Neuronal calcium sensor proteins are direct targets of the insulinotropic agent repaglinide

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The NCS (neuronal calcium sensor) proteins, including neurocalcins, recoverins and visinin-like proteins are members of a family of Ca^{2+}-sensitive regulators, each with three Ca^{2+}-binding EF-hand motifs. In plants, lily CCaMK [chimaeric Ca^{2+}/CaM (calmodulin)-dependent protein kinase] and its PpCaMK (*Physcomitrella patens* CCaMK) homologue are characterized by a visinin-like domain with three EF-hands. In the present study, in an effort to discover NCS antagonists, we screened a total of 43 compounds using Ca^{2+}-dependent drug affinity chromatography and found that the insulinotropic agent repaglinide targets the NCS protein family. Repaglinide was found to bind to NCS proteins, but not to CaM or S100 proteins, in a Ca^{2+}-dependent manner. Furthermore, the drug antagonized the inhibitory action of recoverin in a rhodopsin kinase assay with IC_{50} values of 400 μM. Moreover, repaglinide tightly bound to the visinin-like domain of CCaMK and PpCaMK in a Ca^{2+}-dependent manner and antagonized the regulatory function of the domain with IC_{50} values of 55 and 4 μM for CCaMK and PpCaMK respectively. Although both repaglinide and a potent insulin secretagogue, namely glibenclamide, blocked K_{ATP} channels with similar potency, glibenclamide had no antagonizing effect on the Ca^{2+}-stimulated CCaMK and PpCaMK autophosphorylation, mediated by their visinin-like domain. In addition, a typical CaM antagonist, trifluoperazine, had no effect on the CCaMK and PpCaMK autophosphorylation. Repaglinide appears to be the first antagonist of NCS proteins and visinin-like domain-bearing enzymes. It may serve as a useful tool for evaluating the physiological functions of the NCS protein family. In addition, since repaglinide selectively targets NCS proteins among the EF-hand Ca^{2+}-binding proteins, it is a potential lead compound for the development of more potent NCS antagonists.

Key words: calcium-binding protein, EF-hand motif, visinin-like domain.

INTRODUCTION

A Ca^{2+} signal plays a pivotal role in regulating various cellular responses including cell metabolism, cytoskeletal dynamics, cell cycle, gene expression, neurotransmission and intracellular signal transduction processes [1,2]. The signal-induced change in intracellular free-Ca^{2+} concentration has been portrayed as a switch through a class of Ca^{2+}-binding proteins [3]. The NCS (neuronal calcium sensor) protein family includes more than 40 family members of intracellular Ca^{2+}-binding proteins that are primarily expressed in nerve cells [4]. According to their sequence similarities, they can be grouped into at least five different subfamilies [4]. NCS proteins bind Ca^{2+} with high affinity, i.e. above resting free-Ca^{2+} concentration, and undergo substantial conformational changes on binding, consistent with activity as Ca^{2+} switches [4,5]. Previous reports indicate that NCS proteins have pleiotropic biological actions such as rhodopsin kinase inhibition at high Ca^{2+} levels [6–8], K^+ channel modulation [9], Ca^{2+} channel modulation [10], transcriptional repression [11], presenilin interaction [12], phosphoinositide 4-kinase modulation [13], neurotransmitter release [14] and control of cyclic nucleotide metabolism [15].

Although the biochemistry and molecular biology of Ca^{2+}-binding proteins have progressed over the years, it has been difficult to understand their function in intact cells. For this reason, researchers have long sought the development of a specific antagonist for each of the Ca^{2+}-binding proteins that would permit the definitive determination of the physiological role of the individual Ca^{2+}-binding protein. CaM (calmodulin) antagonists, such as phenothiazines, W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide] [16] and W-66 {*N*-(2-aminoethyl)-*N*-[2-(4-chlorocinnamylamino)ethyl]-5-isoquinolinesulphonamide} [17] are often used as pharmacological tools to study the Ca^{2+}/CaM-dependent reactions.

In the present study, to elucidate intracellular Ca^{2+} sensors and in an effort to discover selective NCS protein antagonists, we have screened a total of 43 compounds using Ca^{2+}-dependent drug affinity chromatography. The present study describes the finding of a K_{ATP} channel inhibitor repaglinide, which interacts with NCS proteins, but not with CaM or S100 proteins, in a Ca^{2+}-dependent fashion. Repaglinide suppressed the Ca^{2+}-dependent inhibitory effects of recoverin on rhodopsin kinase activity. Moreover, repaglinide tightly bound to the visinin-like domain of chimaeric CaM kinases and effectively inhibited autophosphorylation of the enzymes. Repaglinide is the first drug to inhibit the function of NCS proteins. The availability of antagonists should permit understanding of the physiological role of this protein family in cellular responses.

MATERIALS AND METHODS

Materials

Repaglinide was a gift from Novo Nordisk Pharma (Bagsvaerd, Denmark). The chemical structure of repaglinide is presented

Abbreviations used: CaM, calmodulin; CaMKII, Ca^{2+}/CaM-dependent protein kinase II; CCaMK, chimaeric Ca^{2+}/CaM-dependent protein kinase; DMF, *N*,*N*′-dimethylformamide; EAH, epoxy-aminohexyl; NCS, neuronal calcium sensor; PpCaMK, *Physcomitrella patens* CCaMK; SUR, sulphonylurea receptor; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide; W-66, *N*-(2-aminoethyl)-*N*-[2-(4-chlorocinnamylamino)ethyl]-5-isoquinolinesulphonamide; WT, wild-type.

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in Figure 2(A). Tranilast was donated by Kissei Pharmaceutical (Nagano, Japan). Amlexanox was from Takeda Pharmaceutical (Osaka, Japan). FY-609 {3-[4-(8-fluoro-5,11-dihydrobenz-[b]oxepino[4,3-b]pyridin-11-ylidene)piperidino]propionic acid dihydrate} was from Fuji-yakuhin (Saitama, Japan). Olopatadine was from Kyowa Hakko Kogyo (Tokyo, Japan). Pranoprofen was from Yoshitomi Pharmaceutical (Osaka, Japan). Nateglinide was from Ajinomoto (Tokyo, Japan). AD-1590 {2-(8-methyl-10,11-oxodibenz[b,f]oxepin-2-yl)propionic acid} was from Dainippon Pharmaceutical (Osaka, Japan). Epinastine was from Nippon Boehringer Ingelheim (Hyogo, Japan). MY-1250 (5,6-dihydro-7,8-dimethyl-4,5-dioxo-4*H*-pyrano[3,2-c]quinoline-2-carboxylic acid) was from Mitsubishi Chemical (Tokyo, Japan). Glibenclamide and fluphenazine were purchased from Wako (Tokyo, Japan). Trifluoperazine was from Alexis (Lausen, Switzerland). W-7 was from Calbiochem (San Diego, CA, U.S.A.). Disodium chromoglycate, hydroxyzine and propranolol were purchased from Sigma. Compounds 2–23 and 26–31 (Figure 1) were custom-synthesized in our laboratory. Epoxy-activated Sepharose 6B and EAH (epoxy-aminohexyl)–Sepharose 4B were purchased from Amersham Biosciences.

