Flanking Proline Residues Identify the L-Type Ca^{2+} Channel Binding Site of Calciseptine and FS2[†]

R. Manjunatha Kini,*[,‡,||] Ray A. Caldwell,[§] Qi Y. Wu,[§] Clive M. Baumgarten,[§] Joseph J. Feher,[§] and Herbert J. Evans[‡,⊥]

From the Department of Biochemistry and Molecular Biophysics and Department of Physiology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Received February 3, 1998; Revised Manuscript Received March 26, 1998

ABSTRACT: Calciseptine and FS2 are 60-amino acid polypeptides, isolated from venom of the black mamba (*Dendroaspis polylepis polylepis*), that block voltage-dependent L-type Ca^{2+} channels. We predicted that these polypeptides contain an identical functional site between residues 43 and 46 by searching for proline residues that mark the flanks of protein−protein interaction sites [Kini, R. M., and Evans, H. J. (1966) *FEBS Lett. 385*, 81−86]. The predicted Ca^{2+} channel binding site also occurs in closely related toxins, $C_{10}S_2C_2$ and S_4C_8. Therefore, it is likely that these toxins also will block L-type Ca^{2+} channels. To test the proposed binding site on calciseptine and FS2, an eight-residue peptide, named L-calchin (L-type calcium channel inhibitor), was synthesized and examined for biological activity. As expected for an L-type Ca^{2+} channel blocker, L-calchin reduced peak systolic and developed pressure in isolated rat heart Langendorff preparations without affecting diastolic pressure or heart rate. Furthermore, L-calchin caused a voltage-independent block of L-type Ca^{2+} channel currents in whole-cell patch-clamped rabbit ventricular myocytes. Thus the synthetic peptide exhibits the L-type Ca^{2+} channel blocking properties of the parent molecules, calciseptine and FS2, but with a lower potency. These results strongly support the identification of a site in calciseptine and FS2 that is important for binding to L-type Ca^{2+} channels and reinforce the importance of proline brackets flanking protein−protein interaction sites.

Identifying sites of protein−protein interactions remains one of the most difficult and time-consuming challenges in protein chemistry. Recently we examined the flanking segments of more than 1600 continuous peptide segments that comprise protein−protein interaction sites (*1*, *2*). This survey indicated that proline residues are commonly found near sites of interaction. The probability of finding a proline residue in the flanking segments of interaction sites is about 2.5-times greater than elsewhere (*2*). Based on these observations, we proposed a structural role for proline residues in protecting the conformation and integrity of interaction sites and in presenting the sites to their complementary proteins (*1*, *2*). As a corollary, a new and simple method for predicting protein−protein interaction sites was offered (*3*). The complete amino acid sequence is the only requisite for this prediction method. At first the positions of all proline residues in the amino acid sequence of the protein of interest are marked. Then we search for all protein segments that are flanked by proline residues. Short segments of 3−7 residues enclosed by proline brackets are identified as potential interaction sites. The number of such predicted sites are small; a protein of 100 amino acid residues contain 2 potential protein−protein interaction sites.[1,2] Using any other available information these predicted sites can be either strengthened or ruled out (for details, see ref *1*, *3*). Using this method, we have identified several protein−protein interaction sites (see Discussion).

To further test this method for predicting sites of protein−protein interaction and to extend its applicability to another class of interaction, that between an ion channel and a natural peptide toxin, we considered calciseptine and FS2, peptide toxins from the venom of the black mamba (*Dendroaspis polylepis polylepis*). These toxins specifically inhibit voltage-dependent, L-type Ca^{2+} channels (*4*, *5*). Calciseptine blocks spontaneous contractions of rat portal vein and uterus and contractions of rat thoracic aorta and cardiac preparations induced by 40 mM K^+ and Bay K8644, respectively. The mechanism of action of this peptide was confirmed by showing that calciseptine blocked L-type Ca^{2+} channels in voltage-clamped A7r5 cells and ventricular myocytes (*4*). Like nitrendipine, a Ca^{2+} channel antagonist, calciseptine binds to a 1,4-dihydropyridine recognition site on the L-type channel of rat synaptosomal membranes (*6*). FS2, a highly homologous toxin, also inhibits L-type Ca^{2+} channels (*5*). Both calciseptine and FS2 are 60-residue polypeptides with

[†] This work was supported by a grant-in-aid from the American Heart Association (AHA890775) to H.J.E.

* To whom all correspondence should be addressed. Phone: (65) 874-5235. Fax: (65) 777-0052. E-mail: bsckinim@nus.sg.

[‡] Department of Biochemistry and Molecular Biophysics.

[§] Department of Physiology.

[||] Present address: Bioscience Centre, Faculty of Science, National University of Singapore, Singapore 119260.

[⊥] Deceased.

[1] Abbreviations: L-calchin, L-type *cal*cium *ch*annel *in*hibitor synthetic peptide based on the predicted site; S-calchin, peptide with the same amino acid composition as L-calchin, but scrambled sequence; and I_{Ca-L}, L-type Ca^{2+} channel current.

