Excitation-Contraction Coupling in Cardiac Purkinje Fibers

Effects of Cardiotonic Steroids on the Intracellular [Ca^{2+}] Transient, Membrane Potential, and Contraction

W. GIL WIER and PETER HESS

From the Department of Pharmacology, Mayo Foundation, Rochester, Minnesota 55905; and the Department of Physiology, University of Maryland, School of Medicine, Baltimore, Maryland 21201

ABSTRACT The [Ca^{2+}]-activated photoprotein aequorin was used to measure [Ca^{2+}] in canine cardiac Purkinje fibers during the positive inotropic and toxic effects of ouabain, strophanthidin, and acetylstrophanthidin. The positive inotropic effect of these substances was associated with increases in the two components of the aequorin signal, L_1 and L_2. On the average, strophanthidin at 10^{-7} M produced steady, reversible increases in L_1, L_2, and peak twitch tension of 20, 91, and 240%, respectively. This corresponds to increases in the upper-limit spatial average [Ca^{2+}] from 1.9×10^{-6} M to 2.1×10^{-6} M at L_1 and from 1.4×10^{-6} M to 1.8×10^{-6} M at L_2. Elevation of diastolic luminescence above the control level was not detected. At higher concentrations (5×10^{-7} M), strophanthidin produced aftercontractions, diastolic depolarization, and transient depolarizations, all of which were associated with temporally similar changes in [Ca^{2+}]. During these events, diastolic [Ca^{2+}] rose from the normal level of $\sim 3 \times 10^{-7}$ M up to $1\text{–}2 \times 10^{-6}$ M. The negative inotropic effect of 5×10^{-7} M strophanthidin was not associated with a corresponding decrease in the [Ca^{2+}] transient but was associated with a change in the relationship between [Ca^{2+}] and tension. Assuming the Na^+-lag mechanism of cardiotonic steroid action, we conclude the following: at low concentrations of drug, increased Ca^{2+} uptake by the sarcoplasmic reticulum prevents a detectable rise in cytoplasmic [Ca^{2+}] during diastole, but this increased Ca^{2+} uptake results in increased release of Ca^{2+} during the action potential. At higher drug concentrations, observable [Ca^{2+}] changes during diastole activate tension and membrane conductance changes.

INTRODUCTION

Cardiotonic steroids exert a large number of effects in cardiac tissue (recent reviews: Lullman and Peters, 1979; Noble, 1980; Greef, 1981). The reported

Address reprint requests to Dr. Gil Wier, Dept. of Physiology, University of Maryland, School of Medicine, 660 W. Redwood St., Baltimore, MD 21201. Dr. Hess's present address is Dept. of Physiology, Yale University School of Medicine, New Haven, CT 06510.

effects include the well-known positive inotropic effect at moderate doses, a negative inotropic effect at both very low (Hart et al., 1983) and very high doses, aftercontractions at high doses, and effects on membrane electrical activity, including abnormal pacemaker activity. Many recent studies have shown that changes in the chemical activity of intracellular Ca^{2+}, Na^+, H^+, and K^+ occur ($[Ca^{2+}]$: Allen and Blinks, 1978; Wier, 1980*b*; Sheu and Fozzard, 1982; Morgan and Blinks, 1982; $[Na^+]$: Deitmer and Ellis, 1978; Lee et al., 1980; Lee and Dagostino, 1982; Sheu and Fozzard, 1982; $[H^+]$: Deitmer and Ellis, 1980; extracellular $[K^+]$: Cohen et al., 1976). The extent to which all these ion concentration changes are involved in the effects above is presently unclear.

In the present study we have used the Ca^{2+}-activated photoprotein aequorin to measure cytoplasmic $[Ca^{2+}]$, both during diastole and during the twitch contraction in normal muscles and in muscles treated with cardiotonic steroids. We have included the use of relatively low doses of cardiotonic steroids in our study in an attempt to observe truly therapeutic effects. In this respect, 10^{-7} M strophanthidin was satisfactory since it usually produced stable effects that were readily reversible. (10^{-7} M is near the upper limit of the "low-dose" range referred to by Noble [1980].) We used 5×10^{-7} M strophanthidin to produce toxic effects: aftercontractions, a negative inotropic effect, transient depolarizations, and shortened action potentials.

Aequorin signals from canine Purkinje fibers are advantageous for studying the effects of cardiotonic steroids on intracellular $[Ca^{2+}]$ because the signals consist of two distinct components, which arise from different cellular processes, as described by Wier (1980), Wier and Isenberg (1982), and Hess and Wier (1983*a*). The second-occurring component, L_2, has been attributed to Ca^{2+} released from stores, probably the sarcoplasmic reticulum. The processes and source of Ca^{2+} underlying the more rapid initial component, L_1, have not yet been unequivocally identified. L_1 has some of the properties expected for a signal related to Ca^{2+} coming into the cell across the surface membrane via slow inward current (Wier and Isenberg, 1982). L_1 also has some properties not expected for Ca^{2+} entry via slow inward current, such as a strong reduction by caffeine (Hess and Wier, 1984). We also report in this study the use of aequorin to quantify diastolic levels of $[Ca^{2+}]$ and the slowly changing cytoplasmic $[Ca^{2+}]$ during the toxic effects of cardiotonic steroids.

METHODS

The preparations were free-running strands of Purkinje tissue dissected from either ventricle of canine hearts. The strands were selected for transparency, and on the average, were 250 μm in diameter and 3 mm in length. The preparations were mounted in a temperature-controlled bath (35°C) in an oxygenated Hepes-buffered physiological salt solution of standard composition (154 mM NaCl, 5.4 mM KCl, 1.0 mM $MgCl_2$, 2.7 mM $CaCl_2$, 12.0 mM Hepes, 11.0 mM dextrose, pH 7.4). The methods of microinjection of aequorin and the recording of membrane potential, aequorin luminescence, and tension were generally the same as previously described (Wier and Isenberg, 1982). Aequorin luminescence and tension were filtered at 500 and 250 Hz before being recorded on FM tape (passband, DC to 650 Hz). The membrane potential signal was not filtered before recording on the FM tape recorder. However, the overshoots and upstrokes of the action potentials were attenuated and slowed by the tape recorder; thus, our illustrations are not faithful representations of these events. Signal averaging was performed after the experiment by simultaneously playing back the aequorin luminescence, membrane potential, and tension into a multichannel analyzer (TN-1710; Tracor Northern, Middleton, WI). Each channel was digitized at 0.48-ms intervals. The analyzer allowed summation averaging, integration, scaling of data, and production of illustration-quality records through the use of its analog outputs and an *X-Y* recorder.

The use of summation averaging requires the assumption that the signals being averaged are unchanging during the period of averaging. This assumption is difficult to evaluate for aequorin luminescence. We assumed that if tension and membrane potential remained constant or changed within acceptable limits during the period of averaging, the same would be true of aequorin luminescence. With two exceptions (some of the points in Fig. 5, traces *b* and *c*, and Fig. 10; see Results for a discussion of these experiments), the

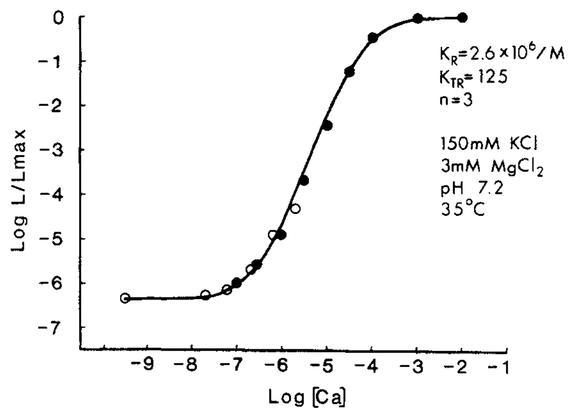

FIGURE 1. The Ca^{2+} concentration effect curve of aequorin. The logarithm of the ratio of the luminescence, L, at a given [Ca^{2+}] to the maximum luminescence obtained in a saturating [Ca^{2+}], L_{max}, has been plotted vs. the logarithm of [Ca^{2+}]. The conditions in the assay apparatus were: 150 mM KCl, 3 mM $MgCl_2$, pH, 7.0; 5 mM PIPES, 35°C. [Ca^{2+}] was achieved either by dilution (filled circles) or by the use of Ca-EGTA buffers (open circles), according to Blinks et al. (1978). The continuous line is a plot of Eq. 1. See text for details of this equation and the best-fitting values of the constants.

tension did not change by more than 10% from beginning to end of the averaging period. We compared individual action potentials with the average action potential to make sure that the averaged signal was truly representative.

