Effects of the Positive Inotropic Agent Org 30029 on Developed Force and Aequorin Light Transients in Intact Canine Ventricular Myocardium

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The action of a novel cardiotonic agent N-hydroxy-5,6-dimethoxy-benzo[b]thiophene-2-carboximide hydrochloride (Org 30029) on intracellular aequorin light transients and isometric contractions was investigated in isolated canine ventricular trabeculae. The positive inotropic effect of Org 30029 (30 μM–3 mM) was consistently associated with prolongation of the duration of contraction and an increase in the amplitude of the intracellular Ca\(^{2+}\) transients. The maximum inotropic response to Org 30029 was approximately 150% of the maximum response to isoproterenol, whereas the maximum increase in the amplitude of Ca\(^{2+}\) transients produced by Org 30029 was only 20% of the isoproterenol-induced maximum. The duration of isometric contractions was prolonged by Org 30029, with no change in the duration of the light transients. The concentration–response curve for the positive inotropic effect of Org 30029 was shifted by carbamylecholine chloride (carbachol, 3 μM) to the right and downward, but the maximum response to Org 30029 was greater than that to isoproterenol even in the presence of carbachol. Carbachol abolished the increase in light transients and cAMP accumulation induced by Org 30029 (1 mM), whereas it only partially attenuated the positive inotropic effect of Org 30029. In the presence of carbachol (3 μM), Org 30029 increased the force of contraction in a concentration-dependent manner without augmenting the aequorin light transients. These results are compatible with the hypothesis that Org 30029 increases cardiac contractility by increasing myofilament Ca\(^{2+}\) responsiveness. (Circulation Research 1993;72:597–606)

KEY WORDS • Org 30029 • isoproterenol • Ca\(^{2+}\) transients • aequorin • positive inotropic effect • ventricular myocardium • dogs

The positive inotropic effects of cardiotonic agents are achieved by increases in intracellular Ca\(^{2+}\) mobilization, by alteration of responsiveness of myofilaments to Ca\(^{2+}\), or by a combination of the two mechanisms.1 The major classes of inotropic agents that are currently used for the treatment of heart failure act through the first mechanism. The cardiac glycosides increase [Ca\(^{2+}\)]\(_{i}\) through the intracellular Na\(^{+}\) accumulation produced by inhibition of Na\(^{+}\), K\(^{+}\)-ATPase and the Na\(^{+}\)/Ca\(^{2+}\) exchange system.2 Catecholamines (through β-adrenoceptor stimulation) and cAMP phosphodiesterase (PDE) inhibitors cause an accumulation of cAMP, which produces phosphorylation of functional proteins such as L-type Ca\(^{2+}\) channel proteins, phospholamban, and troponin I via activation of protein kinase A.3 The characteristics of the cAMP-mediated positive inotropic effect, associated with increases in [Ca\(^{2+}\)]\(_{i}\), and marked acceleration of relaxation, have been well documented.4–7 Thus, all of these inotropic agents, including the cardiac glycosides, catecholamines, and PDE inhibitors, induce a marked increase in [Ca\(^{2+}\)]\(_{i}\] during twitch contraction. At the same time, all of these compounds can readily cause dangerous arrhythmias that may be causally related to an excessive elevation of [Ca\(^{2+}\)]\(_{i}\], and this limits their clinical application.

Therefore, an intensive effort has been devoted to developing new cardiotonic agents that operate via different mechanisms or that can be used in conjunction with cardiac glycosides.7,8 Compounds that produce an increase in myofilament Ca\(^{2+}\) responsiveness have attracted much interest, since they are less likely to produce arrhythmias. Several agents, including methylxanthines and some newly developed PDE inhibitors, such as sulmazole, pimobendan, and MCI 154, have been shown to possess an additional action on myofilament Ca\(^{2+}\) responsiveness in both skinned and intact cardiac muscle.9–17

The present study was undertaken to analyze the mechanism of inotropic action of N-hydroxy-5,6-dimethoxy-benzo[b]thiophene-2-carboximide hydrochloride (Org 30029) (Figure 1) on intact canine ventricular muscle. This compound, recently developed as a cardiotonic agent,18–21 has been shown to possess an action on Ca\(^{2+}\) responsiveness in skinned cardiac muscle and to inhibit selectively PDE isoenzymes in vitro.
it was confirmed that the developed force before starting microinjection (6.98±0.83 mN/mm²) was not significantly different from that after completion of the microinjection (6.73±0.89 mN/mm², n=5 each). After microinjection, we took maximal care to keep the injected cells quiescent; therefore, the muscle preparations were electrically stimulated only for a short duration before transfer to the experimental organ bath.