[2] R. M. Kini, unpublished observations.

S0006-2960(98)00272-4 CCC: $15.00
Published on Web 06/05/1998

four disulfide bridges (*4, 7*). They share significant structural homology with postsynaptic neurotoxins (for details, see Results).

Examination of the amino acid sequence of calciseptine (*4*) revealed a potential protein−protein interaction site consisting of four amino acids (residues 43−46) bracketed by two proline residues. To test this prediction, an eight-residue peptide including the proposed site was synthesized and examined for biological activity. This peptide, designated L-calchin (L-type calcium channel inhibitor),[1] reduced ventricular developed pressure in an isolated, perfused, heart preparation and inhibited L-type Ca^{2+} channel current (I_{Ca-L}) in patch-clamped, isolated, ventricular myocytes in a voltage-independent manner. Thus L-calchin exhibited the L-type Ca^{2+} channel blocking properties of the parent molecules, calciseptine and FS2, but with a lower potency.

These results strongly support the identification of a site on calciseptine and FS2 that binds to L-type Ca^{2+} channels. The synthetic peptide L-calchin can be a prototype for the development of peptidic (or nonpeptidic) cardiovascular agents. Moreover, our ability to predict the site of interaction of this channel toxin reinforces the importance of proline brackets flanking protein−protein interaction sites.

MATERIALS AND METHODS

Peptide Synthesis. The predicted site for calciseptine and FS2 binding to Ca^{2+} channels was TAMW (see Results). A test peptide, designated L-calchin, with the sequence APTAMWPA was synthesized by solid-phase synthesis using *t*-BOC chemistry on Merrifield resin (*8*), with a Milligen/Biosearch Model 9600 peptide synthesizer. The peptide sequence included the flanking prolines, because they enhance the biological activity of short peptides (*9*). An additional flanking residue on each side was also included. We also synthesized a control peptide, TAPAPWMA, with the same amino acid composition as that of L-calchin. This peptide was designated as S-calchin (scrambled calchin). Both L-calchin and S-calchin were purified by reverse-phase HPLC to more than 95% purity, with about 80% yields. The expected amino acid ratios were found after amino acid analysis, and the structures was confirmed by determining their mass from electrospray ionization mass spectra obtained with a Perkin-Elmer/Sciex API 300 mass spectrometer. The measured weights were 844.0 and 843.7 for L-calchin and S-calchin, respectively, whereas the calculated molecular weight was 844.1.

Isolated Heart Langendorff Preparation. Male Sprague−Dawley rats (300−350 g) were anesthetized with 65 mg/kg sodium pentobarbital and injected with 100 U heparin via the femoral vein. The heart was excised and placed in ice-cold Krebs−Henseleit buffer containing (mM) 118.5 NaCl, 25 $NaHCO_3$, 3.2 KCl, 1.4 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4, 11.1 glucose (pH 7.4, gassed with 95% O_2/5% CO_2). The aorta was cannulated, and retrograde perfusion of 37 °C Krebs−Henseleit buffer was begun at a constant pressure, 80 mmHg. Perfusate was selected from separately gassed and warmed reservoirs containing Krebs−Henseleit buffer with no additions, or with 1 μM diltiazem or 5 μM L-calchin. Left ventricular pressure was monitored continuously using a Grass *P*23XL transducer connected to a fluid-filled latex balloon inserted into the left ventricle via the mitral valve. End-diastolic pressure was set at about 4 mmHg by adjusting the initial volume of the balloon. Cardiac temperature was monitored with a needle probe (Physiotemp, Clifton, NJ). All hearts were equilibrated for 20 min before beginning test perfusion. In some experiments, the heart was paced at 340 beats/min (2.5 V, 3 ms pulse) through two platinum electrodes connected to the apex and the ventricle just below the left atrium.

Cardiac Myocyte Isolation. Ventricular myocytes were isolated from New Zealand white rabbits (2.0−3.0 kg) by a collagenase−protease digestion procedure (*10*). Briefly, spontaneously beating hearts were perfused via the aorta with a 37 °C modified Tyrode solution containing (mM): 0.75 $CaCl_2$, 130 NaCl, 5.4 KCl, 3.5 $MgCl_2$, 0.4 NaH_2PO_4, 10 glucose, 20 taurine, 10 creatine, 5 HEPES (pH 7.25, gassed with 100% O_2). Subsequently the perfusate was switched to an isolation solution containing 1 mg/mL collagenase (type II; Worthington Biochemical Corp., Freehold, NJ) and protease (Pronase E, type XIV; Sigma Chemical Co., St. Louis, MO) in 80 μM Ca^{2+}-Tyrodes. Following collection, myocytes were stored in a Kraft−Brühe solution containing (mM) 80 K glutamate, 2.5 KCl, 1.8 $MgSO_4$, 0.5 EGTA, 10 taurine, 11 glucose, 10 HEPES and titrated to pH 7.2 with KOH. A typical isolation yielded 60−70% viable cells. Cells selected for study had clear striations, were free of membrane blebs, and were quiescent. All patch clamp recordings were made at 22−23 °C within 8 h of cell isolation.