Immobilization of the drugs to matrices for drug affinity chromatography

Coupling of the compounds containing carboxy group (compounds 1–34, 38, 40, 42 and 43; Figure 1) to EAH–Sepharose was performed as follows. The resin (0.5 ml) was washed three times with 10 ml of DMF (*N*,*N*′-dimethylformamide) by decantation. The compound (50 mg) dissolved in 1 ml of DMF was added to the gel. *N*-ethyl-*N*′-(3-dimethy aminoproply)carbodiimide hydrochloride (100 mg), suspended in 5 ml DMF, was added to the mixture and the pH of the mixture was adjusted to 5.0. After incubation with gentle shaking for 24 h at 25 °C, the pH of the mixture was readjusted to 5.0 and shaken further for 24 h. The resin was washed with DMF and distilled water and then incubated with 0.2 M sodium acetate (2 ml) and acetic anhydride (1 ml) for 30 min at 0 °C and incubated further for the same duration of time at 25 °C. The resin was then washed successively with water, 0.1 M NaOH and water, and equilibrated with 20 mM Tris/HCl (pH 7.5), containing 0.5 mM $CaCl_2$ (equilibration buffer). The compounds containing primary amine group (50 mg) (compounds 35–37, 39 and 41; Figure 1) were coupled with epoxy-activated Sepharose 6B (0.5 ml) according to the manufacturer's instruction.

Preparation of bovine tissue extracts

Bovine brain and retina were homogenized in 5 vol. of 20 mM Tris/HCl and 0.1 mM EGTA (pH 7.5). The homogenate was centrifuged at 15000 ***g*** for 30 min at 4 °C, and the supernatant fluid was adjusted to a $CaCl_2$ concentration of 0.5 mM, followed by centrifugation at 15000 ***g*** for 30 min at 4 °C. The supernatant was then loaded on to the drug-coupled columns.

Drug screening using calcium-dependent affinity chromatography

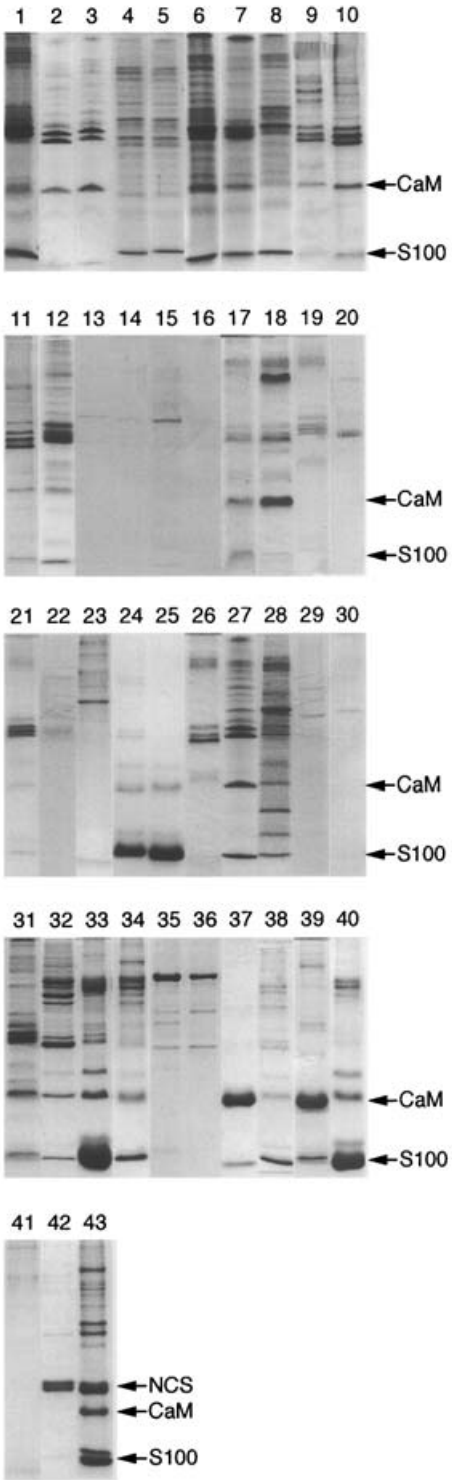
The bovine brain extract obtained above (each 2 ml) was applied to the miniaturized drug affinity columns (0.5 ml bed volume), equilibrated with the equilibration buffer. After the columns were washed with 5 ml of 20 mM Tris/HCl, 0.2 mM $CaCl_2$ (pH 7.5; washing buffer), proteins were eluted with 20 mM Tris/HCl (pH 7.5) containing 4 mM EGTA. The eluted proteins were resolved on 12 % (w/v) Tricine/SDS/PAGE and visualized by staining with Coomassie Brilliant Blue. For large-scale purification of NCS proteins, bovine brain and retina extracts (each 20 ml) were prepared and applied to repaglinide-coupled Sepharose (5 ml bed volume) as described above. After washing the column, NCS proteins were obtained by eluting the column with 40 ml of 20 mM Tris/HCl/4 mM EGTA (pH 7.5) and 40 ml of 20 mM Tris/HCl/4 mM EGTA/2 M NaCl (pH 7.5).

Protein sequencing

Protein samples were subjected to 12 % Tricine/SDS/PAGE and transferred to PVDF membranes. After staining with Ponceau S, the protein bands were cut out and digested with lysylendopeptidase. Following overnight incubation at 37 °C, the proteolytic fragments were separated by HPLC (model LC10A; Shimadzu Kyoto, Japan) with a C_{18} reversed-phase column (TSKgel ODS-80; Tosoh, Tokyo, Japan) with a linear gradient of 0–80 % (v/v) acetonitrile in the presence of 0.1 % (v/v) trifluoroacetic acid at a flow rate of 1 ml/min. The amino acid sequence of each proteolytic fragment was determined with an automated protein sequencer (model 476A; Applied Biosystems, Tokyo, Japan).

Construction of neurocalcins, CCaMK (chimaeric Ca^{2+}/CaM-dependent protein kinase), PpCaMK (*Physcomitrella patens* CCaMK) and deletion mutants of CCaMK and PpCaMK

Neurocalcin α (GenBank® accession no. AB006006) and neurocalcin δ (GenBank® accession no. D10884) were prepared by PCR with bovine brain cDNA library (ClonTech, Palo Alto, CA, U.S.A.) as template and the following specific primers: neurocalcin α, 5′-CATATGGGGAAACAGAATAGCAAACTG-GCC-3′ and 5′-GGATCCTCATTTCTGAATGTCGCACTGCA-G-3′; and neurocalcin δ, 5′-CATATGGGAAAACAGAACAGC-AAGCTGCGC-3′ and 5′-GGATCCTCAGAACTGGCCGGCG-CTGCTGGGGTC-3′. Lily CCaMK cDNA (GenBank® accession no. AAC49008) was prepared by PCR with the lily cDNA library made from developing anthers (kindly provided by Dr Gynheung An, Department of Life Science, Pohang University of Science and Technology, Pohang, South Korea) as template and the specific primers, namely 5′-CATATGTCGAGGCATGAGAGC-AGAAAGCTC-3′ and 5′-GGATCCCTATATCGTTCGCAGCG-AAGATAG-3′. The moss *P. patens* was provided by Dr Mitsuyasu Hasebe (National Institute of Basic Biology, Okazaki, Japan). For isolation of PpCaMK cDNA (GenBank® accession no. AY155462), RT–PCR was performed using RNA isolated from *P. patens* protonemata and degenerate primers 5′-CAYCGYGA-YCTNAARCCNGARAA-3′ (cam-1) and 5′-AGYTTNCGNCG-NGCRTTRAA-3′ (cam-2) corresponding to the conserved amino acids HRDLKPEN and FNARRKL respectively. The amplified PCR product was used as a template for further amplification with 5′-CTNAARATHATGGAYTTYGG-3′ (cam-3) corresponding to amino acids LKIMDFG and cam-2, and the amplified fragment was cloned and sequenced. The amplified 437 bp DNA fragment that was found to have high similarity to lily CCaMK was used as a probe for screening of the λ ZAPII cDNA library of the *P. patens* protonemata to obtain the PpCaMK cDNA clone with an entire coding region. The specific primers, 5′-CATATGA-GTGATCCGTATGGGAGGAGATTG-3′ and 5′-GGATCCTCA-CTGGACC TGACCCTCCAGACG-3′ were used for amplification of the PpCaMK coding region. The mutants CCaMK 341–520 and PpCaMK 326–504 were generated by using the