Aequorin was dissolved at a concentration of approximately 2 mg/ml in a solution containing 150 mM KCl and 5 mM HEPES buffer, pH 7.5. The solution was loaded into fine-tipped (35–50 MΩ in 150 mM KCl) micropipettes through which membrane potential was monitored to determine when cells had been penetrated. Aequorin was injected by the application of gas pressure.24–26 It was estimated that the microinjection in a volume of 1–2 nl aequorin solution into a single skeletal muscle cell would provide a sufficient aequorin light signal in association with contractile activity.27 Since myocardial cells are much smaller, it is difficult to predict the exact volume of aequorin solution that should be microinjected into individual cells. In frog atrial cells, it was estimated that the amount microinjected into each cell should be approximately 5 pL; this amount should be similar for canine ventricular cells.

When a satisfactory light signal was obtained on stimulation (typically after injection into 80–100 cells), the muscle was quickly transferred to an apparatus designed to record light signals with high efficiency and to minimize motion artifacts in the aequorin signals.25 The muscle was stimulated with pulses of 5-msec duration and approximately 20% above threshold intensity at a frequency of 0.33 Hz through the punctate cathode. The top of the muscle was connected with 9–0 Tevdek thread to the arm of a servo-operated electromagnetic muscle lever operated in the isometric mode. During a 30-minute equilibration period after the transfer, the muscle length was adjusted to that at which contractile force was greatest. All experiments were carried out at 37.5°C. The light emitted by the injected aequorin was detected by a photomultiplier (EMI 9635A). Signal averaging was carried out to obtain a satisfactory signal-to-noise ratio in the aequorin signals. In the recordings shown in the present study, 100 or 200 signals were averaged. During the equilibration period, the amplitude of aequorin light transients declined to reach a low steady level, implying that some of the injected cells may have not recovered. Changes in diastolic light emission during the decline of the peak light transients were undetectable. Only trabecular preparations in which the light transients remained stable for many hours were accepted for study. The percentage of successful experiments included in the study was 60% (nine of 15 preparations). In successful preparations, the aequorin signals did not show any sign of Ca²⁺ overload even when the highest concentration of cardiotonic drugs was added, indicating that hypoxia of the aequorin-injected cells may be minimal. We occasionally observed the spontaneous activities and arrhythmias after the administration of isoproterenol, which may be largely ascribed to the presence of Purkinje fibers in the preparation. By carefully trimming the surface of muscle preparation, the occurrence of the arrhythmic activity was markedly reduced.

### Determination of Aequorin Light Signals

The Ca²⁺-sensitive bioluminescent protein aequorin (prepared for injection according to Blinks et al.21 in 1978) was microinjected into the cells of the muscle mounted horizontally in an organ bath constructed for aequorin injection. Bicarbonate-buffered Krebs-Henseleit solution freshly oxygenated with 95% O₂–5% CO₂ was circulated rapidly past the surface of the muscle at 32°C (the composition of the solution was the same as described above). The muscle was stimulated at a frequency of 0.33 Hz with threshold pulses delivered through punctate electrodes and was stretched to a length at which the contractile force was nearly maximum. After an equilibration period of approximately 30 minutes, electrical stimulation was discontinued, and aequorin microinjection was started. Although it took several hours to complete the microinjection of aequorin, the injection procedure only slightly affected the basal developed force. In the preliminary experiments,
Drugs were administered into a 50-ml organ bath in a cumulative manner in volumes of 0.1 or 0.2 ml. All preparations were exposed to bupranolol (10⁻⁶ M) for more than 20 minutes before administration of Org 30029 to exclude the effects of norepinephrine released by test compounds.

