The Calbindin-D_{28k} binding site on inositol monophosphatase may allow inhibition independent of the lithium site of action

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Running title: Inhibiting calbindin-activated IMPase

Abstract

Among numerous reported biochemical effects the lithium-inhibitable enzyme inositol-monophosphatase (IMPase) remains a viable target for lithium's therapeutic mechanism of action. Calbindin-D_{28}k (calbindin) interacts with IMPase enhancing its activity. In the present study *in silico* modeling of IMPase-calbindin binding using the program MolFit indicated that the 55-66 amino acid segment of IMPase anchors calbindin via Lys59 and Lys61 with a glutamate in between (Lys-Glu-Lys motif). The model further suggested that the Lys-Glu-Lys motif interacts with residues Asp24 and Asp26 of calbindin. Indeed, we found that differently from wildtype calbindin, IMPase was not activated by mutated calbindin in which Asp24 and Asp26 were replaced by alanine. Calbindin's effect was significantly reduced by a peptide with the sequence of amino acids 58-63 of IMPase (peptide 1) and by six amino-acid peptides including at least part of the Lys-Glu-Lys motif. The three amino-acid peptide Lys-Glu-Lys or five amino-acid peptides containing this motif were ineffective. Intracerebroventricular administration of peptide 1 resulted in a significant antidepressant-like reduced immobility in the Porsolt forced swim test (FST) compared with mice treated with a scrambled peptide or artificial cerebrospinal fluid. Based on the sequence of peptide 1, and to potentially increase the peptide's stability, cyclic and linear pre-cyclic analog peptides were synthesized. One cyclic and one linear pre-cyclic analog peptides exhibited an inhibitory effect on calbindin-activated brain IMPase activity *in vitro*. These findings may lead to the development of molecules capable of inhibiting IMPase activity at an alternative site than that of lithium.

Keywords: bipolar disorder; inositol monophosphatase; calbindin; lithium; protein-protein docking;

Introduction

Inositol monophosphatase (IMPase) remains a viable target for the therapeutic effect of lithium as a mood stabilizer (1, 2). IMPase has an important role in the phosphatidylinositol (PI) signaling system since it catalyzes the dephosphorylation of *myo*-inositol monophosphates to free *myo*-inositol (3). It has been shown that therapeutically-relevant lithium concentrations (0.5-1.5 mM) exert an uncompetitive inhibition on IMPase with a Ki of 0.1 to 1.0 mM, probably by binding to a Mg^{2+}-binding site of the enzyme (3-5). Reduced activity of IMPase may lead to depletion of intracellular free *myo*-inositol which is required for the re-synthesis of the signaling precursor PI and thus may dampen overactivity of this signaling cascade (6). This possibility is known as the inositol depletion hypothesis of the mechanism of lithium's mood stabilizing effect (7): While several studies question the relevance of this hypothesis (8) other results support it (1, 9, 10).

Based on lithium's inhibition of IMPase activity several pharmaceutical companies have tried to synthesize IMPase inhibitors such as bisphosphonates (11) and terpenoid and tropolone analogues (12) targeted at the substrate site of the enzyme. However, their further development to clinical mood stabilizers was limited for a variety of reasons (11).

Calbindin $D_{28}k$ (calbindin), a member of the vitamin-D-dependent calcium-binding proteins, was found to activate IMPase *in vitro* (13). It was shown that calbindin binds a specific amino-acid sequence of IMPase (residues 55-66) enhancing its activity by several fold (13). Calbindin is common in neurons of the hippocampus (14), temporal cortex (15), anterior cingulate cortex (16) and prefrontal cortex (17) constituting about 1% of total brain soluble protein (18). Calbindin shapes postsynaptic calcium signals (19-21), and is involved in synaptic plasticity (22). Schmidt et al (21) have shown *in situ* that calbindin's interaction with IMPase occurs in spines and dendrites but not in axons of cerebellar Purkinje neurons which depend on PI signaling for synaptic integration. The interaction is enhanced by synaptic activity and results in calbindin's immobilization by membrane- or cytoskeleton-bound IMPase (21). We have previously shown that addition of recombinant human calbindin increases IMPase activity of postmortem human prefrontal cortex crude homogenate by 3.5 fold (23). Given the fact that calbindin

is highly abundant in brain tissue it is conceivable to assume that *in-vivo,* brain IMPase is constitutively bound to calbindin and that this protein-protein interaction is involved in PI-mediated synapse function.

In the present study we explored the possibility to inhibit IMPase activity at the calbindin-IMPase interaction site using competitive peptides which would interfere with the activating interaction. Four six amino-acid linear peptides rationally designed based on *in silico* modeling of the interaction inhibited calbindin-activated brain IMPase activity *in vitro.* One of these peptides was tested *in vivo* in an animal model of depression, the Porsolt forced swim test (FST), and exhibited an antidepressant-like reduced immobility. Based on the backbone of this linear peptide, cyclic and linear pre-cyclic analog peptides were synthesized to potentially increase the peptide's stability. One cyclic peptide and one linear pre-cyclic peptide exhibited an inhibitory effect on calbindin-enhanced brain IMPase activity *in vitro*. These findings may lead to the development of molecules capable of inhibiting IMPase activity at an alternative site than that of lithium.

Materials and Methods

Modeling the structure of the complex between IMPase and calbindin

Figure 1, showing the three dimensional structure of IMPase and of the complex of IMPase with calbindin, was produced using the program PyMol (24). The crystal structure of IMPase (PDB entry 2bji) and the NMR structure of calbindin (PDB entry 2g9b) were used to model the structure of the complex using the protein-protein docking program MolFit (25). For a full and detailed description of the modeling process please refer to the Supplementary Information.

Peptides design based on the modeled complex

We used the resulting model as a basis for designing eleven short linear peptides (Table 1). Peptide 1, a 6-mer peptide, was composed of an identical sequence as residues 58-63 of IMPase (Ile-Lys-Glu-Lys-Tyr-Pro). These six amino acids are the central sequence of the previously reported 12 amino acid

segment (residues 55-66) of IMPase shown to interact with calbindin (18). Furthermore, this specific 6-mer peptide is fully conserved between mouse and human. This segment includes Lys59 and Lys61, separated by Glu60 designated the Lys-Glu-Lys motif. Additional designed three 6-mer peptides included at least part of the Lys-Glu-Lys motif (peptides 2-4). In peptide 2 we permutated Glu60 to alanine. Peptides 3 and 4 are similar to peptide 1 but in each peptide one of the Lys residues in the Lys-Glu-Lys motif was permutated to alanine. Four other 6-mer peptides included only one or none of the amino acids Lys or Glu of the Lys-Glu-Lys motif (peptides 5-8). In peptide 5 one of the lysine residues and Ile58 were permutated to histidine and serine, respectively, and the whole sequence has been scrambled. Peptide 6 was composed of the same sequence as peptide 1 but the two lysine residues were permutated into alanine. Peptide 7 was also composed of the same sequence as peptide 1 but the whole Lys-Glu-Lys motif has been permutated to three alanine residues (Ala-Ala-Ala). Peptide 8 was a random 6-mer peptide containing two lysine residues, albeit, separated by two, rather than a single residue, none of which was a glutamate. Two 5-mer peptides lacking the carboxyterminal proline included either the Lys-Glu-Lys motif (peptide 9) or Lys-Ala-Lys replacement (peptide 10). One 3-mer peptide was composed of the Lys-Glu-Lys motif only (peptide 11). The peptides were custom-synthesized by GeneScript Corporation, Scotch Plains, NJ (Table 1).

Mutated calbindin

Human recombinant calbindin was mutated at the Israel Structural Proteomics Center (ISPC), the Weizmann Institute of Science (Rehovot, Israel) to exchange residues Asp24 and Asp26 into alanine (calbindin D24A, D26A). Calbindin D24A, D26A was generated by site-directed mutagenesis based on the QuikChange site-directed mutagenesis protocol of Stratagene (La Jolla, CA). For a full and detailed description of the preparation of mutated calbindin please refer to the Supplementary Information.

