The calmodulin-binding site of the plasma membrane Ca^{2+} pump interacts with the transduction domain of the enzyme

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Abstract

Calpain proteolysis of the plasma membrane Ca^{2+} pump removes a C-terminal 14-kDa portion which includes the calmodulin-binding domain. This produces a fully activated 124-kDa fragment, which can be inhibited by synthetic versions of the calmodulin-binding domain. The inhibition is strongest when Trp-8 in the latter domain is replaced by a Tyr residue (Falchetto, R., Vorherr, T., Brunner, J., & Carafoli, E., 1991, *J. Biol. Chem.* 266, 2930–2936). In the present study, the N-terminus of the 28-residue synthetic calmodulin-binding domain was acetylated with ^3H -acetic anhydride, and Phe in position 25 was replaced by a phenylalanine derivatized with a diazirine-based, photoactivatable carbene precursor. This peptide (C28WC*) inhibited the fully active 124-kDa fragment of the pump and became cross-linked to it upon photolysis. After proteolysis of the fragment with Asp-N or *Staphylococcus aureus* V8 (Glu-C) protease, labeled peptides were isolated by reversed-phase high-performance liquid chromatography and subjected to Edman sequence analysis. The peptides originated from a region of the pump located within the unit protruding into the cytoplasm between transmembrane domain two and three. This unit has been proposed to be the site of the energy transduction domain, which would couple the ATP hydrolysis to Ca^{2+} translocation.

Keywords: autoinhibitory domain; Ca^{2+} pump; calmodulin-binding domain; calpain; diazirine cross-linker; “receptor” site

The Ca^{2+} affinity and the $V_{\max}$ of the Ca^{2+} pump of the plasma membrane are increased by calmodulin (Niggli et al., 1981). Controlled proteolysis of the pump by trypsin (Benaïm et al., 1984; Zurini et al., 1984) or by the Ca^{2+} -dependent protease calpain (Wang et al., 1988; James et al., 1989b) produces fragments that have lost calmodulin sensitivity and are fully active in its absence. The fragments lack the C-terminal portion of the enzyme (James et al., 1989b; Zvaritch et al., 1990), which contains the calmodulin-binding domain (James et al., 1988).

These studies have led to the proposal that the calmodulin-binding site may function as an internal repressor of the activity of the pump. Synthetic peptides corresponding to the calmodulin-binding domain indeed inhibit the trypsin-activated Ca^{2+} -stimulated ATPase activity of the pump (Enyedi et al., 1989).

The calpain-truncated Ca^{2+} pump is also inhibited by the synthetic calmodulin-binding peptides (Falchetto et al., 1991). The “receptor” region for the N-terminal portion of the calmodulin-binding peptides has been identified with the help of a radioactive, photoactivatable version of the domain in which a phenylalanine in the N-terminal portion of the 28-amino acid peptide (Phe-9) was replaced by a carbene-generating Phe derivative. The calpain-produced 124-kDa fragment of the pump was labeled with the peptide, leading to the identification of a binding site for it in the region between the phosphoenzyme-forming aspartic acid and the FITC-binding site, which is generally assumed to be part of the ATP-binding pocket. In the present study, a radioactive, photoactivat-

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Abbreviations: DCC, dicyclohexylcarbodiimide; DTT, dithiothreitol; DVB, divinylbenzene; EGTA, ethylene glycol bis-(β -aminoethylether) *N,N,N',N'*-tetraacetic acid; FITC, fluorescein-isothiocyanate; Fmoc, 9-fluorenylmethyloxycarbonyl; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; HOBT, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; NMP, *N*-methylpyrrolidone; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid.

able, 28-residue calmodulin-binding peptide similar to that used in the previous investigation (Falchetto et al., 1991) was synthesized. However, the carbene-generating Phe analogue was introduced in this case into the C-terminal half of the peptide (Phe-25). The peptide was tested for its ability to inhibit the Ca^{2+} -ATPase activity of the calpain-truncated 124-kDa fragment and was then cross-linked to it. This led to the identification of a second anchoring point for the calmodulin-binding domain within the unit protruding into the cytoplasm from the second transmembrane helix, i.e., within the portion of the ATPase assumed to be involved in the coupling of ATP hydrolysis to Ca^{2+} translocation, the transduction domain.

