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Evidence of a Role for Calmodulin in the Regulation of Calcium Release from Skeletal Muscle Sarcoplasmic Reticulum†

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ABSTRACT: The effect of calmodulin and calmodulin inhibitors on the "Ca^{2+} release channel" of "heavy" skeletal muscle sarcoplasmic reticulum (SR) vesicles was investigated. SR vesicles were passively loaded with $^{45}Ca^{2+}$ in the presence of calmodulin and its inhibitors, followed by measurement of $^{45}Ca^{2+}$ release rates by means of a rapid-quench–Millipore filtration method. Calmodulin at a concentration of 2–10 μM reduced $^{45}Ca^{2+}$ efflux rates from passively loaded vesicles by a factor of 2–3 in media containing 10^{-6}–10^{-3} M Ca^{2+}. At 10^{-9} M Ca^{2+}, calmodulin was without effect. $^{45}Ca^{2+}$ release rates were varied 1000-fold ($k_1 \simeq 0.1$–100 s^{-1}) by using 10^{-5} M Ca^{2+} with either Mg^{2+} or the ATP analogue adenosine 5′-(β,γ-methylenetriphosphate) in the release medium. In all instances, a similar 2–3-fold reduction in release rates was observed. At 10^{-5} M Ca^{2+}, $^{45}Ca^{2+}$ release was half-maximally inhibited by about 2×10^{-7} M calmodulin, and this inhibition was reversible. Heavy SR vesicle fractions contained 0.1–0.2 μg of endogenous calmodulin/mg of vesicle protein. However, the calmodulin inhibitors trifluoperazine, calmidazolium, and compound 48/80 were without significant effect on $^{45}Ca^{2+}$ release at concentrations which inhibit calmodulin-mediated reactions in other systems. Studies with actively loaded vesicles also suggested that heavy SR vesicles contain a Ca^{2+} permeation system that is inhibited by calmodulin.

Muscle contracts when the free Ca^{2+} concentration of the myofibrillar space reaches 10^{-6}–10^{-5} M by the release of Ca^{2+} stores from sarcoplasmic reticulum (SR)[1] via a putative Ca^{2+} channel (Ebashi, 1976; Endo, 1977; Winegrad, 1982). Muscle relaxes again when Ca^{2+} concentration is decreased below 10^{-7} M through the action of the membrane-bound Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase or Ca^{2+} pump of SR (Tada et al., 1978; Ikemoto, 1982; Martonosi & Beeler, 1983; Inesi, 1985). Release of Ca^{2+} by SR is triggered by an action potential at the neuromuscular junction that is communicated to SR via an extension of the surface membrane, the T system. Although the molecular basis of T-system depolarization-induced Ca^{2+} release by SR has remained obscure, recent studies have indicated the presence of a ligand-gated SR Ca^{2+} release channel which is activated by Ca^{2+} and adenine nucleotides and inhibited by Mg^{2+} (Stephenson, 1981; Onishi, 1981; Yamamoto & Kasai, 1982; Miyamoto & Racker, 1982; Fabiato, 1983; Kirino et al., 1983; Morii & Tonomura, 1983; Nagasaki & Kasai, 1983; Kim, et al., 1983; Meissner, 1984; Meissner et al., 1986).

The influence of calmodulin on Ca^{2+} uptake and release in SR has been studied primarily by using isolated vesicle fractions. In cardiac muscle, Ca^{2+}-stimulated ATPase and Ca^{2+} uptake activities are stimulated by calmodulin- and cAMP-dependent protein kinases. Both kinases exert their activating effects by phosphorylation of a 22 000-dalton protein called phospholamban (Tada et al., 1982). Skeletal muscle SR vesicles also have been found to contain a calmodulin-dependent protein kinase as well as calmodulin; however, activation of the Ca^{2+} transport system by calmodulin has not yet been shown (Campbell & MacLennan, 1981; Chiesi & Carafoli, 1982, 1983; Seiler et al., 1984; Eibschutz et al., 1984).

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[1] Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; AMP-PCP, adenosine 5′-(β,γ-methylenetriphosphate); Pipes, 1,4-piperazinediethanesulfonic acid; cAMP, adenosine cyclic 3′,5′-phosphate.

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Other more indirect approaches involving the use of calmodulin inhibitors have been inconclusive in that these drugs seem to inhibit the ATPase in a reaction that is not mediated by calmodulin (Anderson et al., 1984; Prokopjeva et al., 1984). Tuana & MacLennan (1984) observed that two potent inhibitors of calmodulin, calmidazolium and compound 48/80, inhibit calmodulin-dependent protein phosphorylation and ATP-dependent Ca^{2+} uptake but not Ca^{2+}-stimulated ATPase activity in skeletal muscle SR vesicles. Kim & Ikemoto (1985) recently reported that the amount of Ca^{2+} release decreased in parallel with an increased extent of phosphorylation of a 60 000-dalton protein by calmodulin. These results have led to the suggestion that in skeletal muscle a calmodulin-dependent protein kinase might be involved in the regulation of a Ca^{2+} release channel rather than the Ca^{2+} pump.

In this report, we present evidence that Ca^{2+} release from "heavy" SR vesicles is inhibited by calmodulin. Our results suggest that calmodulin does not exert its effect via a calmodulin-dependent phosphorylation reaction but instead through a direct interaction with the SR Ca^{2+} release channel or a membrane component which acts on the channel. A preliminary account of part of this work has appeared elsewhere (Meissner & Darling, 1985).

Materials and Methods

Materials. Calmodulin isolated with greater than 98% purity from bovine brain, the catalytic subunit of protein kinase from bovine heart, and the ATP analogue AMP-PCP were obtained from Sigma, St. Louis, MO. Calmodulin levels in heavy SR Ca^{2+} release fractions were measured with the use of an ^{125}I-calmodulin radioimmune assay kit from New England Nuclear (Boston, MA). $^{45}Ca^{2+}$ was purchased from ICN Pharmaceuticals, Irvine, CA. All other reagents were of reagent grade.

Isolation of Vesicles. Rabbit skeletal muscle sarcoplasmic reticulum was fractionated into "heavy Ca^{2+} release" and "light control" vesicle fractions by differential and sucrose gradient centrifugation as described in the preceding paper (Meissner et al., 1986).

$^{45}Ca^{2+}$ Flux Measurements with Passively Loaded Vesicles. $^{45}Ca^{2+}$ efflux rates from vesicles passively loaded with $^{45}Ca^{2+}$ were determined with the use of an Update System 1000 chemical quench apparatus (Madison, WI) and by Millipore filtration (Meissner, 1984; Meissner et al., 1986). Vesicles (2–10 mg of protein/mL) were incubated at 22 °C with or without exogenously added calmodulin in a medium containing 20 mM K-Pipes, pH 7, 0.1 M KCl, 0.1 mM EGTA, and 0.106 mM $^{45}Ca^{2+}$. After 5 min, the Ca^{2+} concentration was raised to 1.1 mM, and vesicles were incubated for an additional 2 h at 22 °C.