#	Compound
1	Tranilast
2	N-(3',4'-dimethoxycinnamoyl)m-aminobenzoic acid
3	N-(3',4'-dimethoxycinnamoyl)p-aminobenzoic acid
4	N-acethylanthranilic acid
5	N-benzoylanthranilic acid
6	N-cinnamoylanthranilic acid
7	N-(3',4',5'-trimethoxybenzoyl)anthranilic acid
8	N-(3',4',5'-trimethoxycinnamoyl)anthranilic acid
9	O-(3',4'-dimethoxycinnamoyl)salicylic acid
10	O-(3',4'-dimethoxycinnamoyl)-m-hydroxybenzoic acid
11	O-(3',4'-dimethoxycinnamoyl)-p-hydroxybenzoic acid
12	O-acethylsalicylic acid
13	O-benzoylsalicylic acid
14	O-cinnamoylsalicylic acid
15	O-(3',4',5'-trimethoxybenzoyl)salicylic acid
16	O-(3',4',5'-trimethoxycinnamoyl)salicylic acid
17	benzoic acid
18	cinnamic acid
19	dihydrocinnamic acid
20	3,4,5-trimethoxybenzoic acid
21	p-methoxycinnamic acid
22	3,4-dimethoxycinnamic acid
23	3,4,5-trimethoxycinnamic acid
24	Amlexanox
25	DSCG
26	chromone-2-carboxylic acid
27	chlorogenic acid
28	p-aminohippuric acid
29	N-(3',4'-dimethoxycinnamoyl)-2-aminonicotinic acid
30	dihydrocinnamoylanthranilic acid
31	cis-2-benzamidocyclohexane carboxylic acid
32	3-[4-(8-fluoro-5, 11-dihydrobenz[b] oxepino[4, 3-b]pyridin-11-ylidene) piperidino]propionic acid dihydrate (FY-609)
33	Olopatadine
34	2-[8-methyl-10,11-0xodibenz[b,f]oxepin-2-yl]propionic acid) (AD-1590)
35	Epinastine
36	Hydroxyzine
37	Fluphenazine
38	5,6-dihydro-7,8-dimethyl-4,5-dioxo-4H-pyrano [3,2-c]quinoline-2-carboxylic acid (MY-1250)
39	W-7
40	Propranolol
41	Pranoprofen
42	Repaglinide
43	Nateglinide

Figure 1 Drug screening assay using Ca^{2+}-dependent affinity chromatography

Tricine/SDS/PAGE (12 % gel) of the binding proteins obtained from various drug affinity columns using bovine brain extracts. SDS/PAGE shows 4 mM EGTA eluates (left panel). The names with arrows on the right show major Ca^{2+}-binding proteins of the drugs. The key to the drugs used is shown in the right panel. DSCG, disodium chromoglycate.

following specific primers: CCaMK 341–520, 5′-CATATGAAAGTTTTGTTGAGACAAAGAAA-3′ and 5′-GGATCCCTATATCGTTCGCAGCGAAGATAG-3′; and PpCaMK 326–504, 5′-CATATGAAGTTTCTGCTGCGCACCAG-3′ and 5′-GGATCCTCACTGGACCTGACCCTCCATACG-3′. The PCR product was subcloned into pT7blue T-vector (Novagen, Madison, WI,

U.S.A.) and sequenced using Applied Biosystems 377 DNA Sequencer. *Nde*I–*Bam*HI fragments of neurocalcins, CCaMK and PpCaMK were inserted into the corresponding sites of pET-11a (Novagen). *Nde*I–*Bam*HI fragments of the mutants CCaMK 341–520 and PpCaMK 326–504 were inserted into the corresponding sites of pET-16b (Novagen).

Expression and purification of neurocalcins, CCaMK, PpCaMK and deletion mutants of CCaMK and PpCaMK

The expression vector was introduced into *Escherichia coli* BL21 (DE3). Bacteria were grown at 37 °C in Luria–Bertani medium supplemented with ampicillin (100 mg/l). The proteins were induced by adding 1 mM isopropyl β-D-thiogalactoside when D_{600} reached 1. Cells were collected by centrifugation for 5 h after induction and the proteins were then extracted. Neurocalcin α and δ proteins were purified by ion-exchange chromatography on Q-Sepharose as described previously [18]. CCaMK and PpCaMK proteins were purified by using a CaM–Sepharose 4B (Amersham Biosciences) as described previously [19]. CCaMK 341–520 and PpCaMK 326–504 proteins were purified using Ni^{2+}-resin column (Novagen) according to the manufacturer's instructions. The quality of the purified proteins was checked by SDS/PAGE [12 % (w/v) gel].

Analytical binding assays of neurocalcins, CCaMK, PpCaMK, CCaMK 341–520 and PpCaMK 326–504 to repaglinide–Sepharose

The assays were performed in 150 μl of 20 mM Tris/HCl containing 2 mM $CaCl_2$ (pH 7.5), 25 μg expressed protein and 50 μl of repaglinide-coupled Sepharose. The reaction was incubated at 4 °C for 4 h. The Sepharose beads were collected and washed three times in 1 ml of 20 mM Tris/HCl containing 0.2 mM $CaCl_2$ (pH 7.5). Ca^{2+}-dependent bound proteins were eluted by 20 mM Tris/HCl containing 4 mM EGTA (pH 7.5). The eluted proteins were subjected to SDS/PAGE (12 % gel). In some cases (Figures 6B and 7D), the binding assay was performed in the presence of 2 mM $CaCl_2$ or 2 mM EGTA. The beads were collected and washed three times in 1 ml of 20 mM Tris/HCl containing 2 mM $CaCl_2$ or 2 mM EGTA (pH 7.5). The bound proteins were eluted by SDS sample buffer.

Western blotting

Repaglinide-binding proteins from bovine retina were resolved on SDS/PAGE and then electrophoretically transferred on to a nitrocellulose membrane (Amersham Biosciences). Western blotting was performed using anti-recoverin antibody (1/1000) and horseradish peroxidase-conjugated secondary antibody (1/2000). The signal was detected using a chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA, U.S.A.).

Protein kinase assay

Rat CaMKII (Ca^{2+}/CaM-dependent protein kinase II) holoenzyme was purified from rat forebrain as described previously [20]. The purity of the final preparation was > 95 %. CaMKII (12.5 nM) was incubated with 50 mM Hepes, 10 mM $Mg(Ac)_2$, 1 mM dithiothreitol, 40 μM syntide-2, 2 mM $CaCl_2$ and 1.5 μg CaM (pH 7.5) in the presence of the respective drug. The reaction was initiated by the addition of 100 μM [γ-^{32}P]ATP (6500 c.p.m./pmol; Amersham Biosciences) and terminated by spotting aliquots (15 μl) on to phosphocellulose paper (P-81; Whatman, Maidstone, Kent, U.K.) followed by washing in 75 mM phosphoric acid. Phosphate incorporation into syntide-2 was quantified by liquid-scintillation counting of the filters. CaMKII activity was measured for 5 min at 30 °C under linear conditions.

Rhodopsin phosphorylation assay

Rhodopsin phosphorylation assay was performed as described previously [21]. Briefly, S-modulin, a frog homologue of bovine recoverin, was mixed with frog rod outer segment membranes under complete darkness in a potassium gluconate buffer [115 mM potassium gluconate/2.5 mM KCl/2 mM $MgCl_2$/0.3 mM $CaCl_2$/0.2 mM EGTA/10 mM Hepes (pH 7.5)]. After bleaching of rhodopsin, the phosphorylation reaction was started by mixing 15 μl of the mixture with 10 μl of potassium gluconate buffer, containing 0.25 mM [γ-^{32}P]ATP (24 MBq/μmol) and 1.25 mM GTP. The resultant reaction mixture contained 10 μM rhodopsin, 5 μM expressed protein, 0.1 mM ATP, 0.5 mM GTP and 10 % (v/v) DMSO with or without the respective drug. After 2 min of incubation at room temperature (25 °C), the reaction was terminated by adding 150 μl of 10 % (w/v) trichloroacetic acid. After centrifugation (10000 ***g***, 5 min), the precipitate was washed with 500 μl of potassium gluconate buffer and subjected to SDS/PAGE. The amount of ^{32}P incorporated into rhodopsin was quantified by using an image analyser (BAS 2000; Fuji Film, Tokyo, Japan).