Results

Effect of peptide C28WC on the activity of the 124-kDa fragment of the ATPase*

The photoactivatable peptide C28WC* corresponds to the native calmodulin-binding domain of the plasma membrane Ca^{2+} pump, except for Phe-25, which was substituted by a carbene-generating phenylalanine analogue (see Materials and methods). The peptide reduced the activity of the calpain-truncated Ca^{2+} -ATPase with an IC_{50} of 20 μM (Fig. 1), i.e., about as effectively as the native peptide C28W (Falchetto et al., 1991). This indicates that the peptide specifically interacted with the pump, justifying its use for the identification of its "receptor" region.

Labeling of the calpain-truncated Ca^{2+} -ATPase with the photoactivatable peptide C28WC and identification of the peptide-binding region*

Peptide C28WC* (30 μM) was incubated with the 124-kDa pump fragment (2 μM) and cross-linked to it by photo-

activation as described under Materials and methods. The radioactivity incorporated in the HPLC-purified labeled fragments (see below) ranged between 1 and 2% of the total radioactivity associated with the peptide C28WC* added to the photolysis mixture to start the labeling experiments. This is in the normal range for the incorporation of such a carbene-generating cross-linker into proteins (Brunner & Semenza, 1981). As a further control for the specificity of the interaction of the peptide, the labeling of the 124-kDa fragment was carried out in the presence of peptide C49W (see Materials and methods), i.e., a longer synthetic version of the calmodulin-binding domain of the pump, which also contains an acidic stretch N-terminal to it. The ATPase was first incubated for 5 min at 37 °C in the presence of 100 μM of peptide C49W, then 30 μM of peptide C28WC* were added. After 5 min of incubation at 37 °C, the mixture was subjected to photolysis and subjected to the normal procedure of carboxymethylation and dialysis. The radioactivity remaining in the reaction mixture was in this case only about 0.05% of that originally introduced, i.e., 20–40 times less than in the absence of unlabeled peptide (data not shown). The inhibition indicated that the labeling by peptide C28WC* was indeed specific.

The starting material for each of the two digestion experiments described below was 3.4 nmol of the calpain-produced 124-kDa fragment. In the first, cleavage of the cross-linked material was performed with Asp-N protease. The digest was fractionated on a reversed-phase C4 column (see Materials and methods) using a linear gradient from 0% to 100% buffer B in 40 min. The fractions containing the single radioactive peak were pooled (Fig. 2A) and rechromatographed on a C18 column (see Materials and methods) with a discontinuous gradient (from 0% to 30% buffer B in 10 min and from 30% to 100% buffer B in 40 min). The two peaks eluting at 24 and 25 min, contained in the fractions with the highest radioactivity (Fig. 3A), were sequenced yielding in both cases a structure corresponding to amino acids 251–271 of isoform hPMCA4 of the Ca^{2+} -ATPase (Strehler et al., 1990; Fig. 4).

In the second experiment, cleavage was carried out using *Staphylococcus aureus* V8 (Glu-C) protease. The digest was first separated on the C4 column with the same gradient as for the Asp-N digest. A single radioactive peak eluted at 22 min (Fig. 2B). The pooled fractions in the peak were rechromatographed on the C18 column with the discontinuous gradient described above for the Asp-N digest. The peak eluting at 25 min, contained in the fraction with the highest radioactivity (Fig. 3B), was sequenced yielding a structure corresponding to amino acids 206–235 of the Ca^{2+} -ATPase hPMCA4 isoform (Strehler et al., 1990; Fig. 4). In addition to this sequence the fraction also contained a fragment corresponding to the N-terminal portion of the protease.

