The 'calcium sensitising' effects of ORG30029 in saponin- or Triton-skinned rat cardiac muscle

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1 The effects of a range of concentrations of ORG30029 (1 μ M to 1 mM) were investigated on fully (Triton-treated) or selectively (saponin-treated) 'skinned' ventricle trabeculae from rat. The Ca-sensitivity was increased by 100 μ M and amounted to a mean reduction in the Ca^{2+} necessary for half-maximal activation ($1/K_{\text{app}}$) of 0.174 ± 0.053 (mean $\pm$ s.e.mean) pCa units. ORG30029 (50 μ M) gave a smaller mean shift of 0.05 ± 0.016 pCa units. A slight shallowing of the relationship between $-\log[\text{Ca}^{2+}]$ (pCa) and steady-state tension was also generally found (mean Hill exponent reduction 0.37 ± 0.28 at 100 μ M).

2 The Ca-sensitizing action altered in a dose-dependent fashion. Judged by the ability to enhance force from an initial level of activation of 10–20% of maximum Ca-activated force (C_{max}), the first significant effects occurred near 10 μ M and continued to increase at 1 mM.

3 A small ($3.7 \pm 1.1\%$) but consistent increase in C_{max} was produced by ORG30029 at 100 μ M. Much larger increases were produced by the drug at higher concentrations (up to 20–50% at 1 mM).

4 The consequence of these changes is that at levels of activation likely to occur in the heart, the absolute increase in force by 100 μ M ORG30029 was considerable: it amounted to a 150–200% increase at a $[\text{Ca}^{2+}]$ producing 20% at C_{max} . At this drug concentration virtually all the effect was due to Ca-sensitizing rather than increasing C_{max} .

5 In the saponin-treated preparations, at concentrations up to 1 mM, ORG30029 did not release Ca from the sarcoplasmic reticulum, as indicated by the failure to evoke contracture under conditions where caffeine induced large responses.

6 At 100 μ M, ORG30029 potentiated the transient, caffeine-induced contracture (saponin-treated preparation) to an extent consistent with the Ca-sensitizing effect seen under conditions of steady-state tension development.

Introduction

The treatment of congestive heart failure still relies on the use of cardiac glycosides, diuretics and vasodilators. Their narrow therapeutic window and tendency to arrhythmogenicity have prompted the search for other inotropic agents. One apparently desirable pharmacological strategy involves increasing the Ca-sensitivity of the contractile proteins. This effect would circumvent the undesirable side-effects associated with drug actions that promote contractility by raising $[\text{Ca}^{2+}]$ inside the cardiac muscle cell (Wetzel & Haeufel, 1988). 'Ca-sensitizing' drugs include compounds such as caffeine and other methyl-xanthines (Wendt & Stephenson, 1983), the more potent benzimidazoles such as sulmazole and pimobendan (van Meel, 1987) and MCI-154 (Kitada *et al.*, 1987). Most of these are also potent phosphodiesterase (PDE) inhibitors (Wetzel & Haeufel, 1988), including MCI 154 (Sellin *et al.*, 1988) which was initially thought to be a 'pure' Ca-sensitizer. Evidence remains equivocal about the relative importance of these two modes of action in their overall inotropic effect.

This study was undertaken to test the effectiveness of ORG30029, a new cardiotonic agent (Nicholson *et al.*, 1989a,b; Shahid *et al.*, 1989a,b), on the contractile behaviour of two types of preparation of cardiac muscle. The first is muscle where all the cellular membranes have been functionally and structurally disrupted by the non-ionic detergent Triton X-100. The second is muscle treated with the cholesterol-precipitating agent saponin. This selectively 'skins' the cells leaving the sarcolemma perforated with holes large enough to permit the free passage of small ions and molecules (such as ATP, EGTA etc), but small enough to retain the diffusible sarcoplasmic enzymes: the mitochondrial and sarcoplasmic reticular (SR) membranes remain functional. This permits the study of the Ca-sensitizing drugs under condition of dynamic tension development, since the caffeine-induced

tension transient can be comparable in time course and amplitude with the physiological 'twitch'. This preparation has the advantage that many of the complicating factors acting in the intact cell have been removed. This is essential to clarify the properties of ORG30029 distinct from those of PDE inhibition (Nicholson *et al.*, 1989b; Shahid *et al.*, 1989b).

