Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 348 (2006) 288–294
BBRC
www.elsevier.com/locate/ybbrc

A region of calpastatin domain L that reprimes cardiac L-type Ca^{2+} channels

Etsuko Minobe [a], Li-Ying Hao [a,b,*], Zahangir A. Saud [a], Jian-Jun Xu [a], Asako Kameyama [a], Masatoshi Maki [c], Kirsty K. Jewell [d], Tim Parr [d], Ronald G. Bardsley [d], Masaki Kameyama [a,*]

[a] Department of Physiology, Graduate School of Medical and Dental Sciences, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890-8544, Japan
[b] Department of Pharmaceutical Toxicology, School of Pharmaceutical Sciences, China Medical University, 92 Beier Road, Shenyang 110001, China
[c] Laboratory of Molecular and Cellular Regulation, Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan
[d] Division of Nutritional Sciences, School of Biosciences, Sutton Bonington Campus, University of Nottingham, Loughborough, Leicestershire LE12 5RD, UK

Received 25 June 2006
Available online 20 July 2006

Abstract

Calpastatin, an endogenous inhibitor of calpain, is composed of domain L and four repetitive homologous domains 1–4. Domains 1–4 inhibit calpain, whereas domain L partially reprimes L-type Ca^{2+} channels for voltage-gated activation. In the present study, the effects on Ca^{2+} channel activity of four isoforms and a series of fragments of calpastatin domain L were investigated in guinea-pig ventricular myocytes with the patch–clamp method. With one exception, all the isoforms and fragment peptides that contained amino acid residues 54–64 of domain L reprimed the Ca^{2+} channels to comparable levels (9–15% of control activity) to those observed previously with a full-length form of calpastatin. These results suggest that the region containing amino acid residues 54–64 (EGKPKEHTEPK) is responsible for the Ca^{2+} channel repriming function of calpastatin domain L.

Keywords: Calpastatin; Domain L; Calcium channel; Run-down; Ventricular myocyte

A calpastatin molecule contains a unique N-terminal region (domains L and XL) and four homologous C-terminal regions (domains 1–4) [1–4]. It is well established that domains 1–4 are responsible for the inhibition of calpain, the calcium-dependent cysteine protease [5]. However, the function of the N-terminal region, especially that of domain L, is not clear, although its existence is well conserved in tissue-type calpastatin of human, pig, rabbit, and other mammals [6–8]. We and others have previously suggested that calpastatin has a Ca^{2+} channel repriming function based on the following findings: (1) run-down of L-type Ca^{2+} channel in guinea-pig cardiac myocytes is prevented and/or reversed by cytoplasm extracted from bovine heart, in which one of the effective components was shown to be calpastatin [9–12]. (2) Calpastatin extracted from bovine heart or expressed in *Escherichia coli* (*E. coli*) as a recombinant protein partially reverses the run-down [12–16]. (3) The channel-activating effect of calpastatin is not mimicked by synthetic calpain inhibitors [17]. Furthermore, we have recently confirmed that this channel activating function is localized in domain L [16]. Based on these findings, we have proposed that calpastatin has dual functions, namely a calpain-inhibiting function carried by domains 1–4 and Ca^{2+} channel-activating function carried by domain L [16].

Based on the N-terminal sequences, calpastatin is classified into four types, type I–IV [4]. Types I and II contain the XL region in addition to domains L and 1–4, while type

* Corresponding authors. Fax: +81 99 2755522 (L.-Y. Hao).
E-mail addresses: lyhao@mail.cum.edu.cn (L.-Y. Hao), kame@m.kagoshima-u.ac.jp (M. Kameyama).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.07.052

III starts from domain L and type IV from domain 2. In this study, we have investigated whether these isoforms have different effects on Ca^{2+} channel activity and which specific region of the calpastatin L or XL domains is responsible for the Ca^{2+} channel activating function in guinea-pig cardiac myocytes using the patch–clamp method. We have found that the Ca^{2+} channel activating effect of calpastatin is restricted to domain L and that the sequence corresponding to amino acid residues 54–64 (human cDNA, GenBank Accession No. D16217) may be responsible for this function.

Materials and methods

Preparation of single cardiac myocytes. Ventricular myocytes from guinea-pigs were dispersed by collagenase and protease as described previously [18]. A female guinea-pig (weight 350–500 g) was anaesthetized with sodium pentobarbital (30 mg/kg i.p.), and the aorta was cannulated under artificial respiration. The heart was then dissected out, mounted on a Langendorff apparatus, and perfused with Tyrode solution for 3 min at 37 °C, followed by a nominally Ca^{2+}-free Tyrode solution for 6 min, a solution containing collagenase (0.08 mg/ml, Yakult, Tokyo, Japan) for 7–15 min, and finally washed out with a high-K^+ and low-Ca^{2+} storage solution. The left ventricular myocytes were dispersed and filtered through a stainless steel mesh (105 μm), treated with alkaline protease (Nagase NK-103, 0.03 mg/ml, Katayama, Osaka, Japan) and DNase I (type IV, 0.02 mg/ml, Sigma Aldrich, St. Louis, USA) for 4 min at 37 °C, and washed twice with centrifugation at 800 rpm for 3 min. The isolated cells were stored at 4 °C until used for the patch–clamp experiments.