Autophosphorylation assay

Autophosphorylation assays of CCaMK and PpCaMK (0.4 μM each) were performed for 30 min at 30 °C in the presence of 50 mM Hepes, containing 10 mM $Mg(Ac)_2$, 1 mM dithiothreitol, 10 % DMSO, 100 μM [γ-^{32}P]ATP (6500 c.p.m./pmol; pH 7.5) and either 2.5 mM EGTA or 0.5 mM $CaCl_2$, at various concentrations of repaglinide and trifluoperazine. Aliquots (10 μl) were transferred to SDS sample buffer to stop the reaction and then analysed by SDS/PAGE (12 % gel). The gels were dried and subjected to autoradiography. The amounts of ^{32}P incorporated into CCaMK and PpCaMK were quantified by using an image analyser (LAS-1000 plus; Fuji Film).

RESULTS

Drug screening and Ca^{2+}-dependent affinity chromatography of NCS proteins

EF-hand Ca^{2+}-binding proteins, such as CaM and S100 proteins, interact with CaM antagonists in a Ca^{2+}-dependent manner [16,17]. Although the currently available CaM antagonists tend to be relatively non-selective [22], they are useful probes to study the relationships between structure and function in the CaM and S100 protein family [16,23]. Therefore we created affinity matrices by directly linking a total of 43 drugs to Sepharose to develop a Ca^{2+}-dependent affinity chromatography of NCS proteins. Compounds 35–37, 39 and 41 were immobilized to epoxy-Sepharose via their primary amine group, whereas compounds 1–34, 38, 40, 42 and 43 were immobilized to EAH–Sepharose via their carboxy group (Figure 1A). After the application of bovine brain extracts in the presence of Ca^{2+}, we identified many proteins with molecular masses ranging from 10 to 100 kDa that eluted from these matrices with excess of EGTA. A selective interaction between a Ca^{2+}-binding protein (i.e. CaM, S100 proteins or NCS proteins) and a drug can occur with only

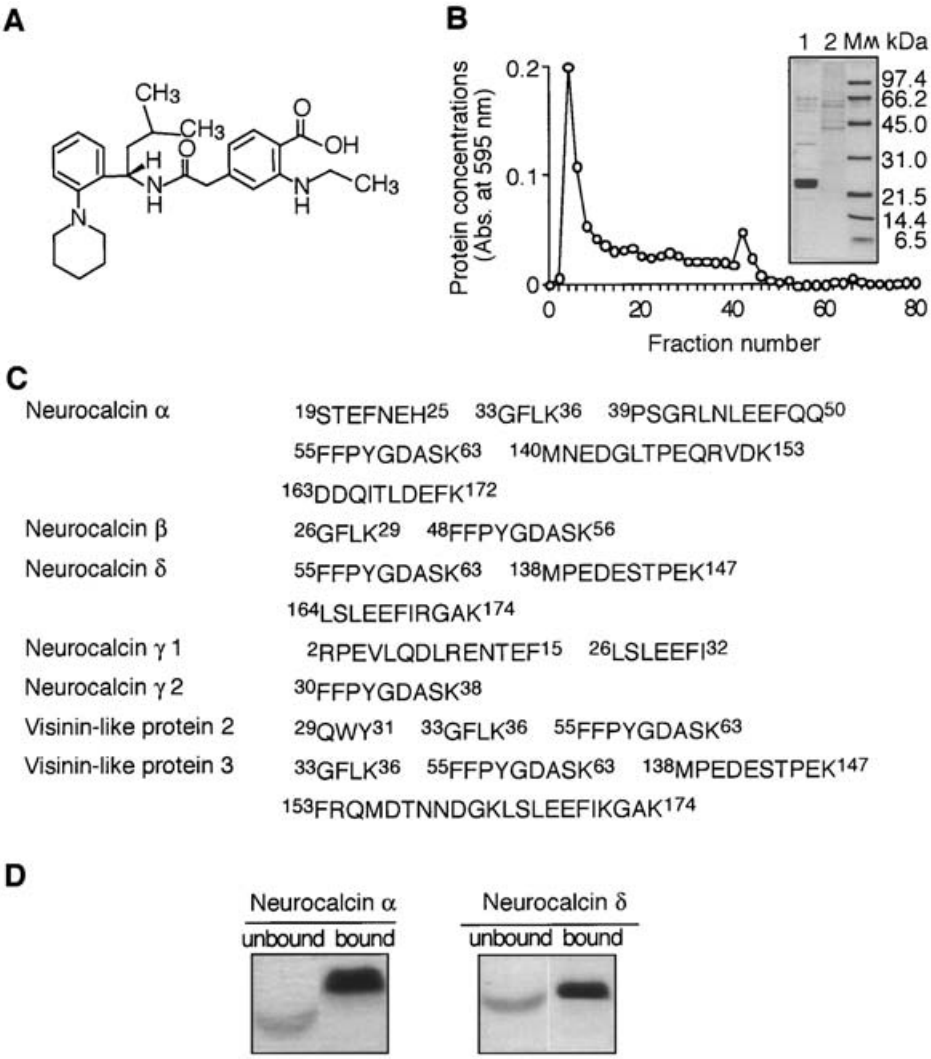


Figure 2 Identification and sequencing of repaglinide-binding proteins and binding assay of recombinant neurocalcin proteins

(**A**) Chemical structure of repaglinide. (**B**) Elution profile and SDS/PAGE of repaglinide-binding proteins obtained on repaglinide affinity chromatography (5 ml bed volume) using 20 ml of bovine brain extract. The eluate (5 μl) was subjected to SDS/PAGE; 4 mM EGTA eluates (lane 1) and 2 M NaCl eluates (lane 2). Molecular masses (Mм) are shown in kDa. (**C**) The amino acid sequences of the proteolytic fragments of repaglinide-binding proteins from repaglinide affinity chromatography using bovine brain extract. Repaglinide-binding proteins were proteolysed by lysylendopeptidase. (**D**) Purified recombinant neurocalcins α and δ binding to repaglinide–Sepharose. Purified recombinant neurocalcin α (25 μg; left panel) and neurocalcin δ (25 μg; right panel) were incubated with repaglinide–Sepharose (50 μl) in the presence of 2 mM $CaCl_2$ in a total volume of 150 μl. After the resins were washed with 20 mM Tris/HCl and 0.2 mM $CaCl_2$ (pH 7.5), the bound proteins were eluted with 100 μl of 20 mM Tris/HCl and 4 mM EGTA (pH 7.5). The unbound and bound fractions (each 5 μl) were subjected to SDS/PAGE.

several drugs, and the strengths of the interactions vary for each drug (Figure 1). The 10 kDa protein was selectively eluted from the amlexanox, disodium chromoglycate, olopatadine and propranolol columns with 4 mM EGTA, whereas the 17 kDa protein was eluted from cinnamic acid, fluphenazine and W-7 columns. The 10 and 17 kDa proteins were sequenced and identified as a mixture of S100A1 and B and CaM respectively (results not shown). The most selective drug will be the one that binds the NCS proteins well while binding only a relatively small proportion of other proteins applied. The EGTA eluate from the repaglinide–Sepharose column showed a doublet protein band at 22 kDa on SDS/PAGE (Figure 1B, compound 42; Figure 2B, lane 1), whereas small amounts of 65 and 42 kDa proteins were detected in the NaCl eluate (Figure 2B, lane 2). These observations indicated that the 22 kDa doublet proteins were specifically bound to repaglinide in a Ca^{2+}-dependent manner. To identify the 22 kDa repaglinide-binding proteins, amino acid sequencing of the proteins was performed. Using the SwissProt database, the sequences of the protein were compared with those of bovine neurocalcins and visinin-like proteins (Figure 2C). Partial amino acid sequence analysis showed that the 22 kDa brain repaglinide-binding proteins had 100% homology with neurocalcins and visinin-like proteins.