Experiments were thus able to test the extent of the increase in Ca-sensitivity of the contractile proteins under both static and transient states of activation and to test for possible effects on the SR in the selectively-skinned preparations.

Methods

A number of features in our experimental regime, such as the measurement of pH, are significantly different from those of other laboratories in the field. Most details of the methods have been published (Miller *et al.*, 1982; 1985; Miller & Smith, 1985; Harrison *et al.*, 1988).

In summary, experiments were carried out on small, free-running trabeculae (2–3 mm long and 802–120 μ m in diameter) isolated from the right ventricle of the rat and mounted for isometric force measurement. The animals were killed by a blow to the head, the heart was rapidly excised and flushed with a saline solution (Solution 'F', Table 1) at about 25°C.

Precise knowledge of sarcomere length (s.l.) is critical in these experiments for the following reasons: (i) the well-established effect of reduced Ca-sensitivity of the contractile proteins at shorter s.l. (e.g. Hibbert & Jewell, 1982; Harrison *et al.*, 1988), (ii) the efficacy of Ca-sensitising by caffeine (de Beer *et al.*, 1988) and sulmazole (D.J. Miller & D.S. Steele, unpublished observations) may be affected by sarcomere length, (iii) the effect of s.l. on the extent of Ca-uptake and/or release by the SR (C. Lamont and D.J. Miller, unpublished observations). Sarcomere length was measured by differential interference contrast light microscopy (see Harrison *et al.*, 1988 for details) and set to 2.1–2.2 μ m for the present study.

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Solutions

The rationale for the solution composition, the method of calculating free ion levels and ionic strength, the choice of ion binding constants for the various ligands, and the precautions for EGTA purity, Ca-contamination determination and the measurement of pH are described in detail elsewhere (Miller & Smith 1984; 1985; Smith & Miller, 1985; Harrison *et al.*, 1988).

Chemical skinning procedure

Once the muscle had been mounted, it was initially exposed to a 'relaxing' solution (B, Table 1) including either the cholesterol-precipitating agent saponin ($50 \mu\text{g ml}^{-1}$) or Triton X-100 (1% v/v) for 20–30 min. The chemical skinning agent was then removed by washing in fresh solution B. Ca-sensitivity was unaltered by the latter treatment but Ca-activated contractures developed with a half rise-time about 1.5 times faster (see Miller & Smith, 1985 for details). The caffeine-induced contracture was completely abolished by Triton, since Triton destroys the integrity of the SR and mitochondrial membranes.

Tension responses are shown calibrated in either absolute force or relative force, or both. For relative force, tension was normalised to maximum Ca-activated force (C_{max}) which was determined at frequent intervals throughout the experiment (see Harrison *et al.*, 1988).

Drugs and solvents

ORG30029 (N-hydroxy-5,6-dimethoxy-benzo[b] thiophene-2-carboximidamide HCl, MW 289, Organon Labs) was dissolved (in dimethylsulphoxide, DMSO) at 100 mM to give a final concentration of DMSO not exceeding 1% (v/v at 1 mM drug) in experimental media. The solubility limit for ORG30029 was close to 1 mM in the present media. Correction for the small acidification by ORG30029 became necessary above 0.2 mM. In several experiments, Milrinone and/or 1-methyl 3-isobutylxanthine (IBMX) were tested for comparison with ORG30029. These compounds were also dissolved in DMSO.