IMPase activity

IMPase activity in mouse brain homogenates was measured as previously described (26). Inorganic phosphate liberated from inositol-1-phosphate was quantified spectrophotometrically in an ELISA

reader (iEMS, Labsystems, Helsinki, Finland) using the malachite green color reagent. To study the effect of the synthetic peptides on the enhancing interaction between calbindin and IMPase the reaction was carried out in the presence of 20 μM human recombinant calbindin (27) (produced by PPS, Rehovot, Israel, using the plasmid kindly donated by S. Linse, Lund, Sweden) and in the presence or absence of 10 μM of each of the peptides. In order to distinguish IMPase activity from non-specific phosphatases the reaction was carried out in the presence and absence of 30 mM LiCl. LiCl is a specific inhibitor of this enzyme (3) and at this concentration totally inhibits IMPase activity. The enzyme activity was calculated as the difference between the activity values in the absence and in the presence of LiCl. Values were normalized to the mean of the control values in each run.

Preparation of liposome-trapped peptides

Liposomes (Liposome kit: Lipid mixtures for the preparation of liposomes, Sigma, St. Louis, MO) were prepared according to the manufacturer's instructions with modification as follows. The reconstituted liposomes were diluted 1:5 with artificial CSF (aCSF, consisting of 145 mM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl and 1 mM $MgCl_2$) or one of the peptides dissolved in aCSF followed by a short sonication for five seconds using a Polytron cell homogenizer (Heat System Ultrasonic INC, Newtown, CT) at 4^{o}C and 50% power capacity.

Animals

Mice used for behavioral and pharmacological studies were housed and kept under 12:12 h light/dark cycles (lights on at 8:00 AM) in a room with constant temperature (22ºC) and food and water *ad libitum*. All experiments were conducted during the light phase of the light/dark cycle. Protocols of the experiments were approved by the Ben-Gurion University of the Negev (Beer-Sheva, Israel) committee for the ethical care and use of animals in research and conducted according to NIH guidelines.

Effect of specific peptides in the Porsolt forced swim test

Eight weeks old ICR male mice (Harlan, Jerusalem, Israel) were anesthetized with 20% isoflurane and a screw guide was implanted 0.2-0.3 mm posterior to the bregma 1 mm lateral to midline, above the lateral ventricle. The incision was closed and the mice were put back in their cage with 5% lidocaine administered locally and 1.25 g dipyrone/200 ml in the drinking water was given for analgesia. After a week of recovery, mice were anesthetized and injected through the guide screw with 5 µl of liposomes containing the test peptide (peptide 1 IKEKYP: 50 nmoles, n=13, peptide 5 SYEHKP: 50 nmoles, n=5) or aCSF as control (n=8). Three hours later mice were placed for 6 minutes in a glass cylinder (height 20 cm, diameter 12 cm) filled with water at a temperature of 24 ± 1ºC to a height of 13 cm (such that the mouse could not touch the bottom or climb out of the cylinder at any point). The session was digitally recorded for later analysis. Analysis of the digitally recorded behavior was done by an experimenter who was blind to a mouse treatment. The duration of immobility was monitored during the last 4 min of the 6-min test. Immobility period was defined as the time spent by the animal floating without struggling and making only those movements necessary to keep its head above the water. In addition, another group of mice received a single i.p. injection of 15 mg⁄kg imipramine hydrochloride (Sigma, St. Louis, MO) 30 min before the test and were used as a positive control of an antidepressant effect. After the test the screw location was confirmed by dye injection.

Synthesis and measurement of metabolic stability of cyclic and linear pre-cyclic analog peptides

Synthesis of cyclic and linear pre-cyclic analog peptides was based on the concept of backbone cyclization (28, 29). The metabolic stability of cyclic peptide 1 was measured using rat brush border membrane vesicles as previously described (30). For a full and detailed description of these methodologies please refer to the Supplementary Information.

Results

A modeled structure of the complex between IMPase and calbindin suggests that residues Lys59, Glu60 and Lys 61 of IMPase play a major role in the interaction

A plausible model of the interaction between IMPase and calbindin was obtained using the docking program MolFit followed by energy minimization. Residues 55-66 of IMPase comprise the segment which has been shown to interact with calbindin (13). In the docking procedure residues 55-66 of IMPase were placed in the interface between the two proteins. In contrast, no restraints were imposed on the interacting surface of calbindin. In the modeled complex depicted in Figure 1 optimal binding requirements were deduced. IMPase binds a groove formed by the N- and C-terminal domains of calbindin and interacts with the calcium binding loops. The 55-66 segment of IMPase anchors calbindin via two lysine residues, Lys59 and Lys61, whereas the residue in between, glutamate (Glu60), is hydrated. The modeled complex further indicates several possible hydrogen bonds between residues 55-66 of IMPase and calbindin. N_ξ (the side chain nitrogen atom) of Lys59 of IMPase is hydrogen bonded to calbindin via three oxygen atoms, the Oδ1 (a side chain oxygen atom) of Asp24, and the main chain carbonyl oxygen atoms of Asp24 and Asp26. The distances between N_ξ and each of Oδ1, the carbonyl oxygen of Asp24 and the carbonyl oxygen of Asp26 are 2.70Å, 2.74Å and 2.77Å, respectively. Additionally, N_ξ of Lys61 of IMPase is hydrogen bonded to calbindin via Oδ1 of Asn158 (2.85Å) and via Oδ1 of Asp155 (2.91Å); Nε2 (a nitrogen atom in the heterocyclic side chain) of His65 of IMPase is involved in a hydrogen bond to Oδ2 of Asp280 of calbindin.

Specific linear peptides inhibit calbindin-activated IMPase

The effect of specifically designed linear peptides was validated by *in vitro* IMPase activity measurements in the presence of calbindin. As shown in Figure 2 IMPase activity of mouse brain crude homogenate is increased by 1.9 ± 1.0 (S.E.M.) fold in the presence of 20 μM human recombinant calbindin. When 10 μM of peptide 1 were added to the reaction mixture the effect of calbindin on IMPase activity was strongly reduced. Peptides 2 through 4, which included the Lys-Glu-Lys motif or

part of it, inhibited the activating interaction similarly to peptide 1 (Figure 2A). Peptides 5 through 8, which included only one or none of the amino acids Lys or Glu, did not interfere with the enhancement of IMPase activity by calbindin (Figure 2B). Peptides 9 and 10, comprised of five amino acids and peptide 11 comprised of three amino acids which contained either an intact Lys-Glu-Lys motif or a Lys-Ala-Lys replacement did not interfere with the enhancement of IMPase activity by calbindin (Figure 2C). Our results suggest that peptides 1 through 4 but not peptides 5 through 11 contain the preferred residues that optimize the competition with IMPase on the interaction with calbindin.

The inhibitory effect of peptide 1 on calbindin-activated IMPase was also demonstrated with postmortem human brain crude homogenate. Human recombinant calbindin increased human brain IMPase activity by 3 fold and peptide 1 abolished this effect (data not shown).

To control for a possible nonspecific effect of linear short peptides on IMPase activity the enzyme's activity in the presence of peptide 1 (but in the absence of calbindin) was assessed. Peptide 1 did not affect the basal activity of IMPase. We further assessed possible protease activity in the homogenate and the possibility that prior interaction between IMPase and calbindin prevents interference by the active peptides. To this end we preincubated calbindin with the homogenate for 10 min prior to the addition of the substrate. No difference in IMPase activity was found in these two experiments (data not shown).