Whole-Cell Patch Clamp. Bath and pipet (internal) solutions were designed to isolate I_{Ca-L}. The control bath solution contained (mM) 120 NaCl, 20 mM CsCl, 0.01 tetrodotoxin, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.73 NaH_2PO_4, 5 glucose, 5 HEPES and was titrated to pH 7.4 with CsOH. Isoproterenol, 0.5 μM, was added to enhance I_{Ca-L} and minimize its rundown. When indicated, 100 μM L-calchin was dissolved directly in the bath solution. The pipet solution contained (mM) 119.8 CsCl, 5 K_2EGTA, 0.062 $CaCl_2$, 4 $MgSO_4$, 3.1 K_2ATP, 5 Na_2-creatine phosphate, 10 HEPES, and was titrated to pH 7.1 with CsOH. Pipets were fabricated from 7740 glass (1.5 mm OD, 0.84 mm ID) using a two-stage pull, coated at least twice with Sylgard 184 (Dow Corning, Midland, MI), and fire-polished to resistances between 2 and 4 MΩ.

An EPC-7 amplifier (List Medical, Darmstadt-Eberstat, FRG) was used to patch clamp myocytes in the whole-cell configuration, and a 150 mM KCl agar bridge served as the ground electrode. The membrane current was low pass filtered at 2 kHz (8 pole Bessel) and digitized at 10 kHz. To inactivate Na^+ channels not blocked by tetrodotoxin and to isolated I_{Ca-L}, myocytes were held at −64 mV after gaining access. Voltage protocols and data acquisition were controlled by custom programs written in ASYST (Keithly-Asyst, Tauton, MA). Capacity transients were partially compensated by analogue circuitry, and additional capacitance cancellation and leak current subtraction was achieved off line by subtracting the appropriately scaled average of 14 10-mV steps. After series resistance compensation, the maximum voltage error was 4.7 ± 0.5 mV. The diffusion potential between pipet and bath solutions, measured as $E_{bath} - E_{pipet}$ (*11*), was 4.1 ± 0.1 mV ($n = 4$), and all reported voltages have been corrected by this amount. Results are reported as mean ± SEM.

	Species	Protein	Biological Activity	1 10
1.	*D. p. polylepis*	Calciseptine	Ca^{2+} Channel Blocker	R I C Y I H K A S L **P** R A T K T C V E
2.	*D. p. polylepis*	FS2	Ca^{2+} Channel Blocker	R I C Y S H K A S L **P** R A T K T C V E
3.	*D. angusticeps*	$C_{10}S_2C_2$	Ca^{2+} Channel Blocker[a]	R I C Y S H K A S L **P** R A T K T C V E
4.	*D. j. kaimosae*	S_4C_8	Ca^{2+} Channel Blocker[a]	R I C Y T H K S L Q A K T T K S C E G
5.	*D. j. kaimosae*	Dendroaspin[b]	Aggregation Inhibitor	R I C Y N H L G T K **P P** T T E T C Q E
6.	*D. p. polylepis*	Toxin α	Postsynaptic Neurotoxin	R I C Y N H Q S T T R A T T K S C E E
7.	*D. j. kaimosae*	V^{I}_{n} 1	Postsynaptic Neurotoxin	R I C Y N H Q S T T **P** A T T K S C G E
8.	*D. angusticeps*	Toxin C	Anti-acetylcholinesterase	T I C Y S H T T T S R A I L K D C G E
9.	*D. p. polylepis*	Fasciculin	Anti-acetylcholinesterase	T M C Y S H T T T S R A I L T N C G E

	20 30 40 50 60	Homology
1.	N T C Y K M F I R T Q R E Y I S E R G C G C **P** T A M W **P** Y Q T E C C K G D R C N K	---
2.	N T C Y K M F I R T H R E Y I S E R G C G C **P** T A M W **P** Y Q T E C C K G D R C N K	96.7%
3.	N S C Y K M F I R T S **P** D Y I S D R G C G C **P** T A M W **P** Y Q T A C C K G D R C **N** K	88.3%
4.	N T C Y K M F I R T S R E Y I S E R G C G C **P** T A M W **P** Y Q T E C C K G D R C N K	81.7%
5.	D S C Y K N I W T F D N I I R R G C G C F T **P** R G D M **P** G **P** Y C C E S D K C N L	44.3%
6.	N S C Y K K Y W R D H R G T I I E R G C G C **P** K V K **P** G V G I H C C Q S D K C N Y	46.7%
7.	N S C Y K K T W S D H R G T I I E R G C G C **P** K V K Q G I H L H C C Q S D K C N Y	46.7%
8.	N S C Y R K S R R H **P P** K M V L G R G C G C **P P** G D D Y L E V K C C T S **P** D K C N Y	37.7%
9.	N S C Y R K S R R H **P P** K M V L G R G C G C **P P** G D D Y L E V K C C T S **P** D K C N Y	34.4%