Results

Effects of ORG30029 on Ca-sensitivity of the contractile proteins

The relationship between pCa and tension for chemically skinned rat and human heart has been thoroughly characterized in this laboratory. The data from all otherwise satisfactory experiments are well fitted by the Hill equation (Equation 1). This allows a convenient and objective description of the effects of ORG30029 and other substances in terms of the alteration required to the two variables (K_{app} and 'h' in

Equation 1), appropriate to each preparation, which define the position and steepness of the curve relating pCa to tension (see Harrison *et al.*, 1988 for details).

$$\frac{C}{C_{\text{max}}} = \frac{K_{\text{app}}[\text{Ca}^{2+}]^h}{1 + K_{\text{app}}[\text{Ca}^{2+}]^h} \quad (\text{Equation 1})$$

K_{app} has the units M^{-h} . $K_{1/2}$, the reciprocal of the $[\text{Ca}^{2+}]$ required for half-maximum activation, is defined as the h th root of K_{app} . The curves were fitted by a non-linear least squares fitting procedure (see Brown & Dennis, 1972 and Brown, 1972).

The fact that we can be confident about the use of the Hill equation in these circumstances permits curve fitting even with relatively few $[\text{Ca}^{2+}]$ s. This is often desirable since more repeat runs of a smaller set of solutions are then possible. Figure 1 shows examples of pCa-tension relationships for the rat preparation, together with effect of $100 \mu\text{M}$ ORG30029. Independent checks with a range of $[\text{Ca}^{2+}]$ s between pCa 4.5

Figure 1 This figure shows two examples (different preparations) of pCa tension relationships with (●) and without (■) ORG30029 ($100 \mu\text{M}$) obtained in Triton-treated rat ventricle trabeculae. (a) Log $K_{1/2}$ 5.40, Hill coeff. 3.76 (no drug); 5.46 and 3.03 (plus drug), respectively. (b) Log $K_{1/2}$ 5.34, Hill coeff. 3.63 (no drug); 5.49 and 3.63 (plus drug), respectively. Steady-state tension achieved at each pCa was plotted, together with that achieved when the drug was added: the protocol employed corresponds to the first part of that illustrated in Figure 2. Tension was normalised to C_{max} , achieved either with or without the drug; this was determined several times during the experiments.

Table 1 Composition of solutions (in mM except where stated; pH = 7.00)

Solution	K^{+}	Mg^{2+}	Ca_{total}	(pCa)	ATP	Creatine PO_4	Na^{+}	EGTA	HDTA	HEPES
A	140	7	10.0	4.25†	5	10	40	10	—	25.0
B	140	7	0.02	9.03	5	15	40	10	—	25.0
C	140	7	0.2‡	5.12	5	15	40	0.2	9.8	25.0
D	140	7	0.02	7.29	5	15	40	0.2	9.8	25.0
E	140	7	10.1	3.99	5	15	40	10	—	25.0
F(Tyrode)	5.4	1	2	2.7	—	—	140	—	—	5

All solutions (except F) included 25 iu creatine kinase (Sigma). * Potassium ions added as KCl and KOH. § Magnesium added as 1 M MgCl_2 ; free Mg^{2+} = 2.13–2.5 mM in all solutions including mixtures of A and B or C and D. ¶ Sodium ions from the salts Na_2ATP and Na_2CrP . † An additional 0.1 mM $CaCl_2$ was added to samples of this solution to yield 'full activating' solution (pCa 3.98). ‡ Contamination levels in the final experimental solution (see Miller & Smith, 1984). Total chloride concentration varied from about 110–120 mM. HDTA = 1,6 diaminoheptane N,N,N',N'-tetraacetic acid.

Figure 2 Tension records obtained at a low level of activation ($<10\%$ maximum Ca-activated force ($C_{\max}$)). The effect of adding and subsequently removing ORG30029 at two concentrations is shown.

and 3.5 confirm that maximum activation is consistently achieved at pCa 4 in these preparations.