To exclude the possibility that these interactions were indirect or non-specific, we performed a binding assay *in vitro* using recombinant neurocalcins α and δ to confirm the above results. *E. coli*-expressed recombinant neurocalcins α and δ strongly bound to the repaglinide affinity matrix in the presence of Ca^{2+} and was eluted by excess of EGTA (Figure 2D). The electrophoretic mobilities of unbound neurocalcins α and δ (i.e. in the presence

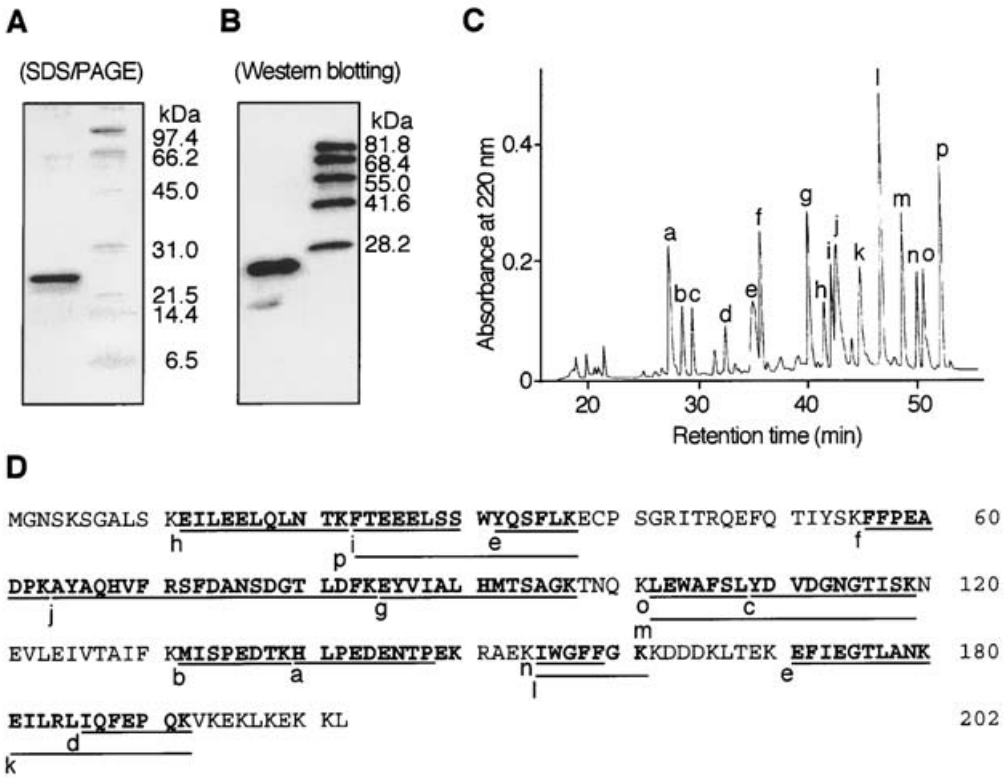


Figure 3 Identification of repaglinide-binding proteins from bovine retina extract

(**A**) SDS/PAGE of repaglinide-binding proteins obtained on repaglinide affinity chromatography (5 ml bed volume) using 20 ml of bovine retina extract. SDS/PAGE shows 4 mM EGTA eluate (5 μl). Molecular masses are indicated in kDa. (**B**) Western blotting of repaglinide-binding proteins obtained on repaglinide affinity chromatography. The proteins obtained on repaglinide affinity chromatography were subjected to SDS/PAGE and then transferred to a PVDF membrane. The transferred proteins were subjected to Western blotting by the anti-recoverin antibody as described in the Materials and methods section. The eluted proteins were immunoblotted with the polyclonal anti-recoverin antibody. Molecular masses are indicated in kDa. (**C**) Separation of the 22 kDa proteolysed protein obtained from repaglinide affinity chromatography by reversed-phase HPLC. The proteolytic fragments of the protein obtained from repaglinide affinity chromatography were isolated by a reversed-phase HPLC. The major peptides are identified by the labels a–p. (**D**) The amino acid sequence of recoverin. The sequences of the 16 peptides (a–p from **C**) are underlined.

of Ca^{2+}) were faster than those of bound fractions (i.e. in the presence of EGTA). This difference in mobility probably reflects a Ca^{2+}-induced conformational change of the protein [24]. Thus repaglinide directly targeted native and recombinant neurocalcins and visinin-like proteins.

Purification of repaglinide-binding protein from bovine retina

The recoverins, i.e. recoverin [25] and S-modulin [6], are the founder members of the family. They are specifically expressed in retinal rod and cone photoreceptor cells [5]. To know whether NCS family proteins other than neurocalcin bind to repaglinide, an extract of bovine retina (as a source of recoverins) was applied to the repaglinide-coupled affinity column and bound proteins were eluted as described in the Materials and methods section. The EGTA eluate of bovine retinal extract showed a single 22 kDa protein band on SDS/PAGE (Figure 3A). Western blotting using an anti-bovine recoverin showed that the retinal 22 kDa protein had reacted with the recoverin antibody (Figure 3B). To identify the retinal repaglinide-binding protein further, proteolytic fragments of the protein were separated by HPLC. Amino acid sequencing of the 22 kDa bovine retinal protein was 100% identical with bovine recoverin (Figures 3C and 3D). These results indicated that the 22 kDa repaglinide-binding protein from bovine retina was identified as recoverin. Although neurocalcins and recoverins belong to different subfamilies of NCS, repaglinide effectively binds to both classes of protein.

Repaglinide is not a CaM antagonist

Trifluoperazine and W-7, which are typical CaM antagonists, inhibit CaM kinases, myosin light-chain kinase and cyclic nucleotide phosphodiesterase by inhibiting activation of the enzymes by CaM [26]. To investigate the possible CaM antagonizing activity of repaglinide, the effect of the drug on Ca^{2+}/CaM-dependent activity of CaMKII was examined. As shown in Figure 4, repaglinide at concentrations of up to 800 μM had only a marginal effect on Ca^{2+}/CaM-dependent CaMKII activity, whereas trifluoperazine, a typical CaM antagonist, inhibited Ca^{2+}/CaM activation of the enzyme in the same concentration range and its IC_{50} value was 13 μM. Although some CaM antagonists can interact with NCS proteins [27], repaglinide was found to have no CaM-antagonizing activity and was also found to belong to a distinct class of EF-hand protein interacting compounds.

Effects of repaglinide on Ca^{2+}/recoverin-dependent suppression of rhodopsin kinase activity

To determine the role of repaglinide on NCS proteins, Ca^{2+}-dependent inhibition of the rhodopsin kinase by recoverin was selected as a typical drug-assessing system. Both recoverin

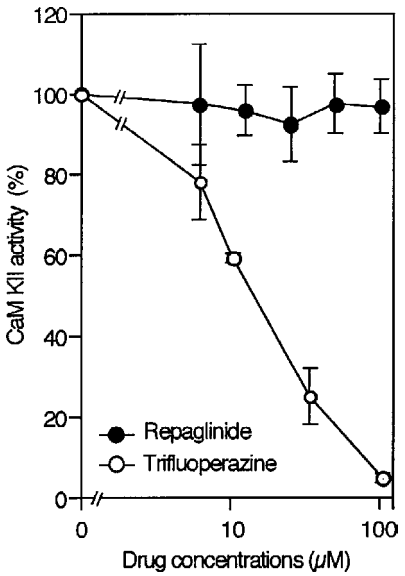


Figure 4 Effect of repaglinide on CaMKII activity

CaMKII activity was measured as described in the Materials and methods section and is represented as a percentage of the maximum CaMKII activity observed in the absence of repaglinide or trifluoperazine. Circles and bars represent means ± S.D. ($n = 3$).

