Limited Carbodiimide Derivatization Modifies Some Functional Properties of the Sarcoplasmic Reticulum Ca^{2+} Release Channel†

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ABSTRACT: Sarcoplasmic reticulum membrane derived from the terminal cisternae region reacts with the carboxyl reagent *N,N'*-dicyclohexylcarbodiimide. The extension of this reaction is dependent on the reagent/protein ratio. By using a low ratio (10 μM reagent and 1 mg of protein/mL), we can selectively prevent the closure of the 450-kDa Ca^{2+} channel. Rapid filtration experiments indicate no alteration in the activating mechanism of Ca^{2+} release induced by Ca^{2+} or Sr^{2+} whereas the Ca^{2+} efflux inhibition by Ca^{2+}, Mg^{2+}, or ruthenium red disappears after the chemical treatment. The activating/inhibitory effect of ryanodine on the Ca^{2+} channel does not appear to be perturbed by *N,N'*-dicyclohexylcarbodiimide. The negligible incorporation of the ^{14}C radioactive reagent to the 450-kDa band (the Ca^{2+} channel subunit) indicates the possibility of protein cross-linking in addition to simple derivatization. The functional alterations produced by this reagent suggest the presence of critical acidic residue(s) in a hydrophobic environment which are involved in the low-affinity cationic binding site. They can be tentatively associated with hydrophobic domains of the channel subunits contributing to the lining of the pore for Ca^{2+} release. The data also indicate that the channel activation by micromolar Ca^{2+} occurs in a different protein domain which is carbodiimide-insensitive under the experimental conditions tested.

The junctional membrane of the sarcoplasmic reticulum network contains the ionic channel involved in the release of Ca^{2+} [for reviews, see Endo (1977), Lai and Meissner (1989), and Fleischer and Inui (1989)] prior to the contraction of the skeletal muscle fiber. The scarce occurrence in terms of the percentage of the Ca^{2+} channel in standard preparations of sarcoplasmic reticulum (SR)[1] heavy fraction (Inui et al., 1987; Imagawa et al., 1987), the very high molecular mass (Pessah et al., 1986; Imagawa et al., 1987; Lai et al., 1988), and the acute proteolytic sensitivity (Seiler et al., 1984; Lai et al., 1988) have been some particular difficulties for biochemical studies on this protein.

Much of the effort devoted up to date for characterizing this Ca^{2+} release pathway has been directed toward the identification of the channel protein (Inui et al., 1987; Imagawa et al., 1987), the oligomeric organization (Ferguson et al., 1984; Lai et al., 1988), the role in the excitation–contraction coupling (Block et al., 1988; Saito et al., 1988), the modulators of the channel function (Yamamoto & Kasai, 1982b; Meissner, 1984; Meissner, 1986; Cifuentes et al., 1989), and even the molecular cloning and sequencing of the cDNA (Takeshima et al., 1989; Marks et al., 1989; Zorzato et al., 1990).

However, one of the most important aspects that deserves more attention is the study of the action mechanism, how some conformational changes in the cytoplasmic activating domain can induce some modification in the transmembrane segments region, leading to the opening of the channel. A previous step could be the characterization of functional domains in the protein. The proteolytic digestion of the Ca^{2+} channel subunit has permitted the identification of surface-exposed peptides (Marks et al., 1990), and long-range conformational changes have already been described for activating Ca^{2+} (Kang et al., 1992). Lately, some studies have identified specific domains involved in the functional activity of the Ca^{2+} release channel (Chen et al., 1992; Orr et al., 1993).

The use of reagents for site-specific modification of proteins is a very promising approach leading to the identification of critical reactive groups and functional protein domains. One possibility is the reagent *N,N'*-dicyclohexylcarbodiimide. This compound has been proved to react covalently and blocks a number of transport systems involved in ionic fluxes of H^+ (Pennington & Fischer, 1981; Phelps & Hatefi, 1981), Ca^{2+} (Pick & Racker, 1979; de Ancos & Inesi, 1988), or Na^+–K^+ (Robinson, 1974; Pedemonte & Kaplan, 1986) across biological membranes. Indeed, the carbodiimide reaction has been shown to be quite specific for carboxyl groups and directed to highly conserved and hydrophobic domains of the proteins (Solioz, 1984). This has attracted our interest in the carboxyl reagent as a tool to investigate the mechanism of Ca^{2+} release through the 450-kDa Ca^{2+} channel in the SR membrane. It must be stressed that this channel is a very peculiar entity showing only a structural kinship with the inositol 1,4,5-trisphosphate receptor (Gill, 1989).

In this study we used a low DCCD/protein ratio to obtain a limited carbodiimide derivatization. Under these experimental conditions we studied by the rapid filtration technique the response of the Ca^{2+} channel activity to several well-established modulators. The present results permit some analysis of the physical location and the functional role of the DCCD-reactive carboxyl group(s).

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[1] Abbreviations: SR, sarcoplasmic reticulum; DCCD, *N,N'*-dicyclohexylcarbodiimide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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MATERIALS AND METHODS

Microsomal Preparation. Junctional sarcoplasmic reticulum vesicles were obtained from rabbit leg and back muscle by a slight modification of the Saito et al. (1984) procedure, as described previously (Soler et al., 1992). The protein concentration was estimated by a colorimetric method (Lowry et al., 1951) using bovine serum albumin as the standard.