Preparation of calpastatin peptides. Calpastatin isoforms and their fragment peptides used in this study are illustrated in Fig. 1. Porcine calpastatin isoforms (type I, II, III and IIIΔ3; GenBank Accession Nos. AJ583407, AJ583408, and AJ583409) were generated by RT PCR from cardiac and skeletal muscle cDNA using forward primers located in exons 1Xa, 1Xb, and 2 and a common reverse primer located in exon 7, essentially as described previously [19], and cloned by into pGEX-4 T-1 (Amersham Biosciences, Uppsala, Sweden). The cDNAs corresponding to amino acid residues 3–148 (L3–148), 3–68 (L3–68), and 57–148 (L57–148) of human calpastatin (GenBank Accession No. D16217) were inserted into pGEX-6P-3 (Amersham Biosciences). The corresponding peptides were expressed as glutathione *S*-transferase (GST) fusion proteins in *E. coli*, BL21 and purified using Glutathione Sepharose 4B (Amersham Biosciences). The GST region of human calpastatin fragments was removed by cleavage using PreScission protease (Amersham Biosciences). Finally, expressed peptides

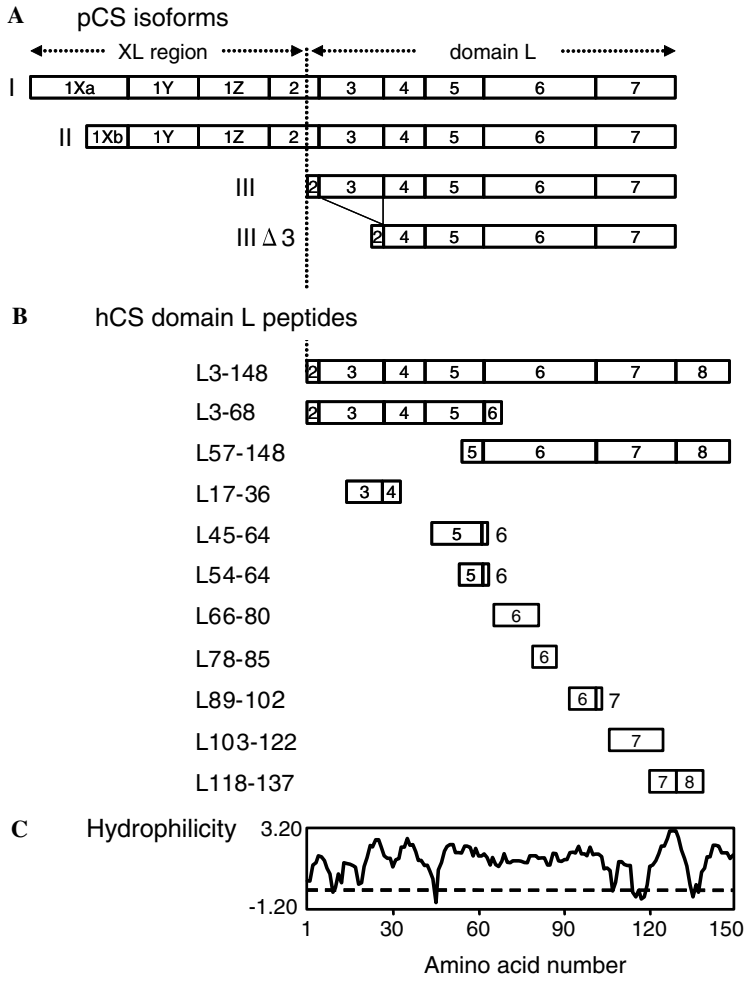

Fig. 1. Schematic representation of fragments of domain L of porcine calpastatin isoforms (A) and human calpastatin (B). The corresponding range and the exon number are shown with open box. Dotted line indicates the beginning of the domain L. Human calpastatin fragments are named with the amino acid number of the first and the last residue. (C) Hydropathy plot in a fixed segment (average span: five residues) was calculated on Hoop & Woods parameter by using a calculation software of Genetyx and plotted for each residue, dotted line shows the 0 level.

were checked on SDS–PAGE and quantified by the Bradford method. Shorter peptides corresponding to amino acid residues 17–36 (named as L17–36), 45–64 (L45–64), 54–64 (L54–64), 66–80 (L66–80), 78–85 (L78–85), 89–102 (L89–102), 103–122 (L103–122), and 118–137 (L118–137) were prepared by custom synthesis (Sigma Aldrich Japan, Ishikari, Japan).