$^{45}Ca^{2+}$ Flux Measurements with Actively Loaded Vesicles. $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ release by actively loaded vesicles were determined as follows. Vesicles (25–35 μg of protein) were incubated for 1 min in the presence or absence of exogenously added calmodulin at 22 °C in 0.5 mL of a medium containing 20 mM K-Pipes, pH 7, 0.1 M KCl, 10 μM free Ca^{2+} (100 μM EGTA plus 106 μM Ca^{2+}), and varying concentrations of Mg^{2+}. Uptake media also contained 5 mM NaN_3 and 1.5 μM carbonyl cyanide *m*-chlorophenylhydrazone in order to inhibit possible Ca^{2+} uptake by contaminating mitochondria (Meissner, 1984). $^{45}Ca^{2+}$ uptake was initiated by the addition of 12.5 μL of 0.2 M ATP. After 1 min, an aliquot of 0.4 mL was placed on a 0.45-μm Millipore filter and rinsed for 10 s with a 20 mM K-Pipes, pH 7, and 0.1 M KCl medium that either inhibited (10 mM Mg^{2+} and 10 μM ruthenium red) or promoted (100 μM EGTA and 106 μM Ca^{2+}) $^{45}Ca^{2+}$ efflux from Ca^{2+} release vesicles. Radioactivity retained by the vesicles on the filters was determined by liquid scintillation counting.

Calmodulin Content of Heavy SR Vesicle Fractions. Heavy SR vesicle fractions at a protein concentration of 1 mg/mL were incubated for 10 min at 22 °C or heated for 10 min at 95 °C in media containing either 20 mM K-Pipes, pH 7, 0.1 M KCl, 100 μM EGTA, and 106 μM Ca^{2+} (10 μM free Ca^{2+}) or 0.125 M borate, pH 8.4, 0.075 M NaCl, 0.2% bovine serum albumin, and 1 mM EGTA ($<10^{-10}$ M free Ca^{2+}). After treatment, vesicles were sedimented for 30 min at 100000*g* in a Beckman airfuge. The supernatants of samples not heated at 95 °C were incubated for 10 min at 95 °C. The calmodulin content of the supernatant fractions was measured with the use of a New England Nuclear ^{125}I-calmodulin radioimmune assay kit.

Biochemical Assays. Protein was determined by the Lowry method using bovine serum albumin as a standard. Free Ca^{2+} and Mg^{2+} concentrations were calculated according to a computer program using binding constants published by Fabiato (1981).

Results

Effect of Calmodulin on Ca^{2+}-Induced $^{45}Ca^{2+}$ Release. The effect of calmodulin on the Ca^{2+} release behavior of heavy SR vesicles was studied by passively loading the vesicles with 1 mM $^{45}Ca^{2+}$ in the presence and absence of 6 μM calmodulin. Vesicles were diluted into a medium which contained either the two Ca^{2+} release inhibitors Mg^{2+} and ruthenium red or 10 μM free Ca^{2+}. Rapid initial $^{45}Ca^{2+}$ efflux rates in Ca^{2+} release promoting medium were determined with the use of a rapid-quench apparatus (Meissner et al., 1986). Between 50 and 70 nmol of $^{45}Ca^{2+}$/mg of protein was retained by the vesicles when these were diluted directly into a medium containing 10 mM Mg^{2+} and 10 μM ruthenium red (Figure 1). In the presence of the two channel inhibitors, $^{45}Ca^{2+}$ was slowly released [0.2 nmol (mg of protein)$^{-1}$ s^{-1}]. In contrast, vesicles rapidly released $^{45}Ca^{2+}$ when placed into the 10 μM Ca^{2+} medium. The inset of Figure 1 shows that Ca^{2+} releasing vesicles not preincubated with calmodulin released $^{45}Ca^{2+}$ with a first-order rate constant of about 1 s^{-1} which corresponds to an initial release rate of about 70 nmol of $^{45}Ca^{2+}$ (mg of protein)$^{-1}$ s^{-1}. Prior exposure of the vesicles to calmodulin slowed down $^{45}Ca^{2+}$ release by a factor of ~2. The amount of rapidly released $^{45}Ca^{2+}$ was not affected by calmodulin (Figure 1).

The effectiveness of calmodulin in decreasing the rate of $^{45}Ca^{2+}$ release was dependent on the free Ca^{2+} concentration in the release medium (Figure 2). As previously observed (Meissner et al., 1986), $^{45}Ca^{2+}$ release was maximal at 10^{-6}–10^{-5} M free Ca^{2+} for vesicles which did not contain exogenously added calmodulin. Addition of calmodulin reduced the rate constant of $^{45}Ca^{2+}$ release at free Ca^{2+} concentrations in excess of 10^{-7} M. In release media containing 10^{-6}–10^{-3} M Ca^{2+}, calmodulin reduced the rate constants by a factor of 2–3. At 10^{-7} M Ca^{2+}, calmodulin was only slightly effective in slowing down $^{45}Ca^{2+}$ release, whereas at 10^{-9} M Ca^{2+}, calmodulin was without effect. The loss of effectiveness of calmodulin as an inhibitor of $^{45}Ca^{2+}$ release at low Ca^{2+} concentrations is in agreement with what has been observed for other calmodulin-regulated proteins and implies that the Ca^{2+}–calmodulin complex may be the inhibitory species (Klee & Vanaman, 1982; Cox, 1984).