Free Cation Concentrations. The free Ca^{2+} or Sr^{2+} concentration in the incubation and/or release media was established by cation–EGTA buffers according to a computer program (Fabiato & Fabiato, 1979) that considered the association constants of the different species present. The protonation equilibria of EGTA were taken from Blinks et al. (1982).

Carbodiimide Derivatization. Junctional vesicles (1 mg of protein/mL) were incubated for 10 min at room temperature in a medium containing 50 mM MES–Tris, pH 6.0, 100 mM KCl, 1 mM EGTA, 0.3 M sucrose, and 10 or 200 μM DCCD. Thereafter, the samples were cooled on an ice bath and centrifuged at 190000*g* and 4 °C for 45 min to stop the reaction. The pellets, once resuspended in 10 mM MOPS, pH 7.0, and 0.3 M sucrose, were aliquotted and frozen at −80 °C until use. Samples exposed to the same treatment in the absence of DCCD were used as a control. In some experiments the incubation pH or the DCCD concentration was modified as described in the corresponding figure legend.

Ca^{2+} Loading Capacity. The passive accumulation of Ca^{2+} was studied after equilibration of DCCD-treated junctional vesicles (1 mg/mL) for 2 h at room temperature in the presence of 20 mM MOPS, pH 6.8, 80 mM KCl, and 5 mM $^{45}CaCl_2$ (~10 000 cpm/nmol). Aliquots of 60 μL were added in 0.94 mL of dilution medium containing 20 mM MOPS, pH 6.8, 80 mM KCl, and 1 mM $LaCl_3$. Then, samples of 0.9 mL (~54 μg of protein) were filtered through nitrocellulose filters (Millipore HAWP, 0.45 μm), and the filters were rinsed with 3 mL of dilution medium before counting by the liquid scintillation technique.

Ca^{2+}–ATPase Activity. The enzymatic activity was assayed by measuring at 25 °C the inorganic phosphate liberated from ATP in a reaction medium containing 20 mM MOPS, pH 6.8, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.827 mM $CaCl_2$ (pCa 5.3), 0.02 mg of DCCD-treated vesicles/mL, 10 μM A23187, and 1 mM ATP. Initial rates were evaluated by stopping the reaction at different time intervals with a molybdovanadate reagent (Lin & Morales, 1977). The basal ATPase activity was evaluated in the presence of 2 mM EGTA and no Ca^{2+} added. The Ca^{2+}-dependent activity was calculated from the difference between the total and the Mg^{2+}-dependent activities.

Assays of Ca^{2+} Release. The kinetics of Ca^{2+} release was studied in DCCD-derivatized vesicles (3 mg/mL) loaded by passive equilibration for 2 h at room temperature with 5 mM $^{45}CaCl_2$ (~8000 cpm/nmol) in the presence of 20 mM MOPS, pH 6.8, 80 mM KCl, and 50 mM sucrose. At the end of the incubation, 40-μL protein aliquots were added in 0.96 mL of dilution medium composed of 20 mM MOPS, pH 6.8, 80 mM KCl, 5 mM $CaCl_2$, and 50 mM sucrose. Samples of 0.9 mL (108 μg of protein) were loaded onto 0.65-μm Millipore filters (DAWP) placed in a rapid filtration apparatus (Bio-Logic Co., Claix, France) and rinsed under vacuum with 4 mL of the nonradioactive Ca^{2+} buffer. Twenty-five seconds after the vesicle dilution, each aliquot was perfused for a different millisecond time-controlled interval with the corresponding medium.

(i) The Ca^{2+}-induced Ca^{2+} release was elicited by using perfusion media containing 20 mM MOPS, pH 6.8, 80 mM KCl, 1 mM EGTA, different $CaCl_2$ concentrations to yield the required free Ca^{2+}, and 50 mM sucrose.

(ii) The release medium induced by Sr^{2+} was composed of 20 mM MOPS, pH 6.8, 80 mM KCl, 2 mM EGTA, 127.7 μM strontium nitrate to provide 10 μM free Sr^{2+}, and 50 mM sucrose. In other experiments the Sr^{2+} concentration was 101.3 μM, and 3 mM $CaCl_2$ was also present.

(iii) The release of Ca^{2+} induced by Ca^{2+} plus ATP was measured in the presence of Ca^{2+} release media supplemented with 2 mM ATP.

(iv) The effect of Mg^{2+} on Ca^{2+} release was studied by using perfusion media containing 20 mM MOPS, pH 6.8, 80 mM KCl, 1 mM EGTA, different $CaCl_2$ concentrations to give a free Ca^{2+} concentration of 1 μM, different $MgCl_2$ concentrations, and 50 mM sucrose.

(v) The ruthenium red effect was measured by including different ruthenium red concentrations in a release medium containing 20 mM MOPS, pH 6.8, 80 mM KCl, 1 mM EGTA, 0.503 mM $CaCl_2$ to yield 1 μM free Ca^{2+}, and 50 mM sucrose.

(vi) The Ca^{2+} release/blocking induced by ryanodine was evaluated by measuring the steady-state Ca^{2+} content of the vesicles under conditions of Ca^{2+} pumping activity. The DCCD-treated vesicles (1 mg/mL) were incubated for 30 min at 37 °C with different concentrations of ryanodine in the presence of 20 mM MOPS, pH 7.0, 150 mM KCl, and 100 μM $CaCl_2$. Aliquots of the incubation mixture (0.06 mL) were diluted 20-fold at room temperature in a solution of 20 mM MOPS, pH 7.0, 150 mM KCl, 10 mM $MgCl_2$, and 100 μM $^{45}CaCl_2$ (~10 000 cpm/nmol), and then, 2 mM acetyl phosphate was added at zero time to initiate the Ca^{2+} uptake. The reaction was allowed to proceed for 2 min prior to filtration of 1-mL samples (50 μg of protein) on DAWP Millipore filters. The filters were rinsed with 4 mL of the medium 20 mM MOPS, pH 7.0, 80 mM KCl, and 1 mM $LaCl_3$ before radioactive counting.