The calibration of intracellular aequorin signals requires knowledge of the relationship between [Ca^{2+}] and aequorin luminescence. This relationship is presented graphically as the concentration effect, or C-E, curve. The C-E curve we used, and the conditions under which it was obtained, are shown in Fig. 1. The curve was obtained by the methods of Blinks et al. (1978). The data were well fit by Eq. 1, which was originally derived by

$$L/L_{max} = \{(1 + K_R[Ca^{2+}])/(1 + K_{TR} + K_R[Ca^{2+}])\}^3 \quad (1)$$

(Allen et al., 1977), according to a two-state model of the Ca^{2+} binding sites on aequorin. Under the conditions used in the experiment of Fig. 1, which mimic the intracellular ionic conditions of canine Purkinje fibers, the best-fitting values of the constants were: K_R, 2.6

$\times 10^6$ M^{-1}; K_{TR}, 126. (K_R represents the equilibrium constant of Ca^{2+} binding to the "effective" or R state of the site, and K_{TR} represents the equilibrium constant of the transition between the "ineffective" or T state and the R state. Ca^{2+} binding to sites in the T state is assumed to be negligible. L_{max} is the luminescence in a saturating [Ca^{2+}].)

The main uncertainty in the use of the C-E curve is the value of intracellular [Mg^{2+}]. In the past, we and others (Allen and Kurihara, 1980) have assumed a value of 2 mM, based on nuclear magnetic resonance studies of muscle. However, Hess et al. (1982) have recently used an Mg-selective microelectrode in Purkinje fibers and have reported a value

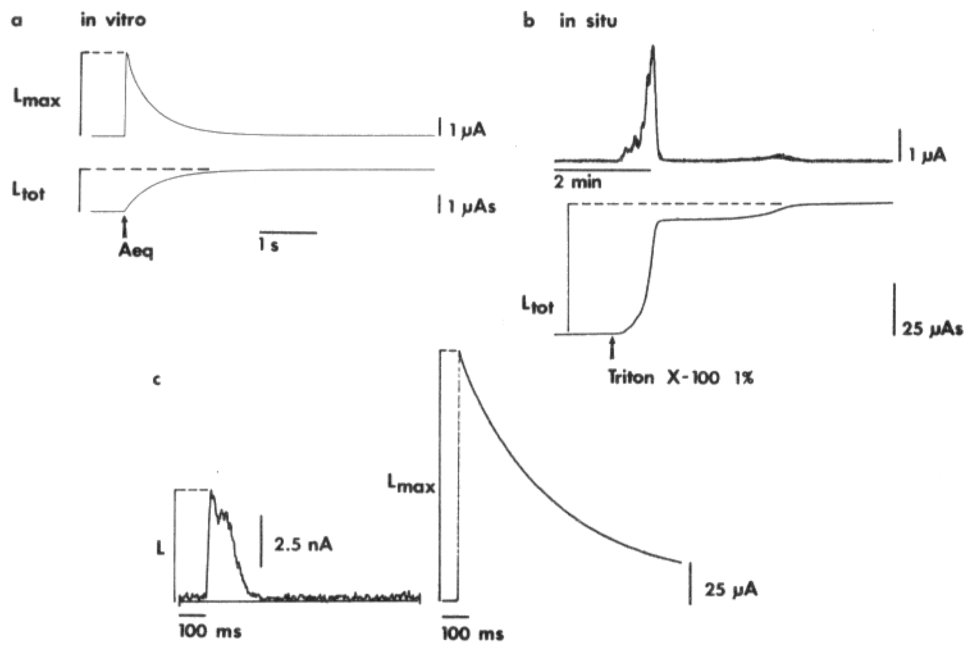

FIGURE 2. Procedures for the calibration of intracellular aequorin signals. Luminescence is proportional to the current of the photomultiplier tube. (*a*) Records of luminescence in the in vitro assay apparatus after injecting aequorin into a solution containing a saturating [Ca^{2+}]. L_{max} is obtained from the peak of the luminscence, L_{tot} is obtained by integrating the luminescence. (*b*) Records of luminescence in situ after exposing the aequorin-injected preparation to TX-100. L_{tot} is obtained by integrating the luminescence (upper trace of *b*). (*c*) The right-hand trace is the calculated L_{max} from the preparation. The left-hand trace is a typical physiological aequorin signal.

of ~3 mM. We therefore consider the C-E curve illustrated in Fig. 1 most appropriate and have used the constants derived from it for our calculations of intracellular [Ca^{2+}]. It should be noted that the influence of other environmental conditions, such as pH, [K^+], and [Na^+], are extensively documented (Blinks et al., 1982), and the slight uncertainties about the concentrations of these ions inside Purkinje fibers do not influence our estimation of intracellular [Ca^{2+}].

To calculate intracellular [Ca^{2+}] from measurements of intracellular luminescence, it is necessary to express the intracellular luminescence in the same terms as the C-E curve. The intracellular value of L_{max} is the luminescence that would be obtained if all the aequorin in the preparation were instantly exposed to a saturating [Ca^{2+}]. To expose the aequorin in the muscle to Ca^{2+}, the method of Allen and Blinks (1978) was used; preparations were exposed to a 1% solution of Triton X-100 (TX-100) in our normal saline solution, and luminescence was detected under the same optical conditions as were used during prior experimentation. Peak luminescence during such a procedure (Fig. 2) was usually 10^3 times higher and 10^2 times longer lasting than that associated with a single contraction. However, the aequorin was evidently not instantly exposed to a saturating [Ca^{2+}]; if it had been, the luminescence would have risen with a time constant of 5 ms. Therefore, we used Eq. 2 to calculate L_{max}, where

$$L_{max} = L_{tot,in\,situ} \times (L_{max,in\,vitro}/L_{tot,in\,vitro}). \quad (2)$$

($L_{max,in\,vitro}/L_{tot,in\,vitro}$) is measured after addition of aequorin to a solution containing a saturating [Ca^{2+}]. This method of calculating L_{max} is slightly different from that used by Allen and Blinks (1978) in that L_{tot} is used rather than the rate constant, K_{max}, of aequorin utilization. Our method of calculation of L_{max} does not give results different from theirs; we use our method only because one less assumption (that of an exponential utilization of aequorin) is made. The light signal remaining after several minutes exposure to TX-100 was assumed to be background signal, that is, signal arising from sources other than aequorin and including the dark current of the photomultiplier tube. To measure the background signal accurately, the signal remaining after exposure to TX-100 was integrated over a period of several minutes and an average value was obtained. This value appears in our figures as a smooth straight line at $L/L_{max} = 0$. After subtraction of the background signal from the luminescence to obtain L, calculation of intracellular [Ca^{2+}] was done with the use of an appropriately rearranged form of Eq 1.

RESULTS

Fig. 3 shows simultaneous recordings of membrane potential, aequorin luminescence, and tension under control conditions in 2.7 mM [Ca^{2+}]. The aequorin signal is calibrated in units of L/L_{max}, as described in Methods. During the diastolic interval, the aequorin luminescence is detectably elevated above the average background signal (smooth line at $L/L_{max} = 0$). The average diastolic L/L_{max}, as obtained by integration of the aequorin luminescence over the last 500 ms of each 1-s sweep, is 1.74×10^{-6}. This value is more than three times higher than the L/L_{max} expected from the Ca^{2+}-independent aequorin luminescence under our experimental conditions (see Fig. 1) and correspond to a [Ca^{2+}] of 2.1×10^{-7} M.

The values of [Ca^{2+}] calculated from the peaks of the two components L_1 and L_2 of the aequorin signal are 1.4×10^{-6} M and 1.3×10^{-6} M, respectively. Because of possible spatial [Ca^{2+}] gradients, these values must be regarded as upper-limit estimates of the "true" spatial average [Ca^{2+}] (Blinks et al., 1982). Value of diastolic [Ca^{2+}] and upper limits of [Ca^{2+}] at L_1 and L_2 are listed in Table I.

Effects of 10^{-7} M Cardiotonic Steroids on the Action Potential, Aequorin Luminescence, and Force of Contraction

The typical effects of 10^{-7} M strophanthidin are illustrated in Figs. 4 and 5. Such low doses of strophanthidin had little effect on the action potential, but markedly influenced the aequorin signal and increased the force of contraction.