Comments on the protocol for activation Curves of this type can be constructed in at least three ways. A 'cumulative' response can be produced by sequentially stepping the $[Ca^{2+}]$ upward over the appropriate range. This approach is widely used in pharmacological studies but has a number of drawbacks. While it is less evident why this approach should be unsatisfactory for Ca^{2+} , where 'desensitization' is not a problem, we have occasionally found appreciably different curves with this approach than those derived from making a series of single activations at a test pCa. It seems most likely to be because the 'cumulative' method requires that the muscle maintain substantial forces for prolonged periods with consequent increase in PO_4 , local reduction in pH etc. $[Ca^{2+}]$ must be raised and not lowered since these preparations can display a marked hysteresis, being less sensitive to $[Ca^{2+}]$ as it rises than as it falls (Harrison *et al.*, 1988). Overall, the cumulative protocol often adversely affects the ability of the preparation to produce force. To make the activation as rapid and uniform as possible throughout the cross-section of the muscle the 'Ca-jump' method is used, whereby Ca-buffer capacity is raised (see Table 1) at the same time as free $[Ca^{2+}]$ itself (Miller, 1975; Moisescu, 1976). Therefore, in the present study, we have used single steps to the test pCa (made by mixing solutions A and B, Table 1) from solution D (Table 1) then switching to a solution with the test drug at the test pCa (see Figures 2, 4 and text later). The reverse step, out of ORG30029, is not of immediate value; again the phenomenon of hysteresis potentially confuses responses which involve a fall in the degree of activation. Examples of the type of sequence used to obtain Figure 1 are illustrated in Figure 2; further details are given in the legend. Figure 2 also serves to illustrate that as little as $10\mu M$ ORG30029 produces a significant Ca-sensitizing effect: at this low level of activation ($\sim 5\%$ $C_{\max}$) force is almost doubled. ORG30029 $1\mu M$ had no significant effect (not illustrated).

Collected data for ORG30029 are presented in Table 2. In 6 experiments (at $100\mu M$) the shift produced by ORG30029 was to the left (lower $[Ca^{2+}]$) in each case. The mean shift in $\log K_{1/2}$ was 0.174 ± 0.053 pCa units. The slope of the relationship, characterized by the Hill exponent 'h' in Equation 1, fell appreciably in most experiments (Table 2). The variation of absolute calcium sensitivity between preparations generally makes an accumulation of all data to a single plot for quantitative purposes invalid. However, the collected data for absolute Ca-sensitivity in the presence and absence of the drug showed almost exactly the same shift of the mean $\log K_{app}$. This probably reflects the fact that the present series of experiments was carried out on preparations of very similar dimensions and closely controlled s.l.

Table 2 Ca sensitizing effect of ORG30029

	$\log K_{1/2}$	Hill coeff.	No. of preps.
Standard	5.401 ± 0.026	3.61 ± 0.1	
ORG30029 $100\mu M$	5.574 ± 0.051	3.24 ± 0.36	
Mean shift	0.174 ± 0.053		6
Standard	5.407 ± 0.011	4.32	
ORG30029 $50\mu M$	5.457 ± 0.015	4.11	
Mean shift	0.05 ± 0.016		3
Mean increase in $C_{\max}$		($100\mu M$) $3.69 \pm 1.12\%$	6
		(1 mM) $20.6 \pm 4.6\%$	7

Data shown are means $\pm$ s.e.mean.

Effects on peak force

The results in the above section were expressed as % of $C_{\max}$ in each case. This obscured the effect that ORG30029 has upon $C_{\max}$ itself. As we noted for sulmazole and pimobendan (Harrison *et al.*, 1986), ORG30029 increased peak force in skinned fibres (see Table 2); an example of the effect of 1 mM ORG30029 is illustrated in Figure 3, where $C_{\max}$ increased by about 30%. (Checks were made at slightly higher $[Ca^{2+}]$ (pCa 3.7) to ensure that force was maximal in these cases). This result is similar to that obtained with sulmazole at the same dose (data not shown). The potential to increase peak force in

Figure 3 Effect of ORG30029 (1 mM) on maximum Ca-activated force ($C_{\max}$) (evoked at pCa 4); it produced a 31% increase in $C_{\max}$.

