shaped: the maximal response to UD-CG 212 Cl was achieved at 10⁻⁵ M, amounted to 17.6 ± 2.43% of ISOmax, and was associated with an increase in Ca²⁺ transients by 6.85 ± 1.68% of ISOmax (n = 7 each). In the control condition at pH 7.4 the EC50 value for the positive inotropic
effect of UD-CG 212 Cl was $3.38 \times 10^{-7}$ M and the $EC_{50}$ for the increase in amplitude of $Ca^{2+}$ transients was $2.35 \times 10^{-6}$ M. Under acidosis the maximal response to UD-CG 212 Cl was not achieved even at $10^{-4}$ M; the positive inotropic effect at $10^{-4}$ M was $7.53 \pm 0.90\%$ of $ISO_{max}$ ($P < 0.01$ versus the increase induced by UD-CG 212 Cl at $10^{-4}$ M at pH 7.4) and was associated with an increase in $Ca^{2+}$ transients by $6.06 \pm 2.84\%$ of $ISO_{max}$ that was not significantly different from the increase at pH 7.4 ($n = 5; \ P > 0.05$ versus the control). The $EC_{50}$ value for the positive inotropic effect of UD-CG 212 Cl was supposed to be higher than $3.18 \times 10^{-6}$ M, and the value for $Ca^{2+}$ transients was $>2.79 \times 10^{-6}$ M. Overall, the extent of increments in $Ca^{2+}$ transients induced by UD-CG 212 Cl under acidosis was essentially similar to that at pH 7.4 ($P > 0.05$), whereas the positive inotropic effect of UD-CG 212 Cl was much smaller under acidic condition ($P < 0.01$).

**Influence of Acidosis on Duration of Aequorin Light Transients and Contraction.** Figure 3 shows alterations of the amplitude and time course of aequorin light transients and isometric contractions induced by acidosis at $2.5$ mM $[Ca^{2+}]_o$ (Fig. 3A) and by UD-CG 212 Cl at $10^{-5}$ M under acidosis (Fig. 3B). Acidosis produced a pronounced depression of contractile force with a small abbreviation of contraction (Fig. 3A, top), while $Ca^{2+}$ transients were markedly prolonged by acidosis (Fig. 3A, bottom). In summarized data the total duration of aequorin light transients was $184.5 \pm 5.67$ ms significantly to $230.1 \pm 5.77$ ms by $24.8 \pm 3.13\%$ ($n = 5; \ P < 0.01$) with a significant prolongation of decline time from $133.1 \pm 8.01$ to $172.1 \pm 8.97$ ms ($n = 5; \ P < 0.01$). The time to peak light was not significantly altered by acidosis: $51.5 \pm 3.05$ ms in the control and $47.8 \pm 3.71$ ms in acidosis ($n = 5; \ P > 0.05$). In contrast, the duration of isometric contractions was rather shortened, although the difference was statistically not significant: the total duration of contraction was $370.6 \pm 31.1$ ms in the control and $322.4 \pm 24.0$ ms in acidosis; the time to peak force was $157.5 \pm 7.33$ ms in the control and $141.6 \pm 8.81$ ms in acidosis; and the relaxation time was $213.1 \pm 24.2$ ms in the control and $180.8 \pm 15.3$ ms in acidosis ($n = 5$ each).

During acidosis UD-CG 212 Cl at $10^{-8}$ M induced a moderate positive inotropic effect in association with a small but definite increase in the amplitude of $Ca^{2+}$ transients (Fig. 3B, top). UD-CG 212 Cl induced little alteration of the time course of $Ca^{2+}$ transients and isometric contractions during acidosis (Fig. 3B, bottom).
Influence of Acidosis on Myofilament Ca²⁺ Sensitivity during Application of Different Inotropic Interventions. Figure 4 shows the relationship between the peak Ca²⁺ transients and contractile force during the elevation of [Ca²⁺]₀ and administration of UD-CG 212 Cl in the control and acidic conditions. The relationship for elevation of [Ca²⁺]₀ under acidosis was shifted to the right and downward compared with the control. In the control condition UD-CG 212 Cl shifted markedly the relationship of the amplitude of Ca²⁺ transients and force to the left and upward, an indication that the compound elicits an increase in myofilament Ca²⁺ sensitivity (Takahashi and Endoh, 2001). In contrast, under acidosis the relationship during administration of UD-CG 212 Cl was superim-
Influence of Acidosis on Myofilament Ca\(^{2+}\) Sensitivity. Acidosis decreases the Ca\(^{2+}\) sensitivity at the process of Ca\(^{2+}\) binding to troponin C (Orchard and Kentish, 1990; Palmer and Kentish, 1994) and decreases also directly the crossbridge cycling (Hulme and Orchard, 1998). Acidosis decreases the Ca\(^{2+}\) binding to troponin C through modulation of C-terminal domain of troponin I (Westfall et al., 1997, 2000). Since EMD 57033 binds the C-terminus of troponin C in a region of interaction with troponin I (Li et al., 2000), the acidosis-induced decrease in Ca\(^{2+}\) sensitivity and the rever-
sal induced by Ca\(^{2+}\) sensitizers may occur through integrated mechanisms of thin filament regulation that ultimately lead to the structural alterations of troponin C to decrease or reverse the Ca\(^{2+}\).