In each preparation, isoproterenol was titrated to establish the maximum response. After application of isoproterenol at the highest concentration, its effect lasted for more than 60 minutes during washout.³⁰

The possibility that Org 30029 elicits a direct action on aequorin bioluminescence was examined by use of an in vitro detection apparatus.²⁴ A small volume of aequorin solution was rapidly injected into a solution with 150 mM KCl and 1 mM CaCl₂, pH 7. The amplitude and time course of aequorin light emission were not influenced by the injection of 1 mM Org 30029 under these experimental conditions nor was the light emission affected by 1 mM Org 30029 in a solution in which [Ca²⁺] was buffered to 0.4 μM with EDTA.

**Determination of cAMP**

The cAMP content of the canine right ventricular trabecula was determined as described previously.³¹,³² Briefly, for the assay of cAMP, muscles were mounted in 20-ml organ baths, stimulated, and stretched in the same experimental conditions as those in the aequorin study. All experiments with Org 30029 were carried out in the presence of 10⁻⁵ M bupranolol. Muscles were removed from the bath 10 minutes after the administration of 10⁻³ M Org 30029 (or 5 minutes after 10⁻⁶ M isoproterenol) when the inotropic response reached a steady level and frozen immediately in liquid nitrogen. Frozen muscles were weighed immediately and stored overnight at -30°C. Before the muscles were homogenized, ice-cold 5% trichloroacetic acid (0.5 ml) was poured on each sample in a Teflon capsule precooled in liquid nitrogen. The frozen muscle sample was then mechanically homogenized in a Mikro-Dismembrator (B. Braun, Melsungen, FRG) in which muscle was shaken for 30 seconds with a 10-g gold ball that had been precooled in liquid nitrogen. The homogenate was thawed and centrifuged at room temperature. After the addition of 10 μl of 1N HCl, aliquots of 100 μl supernatant were extracted five times with 1 ml water-saturated ether, heated at 80°C for 3 minutes to evaporate the residual ether, lyophilized overnight, and resuspended in 100 μl distilled water. The cAMP content was determined by the sensitive radioimmunoassay method (cAMP kit, Yamasa Shoyu Co., Choshi, Japan).

Experimental values were presented as mean±SEM. Significant differences between mean values were estimated by Student’s t test. A value of p<0.05 was considered to indicate a significant difference.

The drugs used in these experiments were as follows: Org 30029 (Organon Laboratories Ltd., Lanarkshire, Scotland), (-)isoproterenol hydrochloride and carbacholchlorine chloride (carbachol) (Sigma Chemical Co., St. Louis, Mo.), (+)-bupranolol hydrochloride (Kaken Kagaku, Tokyo), and sodium pentobarbital (Tokyo Kasei). Aequorin was obtained from Friday Harbor Photoproteins, Friday Harbor, Wash.

**Results**

**Positive Inotropic Effect of Org 30029**

Figure 2A shows the positive inotropic effects produced by the cumulative administration of Org 30029 to canine ventricular muscle in the presence of 10⁻⁶ M bupranolol. Org 30029 elicited a concentration-dependent positive inotropic effect. The E₉⁰ value of Org 30029 was (3.76±0.40)×10⁻⁶ M. In each preparation, the maximum response to isoproterenol was determined after washout of bupranolol for more than 30 minutes, and the inotropic effect of Org 30029 was expressed as a percentage of the maximum response to isoproterenol. Individual variations in the inotropic effects of isoproterenol were small. The maximum positive inotropic effect achieved by Org 30029 at 3×10⁻³ M amounted to 153.9±12.7% of that of isoproterenol (n=8). The total duration of contraction (Figure 2B) was prolonged in a concentration-dependent manner to 320.0±11.2 msec from the control value of 217.7±5.0 msec. Relaxation time was also prolonged (to 191.8±11.5 msec from 109.1±2.5 msec), whereas the time to peak force was prolonged to a lesser extent (from 108.6±5.2 to 128.2±3.3 msec at a concentration of 3×10⁻³ M).
Influence of Org 30029 on the Relation Between Ca^{2+} Transients and Contractile Force