Solutions. Tyrode solution contained (in mM): 135 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 1.0 $MgCl_2$, 5.5 glucose, 1.8 $CaCl_2$, and 10 Hepes–NaOH buffer (pH 7.4). Storage solution contained (in mM): 70 KOH, 50 glutamic acid, 40 KCl, 20 KH_2PO_4, 20 taurine, 3 $MgCl_2$, 10 glucose, 10 Hepes, and 0.5 EGTA-buffer (pH 7.4 by KOH). Pipette solution contained (in mM): 50 $BaCl_2$, 70 TEA–Cl, 0.5 EGTA, 0.003 Bay K 8644, and 10 Hepes–CsOH buffer (pH 7.4). The basic internal solution contained (in mM): 90 K aspartate, 30 KCl, 10 KH_2PO_4, 1 EGTA, 0.5 $MgCl_2$, 0.5 $CaCl_2$, and 10 Hepes–KOH buffer (pH 7.4).

Patch–clamp and data analysis. Ca^{2+} channel activity was recorded in the myocytes superfused with basic internal solution at 23–25 °C using a patch pipette (2–4 MΩ) containing 50 mM Ba^{2+} and 3 μM Bay K 8644 (a dihydropyridine class Ca^{2+} channel activator; a generous gift from Bayer, Leverkusen, Germany). The channel activity was elicited by a depolarizing pulse from a holding potential of −70 mV to 0 mV at a rate of 0.5 Hz with a patch–clamp amplifier EPC-7 (List, Darmstadt, Germany). The current signals were filtered at 1–1.5 Hz and fed to a computer at a sampling rate of 3.3 kHz where the capacity and the leakage currents were digitally subtracted. The *NPo* value was used to represent the channel activity, where *N* is the number of channels in the patch and *Po* is the time-averaged open-state probability of the channels of each depolarizing pulse. The *NPo* was calculated based on the equation $NPo = I/i$, where *I* is the mean current during the period 5–105 ms after the onset of the test pulses, and *i* is the unitary current amplitude. In each experiment, basal activity was recorded in the cell-attached mode for 2 min, and then run-down was induced by excising the patch membrane from the cell into basic internal solution to establish the inside-out patch mode. One minute after the patch excision, test solutions were applied by moving the patch into a small inlet of the perfusion chamber, which was connected to a microinjection system. The test solutions were supplemented with 3 mM MgATP (Sigma), based on our previous finding that ATP was required to reverse run-down [9,20].

Data are presented as means ± SE. Student's *t* test was used to estimate statistical significance and a *p* value less than 0.05 was considered as significant.

The experiments were carried out with permission from the Committee of Animal Experimentation, Kagoshima University, Japan.

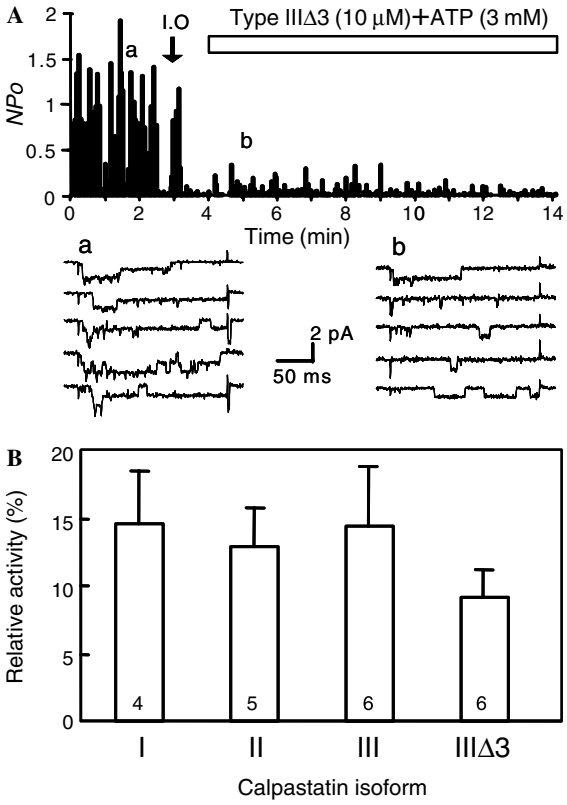

Fig. 2. (A) Effect of 10 μM calpastatin type IIIΔ3 recombinant protein on Ca^{2+} channel activity. The *NPo* value was calculated and plotted against time. Inside-out patch mode (I.O) was initiated at the time indicated by the arrow. Test solutions, supplemented with 3 mM MgATP, were applied as indicated by the box. Consecutive current traces in the cell-attached mode (a) and in the inside-out patch mode in the presence of type IIIΔ3 domain L (b) are shown in the lower panel. (B) Ca^{2+} channel activity (relative values) induced by domain XL-L of porcine calpastatin isoforms in the inside-out patch mode. All the test solutions were supplemented with 3 mM ATP. Data are presented as means ± SE with numbers of experiments inside the data bars.