The change in the aequorin signal always involved differential effects on the two components of the aequorin signal, L_1 and L_2. In the experiment illustrated in Fig. 4, an increase occurred almost exclusively in L_2. The amplitude of L_2 was taken as the amplitude of the aequorin signal 80 ms after the stimulus, a time that corresponded to the beginning of the "plateau" of the control signal. The amplitude of L_1 (taken as the peak amplitude of the aequorin signal) was essentially unaltered during the exposure to the drug, but was depressed in this experiment after recovery from the positive inotropic effect. The time courses of the effects on L_1, L_2, and force of contraction are illustrated in Fig. 5, *a* and *b*. The amplitudes of L_1, L_2, and tension are plotted at 100-s intervals throughout the experiment, and then once at 40 min after washout of the drug. Fig. 5, *a*

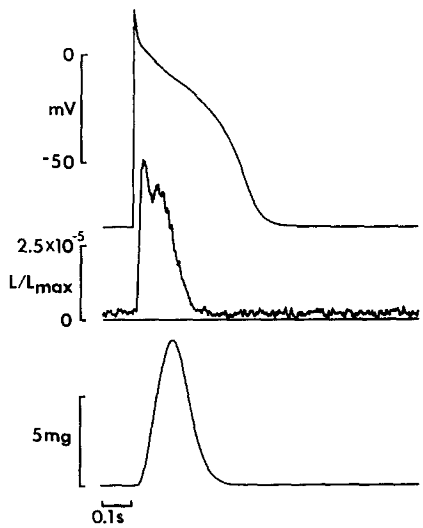

FIGURE 3. Simultaneous, averaged recordings of membrane potential (upper trace), intracellular aequorin luminescence (middle trace), and force of contraction under control conditions. $[Ca^{2+}]_o = 2.7$ mM. 100 sweeps were averaged. Frequency of stimulation was 1 Hz. Calibration of the aequorin signal in units of L/L_{max} was obtained as indicated in Methods. The mean diastolic level of $[Ca^{2+}]$ during the last 500 ms of the cardiac cycle was 2.1×10^{-7} M in this preparation.

and *b*, illustrates the general finding that the increases in L_2 and contraction force occurred along very similar time courses. Comparison of the time course of tension change determined from single tension traces with that obtained from the averaged tension, as in Fig. 5*b*, indicated that the averaged tension differed from that at the beginning or end of the averaging period by a maximum of 15% (during the most rapid change of tension).

Figs. 6–8 illustrate the same type of experiment with 10^{-7} M ouabain. Ouabain was more potent than the other agents and thus effects other than those described above were produced during prolonged exposure. However, the early effects of 10^{-7} M ouabain were identical to those of 10^{-7} M strophanthidin.

In all experiments the increase in L_2 caused by cardiotonic steroids was

TABLE IA

Upper Limit of Spatial Average [Ca^{2+}]$_i$ During Contraction

Experiment	L_1/L_{max}	[Ca^{2+}]	L_2/L_{max}	[Ca^{2+}]	F_T/F_{max}
		M		*M*	
D3	1.5×10^{-4}	2.3×10^{-6}	4.9×10^{-5}	1.5×10^{-6}	0.14
D13	2.6×10^{-4}	2.9×10^{-6}	1.1×10^{-4}	2.1×10^{-6}	0.34
D14	4.1×10^{-5}	1.4×10^{-6}	3.3×10^{-5}	1.2×10^{-6}	0.13
D19	5.6×10^{-5}	1.5×10^{-6}	2.2×10^{-5}	1.0×10^{-6}	0.19
D23	6.9×10^{-5}	1.7×10^{-6}	1.4×10^{-5}	8.1×10^{-7}	0.16
D7	1.8×10^{-4}	2.5×10^{-6}	6.0×10^{-5}	1.6×10^{-6}	—
D8	2.9×10^{-5}	1.2×10^{-6}	1.7×10^{-5}	8.8×10^{-7}	0.42
V1	7.9×10^{-5}	1.8×10^{-6}	5.3×10^{-5}	1.5×10^{-6}	—
V2	9.8×10^{-5}	2.0×10^{-6}	2.7×10^{-5}	1.1×10^{-6}	0.11
D24	1.1×10^{-4}	2.1×10^{-6}	7.0×10^{-5}	1.7×10^{-6}	—
Mean ± SEM:		$1.9 + 0.2 \times 10^{-6}$		$1.3 + 0.1 \times 10^{-6}$	

proportionally greater than the increase in L_1. The data illustrated in Fig. 7 offer a clue to the variability of effects on L_1 seen in different preparations (compare Figs. 4 and 6). Fig. 7*a* contains superimposed recordings of aequorin luminescence at different times during the development of the positive inotropic effect. The difference between the first two recordings in *a* is shown in *b* and clearly shows increases in both L_1 and L_2. However, the difference between the last two recordings (shown in *c*) clearly illustrates that a much greater increase occurred in L_2 during that time. Thus, L_1 seemed to "saturate" after a relatively small increase. The difference trace in *d*, which is the difference between the first and the last recordings in *a*, clearly shows that, overall, the greatest increase occurred in L_2. Therefore, it may be that the variability of effects on L_1 was due to natural variation in the basal state of L_1. In some muscles, the increase in L_2 was so great that the two components of the signal became indistinguishable (Fig. 11), as was also the case when L_2 increased as a result of rest potentiation (Wier, 1980). Fig. 7 also illustrates that the drugs generally had little effect on the time course of the aequorin signal: the time to peak of either L_1 or L_2 was not changed by the

TABLE IB

[Ca^{2+}]$_i$ During the Interval Between Contractions

Experiment	[Ca^{2+}]$_o$	L/L_{max}	[Ca^{2+}]$_i$
	mM		*M*
D3	2.1	2.30×10^{-6}	2.6×10^{-7}
D13	2.7	1.83×10^{-6}	2.2×10^{-7}
D14	2.7	1.74×10^{-6}	2.1×10^{-7}
D19	2.7	2.25×10^{-6}	2.6×10^{-7}
D23	2.7	4.48×10^{-6}	4.3×10^{-7}
V1	2.7	2.96×10^{-6}	3.2×10^{-7}
V2	2.7	1.78×10^{-6}	2.1×10^{-7}
D24	2.7	3.09×10^{-6}	3.3×10^{-7}
Mean ± SEM:			$2.9 + 0.3 \times 10^{-7}$

drug, nor was the half-time of the final decline in luminescence (30 ms in six muscles).

Upper-Limit Estimate of Spatial Average Cytoplasmic [Ca^{2+}] During the [Ca^{2+}] Transient

Table II presents data from four muscles that were exposed to 10^{-7} M or 2×10^{-7} M strophanthidin and which developed steady, reversible, positive inotropic effects with no evidence of "toxicity." Fig. 8 presents graphs of twitch tension vs. [Ca^{2+}] calculated from L_1 and L_2 during the positive inotropic effect of 10^{-7} M ouabain. We estimate that the fractional activation of the muscle increased from ~0.07 to 0.21 in association with a maximum increase of ~0.2 pCa unit. The fractional activation was obtained by division of peak twitch tension by the maximal tension obtained during the TX-100 contracture.

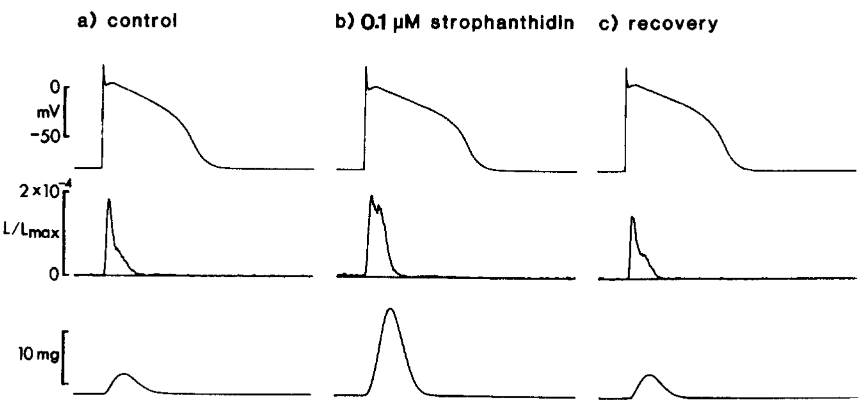

FIGURE 4. Simultaneous, averaged recordings of membrane potential (upper trace), intracellular aequorin luminescence (middle trace), and force of contraction. (*a*) Control; (*b*) the steady state effect of 10^{-7} M strophanthidin; (*c*) recovery after washout of the drug. Overall diameter of the strand, 250 μm. 100 sweeps were averaged in each condition.