Effect of AMP-PCP, Mg^{2+}, and Calmodulin on Ca^{2+}-Induced Ca^{2+} Release. The rate of $^{45}Ca^{2+}$ efflux from heavy SR vesicles was dependent on the presence or absence of

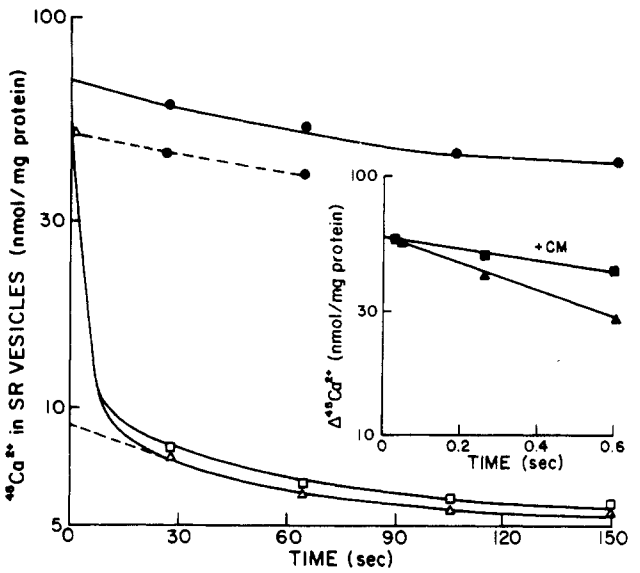

FIGURE 1: Measurement of $^{45}Ca^{2+}$ efflux rates from passively loaded vesicles. A heavy SR Ca^{2+} release fraction (2 mg of protein/mL) was preincubated for 2 h at 22 °C in a medium containing 20 mM K-Pipes, pH 7, 0.1 M KCl, 1.1 mM $^{45}Ca^{2+}$, 0.1 mM EGTA, and either 0 (●, △, ▲) or 6 μM (□, ■) calmodulin (CM). Vesicles were diluted at 22 °C 50-fold into release media containing 20 mM K-Pipes, pH 7, 0.1 M KCl, and 10 mM Mg^{2+} plus 10 μM ruthenium red (●) or 1 mM EGTA plus 0.95 mM Ca^{2+} (10 μM free Ca^{2+} final concentration) (□, △). An Update System 1000 chemical quench apparatus was used to determine rapid $^{45}Ca^{2+}$ efflux rates. Vesicles were mixed with 4 volumes of 6.25 mM EGTA and 5.77 mM Ca^{2+} medium. After the addition of the vesicles, the free Ca^{2+} concentration of the medium was 10 μM (5 mM EGTA plus 4.82 mM Ca^{2+}). Rapid $^{45}Ca^{2+}$ efflux was inhibited at 260 ms by the addition of 4 additional volumes of a quench solution containing 22 mM Mg^{2+} and 22 μM ruthenium red. Vesicles were subsequently placed on 0.45-μm Millipore filters and rinsed with a medium containing 10 mM Mg^{2+} and 10 μM ruthenium red. Amounts of $^{45}Ca^{2+}$ initially trapped by all vesicles (68 nmol/mg of protein) as well as amounts not readily released by a subpopulation of vesicles (9 nmol/mg of protein) were obtained by back-extrapolation to the time of vesicle dilution. In the inset, the time course of $^{45}Ca^{2+}$ efflux from the vesicle population capable of rapid Ca^{2+} release (59 nmol/mg of protein) was obtained by adding at 25, 260, and 600 ms the two Ca^{2+} release inhibitors Mg^{2+} and ruthenium red.

Table I: Effects of Calmodulin on Ca^{2+} Release from Heavy SR Vesicles[a]

additions to release medium	$^{45}Ca^{2+}$ efflux, k (s^{-1}) −CM	+CM
10 μM Ca^{2+}, 10 mM Mg^{2+}, 10 μM RR	0.004	0.004
10 μM Ca^{2+}	0.96	0.40
10 μM Ca^{2+}, 5 mM AMP-PCP	82	46
10 μM Ca^{2+}, 5 mM Mg^{2+}, 5 mM AMP-PCP	7.7	3.0
10 μM Ca^{2+}, 8.5 mM Mg^{2+}, 5 mM AMP-PCP	0.9	0.4
10 μM Ca^{2+}, 1 mM Mg^{2+}	0.023	0.01

[a] Heavy SR vesicles were passively loaded with 1 mM $^{45}Ca^{2+}$ in the absence or presence of 10 μM calmodulin (CM). Release media contained after the addition of the vesicles 0 or 2 μM calmodulin, 10 μM free Ca^{2+}, and the indicated concentrations of Mg^{2+}, AMP-PCP, and ruthenium red (RR). The first-order rate constants of $^{45}Ca^{2+}$ release from the Ca^{2+}-permeable vesicle population were determined as indicated in Figure 1.

AMP-PCP and Mg^{2+} in the release medium (Table I). Nucleotide-stimulated Ca^{2+} release was studied with the use of the nonhydrolyzable ATP analogue AMP-PCP in order to prevent reuptake of the released $^{45}Ca^{2+}$ by the SR Ca^{2+} pump. Addition of 5 mM AMP-PCP to a 10 μM free Ca^{2+} release medium increased the rate constant of $^{45}Ca^{2+}$ release from about 1 to 82 s^{-1}. Mg^{2+} negated the effect of AMP-PCP in

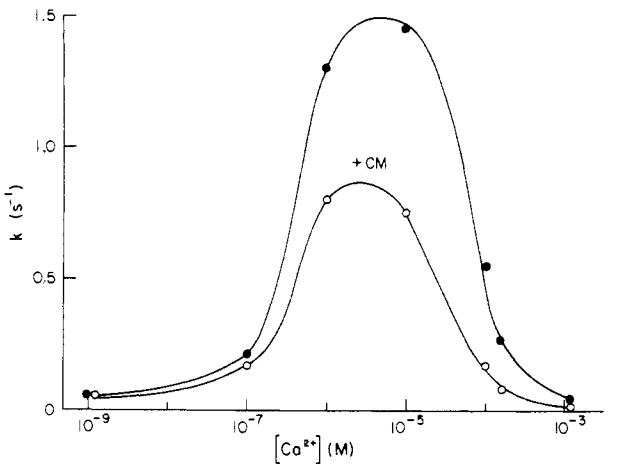

FIGURE 2: Ca^{2+} concentration dependence of calmodulin inhibition of $^{45}Ca^{2+}$ efflux. A heavy SR Ca^{2+} release fraction, preincubated for 2 h at 22 °C with 1 mM $^{45}Ca^{2+}$ in the absence or presence of 6 μM calmodulin, was diluted 5-fold into release media containing amounts of EGTA plus Ca^{2+} to yield the indicated final concentrations of free Ca^{2+}. After addition of vesicles, release media contained 1.2 μM calmodulin. Rapid $^{45}Ca^{2+}$ efflux rates from the Ca^{2+}-permeable vesicle fraction were determined by using the rapid-quench apparatus (cf. Figure 1).

that it reduced release rates to values seen in the absence of the nucleotide. In the absence of nucleotide, Mg^{2+} nearly fully inhibited $^{45}Ca^{2+}$ release at a concentration of 1 mM.

Calmodulin reduced the rate constants of $^{45}Ca^{2+}$ efflux from heavy SR vesicles by a factor of 1.8–2.6 in media containing 10 μM free Ca^{2+} and varying concentrations of AMP-PCP and Mg^{2+} (Table I). Thus, under a wide range of release conditions where $^{45}Ca^{2+}$ release rates differed by a factor of 1000 or more, calmodulin reduced the $^{45}Ca^{2+}$ release rate by approximately the same percentage.