IMPase activity in mouse brain crude homogenate is not enhanced by mutated human recombinant calbindin

We examined whether a change in the interaction site of calbindin with IMPase will affect calbindin's activation of IMPase. Human recombinant calbindin was mutated so that two amino acids in its interaction site with IMPase, Asp24 and Asp26, were replaced by alanine. IMPase activity in mouse brain crude homogenate was measured in the presence of 20 μM mutated *vs.* wildtype calbindin. As shown in Figure 3, differently from wildtype calbindin, IMPase activity was not enhanced by 20 μM mutated calbindin [ANOVA $F_{2, 14} = 21.8$, $p<0.001$; post-hoc analysis: the activity in the presence of

wildtype calbindin was significantly different from both the control ($p<0.001$) and the activity in the presence of mutated calbindin ($p<0.001$)].

Specific cyclic and linear pre-cyclic analog peptides inhibit calbindin-activated IMPase

Out of the two cyclic and two linear pre-cyclic analog peptides (Table 1) cyclic 1 and linear pre-cyclic analog 1, both with m=4 and n=4 (Scheme 1) significantly reduced calbindin's activation of IMPase (Figure 4).

Enhanced metabolic stability of cyclic peptide 1

Comparison of the stability of cyclic peptide 1 with that of linear peptide 1 revealed that cyclic peptide 1 maintained 50% of its initial amount after 60 minutes of incubation with BBMVs while its linear analog was completely degraded after 5 minutes. The enhanced stability contributed by cyclization adds "drug like" properties required for further development for potential clinical utilization (30).

Peptide 1 but not peptide 5 exhibited an *in-vivo* antidepressant-like effect in the FST

We examined the behavioral effect of two linear peptides [(Peptide 1 (IKEKYP) derived from the 12 amino-acid sequence reported to interact with calbindin (13) and peptide 5 (SYEHKP) lacking the Lys-Glu-Lys motif] in the lithium-sensitive animal model of depression, the FST. Intracerebroventricular (ICV) administration of 50 nmoles of peptide 1 in liposomes to mice three hours prior to testing resulted in significantly less immobility compared with mice treated with either peptide 5 in liposomes or with aCSF in liposomes [mean immobility time: peptide 1, n=13, 40 sec ± 14 (SEM); peptide 5, n=5, 125 ± 38; aCSF, n=8, 94 ± 25; ANOVA, $F_{3, 27} = 4.5$, p=0.01, posthoc LSD test, peptide 1 *vs.* peptide 5, p=0.01; peptide 1 *vs.* aCSF, p=0.054]. As expected, imipramine-treated mice (positive control) were significantly less immobile compared with aCSF-treated or peptide 5-treated mice (n=5, 10 ± 7; p=0.02 and p=0.005, respectively) (Figure 5).

Discussion

IMPase, a critical enzyme for brain cell signaling (26: Atack, 1995 #624, 31), is activated by calbindin (13), a ubiquitous protein (32-34) constituting about 1% of brain protein content (35). Lithium, a mainstay treatment in bipolar disorder, inhibits IMPase at therapeutically-relevant concentrations. This inhibition is among the hypotheses of the molecular mechanism of lithium's therapeutic action (7). Since lithium has significant limitations and since repeated efforts by several drug companies to develop alternative IMPase inhibitors directed to the catalytic site of the enzyme were non-successful (11, 12, 31) we studied the possibility to inhibit IMPase activity by targeting another site, namely, the site through which it is being activated by calbindin. This was based on the assumption that *in-vivo*, in neurons of particular brain sub-regions (36) implicated to be involved in mood regulation (16, 17) and where calbindin is abundant, calbindin's interaction with IMPase enhances the enzyme activity.

Interfering with protein-protein interaction is a new paradigm in drug discovery for neurological and psychiatric disorders (37, 38). Small molecule inhibitors designed within this concept include short peptides (39). To rationally design short peptides as potential competitive inhibitors of calbindin-activated IMPase we modeled *in silico* the structure of the IMPase-calbindin complex by docking the NMR structure of calbindin (40) to the crystal structure of bovine IMPase (41)**.** The model suggested that out of the 12 amino acid segment of IMPase which interacts with calbindin (residues 55-66) (13) two IMPase residues, Lys59 and Lys61, are hydrogen bonded to calbindin. The residue in between these two lysine residues, Glu70, also participates in the interaction with calbindin. The two lysine residues interacting with two aspartate residues of calbindin (Asp24 and Asp26) are among the central six amino acids of the 12 amino acid segment which is conserved between human and mouse IMPase.

We experimentally assessed the role of the Lys-Glu-Lys motif of IMPase and the role of Asp24 and Asp26 of calbindin in the interaction between the two proteins by assessing the inhibitory effect of different peptides on the enhancement of mouse brain crude homogenate IMPase activity by wildtype or

mutated human recombinant calbindin. The inhibitory effect of the linear peptides 2 through 4 (Table 1) on calbindin's activation of IMPase was comparable to that of the 6-mer peptide composed of an identical sequence as residues 58-63 of IMPase (peptide 1; Table 1) as long as the permutated peptide included the Lys-Glu-Lys motif, or at least a part of it. Nonetheless, five amino acid peptides which included a Lys-X-Lys motif but lacked the C-terminal proline residue (peptides 9 and 10; Table 1) did not inhibit the activation of IMPase by calbindin indicating a requirement for this specific carboxyl terminal residue. A three amino acid peptide comprised of the Lys-Glu-Lys motif only (peptide 11; Table 1) also did not inhibit IMPase activation by calbindin. This result suggests that the Lys-Glu-Lys motif, or part of it, is necessary but not sufficient for the peptide to inhibit calbindin's activation of IMPase and defines a size constraint of at least six amino acids required to inhibit the activation of IMPase by calbindin. A similar result of gradual decreased length of an inhibitory peptide affecting its potency was observed for peptides inhibiting polyglutamine protein aggregation and cell death in neurodegenerative diseases including Huntington's disease (42). To further evaluate the validity of our *in silico* model we assessed the role of calbindin's Asp24 and Asp26 residues in the interaction with IMPase using a mutated calbindin in which both of these aspartate residues were mutated into alanine. Unlike wildtype calbindin the mutated calbindin did not activate IMPase. This result supports the model's implication that these two aspartate residues of calbindin play a significant role in the interaction of the protein with IMPase.

Berggard et al. (13) found ~100 fold increase of IMPase activity in the presence of calbindin in an assay with pure proteins and at an extremely low substrate concentration (2.5 μM) and ~2-3 fold increase at a physiologically-relevant substrate concentration (80 μM). Similarly to Berggard et al. (13, 18), we have previously reported (23) about 3 fold increase of IMPase activity in postmortem human brain homogenate in the presence of recombinant human calbindin, a finding replicated in the present study. Using mouse brain crude homogenate and recombinant human calbindin, the increase of IMPase activity was of about 2 fold. It is conceivable that the difference in the extent of activation of mouse brain IMPase by calbindin stems from lower compatibility between mouse IMPase and human

calbindin. Indeed, sequence alignment of the full 55-66 segment reveals two amino acid differences between human and mouse IMPase. Ile55 and Ser64 in human IMPase are replaced in the mouse IMPase by methionine and cysteine, respectively.

The biochemical results support the suggested molecular mode of interaction between calbindin and IMPase and provide a proof of concept for the possibility to inhibit IMPase activity at a site different than that of lithium. The next step was to search for a behavioral proof of concept. To this end we compared the effect of ICV administration of peptide 1 *vs*. peptide 5 (Table 1) on the forced swim test in mice. We hypothesized that similarly to lithium peptide 1 but not peptide 5 will exhibit an antidepressant-like reduction of immobility (43, 44). Our results confirmed our hypothesis and provided an *in vivo* behavioral proof of concept for the novel site through which it may become possible to design novel mood stabilizers.