Estimate of a Possible Change in Diastolic [Ca^{2+}]

As long as membrane electrical activity was unaffected by the drug and no abnormal patterns of tension development were present, we were unable to demonstrate any increase in luminescence except that associated with the contraction. The ability to detect such other increases is dependent on the signal-to-noise ratio obtained in each particular experiment. In the experiment of Fig. 5, which was favorable in this respect, a doubling of [Ca^{2+}] from 3.0×10^{-7} M to 6×10^{-7} M would have led to an increase in L/L_{max} from 2.27×10^{-6} to 7.59×10^{-6}, or an increase by a factor of 3.3. Such an increase would have been detectable. Therefore, we conclude that throughout the period illustrated in Fig. 5, [Ca^{2+}] did not increase to levels $>6 \times 10^{-7}$ M. A direct test of our ability to detect small increases in the diastolic [Ca^{2+}] is shown in Fig. 9: a fivefold increase of $[Ca^{2+}]_o$ led to a small but detectable increase in [Ca^{2+}] at rest.

However, subsequent exposure of this preparation to 2×10^{-7} M strophanthidin caused a twofold increase in twitch tension but did not result in a detectable elevation of diastolic [Ca^{2+}]. By measuring [Ca^{2+}] in successive 100-beat intervals in each solution, we obtained a mean value and variance of the estimate of the diastolic levels of [Ca^{2+}]. With the use of a *t* test, we found that in this experiment only the diastolic [Ca^{2+}] in 18 mM [Ca^{2+}]$_o$ and that immediately after discontinued stimulation in the toxic dose of strophanthidin (5.5×10^{-7} M) were significantly higher than the control level. Despite the continuous presence of 5.5×10^{-7} M strophanthidin, the value of [Ca^{2+}] after 10 min rest returned to a value not different from control (not shown).

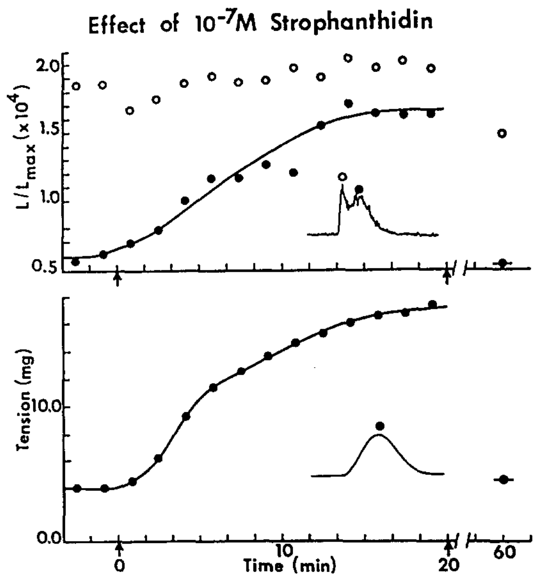

FIGURE 5. The time courses of the effects of 10^{-7} M strophanthidin on L_1 (open symbols) and L_2 (filled symbols) and peak force of contraction. Tic marks on the horizontal axis indicate the successive 100-s periods during each of which 100 luminescence and tension signals were averaged. The average value is plotted in the center of that period.

It was true in all experiments that as long as membrane electrical activity and contraction remained in a "normal" configuration, we were unable to detect an elevation of [Ca^{2+}] during diastole.

Toxic Effects

Some of the "toxic" effects of cardiotonic steroids are illustrated in Fig. 10, in which data from the same experiment as Fig. 6 are presented. 35 min after application of ouabain, marked changes in the action potential and the development of tension during diastole were evident. These changes became more pronounced at 42 and 47 min. Comparison of single tension responses with the averaged tension responses revealed that the average tension response differed by no more than 15% from the tension response at the beginning or end of the averaging period. After the twitch, the changes in diastolic membrane potential, aequorin luminescence, and tension followed very similar time courses. Several other phenomena are illustrated in Fig. 10: a marked shortening of the action potential and a variation in L_2 and tension, but not L_1, at various times during the drug effect. The effects illustrated in Fig. 10 are identical to those always obtained with 5×10^{-7} M strophanthidin.

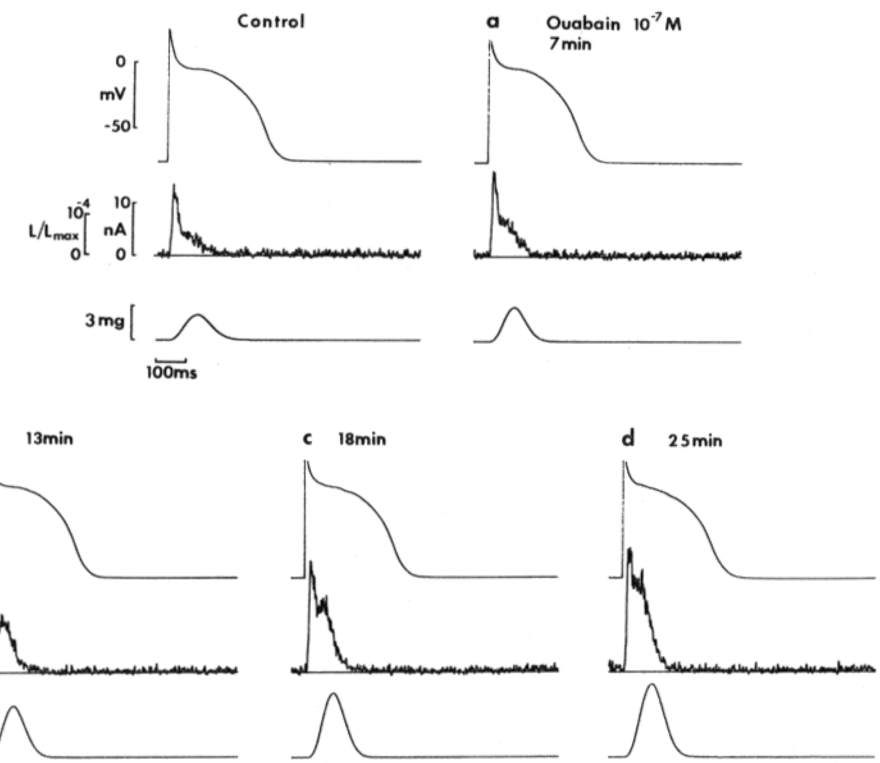

FIGURE 6. Simultaneous, averaged recordings of membrane potential, aequorin luminescence, and force of contraction during the application of 10^{-7} M ouabain. Recordings *a–d* are the average of 100 sweeps (1 Hz) beginning at the times indicated after application of 10^{-7} M ouabain.

Estimate of Spatial Average [Ca^{2+}] During Diastole

We used the C-E curve to calculate [Ca^{2+}] from the slow changes in luminescence during diastole. [Ca^{2+}] varied from the normal resting value of $\sim 3 \times 10^{-7}$ M to between 1 and 2×10^{-6} M during the peak of the aftercontraction.

The Negative Inotropic Effect of High Doses

The force of contraction often passed thorugh a maximum during exposure to high concentrations of cardiotonic steroids. Contraction force sometimes fell to

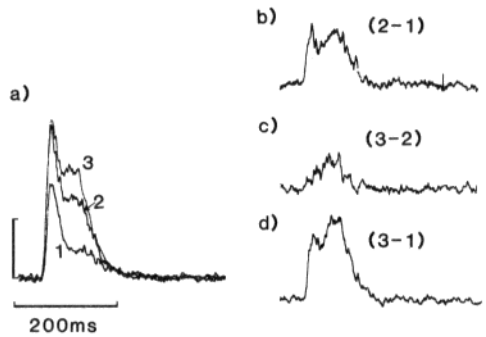

FIGURE 7. The relative changes in the two components of the aequorin signal after application of 10^{-7} M ouabain. (*a*) The averaged traces labeled 1–3 were obtained before, at 20 min, and at 30 min, respectively, after application of the drug. (*b*) The difference between traces 1 and 2. (*c*) The difference between traces 3 and 2. (*d*) The difference between traces 3 and 1.

levels below that of control conditions. This phenomenon is shown by the experiment illustrated in Fig. 11, in which 5×10^{-7} M strophanthidin was applied. A striking finding was that while the peak twitch tension fell, there was not an accompanying decrease in the amplitude of the aequorin signal. The fall in the force of contraction always occurred at the same time as the development of aftercontractions, altered membrane electrical activity, and detectably elevated [Ca^{2+}] during diastole. Fig. 12 illustrates that the same phenomenon is observed during superfusion with a solution containing 18 mM [Ca^{2+}]$_o$. Fig. 12, *a* and *b*, shows qualitatively similar effects for both high [Ca^{2+}]$_o$ and strophanthidin in the same preparation. The slow-speed recordings clearly show that the fall in the force of contraction is not accompanied by a fall in the luminescence in either case, and this effect is maintained throughout the period of exposure, when a steady state is reached. Thus, this phenomenon does not appear to be a direct effect of the drug, but occurs in [Ca^{2+}]-overloaded states.