The dependence of the $^{45}Ca^{2+}$ release rate on calmodulin concentration was determined by adding 0–10 μM calmodulin to incubation and release media. In the absence of exogenous calmodulin, the rate constant of $^{45}Ca^{2+}$ release was 38 s^{-1} for vesicles diluted into release media containing 10 μM free Ca^{2+} and 1 mM AMP-PCP. Ca^{2+} release was half-maximally inhibited at a calmodulin concentration of about 0.2 μM (k = 29 s^{-1}) and maximally inhibited at 2–5 μM calmodulin (k = 20 s^{-1}) (not shown). For comparison, the calmodulin concentration has been reported to be approximately 2 μM in rabbit skeletal muscle (Yagi et al., 1978).

Time Course of Calmodulin Inhibition. The time scale of inhibition and reversal of inhibition of $^{45}Ca^{2+}$ release by calmodulin was determined by preincubating vesicles with and without a saturating calmodulin concentration and dilution into media containing or lacking calmodulin. In Figure 3A, vesicles were diluted into release media containing 10 μM free Ca^{2+}, 5 mM AMP-PCP, and 5 or 8 mM Mg^{2+}. The two Mg^{2+} concentrations were chosen to observe calmodulin inhibition on two different time scales in the presence of adenine nucleotide. In both instances, addition of 6 μM calmodulin to incubation and release media reduced the rate constant of $^{45}Ca^{2+}$ release by a factor of 2 (4.1 vs. 2.1 s^{-1} at 5 mM Mg^{2+}, and 0.75 vs. 0.37 s^{-1} at 8 mM Mg^{2+}). Intermediate amounts of $^{45}Ca^{2+}$ were released by vesicles incubated without calmodulin but diluted into media containing calmodulin. With time, $^{45}Ca^{2+}$ release appeared to approach a rate seen for vesicles exposed to calmodulin for 2 h during passive loading with 1 mM $^{45}Ca^{2+}$.

Reversal of calmodulin inhibition of Ca^{2+} release was investigated in Figure 3B using release media containing 10 μM

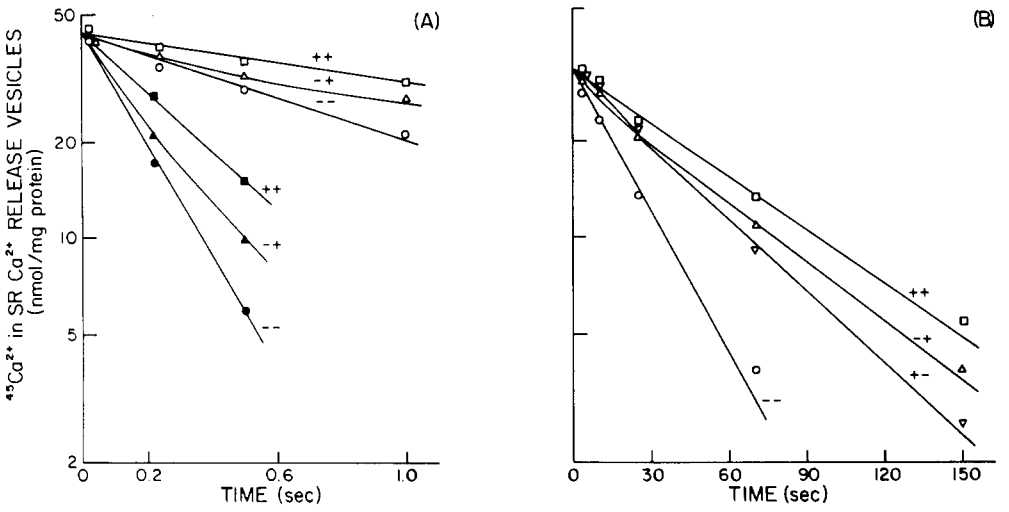

FIGURE 3: Time course of inhibition of $^{45}Ca^{2+}$ release by calmodulin. A heavy SR vesicle fraction [2 mg of protein/mL in (A), 8 mg of protein/mL in (B)] was passively loaded with 1 mM $^{45}Ca^{2+}$ in the absence or presence of exogenous calmodulin as described under Materials and Methods. $^{45}Ca^{2+}$ efflux from the Ca^{2+}-permeable vesicle fraction was determined as described in Figure 1. In panel A, after addition of vesicles, release media contained 10 μM free Ca^{2+}, 5 mM AMP-PCP, and 5 (■, ▲, ●) or 8 mM (□, △, ○) Mg^{2+}. Vesicles incubated in the absence (○, ●, △, ▲) or presence (□, ■) of 6 μM calmodulin were diluted 5-fold into release media containing 0 (○, ●) or 6 μM (□, △, ■, ▲) calmodulin. In panel B, vesicles were diluted into release media containing 10 μM free Ca^{2+} (final concentration) and 0.6 mM Mg^{2+}. Vesicles incubated in the absence (○, △) or presence (□, ▽) of 5 μM calmodulin were diluted 250-fold into media containing 0 (○, ▽) or 5 μM (□, △) calmodulin. Plus and minus signs indicate the presence or absence of exogenously added calmodulin in vesicle and dilution media.

free Ca^{2+} and 0.6 mM Mg^{2+}. The addition of 0.6 mM Mg^{2+} retarded $^{45}Ca^{2+}$ release so that it could be measured without the use of the rapid-quench apparatus. By slowing down $^{45}Ca^{2+}$ release and avoiding the use of the rapid-quench apparatus which affords only a 5-fold dilution, we were able to lower the calmodulin concentration from 5 to 0.02 μM during vesicle dilution. Addition of 5 μM calmodulin to both incubation and release media decreased the rate constant of $^{45}Ca^{2+}$ release 2.5-fold from 0.033 to 0.013 s^{-1}. A similar reduction in $^{45}Ca^{2+}$ release was observed with time for vesicles not preincubated with calmodulin but diluted into the release medium containing 5 μM calmodulin. Therefore, on a time scale of 10–60 s, calmodulin was essentially able to exert its full inhibitory effect regardless of whether vesicles were preincubated in the presence or absence of calmodulin. A partial and gradual loss of calmodulin inhibition of $^{45}Ca^{2+}$ release was observed for vesicles preincubated with 5 μM calmodulin but subsequently diluted 250-fold into a calmodulin-free medium.

