Dissociation of Changes in Apparent Myofibrillar Ca\(^{2+}\) Sensitivity and Twitch Relaxation Induced by Adrenergic and Cholinergic Stimulation in Isolated Ferret Cardiac Muscle

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ABSTRACT In isolated, aequorin-injected ferret cardiac muscle we measured the apparent myofilament Ca\(^{2+}\) sensitivity and its relationship to twitch relaxation time in the presence of autonomic perturbations. The Ca\(^{2+}\)-tension relation was determined from the peak aequorin luminescence and peak twitch tension measured in muscles across a broad range of bathing [Ca\(^{2+}\)] in the presence and absence of acetylcholine (ACh) (1 μM) or isoproterenol (ISN) (1 μM), or both drugs. ACh shifted the relationship of peak tension to peak aequorin light leftward, which suggests an increase in myofilament Ca\(^{2+}\) sensitivity, but it did not alter relaxation, which was measured as the time for peak tension to decay by 50% (\(t_{50\%R}\)). ISN produced its previously documented effects, i.e., a rightward shift of the relationship of peak tension to peak aequorin light and a decrease in \(t_{50\%R}\). ACh abolished the ISN effect on the peak tension-aequorin light relationship but did not reverse the effect of ISN to decrease \(t_{50\%R}\). The effects of ACh and ISN of modulating the apparent myofilament Ca\(^{2+}\) sensitivity in intact muscles, corroborate findings of previous studies in isolated myofibrillar preparations. However, these perturbations of myofilament Ca\(^{2+}\) sensitivity in the intact muscle do not relate to twitch relaxation, measured as \(t_{50\%R}\), since (a) ACh affects the former but not the later and (b) the effect of ISN on the Ca\(^{2+}\)-tension relationship is abolished by ACh, while the relaxant effect persists.

INTRODUCTION

Autonomic agonists produce biochemical changes in both membranes and myofilaments in cardiac muscle. The relative importance of these changes in causing the effects characteristic of adrenergic and cholinergic modulation of cardiac function, particularly the relaxation of twitch tension, has remained unclear. The release of

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Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) into the cytoplasm, and the subsequent binding of Ca\(^{2+}\) to troponin C leads to the formation of actin-myosin crossbridges and the development of tension in cardiac muscle. Unbinding Ca\(^{2+}\) from troponin, and the removal of Ca\(^{2+}\) from the cytoplasm, permits breakage of crossbridges and the relaxation of tension. If Ca\(^{2+}\) unbinds more rapidly under conditions of \(\beta\)-adrenergic receptor stimulation, there could be both a reduction of tension at a given free Ca\(^{2+}\) (as less would be bound) and an increased rate of relaxation. This decreased sensitivity of myofilaments to the effects of Ca\(^{2+}\) could be a mechanism through which relaxation could be regulated. Alternatively, regulation of the release and uptake of Ca\(^{2+}\) from the SR could modulate relaxation by controlling the pool of Ca\(^{2+}\) available for binding to the filaments.

In isolated cardiac myofibrillar preparations, cAMP-dependent phosphorylation of troponin causes a rightward shift in the relationship between Ca\(^{2+}\) and myofilament ATPase activation (Ray and England, 1976; Solaro et al., 1976; Holroyde et al., 1979), suggesting a decreased sensitivity of phosphorylated myofilaments to Ca\(^{2+}\) activation. A similar rightward shift in the relationship between force and Ca\(^{2+}\) induced by cAMP has been reported in cardiac muscles chemically skinned with EGTA or with detergents (McClellan and Winegrad, 1978; McClellan and Winegrad, 1980; Mope et al., 1980; Herzig et al., 1981). This decrease in myofilament sensitivity to Ca\(^{2+}\), resulting from an increase in the off-rate of Ca\(^{2+}\) from the myofilaments (Robertson et al., 1982), has been advanced as a possible mechanism whereby \(\beta\)-adrenergic stimulation increases the relaxation rate of the twitch in intact muscle (McClellan and Winegrad, 1978; Holroyde et al., 1979; McClellan and Winegrad, 1980; Mope et al., 1980; Robertson et al., 1982). However, even under such carefully controlled conditions, phosphorylation of myofilaments by cAMP-dependent kinases and its effects on contractile activity can only be demonstrated after first increasing myofilament sensitivity with detergents or phosphodiesterase inhibitors (Mope et al., 1980; Herzig et al., 1981; Horowitz et al., 1983). The importance of inhibiting phosphatase activity in such experimental preparations is also well known (Reddy and Wyborny, 1976). Similarly, in mechanically skinned cardiac cells fibers bathed in carefully controlled Ca\(^{2+}\) solutions buffered by EGTA, no effect of micromolar concentrations of cAMP on myofilament sensitivity occurred in the absence of phosphodiesterase inhibition (Fabiato and Fabiato, 1975; Fabiato, 1981).