In these experiments the DCCD concentration used for derivatization was always 10 μM. The apparent rate constants for Ca^{2+} release were obtained from semilogarithmic plots of the Ca^{2+} content inside the vesicles as a function of time. Data are the average of at least three independent determinations performed with different membrane preparations. When appropriate, they were represented with the standard error of the mean.

Labeling with [^{14}C]DCCD. The SR membranes (1 mg/mL) were incubated as described for the chemical modification reaction but in the presence of radiolabeled DCCD (50 μCi/μmol). The vesicles were sedimented by centrifugation to remove the unreacted [^{14}C]DCCD and resuspended in 10 mM MOPS, pH 7.0, and 0.3 M sucrose.

Gel Electrophoretic Analysis. Standard minislab gel electrophoresis in the presence of SDS was performed by the method of Laemmli (1970) using 0.75-mm-thick, 5–10% polyacrylamide gradient for the separating gel. A typical electrophoresis was run at 200 V for approximately 45 min. High molecular weight proteins were studied in a 5% separating gel run at a constant current of 120 V for 2 h. The stacking gel contained a 4% polyacrylamide concentration. The staining of the slab gels was carried out with 0.1% Coomassie Blue R-250 by standard methods (Fairbanks, 1971).

Materials and Other Procedures. Ryanodine was purchased from Agri Systems International (Windgap, PA). The molecular weight markers for SDS gel electrophoresis (MW-SDS-200 kit from Sigma) were myosin (205 000), β-galac-

tosidase (116 000), phosphorylase b (97 400), bovine serum albumin (66 000), ovalbumin (45 000), and carbonic anhydrase (29 000). Carbodiimide stock solutions were prepared fresh every day in absolute ethanol. Radioactive [^{14}C]DCCD was dissolved in toluene as purchased from Amersham Iberica (Spain). Appropriate aliquots were dried under a stream of nitrogen gas to evaporate the solvent and then redissolved in ethanol prior to the experiments. Autoradiography for detection of the [^{14}C]DCCD-bound proteins was carried out by the fluorographic technique as previously described (Laskey & Mills, 1975).

RESULTS

To study the phenomenon of Ca^{2+} release, it is essential to get SR vesicles with the Ca^{2+} loading capacity. Thus, our first concern was to study this parameter in the junctional membrane after the chemical treatment with the carboxyl reagent DCCD. Vesicles were initially incubated for 10 min with different concentrations of carbodiimide at pH 6.0 and in the absence of Ca^{2+}, sedimented by centrifugation, and resuspended in a buffered sucrose solution. The derivatized vesicles were subsequently incubated in a 5 mM $^{45}Ca^{2+}$ medium to reach equilibration and then diluted in the presence of La^{3+} before evaluating the Ca^{2+} retained inside. For the DCCD concentrations shown in Figure 1A, the Ca^{2+} content of passively loaded vesicles was around 50–60 nmol/mg protein with a slight tendency to decrease as the carbodiimide concentration increases. These values represent approximately a 60–70% of the total Ca^{2+} loading capacity measured under standard conditions. Hence, the difference in the loading capacity seems to be ascribable to the experimental conditions and not to the chemical modification itself since the Ca^{2+} content was already lower when the vesicles were exposed to the same procedure in the absence of DCCD (see the zero concentration point). High DCCD concentrations (1 mM) collapse the Ca^{2+} loading capacity of the vesicles (data not shown).

The decreased passive loading observed in Figure 1A is not related to the extent of the DCCD chemical reaction as confirmed in Figure 1B. We measured the initial rate of the Ca^{2+}-dependent ATPase activity, and this was taken as an index of the chemical process owing to the relative abundance of this protein in the SR membrane and the well-known reactivity of DCCD toward this protein. The hydrolytic activity of ATP was found to exponentially decrease as the concentration of DCCD was raised, and this tendency was not correlated with the extension of the Ca^{2+} loading.

In the next experiments, we performed a radioactive labeling of the junctional vesicles under conditions of low DCCD/protein ratio (10 μM [^{14}C]DCCD and 1 mg of protein/mL) or high DCCD/protein ratio (200 μM [^{14}C]DCCD and 1 mg of protein/mL) and then the labeled vesicles were analyzed by SDS–polyacrylamide gradient gel electrophoresis. The Coomassie Blue staining of the separating gel shows (Figure 2A) the presence of the typical components of the cisternal–junctional membrane. The protein band marked with an asterisk corresponds to the partial degradation of the Ca^{2+} channel subunit. The electrophoretic pattern of lane 1 (samples treated with 10 μM DCCD) resembles that of untreated vesicles. However, the presence of 200 μM DCCD (lane 2) modifies to a greater extent that pattern. Thus, the Ca^{2+} channel bands and the Ca^{2+} pump protein appear smaller than in lane 1. It should be noted that both lanes were equally loaded with 20 μg of protein. The autoradiographic analysis (Figure 2B) shows the labeling of several proteins including