TABLE II

*Steady State Effects of Low Concentrations of Strophanthidin on the [Ca^{2+}] Transient and Twitch Tension**

Experiment	Control		Drug		$T_{drug}/T_{control}$
	[Ca^{2+}]$_{L_1}$	[Ca^{2+}]$_{L_2}$	[Ca^{2+}]$_{L_1}$	[Ca^{2+}]$_{L_2}$	
D7	2.5×10^{-6}	1.6×10^{-6}	2.6×10^{-6}	2.4×10^{-6}	4.37
D8	1.2×10^{-6}	8.8×10^{-7}	1.4×10^{-6}	1.3×10^{-6}	1.79
D21	1.9×10^{-6}	1.3×10^{-6}	2.1×10^{-6}	1.7×10^{-6}	1.92
D24	2.1×10^{-6}	1.7×10^{-6}	2.2×10^{-6}	1.9×10^{-6}	1.65
Mean ± SEM:	$1.9 + 0.3 \times 10^{-6}$	$1.4 + 0.2 \times 10^{-6}$	$2.1 + 0.2 \times 10^{-6}$	$1.8 + 0.2 \times 10^{-6}$	2.4 + 0.6

* 10^{-7} M strophanthidin was used except in experiment D24 (2×10^{-7} M).

The [Ca^{2+}] During Aftercontractions and Transient Depolarizations

As can be seen from Fig. 12, there is more luminescence associated with the aftercontraction than with the control contraction, even though the control

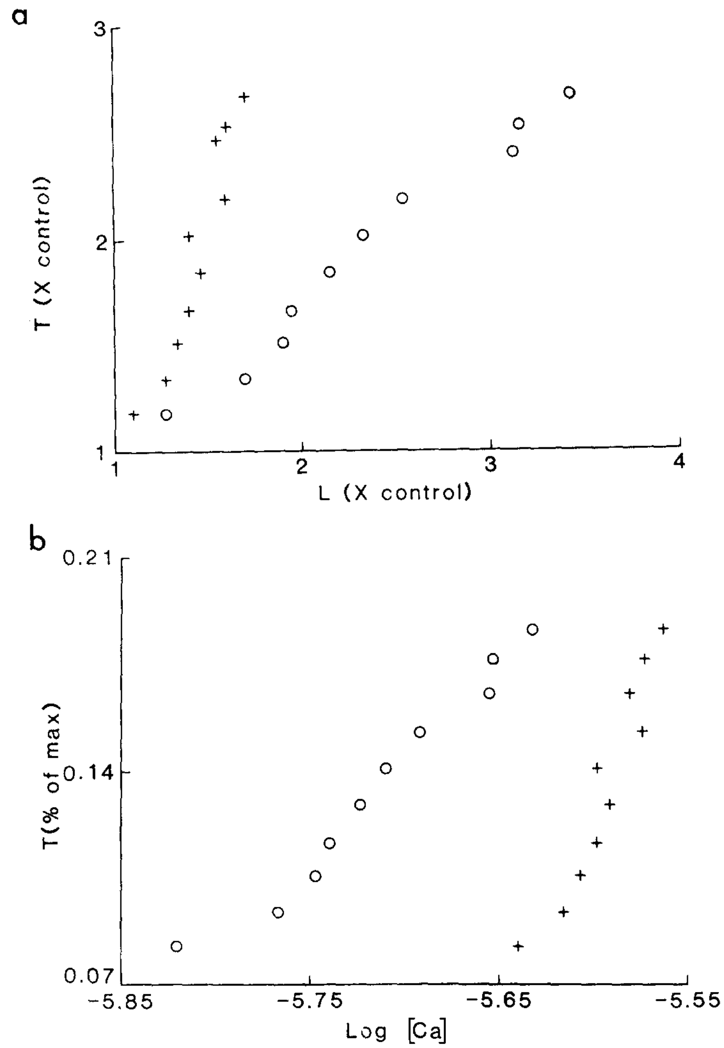

FIGURE 8. (*a*) Peak force of contraction plotted vs. L_1 (+) and L_2 (O) for the experiment illustrated in Fig. 5. All the data have been normalized to their respective control values. (*b*) Peak force of contraction plotted vs. [Ca^{2+}] calculated from L_1 (+) and L_2 (O). The value of [Ca^{2+}] calculated represents an upper limit on the spatial average cytoplasmic [Ca^{2+}] (see text for discussion of details).

contraction is about eight times larger than the aftercontraction. Because the luminescence and tension are changing slowly during the aftercontraction, and because the luminescence and tension changes are in phase, it seems reasonable to believe that spatial gradients of [Ca^{2+}] are not as great during the aftercontraction as they are during a normal contraction. Thus, the estimation of [Ca^{2+}] during the aftercontraction is likely to be quite reliable. We conclude that there is more spatial average [Ca^{2+}] associated with the aftercontraction than with the control contraction.

The reliability of the estimate of [Ca^{2+}] from luminescence associated with aftercontractions means that a [Ca^{2+}]-tension curve can be constructed. Fig. 13

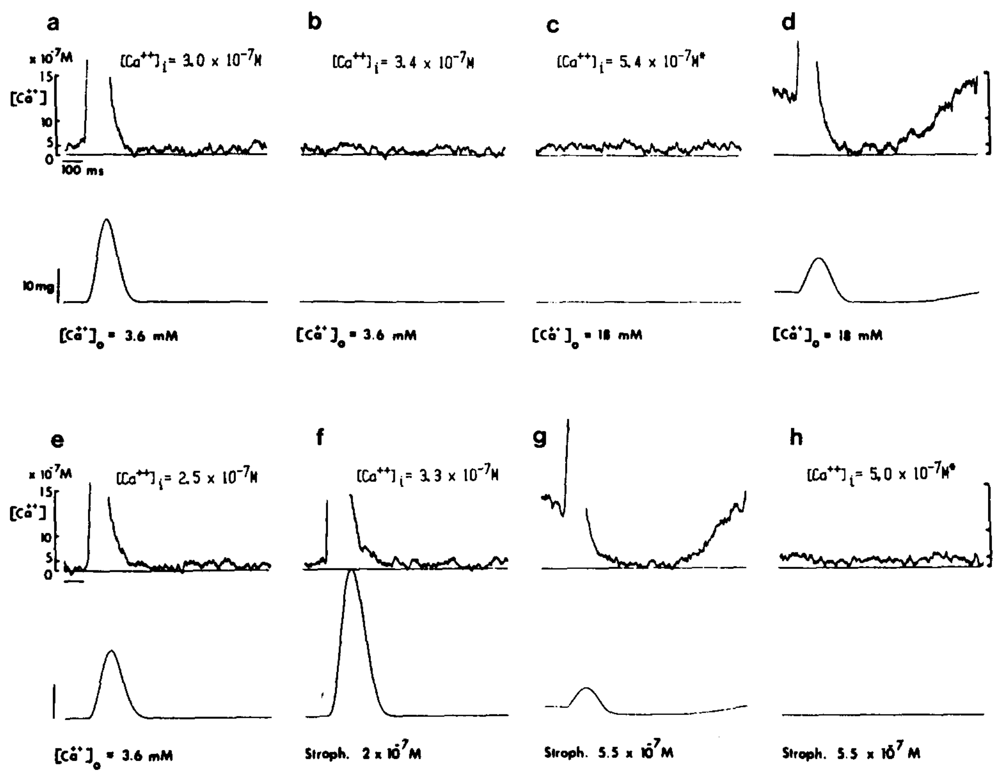

FIGURE 9. Comparison of levels of [Ca^{2+}] during diastole under various conditions in the same preparation. (*a–h*) Simultaneous records of aequorin luminescence (top) and tension (bottom) obtained under steady state conditions during successive interventions. (*a*) Stimulated at 1 Hz in $[Ca]_o$ = 3.6 mM. (*b*) At rest in $[Ca]_o$ = 3.6 mM. (*c*) At rest in $[Ca]_o$ = 18 mM. (*d*) Stimulated at 1 Hz in $[Ca]_o$ = 18 mM. (*e*) Stimulated at 1 Hz in $[Ca]_o$ = 3.6 mM. (*f*) Stimulated at 1 Hz in $[Ca]_o$ and 2.0×10^{-7} M strophanthidin. (*g*) Stimulated at 1 Hz in $[Ca]_o$ = 3.6 mM and 5.5×10^{-7} M strophanthidin. (*h*) Average of first 100 1-s sweeps after stimulation was stopped in $[Ca]_o$ = 3.6 mM and 5.5×10^{-7} M strophanthidin. Each record is the average of 100 sweeps. The values of [Ca^{2+}] above each record represent the mean diastolic [Ca^{2+}] during the last 500 ms of each sweep in *a*, *e*, and *f* and the mean resting [Ca^{2+}] during the whole sweep in *b*, *c*, and *h*. Resting and diastolic values for [Ca^{2+}] were also repetitively obtained during successive 100-beat intervals. The mean and standard deviation of these successive measurements were then used to test for significance of two different values. With this method only the values marked with an asterisk were found to be significantly higher than the control ($P < 0.05$).