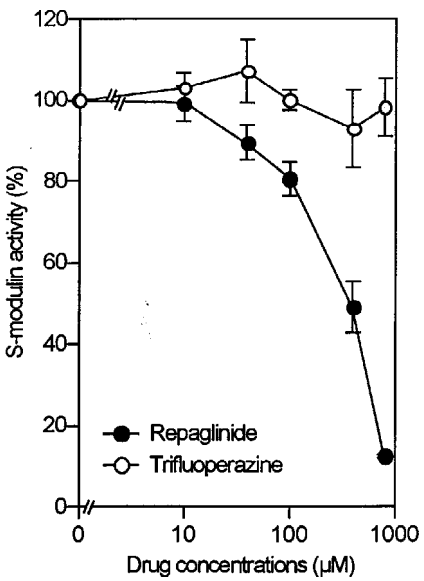


Figure 5 Effect of repaglinide on S-modulin activity

S-modulin activity represents incorporation of ^{32}P into rhodopsin at 100 μM Ca^{2+}. The results are shown as percentages of S-modulin activity in the absence of repaglinide or trifluoperazine. Circles and bars represent means ± S.D. ($n = 3$).

and S-modulin (frog homologue of recoverin) are 23 kDa EF-hand Ca^{2+}-binding proteins found in rod photoreceptors [6,25]. The Ca^{2+}-bound form of S-modulin binds to rhodopsin kinase and inhibits its activity [8]. As shown in Figure 5, repaglinide produced nearly 100 % inhibition of recoverin activity at 800 μM with the IC_{50} of approx. 400 μM, whereas trifluoperazine at concentration up to 800 μM did not inhibit recoverin activity at all.

Effects of repaglinide on Ca^{2+}-dependent autophosphorylation of CCaMK

The structural feature of CCaMK is characterized by a serine/threonine kinase domain, an autoinhibitory domain, a CaM-binding domain and a visinin-like domain [28]. The visinin-like domain at the C-terminal end makes this kinase unique among all the known CaM kinases. The amino acid sequence in the CaM-binding domain of CCaMK is similar to the CaM-binding region of CaMKII α. The visinin-like domain of CCaMK contains three conserved EF-hand motifs, similar to the NCS proteins such as frequenin, neurocalcin and hippocalcin [29]. CCaMK exhibits basal autophosphorylating activity stimulated severalfold by Ca^{2+}. Sathyanarayanan et al. [30] reported that Ca^{2+}-stimulated autophosphorylation, mediated by the visinin-like domain, significantly tightens the binding of CaM to CCaMK. A deletion mutant lacking the visinin-like domain did not show Ca^{2+}-dependent autophosphorylation [19]. The visinin-like domain of CCaMK could function as a Ca^{2+}-sensitive switch regulating the autophosphorylation. This was also indicated by observations of repaglinide having no effect on the kinase activity of CCaMK mutant lacking visinin-like domain (results not shown).

As shown in Figure 6(A), in the presence of EGTA, basal autophosphorylation was detected as a faint band on the autoradiograph and ^{32}P incorporation was greatly stimulated by 0.5 mM Ca^{2+}. Furthermore, CaM (1 μM) inhibited Ca^{2+}-dependent autophosphorylation to the basal level. Ca^{2+}-binding proteins of the EF-hand family are known to show differential electrophoretic mobilities in the presence or absence of Ca^{2+}. Figure 6(A) also shows that CCaMK migrates faster in the presence of Ca^{2+}, whereas in the presence of a Ca^{2+} chelator (EGTA) the enzyme migrates more slowly. To study repaglinide-binding properties of the visinin-like domain of CCaMK, recombinant visinin-like domain (CCaMK 341–520) was expressed in *E. coli* and purified to near homogeneity. Results of the repaglinide-binding assay showed that both CCaMK WT (wild-type) and the C-terminal visinin-like domain of CCaMK 341–520, bind to repaglinide–coupled Sepharose only in the presence of Ca^{2+} and was eluted by SDS sample buffer (Figure 6B). Next we examined the inhibitory effects of repaglinide on the CCaMK autophosphorylation, which is thought to be regulated by Ca^{2+} via the visinin-like domain of the enzyme. As shown in Figure 6(C), repaglinide inhibited the CCaMK autophosphorylation in a concentration-dependent manner with IC_{50} equal to 55 μM. In contrast, trifluoperazine had no effect on the CCaMK autophosphorylation. Interestingly, glibenclamide, which has similar mechanism of action on K_{ATP} channels and potency as repaglinide, had no effect on the CCaMK autophosphorylation (Figure 6C). These results demonstrate that repaglinide tightly binds to the visinin-like domain of CCaMK in a Ca^{2+}-dependent manner and effectively antagonizes the regulatory function of the domain.

Characterization of PpCaMK and effects of repaglinide on Ca^{2+}-stimulated autophosphorylation of PpCaMK

CCaMK has been found in many plant species, such as lily and tobacco [31]. We have cloned and characterized a novel CCaMK-like protein kinase gene termed as PpCaMK from *P. patens*. In Figure 7(A), nucleotide and amino acid sequences of the newly cloned PpCaMK are presented. Sequence comparisons revealed that lily CCaMK and PpCaMK share similar structures including the catalytic domain, CaM-binding domain and the visinin-like domain. PpCaMK and CCaMK share 56.4 % identity and 72 % similarity (Figure 7A). Homology in the visinin-like domain (54 % identity and 74 % similarity) indicates that the visinin-like

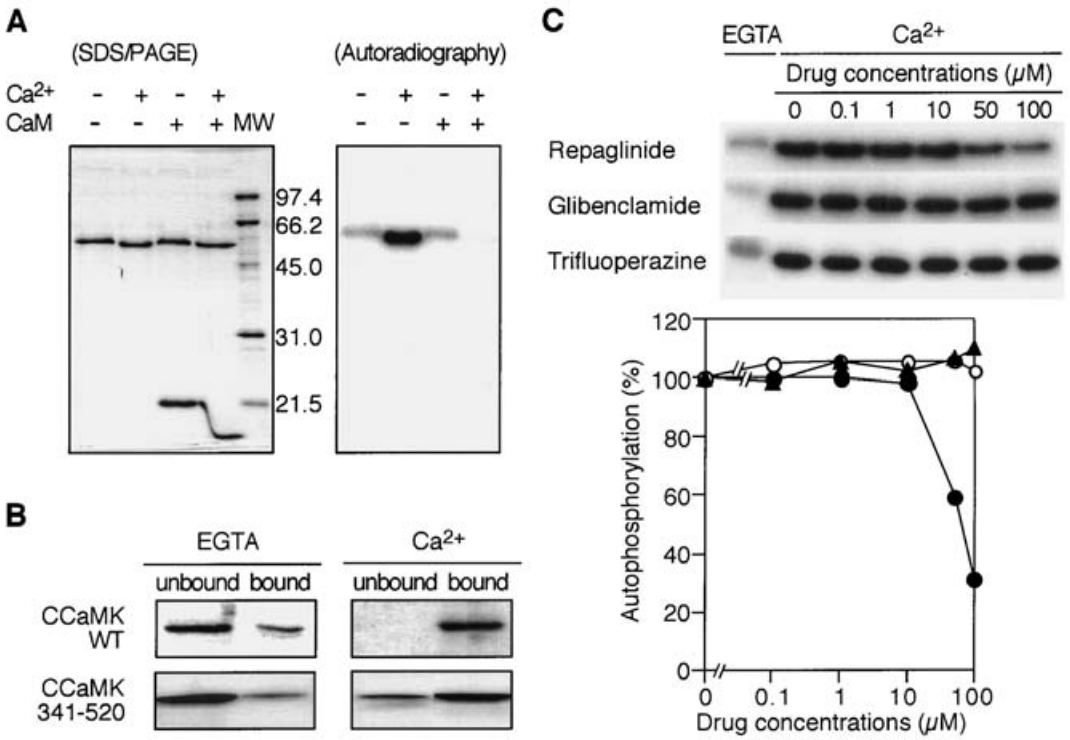


Figure 6 Effect of repaglinide on the binding and Ca^{2+}-stimulated autophosphorylation of CcaMK