FIGURE 1: Amino acid sequences of calciseptine FS2 and related toxins from *Dendroaspis* venoms. Amino acid sequences are taken from the following references: calciseptine (*4*), FS2 (*7*), S_4C_8 (*24*), $C_{10}S_2C_2$ (*25*), dendroaspin (*26*), toxin α (*27*), V^{I}_{n} 1 (*28*), Toxin C (*29*), and fasciculin (*30*). (a) Predicted biological activity based on the presence of the binding site of calciseptine and FS2. (b) Mambin, a platelet aggregation inhibitor isolated from *Dendroaspis jamesonii* venom, has the identical amino acid sequence (*31*), and a similar toxin also was isolated from *D. j. kaimosae* venom (*32*). Functional sites of calciseptine and related toxins (this paper) and of dendroaspin (or mambin) (*26, 31*) are singly and doubly underlined, respectively.

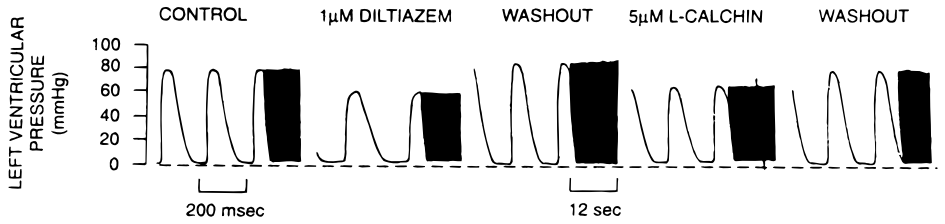

FIGURE 2: Representative trace of the effect of diltiazem and L-calchin on left ventricular pressure in the isolated, perfused rat heart. Tracings were obtained at the end of 20 min equilibration perfusion with Krebs−Henseleit buffer (control), near the end of 5 min perfusion with buffer containing 1 μM diltiazem, near the end of 5 min of diltiazem washout, near the end of 5 min perfusion with buffer containing 5 μM L-calchin, and near the end of 10 min of calchin washout.

RESULTS

Binding Site Prediction. Calciseptine and FS2 belong to the neurotoxin-cardiotoxin family of proteins. The amino acid sequences of several toxins isolated from *Dendroaspis* species are shown in Figure 1. The sequence of calciseptine and FS2 differ at only two residues, positions 5 and 30. Both are highly homologous with $C_{10}S_2C_2$ and S_4C_8 and are about 35−50% homologous to *Dendroaspis* toxins with differing biological activity. The three-dimensional structures of FS2, dendroaspin, α-neurotoxin, and fasciculin 1 exhibit the folding pattern of the three-finger toxin family (*12*−*15*): three loops extend from the crossover region (core) that contains all four disulfide bridges and a series of short loops.

The amino acid sequences of calciseptine (*4*) and FS2 (*7*) show three proline residues, at positions 11, 42, and 47 (see Figure 1). Only the proline residues at position 42 and 47 enclose a small segment of the protein. Therefore, we predicted that the segment between residues 42 and 47, TAMW, is a site on both calciseptine and FS2 which interacts with Ca^{2+} channels. The same site, including the flanking prolines, is present in $C_{10}S_2C_2$ and S_4C_8, and we predict that these toxins also will exhibit L-type Ca^{2+} channel blocking activity. Although the remaining five toxins are phylogenetically related and share a structural folding pattern, they do not possess this homologous binding domain and they exhibit diverse activity against other targets (references in legend of Figure 1).

Effect of L-Calchin on Left Ventricular Function. Inhibitors of L-type Ca^{2+} channels depress cardiac mechanical performance. Therefore, the effect of the synthetic peptide L-calchin on left ventricular function was evaluated in isolated perfused rat hearts. Representative traces comparing the effect of L-calchin and diltiazem, a known L-type Ca^{2+} channel blocker, on left ventricular pressure in a spontaneously beating heart are shown in Figure 2. Records were obtained after perfusion with (a) control buffer for 20 min, (b) 1 μM diltiazem for 5 min, (c) control buffer for 5 min to wash out diltiazem, (d) 5 μM L-calchin for 5 min, and (e) control buffer for 10 min to wash out L-calchin. Both diltiazem and L-calchin reduced peak systolic pressure with little or no change in diastolic pressure. Thus, both drugs reduced left ventricular developed pressure. The effects of diltiazem and L-calchin were readily reversible; wash-out

Table 1: Effect of Calchin on Isolated Perfused Rat Hearts[a]

parameter	control	5 μM L-calchin	recovery
systolic pressure	80.7 ± 3.9	66.3 ± 3.8*	77.6 ± 3.5
diastolic pressure	2.6 ± 0.5	3.1 ± 0.6	2.7 ± 0.6
developed pressure	78.1 ± 4.2	63.2 ± 4.1*	74.8 ± 3.9
heart rate	335 ± 8	334 ± 8	333 ± 9

[a] Values shown are the mean ± SEM for $n = 9$ rat hearts, of which six were paced and three were unpaced.