A digest of the 124-kDa cross-linked product with

Fig. 1. Effect of peptide C28WC* on the activity of the 124-kDa ATPase fragment. The ATPase activity is expressed as a percent of the control (no peptide added). The fragment concentration was 50 nM. Other details are found in Materials and methods.

Fig. 2. HPLC fractionation of the protease digests of the labeled 124-kDa fragment. The digests were fractionated on a C4 HPLC column (see Materials and methods). The gradient was run as described under Results. The flow rate was 500 μ L/min, detection was at 210 nm (solid line), 1-min fractions were collected, and 25 μ L were withdrawn for scintillation counting (dotted line). The solid bar shows the fractions pooled for rechromatography on a C18 HPLC column (see Fig. 3). **A:** Asp-N protease digest. **B:** V8 protease digest.

Fig. 3. Rechromatography of the pooled fractions from the Asp-N and the V8 protease digests fractionated on the C4 column. The pooled radioactive fractions from the HPLC runs shown in Figure 2 were loaded onto the C18 column (see Materials and methods) and run with the gradient described under Results. The flow rate was 500 μ L/min, detection was at 210 nm (solid line), 0.5-min fractions were collected, and 30- μ L aliquots were withdrawn for scintillation counting (dotted line). **A:** Asp-N protease digest. **B:** V8 protease digest.

Lys-C protease as performed in the previous study (Falchetto et al., 1991) was not attempted in this case because this protease, by splitting peptide C28WC* at Lys-20, would have eliminated its radioactive N-terminus. Table 1 summarizes the results of the sequence analysis of the purified cross-linked fragments.

Because the labeled pump fragments had a retention time similar to that of the free peptide C28WC* (see below), the possibility was considered that the radioactivity was contributed by traces of coeluting free peptide and not by the fragments that were sequenced. The fragments labeled with peptide C28W* in the previous study (Falchetto et al., 1991) indeed had the same retention time as that of the free peptide. To rule out this possibility, aliquots of the reaction mixture removed at different steps of the procedure were loaded onto the C4 column and run with a linear gradient from 0% to 100% B in 30 min.

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201 LPVAETVGD IAQVKYDGLL PADGILIQGN DLKIDESSLT GESDHVKSL
      *      *
#####
251 DKDPMLLSGT HVMEGSGRMV VTAVGVSQT GIILTLGVN EDDEGEKKKK
      *      ***
301 GKKGVPENR NKARTQDGA LEIQFINSQE GIDNEEKDKK AVKVPKKEKS
      *
351 VLQGLTRLA VQIGKAGLLM SALTVFILIL YFVIDNEVIN RRPWLPECTP
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Fig. 4. The region of pump isoform hPMCA4 that interacts with the C-terminal portion of peptide C28WC*. Residues 201–400 of the Ca²⁺-ATPase isoform hPMCA4 (Strehler et al., 1990) are shown. The symbols above the amino acid sequence indicate the primary structure of the radioactively labeled, cross-linked peptides identified in the V8 (+) and Asp-N (x) protease digests, the putative region involved in the phospholipid response of the pump (solid line; Zvaritch et al., 1990), and the third putative transmembrane domain of the pump (dotted line). Asterisks below the sequence denote unidentified residues from the sequencing runs (see also Table 1).

Table 1. Sequence analysis of the radioactive fragments obtained from proteolytic digests of the 124-kDa fragment of the pump cross-linked to peptide C28WC*^a