The extension of the in vitro conditions to conditions that pertain to intact muscle, is not straightforward. However, there is some evidence for a cAMP-mediated decrease in myofilament Ca\(^{2+}\) sensitivity in intact muscle. In ferret papillary muscle \(\beta\)-adrenergic stimulation can decrease the steady (tonus) tension during contracture associated with a given Ca\(_i\) measured via a Ca\(^{2+}\)-sensitive microelectrode (Marban et al., 1980) or via aequorin luminescence (Kurihara and Konishi, 1987). However, while the bathing Ca\(^{2+}\) (and therefore tension) of skinned fibers and contractured muscles can be rapidly changed, there is no twitch per se in these experimental models, so twitch relaxation parameters cannot be directly measured. This can only be measured in cardiac muscle during electrical stimulation. In electrically stimulated intact cardiac muscle the "apparent" myofibrillar Ca\(^{2+}\) sensitivity during twitches has been measured by simultaneously monitoring myoplasmic free Ca\(^{2+}\)
(using Ca²⁺ indicators) and twitch tension (Allen and Blinks, 1978; Allen and Kurihara, 1980; Morgan, 1985; Blinks and Endoh, 1986; Endoh 1986; Kurihara and Konishi, 1987; Endoh and Blinks, 1988). The results of these studies, like those in most, but not all, studies in isolated myofibrils, show a rightward shift in the twitch tension–Ca²⁺ relationship in response to β-adrenergic stimulation and an associated acceleration of twitch relaxation. In intact muscle preparations, however, both the membrane and myofilament effects of β-adrenergic stimulation are present, so the relative contributions of each are unknown. Thus, despite the association between an apparent reduction in myofilament Ca²⁺ sensitivity and a reduction in twitch relaxation time in intact muscle, it is still unclear whether myofilament effects or membrane effects mediate the relaxant effect of β-adrenergic agents. For example, cAMP-dependent kinases also phosphorylate phospholamban in the SR, increasing its Ca²⁺ pumping rate (LaRaia and Morkin, 1974; Kirchberger et al., 1974; Tada et al., 1974). An accelerated rate of removal of Ca²⁺ from the cytoplasm could accelerate twitch relaxation time. Indeed, in mechanically skinned cardiac cells under conditions in which the SR can effectively complete with EGTA for Ca²⁺, the relaxation of phasic contractions initiated by Ca²⁺-triggered SR Ca²⁺ release was enhanced by cAMP (Fabiato and Fabiato, 1975). More recent studies of phosphorylation and dephosphorylation of SR phospholamban by isoproterenol (ISN) and acetylcholine (ACh), respectively, in conjunction with relaxation measurements in intact hearts, also suggest that enhanced removal of Ca²⁺ from the cytosol into the SR modulates ISN's relaxant effect (Lindemann and Watanabe, 1985).

Cholinergic agents, in contrast to β-adrenergic agonists, cause a leftward shift in the force-Ca²⁺ relationship in skinned fibers. This has been attributed to an increase in myofilament Ca²⁺ sensitivity (McClellan and Winegrad, 1978; Horowits and Winegrad, 1983). The effect of cholinergic agonists on the apparent force-Ca²⁺ relationship measured during the twitch in intact muscle has not been well characterized, but it has been predicted that cholinergic stimulation should alter relaxation as a direct result of its effects on the myofilaments (Horowits and Winegrad, 1983). If autonomic perturbations do modulate relaxation by shifting the force-Ca²⁺ relationship, one would predict that cholinergic agonists might increase relaxation time in conjunction with a leftward shift of the force-Ca²⁺ relationship, and that they would antagonize both the myofilament and the relaxant effects of β-adrenergic agonists.

The present study tests these predictions. Using the method of Allen and Kurihara (1980), we determined apparent myofilament Ca²⁺ sensitivity by microinjecting the chemiluminescent indicator aequorin into isolated ferret papillary muscles, and simultaneously monitoring the time course and magnitude of the intracellular [Ca²⁺] (Caₜ) transient and twitch force. Peak twitch tension and the Caₜ transient, as well as the relaxation of tension and the Caₜ transient, were studied under control conditions and in the presence of ACh and ISN, alone or in combination.

M E T H O D S

The experimental preparation was essentially the same as described previously (Orchard and Lakatta, 1985). 12–16-wk-old ferrets were anesthetized with chloroform. The hearts were removed and washed in oxygenated Tyrode's solution (see below) at room temperature. Thin
(diameter, mean ± SEM, 0.67 ± 0.03 mm) right ventricular papillary muscles were isolated and small loops of silk thread were tied around each end of the muscle. One loop was attached to a stationary hook in a horizontal plexiglass muscle bath (effective volume, 0.25 ml) and the other loop was attached to a force transducer (UC-2; Gould Instruments, Inc., Cleveland, OH). Over a period of 60 min, the muscles were slowly stretched to the length at which maximum isometric twitch force development occurred ($L_{max}$). The muscles were superfused at 30°C with Tyrodes solution (pH 7.4) containing in millimolar: 135 Na⁺, 98 Cl⁻, 20 HCO₃⁻, 1 HPO₄²⁻, 1 SO₄²⁻, 1 Mg²⁺, 5 K⁺, 10 glucose, 20 acetate, and 4 U/liter insulin. Superfusate [Ca²⁺] (Caₐ) was adjusted with CaCl₂. In some experiments, 6 mM HEPES buffer was substituted for bicarbonate buffer, and 100% oxygen was substituted for a mixture of 95% oxygen and 5% carbon dioxide. All muscles were stimulated electrically at 0.3 Hz by punctate platinum electrodes that were placed within 1 mm of the muscle and delivered 2 ms repetitive pulses at 110-125% of threshold voltage. The low rate of muscle stimulation was chosen so we could be certain that even at the high forces generated in the presence of catecholamines the core of the papillary muscle would not become hypoxic. This would have produced a decrease in developed tension not reflected in the changes in Caₐ as measured in more superficial layers of cells. The resultant false shift in apparent Ca²⁺ sensitivity would obscure the effects of the autonomic interventions being studied. Higher, more physiologic rates of stimulation could result in higher Ca²⁺ flux due to a higher Caₐ, which would maximally stimulate Ca²⁺ release by the SR. By varying Caₐ, we achieved the same range of Caₐ as could be achieved by varying the stimulation rate. In a series of pilot experiments, rates of 0.2, 0.3, 0.4, and 1.0 Hz were shown to produce identical force-Ca relationship, not a shift in the relationship, which confirmed prior results (Allen and Kurihara, 1980). Still the results reported here relate to only one stimulation frequency, and may not be directly applicable to results from experiments carried out at higher rates of stimulation.