Results

Effects of porcine calpastatin isoforms

Four types of calpastatin protein isoforms have been identified in porcine cardiac muscle [19]. Types I and II contain the XL region encoded by alternative exons 1Xa or 1Xb, exon 1Y, and exon 1Z arranged tandemly upstream of domain L. Fig. 1 shows the first nine exons that encode the XL and L domain, out of a total of approximately 30 exons representing the whole gene. Type III starts from an ATG translation start site in exon 2 and it has a splicing variant that is an exon 3-deleted form (type IIIΔ3). The Ca^{2+} channel repriming effects of domain XL-L regions of these four calpastatin isoforms were evaluated as the potency to recover channel activity after run-down of the channels in the inside-out patch mode. Fig. 2A shows an example of the experiments with domain L of type IIIΔ3. The Ca^{2+} channel was first recorded in the cell-attached mode as control, and then the membrane patch was excised to the inside-out patch mode (indicated as I.O). Channel activity was decreased in the inside-out mode due to run-down, but application of domain L partially recovered the channel activity. Channel activity induced by domain L of type IIIΔ3 was small (~10% of that in the cell-attached mode) but was significantly higher than that observed with 10 μM GST (4.0 ± 1.6%, $n = 4$; $p < 0.05$) or 2 mg/ml BSA (5.2 ± 0.9%, $n = 5$; $p < 0.05$).

The effects of domain XL-L of types I–III were similar to or slightly higher than that of type IIIΔ3. Values of relative channel activity for domain XL-L of types I, II, III, and IIIΔ3 (10 μM) were 14.5 ± 3.9% ($n = 4$), 12.8 ± 3.0% ($n = 5$), 14.4 ± 4.4% ($n = 6$), and 9.1 ± 2.0% ($n = 6$), respectively (Fig. 2), and these values were significantly higher than those for controls GST and BSA ($p < 0.05$). However, in mutual comparison, these values are not significantly different from each other ($p > 0.05$). These results suggest that XL region and exon 3 are not necessary for the activation of the Ca^{2+} channel, and therefore that they are not effective regions for repriming the Ca^{2+} channels.

Effects of L3–148

Domain L of human calpastatin is composed of 150 amino acids. L3–148 is a peptide that has 2-amino acid omissions at N- and C-termini. The relative channel activity in the presence of 10 μM L3–148 in the inside-out mode was 10.6 ± 1.7% ($n = 7$) of that in the cell-attached mode (Fig. 4A). This value was not different from that reported previously for the full-size domain L [13,16], suggesting that the omission of the initial and the final two amino acids did not influence the Ca^{2+} channel repriming function of calpastatin and its domain L.

Effects of L3–68 and L57–148

Domain L was then divided into two parts, L3–68 and L57–148, to explore which part contained the functional region. The effect of 10 μM L57–148 (with 3 mM ATP) is shown in Fig. 3A. The activity of the channels decreased in inside-out patch in the basic internal solution, but application of L57–148 recovered the channel activity. The relative channel activity was 13.6 ± 2.2% ($n = 5$), significantly higher than that observed with GST ($p < 0.01$) or BSA ($p < 0.01$). The relative channel activity induced by 10 μM L3–68 (with 3 mM ATP) in the inside-out patch mode was 7.6 ± 1.9% ($n = 5$) (Fig. 4A). This value was not statistically different from those for controls (GST and BSA). These results indicated that the Ca^{2+} channel repriming function is likely to be in a region within L57–148.

Effects of L54–64 and other short peptides

Considering that L57–148 was effective, we designed several shorter peptides (8–20 amino acids) from this region considering hydropathy of the residues and predicted secondary structures, i.e., L54–64, L66–80, L78–85, L89–102, L103–122, and L118–137. Among these peptides, L54–64, with 11 amino acids, showed the highest channel activating effect. As shown in Fig. 3B, after run-down in inside-out patch, the application of 10 μM L54–64 restored the channel activity to 13.9 ± 2.3% ($n = 11$) of that recorded in cell-attached mode ($p < 0.05$ vs. GST and BSA), which is comparable to those observed with L3–148,

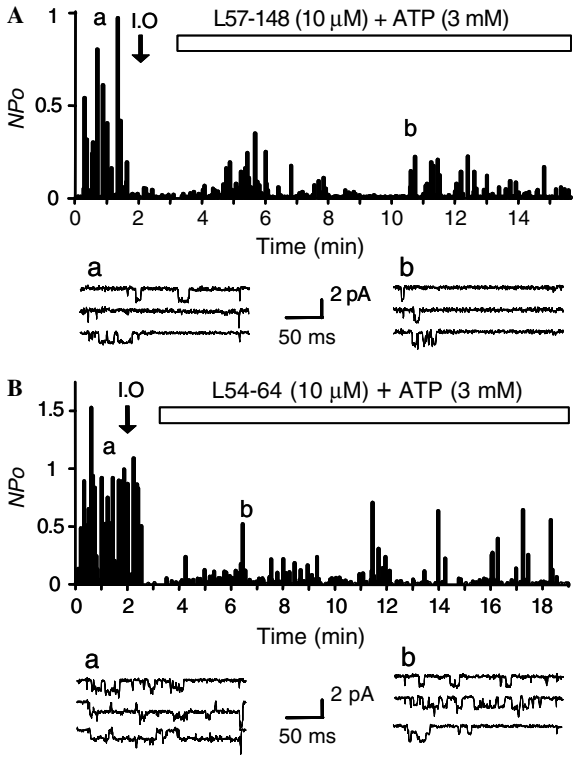

Fig. 3. Effect of 10 μM L57–148 (A) and L54–64 (B) on Ca^{2+} channel activity. The *NPo* value was calculated and plotted against time. Inside-out patch mode (I.O) was initiated at the time indicated by the arrow. Test solutions, supplemented with 3 mM MgATP, were applied as indicated by the box. Consecutive current traces in the cell-attached mode (a), and in the inside-out patch mode in the presence of L57–148 (A,b) and L54–64 (B,b) are shown in the lower panel of (A,B), respectively.