Cycle no.	V8 digest hPMCA4 206–235			Asp-N digest hPMCA4 251–271		
	PTH amino acid	Yield (pmol)	cDNA-deduced sequence	PTH amino acid	Yield (pmol)	cDNA-deduced sequence
1	Ile	25.46	Ile	Asp	99.49	Asp
2	Val	18.66	Val	Lys	34.65	Lys
3	Val	18.02	Val	Asp	33.85	Asp
4	Gly	32.42	Gly	Pro	19.76	Pro
5	Asp	12.93	Asp	Met	8.07	Met
6	Ile	19.88	Ile	Leu	30.50	Leu
7	Ala	15.35	Ala	Leu	32.16	Leu
8	Gln	12.51	Gln	Xaa	—	Ser
9	Val	19.00	Val	Gly	17.47	Gly
10	Lys	13.96	Lys	Thr	7.10	Thr
11	Tyr	10.26	Tyr	His	N.D.	His
12	Gly	28.65	Gly	Val	12.47	Val
13	Asp	15.92	Asp	Met	3.02	Met
14	Leu	12.78	Leu	Glu	4.00	Glu
15	Leu	14.05	Leu	Gly	5.68	Gly
16	Pro	8.21	Pro	Xaa	—	Ser
17	Ala	8.83	Ala	Xaa	—	Gly
18	Asp	7.29	Asp	Xaa	—	Arg
19	Gly	22.03	Gly	Met	1.46	Met
20	Ile	8.58	Ile	Val	9.13	Val
21	Leu	10.12	Leu	Val	10.52	Val
22	Xaa	—	Ile			
23	Gln	5.89	Gln			
24	Gly	19.93	Gly			
25	Asn	5.62	Asn			
26	Xaa	—	Asp			
27	Leu	7.39	Leu			
28	Lys	4.93	Lys			
29	Ile	5.20	Ile			
30	Asp	4.93	Asp			

^a The phenylthiohydantoin (PTH) amino acid yields are the raw data obtained from the sequencer's analysis, and thus the values of several residues, e.g., glycine, also include the background amount of the amino acid. Xaa stands for an unidentified amino acid. N.D.: no pmol value was obtained for this His residue, which was identified by inspection of the HPLC profile. The PTH amino acid yields for the fragment from the Asp-N protease digest are the sum of the yields obtained from the two radioactive peaks (see Results).

Figure 5A shows the radioactivity profile of 75 nmol of free (unphotolyzed) peptide C28WC*: the highest amount of radioactivity eluted from the column after 16 min. The photolyzed free peptide eluted 2 min earlier (not shown), presumably because the diazirine group lost N₂ and the generated carbene reacted with water, making the peptide slightly more hydrophilic. Then 75 nmol of the peptide were photolyzed with 5 nmol of the 124-kDa fragment. Figure 5B shows the radioactivity profile of the reaction mixture after carboxymethylation (see Materials and methods) but before the dialysis step: a major radioactive fraction eluted after 14 min, and the remainder of the counts tailed off between 15 and 25 min. Very likely, the major peak corresponded to free peptide since it had the same retention time. However, after dialysis

of the carboxymethylated mixture (see Materials and methods), this major peak disappeared and most of the remaining radioactivity spread between 15 and 26 min (Fig. 5C). Thus, dialysis evidently eliminated the free peptide C28WC* from the mixture. In a separate experiment 70 µg of Ca²⁺-ATPase were first precipitated with trichloroacetic acid, resuspended in the carboxymethylation buffer (Falchetto et al., 1991), loaded onto the C4 column, and then run with the gradient described above. A broad peak was seen (Fig. 5D) at about the same elution region (15–25 min) of the radioactivity from the dialyzed reaction mixture (Fig. 5C), i.e., the counts detected were indeed associated with the ATPase. Finally, digestion of the labeled ATPase with V8 protease produced a major radioactive fraction eluting after 14–15 min, accompanied

Fig. 5. Elution pattern of the labeled pump fragment and of the free peptide C28WC*. Peptide C28WC* (75 nmol) was photolyzed with 5 nmol of the 124-kDa fragment derived from the Ca²⁺ pump (see Materials and methods). Aliquots of the reaction mixture were loaded onto the C4 column at different steps of the procedure (before and after the dialysis, and after the V8 protease digestion; see the description of the experiment in the Results section). The flow rate was 500 μ L/min; 1-min fractions were collected and withdrawn for scintillation counting. **A:** Radioactivity elution profile of 2.7 nmol of peptide C28WC* normalized to 75 nmol. **B:** Radioactivity elution profile of 1% of the reaction mixture after carboxymethylation, normalized to the total radioactivity of the mixture. **C:** Radioactivity elution profile of 4% of the reaction mixture after carboxymethylation and dialysis, normalized to the total radioactivity of the mixture. **D:** Absorption profile of the eluate of the HPLC column loaded with 70 μ g Ca²⁺-ATPase. **E:** Radioactivity elution profile of a sample of labeled ATPase (Fig. 5C) cleaved with V8 protease. The values in A–C were normalized to the totals used to show the progressive decrease in the radioactivity recovered.