Several superficial cells of some papillary muscles were microinjected with the photoprotein aequorin (purchased from Dr. J. R. Blinks, Mayo Medical School, Rochester, MN). Aequorin light (a function of Caₐ) was monitored using a photomultiplier tube (model RFI/B293; Thorn EMI GENCOM, Plainview, NY), the output of which was displayed on a four-channel pen recorder (Gould Instruments) and recorded on magnetic tape (3968A instrumentation recorder; Hewlett-Packard Co., Mountain View, CA). All measurements were made after twitch tension had reached steady state. Aequorin light and force was averaged during 25-100 twitches using a MINC-11 computer (Digital Equipment Corp., Maynard, MA). The sampling was performed at 1 kHz. Software written for this computer derived peak twitch tension (TT) (twitch force was converted to tension by normalizing the force of each muscle to the cross-sectional area of the muscle), the time from stimulus to peak tension (TPT), the time it took for the tension to relax by half ($t_{1/2}$), the peak of the light transient (PL) (normalized to represent the average light from a single twitch), the time to peak light (TPL), and the time to one-half and three-quarters relaxation of light from PL ($t_{1/2}$PL and $t_{3/4}$PL, respectively). The late phase of relaxation of light in each muscle ($t_{1/2}$PL - $t_{1/2}$PL) was also measured.

In the muscles injected with aequorin, Caₐ was varied from 0.25 to 12 mM, and the aequorin and TT signals were recorded. ISN (Breon) (final concentration 1 μM), ACh (Sigma Chemical Co., St. Louis, MO) (final concentration 1 μM), or both drugs were then added to the superfusate and the parameters of TT and aequorin luminescence were again recorded as superfusate Ca²⁺ was varied over the same range in the presence of drug (Fig. 1). Force-Ca²⁺ relationships could then be constructed relating PL to TT in the presence or absence of autonomic agonists, while simultaneously monitoring twitch relaxation time in the same muscles.

The aequorin light signals obtained from intact muscles are intrinsically noisy. This noise is negligible for large signals, such as the effect of large changes in Caₐ or of the addition of ISN.
to the superfusate, because such large changes in Ca$_i$ are easily discerned despite the noise of the light collection system. Other, smaller effects, such as the measurement of the slower phase of the Ca$_i$ transient, especially during small transients, are more difficult to measure because of the signal to noise ratio. We have chosen, therefore, to use each muscle as its own control in comparing the effect of adrenergic and cholinergic agents on the relaxation of the Ca$_i$ transient. Even so, the noise present during the slower, relaxation phase of the Ca$_i$ transient makes the precise measurement of single points in time, such as the exact $t_{aR}$ or the $t_{aR}$ of light, problematical. Multiple measurements of the relaxation of the transient in a number of muscles under a variety of conditions are therefore given so that the effect of autonomic modulation on the transient can be discerned from the effects of random noise on the transient.

![Graph showing the experimental protocol](image)

**Figure 1.** Representative experiment demonstrating the experimental protocol. Ca$_o$ was varied from 0.25 mM to 12 mM, while the peak aequorin light (PL) and peak twitch tension (TT) were measured. A drug (ACh) was then added and PL and TT were again measured over the same range of Ca$_o$. TT and PL both reached a plateau after each intervention, although the plateau was different under the different conditions.

There appear to be problems with all methods of relating force to Ca$_i$ during a twitch. Since the on-rate of binding Ca$_i$ to troponin C is not rate limiting (Blinks and Endoh, 1986) in the development of TT, relating peak free Ca$_i$ to TT seems reasonable since Ca$_i$ delivered to the myofilaments is the primary determinant of the resultant TT. Since PL and TT occur at different points in time, others (Allen and Kurihara, 1980) have measured the tension existing at the same point in time as PL (TT$_{PL}$), or have plotted the maximum rate of tension development (dT/dt$_{max}$) against PL (Endoh and Blinks, 1988) to make the relationship more straightforward. However, PL has also been related directly to TT (Blinks and Endoh, 1986), and gives the same qualitative result as the PL-dT/dt$_{max}$ relationship (Endoh and Blinks, 1988). So long as the time to PL is constant for all interventions (as it is for ISN, ACh, and control experiments in ferret ventricular muscle), so that different rates of Ca$_i$ removal into the SR do not seem to appreciably affect the peak Ca$_i$ delivered to the myofilaments, either approach seems reasonable. Similar tension--aequorin light Ca$_i$ relationships are seen in our experimental preparation regardless of whether PL and TT are used, or PL and TT$_{PL}$ are used (Fig. 2). Since TT$_{PL}$ can be small and hard to measure, we have elected to relate PL to
FIGURE 2. The measurement of TT and aequorin luminescence during a twitch. The peak aequorin luminescence has a similar relationship to both TT (A) and to the tension measured at the same moment in time as peak light (TTPL) (B). The effect of ISN (1.0 μM) and ACh (1.0 μM) on the tension-aequorin luminescence relationship is qualitatively the same whether the measurement is plotted PL vs. TT or PL vs. TTPL.

TT, and our results are similar not only to those of other laboratories using aequorin in intact muscle with either method, but also to experiments with myofibrillar preparations and skinned fibers as well.

RESULTS

An initial series of experiments was performed in muscles not injected with aequorin to determine the effect of ISN and ACh on the twitch in ferret cardiac muscle under the present experimental conditions. For these muscles, superfusate [Ca2+] was 2 mM. Table I shows that as expected, ISN potentiated TT and abbreviated twitch duration. Both effects of ISN were totally reversed within 2.5 ± 0.5 (± SEM) min after the removal of the drug (results not shown). ACh, in contrast, had a negative inotropic effect without affecting twitch relaxation time. This cholinergic effect is apparently species specific. While ACh has been reported to have a direct negative inotropic effect on rat myocardium (George et al., 1970), in guinea pig preparations cholinergic agents have only antiadrenergic effects, with no direct effect on contractility (Watanabe and Besch, 1975). Consideration was made of the

| Drug          | No | Control | Drug | Control | Drug | Control | Drug | Control | Drug |
|---------------|----|---------|------|---------|------|---------|------|---------|------|
| ISN (1 μM)    | 5  | 2.5 ± 0.3 | 161 ± 7 | 171 ± 8 | 80 ± 3 | 101 ± 6 | 87 ± 4 |
| ACh (1 μM)    | 5  | 2.4 ± 0.7 | 54 ± 8  | 155 ± 3 | 88 ± 4 | 96 ± 6  | 98 ± 2 |
| ISN + ACh (1 μM each) | 9 | 2.6 ± 0.3 | 64 ± 7  | 150 ± 4 | 85 ± 2 | 97 ± 2  | 81 ± 4 |