Figure 4 Direct demonstration of Ca-sensitizing by ORG30029; 1 mM of each compound was applied ($\blacktriangle$) and removed ($\blacktriangledown$) at pCa 5.63 where the muscle was producing about 10% maximum Ca-activated force (C_{max}). The relatively small effect of isobutylmethylxanthine (IBMX) at this concentration provides an index of the efficacy of ORG30029 in promoting Ca-sensitizing. Above the top trace, two index lines indicate the levels of C_{max} (at pCa 4.0) with and without ORG30029. Milrinone 1 mM produced a small reduction of force at pCa 5.63 and also C_{max} (not shown). The lowest trace shows the effects of 200 μ M milrinone and 200 μ M IBMX.

combination with an increased Ca-sensitivity is a unique feature of the action of this type of drug. Higher concentrations gave even greater increases in peak force (60% or more with 10 mM sulmazole). This action, as well as that of increasing Ca-sensitivity, mimics that of certain natural imidazoles such as homocarnosine and carnosine (Harrison *et al.*, 1986 and unpublished data). The results with high concentrations of ORG30029 and sulmazole revealed that there is considerable scope for increasing force by this route (see Discussion). The substantial peak force effect was additional to the Ca-sensitizing effect. Figure 4 shows the overall effect of ORG30029 at submaximally activating levels of $[Ca^{2+}]$, together with that of milrinone and IBMX. Thus the potent PDE inhibitors failed to influence force significantly, as expected in the Triton-treated state (Wendt & Stephenson, 1984, and unpublished observations). The small potentiating effect of IBMX is in line with that expected (Chapman & Miller, 1974; Miller & Thieleczek, 1977) for other methylxanthines such as caffeine and theophylline (Wendt & Stephenson, 1983) which act as Ca-sensitisers under these circumstances.

Effects of ORG30029 upon Ca-release and during Ca-release by the sarcoplasmic reticulum

At 1 mM, ORG30029 had no detectable ability to provoke Ca-release from the SR. This was estimated by direct application of the drug in conditions where caffeine (0.5–10 mM) can produce large contractures. The protocol for these experiments is described in the legend of Figure 5. An initial exposure to a supra-threshold $[Ca^{2+}]$ served to load the SR (not illustrated). A series of caffeine contractures could then be evoked which fell steadily in amplitude unless 'reloading' of the SR took place.

An alternative protocol (see Steele *et al.*, 1990) permitted assessment of the effect of ORG30029 on the caffeine-evoked contracture. A short loading period (typically 1 min) at a sub-threshold $[Ca^{2+}]$ (weakly Ca-buffered with 0.2 mM EGTA_{total})

Figure 5 The lack of response of a saponin-treated preparation to ORG30029 (1 mM) in contrast to that produced by caffeine (10 mM). The sarcoplasmic reticulum (SR) was first loaded with Ca by exposing the preparation to a suprathreshold pCa (10 mM EGTA_{total}) for 2 min. Then the Ca-buffer capacity was reduced to 0.2 mM (two washes). The solution change artefacts indicate when ORG30029 was applied and removed. Under these conditions, the ability of the SR to release significant quantities of Ca^{2+} was exemplified by the response to the subsequent challenge with caffeine, which reached nearly 50% of C_{max} . (In other experiments, we have shown that as little as 0.5 mM caffeine will produce significant contractures under these conditions.)

was immediately followed by a challenge with caffeine. This evoked a brief contracture which typically peaked within 200 ms (Figure 6a and b). The loading-release sequence could be repeated indefinitely and produced caffeine-contractures which were very consistent in amplitude. Figure 6 shows an example of a sequence of this kind. When ORG30029 was included (in both 'loading' and 'release' solutions) the contractures were immediately potentiated (Figure 6a and b). The extent of the potentiation (from 33% to 49% of maximum Ca-activated force in this case) was entirely consistent with the degree of Ca-sensitizing produced by the drug (see e.g. Figure 1: this degree of potentiation would require an increase in log $K_{1/2}$ of only 0.08). When the contractures were normalised (Figure 6) it became clear that the speed of tension develop-