Table 2: Effect of Diltiazem on Isolated Perfused Rat Hearts[a]

parameter	control	1 μM diltiazem	recovery
systolic pressure	85.6 ± 4.3	71.0 ± 3.8*	84.8 ± 4.8
diastolic pressure	2.5 ± 0.6	5.3 ± 0.6	2.6 ± 0.5
developed pressure	83.1 ± 4.8	65.8 ± 4.0*	82.1 ± 5.0
heart rate	335 ± 11	315 ± 22	318 ± 22

[a] Values shown are the mean ± SEM for $n = 8$ rat hearts, of which four were paced and four were unpaced; (*), significantly different from control, $p < 0.0001$, using a paired *T*-test.

with control buffer for 5 and 10 min, respectively, was sufficient to reestablish baseline ventricular function.

The increased interval between pressure pulses in the presence of diltiazem (Figure 2) indicates that diltiazem slowed the spontaneous heart rate. In this example, heart rate was 306 bpm in the control, 223 bpm at the end of the 5 min diltiazem perfusion, and 300 bpm at the end of the washout period. Diltiazem decreased heart rate in 2 of 4 spontaneously beating hearts. In contrast, L-calchin did not alter the heart rate in any of the unpaced hearts.

To ensure that subtle changes in heart rate did not contribute to the negative inotropic effect of L-calchin, the experiment was repeated in hearts electrically paced at 340 beats/min. Pacing altered neither diastolic pressure nor the effects of L-calchin and diltiazem on ventricular mechanical function. Mechanical data from spontaneously beating and paced hearts were indistinguishable, and the data are combined in the summary presented in Tables 1 and 2.

Because control developed pressure varied according to the size of the heart and fit of the balloon, the effects of these agents are better seen by normalizing the developed pressure to that observed during perfusion with control buffer. Expressed this way, 5 μM L-calchin depressed developed pressure by 19.2 ± 2.2% ($p < 0.0001$), whereas 1 μM diltiazem depressed developed pressure by 20.8 ± 2.1% ($p < 0.0001$). This suggests that L-calchin is about 5-times less potent than diltiazem in suppressing cardiac mechanical performance. Developed pressure fully recovered after wash-out of diltiazem to 98.7 ± 1.8% of control ($p < 0.50$), but remained slightly depressed, 96.1 ± 1.4% of control ($p < 0.025$), after wash-out of L-calchin. A slow rundown of the preparation may have contributed to the small residual reduction of developed pressure after wash-out of L-calchin.

Block of L-Type Ca^{2+} Channels by L-Calchin. The results of studies on mechanical function were consistent with the idea that L-calchin inhibits L-type Ca^{2+} current, which is responsible for triggering contraction in the heart, but a number of alternative explanations could be offered. To directly test effects of L-calchin on the L-type channel, we examined its effects on I_{Ca-L} in ventricular myocytes using whole-cell patch clamp. Figure 3A illustrates the capacitance and leak-corrected I_{Ca-L} observed in a ventricular myocyte treated with 0.5 μM isoproterenol. I_{Ca-L} was elicited by a 200-ms, 70-mV step depolarization from a holding potential of −64 mV (upper trace). Exposing the myocyte to 100 μM L-calchin for 6 min (middle trace) reduced peak current to 46% of the control current in this cell. I_{Ca-L} recovered to 98% of control after 6 min of wash-out in L-calchin-free buffer (lower trace). In eight cells, L-calchin decreased I_{Ca-L} by 43 ± 3% ($p < 0.005$), and following washout, the current recovered to 101 ± 2% of its control value in all but one myocyte. In the remaining myocyte, only 80% of the initial current returned on wash-out. Calciseptine inhibited 50% of I_{Ca-L} at 430 nM in A7r5 cells (*4*), and FS2 appears to be generally equipotent with calciseptine (*5*). Thus, L-calchin appeared to be less potent than the parent molecules. S-calchin, however, did not affect the I_{Ca-L} at 100 μM concentration indicating the effect of L-calchin is sequence-specific.