Figure 3 shows the effects of increasing concentrations of Org 30029 on aequorin light signals and isometric contractions in the presence of bupranolol (10^{-6} M) in an isolated canine ventricular trabecula. Org 30029 increased both the amplitude of light transients and developed force in a concentration-dependent manner (Figure 3, right panel). The changes in the time course of contraction were characteristic of β-adrenoceptor stimulation; i.e., they were associated with abbreviation of contraction and acceleration of relaxation. The increase in the amplitude of the light transients induced by isoproterenol was much greater than that caused by Org 30029, whereas the maximum positive inotropic effect of isoproterenol was less than that of Org 30029. In Figure 4, summarized data on the effect of Org 30029 (left panel) and isoproterenol (right panel) on peak force and light are shown. The ordinate has been expressed in terms of the 2.5th root of peak light intensity because, over a large

**Figure 3.** Effects of the cumulative administration of Org 30029 (left panel) and (-)-isoproterenol (right panel) on aequorin signals and isometric contractions in the isolated canine ventricular trabecula. Signal-averaged recordings of 100 successive contractions with aequorin signals (noisy recordings) and isometric contraction recordings at various concentrations of respective agents were superimposed. Numbers adjacent to the curves indicate the molar concentration. The response to Org 30029 was determined in the presence of 10^{-6} M bupranolol. After an extensive washout of the drugs used, the response to isoproterenol was determined. The concentration–response curve of isoproterenol in this series was slightly shifted to the right even after an extensive washout of bupranolol compared with the curve determined without bupranolol.

**Figure 4.** Graphs showing the effects of Org 30029 (left panel) and (-)-isoproterenol (right panel) on the amplitude of aequorin light signals and isometric contractions of isolated canine ventricular trabeculae. The response to Org 30029 was determined in the presence of 10^{-6} M bupranolol. Data were calculated by use of signal-averaged recordings from 100 or 200 successive contractions with aequorin signals and isometric contractions at various concentrations of respective agents. The basal force of contraction was 5.43±0.70 mN/mm^2; the maximum inotropic response to isoproterenol (the change in force in response to isoproterenol) was 13.66±0.71 mN/mm^2 (n=5). The control amplitude of aequorin light signals was 1.84±0.71 nA; the maximum response to isoproterenol (the change in light in response to isoproterenol) was 10.08±3.79 nA (n=5).
Figure 5 shows the relation between the amplitude of Ca\(^{2+}\) transients and developed force after the administration of Org 30029 and isoproterenol. For a given increase in the developed force, Org 30029 increased the amplitude of Ca\(^{2+}\) transients much less than isoproterenol. This suggests that Org 30029 may affect the process subsequent to the increase in intracellular Ca\(^{2+}\) mobilization.

It has been demonstrated that isoproterenol decreases the myofilament Ca\(^{2+}\) responsiveness through accumulation of cAMP in relation to the resultant phosphorylation of troponin I and C protein and that the extent of this regulation varies widely with species.\(^4\)-\(^7\) Therefore, the effect of isoproterenol on the relation between the peak Ca\(^{2+}\) transient and the developed force was compared with that observed after alteration of [Ca\(^{2+}\)]\(_i\). When [Ca\(^{2+}\)]\(_i\) was increased, the relation between the Ca\(^{2+}\) transient and developed force was slightly shifted to the left compared with the relation observed during the administration of isoproterenol (Figure 5).

Figure 6 shows a comparison of the changes in the time courses of the isometric contractions and light transients induced by Org 30029 (left panel) and isoproterenol (right panel). The amplitude of control signals and those recorded in the presence of the drugs was adjusted and superimposed to facilitate the comparison. Org 30029 prolonged the contraction with little change in the time course of the light transient, whereas isoproterenol abbreviated both the light transient and the isometric contraction.

Influence of Carbachol on the Effects of Org 30029

It has been shown in mammalian ventricular myocardium that the muscarinic receptor activation selectively inhibits the positive inotropic effects associated with accumulation of cAMP metabolism, whereas it scarcely affects the cAMP-independent inotropic such as basal force of contraction and the effects of elevation of [Ca\(^{2+}\)]\(_i\), cardiac glycosides, and myocardial \(\alpha\)-adrenoceptor stimulation.\(^31\)-\(^34\) The muscarinic inhibition induced by carbachol has therefore been proposed to be used as a pharmacological tool to differentiate the involvement of cAMP in the inotropic action of newly developed cardioactive agents.\(^34\),\(^35\) In the canine ventricular myocardium, the increases in developed force, aequorin light