illustrates such a curve. However, it must be stressed that this curve may not apply to control conditions because of the phenomenon noted above. It is, however, expected to be a steady state relationship because of the slowness and phase relationships of the changes in tension and luminescence (see inset). In the experiment illustrated, the maximum of the aftercontraction was estimated to be no more than 5% of the maximum force of which this muscle was capable (judged from the force of the TX-100 contracture). Thus, the [Ca^{2+}]-tension

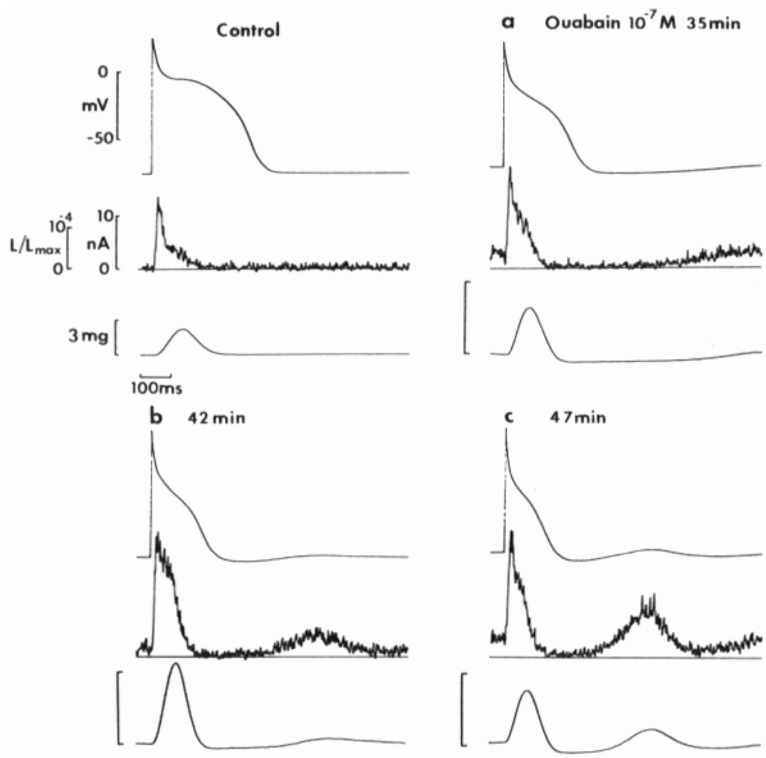

FIGURE 10. The toxic effects of 10^{-7} M ouabain. Simultaneous, averaged recordings of membrane potential, aequorin luminescence, and force of contraction. Recordings *a–c* are the result of averaging 100 sweeps (1 Hz), beginning at the times indicated after the application of 10^{-7} M ouabain. Same preparation as illustrated in Fig. 6.

curve illustrated represents only the very bottom part of the relationship and is probably shifted to the right compared with control conditions.

DISCUSSION

The quantification of intracellular [Ca^{2+}] with aequorin requires a number of assumptions (Blinks et al., 1982). We will discuss here the most important ones for our measurements. The quantification of diastolic [Ca^{2+}] with the method we have used requires that intracellular [Ca^{2+}] be steady and the same in the last

500 ms of each 1-s cardiac cycle. We were never able, even by analysis of average signals resulting from 1,000 sweeps, to detect any change in the aequorin luminescence during this period in normal muscles. Another assumption is that there are no damaged cells that contribute an abnormally high luminescence. We have observed that Purkinje fibers of diameter >300 μm and canine papillary muscles do not depolarize when impaled and microinjected with the same microelectrode which depolarizes small, short Purkinje fibers (unpublished data). Thus, the fibers we used permitted the detection of damaging microinjections. We do not report results for any Purkinje fiber that became depolarized during microinjection or that was >300 μm in diameter.

The values of $[Ca^{2+}]$ during diastole under control conditions measured with aequorin are in reasonably good agreement with those reported by other workers

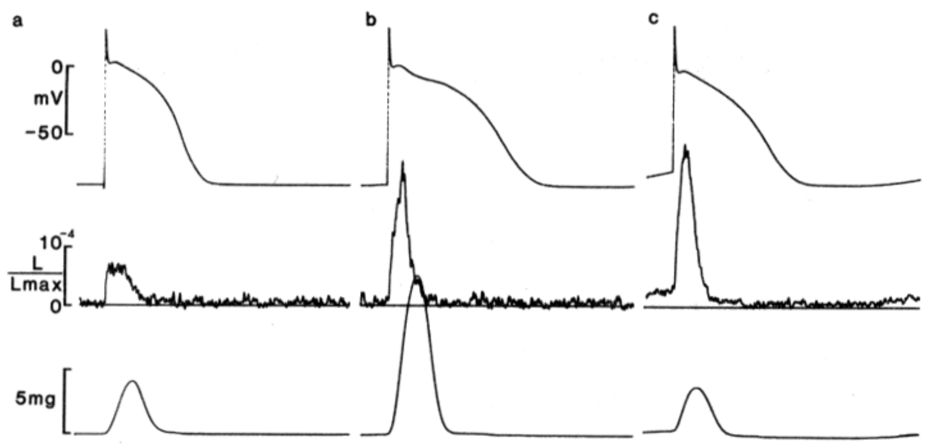

FIGURE 11. The toxic effects of 5×10^{-7} M strophanthidin. Simultaneous, averaged recordings of membrane potential, aequorin luminescence, and force of contraction. (*a*) Control; (*b*) the maximum positive inotropic effect; (*c*) the negative inotropic effect, and diastolic force development and depolarization. 100 sweeps at 1 Hz for all recordings.

who have used Ca-selective microelectrodes in resting cardiac preparations. For a complete list of references and a table of all the values, see Blinks et al. (1982). The values reported in cardiac Purkinje fibers are: 1.7×10^{-7} M (Coray et al., 1980), 2.1×10^{-7} M (Sokol et al., 1980), 3.1×10^{-7} M (Sheu, 1981), and 2.7×10^{-7} M (Sheu and Fozzard, 1982).

An approximately linear relationship between peak force of contraction and component L_2 of the aequorin signal was found in all experiments during the development of the positive inotropic effect at low doses or in the initial stages of a higher concentration drug effect. Wier and Isenberg (1982) found a similar linear relation between the amplitude of the aequorin signal and the force of contraction when the amplitude of voltage-clamp pulses was varied. They noted that the pCa-tension curve of Fabiato and Fabiato (1978) is nearly superimposable on the pCa-aequorin luminescence curve up to 60% of peak tension and that this might explain the linearity of the relationship between peak aequorin luminescence and peak tension. It would also have to be true that spatial gradients of $[Ca^{2+}]$ were neglible. With regard to this point, Fabiato (1981) has recently reported that $[Ca^{2+}]$ calculated from aequorin luminescence during the twitch of skinned cardiac cells is the same as that calculated by referring the twitch force to a steady state pCa-tension curve. While both the development of force and the change in aequorin luminescence in skinned cells are much slower than those in intact cells, the signals are still markedly out of phase, as they are during