Figure 6 Effect of ORG30029 on a train of regular caffeine-induced contractures. The lowest panel shows the responses of a saponin-treated preparation to a sequence of challenges with 10 mM caffeine (for 15 s, ▲). Between the caffeine contractures, the sarcoplasmic reticulum (SR) was able to 'reload' with Ca^{2+} as the trabecula was exposed to a subthreshold $[\text{Ca}^{2+}]$, weakly Ca-buffered, for 1 min (0.2 mM EGTA_{total} throughout). In this example, once a consistent train of responses was established, 100 μM ORG30029 was applied to both loading and caffeine solutions. The two contractures indicated (●, on the lowest trace) superimposed and on a faster time-base. Tension developed as soon as the preparation was immersed in the caffeine solution (indicated by the end of the solution change artefact): the contractures peaked within about 150 ms and relaxed fully within 2 s. The delayed fall of the ORG30029-potentiated response was revealed in the middle trace where the two responses have been normalised.

ment was little affected, but relaxation was delayed considerably, by ORG30029.

Discussion

The main features of the action of ORG30029 observed in this study reveal that the drug has pronounced effects on muscle Ca-sensitivity by acting directly on the contractile proteins. Up to 1 mM these compounds have no significant ability to release Ca from the SR. These basic properties can thus be considered in conjunction with those described previously (Shahid *et al.*, 1989a,b; Nicholson *et al.*, 1989a,b) which relate to the type III and IV PDE inhibition. The results of the present study show that no intermediation of the effects of ORG30029 by adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent processes need be invoked to account for the Ca-sensitizing action. This follows from the relatively insignificant Ca-sensitizing effect of known PDE inhibitors (e.g. Figure 4) together with the effectiveness of ORG30029 in the fully disrupted state (Triton extracted). However, this conclusion does not preclude interactions involving both types of process in the intact cell.

The ability to facilitate the transient caffeine contracture (Figure 6) can be directly compared with that obtained with another new Ca-sensitizer, EMD 53998, in the intact cell (Allen & Lee, 1989). The time course of the 'twitch-like' con-

tracture is comparable to that of action potential triggered contraction in the intact cell at this temperature. The result serves to demonstrate that the Ca-sensitizing effect occurs during the dynamic, twitch-like caffeine contractures as well as during steady-state force development where a suprathreshold $[\text{Ca}^{2+}]$ is applied under strongly Ca-buffered conditions. The time-course changes seen are consistent with an increase in the affinity of Ca^{2+} -binding by the regulatory proteins (see below).

Complete or partial chemical skinning

The effects described above are obtained whether the muscles are selectively skinned (with saponin) or where membrane disruption is complete (with Triton X-100). In the former state, diffusible enzymes and the intracellular organelles remain present and active (e.g. Endo & Kitazawa, 1978; Fry & Miller, 1985), whereas in the latter they do not. Thus, we can conclude that the actions of ORG30029 in enhancing peak force, increasing Ca-sensitivity and altering the steepness of the pCa-tension relationship do not require the intermediation of any cytoplasmic processes, nor are they substantially modified by those that persist in the saponin-treated state.

Peak force enhancement

Thus, this study of the nature of the increase in Ca-sensitivity reveals two important features of the action of drugs of the ORG30029 type. First, we consider the alteration in peak Ca-activated force. This is a mode of action unique to the Ca-sensitizing drugs, as far as we are aware. An increase in C_{max} seems to be associated with the action of most of them, although caffeine and theophylline depress it significantly (Wendt & Stephenson, 1983). It indicates that cross-bridge cycle kinetics must be affected if the results are to be interpreted within the current paradigm for force generation by vertebrate striated muscle (a sliding filament mechanism with myosin cross-bridges as independent force generators controlled by thin-filament based Ca-regulation). The only straightforward way that maximum force can be increased must be by increasing the 'dwell time', that fraction of the cycle time for which the bridge is attached. The effect can be quite substantial, as is revealed by higher doses of ORG30029, reaching 20% or more at 1 mM (Figure 4, Table 2). Although at likely therapeutic doses this effect will be very small for ORG30029 and its relatives, and the other Ca-sensitizers described in the literature, it clearly points to another mode of potential action for positive inotropic drugs which merits exploration.