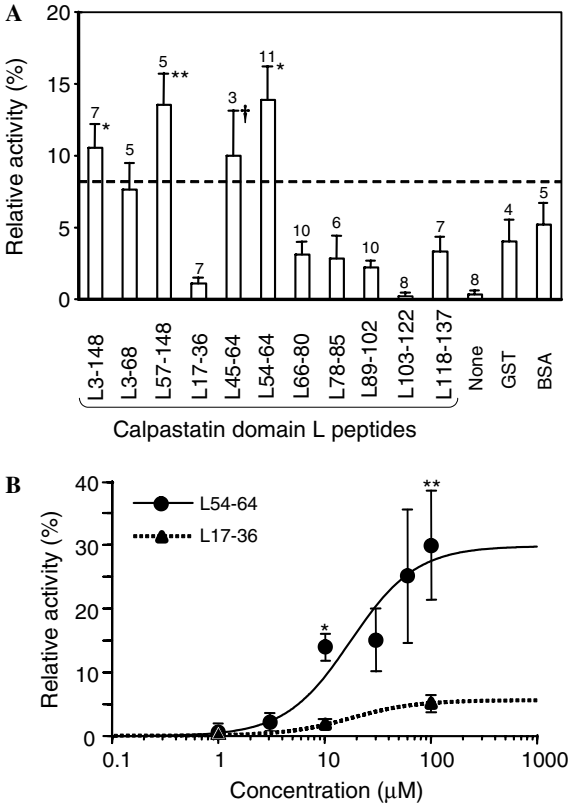

Fig. 4. Effects of fragments of human calpastatin domain L on Ca^{2+} channel activity. (A) Summary of the repriming effects of domain L fragments (10 μM). Control values were recorded with no protein/peptide (none), 10 μM GST, and 2 mg/ml BSA. Data are presented as means ± SE with numbers of experiments. Significant differences are marked by $*p < 0.05$ or $**p < 0.01$ vs. GST and BSA. †In L45–64, the effect was not significant at 10 μM, but significant ($p < 0.05$) at 80 μM. Broken line indicates 8% level in the relative channel activity. (B) Concentration-dependence of the channel-repriming effect of L54–64 and L17–36. Data are presented as mean ± SE ($n = 3$–11). Significant differences are marked by $*p < 0.05$ or $**p < 0.01$. All the test solutions were supplemented with 3 mM ATP in (A,B).

L57–148 (Fig. 4), as well as full-size calpastatin and its domain L [11,12,15,16].

The other peptides, L66–80, L78–85, L89–102, L103–122, and L118–137, each at 10 μM were less effective, with the relative channel activities in the presence of these peptides being less than 5%. When the concentrations of these peptides were increased to 50–100 μM, the relative channel activities were slightly increased: $9.7 \pm 1.6\%$ ($n = 5$) for 100 μM L66–80, $6.7 \pm 2.1\%$ ($n = 5$) for 50 μM L78–85, $9.4 \pm 2.6\%$ ($n = 7$) for 100 μM L89–102, $5.9 \pm 2.7\%$ ($n = 6$) for 50 μM L103–122, and $8.2 \pm 2.1\%$ ($n = 5$) for 100 μM L118–137. Nevertheless none of these effects was statistically significant ($p > 0.05$ vs. GST or BSA).

L45–64, which overlaps with L54–64, also recovered the channel activity. Although the relative channel activity in the presence of L45–64 (10 μM) was not significant ($10.0 \pm 3.3\%$, $n = 3$, $p > 0.05$ vs. GST or BSA), it was significantly higher with 80 μM L45–64 ($9.6 \pm 1.2\%$, $n = 5$, $p < 0.05$). These results suggest that the Ca^{2+} channel activating function of calpastatin domain L with 150 amino acids in length can be covered by a short peptide of only 11 amino acids.

As summarized in Fig. 4A, the peptides that contain the region of amino acid residues 54–64 show the Ca^{2+}-channel repriming effect, with exception of L3–68. This finding strongly suggested that L54–64 carried the Ca^{2+}-channel repriming effect.

Dose-dependence of L54–64

Finally, we investigated concentration-dependence of the effect of L54–64. For comparison, L17–36, a shorter peptide derived from the N-terminal regions of L3–68, was also examined. As shown in Fig. 4B, the channel repriming effect increased with increasing concentration of L54–64 up to 100 μM. On the other hand, the effect of L17–36 was not significant even at 100 μM ($5.1 \pm 1.3\%$, $n = 5$), although a slight concentration-dependent effect was seen. These results further support the view that the Ca^{2+} channel repriming effect is located in the region of amino acid residues 54–64 of domain L.

