Effects of a Nonionic Detergent on Calcium Uptake by Cardiac Microsomes†

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ABSTRACT: We investigated the effects of the nonionic detergent octaethylene glycol monododecyl ether ($C_{12}E_8$) on the sarcoplasmic reticulum calcium pump in cardiac microsomes in view of its specific effects on different ATP-accelerated steps in the catalytic cycle of the Ca-ATPase in leaky fast skeletal muscle microsomes. At low concentrations of $MgATP^{2-}$ (<2.5 μM), a nonsolubilizing concentration of added $C_{12}E_8$ (15 μM) increased apparent $V_{max(MgATP)}$ of oxalate-facilitated calcium uptake associated with $MgATP^{2-}$ binding to the high affinity catalytic site. An ATP induced acceleration of calcium uptake, attributable to regulatory nucleotide binding, was seen between 2 and 3 μM $MgATP^{2-}$ in both $C_{12}E_8$-treated and control microsomes. These effects of $C_{12}E_8$ are similar to those seen previously with trypsin treatment of microsomes [Lu, Y.-Z., Xu, Z.-C., & Kirchberger, M. A. (1993) *Biochemistry 32*, 3105–3111]. However, at a saturating Ca^{2+} between 3 and 10 μM $MgATP^{2-}$, $C_{12}E_8$ produced a greater reduction in the magnitude of the ATP-induced acceleration of calcium uptake seen with trypsin. At 1 mM $MgATP^{2-}$, $C_{12}E_8$ and trypsin as well as protein kinase A-catalyzed microsomal phosphorylation all increased the Ca^{2+} affinity of the pump, but only the latter two treatments significantly increased apparent $V_{max(Ca)}$. In fact in trypsin-treated and phosphorylated microsomes, $C_{12}E_8$ reduced $V_{max(Ca)}$ to close to the control values; it reduced $V_{max(Ca)}$ only slightly in control microsomes. Under our experimental conditions, comparable effects of 15 μM $C_{12}E_8$ on calcium uptake were absent in fast skeletal muscle microsomes, which lack phospholamban. The present results show that the inhibitory effects of phospholamban with respect to Ca^{2+} affinity and $V_{max(Ca)}$ of the calcium pump in cardiac microsomes may be dissociated by $C_{12}E_8$, which allows rationalizing these effects in terms of ligand-induced changes in the rate-limiting step of the reaction cycle.

Mild trypsin treatment of cardiac microsomes, which cleaves the cytoplasmic domain of phospholamban, resembles the effects of phospholamban phosphorylation by protein kinase A and provides a useful model for studying the regulation of the sarcoplasmic reticulum (SR)[1] calcium pump by phospholamban (Kirchberger et al., 1986; Lu et al., 1993). Protein kinase A-catalyzed phospholamban phosphorylation increases the affinity of the pump for Ca^{2+} and has been reported also to increase $V_{max(Ca)}$ (Tada et al., 1979; Kirchberger et al., 1986), although technical difficulties associated with the assay have sometimes interfered with determination of the latter parameter (Hicks et al., 1979). Monoclonal antibody to phospholamban also produces an increase in Ca^{2+} pump affinity but does not increase $V_{max(Ca)}$ measured at millimolar ATP (Jones & Field, 1993). The use of trypsin-treated cardiac microsomes overcomes possible problems associated with microsomal phosphorylation or the use of monoclonal antibody and has provided strong evidence for an inhibitory effect of phospholamban on maximal pump velocity measured at saturating Ca^{2+} and millimolar ATP (Lu et al., 1993).

In our recent study with trypsin-treated cardiac microsomes (Lu et al., 1993), we presented evidence for an inhibition by phospholamban of the interaction of the SR calcium pump with its nucleotide substrate. Our data were consistent with a proposed interference by phospholamban of the acceleratory effects of ATP in the reaction cycle of the calcium pump as well as a decrease in the apparent $V_{max(ATP)}$ associated with nucleotide binding at the high-affinity (catalytic) site. Other investigators have shown that nonsolubilizing concentrations of the nonionic detergent $C_{12}E_8$ produce specific effects on different steps in the reaction cycle of the Ca-ATPase in leaky fast skeletal muscle microsomes: all ATP-accelerated steps tested are accelerated by the detergent except E_2P decomposition, which is inhibited (Andersen et al., 1983; Champeil et al., 1986; Gould et al., 1986; de Foresta et al., 1989). It was suggested that $C_{12}E_8$ provides a useful tool for identifying rate-limiting steps in the reaction cycle of the Ca-ATPase under various experimental conditions (Champeil et al., 1986). In the present study, we determined the effects of nonsolubilizing concentrations of $C_{12}E_8$ on calcium uptake in cardiac microsomes on the basis of the detergent's known effects in the permeabilized skeletal muscle microsomes. At the extremely low effective concentration of $C_{12}E_8$ used in our study, microsomal membrane integrity was not detectably impaired, based on the ability of the microsomes to accumulate calcium.

EXPERIMENTAL PROCEDURES

Materials. Canine cardiac microsomes and fast skeletal muscle microsomes, derived from vastus lateralis muscle of New Zealand White rabbits, were obtained as described previously and stored in liquid nitrogen (Kirchberger et al., 1986). Protein kinase A was partially purified from bovine heart (Kirchberger & Tada, 1976). $C_{12}E_8$, Brij-35, -56, and -58, and digitonin were purchased from Sigma Chemical Co. Soybean trypsin inhibitor was from Worthington Biochemical

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[1] Abbreviations: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $C_{12}E_8$, octaethylene glycol dodecyl monoether; Brij-35, poly(oxyethylene glycol) [23] dodecyl alcohol; Brij-56, poly(oxyethylene glycol) [10] cetyl alcohol; Brij-58, poly(oxyethylene glycol) [20] cetyl alcohol.