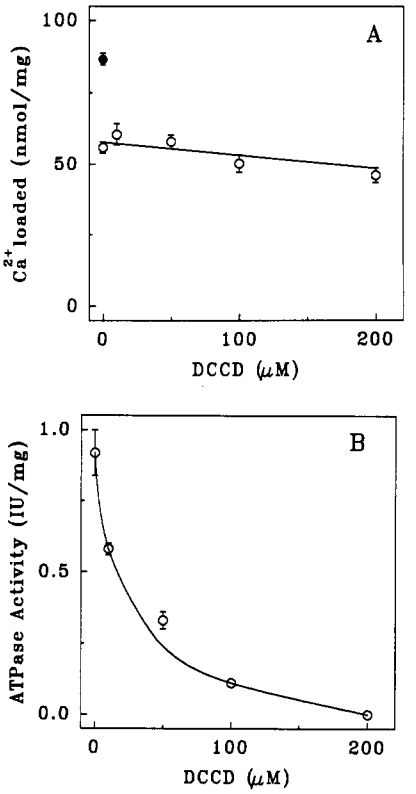

FIGURE 1: Passive Ca^{2+} loading (A) and Ca^{2+}–ATPase activity (B) in cisternal–junctional vesicles after preincubation with DCCD. Microsomal vesicles (1 mg/mL) were treated for 10 min at room temperature in a medium composed of 50 mM MES–Tris, pH 6.0, 100 mM KCl, 1 mM EGTA, 0.3 M sucrose, and the DCCD concentrations indicated in the abscissa axis. The free reagent was eliminated after centrifugation–resuspension. The Ca^{2+} content (panel A) was evaluated after equilibration (2 h at room temperature) of the modified vesicles in the medium 20 mM MOPS, pH 6.8, 80 mM KCl, and 5 mM $^{45}CaCl_2$. Aliquots of 60 μL were diluted in 0.94 mL of the medium 20 mM MOPS, pH 6.8, 80 mM KCl, and 1 mM $LaCl_3$, and then, samples of 0.9 mL were filtered (0.45-μm pore size) prior to the rinsing and counting of the filters (O). For the zero concentration point the vesicles were exposed to the same treatment in the absence of DCCD. The closed circle represents the current level of Ca^{2+} accumulation in native untreated vesicles. The total ATPase activity was measured in the medium 20 mM MOPS, pH 6.8, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.827 mM $CaCl_2$, 0.02 mg/mL of DCCD-treated vesicles, 10 μM A23187, and 1 mM ATP. The Ca^{2+}-dependent activity, expressed as micromoles of P_i per minute and milligrams of protein (panel B), was obtained by subtracting the activity measured in the presence of excess EGTA.

the doublet of the Ca^{2+} channel subunit (asterisk) when the reagent concentration was 200 μM (lane 2). The incubation of the vesicles in the presence of 10 μM radioactive DCCD (lane 1) provides a considerably lower radioactive signal. A careful visual inspection of the original film permits a very slight incorporation of radioactivity into the channel protein to be detected; however, it can no longer be observed after the photographic processing.

Some clues of the DCCD modification process can be obtained by studying more carefully the protein bands after the Coomassie staining. The experimental conditions included a 4% polyacrylamide stacking and 5% polyacrylamide separating gel and overloading of the wells in order to facilitate the identification of high molecular weight bands. A set of samples were treated, at different DCCD concentrations, and then subjected to SDS electrophoresis. Thus, we can see in Figure 3 that at the lowest reagent concentration (10 μM DCCD in lane 2) the protein bands appear practically as in nontreated vesicles (lane 1). However, as the DCCD concentration increases (lanes 3–5) a decrease in the amount of

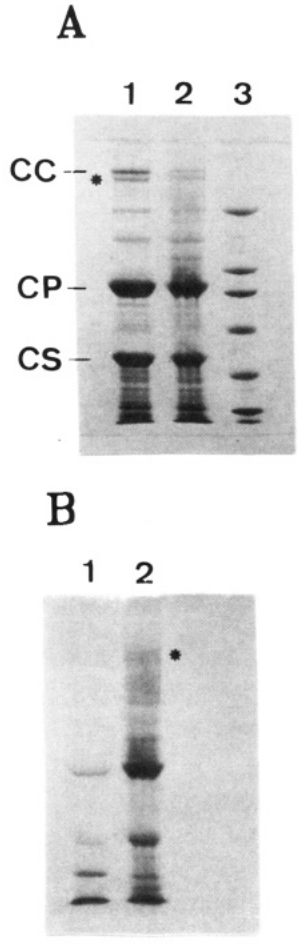

FIGURE 2: Effect of the DCCD/protein ratio on the electrophoresis (A) and autoradiographic (B) patterns of junctional SR. Microsomal vesicles (1 mg/mL) were incubated with 10 μM [^{14}C]DCCD (low DCCD/protein ratio) or 200 μM [^{14}C]DCCD (high DCCD/protein ratio) as described under Materials and Methods. Samples were then subjected to polyacrylamide gradient gel electrophoresis in the presence of SDS. Panel A shows the Coomassie Blue staining of the separating gel at a low (lane 1) or high (lane 2) DCCD/protein ratio. The Ca^{2+} channel subunit (CC), Ca^{2+} pump protein (CP), and calsequestrin (CS) are marked on the gel. Molecular weight standards are shown in lane 3. From top to bottom they are myosin (205K), β-galactosidase (116K), phosphorylase b (97.4K), bovine serum albumin (66K), ovalbumin (45K), and carbonic anhydrase (29K). Panel B shows the corresponding autoradiogram at a low (lane 1) or high (lane 2) DCCD/protein ratio. The labeling of the Ca^{2+} channel subunit (doublet) is marked by an asterisk.