*Values are mean ± SEM.
possibility that the substantial decrease in TT seen in response to ACh in these ferret muscles could be due to the release of endogenous catecholamines in response to the electrical stimulation of the muscles. In such a case, our findings would reflect the antiadrenergic effect of ACh rather than an independent cholinergic effect on contractility. However, the addition of 10 μM propanolol to the superfusate in four ferret papillary muscles under our conditions reduced TT by only 14 ± 0.44%, which suggests that endogenous catecholamine release during these experiments was minimal, and that observed effects of ACh on twitch parameters were indeed direct cholinergic effects.

In addition to its direct effects, ACh also antagonized some of the effects of ISN on the twitch. In the presence of both ISN and ACh, TT was depressed to levels below control, while the abbreviation of twitch relaxation time due to ISN persisted.

These effects were seen whether the drugs were added sequentially as illustrated in Fig. 1, or if they were added simultaneously. Thus ACh completely negated the effect of ISN on TT without altering its effects on twitch relaxation time (Fig. 3, Table I).

These data suggested that ACh could separate the inotropic from the relaxant effects of ISN. Further, the known opposing effects of cAMP and cGMP (the purported intracellular mediators of β-adrenergic and cholinergic effects, respectively) on myofilament Ca²⁺ sensitivity suggested that ACh might be a useful tool in dissecting out the mechanism of ISN’s relaxant effect.

The Effect of Ca₀ on Ca₆ and TT

In a series of muscles injected with aequorin, TPL, tᵦ₆, T₆ PL, TT, TPT, and tᵦ₆ R were measured while Ca₀ was progressively varied from 0.25 to 12 mM. Increasing
Ca$_o$ was found to increase TT, with an accompanying increase in the Ca$_i$ transient, as shown in the example in Fig. 4 A. Varying Ca$_o$ had no consistent effect on twitch relaxation or on the relaxation times of the Ca$_i$ transient (Table II). The relationship between TT and the peak of the light transient over the range of Ca$_o$ listed in Table II in a representative experiment. A sigmoidal relationship between these two variables is seen.

A possible confounding phenomenon in the interpretation of force-Ca$_i$ relationships in intact muscles is the presence of diastolic oscillations of Ca$_i$ (Kort et al., 1985). The oscillations of Ca$_i$ reflect cyclic release of Ca$^{2+}$ from the SR in the absence of action potentials or calcium signals from the sarcolemma (Fabiato and Fabiato, 1975). Because the oscillations occur irrespective of the coordinated cal-

**TABLE II**

The Effect of Ca$_o$ on Twitch Duration and the Ca$_i$ Transient*

| Ca$_o$ (mM) | No. | TPT (ms) | $t_{R}$ (ms) | TPL (ms) | $t_{PL}$ (ms) | $t_{PL} - t_{PL}$ (ms) |
|-------------|-----|----------|-------------|----------|---------------|-------------------------|
| 0.25        | 4   | 145 ± 8  | 104 ± 11    | 40 ± 2   | 25 ± 4        | 26 ± 8                  |
| 0.5         | 8   | 144 ± 3  | 95 ± 3      | 44 ± 2   | 27 ± 3        | 25 ± 2                  |
| 1.0         | 8   | 147 ± 3  | 95 ± 4      | 42 ± 2   | 27 ± 3        | 26 ± 2                  |
| 2.0         | 8   | 150 ± 3  | 94 ± 2      | 42 ± 3   | 30 ± 2        | 25 ± 2                  |
| 5.0         | 12  | 154 ± 3  | 99 ± 2      | 43 ± 2   | 28 ± 2        | 27 ± 2                  |
| 6.0         | 3   | 152 ± 2  | 96 ± 6      | 40 ± 2   | 29 ± 2        | 19 ± 1                  |
| 8.0         | 4   | 155 ± 5  | 101 ± 7     | 43 ± 3   | 28 ± 4        | 19 ± 1                  |
| 10.0        | 4   | 150 ± 3  | 101 ± 10    | 38 ± 1   | 27 ± 1        | 25 ± 2                  |
| 12.0        | 4   | 145 ± 2  | 105 ± 9     | 38 ± 4   | 26 ± 2        | 20 ± 4                  |

*Values are mean ± SEM.
calcium release triggered by electrical stimulation in our preparation, they can alter the Ca\(^{2+}\) loading of the cell and the releasable Ca\(^{2+}\) within the SR on a beat to beat basis, and thus lower or enhance subsequent Ca\(^{2+}\) transients during a twitch (Kort et al., 1985). In the presence of increased spontaneous oscillations of Ca\(_s\), changes in the Ca\(_s\) transient associated with a given twitch force could be mistakenly interpreted as a change in myofilament Ca\(^{2+}\) sensitivity, while, in fact, no such change had occurred (Kort et al., 1985). We examined this possibility in our preparation by analyzing nonaveraged records of aequorin light from several muscles. Photons counted by the photomultiplier tube were summed every 100 ms between twitches and averaged for several twitches in varied Ca\(_o\) concentrations in the presence and absence of drugs. When Ca\(_o\) was between 0.5 and 5.0 mM (i.e., the range used in the experiments are described in Tables III–V), no diastolic oscillations could be detected in the presence or absence of drugs. This is consistent with other observations in this species, i.e., that relatively high Ca\(_o\) is required for oscillatory activity to be seen in ferret ventricle (Kort and Lakatta, 1984).