The use of peptides as drugs endures both advantages and disadvantages. Although peptides are highly selective, can be synthetically produced and optimized, *in vivo* they exhibit short half-lives, poor bioavailability and decreased cell permeability, limiting their use in the clinics (45, 46). This may be due to lack of solubility, rapid enzymatic degradation by the intestinal proteolytic enzymes or poor intestinal permeability. Different pharmaceutical and chemical approaches were developed to enhance oral bioavailability of peptides while maintaining their pharmacological activity. Peptide cyclization or blocking their N- and C-terminal amino acids has been shown to address these limitations rendering the peptides to exhibit increased potency, increased stability to proteolytic degradation and enable their binding to the target at decreased effective concentrations (47, 48). In the present study the cycloscan and backbone cyclization methods (29, 30, 49) were used to circumvent the problems that hamper the conversion of peptides into drugs. Utilizing cyclic or linear pre-cyclic analog peptides in which the pharmacophors are preserved in the same sequence and constitution as in the parent peptide 1 we were able to mimic peptide 1's inhibitory effect on the activation of IMPase by calbindin *in vitro*. The enhanced stability of cyclic peptide 1 compared with it's linear analog adds "drug like" property required for further development for potential clinical utilization (30).

Obviously, further studies should investigate whether cyclic or linear pre-cyclic peptides modified to exhibit membrane permeability and biological stability (48), or nonpeptide small molecules (38) based on our *in silico* model, can mimic behavioral effects of lithium in inositol-related paradigms (50, 51) or other lithium-induced behavioral effects in animals (52).

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Conflict of interest

Neither of the authors has any competing financial interests in relation to this work.

Table 1: Synthetic peptides

IKEKYP peptide 1	composed of an identical sequence as residues 58-63 of IMPase
IKAKYP peptide 2	peptide 1 in which Glu60 was permutated to Ala
IKEAYP peptide 3	peptide 1 in which Lys61 was permutated to Ala
IAEKYP peptide 4	peptide 1 in which Lys59 was permutated to Ala
SYEHKP peptide 5	peptide 1 in which Ile58 and Lys61 were permutated to histidine and serine, respectively, and the whole sequence has been scrambled
IAEAYP peptide 6	peptide 1 in which both Lys59 and Lys61 were permutated to Ala
IAAAYP peptide 7	peptide 1 in which the whole Lys-Glu-Lys motif has been permutated to three Ala residues
KHIKPS peptide 8	a random 6-mer peptide containing two lysine residues albeit, separated by two, rather than a single residue, none of which was glutamate
IKEKY peptide 9	5-mer peptide lacking the carboxyterminal Pro and including the Lys-Glu-Lys motif
IKAKY peptide 10	5-mer peptide lacking the carboxyterminal Pro and including a Lys-Ala-Lys replacement
KEK peptide 11	the Lys-Glu-Lys motif only
Linear pre-cyclic analog 1	based on peptide1 (see Scheme 1)
Cyclic 1	
Linear pre-cyclic analog 2	
Cyclic 2	

Figure legends

Figure 1: **Three dimensional structure of IMPase (a) and the modeled complex of IMPase and calbindin (b)**

a. The crystal structure of IMPase shown as a green ribbon, residues 55-66 are colored in purple and the central six amino acids (residues 58-63) are colored in red. Magnesium ions at the active site are shown as blue balls; the side chain of Glu70 at the active site is shown in yellow.

b. The docking model of IMPase (in green) and calbindin (in blue). Calcium ions are shown as orange balls. The side chains of possible interacting residues are shown as sticks (Lys59 and Lys61 of IMPase - in magenta and Asp24 and Asp26 of calbindin - in yellow).

The figure was produced using the program PyMol.

Figure 2: **The effect of linear peptides on calbindin-activated IMPase activity**
Results are means $\pm$ S.E.M.

A. The effect of six amino acid linear peptides containing the Lys-Glu-Lys motif or part of it (see Table 1)

ANOVA: $F(5, 74)=3.28$, $p=0.01$
Post hoc LSD test:
* control *vs.* calbindin, $p<0.001$
** calbindin *vs.* peptide 1, $p=0.008$,
** calbindin *vs.* peptide 2, $p=0.001$
** calbindin *vs.* peptide 3, $p=0.03$ and peptide 4, $p=0.02$

B. The effect of six amino acid linear peptides which include only one or none of the amino acids Lys or Glu of the Lys-Glu-Lys motif compared to peptide 1 (see Table 1)

ANOVA: $F(6, 55)=1.8$, $p=0.11$
Post hoc LSD test:
* control *vs.* calbindin, $p=0.01$
** calbindin *vs.* peptide 1, $p=0.007$

C. The effect of two five amino acid peptides (peptides 9 and 10) and a three amino acid peptide containing the Lys-Glu-Lys motif or a Lys-Ala-Lys replacement compared with peptide 1 (see Table 1)

ANOVA: $F(5, 50)=3.79$, $p=0.005$

Post hoc LSD test:
* control *vs.* calbindin, p=0.002,
** calbindin *vs.* peptide 1, p<0.001

Figure 3: **Mutated calbindin in which Asp24 and Asp26 were mutated to Ala did not activate IMPase**
Results are means ± S.E.M.; n=5 in all groups

ANOVA: F(2,14)=21.8, p<0.001
Post-hoc LSD test:
wildtype calbindin *vs.* control, p<0.001
wildtype calbindin *vs.* mutated calbindin, p<0.001

Figure 4: **The effect of cyclic or linear pre-cyclic analog (LPCA) peptides on calbindin-activated IMPase activity**
Results are means ± S.E.M.

Student's t-tests:
calbindin *vs.* control, p<0.0001
* peptide 1 *vs.* calbindin, p< 0.001
** LPCA-1 and Cyclic-1 *vs.* calbindin, p< 0.05

Figure 5: **Peptide 1 (IKEKYP) exhibits an antidepressant-like effect in the forced swim test**
Results are mean ± S.E.M.

ANOVA, F3,27=4.52, p=0.011
Post-hoc LSD test:
* peptide 1 *vs.* aCSF, p=0.05, and *vs.* peptide 5, p=0.01
** Positive control, imipramine *vs.* aCSF, p=0.02

References

1. Harwood AJ, Agam G. Search for a common mechanism of mood stabilizers. *Biochem Pharmacol*. 2003;66(2):179-89.
2. Ohnishi T, Ohba H, Seo KC, Im J, Sato Y, Iwayama Y, et al. Spatial expression patterns and biochemical properties distinguish a second myo-inositol monophosphatase IMPA2 from IMPA1. *J Biol Chem*. 2007;282(1):637-46.
3. Hallcher LM, Sherman WR. The effects of lithium ion and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. *J Biol Chem*. 1980;255(22):10896-901.
4. Leech AP, Baker GR, Shute JK, Cohen MA, Gani D. Chemical and kinetic mechanism of the inositol monophosphatase reaction and its inhibition by Li+. *Eur J Biochem*. 1993;212(3):693-704.
5. Ganzhorn AJ, Hoflack J, Pelton PD, Strasser F, Chanal MC, Piettre SR. Inhibition of myo-inositol monophosphatase isoforms by aromatic phosphonates. *Bioorg Med Chem*. 1998;6(10):1865-74.
6. Berridge MJ, Downes CP, Hanley MR. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem J*. 1982;206(3):587-95.
7. Berridge MJ. The Albert Lasker Medical Awards. Inositol trisphosphate, calcium, lithium, and cell signaling. *Jama*. 1989;262(13):1834-41.
8. Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci U S A*. 1996;93(16):8455-9.
9. Shaltiel G, Shamir A, Nemanov L, Yaroslavsky Y, Nemets B, Ebstein RP, et al. Inositol Monophosphatase Activity in Brain and Lymphocyte-derived Cell Lines of Bipolar, Schizophrenic and Unipolar Patients. *World J Biol Psychiatry*. 2001;2:95-8.
10. Williams RS, Cheng L, Mudge AW, Harwood AJ. A common mechanism of action for three mood-stabilizing drugs. *Nature*. 2002;417(6886):292-5.
11. Atack JR. Inositol monophosphatase inhibitors--lithium mimetics? *Med Res Rev*. 1997;17(2):215-24.