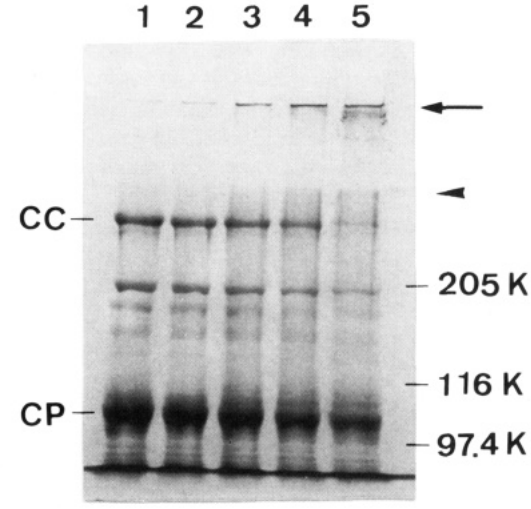

FIGURE 3: Effect of the DCCD treatment on the high molecular weight proteins from junctional SR (stacking–separating gel). Microsomal vesicles (1 mg of protein/mL) were incubated with different DCCD concentrations as detailed in the description of the experimental procedures. Aliquots were dissolved in the Laemmli sample buffer and then analyzed by SDS electrophoresis in a 4% stacking–5% separating gel. After 2 h at 120 V the slab gel was stained with Coomassie Blue. Each well was loaded with 60 μg of protein. The DCCD concentrations were 10 (lane 2), 50 (lane 3), 100 (lane 4), and 200 μM (lane 5). Lane 1 contains untreated vesicles. CC and CP stand for the Ca^{2+} channel and Ca^{2+} pump proteins, respectively. The arrow marks the position of the aggregated protein at the top of the stacking gel, and the arrowhead indicates the interphase between the stacking and separating gel.

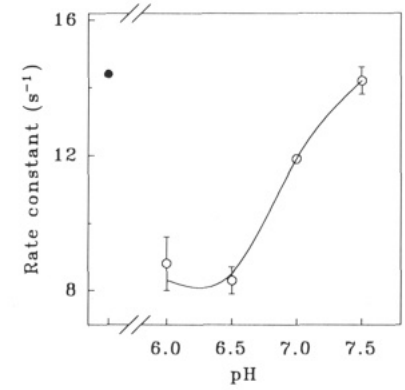

FIGURE 4: pH dependence on the DCCD reaction with junctional vesicles. SR membranes (1 mg/mL) were derivatized for 10 min at room temperature with 10 μM DCCD as described for Figure 1 although at different pH (between 6 and 7.5) by using MES or MOPS buffer. The extent of derivatization at different pH (O) was estimated by measuring the release of Ca^{2+} induced by Ca^{2+} plus ATP at pH 6.8. The perfusion medium was 20 mM MOPS, pH 6.8, 80 mM KCl, 1 mM EGTA, 0.518 mM $CaCl_2$ (1 μM free Ca^{2+}), 5 mM ATP, and 50 mM sucrose. The closed circle gives the apparent rate constant at pH 6.8 of native vesicles.

the Ca^{2+} channel subunit and other proteins and the appearance of a new band at the top of the stacking gel become apparent. By using a 3% stacking gel, the cross-linked protein is able to reach the interphase between the stacking and the separating gel (not shown).

It is of interest that the DCCD reaction requires the presence of protonated carboxyl group(s) (Kurzer & Dovraghi-Zadeh, 1967). In order to estimate the pH dependence of the protein reactive group(s), we varied the incubation pH to carry out the DCCD modification and then we monitored the release of Ca^{2+} induced by Ca^{2+} and ATP. The results of Figure 4 indicate that DCCD decreases the activating effect of Ca^{2+} plus ATP on Ca^{2+} release. The maximal inhibitory effect of DCCD on the process of Ca^{2+} release (i.e., maximal derivatization) was obtained at the lower pH tested, and then as the pH was raised the effect progressively decreased. The midpoint effect was observed at pH around neutrality.

Therefore, vesicles derivatized at a low DCCD/protein ratio in a buffered medium at pH 6.0 were used for the kinetic characterization of Ca^{2+} release. One of the mechanisms proved to activate the Ca^{2+} channel in microsomal preparations is the presence of extravesicular Ca^{2+} (Miyamoto & Racker, 1982; Sumbilla & Inesi, 1987). This process can be studied with radiolabeled Ca^{2+}, showing that the apparent first-order rate constant for Ca^{2+} release is dependent on the external Ca^{2+} concentration (Meissner et al., 1986). It shows a bell-shaped profile (Figure 5), indicating that lower concentrations of Ca^{2+} activate the channel until a maximum is reached (pCa 6) and that higher Ca^{2+} concentrations act as inhibitors of the process. When the junctional vesicles were treated with DCCD before the studies of Ca^{2+} release, it was found that the activation of the Ca^{2+} channel by submicromolar