Discussion

We have found that a short peptide of calpastatin domain L, L54–64, shows a comparable effect on Ca^{2+} channel activity to that of full-size domain L. This provides strong support that domain L, whose function is unknown, has a Ca^{2+}-channel repriming function, and hence calpastatin has dual functions, namely inhibition of calpain (domains 1–4) and the regulation of the Ca^{2+} channel (domain L).

Based on the N-terminal sequences, calpastatin is classified into four types, I–IV [4]. Types I and II contain the XL region (reflecting different promoter usage or alternative splicing) in addition to domain L and inhibitory domains 1–4, while type III mRNA is generated from a promoter upstream of exon 1U and is translated from exon 2 (part of domain L). Type IV arises from a promoter upstream of exon 14 (part of inhibitory domain 2). Accordingly types I–III each have unique N-terminal sequences and are expressed widely in tissues, while type IV is expressed only in testis [3,19,21]. In this study, XL-L regions of type I–III of porcine calpastatin showed repriming effects on Ca^{2+} channel run-down in inside-out patches, to an extent similar to that of domain L. This result suggests that the XL region is not necessary for the effect on Ca^{2+} channels.

A possible role of exon 3 on channel activity was also examined by using type IIIΔ3 isoform, in which exon 3 is deleted by alternative splicing [4,19]. The exon 3 has been suggested to confer membrane binding characteristics or to be a nuclear translocation signal [22]. It is mostly spliced out in skeletal muscle but not in cardiac muscle [23], suggesting a difference between these muscle types in the function of domain L. In this study, however, it is found that type IIIΔ3 reprimes the channel to a comparable level to that observed with other isoforms. This result suggests that a functional role of exon 3 cannot be attributed to the channel repriming effect.

We have found that four peptides that contain amino acid residues 54–64, but not L3–68, show the Ca^{2+}-channel repriming effect. Furthermore, L54–64 showed a concentration-dependent effect with the EC_{50} of about 10 μM. This value is comparable to that of domain L (2.6 μM) [16]. These findings strongly suggest that the channel-repriming effect of calpastatin can be mapped to L54–64, a region corresponding to exon 5. It is interesting to note that exon 5 is frequently spliced out in tissues such as in tumor cells, but not in cardiac muscle [22].

Amino acid sequence analysis of domain L for secondary structures by New Joint method [24] predicts that only four regions can form higher structures (^{29}QAVK^{32}, ^{43}KLSVVH^{48}, ^{81}KKAV^{84}, and ^{114}VAGITAI^{120}), while other regions including L54–64 seem to form random coils. Hydropathy analysis of domain L shows that there is a hydrophilic region spanning 62 amino acid residues from 44 to 105, including L54–64 (Fig. 1). These analyses suggest that the region of L54–64 may be exposed on the surface of calpastatin molecule and ready to interact with other proteins such as ion channels. Domain L of calpastatin is rich in basic amino acid residues, arginine, and lysine [22]. However, the channel-repriming effect of domain L cannot be explained simply by the basic nature (high p*I*) of the peptides (Table 1). For example L3–68, L17–36, L78–85, and L118–137 have p*I* values higher than 10, but have no significant effect on the channel. It seems that the effect of L54–64 on the Ca^{2+} channel is specific and is based on its amino acid sequence. Although overall amino-acid sequence homology of domain L among species (human, monkey, cow, sheep, pig, rabbit, rat, and mouse) is low compared to that of domains 1–4, the sequence of exon 5 is relatively conserved (amino acid homology ~40%).

Besides L-type Ca^{2+} channels, there is no report of the direct activation of other ion channels by calpastatin

Table 1
Distribution of charged residues in human calpastatin fragments

Peptide	No. of amino acid residues	Arg	Lys	His	Asp	Glu	Sum of charge values[b]	p*I*[c]
L3–148[a]	146	1	29	5	5	14	+13.5	10.3
L3–68	66	0	16	4	0	8	+10	10.5
L57–148[a]	92	1	17	2	5	8	+6	10.0
L17–36	20	0	6	2	0	1	+4	10.5
L45–64[a]	20	0	5	2	0	4	+2	9.4
L54–64[a]	11	0	3	1	0	3	+0.5	7.8
L66–80	15	0	1	1	2	0	−0.5	5.1
L78–85	8	0	2	1	0	0	+2.5	10.6
L89–102	14	0	3	0	0	2	+1	7.2
L103–122	20	0	0	0	1	1	−2	3.1
L118–137	20	0	6	0	1	1	+4	10.5

[a] Peptides showing a statistically significant effect on Ca^{2+} channel activity.
[b] Charge values were calculated with Arg and Lys as +1, His as +0.5, and Asp and Glu as −1.
[c] Isoelectric points (p*I*) were calculated by Genetyx software.

domain L, although calpastain seems to regulate activity of some ion channels through inhibition of calpain (e.g., [25]). It is possible that calpastatin might regulate a wide variety of ion channels via domain 1–4 (calpain inhibition) or domain L (possibly direct action), especially for the Ca^{2+}-permeable channels.