Figure 5. Graph showing the relation between the amplitude of aequorin light signal and that of the isometric contraction as contractility was varied by Org 30029 and by isoproterenol in the same canine ventricular trabecula. The 2.5th root of the amplitude of the aequorin signals has been used as an indicator of the amplitude of the Ca\(^{2+}\) transient. (For discussion see text.) Data presented in Figure 4 are plotted (n=5 for each symbol). Open squares with dotted line represent the relation during alteration of [Ca\(^{2+}\)]\(_i\) (3.75, 5.0, 6.25, 7.5, 8.75, 10.0, 11.25, and 12.5 mM). The curve of [Ca\(^{2+}\)]\(_i\) was obtained before that of isoproterenol.

Figure 6. Comparison of the changes in time course of aequorin signals and isometric contractions induced by Org 30029 (left panel) and (-)-isoproterenol (right panel). To facilitate the comparison, signals in the absence and presence of respective agents (concentrations are presented in the figure) were normalized. The response to Org 30029 was determined in the presence of 10\(^{-6}\) M bupranolol. Actual values of force developed were 6.3, 16.0, and 23.1 mN/mm\(^2\) (Org 30029) and 6.3 and 18.7 mN/mm\(^2\) (isoproterenol); peak aequorin light values were 2.40, 4.45, and 4.62 nA (Org 30029) and 2.52 and 13.80 nA (isoproterenol). Signal-averaged recordings of 100 successive contractions with aequorin signals (noisy recordings) and isometric contraction recordings were superimposed.

range of [Ca\(^{2+}\)] in vitro, the light emission from aequorin varies approximately in proportion to the 2.5th power of [Ca\(^{2+}\)].\(^24\)-\(^27\) The maximum inotropic effect of Org 30029 was 140.4±14.6% of that of isoproterenol, and the maximum increase in the amplitude of the Ca\(^{2+}\) transients was 15.5±2.0% (n=5). The force increased further when 10\(^{-3}\) M Org 30029 was applied, and the amplitude of Ca\(^{2+}\) transients reached a maximum at 3x10\(^{-4}\) M. The concentration–response curve for the isoproterenol-induced inotropic response in this series of experiments was slightly shifted to the right compared with the curve determined in the absence of bupranolol,\(^30\) since it is difficult to completely wash out a potent \(\beta\)-adrenoceptor blocking agent such as bupranolol.
transients, and cAMP levels induced by forskolin, β-adrenoceptor agonists, and PDE inhibitors have been shown to be markedly attenuated by carbachol.34 In the presence of carbachol, the concentration–response curve for the positive inotropic effect of Org 30029 was shifted to the right, and the maximum response was depressed (Figure 7). The EC50 of Org 30029 was increased slightly but significantly by carbachol, from the control value of (3.76±0.40)×10−4 M to (5.79±0.45)×10−4 M (n=8). However, the maximum inotropic effect of Org 30029 was 120% of that induced by isoproterenol in the presence of carbachol.

Figure 8 illustrates the effect of carbachol (3×10−6 M) on the aequorin signals and isometric contractions recorded in the presence of 10−3 M Org 30029. The increase in light transients induced by Org 30029 was abolished by carbachol, whereas the positive inotropic effect was attenuated but not eliminated. The summarized data on the influence of carbachol on the Org 30029–induced effects are presented in Figure 9. The developed force in the presence of Org 30029 was reduced by carbachol from 412±46% to 298±38% of the basal force of contraction. The increase in the Ca2+ transients induced by Org 30029 was abolished by carbachol, and the amplitude of the Ca2+ transients in the presence of Org 30029 and carbachol was not significantly different from the control amplitude.

In another series of experiments, the full concentration–response relation for Org 30029 was determined in

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**Figure 7.** Graph showing the influence of 3×10−6 M carbachol on the positive inotropic effect of Org 30029 in isolated canine ventricular trabeculae. The response to Org 30029 was determined in the presence of 10−6 M bupranolol. Experiments were carried out in different muscle groups in the absence or presence of carbachol. In the carbachol group, the basal force of contraction was 3.49±0.80 mN/mm2; the maximum inotropic response to isoproterenol (the change in force in response to isoproterenol) was 15.62±1.32 mN/mm2 (n=8). *p<0.05 vs. the corresponding control responses (n=8).