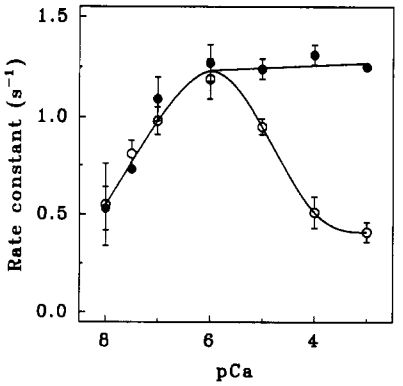

FIGURE 5: Effect of DCCD on the Ca^{2+}-dependent Ca^{2+} release. SR vesicles were treated with 10 μM DCCD as described (see Materials and Methods). The modified vesicles (3 mg/mL) were subjected for 2 h at room temperature to passive equilibration in the presence of 20 mM MOPS, pH 6.8, 80 mM KCl, 50 mM sucrose, and 5 mM $^{45}Ca^{2+}$. Samples of 40 μL were diluted in 0.96 mL of the same medium without radioactive tracer. Thereafter, 0.9-mL aliquots were loaded on the rapid filtration device (0.65-μm filters) and rinsed with 4 mL of the unlabeled medium. The release of Ca^{2+} was triggered by flushing media containing 20 mM MOPS, pH 6.8, 80 mM KCl, 1 mM EGTA, different $CaCl_2$ concentrations, and 50 mM sucrose. Closed circles stand for derivatized sample experiments, and open circles correspond to untreated vesicles (control).

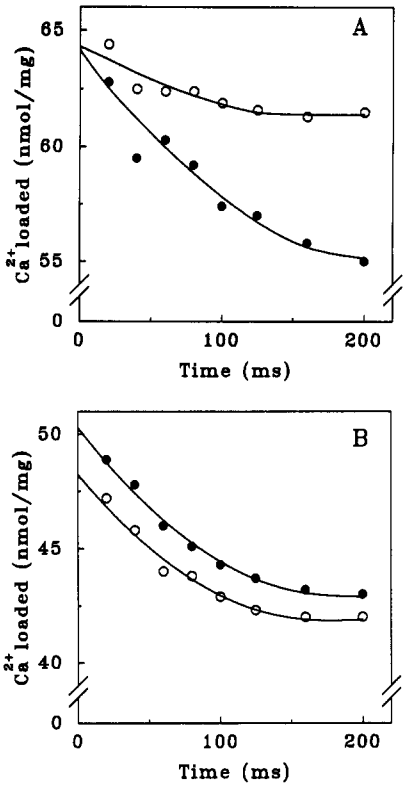

FIGURE 6: External effect of Sr^{2+} and Ca^{2+} on the kinetics of Ca^{2+} release in DCCD-treated vesicles. The time course of the Sr^{2+}-induced Ca^{2+} release was studied by the rapid filtration technique in native vesicles (A) or vesicles derivatized with 10 μM DCCD (B). The passive loading with $^{45}Ca^{2+}$ and the processing of aliquots to measure Ca^{2+} release was as described in the legend of Figure 5. The release medium was 20 mM MOPS, pH 6.8, 80 mM KCl, 2 mM EGTA, 0.127 mM strontium nitrate (10 μM Sr^{2+} free), and 50 mM sucrose (●). In some experiments the release medium was supplemented with 3 mM $CaCl_2$ (○).

Ca^{2+} remains unchanged. Interestingly, once the Ca^{2+} channel is fully activated by Ca^{2+} it cannot be inhibited by submillimolar concentrations of Ca^{2+} as in the native vesicles.

Ca^{2+} can be successfully substituted by Sr^{2+} as the activating cation of the Ca^{2+} channel (Soler et al., 1992). Therefore, addition of 10 μM free Sr^{2+} to the external medium induces a rapid Ca^{2+} release from the interior of the vesicles. The Sr^{2+}-induced Ca^{2+} release measured in Figure 6A shows an apparent rate constant of approximately 1.2 s^{-1} which is similar to the maximal rate constant measured for Ca^{2+} as an inducer of Ca^{2+} release under optimal conditions (cf. Figure 5). This process is inhibited when a millimolar Ca^{2+} concentration is included in the Sr^{2+} release medium.

Experiments performed with DCCD-modified vesicles provide further evidence on the derivatization effect, i.e., Ca^{2+} or a Ca^{2+} analog cation is able to activate the opening of the channel whereas the blocking capacity of the channel by these cations has been lost (Figure 6B).

A question of interest is to know the modulating capacities that have been altered as a consequence of the chemical treatment and eventually to identify the functional groups of the protein that are involved. Accordingly, we studied the effect of other relevant Ca^{2+} channel inhibitors. In this case the loaded vesicles were flushed with a medium containing 1 μM free Ca^{2+} to elicit the release of Ca^{2+}, and this release medium was supplemented with different concentrations of Mg^{2+} or ruthenium red. Both cations are able to block the Ca^{2+}-induced Ca^{2+} release. From the kinetic evaluation of this effect an apparent $K_{0.5}$ for Mg^{2+} at the inhibitory site of about 1 mM (Figure 7A) can be deduced while that of ruthenium red can be established around 1 μM (Figure 7B). Here again, the sarcoplasmic vesicles that have been treated with carbodiimide are no longer able to block the Ca^{2+} efflux stimulated by Ca^{2+}.