The Effect of ACh on Ca\(_s\) and Apparent Myofilament Ca\(^{2+}\) Sensitivity

The effect of ACh on TT and Ca\(_s\) measured simultaneously in an aequorin-injected papillary muscle is illustrated in Fig 3 A. ACh diminished both TT and Ca\(_s\) without affecting the relaxation of either the twitch or the light transient. Because both TT and Ca\(_s\) were lowered by ACh, it was difficult to determine directly whether ACh
| Ca, (mM) | Drug | No. | TPT | t_{iR} | TPL | t_{iPL} | t_{aPL} | t_{aPL} - t_{iPL} |
|---------|------|-----|-----|--------|-----|---------|---------|------------------|
| 0.5     | Control | 4   | 140 ± 3 | 87 ± 3 | 42 ± 3 | 26 ± 4 | 44 ± 4 | 40 ± 2 |
|         | ACh   | 4   | 121 ± 6 | 82 ± 8 | 94 ± 3 | 99 ± 3 | 42 ± 2 | 100 ± 6 | 25 ± 4 | 94 ± 20 | 41 ± 4 | 95 ± 7 | 125 ± 37 |
| 1.0     | Control | 4   | 150 ± 4 | 94 ± 6 | 40 ± 5 | 27 ± 4 | 48 ± 4 | 60 ± 2 |
|         | ACh   | 4   | 126 ± 13 | 82 ± 4 | 89 ± 7 | 98 ± 3 | 36 ± 2 | 89 ± 5 | 23 ± 5 | 84 ± 16 | 42 ± 2 | 90 ± 10 | 98 ± 2 |
| 2.0     | Control | 4   | 156 ± 5 | 94 ± 3 | 40 ± 2 | 26 ± 3 | 50 ± 3 | 22 ± 3 |
|         | ACh   | 4   | 145 ± 10 | 88 ± 5 | 91 ± 5 | 98 ± 5 | 42 ± 1 | 109 ± 4 | 22 ± 3 | 90 ± 10 | 38 ± 3 | 80 ± 6 | 16 ± 2 | 86 ± 20 |
| 5.0     | Control | 6   | 154 ± 4 | 96 ± 3 | 48 ± 6 | 24 ± 5 | 40 ± 7 | 18 ± 2 |
|         | ACh   | 6   | 141 ± 6 | 94 ± 3 | 93 ± 1 | 105 ± 4 | 46 ± 1 | 91 ± 4 | 21 ± 2 | 93 ± 4 | 58 ± 3 | 99 ± 8 | 17 ± 2 | 112 ± 21 |

*Values are mean ± SEM.*
had any effect on myofilament Ca\textsuperscript{2+} sensitivity. However, because data was collected at a full range of Ca\textsubscript{o} before the addition of a drug, a broad range of TT was available to compare with twitches obtained in the presence of ACh. The effect of ACh could then be determined by comparing twitches under control conditions with those obtained after the addition of ACh. For a given TT, a smaller Ca\textsubscript{o} transient was observed in the presence of ACh, than under control conditions, as seen in Figs. 1 and 5 A. Conversely, for a given level of Ca\textsubscript{o}, more TT was generated in the presence of ACh than in the absence of the drug. This would be consistent with the finding that in skinned fibers ACh, presumably through the second messenger of cyclic GMP, increases the sensitivity of the myofilaments to Ca\textsubscript{i} (McClellan and Winegrad, 1978; Horowits and Winegrad, 1983). When TT was measured over a range of Ca\textsubscript{o} and plotted against the peak Ca\textsubscript{i} associated with each respective twitch, there was a shift of the relationship to the left in the presence of ACh compared with control conditions. (Representative example in Fig. 5 B, average data for six experiments in Fig. 7 B.) This leftward shift in the Ca\textsuperscript{2+} tension relationship was not accompanied by a change in twitch relaxation time in these muscles (Table III).

ACH had no consistent effect on the time course of the Ca\textsubscript{i} transient. TPL, t\textsubscript{PL}, t\textsubscript{PL} and (t\textsubscript{PL} - t\textsubscript{PL}) were all unaffected by cholinergic stimulation (Table III).
Because ACh and ISN are known to have opposing effects on the sensitivity of the myofilaments to Ca\(^{2+}\), the lack of an effect of ACh on twitch relaxation suggested that the relationship between twitch relaxation time and apparent myofilament Ca\(^{2+}\) sensitivity is not straightforward. Further, by comparing the relationship between TT and Ca\(_{\text{app}}\) in the presence or absence of either or both autonomic agents, we were able to test whether the relaxant effect of ISN could be dissociated from, and therefore was independent of, its effect on apparent myofilament sensitivity.