**Figure 8.** Influence of carbachol on the changes in aequorin signals (noisy recordings) and isometric contractions induced by 10−3 M Org 30029 in an isolated canine ventricular trabecula. The response to Org 30029 was determined in the presence of 10−6 M bupranolol. Signal-averaged recordings of 100 successive contractions with aequorin signals (noisy recordings) and isometric contraction recordings were superimposed.

**Figure 9.** Bar graph showing the influence of carbachol (3×10−6 M) on the positive inotropic effect and the increase in the amplitude of Ca2+ transients induced by 10−3 M Org 30029 in isolated canine ventricular trabeculae (0.33 Hz, 37.5°C). Experiments were performed in the presence of 10−6 M bupranolol. The basal force of contraction was 6.38±0.42 mN/mm2; the control aequorin signal was 2.43±0.68 nA (n=5). *p<0.05 vs. control values; †p<0.05 vs. the respective values in the presence of Org 30029 alone.
the presence of 3×10⁻⁶ M carbachol. In the presence of carbachol, Org 30029 increased the force of contraction in a concentration-dependent manner, whereas the amplitude of the light transients was decreased by Org 30029 up to 10⁻⁶ M (Figure 10). The duration of light transients was increased after application of 10⁻³ M Org 30029. The amplitude of the light transient was increased slightly at 3×10⁻³ M Org 30029 but was still lower than the control level. In the same preparation, increases in the developed force in response to 10⁻⁸ M isoproterenol or to an elevation of [Ca²⁺], (15 mM) were associated with pronounced increases in the amplitude of the light transients (Figure 10, bottom panel). Similar results were obtained in three other preparations. Org 30029 up to a concentration of 10⁻³ M induced a positive inotropic effect in association with a decrease in the amplitude of the light transients.

The positive inotropic effect of Org 30029 (10⁻³ M) was associated with a significant increase in cAMP level (Figure 11). Carbachol (3×10⁻⁶ M) abolished the Org 30029–induced accumulation of cAMP, whereas the positive inotropic effect of Org 30029 was not affected significantly by carbachol (Figure 11). The absence of an inhibitory action of carbachol on the positive inotropic effect of Org 30029 in this experimental protocol may be due to individual variations of the Org 30029–induced inotropic response (the responses to Org 30029 in the presence and absence of carbachol were determined in different muscle preparations).

**Discussion**

**Interpretation of Aequorin Light Transients**

Simultaneous recordings of the effects of Org 30029 on aequorin light transients and developed force were obtained from the aequorin-injected canine intact right ventricular muscle. Aequorin has been widely used as an indicator of [Ca²⁺], in a variety of cardiac preparations including single myocytes, isolated papillary muscles, and perfused heart. The limitations of aequorin as a Ca²⁺ indicator and the problems involved in the interpretation of aequorin light signals have been discussed extensively elsewhere. Inotropic interventions including elevation of [Ca²⁺], changes in frequency of stimulation, and cardiotoxic drugs such as cardiac glycosides produce approximately parallel transients. Effects of Org 30029 on aequorin signals and isometric contractions of an isolated canine ventricular trabecula (0.33 Hz, 37.5°C) in the presence of 3×10⁻⁶ M carbachol. The response to Org 30029 was determined in the presence of 10⁻⁶ M bupranolol. Signal-averaged recordings of 100 successive contractions with aequorin signals (noisy recordings) and isometric contraction recordings were superimposed.

**Figure 10.** Effects of Org 30029 on aequorin signals and isometric contractions of an isolated canine ventricular trabecula (0.33 Hz, 37.5°C) in the presence of 3×10⁻⁶ M carbachol. The response to Org 30029 was determined in the presence of 10⁻⁶ M bupranolol. Signal-averaged recordings of 100 successive contractions with aequorin signals (noisy recordings) and isometric contraction recordings were superimposed.