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Corp. Other reagents were obtained as reported previously (Lu et al., 1993).

Calcium Uptake Assay. Calcium uptake by microsomes (10 μg of protein/mL) was measured in a standard assay mixture consisting of 40 mM histidine hydrochloride buffer, pH 6.8 at 25 °C, 0.12 M KCl, 5 mM sodium azide, 2.5 mM oxalate-Tris, 0.2 mg/mL pyruvate kinase, 4 mM phosphoenolpyruvate, equimolar concentrations of ATP and $MgCl_2$ to give the $MgATP^{2-}$ concentrations indicated in the text in the presence of an additional 1 mM $MgCl_2$, and a $CaCl_2$–EGTA buffer system consisting of 125 μM $CaCl_2$ and different EGTA concentrations to obtain the specified Ca^{2+} concentration. $MgATP^{2-}$ and Ca^{2+} concentrations were calculated as described previously (Katz et al., 1970) except that an apparent binding constant of Ca^{2+} to EGTA of 10^6 M^{-1} was used. ^{45}Ca was included in the $CaCl_2$–EGTA buffer system at various specific radioactivities decreasing from 41 μCi/μmol calcium at 0.02 μM Ca^{2+} to 4.5 μCi/μmol calcium at 12 μM Ca^{2+}. Various concentrations of detergents were included in the reaction mixture as specified in the text. The indicated concentrations represent added (total) detergent rather than free (unbound) detergent. Microsomes were added to the temperature-equilibrated reaction mixture minus the Ca^{2+} buffer, and after a 2-min incubation, the calcium-uptake reaction was started by the addition of the Ca^{2+} buffer. Aliquots of reaction mixture were filtered after a further 2–6-min incubation. The filters were processed as described previously in order to obtain initial (linear) rates of microsomal calcium uptake (Kirchberger et al., 1986). Zero time samples were obtained from reaction mixtures from which ATP had been omitted. Microsomal protein was determined by the biuret procedure with bovine serum albumin as the standard. $C_{12}E_8$ at the concentrations used in this study does not interfere with the ATP regenerating system (Champeil et al., 1986). The inclusion of an ATP regenerating system in the reaction mixture resulted in significantly enhanced rates of calcium uptake by cardiac microsomes as shown previously by others (Penpargkul, 1979). The use of subsaturating concentrations of Ca^{2+} (0.2 and 2.2 μM) in the assay of calcium uptake by fast skeletal muscle microsomes minimized interference by calcium-release channels at higher Ca^{2+} concentrations (Wimsatt et al., 1990).

Stock solutions of the detergents were prepared as follows. $C_{12}E_8$, Brij-35, and Brij-58 were dissolved in distilled water to obtain a concentration of 0.28 mg/mL. Brij-56 was dissolved in distilled water at a concentration of 1.35 mg/mL and heated gently until a clear solution was obtained and then diluted to 0.28 mg/mL. Digitonin was prepared by heating in distilled water at a concentration of 3.5 mg/mL and diluted further.

Trypsin Treatment of Microsomes. Cardiac microsomes (0.5 mg/mL) were added to the temperature-equilibrated reaction mixture consisting of 40 mM histidine hydrochloride, pH 6.8 at 25 °C, 0.12 M KCl, 5 mM sodium azide, 0.01 mg/mL trypsin, and either 0.12 mg/mL trypsin inhibitor or no further addition. After a 2-min incubation of both reaction tubes, trypsin inhibitor was added to the latter tube, and both tubes were maintained at 25 °C for an additional 2 min, at which time ice-cold buffer B (10 mM Hepes–KOH, pH 6.8, 0.25 M sucrose, 60 mM KCl, and 1 mM dithiothreitol), in which the microsomes are routinely suspended, was added to yield a final microsomal protein concentration of 0.20 mg/mL. The two tubes containing the trypsin-treated and control microsomes were maintained on ice during the time they were being used in the calcium-uptake assay, which was carried out immediately following the trypsin treatment.

Phosphorylation of Microsomes. Microsomes (10 μg/mL) were incubated in the complete reaction mixture described above for the assay of calcium uptake except that the Ca^{2+} buffer was omitted and both 2 μM cyclic AMP and 1 mg/mL partially purified protein kinase A were added to one set of tubes and omitted from the control set. To both sets was added either 15 μM $C_{12}E_8$ or an equivalent volume of distilled water. The phosphorylation reaction was started by addition of microsomes. After 2 min of incubation at 25 °C, the calcium uptake reaction was started by addition of the Ca^{2+} buffer. The samples were then processed as described above for the calcium-uptake assay.

RESULTS

Very low concentrations of $C_{12}E_8$ produced a marked *increase* in calcium uptake by cardiac microsomal vesicles when assayed at subsaturating (0.32 μM) Ca^{2+} (Figure 1A). Maximal stimulation of the calcium uptake rate was obtained at a detergent concentration of about 15 μM and was followed by a precipitous decline in rate with increasing concentration above 18 μM, consistent with a deleterious modification of the membrane. At saturating Ca^{2+} (12 μM), there was a small decrease in calcium uptake with $C_{12}E_8$ concentrations up to 20 μM, followed by a pronounced decrease at higher concentrations.