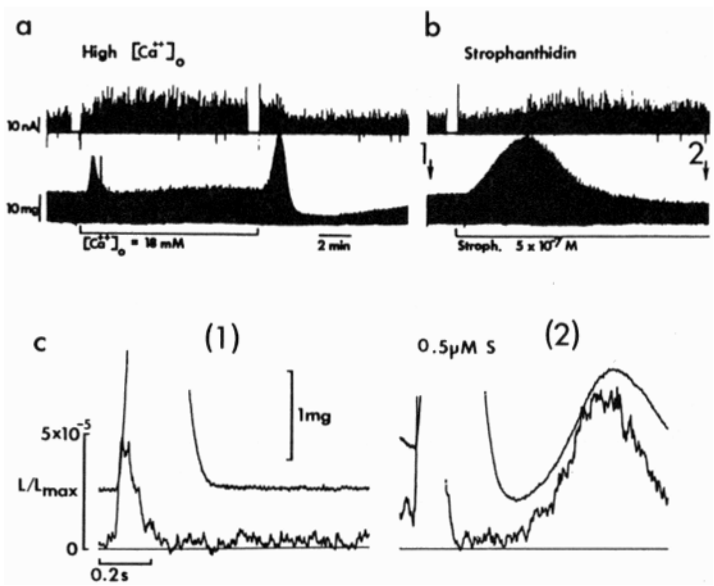

FIGURE 12. The toxic effects of high extracellular $[Ca^{2+}]$ are similar to those of 5×10^{-7} M strophanthidin. (*a*) Continuous recording of aequorin luminescence and force of contraction before, during, and after superfusion with a solution containing 18 mM $CaCl_2$. (*b*) Continuous recording of aequorin luminescence in the same muscle as in *a* before and during application of 5×10^{-7} M strophanthidin. (*c*) Averaged recordings (100 sweeps at 1 Hz) of aequorin luminescence and force of contraction with averaging beginning at the times indicated by the arrows marked 1 and 2 in *b*.

the twitch of an intact Purkinje fiber. Fabiato (1982) has also recently determined the steady state force-pCa curve for skinned canine Purkinje fibers at the same free $[Mg^{2+}]$ as we have assumed. The data for L_2 in Fig. 8 lie ~0.1 pCa unit to the right of that curve. Thus, it would appear that the fact that the aequorin signal and the tension development are out of phase does not necessarily indicate the presence of large spatial $[Ca^{2+}]$ gradients, nor, apparently, does it necessarily preclude in some other way the possibility that the aequorin signal is an accurate measure of $[Ca^{2+}]$ around the myofilaments. However, the data for L_1 lie ~0.1 pCa unit to the right of that for L_2; this could be accounted for by the presence of larger spatial gradients of $[Ca^{2+}]$ during L_1 than during L_2.

The effect of cardiotonic steroids in L_1 was variable. In some experiments, the increase in L_1 was very small (Figs. 4 and 5). Since a large positive inotropic effect was observed even in these experiments, we conclude that the positive inotropic effect is not directly related to an increase in L_1.

While the physiological interpretation of aequorin signals in canine Purkinje fibers is not yet completely clear, some points can be made. L_2 has now been correlated with the inotropic state of the muscle under a variety of conditions. These include the positive inotropic effect of rest or paired stimulation (Wier, 1980*a*), the transient positive inotropic effect of caffeine (Hess and Wier, 1984), and the positive inotropic effect of long voltage-clamp pulses (Wier and Isenberg, 1982). The above effects are most easily explained by the hypothesis that L_2

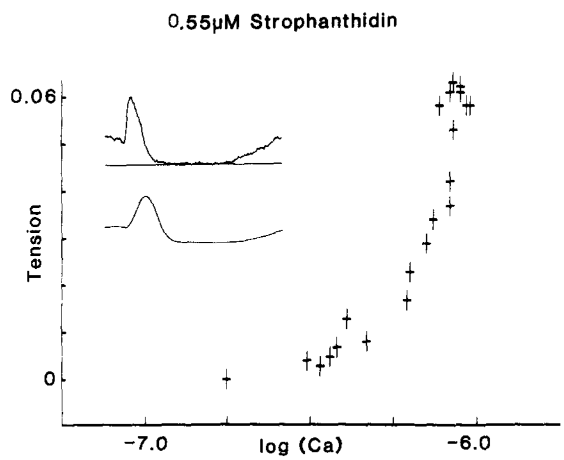

FIGURE 13. The relation between [Ca^{2+}] and force during diastole in a muscle intoxicated with strophanthidin. Force is expressed as a fraction of that obtained at the peak of the contracture in TX-100. The inset illustrates the averaged recordings from which the plotted data were obtained. Aequorin luminescence was filtered at 50 Hz. The muscle was stimulated at 1 Hz and averaging of both signals was performed over 200 s, during which there was no change in the configuration of the twitch and aftercontraction.

arises from Ca^{2+} released from an internal store, probably the sarcoplasmic reticulum. Therefore, we conclude that the positive inotropic effect of cardiotonic steroids is related to an increase in Ca^{2+} released from the sarcoplasmic reticulum, though not as a result of a direct action of the drug on the sarcoplasmic reticulum (see further discussion of this below).

The amplitude of L_1 does not correlate with the positive inotropic effects mentioned above, nor does it always correlate with the positive inotropic effect of cardiotonic steroids. The measured peak of L_1 is not likely to be a perfect measure of the processes underlying L_1, since there is certainly some overlap of the two components of the signal. Therefore, it is difficult to be certain of any effects on the processes underlying L_1. It has recently been reported that strophanthidin or other agents or conditions that increase intracellular [Ca^{2+}]

and [Na^+] increase slow inward current (Marban and Tsien, 1982; Weingart et al., 1978). The reported effect of strophanthidin was quite small. Thus, the relatively small effect of strophanthidin on L_1 is not incompatible with the idea that L_1 is closely associated with Ca^{2+} entering via the surface membrane Ca^{2+} channels (for further discussion of the source of Ca^{2+} for L_1, see Wier and Isenberg [1982]).

We were never able to detect any elevation of diastolic [Ca^{2+}] in association with a positive inotropic effect in the absence of aftercontractions or some tension change during diastole. Also, in no experiment was a tension change during diastole observed without some corresponding change in aequorin luminescence. It is difficult to be certain of our limit of detection, and it was slightly different in every experiment. However, elevation of [Ca^{2+}] to levels above 5×10^{-7} M would always have been detectable. Therefore, we will only conclude at this point that the development of a positive inotropic effect does not require that cytoplasmic [Ca^{2+}] during diastole be elevated to levels above 5×10^{-7} M, and that when [Ca^{2+}] is elevated to this level, other effects are apparent, i.e., small changes in tension and membrane potential during diastole.

While these experiments make it clear that the positive inotropic effect of cardiotonic steroids is associated with an increased cytoplasmic [Ca^{2+}] transient, they do little to elucidate the mechanism of that increase. A small change in diastolic [Ca^{2+}] could have occurred in association with a positive inotropic effect and gone undetected. Similarly, a small change in Ca^{2+} entry could have occurred and gone undetected. It is important to note that both the effects of positive inotropic and toxic doses of cardiac glycosides on [Ca^{2+}] are exactly mimicked (although with a different time course) by increased extracellular [Ca^{2+}]. This strongly emphasizes the indirect action of the drugs, via increased Ca uptake of the cells, but it does not permit us to decide whether this increased uptake occurs through enhanced transmembrane slow inward current, inhibition of Na/Ca exchange (secondary to Na-pump inhibition), or a combination of both. If the Na^+-lag hypothesis (Baker et al., 1969) is accepted as one component of the mechanism of cardiotonic steroid action, then our results suggest that at low concentrations of drug, the sarcoplasmic reticulum avidly takes up Ca^{2+} from the cytoplasm, thereby preventing a rise in cytoplasmic [Ca^{2+}] but producing an increased release of Ca^{2+} during the action potential.

The negative inotropic effect at high doses is not necessarily associated with a decreased amount of Ca^{2+} released from stores (Fig. 11). There is also apparently a shift in the relation between [Ca^{2+}] and tension. Recently, a decrease in intracellular pH has been measured during the action of cardiac glycosides (Deitmer and Ellis, 1980). Such changes can be expected any time intracellular [Ca^{2+}] rises to high levels (Vaughan-Jones et al., 1983). This phenomenon seems a likely candidate to contribute to the shifts in the [Ca^{2+}]-tension relation that we observed, by decreasing the affinity of the myofilaments for Ca^{2+}. Changes in cytoplasmic buffering of Ca^{2+} as a result of intracellular pH changes will also influence the cytoplasmic [Ca^{2+}] transient itself. However, as long as the aequorin signal represents the change in free [Ca^{2+}] in the cytoplasm, the relationship between [Ca^{2+}] and tension can still be assessed.