**Figure 11.** Bar graph showing the influence of carbachol (3×10⁻⁶ M) on the Org 30029–induced positive inotropic effect and cAMP accumulation in isolated canine ventricular trabeculae (0.33 Hz, 37.5°C). The cAMP level was determined 10 minutes after the administration of 10⁻³ M Org 30029. Carbachol (3×10⁻⁶ M) was allowed to act for 3 minutes in the presence of Org 30029. The response to Org 30029 was determined in the presence of 10⁻⁶ M bupranolol. The basal force of contraction was 6.65±1.25 mN/mm² (n=10). Isoproterenol (10⁻⁶ M) increased the force of contraction by 334±79.2% (by 21.67±3.61 mN/mm²) and the cAMP content to 2.21±0.07 pmol/mg wet wt (n=3 for each) in experiments carried out in parallel. Numbers in parentheses indicate the number of preparations. *p<0.05 vs. the respective control values; †p<0.05 vs. the cAMP content after application of Org 30029 alone.
changes in the amplitude and time course of the aequorin light transients and isometric contractions. The inotropic effect of these interventions has been interpreted to be ascribed essentially to the change in the intracellular Ca\(^{2+}\) transient. In contrast, there are the inotropic interventions such as catecholamines, \(^5\) methyloxanthines, \(^1\) newly developed cardiotonic agents (e.g., sulmazoline \(^1\) and EMD 53998 \(^40\), \(^41\)), endothelin, \(^42\) changes in muscle length, \(^43\), \(^44\) and hypoxia and acidosis \(^37\), \(^45\) that lead to changes in the amplitude and time course of isometric contractions that are not accompanied by proportional changes in the light transient. Thus, in addition to any changes in the Ca\(^{2+}\) transient that may occur, these interventions have been suggested to lead to changes in the myofilament Ca\(^{2+}\) responsiveness. In interpreting the effects of Org 30029, we have used the term “myofilament Ca\(^{2+}\) responsiveness” according to these previous studies. It is suggested that the modulation of two processes of cardiac excitation–contraction coupling may contribute to the change in Ca\(^{2+}\) responsiveness, i.e., (1) alteration of the affinity of troponin C for Ca\(^{2+}\) or 2) alteration of the response of the myofilaments to a given level of occupancy of the Ca\(^{2+}\)-binding sites on troponin C (“downstream” mechanisms). \(^1\) However, it is difficult to differentiate these mechanisms even by use of computer modeling. \(^41\)

**Effects of Org 30029**

Org 30029 apparently increases the responsiveness of the myocardial contractile apparatus to Ca\(^{2+}\). It is tempting to speculate that this mechanism is involved in the ability of Org 30029 to produce greater inotropy than that produced by \(\beta\)-adrenoceptor agonists in intact canine ventricular myocardium. This cannot be the only mechanism involved in the positive inotropic action of Org 30029, because this compound also produces a modest increase in the amplitude of the intracellular Ca\(^{2+}\) transient. Nevertheless, the increase in the amplitude of the Ca\(^{2+}\) transient is much smaller than that associated with comparably inotropic concentrations of isoproterenol or elevation of [Ca\(^{2+}\)]. In contrast to isoproterenol, Org 30029 does not produce changes in the time course of the aequorin light transient that could account for this dissociation.

Since an increase in the affinity of troponin C for Ca\(^{2+}\) alone would be expected to reduce the amplitude and duration of the light transient, \(^42\) the fact that Org 30029 increases the amplitude of the light transient suggests that this drug increases the amount of Ca\(^{2+}\) participating in excitation–contraction coupling. This effect of Org 30029 on intracellular Ca\(^{2+}\) levels could be due to its PDE inhibitory effect. \(^19\), \(^21\) The observations that the increase in the amplitude of light transients and cAMP accumulation induced by Org 30029 was abolished by the muscarinic agonist carbachol and that, under the influence of carbachol, Org 30029 produced a positive inotropic effect without increasing the light transients and the cAMP content support the view described above.