In fast skeletal muscle SR, which lacks phospholamban (Kirchberger & Tada, 1976; Jorgensen & Jones, 1986), a different pattern of effects of $C_{12}E_8$ was seen (Figure 1B). At both low (0.2 μM) and near-saturation (2.2 μM) Ca^{2+} concentrations, microsomal calcium uptake remained essentially unchanged from control values with inclusion of up to 15 μM $C_{12}E_8$ in the assay medium. These results suggest that SR membrane integrity is minimally impaired at a detergent concentration of 15 μM, which was chosen for further experiments that were carried out exclusively with cardiac microsomes.

Effect of Duration of Preincubation in $C_{12}E_8$. The effects of 15 μM $C_{12}E_8$ on calcium uptake by microsomes were immediate within our limits of detection. The magnitude of the approximately 2-fold stimulation of calcium uptake at 0.32 μM Ca^{2+} remained relatively constant over a 12-min preincubation period. At 12 μM Ca^{2+}, $C_{12}E_8$ produced a slight decrease in calcium uptake (seen also in Figure 1A), which also remained relatively constant during the entire preincubation period. It was convenient to use a preincubation time of 2 min in further experiments.

Effects of $C_{12}E_8$ on Calcium Uptake Assayed at Micromolar $MgATP^{2-}$ Concentrations. In order to investigate the effect of $C_{12}E_8$ on nucleotide interaction with the calcium pump, calcium uptake was initially assayed at 0–10 μM $MgATP^{2-}$ in the presence of 0.32 or 12 μM Ca^{2+} (Figure 2). At both Ca^{2+} concentrations in this nucleotide concentration range, calcium uptake was significantly increased by the detergent. At 0.32 μM Ca^{2+}, control rates of calcium uptake and the enhancement of calcium uptake produced by $C_{12}E_8$ in the present study and by trypsin treatment, reported in the previous study (Lu et al., 1993), were almost identical. At 12 μM Ca^{2+}, control rates of calcium uptake also were similar in the two studies when measured at 0–3 μM $MgATP^{2-}$, but between 3 and 10 μM nucleotide, the control rates of calcium uptake were somewhat higher than those seen previously with the trypsin inhibitor/trypsin-treated (control) microsomes. The stimulation of calcium uptake by $C_{12}E_8$ between 0 and

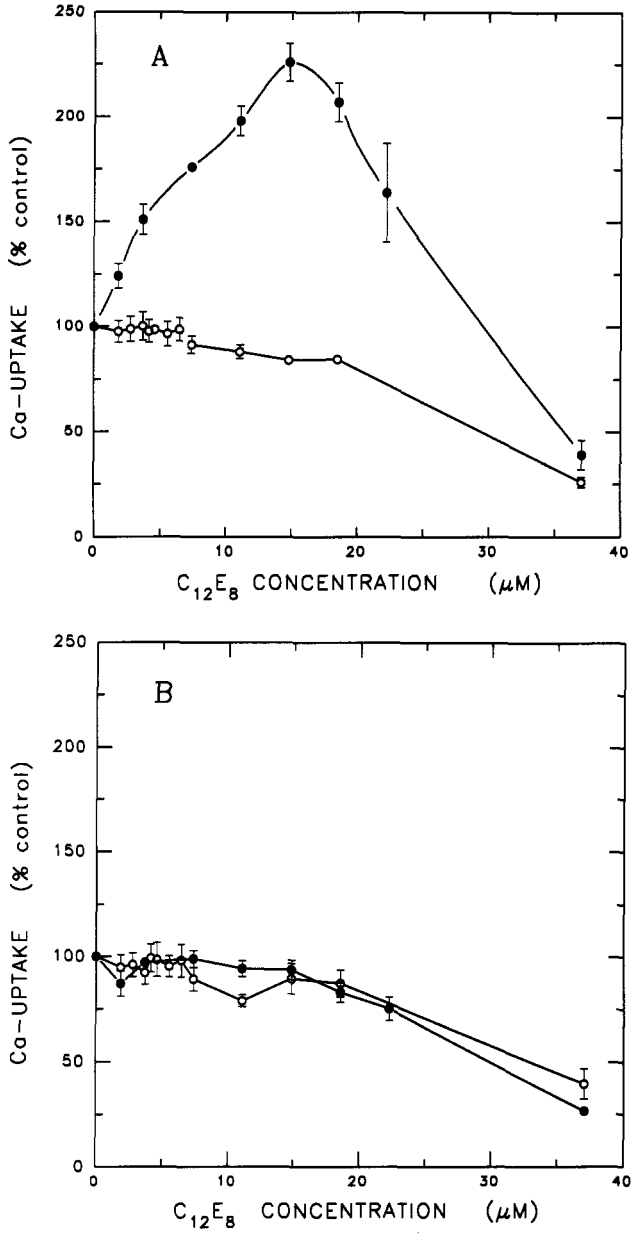

FIGURE 1: Concentration dependence of the effects of $C_{12}E_8$ on calcium uptake by (A) cardiac and (B) fast skeletal muscle microsomes. Calcium uptake was measured in a standard reaction medium including 1 mM $MgATP^{2-}$, the indicated concentrations of detergent, and (A) 0.32 μM Ca^{2+} (●) or 12 μM Ca^{2+} (○) or (B) 0.20 μM Ca^{2+} (●) or 2.2 μM Ca^{2+} (○). Shown are means ± SE of three experiments with different microsome preparations; error bars are omitted when their size is equal to or smaller than the height of the symbol. 100% calcium uptake represents (μmol/mg·min): (A) (●) 0.048; (○) 0.354; (B) (●) 0.500; (○) 1.437. In cardiac but not skeletal muscle microsomes, the difference between calcium uptake in the presence and absence of 15 μM $C_{12}E_8$ is significant at the 0.05 level at both Ca^{2+} concentrations when tested by Student's *t* test for paired variates.