According to our findings and other reports, there are several factors that may be involved in the reversion of Ca^{2+} channel run-down including crude cytoplasm of bovine heart [9–11], a high molecular weight fraction of bovine heart extract [12], calpastatin [12,14,16], ATP [20], PKA [26], G-protein [27], calmodulin [28], and calmodulin-dependent protein kinase II (Hao et al., unpublished data). The mechanisms by which these factors act on the channel are complicated and have not been fully determined. The short peptide L54–64 can be a good tool to investigate interactions and functional cross-talk among the regulating factors and the channels.

Among the above-mentioned Ca^{2+}-channel repriming factors, compared with the effects of crude cytoplasm and calmodulin, the channel-repriming effect of calpastain is limited. Full-length calpastatin, purified from bovine heart or produced as a recombinant protein, can reprime the channels to only 20–40% of original activity [12,14–16]. This incomplete potency of calpastatin is seen also with domain L (devoid of domains 1–4), and its peptide fragments, even though the concentration was increased to 100 μM (Fig. 4B). These results suggest that the limited effect on the channel may be an intrinsic character of calpastatin. In contrast to the limited effect of calpastatin on the run-down channels, calpastatin can maintain channel activity when applied before the channel runs down [13], or it can reactivate the channels to about 60–90% level when applied together with ''H fraction'', a high-molecular fraction of cardiac cytoplasm [12]. Therefore, the effect of calpastatin seems to depend on a particular state of the channel, the molecular basis of which is not clear. Further studies are needed to clarify this channel status dependency of calpastatin's effectiveness.

Calpastain, as the endogenous inhibitor of calpain, can also be digested by calpain [29,30] and other protease such as caspase [31]. At low Ca^{2+} concentration (<20 μM), moderate degradation of calpastatin by μ-calpain occurs and produces a 15 kDa protein with enhanced calpain inhibitory activity and some other fragments with low molecular mass [29]. At higher Ca^{2+} concentration, extensive degradation of calpastatin by m-calpain occurs and the calpain inhibitory activity disappears completely [29,30]. Our current result suggests that even if calpastatin was digested to small fragments, the Ca^{2+} channel activating function might still remain. The small but persistent effect of calpastatin might be important to the cell function.

Acknowledgments

We thank T. Imaichi for technical assistance and E. Iwasaki for secretarial work on the manuscript. This work was supported by research grants from the Japan Society for the Promotion of Science to L.-Y.H. and to M.K., the Kodama Memorial Foundation to E.M., the Inamori Foundation to L.-Y.H., and the Biotechnology and Biological Sciences Research Council, United Kingdom to R.G.B.

References

[1] T. Murachi, Intracellular regulatory system involving calpain and calpastatin, Biochem. Int. 18 (1989) 263–294.
[2] E. Melloni, F. Salamino, B. Sparatore, The calpain–calpastatin system in mammalian cells: properties and possible functions, Biochimie 74 (1992) 217–223.
[3] J. Takano, T. Kawamura, M. Murase, K. Hitomi, M. Maki, Structure of mouse calpastatin isoforms: implications of species-common and species-specific alternative splicing, Biochem. Biophys. Res. Commun. 260 (1999) 339–345.
[4] J. Takano, M. Watanabe, K. Hitomi, M. Maki, Four types of calpastatin isoforms with distinct amino-terminal sequences are specified by alternative first exons and differentially expressed in mouse tissues, J. Biochem. 128 (2000) 83–92.
[5] Y. Emori, H. Kawasaki, S. Imajoh, K. Imahori, K. Suzuki, Endogenous inhibitor for calcium-dependent cysteine protease