The following findings are compatible with the hypothesis that Org 30029 increases cardiac contractility by increasing myofilament Ca\(^{2+}\) responsiveness: 1) The relation between the amplitude of Ca\(^{2+}\) transients and developed force after administration of Org 30029 over a wide range of concentrations is shifted to the left and upward compared with that produced by isoproterenol or by elevation of [Ca\(^{2+}\)]. Whereas \(\beta\)-adrenoceptor stimulation has been suggested to decrease the myofilament Ca\(^{2+}\) responsiveness in other species, \(^4\), \(^7\) isoproterenol did not produce a prominent shift of the relation in the canine ventricular myocardium (Figure 5). 2) Org 30029 prolonged the duration of isometric contractions without changing the duration of light transients. A simple explanation of this effect could be that Org 30029 decreased sarcoplasmic or sarcolemmal Ca\(^{2+}\) transport, in which case it could have nothing to do with changes in myofilament Ca\(^{2+}\) responsiveness. However, since the modulation of Ca\(^{2+}\) transport by inotropic interventions generally produces a parallel change in force and light transients as discussed above, the dissociation of changes in time course of force and light transients may be ascribed to the change in the excitation–contraction coupling process consequent to mobilization of intracellular Ca\(^{2+}\): the increase in Ca\(^{2+}\) binding affinity for troponin may prolong the duration of contraction but abbreviate the light transients. \(^8\), \(^16\), \(^28\), \(^42\) Because the on-rate value for the Ca\(^{2+}\) binding to troponin is diffusion limited, \(^46\) a decrease in the off-rate constant may be likely to be responsible for the effect of Org 30029. 3) In the presence of carbachol, the positive inotropic effect of Org 30029 was associated with a decrease in the amplitude of the light transients. These results in intact ventricular myocardium are consistent with the previous findings in skinned ventricular fibers showing that Org 30029 produces an increase in myofilament Ca\(^{2+}\) responsiveness. \(^18\)

Among newly developed positive inotropic agents that generate cAMP by selective inhibition of PDE-III, several agents such as sulmazoline, \(^11\), \(^12\) pimobendan, \(^13\)–\(^15\) MCI 154, \(^36\), \(^17\) and EMD 53998 \(^40\), \(^41\) have been found to affect myocardial contractility by the mechanism subsequent to an increase in Ca\(^{2+}\) mobilization. Org 30029 belongs to this class of agents. These drugs have potential importance in the treatment of heart failure from several points of view. They may have less arrhythmogenic action than drugs that act primarily through an increase in intracellular Ca\(^{2+}\) mobilization. There is also the possibility that the energy required for the intracellular mobilization of a larger amount of Ca\(^{2+}\) would not be needed. This is borne out by the finding that pimobendan, which also increases myofilament Ca\(^{2+}\) responsiveness, \(^13\)–\(^15\) results in a smaller increase in heat production for a given increase in developed force than does the \(\beta\)-adrenoceptor agonist isoproterenol. \(^46\) Furthermore, moderate reduction of the afterload to the heart produced by vasodilation mediated through cAMP accumulation in vascular smooth muscle may be beneficial for heart failure patients.

The extent of the contribution of the Ca\(^{2+}\) responsiveness and PDE inhibitory action appears to vary markedly among these different agents. The former mechanism is more pronounced in EMD 53998 than in pimobendan. \(^81\) The observation that the positive chronotropic effect of Org 30029 in rabbit and guinea pig right atria is much less pronounced than that of milrinone implies that the contribution of the PDE inhibitory action to the effect of Org 30029 may be relatively less. It has been shown that Org 30029 elicits a negative chronotropic effect in guinea pig right atria, \(^20\) in contrast to milrinone.
The molecular mechanisms through which these compounds augment myocardial contractility are not yet understood. MCI 154 has been shown to increase the maximum Ca\(^{2+}\)-induced tension in skinned cardiac fibers,\(^{16}\) whereas EMD 53998 appears to increase myofilament Ca\(^{2+}\) responsiveness mainly by increasing the affinity of Ca\(^{2+}\) for troponin C.\(^{40,41}\) The action of Org 30029 to produce a greater maximum response than isoproterenol in intact ventricular muscle may be related to the ability of the compound to increase the maximum tension in skinned cardiac fibers as much as 20–50%.\(^{18}\)

In conclusion, Org 30029 is a unique cardioactive agent that may produce a positive inotropic action mainly by increasing the Ca\(^{2+}\) responsiveness of the contractile proteins in intact myocardial cells. Novel cardioactive agents belonging to this class may be worthy of evaluation for their effectiveness as potential therapeutic drugs for the treatment of congestive heart failure.

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