In other systems, calmodulin exerts its regulatory effect by binding to proteins at micromolar concentrations of Ca^{2+} and dissociating again at $<10^{-8}$ M Ca^{2+} (Klee & Vanaman, 1982; Cox, 1984). We were not able to do experiments at low Ca^{2+} concentrations since the effects of calmodulin on the Ca^{2+} release behavior of the vesicles could only be clearly monitored in release media containing micromolar concentrations of free Ca^{2+} (cf. Figure 2). Despite this limitation, two tentative conclutions may be drawn from the flux data of Figure 3. First, calmodulin appears, at least under our experimental conditions, to exert its full inhibitory effect only after several seconds of incubation. Second, calmodulin inhibition of Ca^{2+} release from sarcoplasmic reticulum vesicles appears to be reversible.

Effect of Calmodulin Inhibitors on $^{45}Ca^{2+}$ Release. The calmodulin content of heavy SR vesicle fractions was determined by heating the samples and measuring the solubilized calmodulin by a radioimmune assay (see Materials and Methods). Between 0.1 and 0.2 μg of calmodulin/mg of vesicle protein was solubilized when heavy SR vesicle fractions were heated at pH 8.4 in the presence of 1 mM EGTA. Heating at pH 7 in the presence of 10 μM free Ca^{2+} or treatment at pH 8.4 with 1 mM EGTA without heating released only between 0.05 and 0.10 μg of calmodulin/mg of vesicle protein, suggesting that significant amounts of calmodulin may be tightly bound by the release vesicle fractions. The amount of calmodulin released at pH 8.4 in 1 mM EGTA following heating was, in principle, sufficient to occupy all of the Ca^{2+} release channels, assuming that the channel is made up of protein(s) with a molecular weight of 200 000, that the vesicles have a diameter of 0.015 μm, and that there is on average one channel per vesicle.

A possible effect of endogenous calmodulin on the Ca^{2+} release process from heavy SR vesicles was assessed by using the calmodulin inhibitors trifluoperazine (25 μM), calmodulin (5 and 25 μM), and compound 48/80 (5 and 25 μg/mL) at concentrations which in other systems (Tuana & MacLennan, 1984) have been reported to inhibit calmodulin-mediated reactions. The drugs were added to the vesicles (2 mg of protein/mL) 10–15 min prior to the rapid-quench experiments, and $^{45}Ca^{2+}$ release was determined in a 10 μM free Ca^{2+} release medium containing the inhibitors. None of the drugs significantly affected the time course of $^{45}Ca^{2+}$ release. In the rapid-quench experiments, relatively high vesicle concentrations of 2 and 0.4 mg of protein/mL were used in the incubation and release media, respectively. As shown for calmidazolium in Figure 4, the drugs increased the permeability of the vesicles to $^{45}Ca^{2+}$ in a 10 μM free Ca^{2+}–5 mM Mg^{2+} release medium when the vesicle concentration was decreased from 100 to 10 μg of protein/mL. At the reduced vesicle concentration, calmidazolium rendered Ca^{2+} release vesicles readily permeable to $^{45}Ca^{2+}$, even when these were directly diluted into release media containing the channel blockers Mg^{2+} and ruthenium red. Light control vesicles which lack the channel also were unable to retain $^{45}Ca^{2+}$ when present at a protein concentration of 10 μg/mL (not shown). Further, Ca^{2+} release vesicle fractions became permeable to [^{3}H]sucrose, a solute which in the absence of calmidazolium only slowly permeates across the membranes of heavy SR vesicles (not shown). Taken together, these results suggested a nonspecific breakdown of the permeability barrier of the vesicles. Since calmidazolium is lipophilic, it appeared that the drug rendered

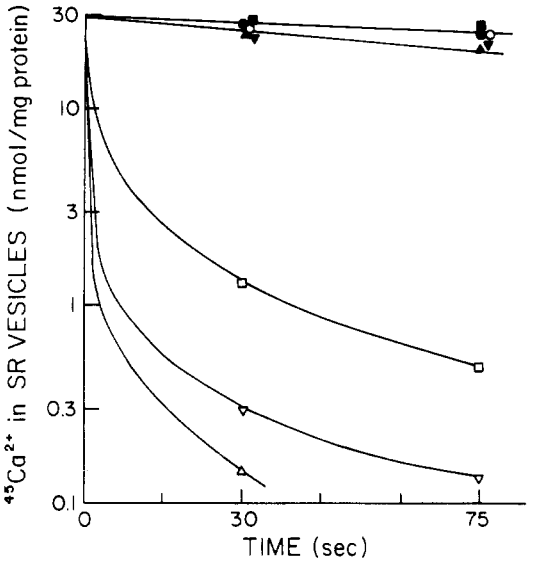

FIGURE 4: Effect of calmidazolium on $^{45}Ca^{2+}$ efflux from heavy SR Ca^{2+} release vesicles. A heavy SR Ca^{2+} release vesicle fraction was incubated at a protein concentration of 10 mg/mL for 2 h at 22 °C in a medium containing 20 mM K-Pipes, pH 7, 0.1 M KCl, 0.1 mM EGTA, and 1.1 mM $^{45}Ca^{2+}$. After 2 h, calmidazolium (2 mM in dimethyl sulfoxide) was added at a concentration of 0 (○, ●), 5 (□, ■), or 20 μM (△, ▲, ▽, ▼), and vesicles were incubated for an additional 15 min. $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles 100-fold [final protein concentration 100 μg/mL (●, ■, ▲, ▼)] or 1000-fold [final protein concentration 10 μg/mL (○, □, △, ▽)] into release media containing 20 mM K-Pipes, pH 7, 0.1 M KCl, 1 mM EGTA, 0.97 mM Ca^{2+}, 5 mM Mg^{2+}, and either 0 (○, ●), 5 (□, ■), or 20 μM (△, ▲) calmidazolium or 20 μM calmidazolium plus 10 μM ruthenium red (▽, ▼). Amounts of $^{45}Ca^{2+}$ remaining with the vesicles were determined by placing vesicle suspensions on 0.45-μm Millipore filters followed by rapid rinsing to remove extravesicular $^{45}Ca^{2+}$.

the vesicles leaky by partitioning into the lipid bilayer and disrupting the membrane.

$^{45}Ca^{2+}$ Uptake and $^{45}Ca^{2+}$ Release by Actively Loaded Vesicles. The effect of calmodulin on the Ca^{2+} release behavior of sarcoplasmic reticulum was also investigated by using actively loaded SR vesicle fractions. Heavy SR Ca^{2+} release vesicle fractions (Figure 5) as well as light SR control vesicle fractions lacking the Ca^{2+} release channel (Meissner, 1984) were actively loaded in media containing 10 μM free $^{45}Ca^{2+}$, 5 mM ATP, 7.5 mM Mg^{2+}, and varying concentrations of calmodulin. Amounts of $^{45}Ca^{2+}$ sequestered by the Ca^{2+}-impermeable and -permeable vesicle populations of the release fraction (cf. Figure 1) were determined by Millipore filtration and rinsing for 10 s with a washing solution which either blocked (10 mM Mg^{2+} plus 10 μM ruthenium red) or activated (10 μM free Ca^{2+}) the SR Ca^{2+} release channel. The difference in $^{45}Ca^{2+}$ retained by the vesicles under the two washing conditions indicated the amount of $^{45}Ca^{2+}$ taken up by the Ca^{2+}-permeable vesicle population.