(**A**) Ca^{2+}-dependent autophosphorylation of CCaMK. The purified proteins were analysed by SDS/PAGE (left panel) and autoradiography (right panel) in the absence of Ca^{2+} (2.5 mM EGTA), presence of Ca^{2+} (0.5 mM), absence of Ca^{2+} (2.5 mM EGTA) and presence of CaM (1 μM), presence of Ca^{2+} (0.5 mM) and CaM (1 μM). Molecular masses (MW) are given in kDa. (**B**) Binding of CCaMK WT and the 341–520 mutant to repaglinide–Sepharose in the presence of 2 mM $CaCl_2$ or 2 mM EGTA. (**C**) Effects of repaglinide, glibenclamide and trifluoperazine on Ca^{2+}-dependent autophosphorylation of CCaMK. CCaMK was autophosphorylated in the presence of $CaCl_2$ (0.5 mM) and at various concentrations (0–100 μM) of repaglinide (●), glibenclamide (○) and trifluoperazine (▲). After SDS/PAGE, incorporation of ^{32}P was detected by autoradiography. The amount of ^{32}P incorporated into CCaMK was quantified by densitometry.

domain has been conserved and may have a unique function in controlling CCaMK activity. Putative CaM-binding region of PpCaMK (ARRKFRATARASI, residues 310–322) has 76% similarity to the CaM-binding domain of lily CCaMK (ARRKLRAAAIASV, residues 326–338). The predicted structure of PpCaMK contains a catalytic domain followed by two regulatory domains, a CaM-binding domain and a visinin-like domain (Figure 7B). The *E. coli*-expressed PpCaMK protein bound to CaM (results not shown). Ca^{2+}-dependent mobility-shift assay on SDS/PAGE gels suggested that PpCaMK directly bound to Ca^{2+} (Figure 7C). In the presence of EGTA, PpCaMK exhibited basal autophosphorylation (i.e. in the presence of EGTA), which was greatly stimulated by Ca^{2+}. Furthermore, Ca^{2+}-dependent autophosphorylation of PpCaMK was inhibited to the basal level in the presence of 1 μM CaM (Figure 7C). These results suggest that PpCaMK is very similar to lily CCaMK in its structural components and in Ca^{2+}-dependent regulatory mechanisms. To identify the repaglinide-binding region of PpCaMK, a truncated mutant was prepared. The PpCaMK mutant 326–504 contained only a visinin-like domain and lacked the catalytic and CaM-binding domains. Both PpCaMK WT and a visinin-like domain of 326–504 mutant strongly bound to repaglinide in the presence of Ca^{2+} but not in the presence of EGTA (Figure 7D). Thus repaglinide bound to PpCaMK via its visinin-like domain in the presence of Ca^{2+}. This was also indicated by the observation of repaglinide having no effect on the kinase activity of PpCaMK mutant lacking a visinin-like domain (results not shown). Next, we examined the effects of repaglinide on Ca^{2+}-dependent autophosphorylation of PpCaMK. As shown in Figure 7(E), repaglinide inhibited Ca^{2+}-dependent autophosphorylation of PpCaMK in a concentration-dependent manner and 50% inhibition was attained at a concentration of 4 μM. Thus repaglinide was more effective in the inhibition of Ca^{2+}-dependent autophosphorylation of PpCaMK as compared with that of lily CCaMK. Both trifluoperazine and glibenclamide had no effect on the PpCaMK autophosphorylation (Figure 7E). The differential inhibition of CCaMK and PpCaMK may be due to their different primary structures in the visinin-like domain of the two enzymes.

DISCUSSION

Ca^{2+}-binding proteins frequently serve as modulator proteins to transduce Ca^{2+} signals into appropriate physiological functions, including neurotransmitter release, ion-channel modulation, cellular metabolism, cytoskeletal dynamics, cell death and cell cycle [1]. Ca^{2+} sensor proteins, like the ubiquitous Ca^{2+}-binding protein CaM, change their conformation on Ca^{2+} binding, thereby enabling their interaction with intracellular target proteins [32,33]. NCS proteins constitute a rapidly growing family of Ca^{2+}-binding proteins, which belong to the superfamily of EF-hand proteins and are expressed predominantly in retinal photoreceptors, neurons and neuroendocrine cells [4,5]. Recent work has demonstrated the role of NCS proteins in the regulation of neurotransmitter release, phototransduction, regulation of ion channels, control of gene expression, regulation of mRNAs' translation, protein kinase