12. Fauroux CM, Freeman S. Inhibitors of inositol monophosphatase. *J Enzyme Inhib*. 1999;14(2):97-108.

13. Berggard T, Szczepankiewicz O, Thulin E, Linse S. Myo-inositol monophosphatase is an activated target of calbindin D28k. *J Biol Chem*. 2002;277(44):41954-9.

14. Iritani S, Niizato K, Emson PC. Relationship of calbindin D28K-immunoreactive cells and neuropathological changes in the hippocampal formation of Alzheimer's disease. *Neuropathology*. 2001;21(3):162-7.

15. Gonzalez-Albo MC, Elston GN, DeFelipe J. The human temporal cortex: characterization of neurons expressing nitric oxide synthase, neuropeptides and calcium-binding proteins, and their glutamate receptor subunit profiles. *Cereb Cortex*. 2001;11(12):1170-81.

16. Cotter D, Landau S, Beasley C, Stevenson R, Chana G, MacMillan L, et al. The density and spatial distribution of GABAergic neurons, labelled using calcium binding proteins, in the anterior cingulate cortex in major depressive disorder, bipolar disorder, and schizophrenia. *Biol Psychiatry*. 2002;51(5):377-86.

17. Beasley CL, Zhang ZJ, Patten I, Reynolds GP. Selective deficits in prefrontal cortical GABAergic neurons in schizophrenia defined by the presence of calcium-binding proteins. *Biol Psychiatry*. 2002;52(7):708-15.

18. Berggard T, Miron S, Onnerfjord P, Thulin E, Akerfeldt KS, Enghild JJ, et al. Calbindin D28k exhibits properties characteristic of a Ca2+ sensor. *J Biol Chem*. 2002;277(19):16662-72.

19. Airaksinen MS, Eilers J, Garaschuk O, Thoenen H, Konnerth A, Meyer M. Ataxia and altered dendritic calcium signaling in mice carrying a targeted null mutation of the calbindin D28k gene. *Proc Natl Acad Sci U S A*. 1997;94(4):1488-93.

20. Barski JJ, Hartmann J, Rose CR, Hoebeek F, Morl K, Noll-Hussong M, et al. Calbindin in cerebellar Purkinje cells is a critical determinant of the precision of motor coordination. *J Neurosci*. 2003;23(8):3469-77.

21. Schmidt H, Schwaller B, Eilers J. Calbindin D28k targets myo-inositol monophosphatase in spines and dendrites of cerebellar Purkinje neurons. *Proc Natl Acad Sci U S A*. 2005;102(16):5850-5.

22. Blatow M, Caputi A, Burnashev N, Monyer H, Rozov A. Ca2+ buffer saturation underlies paired pulse facilitation in calbindin-D28k-containing terminals. *Neuron*. 2003;38(1):79-88.

23. Shamir A, Elhadad N, Belmaker RH, Agam G. Interaction of calbindin D and inositol monophosphatase in human postmortem cortex: possible implications for bipolar disorder. *Bipolar Disord*. 2005;7(1):42-8.

24. DeLano WL. The PyMOL Molecular Graphics System DeLano Scientific: San Carlos, CA, USA; 2002.

25. Katchalski-Katzir E, Shariv I, Eisenstein M, Friesem AA, Aflalo C, Vakser IA. Molecular surface recognition: determination of geometric fit between proteins and their ligands by correlation techniques. *Proc Natl Acad Sci U S A*. 1992;89(6):2195-9.

26. Cryns K, Shamir A, Van Acker N, Levi I, Daneels G, Goris I, et al. IMPA1 is essential for embryonic development and lithium-like pilocarpine sensitivity. *Neuropsychopharmacology*. 2008;33(3):674-84.

27. Thulin E, Linse S. Expression and purification of human calbindin D28k. *Protein Expr Purif*. 1999;15(3):265-70.

28. Gilon C, Halle D, Chorev M, Selinger Z, Byk G. Backbone cyclization: A new method for conferring conformational constraint on peptides. *Biopolymers*. 1991;31(6):745-50.

29. Kasher R, Oren DA, Barda Y, Gilon C. Miniaturized proteins: the backbone cyclic proteinomimetic approach. *J Mol Biol*. 1999;292(2):421-9.

30. Ovadia O, Linde Y, Haskell-Luevano C, Dirain ML, Sheynis T, Jelinek R, et al. The effect of backbone cyclization on PK/PD properties of bioactive peptide-peptoid hybrids: the melanocortin agonist paradigm. *Bioorg Med Chem*. 2009;18(2):580-9.

31. Atack JR, Broughton HB, Pollack SJ. Structure and mechanism of inositol monophosphatase. *FEBS Lett*. 1995;361(1):1-7.

32. Celio MR. Calbindin D-28k and parvalbumin in the rat nervous system. *Neuroscience*. 1990;35(2):375-475.

33. Lambers TT, Mahieu F, Oancea E, Hoofd L, de Lange F, Mensenkamp AR, et al. Calbindin-D28K dynamically controls TRPV5-mediated Ca2+ transport. *Embo J*. 2006;25(13):2978-88.

34. Sooy K, Schermerhorn T, Noda M, Surana M, Rhoten WB, Meyer M, et al. Calbindin-D(28k) controls [Ca(2+)](i) and insulin release. Evidence obtained from calbindin-d(28k) knockout mice and beta cell lines. *J Biol Chem*. 1999;274(48):34343-9.

35. Mattson MP, Rychlik B, Chu C, Christakos S. Evidence for calcium-reducing and excito-protective roles for the calcium-binding protein calbindin-D28k in cultured hippocampal neurons. *Neuron*. 1991;6(1):41-51.

36. Mikkonen M, Soininen H, Pitkanen A. Distribution of parvalbumin-, calretinin-, and calbindin-D28k-immunoreactive neurons and fibers in the human entorhinal cortex. *J Comp Neurol*. 1997;388(1):64-88.

37. Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. *Nature*. 2007;450(7172):1001-9.

38. Reilly MT, Cunningham KA, Natarajan A. Protein-protein interactions as therapeutic targets in neuropsychopharmacology. *Neuropsychopharmacology*. 2009;34(1):247-8.

39. Sharma SC, Rupasinghe CN, Parisien RB, Spaller MR. Design, synthesis, and evaluation of linear and cyclic peptide ligands for PDZ10 of the multi-PDZ domain protein MUPP1. *Biochemistry*. 2007;46(44):12709-20.

40. Kojetin DJ, Venters RA, Kordys DR, Thompson RJ, Kumar R, Cavanagh J. Structure, binding interface and hydrophobic transitions of Ca2+-loaded calbindin-D(28K). *Nat Struct Mol Biol*. 2006;13(7):641-7.

41. Gill R, Mohammed F, Badyal R, Coates L, Erskine P, Thompson D, et al. High-resolution structure of myo-inositol monophosphatase, the putative target of lithium therapy. *Acta Crystallogr D Biol Crystallogr*. 2005;61(Pt 5):545-55.

42. Ren H, Nagai Y, Tucker T, Strittmatter WJ, Burke JR. Amino acid sequence requirements of peptides that inhibit polyglutamine-protein aggregation and cell death. *Biochem Biophys Res Commun*. 2001;288(3):703-10.

43. O'Brien WT, Harper AD, Jove F, Woodgett JR, Maretto S, Piccolo S, et al. Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium. *J Neurosci*. 2004;24(30):6791-8.

44. Bersudsky Y, Shaldubina A, Belmaker RH. Lithium's effect in forced-swim test is blood level dependent but not dependent on weight loss. *Behav Pharmacol*. 2007;18(1):77-80.

45. Pauletti GM, Gangwar S, Wang B, Borchardt RT. Esterase-sensitive cyclic prodrugs of peptides: evaluation of a phenylpropionic acid promoiety in a model hexapeptide. *Pharm Res*. 1997;14(1):11-7.