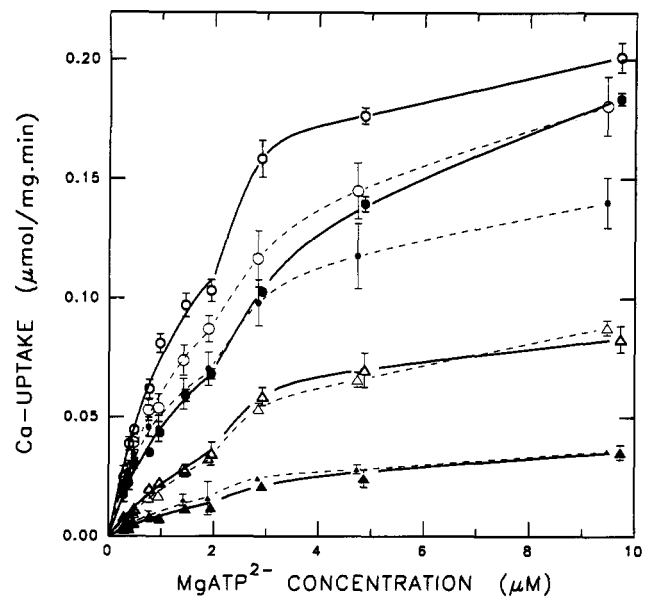

FIGURE 2: Effect of $C_{12}E_8$ on the $MgATP^{2-}$ concentration dependence of calcium uptake by cardiac microsomes. Calcium uptake was measured in a standard medium containing 0–10 μM $MgATP^{2-}$ and 0.32 μM (▲, △) or 12 μM (●, ○) Ca^{2+} in the presence (△, ○) and absence (▲, ●) of 15 μM $C_{12}E_8$. Values represent means ±SE of three experiments with different microsome preparations; error bars are absent when their size is equal to or smaller than the height of the symbol. The mean values were fit to the equation $V = V_{max}/[1 + (K_m/[MgATP^{2-}])^N]$ by a nonlinear least-squares procedure; N is the Hill coefficient. The *solid lines* represent the results of this fit for concentrations up to 2 μM $MgATP^{2-}$ whereas the lines between 2 and 10 μM $MgATP^{2-}$ are included as a visual aid and do not result from the same fit. The *broken lines* were similarly obtained except that the data points used were those from our previous study (Lu et al., 1993) of the effect of trypsin treatment (△, ○) of microsomes on calcium uptake measured at 0.32 μM Ca^{2+} (small ▲, △) and 11 μM Ca^{2+} (•, ○); control microsomes (small ▲, •) had been treated with trypsin inhibitor-inactivated trypsin. The data sets for $C_{12}E_8$- and trypsin-treated microsomes represent different microsome preparations. (Data for trypsin-treated and control microsomes reproduced from Lu et al. (1993) with permission from the American Chemical Society.)

Table 1: Effect of $C_{12}E_8$ on the Apparent Kinetic Parameters for Calcium Uptake by Control and Trypsin-Treated Microsomes Measured at 0–2 μM $MgATP^{2-}$ [a]

parameter	$C_{12}E_8$ (15 μM) –	$C_{12}E_8$ (15 μM) +	+/− ratio	*p*
$V_{max(MgATP)}$ (μmol/mg·min)	0.13 ± 0.00	0.17 ± 0.01	1.32	<0.05
$K_{m(MgATP)}$ (μM)	1.87 ± 0.03	1.60 ± 0.19	0.86	NS

[a] The values are the means ± SE of the optimized parameters obtained by separate fits of each of three independent experiments, represented in Figure 2, to the equation given in the figure legend when N was constrained to 1.0. *p* was determined by Student's *t* test for paired and unpaired variates. NS, not significant at the 0.05 level.

3 μM $MgATP^{2-}$ also was higher. Notable, however, is the fact that $C_{12}E_8$ produced a significant increase in apparent $V_{max(MgATP)}$, associated with nucleotide binding to the high-affinity (catalytic) site. A similar result had been obtained in the previous study with trypsin treatment of microsomes (*cf.* apparent $V_{max(MgATP)}$ from Table 1 with apparant $V_{max(MgATP)}$ of 0.11 ± 0.01 μmol of Ca/mg·min in trypsin inhibitor/trypsin-treated (control) microsomes and of 0.17 ± 0.02 μmol of Ca/mg·min in trypsin-treated microsomes in Table III of Lu et al., 1993). No significant change in apparent $MgATP^{2-}$ affinity with $C_{12}E_8$ was detectable in the present study (Table 1) or with trypsin treatment previously. This is consistent with the lack of effect of phospholamban phosphorylation on nucleotide binding affinity to the catalytic site when estimated from measurement of phosphoenzyme intermediate formation (Tada et al., 1979). The derived parameters shown in Table 1 represent approximations because the acceleration of calcium uptake between 2 and 3 μM ATP^{2-} precludes the determination of the apparent kinetic parameters of the high-affinity catalytic site over a wider range of appropriate substrate concentrations. This acceleration was seen under all conditions tested, both in the present experiments

Table 2: Apparent Kinetic Parameters for Calcium Uptake by Control and Trypsin-Treated Microsomes Measured in the Presence and Absence of $C_{12}E_8$[a]

microsomes	$C_{12}E_8$	$V_{max(Ca)}$ (μmol/mg·min)	%	$K_{m(Ca)}$ (μM)	%	Hill coefficient	%
control	–	0.288 ± 0.032[b]	100	0.84 ± 0.07[d-f]	100	1.75 ± 0.12	100
control	+	0.277 ± 0.028	96	0.43 ± 0.02[e]	51	1.43 ± 0.09	82
trypsin treated	–	0.399 ± 0.040[b,c]	138	0.52 ± 0.01[d,g]	62	1.77 ± 0.07[h]	101
trypsin treated	+	0.303 ± 0.023[c]	105	0.37 ± 0.02[f,g]	44	1.46 ± 0.07[h]	83

[a] Values represent the means ± SE of the optimized parameters that were obtained in separate unweighted fits of three independent experiments to the equation given in the legend to Figure 3. [b–h] Differences between the parameters identified by the same superscript in each column are significant at a p level of <0.05 when tested by Student's t test for paired (*b*, *c*), unpaired (*d*), or both paired and unpaired (*e–h*) variates.