The Ca^{2+} release vesicle fraction retained optimal amounts of $^{45}Ca^{2+}$ (45 nmol/mg of protein) when loaded in and washed with media containing ruthenium red (Figure 5). Amounts sequestered in the presence of ruthenium red were essentially the same whether calmodulin was added to the uptake medium or not. About 10 nmol of $^{45}Ca^{2+}$/mg of protein remained with the vesicles when these were loaded in the absence of ruthenium red and subsequently rinsed with the 10 μM free Ca^{2+} medium which promoted Ca^{2+} release via the channel. Accordingly, about 25% of the sequestered $^{45}Ca^{2+}$ seemed to be taken up by vesicles which lacked the Ca^{2+} release channel. Calmodulin did not significantly affect the amount of $^{45}Ca^{2+}$ retained by the Ca^{2+}-impermeable vesicle fraction. A similar independence on exogenously added calmodulin was observed when a light SR control vesicle fraction was used which lacked the Ca^{2+} release channel (not shown). In contrast, calmodulin increased about 2-fold the amount of $^{45}Ca^{2+}$ taken up by the Ca^{2+}-permeable vesicle population of the release fraction (Figure 5). The Ca^{2+} sequestering capability of the Ca^{2+} release vesicles was increased half-maximally at a calmodulin concentration of ~0.3 μM.

One explanation of the data of Figure 5 is that calmodulin slowed $^{45}Ca^{2+}$ release from heavy SR vesicles, thereby increasing their Ca^{2+} sequestering capability. Another possibility, i.e., activation of the SR Ca^{2+} pump by calmodulin, appeared to be less likely. We found that prior incubation with calmodulin did not increase the Ca^{2+}-stimulated ATPase activity of the vesicles (not shown). The ATP hydrolytic activity of the vesicles was determined under the uptake conditions of Figure 5 except that the vesicles were rendered permeable to Ca^{2+} by the addition of the ionophore A23187 (2 μg/mL).

As shown above, Ca^{2+}- and nucleotide-induced $^{45}Ca^{2+}$ release from passively loaded vesicles is dependent not only on calmodulin concentration but also on the Mg^{2+} concentration in the release medium (Table I). Figure 6 illustrates a similar experiment with actively loaded vesicles. Heavy SR Ca^{2+} release vesicles were actively loaded with $^{45}Ca^{2+}$ in media containing 0 or 2 μM exogenously added calmodulin, 10 μM free $^{45}Ca^{2+}$, 5 mM ATP, and varying concentrations of Mg^{2+}. Increasing amounts of $^{45}Ca^{2+}$ were sequestered by the vesicles as the Mg^{2+} concentration in the uptake medium was increased from 5 to 9 mM Mg^{2+} (0.7–4.1 mM free Mg^{2+}). At the various Mg^{2+} concentrations, inclusion of calmodulin in the uptake medium nearly doubled the Ca^{2+} sequestering capability of the Ca^{2+}-permeable vesicle population. An amount close to that seen in the presence of ruthenium red was sequestered when vesicles were loaded in a medium containing 4.1 mM free Mg^{2+} (9 mM Mg^{2+} plus 5 mM ATP) and 2 μM calmodulin.

That calmodulin affected the Ca^{2+} release mechanism in actively loaded vesicles could be shown by measurement of the $^{45}Ca^{2+}$ efflux rates of the vesicles. Heavy SR Ca^{2+} release vesicles were loaded in media containing 10 μM free $^{45}Ca^{2+}$, 8.5 mM Mg^{2+}, and 5 mM ATP. $^{45}Ca^{2+}$ efflux rates were measured by placing the vesicles on Millipore filters and rinsing them for 10 or 20 s with media containing 10 mM Mg^{2+} plus 10 μM ruthenium red or 10 μM free Ca^{2+} plus 0.6 mM Mg^{2+}. Addition of 0.6 mM Mg^{2+} to the Ca^{2+} release promoting medium slowed $^{45}Ca^{2+}$ efflux so that its rate could be accurately determined by Millipore filtration. The addition of 2.5 μM calmodulin to the uptake and release media reduced the rate constant of $^{45}Ca^{2+}$ release from 0.06 to 0.025 s^{-1}. By comparison, addition of the catalytic subunit of protein kinase (10 units/mL), in the presence or absence of 2.5 μM calmodulin, was without an appreciable effect on the $^{45}Ca^{2+}$ release rate from actively loaded vesicles. The release rate was increased about 2-fold when calmidazolium was present at a concentration of 2 μM in $^{45}Ca^{2+}$ uptake and release media. However, this increase appeared to be due to an increase in general membrane permeability, since a similar increase in $^{45}Ca^{2+}$ efflux was observed when release media contained 10 mM Mg^{2+} and 10 μM ruthenium red.

Discussion

Calmodulin is a ubiquitous intracellular protein which in the presence of Ca^{2+} regulates a multitude of enzymes including the (Ca^{2+} + Mg^{2+})-dependent ATPase of sarcoplasmic reticulum in heart as well as the (Ca^{2+} + Mg^{2+})-dependent

ATPase of the surface membranes in heart and skeletal muscle (Tada et al., 1982; Carafoli & Zurini, 1982; Michalak et al., 1984). The data presented here suggest that calmodulin affects another process involved in the regulation of intracellular Ca^{2+} distribution in muscle, i.e., the Ca^{2+} release step in excitation–contraction coupling in skeletal muscle.