46. Okumu FW, Pauletti GM, Vander Velde DG, Siahaan TJ, Borchardt RT. Effect of restricted conformational flexibility on the permeation of model hexapeptides across Caco-2 cell monolayers. *Pharm Res*. 1997;14(2):169-75.

47. Hess S, Linde Y, Ovadia O, Safrai E, Shalev DE, Swed A, et al. Backbone cyclic peptidomimetic melanocortin-4 receptor agonist as a novel orally administrated drug lead for treating obesity. *J Med Chem*. 2008;51(4):1026-34.

48. Hess S, Ovadia O, Shalev DE, Senderovich H, Qadri B, Yehezkel T, et al. Effect of structural and conformation modifications, including backbone cyclization, of hydrophilic hexapeptides on their intestinal permeability and enzymatic stability. *J Med Chem*. 2007;50(24):6201-11.

49. Kessler H, Ovadia O, Greenberg S, Laufer B, Gilon C, Hoffman A. Improvement of drug-like properties of peptides: the somatostatin paradigm. *Expert Opinion on Drug Discovery*. 2010;5(7):655-71.

50. Belmaker RH, Bersudsky Y. Lithium-pilocarpine seizures as a model for lithium action in mania. *Neurosci Biobehav Rev*. 2007;31(6):843-9.

51. Kofman O, Belmaker RH. Ziskind-Somerfeld Research Award 1993. Biochemical, behavioral, and clinical studies of the role of inositol in lithium treatment and depression. *Biol Psychiatry*. 1993;34(12):839-52.

52. Gould TD, Einat H. Animal models of bipolar disorder and mood stabilizer efficacy: a critical need for improvement. *Neurosci Biobehav Rev*. 2007;31(6):825-31.

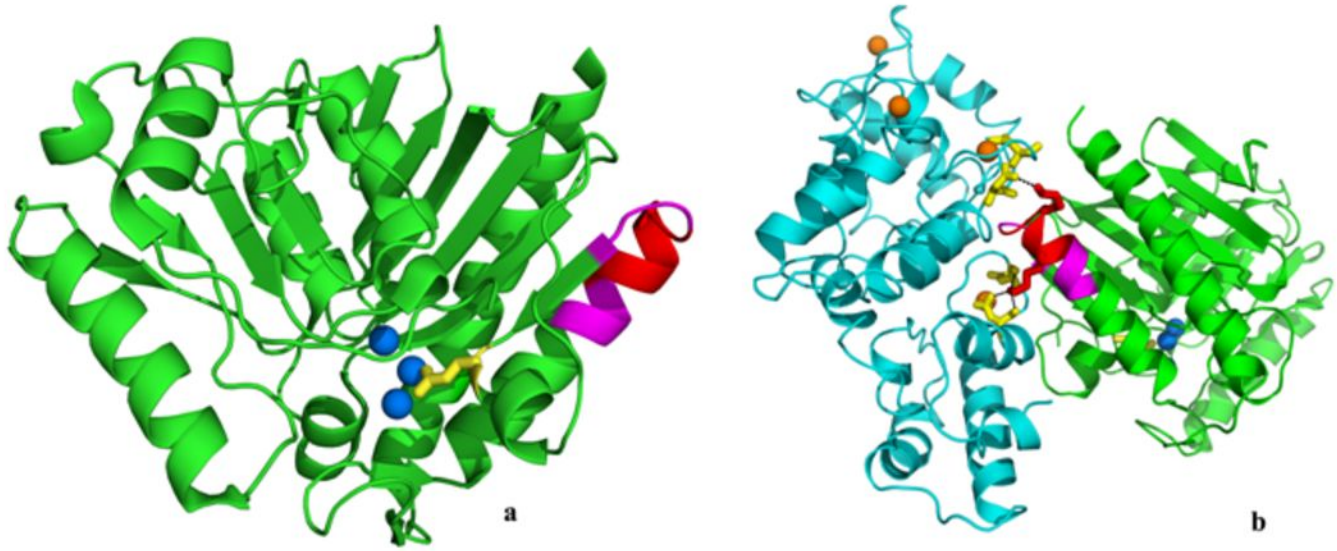
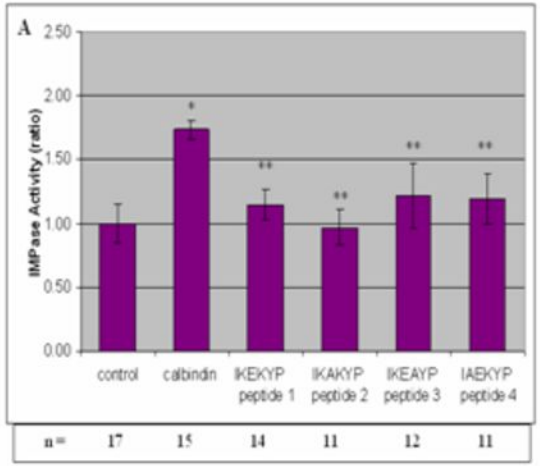


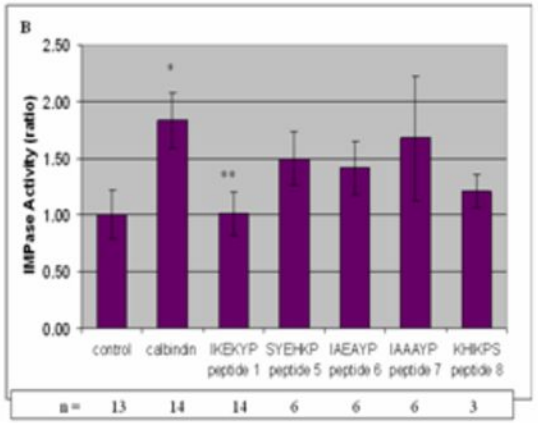
Figure 1: **Three dimensional structure of IMPase (a) and the modeled complex of IMPase and calbindin (b)**

a. The crystal structure of IMPase shown as a green ribbon, residues 55-66 are colored in purple and the central six amino acids (residues 58-63) are colored in red. Magnesium ions at the active site are shown as blue balls; the side chain of Glu70 at the active site is shown in yellow.

b. The docking model of IMPase (in green) and calbindin (in blue). Calcium ions are shown as orange balls. The side chains of possible interacting residues are shown as sticks (Lys59 and Lys61 of IMPase - in magenta and Asp24 and Asp26 of calbindin - in yellow).

The figure was produced using the program PyMol.





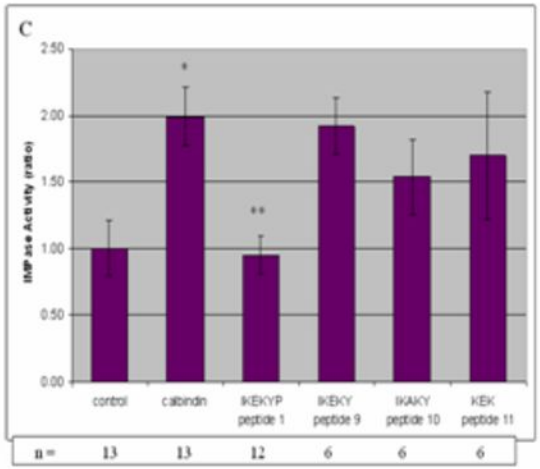


Figure 2: **The effect of linear peptides on calbindin-activated IMPase activity**

Results are means ± S.E.M.