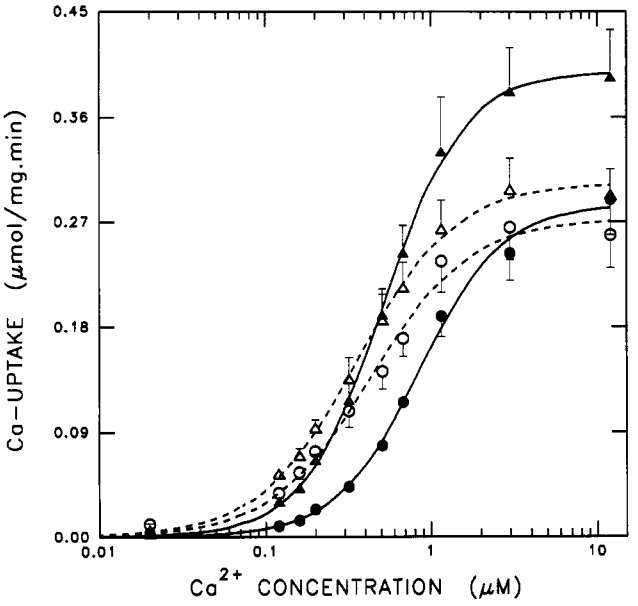

FIGURE 3: Effect of $C_{12}E_8$ on the Ca^{2+} concentration dependence of calcium uptake by control and trypsin-treated cardiac microsomes. Calcium uptake was measured under standard assay conditions in trypsin treated (▲, △) and control (●, ○) microsomes in the presence (○, △) and absence (●, ▲) of 15 μM $C_{12}E_8$. Symbols represent means ± SE of calcium uptake measured at each Ca^{2+} concentration in three experiments with different microsome preparations. For clarity, only the positive or negative error bars are shown; error bars are omitted when their size is equal to or less than the height of the symbol. The mean values are shown together with the unweighted fit to the equation $V = V_{max}/[1 + (K_m/[Ca^{2+}])^N]$ where N is the Hill coefficient. The results of the fit are indicated by *solid* (no detergent present) or *broken* (detergent present) *lines*. The averages of the optimized kinetic parameters obtained from each of the three experiments are shown in Table 2.

and those reported previously, and in each case was proportional to the prevailing calcium-uptake rate.

A distinct difference between the effects of $C_{12}E_8$ and trypsin on calcium uptake is evident at 12 μM Ca^{2+} between 3 and 10 μM $MgATP^{2-}$: the acceleration of calcium uptake seen between 2 and 3 μM $MgATP^{2-}$ diminishes to different extents. In trypsin-treated microsomes and their corresponding controls, rates of calcium uptake increase more nearly in parallel with increasing $MgATP^{2-}$ concentration than is found in microsomes treated with $C_{12}E_8$, where rates appear to converge. This diminution in the stimulation of calcium uptake by $C_{12}E_8$ is consistent with the even lower rate of Ca uptake seen in the presence of $C_{12}E_8$ at 1 mM nucleotide (and 12 μM Ca^{2+}) in Figure 1A compared to the control microsomes. An inhibitory effect of $C_{12}E_8$ on calcium uptake becomes more apparent in Figure 3, discussed below.

Effects of $C_{12}E_8$, Trypsin Treatment, and Phosphorylation on Calcium Uptake Measured at 1 mM $MgATP^{2-}$. In Figure 3 are shown the effects of $C_{12}E_8$ on the Ca^{2+} concentration dependence of calcium uptake measured at 1 mM $MgATP^{2-}$ in control and trypsin-treated microsomes. From 0.02 to 0.32 μM Ca^{2+}, there is little difference between the stimulatory effects of $C_{12}E_8$ or trypsin treatment on calcium uptake, but the combination of the two treatments is more stimulatory. Above 0.32 μM Ca^{2+}, $C_{12}E_8$, unlike trypsin, becomes clearly inhibitory. Thus while there are common aspects in the stimulation of calcium uptake by $C_{12}E_8$ and trypsin treatment, there are also differences, as seen already in Figure 2 at $MgATP^{2-}$ concentrations greater than 3 μM or calcium-uptake rates greater than approximately 0.16 μmol/mg·min.

Trypsin treatment of microsomes in the present study produced the expected increases in Ca^{2+} affinity and apparent $V_{max(Ca)}$ of calcium uptake at 1 mM $MgATP^{2-}$ (Figure 3 and Table 2). $C_{12}E_8$, also, produced a decrease in apparent $K_{m(Ca)}$ and a still greater decrease in this parameter when added to trypsin treated microsmoes so that the apparent $K_{m(Ca)}$ of the microsomes is no longer significantly different from control microsomes treated with $C_{12}E_8$ (Table 2). However, the detergent produced a marked decrease in $V_{max(Ca)}$ in trypsin-treated microsomes but only a slight decrease in trypsin inhibitor/trypsin-treated (control) microsomes that did not reach statistical significance in this set of experiments (but *cf.* Figure 1A). The Hill coefficients in each instance reflect the cooperative nature of calcium uptake with respect to Ca^{2+}. The significance of any possible changes in this parameter with detergent treatment is not readily apparent.