The Ca^{2+} channel activity can be modulated by the plant alkaloid ryanodine, the effect being dependent on the concentration used (Meissner, 1986; Carroll et al., 1991). One way to evaluate the effect of ryanodine on Ca^{2+} release is by monitoring the steady-state level of Ca^{2+} accumulation when SR vesicles are supporting active Ca^{2+} transport (Chu et al., 1988). In these experiments the native or DCCD-treated vesicles were first incubated in the presence of various ryanodine concentrations and then transferred to a medium containing $^{45}Ca^{2+}$, acetyl phosphate, and other ionic requirements to stimulate the active accumulation of Ca^{2+}. The effect of increasing ryanodine concentrations (Figure 8) is initially associated with a progressive decrease in the Ca^{2+} uptake capacity, reaching the lower level of accumulation in the concentration range of 1–10 μM. A further increase in the ryanodine concentration allows the recovery of the net steady-state Ca^{2+} levels. The observed effects of ryanodine on Ca^{2+} uptake have been related to selective interactions of the drug with the Ca^{2+} channel and not with the Ca^{2+}–ATPase protein (Chu et al., 1988).

Our data also show that the vesicles that have been incubated with DCCD retain the sensitivity to ryanodine, in the same concentration range, and therefore the opening and closing effects of ryanodine are still observable. In the control experiments, corresponding to the zero ryanodine concentration, it is observed that the derivatization of the vesicles reduces significantly the level of Ca^{2+} accumulated, and this effect can be correlated with a DCCD effect on the Ca^{2+}–ATPase activity. Therefore, our data can be more properly expressed in a relative scale (inset of Figure 8) for a correct interpretation of the ryanodine effect on DCCD-treated vesicles. The modification of specific residues by DCCD does not seem to alter the modulating capacity of ryanodine on the Ca^{2+} release channel.

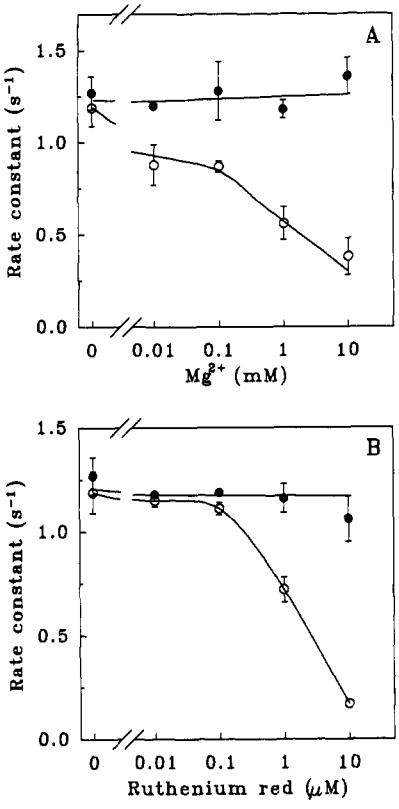

FIGURE 7: Lack of blocking action of Mg^{2+} (A) or ruthenium red (B) in SR vesicles after incubation with 10 μM DCCD. Derivatization, $^{45}Ca^{2+}$ loading, and handling of aliquots were as described in the legend of Figure 5. The flushing media used in the rapid filtration experiments of panel A consisted of 20 mM MOPS, pH 6.8, 80 mM KCl, 50 mM sucrose, 1 mM EGTA, different $CaCl_2$ concentrations to give 1 μM free Ca^{2+}, and different $MgCl_2$ concentrations (●). The flushing media used in panel B contained 20 mM MOPS, pH 6.8, 80 mM KCl, 1 mM EGTA, 0.503 mM $CaCl_2$ (1 μM free Ca^{2+}), 50 mM sucrose, and different ruthenium red concentrations (●). Open circles represent experiments performed with nonderivatized vesicles.

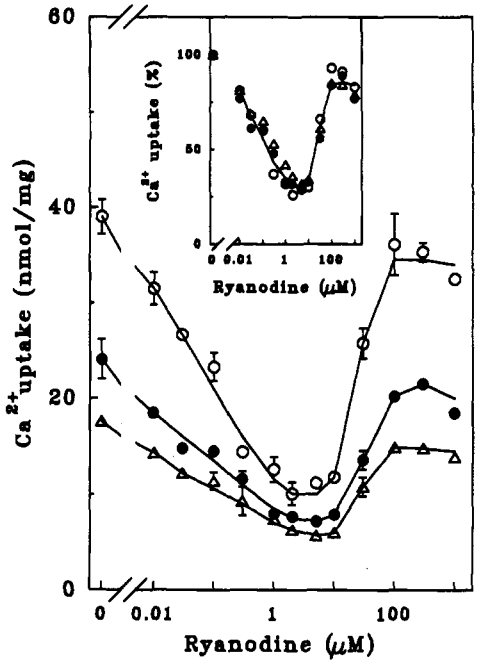

FIGURE 8: Ryanodine concentration dependence on the Ca^{2+} channel activity in DCCD-treated SR vesicles. After membrane derivatization in the presence of 10 (●) or 25 (Δ), μM DCCD, the vesicles (1 mg/mL) were incubated for 30 min at 37 °C in a medium of 20 mM MOPS, pH 7.0, 150 mM KCl, and 100 μM $^{45}CaCl_2$ containing the ryanodine concentrations tested. Aliquots of 0.06 mL were diluted in 1.14 mL of the medium 20 mM MOPS, pH 7.0, 150 mM KCl, 10 mM $MgCl_2$, and 100 μM $^{45}CaCl_2$ to measure Ca^{2+} uptake. The reaction was started by adding 2 mM acetyl phosphate and stopped 2 min later by filtration. Filters were rinsed with 4 mL of the medium 20 mM MOPS, pH 7.0, 80 mM KCl, and 1 mM $LaCl_3$ before radioactive counting. Open circles give the effect of ryanodine on the level of Ca^{2+} accumulation in SR vesicles without the DCCD treatment. The inset of the figure shows the experimental data of the main frame represented in a relative scale with respect to the steady-state Ca^{2+} content at zero ryanodine concentration.