by residual radioactivity, probably belonging to undigested ATPase, tailing off at later elution times (Fig. 5E). The retention time of the labeled V8 fragment was similar to that of the free peptide C28WC*; because the latter was efficiently removed by the dialysis procedure (Fig. 5C), accidental coelution of the free peptide with the fragment evidently did not occur.

Discussion

The original proposal that the calmodulin-binding domain acts as an endogenous inhibitor of calmodulin-modulated enzymes (Klee, 1980) was verified for the calmodulin-stimulated Ca²⁺ pump of the plasma membrane in 1984 (Benaim et al., 1984), and then extended to other calmodulin-regulated enzymes, e.g., the myosin light chain kinase (Kemp et al., 1987). The recently identified “receptor” site for the N-terminal portion of the calmodulin-binding domain of the Ca²⁺ pump lies between two functionally important sites of the Ca²⁺ pump, the phosphoenzyme-forming aspartic acid and the FITC (ATP)-binding domain (Falchetto et al., 1991). This led to the proposal that the calmodulin-binding site would restrict the substrate accessibility to the active site. The interaction between the C-terminal portion of the calmodulin-binding domain of the Ca²⁺-ATPase and the main structure of the molecule has been investigated in the present work.

The introduction of a carbene-generating phenylalanine analogue in the N-terminal portion of the synthetic calmodulin-binding domain (Falchetto et al., 1991) has led to the identification of one interaction site of the domain. Due to the high reactivity of the photogenerated carbene and to the relatively small size and limited mobility of the Phe analogue, the pump could only be cross-linked within a short range of the interaction site. This insured an accurate spatial definition of the “receptor” site for the N-terminal portion of the domain. In the work described here the diazirine-based Phe analogue was introduced into the C-terminal half of the synthetic, 28-residue calmodulin-binding domain, i.e., on Phe-25. This new, derivatized peptide (C28WC*) inhibited the ATPase activity of the 124-kDa calpain fragment of the pump like the nonderivatized peptide, suggesting analogous interaction mechanisms for the two. The radioactive fragments found in the digests of the cross-linked calpain-truncated pump with two proteases derived from the same region of the molecule. The sequenced residues obtained from the *S. aureus* V8 protease and the Asp-N protease fragments, however, did not overlap. The cross-linked peptide probably limited the accessibility of the V8 protease to some of the glutamate residues leading to incomplete cleavage of the interaction region and thus to the production of a relatively large cleavage fragment. The presence of a double radioactive peak in the Asp-N protease digest could have been due to incomplete cleavage of the region yielding labeled peptides with different C-termini but an identical N-terminus. Alternatively, the different peaks could have arisen from fragments having the same length but differing in the insertion mode of the cross-linker, e.g., multiple residue insertions or insertions in different positions within the same residue. The amino acids that were not identified in the sequencing runs are possible candidates for the attachment sites of the cross-linker. In the Asp-N protease fragments the

yield of the fifth residue (Met-255 in the cDNA-deduced sequence of the hPMCA4 isoform) was greatly reduced as compared to the preceding and following amino acids (see Table 1), making this methionine a prime candidate for the insertion site of the cross-linker, since diazirines label preferentially sulfur-containing residues, as discussed in Falchetto et al. (1991).