Ca^{2+} release by SR in muscle is a complex process which in vitro is affected by numerous parameters. In isolated heavy SR vesicles, Ca^{2+} release could be partially regulated by varying Ca^{2+}, Mg^{2+}, and adenine nucleotide concentrations in release media. In the absence of Mg^{2+}, Ca^{2+} is released with a rate which appears to be rapid enough to account for the rate of release in muscle (Table I). On the other hand, in the absence of nucleotide, Mg^{2+} at a concentration of 1 mM nearly fully inhibits the Ca^{2+} channel. However, it is unlikely that Ca^{2+} release in muscle is regulated only by Ca^{2+}, Mg^{2+}, and adenine nucleotides. First, as shown in this study, Ca^{2+} release from heavy SR vesicles is slowed down by exogenously added calmodulin. Second, under assay conditions considered to approximate those in frog skeletal muscle following an action potential [10 μM free Ca^{2+}, 0.2–4 mM free Mg^{2+} (Gupta & Moore, 1980; Baylor et al., 1982), and 5 mM ATP or AMP-PCP], Ca^{2+} release was, at a given Mg^{2+} concentration, either too slow when compared with rates expected to occur in muscle or too rapid to permit retention of transported Ca^{2+} (Table I and Figure 6). That Mg^{2+} and ATP act like second messengers, as Ca^{2+} is thought to, appears to be unlikely. In contrast to Ca^{2+}, there is no evidence that Mg^{2+} or ATP concentrations undergo significant changes during muscle contraction. Third, studies with "skinned" muscle fibers and isolated SR vesicle fractions have shown that Ca^{2+} release is affected by additional factors such as an alteration in pH (Nakamura & Schwartz, 1972; Shoshan et al., 1981) or a change in the ionic environment thought to promote surface membrane charge redistribution or transmembrane potential changes across T-system and/or SR membranes (Stephenson, 1978; Campbell & Shamoo, 1980; Caswell & Brandt, 1981; Shoshan et al., 1983; Ikemoto et al., 1984).

Heavy SR vesicles contained a significant amount of calmodulin which was not solubilized during vesicle isolation and which was sufficient to inhibit most, if not all, Ca^{2+} release channels. As indicated by the ineffectiveness of calmodulin inhibitors at high vesicle concentrations, the endogenous calmodulin did not appear to affect the Ca^{2+} release process of isolated SR vesicles. However, the use of calmodulin inhibitors is problematic in implicating a role for calmodulin in Ca^{2+} release channel function. These drugs are lipophilic molecules which at low concentrations likely decrease their own apparent effectiveness by partitioning into the bilayer. At high drug concentrations or low vesicle concentrations, the drugs become ineffective as diagnostic tools by rendering the membranes "leaky" to small solutes and ions such as [^{3}H]-sucrose and Ca^{2+}. Partitioning of the drugs into the bilayer has been reported to cause alterations in SR membrane structure, thereby promoting Ca^{2+} efflux as well as inhibition of the (Ca^{2+} + Mg^{2+})-dependent ATPase of sarcoplasmic reticulum (Ho et al., 1983; Volpe et al., 1984; Anderson et al., 1984; Prokopjeva et al., 1984).

Calmidazolium (10–20 μM) and compound 48/80 (10–20 μg/mL) inhibited calmodulin-dependent protein phosphorylation and ATP-dependent Ca^{2+} uptake in heavy and light skeletal SR vesicle fractions but did not affect Ca^{2+}-stimulated ATPase activity (Tuana & MacLennan, 1984). In control experiments, using 5–7.5 times higher protein concentrations than in the Ca^{2+} uptake and ATPase experiments, these two drugs did not affect the Ca^{2+} permeability of the vesicles. These results led Tuana & MacLennan (1984) to suggest that in skeletal muscle a calmodulin-dependent protein kinase might be involved in the regulation of a Ca^{2+} release channel. In our studies with passively loaded vesicles, calmodulin inhibition of $^{45}Ca^{2+}$ release occurred in the absence of ATP, suggesting that calmodulin did not exert its effect via an endogenous calmodulin-dependent protein kinase. Rather, the results of this study support the proposal of a direct interaction of calmodulin with the SR Ca^{2+} release channel or a membrane component tightly coupled to channel function.

Calmodulin inhibition of $^{45}Ca^{2+}$ release was reversible and saturable, yet not complete. Calmodulin partially inhibited $^{45}Ca^{2+}$ release by reducing the $^{45}Ca^{2+}$ release rate 2–3-fold (Table I). The amount of rapidly released $^{45}Ca^{2+}$ was not affected by calmodulin (Figure 1). Therefore, calmodulin does not lower the release rate by inhibiting a portion of the vesicles. It is also unlikely that calmodulin inhibited only a portion of the channels, since there is no evidence for the existence of two ligand-gated release channels that may be affected differently by calmodulin. The most likely explanation is that calmodulin lowers the Ca^{2+} release rate by decreasing the conductance and/or "open" time of the Ca^{2+} release channel. In this regard, it should be noted that in single channel recordings (Smith et al., 1985), the open time but not the conductance of the Ca^{2+} release channel has been found to be dependent on AMP-PCP concentration.

Interpretation of our experimental data in terms of a possible physiological function of calmodulin in excitation–contraction coupling is difficult in that we lack direct binding data and do not know the structural relationship of calmodulin and the Ca^{2+} release channel in muscle. Calmodulin binding can only be well studied after the channel components have been identified and isolated. The Ca^{2+} release channel has been found to be present predominantly in heavy SR vesicles, i.e., a subfraction of SR vesicles which is thought to be derived from the terminal cisternae region of SR. However, whether the channel is equally distributed in the terminal cisternae or limited to the junctional region between SR and the T system is not known.

One possible interpretation is that calmodulin has a role in the process of Ca^{2+} release channel inactivation. Calmodulin might rapidly bind to the channel when a high local Ca^{2+} concentration is achieved during excitation–contraction coupling. Binding of calmodulin in turn would slow down further Ca^{2+} release so that the Ca^{2+} pump of SR could lower the myoplasmic Ca^{2+} concentration to a level which results in effective closing of the channel. In support of this hypothesis, we found that the Ca^{2+} sequestering capability of the vesicles is greatly increased by calmodulin. Inhibition of Ca^{2+} release was a slow process when compared with the excitation–contraction cycle in muscle. It can be argued, however, that our calmodulin inhibition studies were carried out under conditions less favorable than those encountered in the highly structured environment in muscle.

A strong argument against an essential role for calmodulin in Ca^{2+} release channel inactivation is that in all of our experiments we noted only a partial reduction of the Ca^{2+} release rate by calmodulin. This observation leads us to propose that a predominant role of calmodulin in excitation–contraction coupling may be modulation of Ca^{2+} release channel function. It is conceivable that calmodulin, by slowing down release of Ca^{2+} from SR, compensates for incomplete removal of Ca^{2+} from the myoplasmic space during prolonged muscle activity.