DISCUSSION

Earlier reports have indicated that DCCD blocks the functional activity of a number of transport systems including cation pumps for H^+, Ca^{2+}, or Na^+–K^+ (see references above) and the Ca^{2+} channel protein could also be a putative candidate for the action of this reagent. Therefore, in order to determine whether DCCD directly affects the Ca^{2+} permeability of the junctional vesicles in a manner consistent with alteration of the Ca^{2+} channel activity, we evaluated the effect of this reagent on the mechanism of Ca^{2+} release through the 450-kDa cation channel. It is noteworthy that the functional group(s) altered after derivatization with DCCD could be different depending on the reagent/protein ratio (de Ancos & Inesi, 1988). Consistent with this, previous studies (Yamamoto & Kasai, 1982a,c; Argaman & Shoshan-Barmatz, 1988) using a relatively high DCCD/protein ratio (0.2 mM DCCD and 0.5 mg of protein/mL, or 1 mM DCCD and 1 mg of protein/mL) have shown inhibition of Ca^{2+} release induced by different mechanisms. It should be noted that the use of Ca^{2+} activators such as alkaline pH, tetraphenylboron, ATP plus inorganic phosphate, or chemical modification with acetic anhydride (Argaman & Shoshan-Barmatz, 1988) render it difficult to know whether or not the 450-kDa channel is in fact involved. We found, however, that a low reagent/protein ratio (10 μM DCCD and 1 mg of protein/mL) produces the opposite effect; i.e., the chemical reaction interferes selectively with the blocking mechanism, and therefore the Ca^{2+} channel cannot reach the closed state in the presence of typical inhibitors such as di- or polyvalent cations.

The combined effect of micromolar Sr^{2+} and millimolar Ca^{2+} raises the issue of specific functional domains in the three-dimensional architecture of the channel. We know that DCCD preferentially interacts with hydrophobic pockets; therefore, the absence of an effect on the micromolar Ca^{2+}- or Sr^{2+}-dependent Ca^{2+} release is an indication of the nonhydrophobic (hydrophilic) nature of the activating channel domain. At the same time, it seems clear that the existence of carboxyl group(s) plays an essential role in the blocking capacity of di- and polyvalent cations. It is reasonable to assume that this carboxyl residue(s) should be located in a hydrophobic (internal) region of the channel.

The pH characteristics of the DCCD modification with an apparent pK_a above the expected values for carboxyl groups is a further indication of a nonpolar environment. The presence of reactive residue(s) of aspartic or glutamic acid involved in the mechanism for the closure of the Ca^{2+} channel is suggested.

We also tried to obtain more information on the ryanodine binding sites in relation with the hydrophobic DCCD-reactive site. Our data provide evidence that the ryanodine sites are distinct from that labeled by carbodiimide which interferes with the blocking action of cations. This is in agreement with the observation that the addition of ryanodine at low concentrations to open the channel cannot be blocked by the addition of ruthenium red or Mg^{2+} (Fleischer et al., 1985; Imagawa et al., 1987) and supports the view of an altered conformation of the channel protein producing a subconduc-

tance (open) state after binding of ryanodine at low concentrations (Carroll et al., 1991). In this connection, site-directed fluorescent labeling of the Ca^{2+} channel has shown that addition of ryanodine in the concentration range to open the channel induces a local conformational change at the Ca^{2+} blocking domain but not at the Ca^{2+} triggering domain (Kang et al., 1992).

The analysis of the carbodiimide reaction poses some difficulties owing to the possibility of side reactions. The formation of an initial unstable *O*-acylisourea allows the appearance of rearrangement, hydrolysis, and cross-linking products (Williams & Ibrahim, 1981). We could observe that, under conditions for modifying the Ca^{2+} channel closure (10 μM DCCD and 1 mg of protein/mL), there is no significant incorporation of the ^{14}C radioactive label. This effect can be attributed to internal protein cross-linking with elimination of the label. In fact it was reported that carbodiimide inactivation of the Na^+,K^+–ATPase was due to the reaction of the initial DCCD adduct with endogenous nucleophiles (Pedemonte & Kaplan, 1986). This also appears to be the case for the SR Ca^{2+}–ATPase inhibition in the presence of a low DCCD/protein ratio (de Ancos & Inesi, 1988). Under more drastic conditions there is a very clear indication of intermolecular cross-linking with the appearance of a protein band on the top of the stacking gel (Figure 3) which is concomitant with the disappearance of the Ca^{2+} channel subunit. These conditions produced inhibition of the Ca^{2+} release process (Argaman & Shoshan-Barmatz, 1988).

We searched for the consensus sequence of the DCCD-sensitive domains from the H^+-, Ca^{2+}-, and Na^+,K^+-transport ATPases on the primary structure of the Ca^{2+} channel subunit (Takeshima et al., 1989) without success. This is not surprising for proteins that are structurally unrelated. Nevertheless, according to general rules in the design of the transmembrane channels (Betz, 1990), it is expected that the DCCD-reactive groups should be located in α-helices of bulky hydrophobic amino acids oriented on the lining of the channel pore. The site-directed mutagenesis technique can contribute to clarify this issue, pinpointing the target of the DCCD reagent.

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