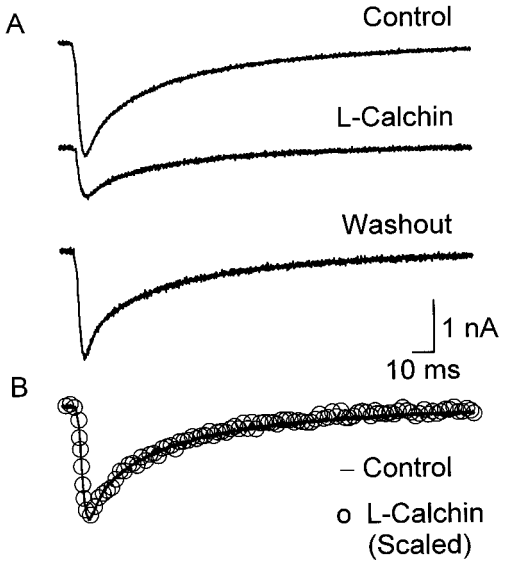

FIGURE 3: I_{Ca-L} in isolated rabbit ventricular myocytes during 200-ms step depolarizations from −64 to +6 mV. (A) Control (top trace), after a 6-min exposure to 100 μM L-calchin (middle trace), and after 6 min of wash-out (bottom trace). L-calchin reduced peak I_{Ca-L} to 46% of its control value, and wash-out restored 98% of the current in this cell. Solutions containing 0.5 μM isoproterenol to enhance I_{Ca-L} and slow its rundown. (B) L-calchin did not affect I_{Ca} kinetics. I_{Ca-L} after block was scaled so that peak current equalled that in control. The scaled current (open circle) superimposes on the control (solid line). For clarity, every 20th scaled point was plotted. During the rising phase of I_{Ca} more frequent points, every fifth point was plotted.

To investigate whether L-calchin affected I_{Ca-L} kinetics, the peak current during L-calchin exposure was scaled to its control value. The resulting trace (Figure 3B, open circle) superimposed on the control current (solid line) throughout the duration of the voltage step. This indicates that L-calchin did not affect the macroscopic activation or inactivation kinetics of L-type Ca^{2+} channels.

Figure 4 shows the $I-V$ curves obtained from the same myocyte as in Figure 3. Block by L-calchin was not itself voltage-dependent. Inhibition of I_{Ca-L} was by a constant fraction throughout the range of potentials studied (−40 to +50 mV). This is not unexpected because the charge on the postulated binding segment TAMW is neutral near physiological pH. Furthermore, L-calchin apparently did not

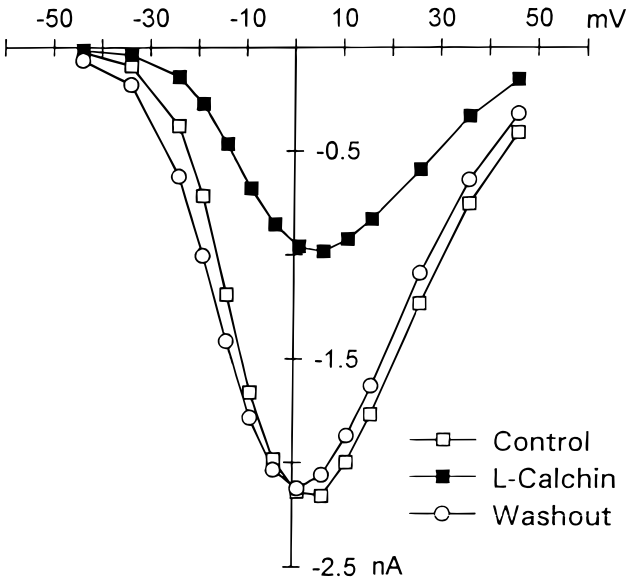

FIGURE 4: I_{Ca-L} current−voltage relationships show that block by L-calchin was voltage-independent and did not affect the voltage-dependence of activation.

affect the voltage dependence of Ca^{2+} channel activation. The voltage corresponding to peak I_{Ca-L} was $+4 \pm 1$ mV ($n = 8$) under control conditions and was unchanged, $+5 \pm 1$ mV, after exposure to L-calchin. Following wash-out of L-calchin, however, the voltage at which peak I_{Ca-L} was elicited shifted slightly negative (-4 ± 2 mV, $n = 8$). This shift probably reflects the well-known shift in the voltage dependence of channel activation that often occurs under whole-cell patch clamp conditions (*16*). Taken together, the electrophysiological data show that L-calchin inhibits L-type Ca^{2+} channels and suggest that its mechanism of action is simply to block the pore. A similar voltage-independent block of I_{Ca-L} was observed with the parent molecule calciseptine in A7r5 cells (*4*).

DISCUSSION

Protein−protein interactions are crucial to almost every physiological and pharmacological process. They impart specificity to allow discrimination among closely related structures, and their high affinity derives in part from the precise fit between the interacting surfaces. Identifying sites of protein−protein interaction is a difficult task, but it is essential to the final understanding of function. Several methods have been used to help identify these interaction sites, including structural homology with other proteins, molecular modeling using computer graphics, and theoretical deductive methods. These methods are used to obtain an initial indication of possible interaction sites.