The labeled fragments described in this work originate from the first cytoplasmic domain of the ATPase (residues 171–367 of the hPMCA4 isoform) protruding between transmembrane domain one and two. The larger unit protruding from transmembrane domain four includes the phosphoenzyme-forming aspartic acid and the nucleotide-binding pocket (approximately, residues 421–842). The first cytoplasmic unit has been defined as the transduction domain, i.e., a domain involved in the coupling of ATP hydrolysis to Ca^{2+} transport (Brandl et al., 1986; Green & MacLennan, 1989). Several findings indicate that this unit is an essential region to ion-motive ATPases of the P-type (Pedersen & Carafoli, 1987a,b). Firstly, the domain contains one of the few stretches with a primary structure conservation greater than 30% among all P-type ion-motive ATPases from different organisms (Green, 1989). The domain has been predicted to consist of anti-parallel β -strands in which several conserved glycines would form the hairpin turns. Mutations of these glycines (Ghislain et al., 1987; Andersen et al., 1989) produce enzymes with defective ATPase activity that suggest an impaired E_1 to E_2 transition. Furthermore, several trypsin-sensitive sites are located in this domain. In the Na^+/K^+ -ATPase cleavage at one of these sites blocks the E_1 to E_2 conversion (Jørgensen & Petersen, 1985). The trypsin-cuts in the sarcoplasmic reticulum Ca^{2+} -ATPase produce fragments that can be phosphorylated by ATP to form the $E_1 \sim P$ state, but which are not able to convert to the $E_2 \sim P$ state (Imamura & Kawakita, 1986). The plasma

membrane Ca^{2+} -ATPase also contains several trypsin-sensitive sites in this region: trypsinolysis yields active fragments ranging in mass from 90 to 76 kDa and an inactive fragment of about 33 kDa, which contains most of the small first cytoplasmic domain (Zvaritch et al., 1990). Several findings indicate that the 33-kDa fragment remains tightly associated with the main body of the enzyme after trypsin cleavage. Work currently under way attempts to clarify whether, and how, its separation from the other fragments affects the activity of the pump.

If the first cytoplasmic protruding unit indeed functions as a transducing domain, it is likely to interact with the larger cytoplasmic domain containing the active site, probably in the hinge region, which is assumed to permit enough mobility of the unit to occur during the catalytic cycle to bring the catalytic aspartic acid and the ATP-binding site in close spatial contact. During the process of coupling of the ATP hydrolysis to Ca^{2+} transport, large conformational changes and interdomain movements are thus likely to occur. The calmodulin-binding sequence is proposed here to hinder these motions by interacting with the first and the second cytoplasmic domain, bridging them in a way that would restrict their mobility. As a result, Ca^{2+} transport and ATPase activity would be inhibited; Figure 6 offers a schematic interpretation of the findings and of their functional consequences.

A peculiar structural feature of the plasma membrane Ca^{2+} -ATPase is a stretch of 44 amino acids within the first cytoplasmic domain, which has been proposed to contain the acidic phospholipid-sensitive site (Zvaritch et al., 1990). Significantly, this region lies close to the second "receptor" site for the calmodulin-binding domain described here. Like the calmodulin-binding sequence, it could somehow restrict interdomain movements, and binding of acidic phospholipids could remove this effect.

Fig. 6. A schematic representation of the interaction of the calmodulin-binding domain with the Ca^{2+} pump. The shaded cylinder is the calmodulin-binding site. D 475 and K 609 are the residues that form the phosphoenzyme and that bind FITC, respectively. The positions are those determined for isoform hPMCA4 (Strehler et al., 1990). Arrows point to the two "receptor" sites interacting with the calmodulin-binding domain identified by the photolabeling experiments.

It is pertinent to mention in this context recent work in this laboratory (Brodin et al., 1992), which has shown that the C-terminal calmodulin-binding domain also binds efficiently acidic phospholipids. Thus, all activation mechanisms of the plasma membrane Ca²⁺ pump would apparently involve the calmodulin-binding domain or its immediate vicinity. Apart from calmodulin itself and acidic phospholipids, this has also been shown to be true for the activating dimerization process (Vorherr et al., 1991) and for the kinase-mediated phosphorylation process (James et al., 1989a; Wang et al., 1991).