It has been proposed previously that proteolytic cleavage of phospholamban's inhibitory cytoplasmic domain produces effects similar to those of protein kinase A-catalyzed phosphorylation of this domain (Kirchberger et al., 1986). It should not be inferred that the effects of the two treatments are necessarily identical in every respect, and a number of considerations apply. The conditions chosen for trypsin treatment of the microsomes represent a compromise between complete cleavage of phospholamban's cytoplasmic domain and the beginning of a deleterious effect associated with hydrolytic cleavage at the T2 site of the calcium pump protein as the trypsin concentration is increased. Therefore, in this context, it is not surprising that $C_{12}E_8$ produced an additional increase in Ca^{2+} affinity in trypsin-treated microsomes (Figure 3 and Table 2). Likewise, the effects of $C_{12}E_8$ on trypsin-treated and trypsin inhibitor/trypsin-treated microsomes, as described above, are not necessarily expected to be identical in phosphorylated microsomes and their corresponding controls, which were tested next.

$C_{12}E_8$ produced a clear decrease in calcium uptake by phosphorylated microsomes when measured at the high end of the Ca^{2+} concentration range (Figure 4). Its predominant effect on the control microsomes was to produce a leftward shift in the Ca^{2+} concentration dependence of calcium uptake toward lower Ca^{2+} concentrations. These effects of $C_{12}E_8$ are manifest also in changes in the optimized kinetic parameters, apparent $V_{max(Ca)}$ and $K_{m(Ca)}$, that were obtained in a fit of the data shown in Figure 4 (Table 3). In these

Table 3: Apparent Kinetic Parameters for Calcium Uptake by Control and Phosphorylated Microsomes Measured in the Presence and Absence of 15 μM $C_{12}E_8$[a]

microsomes	$C_{12}E_8$	$V_{max(Ca)}$ (μmol/mg·min)	%	$K_{m(Ca)}$ (μM)	%	Hill coefficient	%
control	–	0.325	100	1.20	100	1.41	100
control	+	0.304	94	0.73	61	1.24	88
phosphorylated	–	0.386	119	0.47	39	1.52	108
phosphorylated	+	0.328	101	0.40	33	1.49	106

[a] Values represent the optimized parameters that were obtained in an unweighted fit of the data shown in Figure 4 to the equation given in the legend to Figure 3.

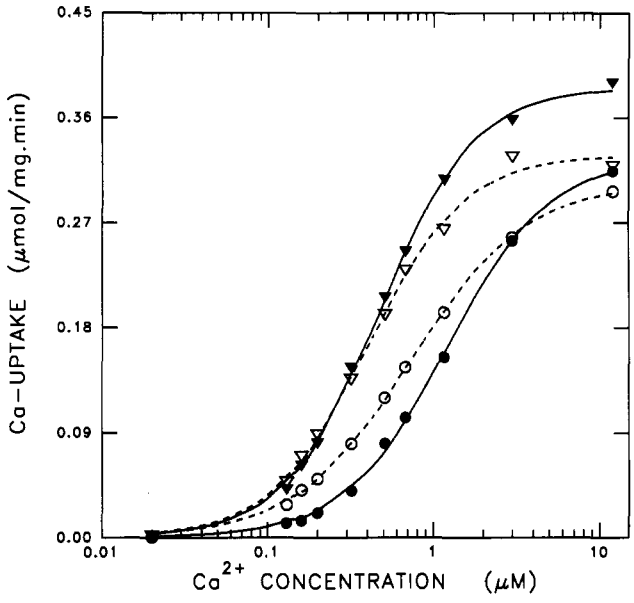

FIGURE 4: Effect of $C_{12}E_8$ on the Ca^{2+} concentration dependence of Ca uptake by control and phosphorylated cardiac microsomes. Microsomes were incubated for 2 min in the presence (▼, ▽) and absence (●, ○) of 2 μM cyclic AMP and 1 mg/mL protein kinase with (○, ▽) and without (●, ▼) 15 μM $C_{12}E_8$ prior to the addition of different concentrations of Ca^{2+} in order to initiate the calcium uptake reaction. The optimized kinetic parameters obtained from the data represented in the figure are shown in Table 3.

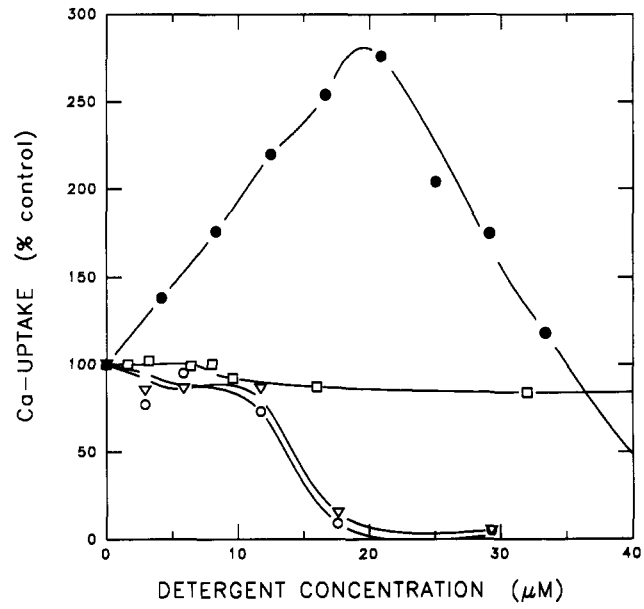

FIGURE 5: Comparative effects of detergents on calcium uptake. Microsomes were preincubated for 2 min with Brij-35 (●), Brij-56 (○), Brij-58 (▽) or digitonin (□) prior to initiation of the calcium-uptake reaction by the addition of Ca^{2+}. Digitonin and Brij-35, like Brij-56 and -58, also resulted in complete loss of calcium accumulation when their concentration was increased beyond the concentrations indicated in the figure. The data represent the averages of two experiments (Brij-35, digitonin) or single experiments (Brij-56, Brij-58) that were repeated under slightly different conditions with similar results.