It has been controversial whether UD-CG 212 Cl increases Ca\(^{2+}\) sensitivity in cardiac muscle. In skinned cardiac cells, UD-CG 212 Cl increased Ca\(^{2+}\) sensitivity under the condition where inorganic phosphate level was elevated (Westfall et al., 1993; Fraker et al., 1997) but not under normal condition (Böhme et al., 1991; Komukai and Kurihara, 1996). These findings suggest that the effect of the compound may be preferentially exerted under pathophysiological conditions such as ischemia-reperfusion where acidosis plays an important pathological role (Vanheel et al., 1989). The present observation, however, does not support such a beneficial effect of UD-CG 212 Cl and indicates that the expression of Ca\(^{2+}\) sensitization induced by UD-CG 212 Cl may be extremely sensitive to the experimental conditions. Since the regulation of Ca\(^{2+}\) sensitivity in intact cells is different from that in skinned cardiac fibers (Gao et al., 1994; Hulme and Orchard, 1998; Komukai et al., 1998), further study is necessary to identify the site of Ca\(^{2+}\)-sensitizing action of the compound in relation to acidosis- and/or inorganic phosphate-induced Ca\(^{2+}\) desensitization mechanisms.

Myofilament Ca\(^{2+}\) Sensitization and Cyclic AMP. It is noteworthy that the Ca\(^{2+}\)-sensitizing action of UD-CG 212 Cl is inhibitable with the muscarinic receptor agonist carbachol (Takahashi and Endoh, 2001). Carbachol has been used as a pharmacological tool to differentiate the cyclic AMP-independent from cyclic AMP-mediated process, in which the former has been defined as Ca\(^{2+}\) sensitizers (Endoh, 1987, 1999), since it has been established that cyclic AMP decreases Ca\(^{2+}\) sensitivity due to phosphorylation of phospholamban and troponin I. More recently, however, we found that the Ca\(^{2+}\)-sensitizing effect of certain agents is susceptible to carbachol. Carbachol abolished the Ca\(^{2+}\)-sensitizing effect of levosimendan (Sato et al., 1998), OR-1896, the active metabolite of levosimendan (Takahashi et al., 2000a,b) and UD-CG 212 Cl (Takahashi and Endoh, 2001) in dog and rabbit ventricular myocardium. These findings together indicate that the subcellular mechanism for the above-mentioned agents may involve cyclic AMP for the expression of the Ca\(^{2+}\)-sensitizing effect. While the role of cyclic AMP in Ca\(^{2+}\) sensitization has been unknown, myosin binding protein C that is phosphorylated by protein kinase A to lead to an activation of actomyosin ATPase activity (Winograd, 1999) may be a potential candidate. In a clinical setting it is postulated that these agents are free from a risk of serious adverse effect on diastolic function because an acceleration of relaxation induced by moderate accumulation of cyclic AMP resulting from phosphodiesterase III inhibition may counteract the Ca\(^{2+}\) sensitization at diastole (Sugawara and Endoh, 1999).