Materials and methods

Materials

[³H]acetic anhydride was purchased from Amersham International (Amersham, UK). Amino acid derivatives were from Novabiochem AG (Laufelfingen, Switzerland). The reagents for peptide synthesis were obtained from Applied Biosystems (Foster City, California). Iodoacetic acid and the solvents not specified in this section were of the highest purity grade available from Fluka AG (Buchs, Switzerland). Asp-N and *S. aureus* V8 (Glu-C) proteases were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). ATP was obtained from Pharmacia (Uppsala, Sweden). Calmodulin was isolated from bovine brain as described by Guerini et al. (1984). The Ca²⁺-ATPase was purified from human erythrocytes and digested with calpain as described in Falchetto et al. (1991). Polyacrylamide gel runs showed that at the end of the calpain-digestion procedure (3 h) the preparation consisted essentially of the 124-kDa product. L-N-9-fluorenylmethoxycarbonyl-4'-(1-azi-2,2,2-trifluor-ethyl)phenylalanine was synthesized from L-4'-(1-azi-2,2,2-trifluor-ethyl)phenylalanine as described in Falchetto et al. (1991). L-4'-(1-azi-2,2,2-trifluor-ethyl)phenylalanine was a kind gift from Dr. J. Brunner, Zürich, Switzerland. Procalpain isolated from human erythrocytes was a kind gift from Dr. E. Melloni, Genova, Italy. All other reagents were of the highest purity grade commercially available.

Synthesis of peptide C28WC*

The peptide [³H]Ac-L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F*-S-S-S (C28WC*; the * stands for the azitrifluorethyl-phenylalanine in position 25) was synthesized and purified according to the following procedure: the first amino acid was attached to *p*-hydroxymethylphenoxymethylpolystyrene (1 mmol/g resin, 1% DVB) using dimethylaminopyridine as a catalyst according to the standard procedure for the Applied Biosystems peptide synthesizer model 431. The fully protected peptide resin (sequence S-S-S) was automatically synthesized using a 10-fold excess of DCC/HOBt-activated Fmoc

amino acid derivatives and a capping cycle employing acetic anhydride after each coupling according to the instructions of the manufacturer. The Pmc-protected arginine and trityl-protected Asn and Gln were used for this synthesis. After deblocking of the N-terminus, the diazirine-labeled Fmoc-Phe was coupled manually. A twofold excess of diazirine derivative was activated with DCC/HOBt and coupled to 30 μ mol of peptide resin in 600 μ L NMP for 3.5 h. The ninhydrin test (Kaiser et al., 1970) showed nearly complete reaction (light blue resin beads). Further synthesis prior to capping after coupling the diazirine-labeled Fmoc-Phe was completed again with a 10-fold excess of amino acid derivative. After the last Fmoc deprotection, 75 μ L [³H]acetic anhydride (25 mCi/mL, 50 μ mol/mL) in 850 μ L methylene chloride were used to radioactively label the N-terminal amino acid. Complete acetylation was achieved using the standard capping protocol. Sixty milligrams of acetylated protected peptide resin were cleaved in 1 mL TFA, 30 μ L ethanedithiol, 30 μ L thioanisole, and 30 μ L water for 2.5 h at room temperature following filtration, precipitation, and washing with diethylether. Sixteen milligrams of crude product were obtained. Analytical HPLC indicated that about 65% of the radioactively labeled target peptide was present in the crude material. Amino acid analysis showed the expected ratios among residues (Trp was not determined).

Synthesis of peptide C49W

The synthesis of peptide C49W (E-E-I-P-E-E-E-L-A-E-D-V-E-E-I-D-H-A-E-R-E-L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-R-V-V-N-A-F-R-S-S-NH₂) was carried out as described in Kataoka et al. (1991) for peptide C24W, which represents the 24 C-terminal residues of peptide C49W. The synthesis was continued on the Tentagel resin (Rapp Polymere, Tübingen, Germany). Cleavage of the peptide was performed in a mixture of 4 mL TFA, 50 μ L ethanedithiol, 50 μ L thioanisole, 50 μ L water, 50 μ L methylsulfide, and 150 mg phenol for 3 h 15 min at room temperature. From 190 mg of Fmoc deprotected peptide resin, 89 mg of crude product were obtained after precipitation and washing with ethyl ether. From 81 mg of crude peptide, 13.4 mg of purified peptide were obtained after preparative HPLC using 0.1% TFA in water and 0.05% TFA, 50% *n*-propanol in water. The amino acid analysis agreed with the expected ratios (Trp was not determined).