The experiments showed a clear correlation between the diastolic oscillations of membrane potential, tension, and aequorin luminescence observed with toxic doses of cardiac glycosides or high $[Ca^{2+}]_o$. Our results are in direct conflict with the conclusions of Vassalle and Lin (1979) that electrical toxicity may occur independently of an intracellular $[Ca^{2+}]$ accumulation. In no experiment was any change in one of the three signals observed without a corresponding change in the other two. The temporal correlation of the three signals was always close. Both the oscillations of tension and membrane potential are likely to be consequences of the oscillation in $[Ca^{2+}]$, which presumably represents cyclic Ca^{2+} release from the SR. The results very strongly support the idea of Ca^{2+}-induced changes in a nonselective membrane conductance for monovalent cations, as originally proposed by Kass et al. (1978*a*, *b*) and recently demonstrated in single-channel recordings from cardiac (Colquhoun et al., 1981) as well as nerve cells (Yellen, 1982). Our results, and the cited single-channel recordings, indicate that such channels are activated at a $[Ca^{2+}]$ of 5×10^{-7} M or higher. Thus, it seems possible that such a channel will be activated during the action potential. However, during the positive inotropic effect of cardiotonic steroids, we observed large increases in the cytoplasmic $[Ca^{2+}]$ transient with little or no consistent change in the plateau or repolarization of the action potential. Thus, it may be that the rapid transient elevation of $[Ca^{2+}]$ during the twitch contraction is not of sufficient duration to activate ion channels.

We thank J. R. Blinks for providing aequorin, laboratory space, equipment, and helpful discussion during the portion of the work performed at Mayo Foundation.
This work was supported by U. S. Public Health Service grants HL 12186, HL 27403, HL 29473, and HL 07111, and by a fellowship (to P. H.) from the Swiss National Science Foundation.

Received for publication 15 May 1983 and in revised form 19 August 1983.

REFERENCES

Allen, D. G., and J. R. Blinks. 1978. Calcium transients in aequorin-injected frog cardiac muscle. *Nature* (*Lond.*). 273:509–513.

Allen, D. G., J. R. Blinks, and F. G. Prendergast. 1977. Aequorin luminescence: relation of light emission to calcium concentration—a calcium-independent component. *Science* (*Wash. DC*). 196:996–998.

Allen, D. G., and S. Kurihara. 1980. Calcium transients in mammalian ventricular muscle. *Eur. Heart J.* 1(Suppl. A):5–15.

Baker, P. F., M. P. Blaustein, A. L. Hodgkin, and R. A. Steinhardt. 1969. The influence of calcium on sodium efflux in squid axons. *J. Physiol.* (*Lond.*). 200:431–458.

Blinks, J. R., P. H. Mattingly, B. R. Jewell, M. van Leeuwen, G. C. Harrer, and D. G. Allen. 1978. Practical aspects of the use of aequorin as a calcium indicator: assay, preparation, microinjection, and interpretation of signals. *Methods Enzymol.* 57:292–328.

Blinks, J. R., W. G. Wier, P. Hess, and F. G. Prendergast. 1982. Measurement of Ca^{2+} concentrations in living cells. *Prog. Biophys. Mol. Biol.* 40:1–114.

Cohen, I., J. Daut, and D. Noble. 1976. An analysis of the actions of low concentrations of ouabain on membrane currents in Purkinje fibers. *J. Physiol.* (*Lond.*). 260:75–103.

Colquhoun, D., E. Neher, H. Reuter, and C. F. Stevens. 1981. Inward current channels activated by intracellular Ca^{2+} in cultured cardiac cells. *Nature* (*Lond.*). 294:752–754.

Coray, A., C. H. Fry, P. Hess, J. A. S. McGuigan, and R. Weingart. 1980. Resting calcium in sheep cardiac tissue and in frog skeletal muscle measured with ion-selective microelectrodes. *J. Physiol.* (*Lond.*). 305:60P–61P.

Deitmer, J. W., and D. Ellis. 1978. The intracellular sodium activity of cardiac Purkinje fibers during inhibition and re-activation of the Na-K pump. *J. Physiol.* (*Lond.*). 284:241–259.

Deitmer, J. W., and D. Ellis. 1980. Interactions between the regulation of the intracellular pH and sodium activity of sheep cardiac Purkinje fibres. *J. Physiol.* (*Lond.*). 304:471–488.

Fabiato, A. 1981. Myoplasmic free calcium concentration reached during the twitch of an intact isolated cardiac cell and during calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. *J. Gen. Physiol.* 78:457–497.

Fabiato, A. 1982. Calcium release in skinned cardiac cells: variations with species, tissues and development. *Fed. Proc.* 41:2238–2244.

Fabiato, A., and F. Fabiato. 1978. Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *J. Physiol.* (*Lond.*). 276:233–255.

Greef, K. 1981. Cardiac Glycosides, Part I: Experimental Pharmacology. Springer-Verlag, Berlin.

Hart, G., D. Noble, and Y. Shimoni. 1983. The effects of low concentrations of cardiotonic steroids on membrane currents and tension in sheep Purkinje fibres. *J. Physiol.* (*Lond.*). 334:103–131.

Hess, P., P. Metzger, and R. Weingart. 1982. Free magnesium in sheep, ferret, and frog striated muscle at rest measured with ion-selective microelectrodes. *J. Physiol.* (*Lond.*). 333:173–188.

Hess, P., and W. G. Wier. 1984. Excitation-contraction coupling in cardiac Purkinje fibers. Effects of caffeine on the intracellular [Ca^{2+}] transient, membrane currents, and contraction. *J. Gen. Physiol.* 83:417–433.

Kass, R. S., W. Lederer, R. W. Tsien, and R. Weingart. 1978*a*. Role of calcium ions in transient inward currents and aftercontractions induced by strophanthidin in cardiac Purkinje fibers. *J. Physiol.* (*Lond.*). 281:187–208.

Kass, R. S., R. W. Tsien, and R. Weingart. 1978*b*. Ionic basis of transient inward current induced by strophanthidin in cardiac Purkinje fibers. *J. Physiol.* (*Lond.*). 281:209–226.

Lee, C. O., and M. Dagostino. 1982. Effect of strophanthidin on intracellular Na ion activity and twitch tension of constantly driven canine cardiac Purkinje fibers. *J. Physiol.* (*Lond.*). 40:185–198.

Lee, C. O., D. H. Kang, J. H. Sokol, and K. S. Lee. 1980. Relation between intracellular Na ion activity and tension of sheep cardiac Purkinje fibers exposed to dihydro-ouabain. *Biophys. J.* 29:315–330.

Lullman, H., and J. Peters. 1979. Action of cardiac glycosides on excitation-contraction coupling in heart muscle. *Prog. Pharmacol.* 2:1–57.

Marban, E., and R. W. Tsien. 1982. Enhancement of calcium current during digitalis inotropy in mammalian heart: positive feed-back regulation by intracellular calcium? *J. Physiol.* (*Lond.*). 329:589–614.

Morgan, J. P., and J. R. Blinks. 1982. Intracellular Ca^{2+} transients in the cat papillary muscle. *Can. J. Physiol. Pharmacol.* 60:520–528.

Noble, D. 1980. Review: mechanism of action of therapeutic levels of cardiac glycosides. *Cardiovasc. Res.* 9:495–514.

Sheu, S. S. 1981. Effects of ouabain on intracellular Na and Ca activities in sheep cardiac Purkinje fibers. *Fed. Proc.* 40:562.

Sheu, S. S., and H. A. Fozzard. 1982. Transmembrane Na^+ and Ca^+ electrochemical gradients in cardiac muscle and their relationships to force development. *J. Gen. Physiol.* 80:325–352.

Sokol, J. H., C. O. Lee, and F. J. Lupo. 1979. Measurement of the free calcium-ion concentration in sheep cardiac Purkinje fibers with neutral carrier Ca-selective microelectrodes. *Biophys. J.* 25:143*a*. (Abstr.)

Vassalle, M., and C. I. Lin. 1979. Effect of calcium on strophanthidin-induced electrical and mechanical toxicity in cardiac Purkinje fibers. *Science (Wash. DC)*. 207:1085–1087.

Vaughan-Jones, R. D., W. J. Lederer, and D. A. Eisner. 1983. Ca^{2+} ions can affect intracellular pH in mammalian cardiac muscle. *Nature (Lond.)*. 301:522–524.

Weingart, R., R. S. Kass, and R. W. Tsien. 1978. Is digitalis inotropy associated with enhanced slow inward calcium current? *Nature (Lond.)*. 273:389–392.

Wier, W. G. 1980*a*. Calcium transients during excitation-contraction coupling in mammalian heart: aequorin signals of canine Purkinje fibers. *Science (Wash. DC)*. 207:1085–1087.

Wier, W. G. 1980*b*. Acetylstrophanthidin effects on intracellular [Ca^{2+}] in aequorin-injected canine Purkinje fibers. *Fed. Proc.* 39:1732. (Abstr.)

Wier, W. G., and G. Isenberg. 1982. Intracellular [Ca^{2+}] transients in voltage clamped cardiac Purkinje fibers. Pflügers Arch. *Eur. J. Physiol.* 392:284–290.

Yellen, G. 1982. Single Ca-activated non-selective cation channels in neuroblastoma. *Nature (Lond.)*. 296:357–359.