contains four internal repeats that could be responsible for its multiple reactive sites, Proc. Natl. Acad. Sci. USA. 84 (1987) 3590–3594.
[6] K. Asada, Y. Ishino, M. Shimada, T. Shimojo, M. Endo, F. Kimizuka, I. Kato, M. Maki, M. Hatanaka, T. Murachi, cDNA cloning of human calpastatin: sequence homology among human, pig, and rabbit calpastatins, J. Enzyme Inhib. 3 (1989) 49–56.
[7] S. Ishida, Y. Emori, K. Suzuki, Rat calpastain has diverged primary sequence from other mammalian calpastatins but retains functionally important sequences, Biochim. Biophys. Acta 26 (1991) 436–438.
[8] J. Killefer, M. Koohmaraie, Bovine skeletal muscle calpastatin: cloning, sequence analysis, and steady-state mRNA expression, J. Anim. Sci. 72 (1994) 606–614.
[9] M. Kameyama, A. Kameyama, T. Nakayama, M. Kaibara, Tissue extract recovers cardiac calcium channels from 'run-down', Pflügers Arch. 412 (1988) 328–330.
[10] A. Kameyama, K. Yazawa, M. Kaibara, K. Ozono, M. Kameyama, Run-down of the cardiac Ca^{2+} channel: characterization and restoration of channel activity by cytoplasmic factors, Pflügers Arch. 433 (1997) 547–556.
[11] A. Kameyama, L.-Y. Hao, E. Takano, M. Kameyama, Characterization and partial purification of the cytoplasmic factor that maintains cardiac Ca^{2+} channel activity, Pflügers Arch. 435 (1998) 338–343.
[12] L.-Y. Hao, A. Kameyama, M. Kameyama, A cytoplasmic factor, calapastatin and ATP together reverse run-down of Ca^{2+} channel activity in guinea-pig heart, J. Physiol. 514 (1999) 687–699.
[13] C. Romanin, P. Grosswagen, H. Schindler, Calpastatin and nucleotides stabilize cardiac calcium channel activity in excised patches, Pflügers Arch. 418 (1991) 86–92.
[14] M. Kameyama, A. Kameyama, E. Takano, M. Maki, Run-down of the cardiac L-type Ca^{2+} channel: partial restoration of channel activity in cell-free patches by calpastatin, Pflügers Arch. 435 (1998) 344–349.
[15] L.-Y. Hao, A. Kameyama, S. Kuroki, S. Nishimura, M. Kameyama, Run-down of L-type Ca^{2+} channels occurs on the α1 subunit, Biochem. Biophys. Res. Commun. 247 (1998) 844–850.
[16] L.-Y. Hao, A. Kameyama, S. Kuroki, J. Takano, E. Takano, M. Maki, M. Kameyama, Calpastatin domain L is involved in the regulation L-type of Ca^{2+} channels in guinea pig cardiac myocytes, Biochem. Biophys. Res. Commun. 279 (2000) 756–761.
[17] K. Seydl, J.O. Karlsson, A. Dominik, H. Gruber, C. Romanin, Action of calpastatin in prevention of cardiac L-type Ca^{2+} channel run-down cannot be mimicked by synthetic calpain inhibitors, Pflügers Arch. 429 (1995) 503–510.
[18] K. Yazawa, M. Kaibara, M. Ohara, M. Kameyama, An improved method for isolating cardiac myocytes useful for patch–clamp studies, Jpn. J. Physiol. 40 (1990) 157–163.
[19] T. Parr, P.L. Sensky, R.G. Bardsley, P.J. Buttery, Calpastatin expression in porcine cardiac and skeletal muscle and partial gene structure, Arch. Biochem. Biophys. 395 (2001) 1–13.
[20] K. Yazawa, A. Kameyama, K. Yasui, J-M. Li, M. Kameyama, ATP regulates cardiac Ca^{2+} channel activity via a mechanism independent of protein phosphorylation, Pflügers Arch. 433 (1997) 557–562.
[21] T. Parr, K.K. Jewell, P.L. Sensky, J.M. Brameld, R.G. Bardsley, P.J. Buttery, Expression of calpastatin isoforms in muscle and functionality of multiple calpastatin promoters, Arch. Biochem. Biophys. 427 (2004) 8–15.
[22] W.J. Lee, H. Ma, E. Takano, H.Q. Yang, M. Hatanaka, M. Maki, Molecular diversity in amino-terminal domains of human calpastatin by exon skipping, J. Biol. Chem. 267 (1992) 8437–8442.
[23] G.H. Geesink, D. Nonneman, M. Koohmaraie, An improved purification protocol for heart and skeletal muscle calpastatin reveals two isoforms resulting from alternative splicing, Arch. Biochem. Biophys. 356 (1998) 19–24.
[24] K. Nishikawa, T. Noguchi, Predicting protein secondary structure based on amino acid sequence, Methods Enzymol. 202 (1991) 31–44.
[25] M. Suzuki, G. Ohki, T. Mochizuki, S. Somlo, K. Ishibashi, M. Imai, Opening of ligand-gated cation channel families by calpain inhibitors, FEBS Lett. 517 (2002) 219–224.
[26] K. Ono, H.A. Fozzard, Phosphorylation restores activity of L-type calcium channels after rundown in inside-out patches from rabbit cardiac cells, J. Physiol. 454 (1992) 673–688.
[27] R.C. Lambert, A. Feltz, Maintained L-type Ca^{2+} channel activity in excised patches of PTX-treated granule cells of the cerebellum, J. Neurosci. 15 (1995) 6014–6022.
[28] J-J. Xu, L.-Y. Hao, A. Kameyama, M. Kameyama, Calmodulin reverses rundown of L-type Ca^{2+} channels in guinea pig ventricular myocytes, Am. J. Physiol. Cell. Physiol. 287 (2004) 1717–1724.
[29] R. De Tullio, M. Averna, F. Salamino, S. Pontremoli, E. Melloni, Differential degradation of calpastatin by μ- and m-calpain in Ca^{2+}-enriched human neuroblastoma LAN-5 cells, FEBS Lett. 475 (2000) 17–21.
[30] F. Salamino, M. Averna, I. Tedesco, R. De Tullio, E. Melloni, S. Pontremoli, Modulation of rat brain calpastatin efficiency by post-translational modifications, FEBS Lett. 412 (1997) 433–438.
[31] S. Barnoy, N.S. Kosower, Caspase-1-induced calpastatin degradation in myoblast differentiation and fusion: cross-talk between the caspase and calpain systems, FEBS Lett. 546 (2003) 213–217.