Recently, we developed an alternative, simple method for predicting protein−protein interaction sites from the amino acid sequence. This method recognizes the importance of the flanking segments of interaction sites, and relies on the presence of a common structural feature, proline residues bracketing the interaction site (*1*, *2*). The proline bracket method successfully identified a fibrin polymerization site on the Aα-chain of fibrinogen (*3*). A synthetic peptide, PSP-9E9 (*P*olymerization *S*ite *P*eptide based on the identified site in the epitope recognized by 9E9 monoclonal antibody), binds to fibrin monomers but not to fibrinogen, and interferes allosterically with fibrin polymerization. The method has also been applied to a new class of serine proteinase inhibitor isolated from mustard seed (*17*), and led to identification of a reactive site that acts like a substrate analogue inhibitor.[3] We have also identified the functional site and developed an analgesic peptide based on the structure of another neurotoxin-cardiotoxin structure, and an antiplatelet peptide based on a phospholipase A_2 structure.[4]

The rationale for the present study was to further test the proline bracket method using snake venom proteins of the neurotoxin-cardiotoxin type. We showed that the resultant peptide, L-calchin, does in fact block the L-type Ca^{2+} current and thereby reduces the developed pressure in the intact, perfused heart. In other studies,[4] we found that L-calchin inhibits K^+-induced contraction of smooth muscle. The sequence of the indicated region, PTAMWP, has been found to occur only in calciseptine, FS2, S_4C_8, and $C_{10}S_2C_2$ by a search using the nonredundant BLASTP database.

L-calchin is less potent than calciseptine. The peptide is likely to be flexible in solution, whereas segments of native proteins often are rigidly positioned by interactions with other residues. It is important, therefore, to consider whether the identified site in native calciseptine and FS2 is available for interaction with L-type Ca^{2+} channels. The 3-D structure of FS2 (*12*) shows that the identified site, residues 42−47, is located on the outer strand of loop III. This strand does not form hydrogen bonds with the inner strand of loop III (*12*), unlike the situation for other neurotoxins (*18*, *19*). Thus the segment is flexible and exists in several conformations. The loop is terminated by a type VIa *cis*-proline turn (*20*) due to the presence of the sequence WPY (*21*). Further, residues 40−46 tilt the plane of loop III orthogonally to the plane of the molecule, making the segment fully exposed and available for interaction with the L-type Ca^{2+} channel protein. The three-dimensional structure of calciseptine has not yet been established. Given the nature and location of the two amino acid substitutions that distinguish FS2 and calciseptine, their tertiary structures are likely to be very similar. It is interesting that Albrand et al. (*12*) previously suggested that W46 in FS2 is a potential site for interaction since it is completely exposed to the solvent as a result of the local conformation. Similar arguments should apply to S_4C_8 and $C_{10}S_2C_2$, which have over 80% homology with calciseptine.

The functional site of erabutoxin, a short-chain neurotoxin, has recently been determined with binding studies to a nicotinic acetylcholine receptor, using several mutant forms of the toxin (*22*, *23*). This is a discontinuous site, with the important residues located in all three loops. The involved segments on two of the three loops of this toxin are flanked by proline residues; the segment in loop I is flanked by proline at residue 11, and the segment in loop III is flanked by prolines at residues 44 and 48. Loop II has no prolines in the vicinity of its interaction region. This example indicates that proline residues may also help in identification of discontinuous protein−protein interaction sites. The calciseptine site in loop III, PTAMWP, is its interaction site. The corresponding region in loop III of erabutoxin is PTVKP. In addition to the sequence differences, the positioning of

[3] R. M. Kini and H. J. Evans, unpublished observations.
[4] R. M. Kini et al., unpublished observations.

this segment is in the plane of the molecule in erabutoxin and other short-chain neurotoxins, whereas it is orthogonal to the plane of the molecule in calciseptine.

Thus, we have identified the functional site of calciseptine and FS2 using a new method involving the primary amino acid sequence and the presence of proline residues in the flanking segments of the interaction site (*3*). The synthetic peptide corresponding to this region, L-calchin, specifically inhibits the L-type Ca^{2+} channel by a simple voltage-independent blockade without affecting channel kinetics, and thereby reduces left ventricular developed pressure in the intact heart. These results strongly support that the predicted region indeed plays an important role in the interaction between the toxin and the L-type Ca^{2+} channel protein. Further studies are needed to determine whether any other part of the toxin molecule is involved in the interaction with the L-type Ca^{2+} channel protein. These studies, however, validate the prediction method and thus reinforce the importance of proline residues in the flanking segments of protein−protein interaction sites (*1*−*3*).

Our new method to identify protein−protein interaction sites is simple, straightforward, and fairly robust. We have used this method to identify new interaction sites in several structurally, functionally, and phylogenetically unrelated proteins (see above). This is the first method based on the recognition of importance of proline residues in the flanking segments of interaction sites (*1, 2*), rather than the interaction sites themselves. This method is fast requiring very little time to identify and test the predicted site(s) and hence should have considerable impact on the economics of research and development of novel bioactive peptides (for details, see refs *1*, *3*). Thus we believe that this new method should contribute significantly to protein chemistry, particularly for solving structure−function relationships of proteins.