**The Effect of ISN on Ca\(_{\text{app}}\) and Apparent Myofilament Ca\(^{2+}\) Sensitivity**

ISN increased both TT and the peak of the Ca\(_{\text{app}}\) transient (Fig. 6). However, less force was developed for a given Ca\(_{\text{app}}\) transient in the presence of ISN than in the absence of the drug, i.e., a larger Ca\(_{\text{app}}\) transient was required to produce a given TT in the presence of ISN than under control conditions (Fig. 6 A). This resulted in a shift to the right of the relationship between TT and the peak of the Ca\(_{\text{app}}\) transient (representative experiment in Fig. 6 B, mean data Fig. 7 A). This finding was consistent with the hypothesis that ISN causes a decrease in myofilament sensitivity, and it would be expected if twitch relaxation, which is accelerated by ISN (Tables I and...
| Cao$_{\text{a}}$ | Drug No. | TPT | $t_{\text{aR}}$ | TPL | $t_{\text{aPL}}$ | $t_{\text{aPL}} - t_{\text{aPL}}$ |
|---|---|---|---|---|---|---|
| mM | | ms | % Control | ms | % Control | ms | % Control | ms | % Control | ms | % Control |
| 0.5 Control | 4 | 144 ± 7 | 94 ± 6 | 44 ± 4 | 26 ± 5 | 56 ± 6 | 18 ± 5 |
| ISN | 4 | 113 ± 5 | 76 ± 5 | 68 ± 2 | 77 ± 4 | 40 ± 2 | 91 ± 6 | 24 ± 1 | 97 ± 6 | 37 ± 1 | 74 ± 6 | 18 ± 1 | 52 ± 6 |
| 1.0 Control | 4 | 142 ± 8 | 98 ± 6 | 43 ± 3 | 25 ± 9 | 48 ± 6 | 21 ± 5 |
| ISN | 4 | 111 ± 3 | 78 ± 3 | 65 ± 2 | 70 ± 6 | 38 ± 1 | 85 ± 3 | 23 ± 2 | 88 ± 15 | 37 ± 3 | 79 ± 4 | 14 ± 1 | 70 ± 6 |
| 2.0 Control | 4 | 148 ± 6 | 94 ± 4 | 44 ± 4 | 30 ± 3 | 53 ± 4 | 23 ± 4 |
| ISN | 4 | 121 ± 6 | 85 ± 4 | 72 ± 5 | 78 ± 4 | 41 ± 2 | 98 ± 8 | 25 ± 3 | 89 ± 9 | 41 ± 3 | 68 ± 5 | 17 ± 1 | 64 ± 7 |
| 5.0 Control | 4 | 151 ± 9 | 101 ± 5 | 40 ± 1 | 30 ± 4 | 53 ± 5 | 19 ± 3 |
| ISN | 4 | 114 ± 3 | 82 ± 7 | 69 ± 2 | 68 ± 6 | 39 ± 1 | 96 ± 4 | 25 ± 1 | 90 ± 9 | 40 ± 2 | 77 ± 5 | 15 ± 1 | 72 ± 8 |

*Values are mean ± SEM.
IV) is predominantly modulated through myofilament effects. However, unlike ACh, ISN altered the time course of the Ca$_i$ transient. While the time to peak light was not affected, ISN did abbreviate the relaxation of the Ca$_i$ transient. This effect could not be seen in the $t_{\text{PL}}$ but could be discerned in the time between half relaxation and three quarters relaxation of the Ca$_i$ transient ($t_{\text{PL}} - t_{\frac{3}{4}\text{PL}}$). In addition to its effects on apparent myofilament Ca$^{2+}$ sensitivity, then, ISN accelerated the uptake of Ca$_i$ from the sarcoplasm. Thus, the effects of ISN in the intact muscle are consistent with effects on both troponin and phospholamban. However, whether either effect predominated in the reduction of relaxation time cannot be determined from experiments in which ISN alone is added to the superfusate bathing the muscle.

![Tension and Light Traces](image)

**FIGURE 8.** The effect of ACh (1.0 $\mu$M) or ISN (1.0 $\mu$M) on Ca$_i$, TT, $t_{\text{PL}}$. Upper traces show an isometric twitch before (a), after (b), the addition of 1 $\mu$M ISN, and after (c) the addition of 1 $\mu$M ACh in the presence of ISN. Ca$_i$ = 0.5 mM for all three tracings. ACh abolished the effect of ISN on TT but not its effect on $t_{\text{PL}}$. Lower traces show the corresponding aequorin transients.

**Effect of the Autonomic Modulation of Apparent Myofilament Ca$^{2+}$ Sensitivity on Twitch Relaxation Time**

When ACh was added to muscles already bathed in ISN, the relationship between TT and peak Ca$_i$ was not significantly different than under control conditions. That is, ACh completely negated the effect of ISN on the myofilaments (Figs. 7 C and 8). However, in spite of the normalization of apparent myofilament sensitivity to Ca$_i$, the acceleration of the relaxation of the Ca$_i$ transient by ISN persisted in the presence of ACH, as did the acceleration of twitch relaxation time (Table V). These effects were observed when ACh was added after ISN (Figs. 3 and 8), simultaneously with ISN, or before ISN. The finding that the relaxant and myofilament effects of ISN could be dissociated, makes it unlikely that the decrease in $t_{\text{PL}}$ mediated by ISN is regulated at the level of the myofilaments.

**DISCUSSION**

The relaxation of TT in cardiac muscle is a complex and incompletely understood phenomenon that involves several levels of regulation. At the intracellular level,
| Ca^2+ | Drug No. | TPT | t\text{\textsubscript{R}} | TPL | t\text{\textsubscript{PL}} | t\text{\textsubscript{PL}} - t\text{\textsubscript{PL}} |
|-------|---------|-----|----------------|-----|----------------|------------------|
|       |         | ms  | % Control      | ms  | % Control      | ms              | % Control      | ms  | % Control      | ms  | % Control      | ms  | % Control      |
| 0.5   | Control  | 8   | 144 ± 3        | 95 ± 3 | 44 ± 2       | 27 ± 3                          | 50 ± 3          | 27 ± 2        |
|       | ACh + ISN |     | 115 ± 5        | 78 ± 4 | 75 ± 2       | 40 ± 6                          | 21 ± 2          | 84 ± 16 | 36 ± 2        | 84 ± 9 | 15 ± 1          | 71 ± 6          |
| 1.0   | Control  | 8   | 147 ± 3        | 95 ± 4 | 42 ± 2       | 27 ± 3                          | 49 ± 4          | 29 ± 2        |
|       | ACh + ISN |     | 117 ± 4        | 79 ± 2 | 71 ± 7       | 44 ± 4                          | 29 ± 4          | 111 ± 12 | 42 ± 6        | 89 ± 10 | 12 ± 2          | 81 ± 5          |
| 2.0   | Control  | 8   | 150 ± 3        | 94 ± 2 | 42 ± 2       | 90 ± 2                          | 55 ± 2          | 23 ± 2        |
|       | ACh + ISN |     | 121 ± 6        | 82 ± 3 | 75 ± 7       | 42 ± 2                          | 90 ± 5          | 82 ± 13 | 16 ± 1        | 73 ± 9             |
| 5.0   | Control  | 12  | 154 ± 3        | 99 ± 2 | 45 ± 2       | 28 ± 2                          | 47 ± 2          | 19 ± 1        |
|       | ACh + ISN |     | 125 ± 2        | 86 ± 3 | 76 ± 1       | 42 ± 2                          | 87 ± 8          | 39 ± 2        | 83 ± 8 | 14 ± 1          | 77 ± 10          |