respects, the effects of detergent were similar in the phosphorylated and trypsin-treated microsomes and in their respective controls. A difference, however, is that the decrease in apparent $K_{m(Ca)}$ that is seen in Table 3 is less than the decrease seen in Table 2, which is probably associated with the higher apparent $K_{m(Ca)}$ of the control microsomes in the present experiment. Nevertheless, phosphorylation markedly reduced the apparent $K_{m(Ca)}$ and increased apparent $V_{max(Ca)}$ to values similar to those obtained with trypsin. These results suggest that the higher apparent $K_{m(Ca)}$ of the control microsomes, seen in Table 3, is probably not associated with a difference in the homogeneity of the membrane preparations but rather is due to other factors including possible differences in the extent of *in vivo* phospholamban phosphorylation, which was previously shown to be correlated with rates of calcium uptake by untreated microsomes (Kasinathan et al., 1988). This variability in *in vivo* phospholamban phosphorylation as well as minor differences in the extent of hydrolytic cleavage of the cytoplasmic domain of phospholamban from the membrane could, in turn, affect an interaction of the detergent with the membrane and the calcium pump protein. Although a large amount of phospholamban in the unphosphorylated state can account for a relatively high $K_{m(Ca)}$ in the control microsomes, it cannot account for their relatively high calcium-uptake rate at 12 μM Ca^{2+} (Figure 4) and apparent $V_{max(Ca)}$ (Table 3). Such differences in cardiac microsomes might be related to the wide variation found by others in fast skeletal muscle Ca-ATPase activity when measured at high ATP concentration but not at low ATP concentrations and attributed to different degrees of stimulation produced by ATP (Gould et al., 1986; Michelangeli et al., 1991).

Comparative Effects of Detergents. In order to investigate the specificity of the effects of $C_{12}E_8$ on calcium uptake, several other nonionic detergents were tested. At a concentration of about 21 μM, Brij-35 ($C_{12}E_{\langle 23\rangle}$[2]) produced a marked stimulation of calcium uptake when microsomes were assayed at 0.32 μM Ca^{2+} while higher detergent concentrations resulted in an apparent loss of the ability of the microsomes to accumulate calcium (Figure 5). Brij-56 ($C_{16}E_{\langle 10\rangle}$) and Brij-58 ($C_{16}E_{\langle 20\rangle}$) both failed to stimulate calcium uptake, and at concentrations higher than about 12 μM produced a sharp loss of the ability of microsomes to accumulate calcium. These data suggest that unlike an increase in the number of carbons from 12 to 16 in the hydrophobic portion of the detergent, an increase in the number of the oxyethylene units in the hydrophilic portion of the detergent is well tolerated. Digitonin, a bulky steroid-based molecule, was ineffective in stimulating calcium uptake when measured at 0.32 μM Ca^{2+} and resulted in a more gradual loss of the ability of the

[2] $\langle\chi\rangle$ indicates a heterogeneous head group composition with an average of χ oxyethylene units.

microsomes to accumulate calcium composed to the other detergents, with complete loss occurring at about 160 μM.

DISCUSSION

The present study demonstrates that $C_{12}E_8$ can be used to dissociate an effect of phospholamban on apparent $V_{max(Ca)}$ of the calcium pump measured at millimolar ATP from its effect on apparent $K_{m(Ca)}$. Thus at 1 mM $MgATP^{2-}$, 15 μM $C_{12}E_8$ decreased apparent $V_{max(Ca)}$ of calcium uptake in trypsin-treated or phosphorylated microsomes to close to the significantly lower $V_{max(Ca)}$ found in control microsomes, in which $C_{12}E_8$ decreased $V_{max(Ca)}$ only slightly; yet, like trypsin treatment or phosphorylation, $C_{12}E_8$ significantly increased Ca^{2+} affinity in the control microsomes (Figures 3 and 4). However, at 0–3.0 μM $MgATP^{2-}$, the effects of $C_{12}E_8$ on calcium uptake were strikingly similar to those previously seen to be produced by trypsin (Figure 2). The implications of these findings, on the basis of the detergent's actions on the Ca-ATPase activity measured by other investigators in fast skeletal muscle microsomes (Anderson et al., 1983; Champeil et al., 1986; Gould et al., 1986; de Foresta et al., 1989), are discussed below.

The nonsolubilizing concentration (15 μM) of added $C_{12}E_8$ present in the reaction mixture is below its critical micellar concentration and appears to produce minimal, negligible loss of transported calcium from the vesicles. It produced, in fact, a 2–3-fold increase in calcium uptake at subsaturating Ca^{2+} in cardiac microsomes but failed to produce a comparable effect on calcium uptake in fast skeletal muscle microsomes when tested at subsaturating Ca^{2+} concentrations under similar conditions (Figure 1). A $C_{12}E_8$-induced increase in Ca^{2+} affinity when measuring cardiac microsomal Ca-ATPase activity was previously reported by Tate et al. (1989).