Ca²⁺-ATPase assay

The ATPase activity was measured by the method of Lanzetta et al. (1979). The reaction was carried out at 37 °C in the following standard reaction buffer: 20 mM HEPES, pH 7.2, 100 mM KCl, 5 mM MgCl₂, 500 μ M CaCl₂, and 500 μ M EGTA; the free calcium concentration was 10 μ M, as calculated using a computer program that assumes a

K_d for the Ca^{2+} -EGTA complex of $0.21 \mu\text{M}$ at pH 7.0 (Martell & Smith, 1974). All samples were preincubated for 5 min at 37°C ; the reaction was started by the addition to 1 mM ATP, carried out for 10 min at 37°C , and then stopped by the addition of the acidic color reagent.

Photocoupling of peptide C28WC* to the 124-kDa fragment

Samples of the calpain-activated Ca^{2+} pump, containing the 124-kDa fragment at a concentration of about $2 \mu\text{M}$, were incubated for 5 min at 37°C in the dark with peptide C28WC* (final concentration $30 \mu\text{M}$) and photolyzed for 2 min with the 450-W lamp of a SPEX Fluorolog 1680 (Metuchen, New Jersey).

Enzymatic digestion of the labeled 124-kDa fragment

After photocoupling, the 124-kDa fragment was carboxymethylated as described by Falchetto et al. (1991). The carboxymethylation mixture was dialyzed first against 50 mM sodium phosphate, pH 7.8, 2 M guanidinium chloride, 2 mM DTT, then against 50 mM sodium phosphate, pH 7.8, 1 M guanidinium chloride. The dialysis membrane (Spectrapor, Los Angeles) had a cut-off of 50 kDa and was saturated with polyvinylpyrrolidone as described by Falchetto et al. (1991). After dialysis, isopropanol was added to a final concentration of 10% and cleavage was carried out at 37°C for 48 h with either $1 \mu\text{g}$ of Asp-N protease per $100 \mu\text{g}$ of 124-kDa fragment or $1 \mu\text{g}$ of *S. aureus* V8 protease per $9 \mu\text{g}$ of 124-kDa fragment. The proteases were added in two separate, equal portions; the second addition was made 24 h after the first.

HPLC analysis and amino acid sequencing

The digestion mixtures were resolved by reversed-phase HPLC using a Nucleosil C4 (Macherey & Nagel, Oensingen, Switzerland), $50 \times 4\text{-mm}$, $5\text{-}\mu\text{m}$, $300\text{-}\text{\AA}$ column and a Nucleosil C18, $50 \times 4\text{-mm}$, $3\text{-}\mu\text{m}$, $120\text{-}\text{\AA}$ column. The reversed-phase buffers used were: A, 0.1% TFA in water; B, 0.1% TFA, 70% acetonitrile in water. Chromatography was performed on an Applied Biosystems 140A system at 40°C ; the columns were thermostatted inside a Chrompack SpH 99 oven (Chrompack Instruments AG, Neuheim, Switzerland). Radioactivity was measured using a Ready Safe Liquid Scintillation Cocktail (Beckman Instruments Inc., Fullerton, California) with a Beckman LS 1801 counter. Sequence analysis was performed on an Applied Biosystems 470A Sequencer and a 120A phenylthiohydantoin derivative analyzer using the standard cycles supplied by the manufacturer.

Determination of the protein concentrations

The protein concentrations were determined by a modification of the method of Lowry et al. (1951) as described in Falchetto et al. (1991).

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