*Values are mean ± SEM.
relaxation is modulated by the unbinding of Ca\(^{2+}\) from troponin, which in turn modulates the availability of actin, which interacts with myosin and develops tension. The Ca\(^{2+}\)-troponin interaction can either be controlled by changing the affinity of troponin for Ca\(^{2+}\), or by regulating the available free Ca\(^{2+}\) in the sarcoplasm by controlling the rate of Ca\(^{2+}\) translocation from the sarcoplasm into intracellular stores, especially into the SR or out of the cell across the sarcolemma. Additionally, the rate of dissociation of actin and myosin is also determined by the load on the cross bridges at the time that Ca\(_i\) is reduced (Brutseart et al., 1980). Our experiments were performed in isometric muscle under a variety of Ca\(_o\) concentrations in the presence and absence of drugs, so that we could examine the effect of the drugs on twitch relaxation at the same level of tension. The effects of internal shortening, sarcomere length, load-dependent relaxation, etc., are therefore constant to a first approximation. Our discussion, then, will focus on Ca\(^{2+}\) modulation of relaxation.

By using papillary muscles microinjected with aequorin, we were able to measure simultaneously twitch relaxation time and intracellular Ca\(^{2+}\). This allowed us to determine the relationship between TT and Ca\(^{2+}\) under a variety of conditions, as has been previously described (Allen and Kurihara, 1980). Changes in the relationship between TT and Ca\(_i\) induced by ISN and ACh were then used to measure changes in the apparent sensitivity of the myofilaments to Ca\(_i\). Because twitch relaxation time and the Ca\(_i\) transient were measured simultaneously in these muscles, changes in apparent myofilament sensitivity could be related to changes in twitch relaxation in the same preparation. Thus, the finding that ACh shifts the Ca\(^{2+}\) tension relationship without simultaneously affecting relaxation, challenges the hypothesis that relaxation is regulated solely at the level of myofilaments. The loading of these muscles with aequorin also allowed the measurement of the time course Ca\(_i\) during the twitch. The time course of the decay of the Ca\(_i\) transient was used to monitor the uptake of Ca\(_i\) during the twitch into other Ca\(^{2+}\) stores of the cell. Abbreviation of the relaxation of aequorin light by ISN was interpreted as acceleration of Ca\(^{2+}\) sequestration into pools, e.g., into the SR, which made the Ca\(^{2+}\) less available to the myofilaments for tension generation. While ACh negated ISN’s myofilament effects, ACh did not alter ISN’s effect on the time course of the Ca\(_i\) transient. The persistence of ISN’s effects on the relaxation of both tension and the Ca\(_i\) transient, even in the presence of ACh, suggests that the relaxant effect of ISN on TT is modulated at the level of the SR. This is in agreement with the interpretation of a previous study in mechanically skinned cardiac cells (Fabiato and Fabiato, 1975).

The existence of an association between the myofilament effects of ISN and twitch relaxant effects cannot be studied directly in myofibrillar and skinned fiber preparations under conditions where no twitch occurs. In those particular studies, [Ca\(^{2+}\)] bathing the myofilaments is varied directly, so the tension-Ca\(^{2+}\) relationship is measured under steady state conditions of buffered free [Ca\(^{2+}\)] and steady state tension. Myofilament Ca\(^{2+}\) sensitivity can be carefully quantitated under these conditions. Twitch relaxation cannot be directly studied since there is no twitch. By definition, Ca\(_i\) is dynamic during a twitch, so tension, so the tension-Ca\(^{2+}\) relationship cannot be measured under equilibrium steady state conditions. The present study in intact muscle, which simultaneously measured the transient tension-Ca\(^{2+}\) relation-
ship, the time course of the Ca transient, and twitch relaxation is therefore complementary to the studies in skinned fibers. The results in both experimental preparations are consistent, albeit that in "skinned" preparations, prior exposure to phosphodiesterase inhibitors is required to demonstrate a cAMP-dependent shift in the tension-Ca relationship.

There are limitations associated with the experimental preparation used in our studies. Aequorin is a nonlinear indicator of [Ca], (aequorin luminescence varies as a function of [Ca]^{2+}5/2); and while it gives good time resolution, it also gives a spatially averaged estimate of Ca. Any intracellular gradient of Ca would, therefore, cause an overestimation of Ca, as the areas of increased [Ca] would be over-represented in the averaged signal. If the gradients were small or constant, then the problem would be negligible for comparisons of muscles under different conditions in which each muscle was used as its own control. For skeletal muscle the Ca transient is so fast and the gradients so steep that these problems may become significant. However, in cardiac muscle, with its slower twitch and shorter diffusion distances for Ca from Ca stores to the myofilaments, these issues are of apparently less concern (Weir and Yue, 1986), even in the presence of ISN, which could sharpen any existing gradients. It is reasonable, then, to assume that the limitations of aequorin might serve only to exaggerate the Ca measurement, making it easier to show a shift in the TT-peak Ca relationship to the right in the presence of ISN alone, but more difficult to show a shift to the left in the presence of ACh alone, or to show no shift at all in the presence of both ISN and ACh.