REFERENCES

1. Kini, R. M., and Evans, H. J. (1994) in *Current Topics in Peptide and Protein Research* (Menon, J., Ed.) Vol. 1, pp 297−311, Council of Scientific Information, Trivandrum, India.
2. Kini, R. M., and Evans, H. J. (1995) *Biochem. Biophys. Res. Commun*. *212*, 1115−1124.
3. Kini, R. M., and Evans, H. J. (1996) *FEBS Lett*. *385*, 81−86.
4. de Weille, J. R., Schweitz, H., Maes, P., Tartar, A., and Lazdunski, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2437−2440.
5. Watanabe, T. X., Itahara, Y., Kuroda, H., Chen, Y. N., Kimura, T., and Sakakibara, S. (1995) *Jpn. J. Pharmacol. 68*, 305−313.
6. Yasuda, O., Morimoto, S., Chen, Y., Jiang, B., Kimura, T., Sakakibara, S., Koh, E., Fukuo, K., Kitano, S., and Ogihara, T. (1993) *Biochem. Biophys. Res. Commun. 194*, 587−594.
7. Strydom, D. J. (1977) *Eur. J. Biochem. 76*, 99−106.
8. Stewart, J. M., and Young, J. D. (1985) *Solid-Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co., Rockford, IL.
9. Kini, R. M., and Evans, H. J. (1995) *FEBS Lett. 375*, 15−17.
10. Dudley, S. C., and Baumgarten, C. M. (1993) *J. Gen. Physiol. 101*, 651−671.
11. Neher, E. (1992) *Methods Enzymol. 207*, 123−131.
12. Albrand, J. P., Blackledge, M. J., Pascaud, F., Hollecker, M., and Marion, D. (1995) *Biochemistry 34*, 5923−5937.
13. Jaseja, M., Lu, X., Williams, J. A., Sutcliff, M. J., Kakkar, V. V., Parslow, R. A., and Hyde, E. I. (1994) *Eur. J. Biochem. 226*, 861−868.
14. Labhart, A. M., Hunziker-Kwik, E. H., and Wuthrich, K. (1988) *Eur. J. Biochem. 177*, 295−305.
15. le Du, M. H., Marchot, P., Bougis, P. E., and Fontecilla-Camps, J. C. (1992) *J. Biol. Chem. 267*, 22122−22130.
16. Hanck, D. A., and Sheets, M. F. (1992) *Am. J. Physiol. 262*, H1197−H1207.
17. Menegatti, E., Tedeschi, G., Ronchi, S., Borlotti, F., Ascenzi, P., Thomas, R. N., Bolognesi, M., and Palmieri, S. (1992) *FEBS Lett. 301*, 10−14.
18. Brown, L. R., and Wuthrich, K. (1992) *J. Mol. Biol. 227*, 1118−1135.
19. Hatanaka, H., Oka, M., Kohda, D., Tate, S., Suda, A., Tamiya, N., and Inagaki, F. (1994) *J. Mol. Biol. 240*, 155−166.
20. Richardson, J. (1981) *Adv. Protein Chem. 34*, 167−330.
21. Yao, J., Feher, V. A., Espejo, B. F., Reymond, M. T., Wright, P. E., and Dyson, H. J. (1994) *J. Mol. Biol. 243*, 736−753.
22. Pillet, L., Tremeau, O., Ducancel, F., Drevet, P., Zinn-Justin, S., Pinkasfeld, S., Boulain, J. C., and Menez, A. (1993) *J. Biol. Chem. 268*, 909−916.
23. Tremeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J. C., and Menez, A. (1995) *J. Biol. Chem. 270*, 9362−9369.
24. Joubert, F. J., and Taljaard, N. (1980) *Int. J. Biochem. 12*, 567−574.
25. Joubert, F. J., and Taljaard, N. (1980) *Biochim. Biophys. Acta 623*, 449−456.
26. Williams, J. A., Lu, X., Rahman, S., Keating, C., and Kakkar, V. (1992) *Biochem. Soc. Trans. 21*, 73S.
27. Strydom, D. J. (1972) *J. Biol. Chem. 247*, 4029−4042.
28. Strydom, D. J. (1973) *Biochim. Biophys. Acta 328*, 491−509.
29. Joubert, F. J., and Taljaard, N. (1978) *S. Afr. J. Chem. 31*, 107−110.
30. Viljoen, C. C., and Botes, D. P. (1973) *J. Biol. Chem. 248*, 4915−4919.
31. McDowell, R. S., Dennis, M. S., Louie, A., Shuster, M., Mulkerrin, M. G., and Lazarus, R. A. (1992) *Biochemistry 31*, 4766−4772.
32. Joubert, F. J., and Taljaard, N. (1979) *Biochim. Biophys. Acta 579*, 228−233.

BI9802723