Overall effect on force production

It is useful to assess the effectiveness of ORG30029 on absolute as well as on relative force production to gain a proper impression of one of its inotropic actions. It is widely accepted that *in vivo* cardiac muscle is only partially activated even under physiologically optimal conditions (Fabiato, 1981). Thus the modest increase in Ca-sensitivity and the reduction of slope produced by ORG30029 translate into a large change in relative force at lower activation levels. At 100 μM , ORG30029 will double force achieved an initial level of activation of 25% C_{max} (see e.g. Figure 2): mean force at pCa 5.52 was $26.2 \pm 7.4\%$ which increased to $52.0 \pm 4.5\%$ ($n = 5$ muscles). It remains to be established that particularly low activation occurs significantly in failing myocardium. However, physiological compensation mechanisms may result in the opposite being the case. Nevertheless, the tendency of these drugs to be most effective in their degree of potentiation at lower levels of activation could be a valuable property. In effect, the degree of physiological compensation for reduced contractility is given scope disproportionately to reduce in the presence of a drug which makes the pCa-tension relationship shallower. This characteristic is shared with other Ca-

sensitizers (physiological and pharmacological) in our experience, although the point is not well documented in the literature.

Mode of action on the regulatory proteins

By analogy with the effects of Ca-sensitizers such as sulmazole and pimobendan, it seems likely that ORG30029 increases Ca binding to troponin C (Solaro & Rüegg, 1982; Jacquet & Heilmeyer, 1987). However, it has recently been demonstrated that caffeine does not affect Ca-binding by isolated cardiac or skeletal troponin-C (Kentish & Palmer, 1989). Differences in the behaviour of the proteins between the isolated state and when incorporated in the complex physico-chemical environment of the myofilament lattice are perhaps not surprising, although they are currently impossible to explain. In any case, functional experiments of the type described here cannot give direct evidence about the nature of the chemical interactions involved. Nevertheless, some clues are provided which may guide experimentation by biochemical techniques. The ability to alter the steepness of the pCa-tension relationship implies an effect on the co-operativity between the troponin-C Ca-binding subunits (see Jacquet & Heilmeyer, 1987), the Hill exponent giving an indication of co-operativity. Indeed, an alteration of Ca-affinity which is different in character from competitive actions, such as those of Mg^{2+} or H^+ , might be expected to be allosteric and modify co-operativity too.

A different line of reasoning might also apply. The shifts in Ca-sensitivity are illustrated here as graphs of pCa vs normalised tension. The assumption behind such an approach is that the cross-bridges act as independent force generators. The assumption (of independence) is widespread in the treatment of drug effects in general. It then follows that in the presence of

ORG30029 the likelihood of any bridge being active at a given $[Ca^{2+}]$ is increased, so greater force is produced until all the bridges are recruited. However, one obvious departure from this model is that C_{max} itself is positively influenced by many Ca-sensitizers, including the one tested here (see above). This requires that the drug additionally acts, directly or indirectly, on the cross-bridge itself. Thus, independently of the proposed action to favour Ca-binding, to regulatory proteins, either the force that the individual bridge produces is higher or more bridges are made available for attachment at any one time. As described earlier, this result cannot be interpreted within current ideas without invoking changes in cross-bridge kinetics. Once this possibility is conceded, it becomes conceivable that all the effects of the 'Ca-sensitizers' could be accounted for by actions on the cross bridge, including the apparent Ca-sensitizing. There is increasing evidence that cross-bridges may interact, perhaps via the force that they produce through long-range effects transmitted through the filaments (Brandt *et al.*, 1980; Shiner & Solaro, 1984). These ideas cannot be satisfactorily tested on extracted proteins, since the three-dimensional structure of the sarcomere is crucial to the phenomena. Further studies with the various types of chemically skinned fibre and Ca-indicators (such as Fura-2) in intact cells presently offer the best hope of progress in understanding the mechanism of action of the 'Ca-sensitizers'.

The ORG30029 type of drug may offer a valuable tool for a better understanding of the mechanism of force production itself because of its potent actions. The present study confirms that there is still considerable scope for development of inotropic agents which follow the general lines of action of ORG30029 as Ca-sensitizers and potentiators of maximum Ca-activated force.

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