Measurement of the time courses of the Ca transient was an important part of this experimental preparation. The abbreviation of the late phase of relaxation of the Ca transient argues strongly for the fact that Ca sequestration is accelerated by ISN. A decrease in myofilament length also decreases the apparent myofilament Ca sensitivity (Hibberd and Jewell, 1982; Allen and Kentish, 1985), but prolongs the Ca transient (Allen and Kurihara, 1982; Housman and Blinks, 1983; Lab et al., 1984). Since the ISN-induced increase in cAMP increases the off-rate of Ca from the myofilaments (Robertson et al., 1982) one might expect the relaxation of the Ca transient to be similarly prolonged by ISN, unless Ca uptake were enhanced to compensate. The fact that ISN enhances the relaxation of the Ca transient despite these confounding factors suggests that the increased Ca uptake is not a small effect. Our results show that ISN affected only a later phase of the Ca transient, i.e., it did not affect the half time of the relaxation of the Ca transient, but rather affected only the time from half time to three-quarters relaxation of Ca. This can be explained on the basis of biochemical studies that have shown that the effect of phosphorylated phospholamban is not uniform throughout all ranges of Ca. Whereas there is a marked acceleration of Ca pumping rate at low [Ca], the effects become relatively less pronounced as [Ca] is increased (Tada et al., 1974; Hicks et al., 1979). Thus El, which measures the relaxation of the Ca transient while Ca is still high, might be expected to be less affected by ISN than the late phase of the Ca transient, when Ca is lower. The fact that ACh did not abolish ISN's effect on the Ca transient suggests that the enhanced Ca uptake induced by ISN in the present experiments in ferret cardiac muscle is not affected by cholinergic agents.
The notion that cAMP-dependent phosphorylation of the SR mediates a shortening of relaxation time by accelerating Ca\(^{2+}\) pumping out of the sarcoplasm is consistent with the interpretation of studies in guinea pig hearts (Lindemann et al., 1983; Lindemann and Watanabe, 1985). Our findings are not in complete accord with the findings of those studies, however, since in guinea pigs ACh antagonizes all of the effects of ISN and has no direct effect in the absence of ISN. In addition to a species difference, there are differences in method between the studies reported here and those done previously in guinea pigs, in that the present studies measure autonomic effects with muscles in steady state during continuous superfusion with drug, while the studies done in guinea pigs used pulses of drug. The specific reasons for the differences between the two studies is unclear. The direct effects of ACh that we observed cannot be explained by any baseline adrenergic effects from our method of muscle stimulation, since propanolol had little effect on the muscles, and certainly had less effect than ACh. It could be suggested that since we used prolonged applications of the drugs, some of ISN's effects might be irreversible or slowly reversible by later additions of ACh. However, the effects we are reporting were present whether the muscle was exposed to ISN initially, ACh initially, or to both drugs simultaneously.

The alteration in the time course of the Ca\(^{2+}\) transient, while supportive of the hypothesis of increased Ca\(^{2+}\) uptake underlying the relaxant effects of ISN, could complicate the interpretation of other portions of the present results. In particular, there is the concern that the shortened time course of the Ca\(^{2+}\) transient may have caused the apparent rightward shift of the tension-Ca\(^{2+}\) relationship even though neither ISN nor ACh alone, or in combination, affected the time to peak light. That is, if Ca\(^{2+}\) was available to the myofilaments for an abbreviated time because of the shorter Ca\(^{2+}\) transient, force generation as measured by peak tension may have been decreased. If the shortened Ca\(^{2+}\) transient resulted in a decreased TT, then it would only artifically appear that a given peak of Ca\(^{2+}\) produced a decreased TT in the presence of ISN, and the relationship between peak Ca\(^{2+}\) and peak tension would not be valid. The assumption behind the stated interpretation of the data, however, is that the binding of Ca\(^{2+}\) to the troponin C is rapid and is therefore largely completed by the time of the late phase of the Ca\(^{2+}\) transient. This is supported by the observation that in the presence of both ISN and ACh together, the late phase of the Ca\(^{2+}\) transient is shortened as it is with ISN alone (Table V), yet the relationship between TT and peak Ca\(^{2+}\) is unchanged in the presence of both drugs compared with control, which is in contrast to the effect of ISN alone (Fig. 7). The fact that ACh given alone caused no change in the time course of the Ca\(^{2+}\) transient, yet shifted the TT-Ca\(^{2+}\) relationship to the left, provides additional evidence that the late phase of the Ca\(^{2+}\) transient is not a major determinant of the apparent shifts in the TT-Ca\(^{2+}\) relationship. Additionally, as noted above, the effects of ISN and ACh seen here in intact muscle are the same as those seen in myofibrillar preparations and in hyperpermeable muscle. Finally, at least two studies have demonstrated that the tonic tension-Ca\(^{2+}\) relationship in intact cardiac muscle is shifted by \(\beta\)-adrenergic agents (Marban, et al., 1980; Kurihara and Konishi, 1987).

In summary, when the data available from other systems documenting the effects of autonomic agonists on Ca\(^{2+}\)-myofilament interactions (in which results must be
extrapolated from the in vitro situation to that of a contracting and relaxing muscle), are combined with the present results in intact muscle (in which the dynamic nature of contraction and relaxation during a twitch present other obstacles to experimental design), three conclusions can be drawn. First, that ACh has no effect on relaxation in ferret cardiac muscle. This is not consistent with the hypothesis that relaxation is modulated via an altered myofilament-Ca$^{2+}$-tension relationship, since ACh alters this relationship. Second, that ISN’s relaxant effect is accompanied by an apparent desensitization of the myofilaments to Ca$^{2+}$, which was predicted by the in vitro studies in most myofibrillar preparations. The problem with this hypothesis is that these two effects appear to be dissociated by the addition of ACh to ISN-stimulated muscle. Third, that the relaxant effect of ISN that augments TT is also accompanied by an acceleration of the late phase of the relaxation of Ca$_{o}$, and that these effects are not abolished by ACh, which is consistent with the notion that the rate of Ca$_{i}$ uptake is one mechanism by which ISN modulates twitch relaxation.

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