A. The effect of six amino acid linear peptides containing the Lys-Glu-Lys motif or part of it (see Table 1)

ANOVA: $F(5, 74)=3.28$, $p=0.01$

Post hoc LSD test:

* control *vs.* calbindin, $p<0.001$

** calbindin *vs.* peptide 1, $p=0.008$,

** calbindin *vs.* peptide 2, $p=0.001$

** calbindin *vs.* peptide 3, $p=0.03$ and peptide 4, $p=0.02$

B. The effect of six amino acid linear peptides which include only one or none of the amino acids Lys or Glu of the Lys-Glu-Lys motif compared to peptide 1 (see Table 1)

ANOVA: $F(6, 55)=1.8$, $p=0.11$

Post hoc LSD test:

* control *vs.* calbindin, $p=0.01$

** calbindin *vs.* peptide 1, $p=0.007$

C. The effect of two five amino acid peptides (peptides 9 and 10) and a three amino acid peptide containing the Lys-Glu-Lys motif or a Lys-Ala-Lys replacement compared with peptide 1 (see Table 1)

005, p=0.3.79)=0, 55ANOVA: F(

Post hoc LSD test:

* control *vs.* calbindin, $p=0.002$,

** calbindin *vs.* peptide 1, $p<0.001$

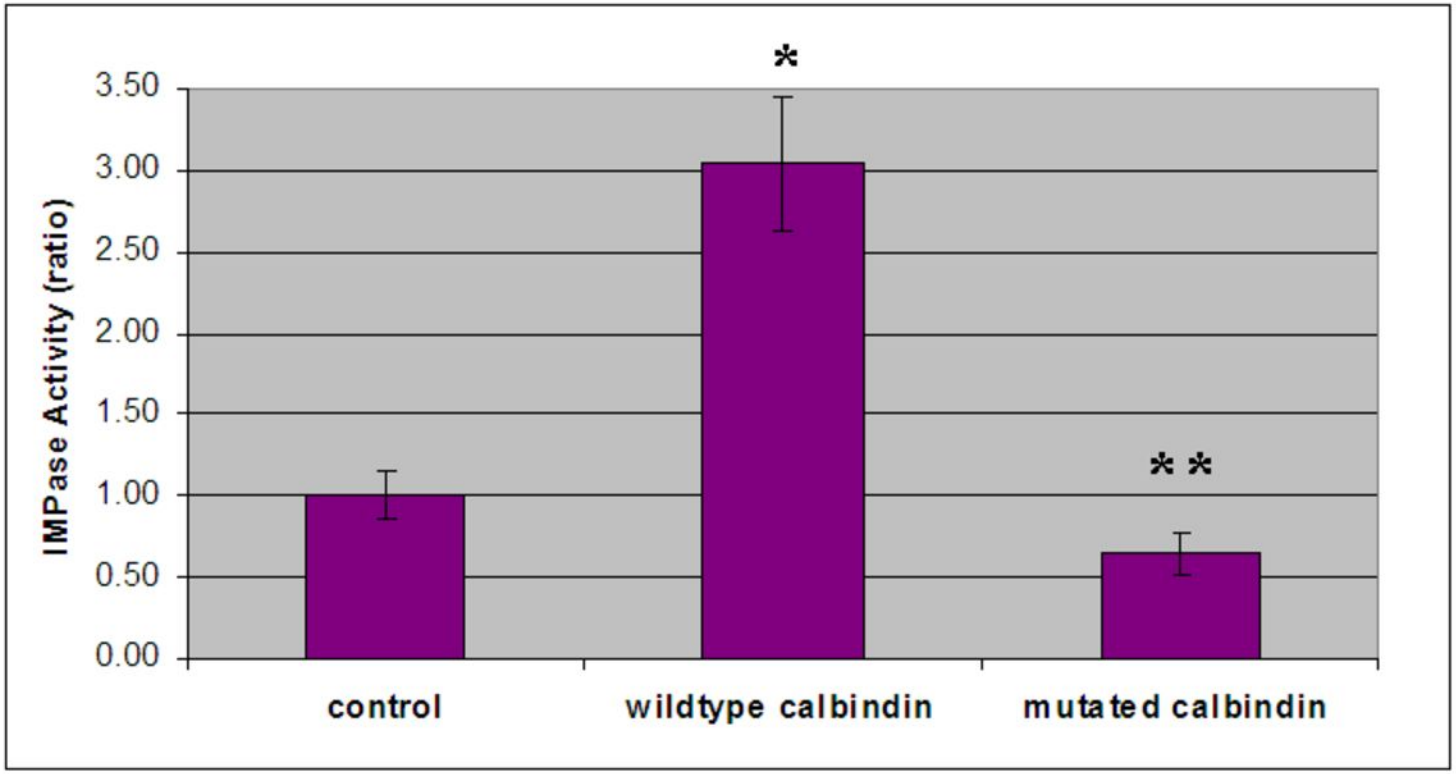


Figure 3: **Mutated calbindin in which Asp24 and Asp26 were mutated to Ala did not activate IMPase**

Results are means $\pm$ S.E.M.; n=5 in all groups

ANOVA: $F(2,14)=21.8$, $p<0.001$

Post-hoc LSD test:

wildtype calbindin *vs.* control, $p<0.001$

wildtype calbindin *vs.* mutated calbindin, $p<0.001$

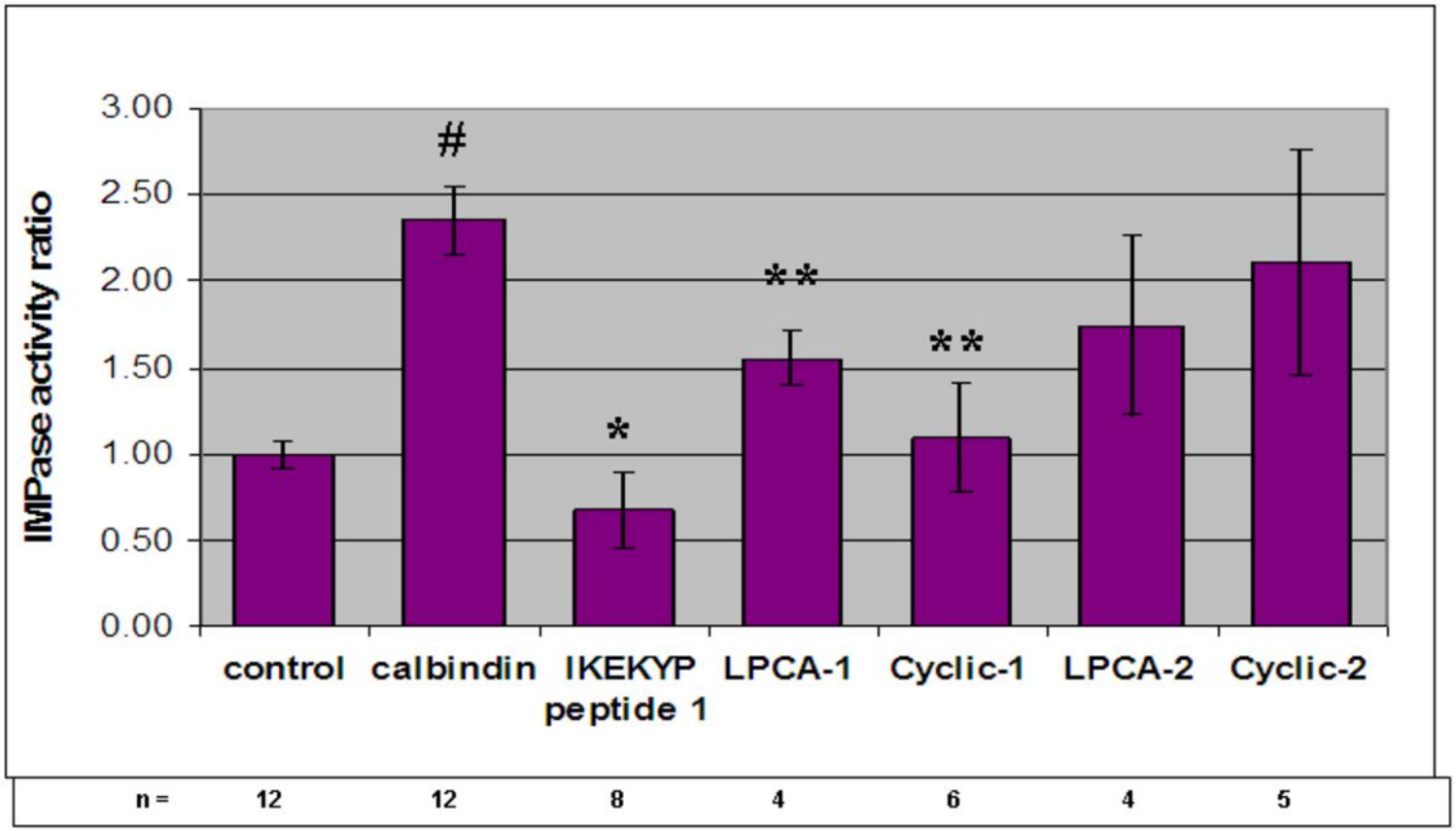


Figure 4: **The effect of cyclic or linear pre-cyclic analog (LPCA) peptides on calbindin-activated IMPase activity**

Results are means ± S.E.M.