As a potential mechanism for alteration of cyclic AMP-mediated regulation under acidosis, it is noteworthy that Mundina-Weilenmann et al. (1996) have shown that the phosphorylation of phospholamban and troponin I is facilitated under acidosis probably due to acidosis-induced inhibition of type 1 phosphatase in rat cardiac muscle. The Ca\(^{2+}\)-sensitizing effect of levosimendan that is also sensitive to carbachol (Sato et al., 1998) has been shown to be attenuated by pretreatment with isoproterenol (Haikala et al., 1997). This finding indicates that a strong activation of cyclic AMP-
mediated signaling process is able to suppress the cyclic AMP-related Ca\(^{2+}\) sensitization. Such a mechanism could contribute also to the acidosis-induced attenuation of the UD-CG 212 Cl-induced Ca\(^{2+}\) sensitization. Actually, accumulation of cyclic AMP induced by UD-CG 212 Cl at pH 7.4 reached first a significant level of approximately 30% of the baseline level at the highest concentration of 3 × 10\(^{-4}\) M, which was much less than the accumulation induced by isoproterenol (>100% at 10\(^{-5}\) M) in canine ventricular muscle (Endoh et al., 1991).

General Considerations. The relationship of Ca\(^{2+}\) and force was examined by the use of plotting the relation of developed force and peak Ca\(^{2+}\) transients (Blinks, 1993). Analysis and explanation of the graph such as that in Fig. 4, however, require great care when there are changes in the time course of Ca\(^{2+}\) transients because the alteration of equilibration kinetics between Ca\(^{2+}\) and the myofilaments in intact cardiac cells can elicit an apparent shift of the relation without changing Ca\(^{2+}\) sensitivity (Yue, 1987). Namely, \(\beta\)-adrenergic stimulation produces apparent shift of the relation to the direction of Ca\(^{2+}\) desensitization due to the abbreviation of duration of Ca\(^{2+}\) transients (Endoh and Blinks, 1988). In the present study acidosis prolonged Ca\(^{2+}\) transients that could cause an apparent increase in Ca\(^{2+}\) sensitivity. Nonetheless acidosis shifted the relationship to the direction of Ca\(^{2+}\) desensitization (Fig. 4), an indication that the acidosis-induced Ca\(^{2+}\) desensitization may have overcome the apparent shift due to equilibration kinetics in the present study. UD-CG 212 Cl did not affect the time course of Ca\(^{2+}\) transients, which may have no influence on the equilibration kinetics of Ca\(^{2+}\)-troponin C binding during twitch contraction in intact cardiac cells.

The present study was carried out in canine ventricular trabeculae. Given the size of the muscles used, the temperature, and the stimulation rate, it is not completely excluded that the core of the muscle preparation could possibly be hypoxic. Thus, the core of the muscle would have a raised inorganic phosphate, which has been shown to alter the response to UD-CG 212 Cl (Westfall et al., 1993; van Meel et al., 1995; Fraker et al., 1997). Therefore, it is likely that the cells in the muscle core that contribute to the force response are responding in a manner different from the surface cells, from which aequorin signals were recorded. To get insight into this important issue we compared the effects of levosimendan in indo-1-loaded rabbit ventricular myocytes and aequorin-loaded papillary muscles (Sato et al., 1998). Levosimendan elicited an identical Ca\(^{2+}\)-sensitizing effect on both preparations. Furthermore, the response of aequorin-loaded rabbit and dog ventricular trabeculae to the Ca\(^{2+}\) sensitizer OR-1896 was very similar (Takahashi et al., 2000a,b). These observations altogether imply that hypoxia in cells in the core of the muscle preparation used may not have crucial influence on the findings in the current study.

In this study the acidic solution contained a different [Cl\(^-\)] than the control solution. Given that Cl\(^-\) is involved in pH regulation, this could complicate the interpretation of the data. Although this is an important issue, the present study could not determine the role of [Cl\(^-\)] in this respect. Nevertheless, it was revealed that 1) the results obtained in the current study are qualitatively very similar to those reported previously in the acidosis induced by alteration of CO\(_2\) levels (Allen and Orchard, 1983; Orchard and Kentish, 1990; Komukai et al., 1998); and 2) acidosis elicited a differential effect on the action of Org-30029 (Watanabe et al., 1996) and UD-CG 212 Cl under the same experimental condition.

In conclusion, the increase in myofilament Ca\(^{2+}\) sensitivity induced by UD-CG 212 Cl, the active metabolite of pimobendan, was abolished under acidic condition in canine ventricular myocardium. Such a modification of the positive inotropic effect of cardiotonic agents that act through an increase in myofilament Ca\(^{2+}\) sensitivity have to be taken into consideration when these agents are applied under pathophysiological condition.

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References


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