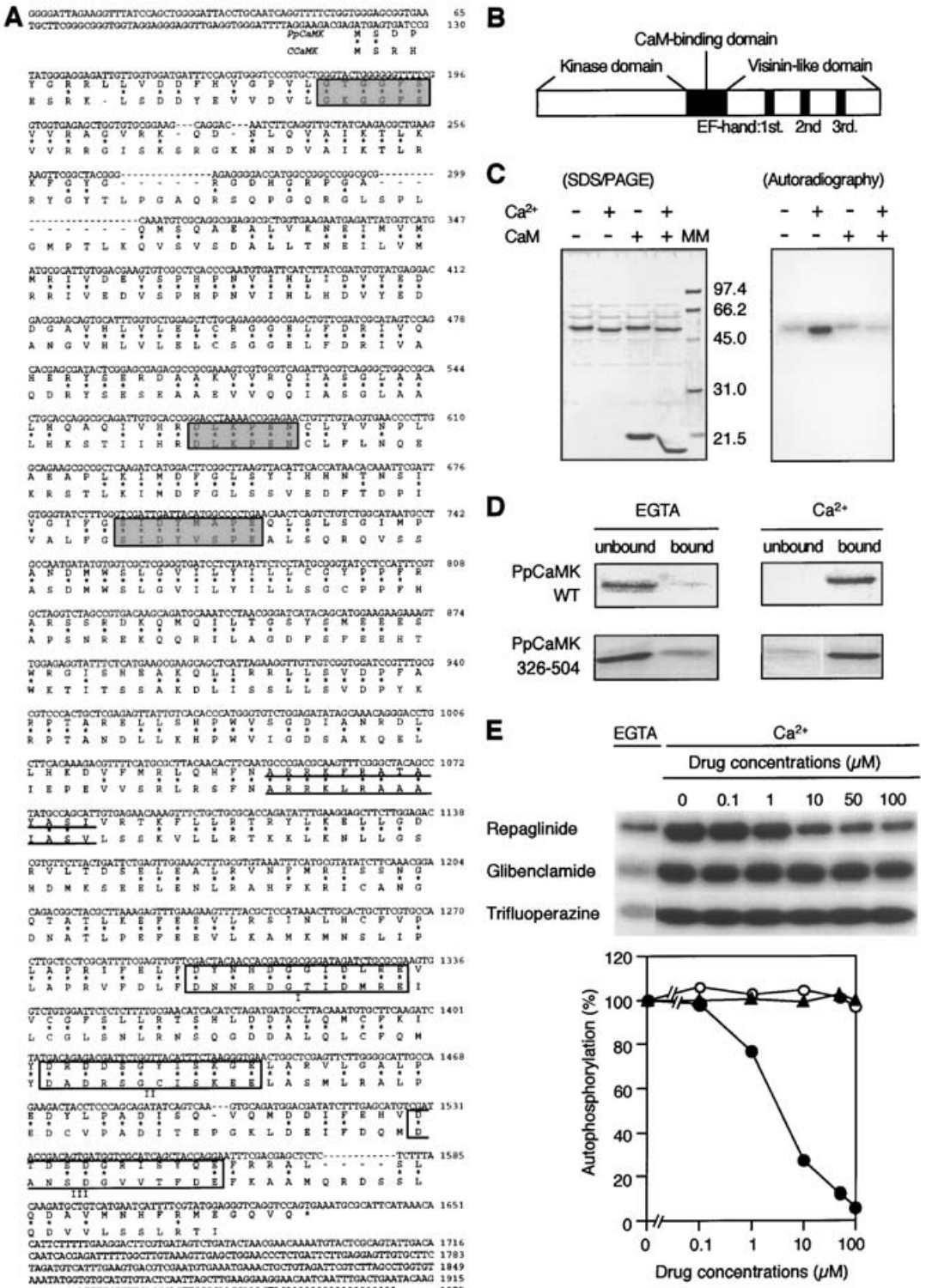


Figure 7 Characterization of PpCaMK and effect of repaglinide on the binding and Ca^{2+}-stimulated autophosphorylation of PpCaMK

(**A**) Nucleotide and deduced amino acid sequences of PpCaMK and comparison of deduced amino acid sequences of PpCaMK (*P. patens*) and CCaMK (lily). The nucleotide numbers are listed on the right. Diagnostic sequences of serine/threonine kinases are shown by shaded boxes. The CaM-binding domain is underlined, and the Ca^{2+}-binding EF-hand motifs (I–III) are boxed. Asterisks represent identical residues. (**B**) Schematic diagram of PpCaMK WT. (**C**) Ca^{2+}-dependent autophosphorylation of PpCaMK. The purified proteins were analysed by SDS/PAGE (left panel) and autoradiography (right panel) in the absence of Ca^{2+} (2.5 mM EGTA), presence of Ca^{2+} (0.5 mM), absence of Ca^{2+} (2.5 mM EGTA) and presence of CaM (1 μM), presence of Ca^{2+} (0.5 mM) and CaM (1 μM). Molecular masses (MM) are indicated in kDa. (**D**) The binding of PpCaMK WT and 326–504 mutant to repaglinide–Sepharose in the presence of 2 mM $CaCl_2$ or 2 mM EGTA. (**E**) Effects of repaglinide, glibenclamide and trifluoperazine on Ca^{2+}-dependent autophosphorylation of PpCaMK. PpCaMK was autophosphorylated in the presence of $CaCl_2$ (0.5 mM) and at various concentrations (0–100 μM) of repaglinide (●), glibenclamide (○) and trifluoperazine (▲). After SDS/PAGE, incorporation of ^{32}P was detected by autoradiography. The amount of ^{32}P incorporated into the PpCaMK was quantified by densitometry.

regulation and phosphoinositide metabolism [4,5]. NCS proteins are also likely to have other, as yet unknown functions. Since the physiological function of NCS proteins is still not fully understood, the development of the specific antagonists might open new lines of investigation on the role of NCS proteins *in vivo*.

In an effort to discover selective NCS antagonists, we have found a new carbamoylbenzoic acid derivative repaglinide which targets NCS proteins such as neurocalcins, recoverins and visinin-like domains of plant CCaMKs.

To screen NCS antagonists, we have used drug-Sepharose conjugates for the Ca^{2+}-dependent binding of the NCS proteins. Previous reports indicated that Ca^{2+}-binding proteins other than CaM interact with immobilized phenothiazine and W-7, typical CaM antagonists, in a Ca^{2+}-dependent manner [16,23]. These results suggest that EF-hand Ca^{2+}-binding proteins, such as CaM, troponin C and S100 proteins, contain similar structural domains for drug binding [23]. A direct comparison of the ability of EF-hand Ca^{2+}-binding proteins including NCS proteins to interact with the antagonist-Sepharose conjugates would facilitate interpretation of biological studies using drugs which inhibit the Ca^{2+}-dependent cellular processes. The results of such studies would also demonstrate the feasibility of using an immobilized drug for the rapid purification of NCS proteins. Results of the present study indicated that repaglinide bound to NCS proteins and effectively inhibited the biological functions of NCS proteins *in vitro*. In the present study, in contrast with immobilized phenothiazine (Figure 1, #37) [23] and W-7 (Figure 1, #39) [16], repaglinide–Sepharose interacted solely with NCS proteins, such as neurocalcins and recoverin, in a Ca^{2+}-dependent manner. Repaglinide antagonized the inhibitory activity of recoverin in rhodopsin kinase assay system. Since a high amount of recoverin (10 μM) was required to inhibit rhodopsin kinase activity, the IC_{50} value of repaglinide (400 μM) is relatively high. However, the molar ratio of recoverin/repaglinide is approx. 1:40 at this IC_{50} value. This molar ratio is quite reasonable, since several CaM antagonists, such as trifluoperazine and W-7 need a similar molar ratio (CaM versus CaM antagonist) to antagonize CaM functions *in vitro*. For example, in the case of W-7 and W-66 which are typical and moderately potent CaM antagonists, the molar ratio of CaM (0.2 μM)/W-7 ($IC_{50} = 15$ μM) is 1:75 and that of CaM (0.2 μM)/W-66 ($IC_{50} = 7.5$ μM) is 1:37.5 [17]. The visinin-like domain of CCaMK and PpCaMK acts as a Ca^{2+}-sensitive switch and the third EF-hands are important in regulating Ca^{2+}-stimulated autophosphorylation [28]. In the present study, the visinin-like domain of PpCaMK (residues 326–504) had 72% similarity to that of lily CCaMK (residues 340–520). Moreover, on assay of PpCaMK autophosphorylation, repaglinide was much more effective with the IC_{50} value of 4 μM.

Repaglinide represents a new class of insulin secretagogues, structurally unrelated to sulphonylureas, but sharing their primary mechanism, that was developed for the treatment of type 2 diabetes [34]. Repaglinide has been reported to be a potent insulinotropic agent with an IC_{50} of approx. 10 nM for closing K_{ATP} channels [35]. However, in the present study, repaglinide concentrations between 4 and 400 μM were needed to antagonize effectively the effect of NCS proteins, lily CCaMK and PpCaMK. The need for relatively high concentrations of repaglinide may limit the usefulness of the drug in evaluating the biological significance of the NCS protein family in intact cells. Dabrowski et al. [36] demonstrated that both repaglinide and glibenclamide block recombinant Kir6.2/SUR1 (where SUR represents sulphonylurea receptor), Kir6.2/SUR2A and Kir6.2/SUR2B channels with similar potency by binding to a site that is located on the SUR subunit. The IC_{50} values for K_{ATP} channel blocking effect were approx. 10 nM for both repaglinide and glibenclamide [36]. However, in the present study, glibenclamide had no antagonizing effect on CCaMK and PpCaMK autophosphorylations via their visinin-like domain. Thus glibenclamide appears to be a suitable negative control compound for physiological studies concerning the involvement of NCS proteins in intracellular Ca^{2+} signalling pathways. In addition, since repaglinide selectively targets NCS proteins among the EF-hand Ca^{2+}-binding proteins, it is a potential lead compound for the development of more potent NCS antagonists.

In conclusion, the present study shows for the first time that repaglinide is an antagonist of the NCS family proteins and of the visinin-like-domain-bearing plant protein kinases. Repaglinide may serve as a useful pharmacological tool for elucidating the roles of the NCS protein family.

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