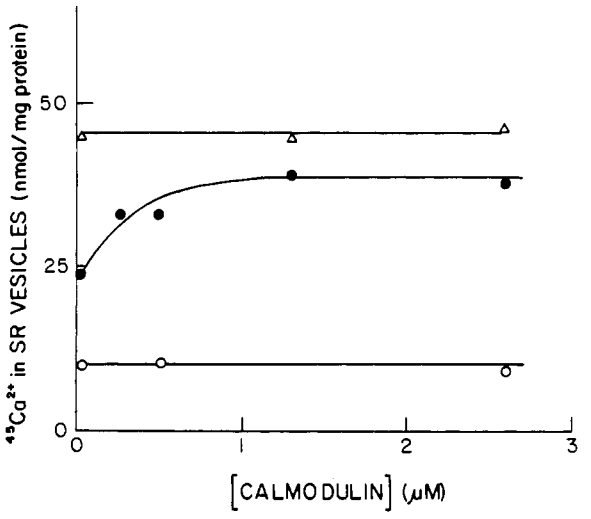

FIGURE 5: Effect of calmodulin concentration on Ca^{2+} uptake by heavy SR Ca^{2+} release vesicles. A heavy SR Ca^{2+} release vesicle fraction was actively loaded as described under Materials and Methods in media containing 10 μM free Ca^{2+}, 5 mM ATP, 7.5 mM Mg^{2+}, the indicated concentrations of exogenously added calmodulin, and 0 (○, ●) or 10 μM ruthenium red (△). After 1 min, vesicles were placed on 0.45-μm Millipore filters and rinsed for 10 s with a medium containing either 10 mM Mg^{2+} plus 10 μM ruthenium red (△, ●) or 10 μM free Ca^{2+} (○). Radioactivity retained by the vesicles on the filters was determined.

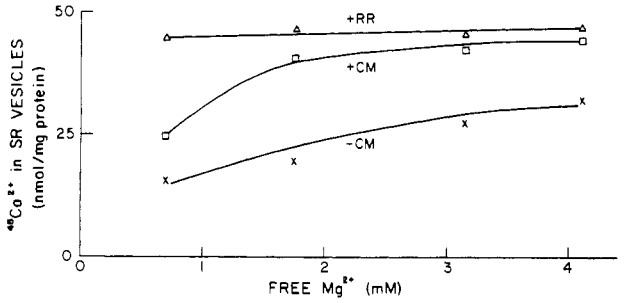

FIGURE 6: Effect of Mg^{2+} concentration on Ca^{2+} uptake in heavy SR Ca^{2+} release vesicles in the absence and presence of calmodulin. Heavy SR vesicles were actively loaded in media containing the indicated concentrations of free Mg^{2+} (×) and either 2 μM calmodulin (□) or 10 μM ruthenium red (△). After 1 min, vesicles were placed on Millipore filters and rinsed for 10 s with a medium containing 10 mM Mg^{2+} plus 10 μM ruthenium red. The amount of $^{45}Ca^{2+}$ taken up by the Ca^{2+}-impermeable vesicle population was 10 nmol/mg of protein and was determined by rinsing the vesicles (×, □) with 10 μM free Ca^{2+} medium. Radioactivity remaining with the vesicles on the filters was determined.

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Registry No. AMP-PCP, 3469-78-1; Ca, 7440-70-2; Mg, 7439-95-4.

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Bacteriophage P22 Cro Protein: Sequence, Purification, and Properties†

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ABSTRACT: The DNA sequence of part of the bacteriophage P22 early regulatory region, including genes *cro* and *c*1, was determined. The protein product of the *cro* gene consists of 61 amino acid residues, and that of *c*1, 92 amino acid residues. Both genes were placed separately in plasmids from which they are expressed from a controllable promoter in vivo. Induced cells bearing the *cro*-expressing plasmid were used as a source for purifying and characterizing the Cro protein. The amino-terminal sequence of this protein was found to be as predicted by the DNA sequence; close agreement was also observed between its predicted and experimentally determined amino acid composition and molar extinction coefficient at 280 nm. In gel filtration experiments, Cro protein at concentrations around 10^{-5} M appears to have a molecular weight of 8600, which is more consistent with monomers (6800) than with dimers (13 600). Cro protein binds specifically to the three repressor binding sites in the P22 right operator; in order of decreasing affinity, these are O_R3, O_R1, and O_R2.

Salmonella phage P22 has a regulatory gene called *cro* that is responsible for turning down synthesis of early phage genes during infection (Winston & Botstein, 1981). Phages that are defective in *cro* function are unable to grow lytically; upon infection of a normal host cell, they overproduce repressor (the product of gene *c*2) and are channeled into lysogeny. The *cro* gene is located between *c*2 and another regulatory gene, *c*1. The *c*1 gene has a role opposite to that of *cro*: it is required for the establishment of lysogeny, though not for lytic growth (Levine, 1957).

Studies of λ, a related phage that grows in *Escherichia coli*, have led to a detailed understanding of the mechanisms that regulate expression of its genes [see Hendrix et al. (1983) for a review]. The basic form of this regulation is the same for both λ and P22 (Susskind & Botstein, 1978). The clusters of early regulatory genes and sites of λ and P22 constitute modules that can be exchanged between the two phages by recombination. In particular, both the *cro* and *c*1 genes of P22 have counterparts in λ, called *cro* and *c*II, respectively. Where the mechanisms of action of P22 regulatory elements have been examined in detail, they are strikingly similar to those of their λ counterparts (Poteete & Ptashne, 1982). It is reasonable to expect that P22 *cro* and *c*1 fall into this pattern. We can predict that P22 Cro protein will be found to exert its regulatory effects by binding to the left and right operators of the phage. In particular, it should bind to the *c*2 repressor binding sites of the right operator with O_R3, the left-most site, having a higher affinity than the other two sites. [The rationale for this regulatory scheme in λ and its generalization to P22 have been discussed by Johnson et al. (1981).]

Although we expect that the Cro proteins of λ and P22 function similarly, the two proteins should exhibit completely distinct specificities. The operator sites of the two phages are quite different in sequence (Maniatis et al., 1975; Poteete et al., 1980); P22 *c*2 repressor does not recognize λ operators, and λ *c*I repressor does not recognize P22 operators (Poteete & Roberts, 1981).

The repressor and Cro proteins of λ and its relatives P22 and 434 constitute an interesting group. Studies of these regulatory proteins have led to insights into the structural basis of sequence-specific DNA binding and the mechanisms of both positive and negative regulation of transcription initiation. In this paper, we present the P22 DNA sequence from the early regulatory region, including the *cro* and *c*1 genes. In addition, we describe the construction of plasmids that express *cro* at high levels and the purification and properties of P22 Cro protein.

Materials and Methods

DNA Sequencing. DNA sequencing was performed by using the chemical method of Maxam & Gilbert (1980) and standard procedures for 3′ and 5′ end-labeling, restriction digestion, and polyacrylamide electrophoresis (Maniatis et al., 1982).

Plasmids. General procedures for plasmid construction and purification were as described previously (Poteete & Roberts, 1981; Poteete, 1982). pBR322 (Bolivar et al., 1977) was the

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