Nonsolubilizing concentrations of $C_{12}E_8$ are known to produce distinct effects on different steps in the catalytic cycle of the Ca^{2+}-ATPase of fast skeletal muscle SR when studied in permeabilized vesicles (Anderson et al., 1983; Champeil et al., 1986; Gould et al., 1986; de Foresta et al., 1989). Such effects could not be accounted for by insertion of the detergent into the bulk lipid of the bilayer (le Maire et al., 1987). Specifically, $C_{12}E_8$ has been shown to increase the rate constants of the transitions of E_2 to E_1 and of E_1P to E_2P and to decrease the rate constant of E_2P decomposition ($E_2P \rightarrow E_2 + P_i$) (Champeil et al., 1986). A correlation between the perturbation by $C_{12}E_8$ of Ca^{2+}–ATPase activity in fast skeletal muscle SR and the rate-limiting step in the catalytic cycle of this ATPase under a particular set of incubation conditions supports the usefulness of the detergent in identifying the rate-limiting step of the reaction cycle.

Our present findings with cardiac microsomes can be interpreted in light of the considerable information available on the effects of $C_{12}E_8$ on different steps in the reaction cycle of the skeletal muscle Ca^{2+}-ATPase. At low $MgATP^{2-}$ concentrations (and saturating Ca^{2+}) when only the catalytic site of the enzyme is occupied, the rate-limiting step in the reaction cycle of the SR calcium pump is likely to be the E_2 to E_1 (or E* to E) transition (Barrabin & de Meis, 1982; Wakabayashi & Shigekawa, 1990). An increase in the rate of this transition by $C_{12}E_8$ has been associated with an increase in Ca^{2+} affinity of fast skeletal Ca^{2+}–ATPase in permeabilized microsomes (Andersen et al., 1983). A $C_{12}E_8$-induced increase in Ca^{2+} affinity was demonstrated in the present study with cardiac microsomes (Figures 3 and 4, and Tables 2 and 3). The $C_{12}E_8$-induced increase in the rate of calcium uptake that is seen at saturating Ca^{2+} between 0 and 2 μM $MgATP^{2-}$ is also consistent with a stimulatory effect at this step in the reaction cycle, which is considered rate limiting in skeletal SR at these nucleotide concentrations (see above). However the lack of a stimulatory effect of the detergent in fast skeletal muscle microsomes under the conditions of our study (Figure 1B) suggests that the stimulatory effect observed in cardiac microsomes in the 0–10 μM range of $MgATP^{2-}$ concentration (Figure 3) is attributable to an inhibition of the E_2 to E_1 transition by unphosphorylated phospholamban in control microsomes and a release from this inhibition as a result of dissociation of phospholamban from the pump by very low concentrations of $C_{12}E_8$.

The rate of the E_2 to E_1 transition is accelerated when the ATP concentration is increased to a range favorable for binding of regulatory nucleotide to the E_2 form of the skeletal muscle enzyme (Wakabayashi & Shigekawa, 1990). In cardiac SR also, the binding of regulatory nucleotide to the E_2 conformation of the pump has been associated with an acceleration of the E_2 to E_1 transition (Pang & Briggs, 1973; Cable & Briggs, 1988). The acceleration of calcium uptake seen in Figure 2 between 2 and 3 μM ATP^{2-} may be attributable to such an effect. Similarly, a marked acceleration in the apparent initial rate of EP formation was seen by Tada et al. (1980) in phosphorylated Ca^{2+}-free microsomes when measured at low micromolar nucleotide concentrations and was attributed to regulatory nucleotide binding to the E_2 conformation of the Ca^{2+}-ATPase protein.

At higher ATP concentrations, particularly at reduced temperature or low Mg^{2+}, E_2P decomposition may become rate limiting (Pang & Briggs, 1973; Champeil et al., 1986). Our data suggest that at high enzyme velocity the effect of phospholamban resembles the effect of $C_{12}E_8$ to selectively inhibit E_2P decomposition under some conditions (Champeil et al., 1986). In trypsin-treated microsomes (in which the cytoplasmic domain of phospholamban has been cleaved), $C_{12}E_8$ reduced calcium uptake to close to control microsomes, presumably by inserting into the membrane bilayer between the calcium pump protein and the cleaved phospholamban. However, in control microsomes at saturating Ca^{2+} and millimolar ATP, E_2P decomposition is presumably already largely inhibited by phospholamban. The absence of an inhibitory effect of $C_{12}E_8$ on calcium uptake at 0–3 μM $MgATP^{2-}$ suggests that E_2P decomposition is not rate limiting under these conditions but that, rather, the E_2 to E_1 transition is rate limiting, as discussed above. Because E_2P decomposition appears only moderately sensitive to ATP concentration (Gould et al., 1986), an ATP-induced acceleration of this step in the reaction cycle would require higher ATP concentrations than an acceleration of the E_2 to E_1 transition, which we suggest occurs at 2–3 μM $MgATP^{2-}$. Squier and Thomas (1988) have correlated decreased phosphoenzyme decomposition in fast skeletal sarcoplasmic reticulum with a decreased protein mobility that may be the result of aggregation (oligomerization) of Ca^{2+}-ATPase protein. Evidence for a more aggregated state of the cardiac Ca-ATPase than of fast skeletal muscle Ca-ATPase in SR membranes was recently provided (Birmachu et al., 1993) and attributed to electrostatic interaction of phospholamban with the Ca^{2+}–ATPase protein in cardiac SR membranes. Further investigation is necessary in order to reconcile these studies with the presently suggested inhibition of E_2P decomposition in trypsin-treated cardiac microsomes by a nonionic detergent.

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