Student's t-tests:

calbindin *vs.* control, $p<0.0001$

* peptide 1 *vs.* calbindin, $p< 0.001$

** LPCA-1 and Cyclic-1 *vs.* calbindin, $p< 0.05$

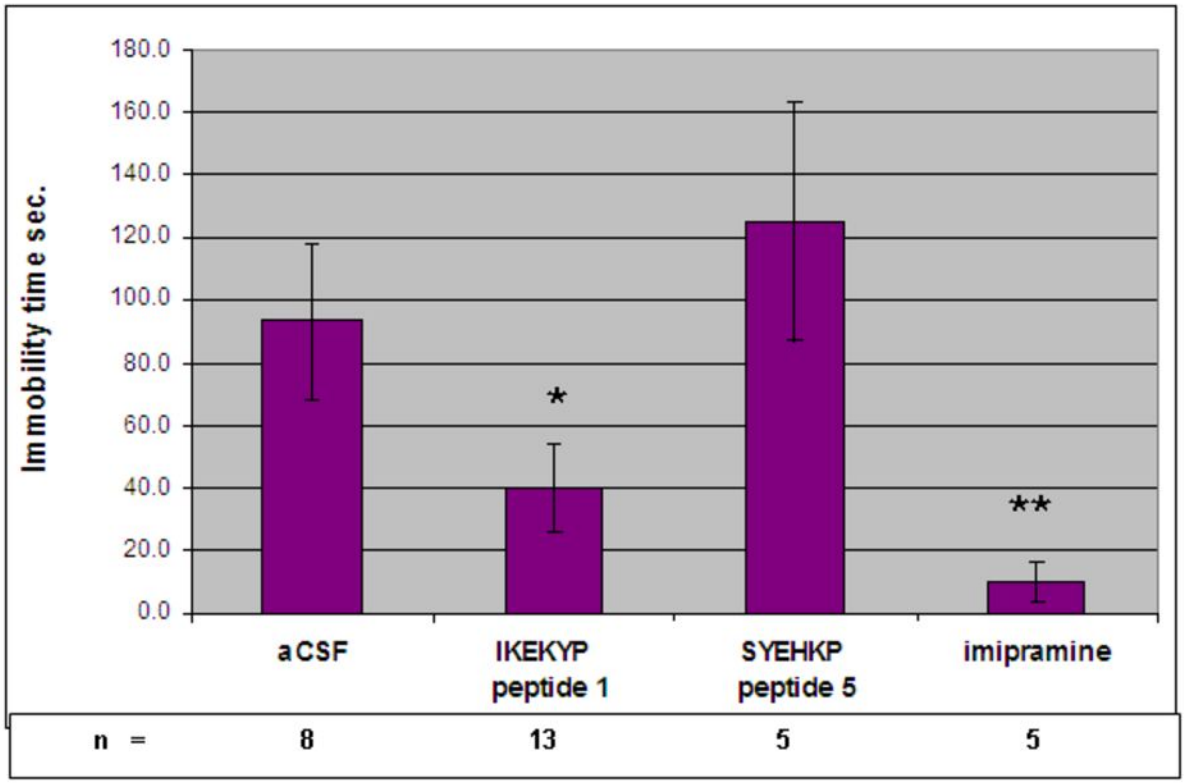


Figure 5: **Peptide 1 (IKEKYP) exhibits an antidepressant-like effect in the forced swim test**

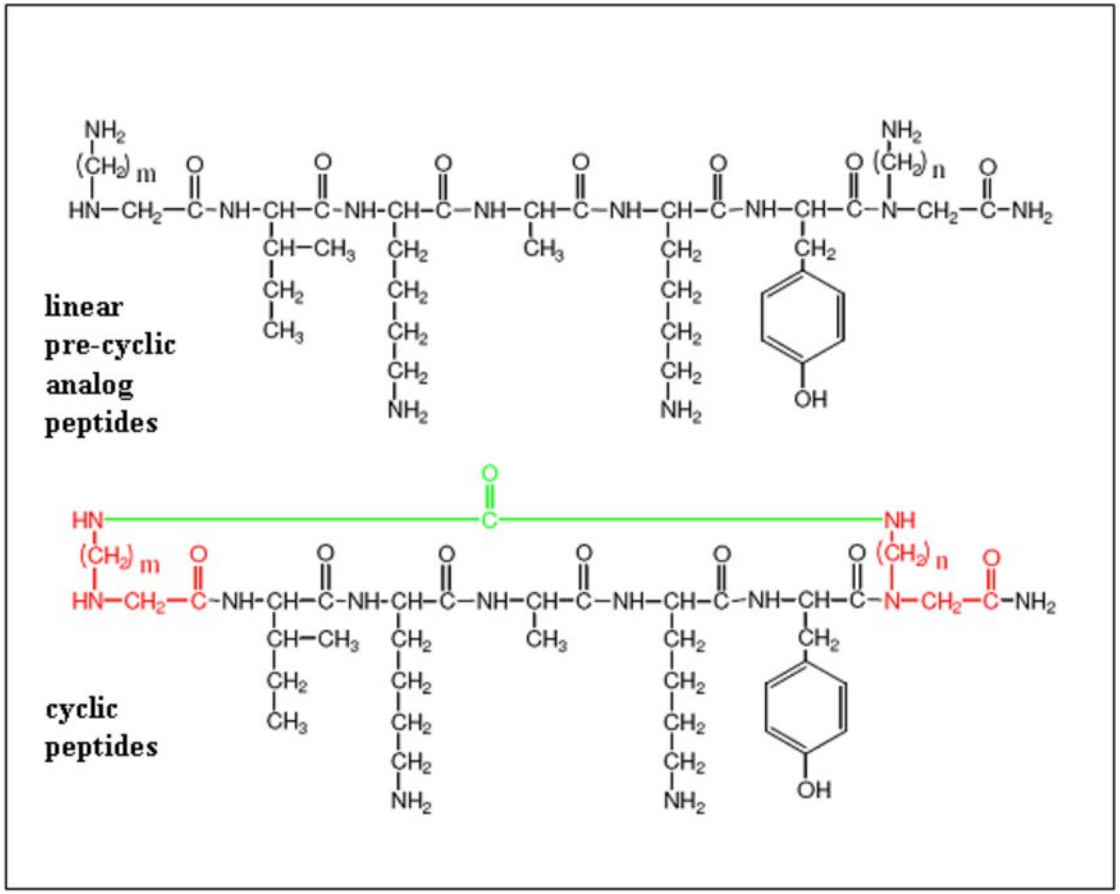
Results are mean ± S.E.M.

ANOVA, $F_{3,27}=4.52$, $p=0.011$

Post-hoc LSD test:

* peptide 1 *vs.* aCSF, $p=0.05$, and *vs.* peptide 5, $p=0.01$

** Positive control, imipramine *vs.* aCSF, $p=0.02$



Scheme 1: The molecular structure of the cyclic and the linear pre-cyclic analog peptides

linear pre-cyclic analog 1: m=4, n=4; cyclic 1: m=4, n=4;
linear pre-cyclic analog 2: m=2, n=4; cyclic 2: m=2, n=4)