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Curcumin is an inhibitor of calcium/calmodulin dependent protein kinase II

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ABSTRACT

Calcium/calmodulin dependent protein kinase II (CaMKII) is involved in the mechanisms underlying higher order brain functions such as learning and memory. CaMKII participates in pathological glutamate signaling also, since it is activated by calcium influx through the *N*-methyl-D-aspartate type glutamate receptor (NMDAR). In our attempt to identify phytomodulators of CaMKII, we observed that curcumin, a constituent of turmeric and its analogs inhibit the Ca^{2+}-dependent and independent kinase activities of CaMKII. We further report that a heterocyclic analog of curcumin I, (3,5-bis[β-(4-hydroxy-3-methoxyphenyl)ethenyl]pyrazole), named as pyrazole-curcumin, is a more potent inhibitor of CaMKII than curcumin. Microwave assisted, rapid synthesis of curcumin I and its heterocyclic analogues is also reported.

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1. Introduction

Calcium/calmodulin dependent protein kinase II (CaMKII) is a major mediator of Ca^{2+} signaling, which is found in brain, heart and other tissues. CaMKII is a multifunctional serine/threonine kinase with a broad range of substrates. CaMKII comprises of a family of isoforms, α, β, γ and δ, derived from four closely related but distinct genes. The α and β forms are restricted to nervous tissue, whereas γ and δ forms are found in most tissues including brain.[1] CaMKII is highly expressed in the forebrain and it forms two percent of the total protein in the hippocampus.[2] Many of the neuronal functions such as neurotransmitter synthesis, regulation of ion channels and many signaling pathways are influenced by CaMKII. CaMKII plays a crucial role in the mechanisms that underlie long term potentiation (LTP) which serves as a cellular model for learning and memory.[3,4] CaMKII exists as a dodecameric holoenzyme, whose activation is regulated by the amplitude and frequency of Ca^{2+} spikes.[5] Each isoform of CaMKII has an N-terminal catalytic domain, followed by a regulatory domain and a C-terminal association domain. The kinase domains form dimers in which the catalytic domains are held together by antiparallel coiled-coil formed by the regulatory domains retaining the enzyme in an inactive conformation.[6] The activation of the *N*-methyl-D-aspartate type glutamate receptor (NMDAR) promotes an influx of Ca^{2+} activating CaMKII. The binding of Ca^{2+}/calmodulin disrupts the interaction of the regulatory domain with kinase domain, thereby converting the enzyme into an active state.[7,8] Once relieved of the inhibition, CaMKII phosphorylates substrates which include receptors, ion channels, cytoskeletal and scaffolding proteins and it could also undergo autophosphorylation. The autophosphorylation at Thr^{286} converts the enzyme into an autonomously active state, the functional consequence of which allows it to function as a memory molecule.[9] CaMKII translocates to postsynaptic density, where it regulates many signaling pathways.[10]

CaMKII, being a downstream target of glutamate induced calcium signaling, may also play a role in pathological glutamate signaling. Conflicting data regarding the role of CaMKII in promoting[11] or inhibiting neuronal cell death[12] have been reported. Currently known CaMKII inhibitors are mainly used as tools for studying the enzymatic function of the kinase. Among these, the KN series, which includes KN62 and KN93, do not inhibit the autonomous activity.[13,14] The peptides derived from the autoinhibitory region of CaMKII, such as AIP (autoinhibitory peptide) or AC3, also serve as CaMKII inhibitors. CaMKIIN has been recently identified as a CaMKII specific, natural inhibitor[15,16] and tatCN21 inhibitor is a peptide derived from the natural inhibitor.[17] CaMKIINα and CaMKIINβ are endogenous CaMKII inhibitors, the roles of which are not very clear. During contextual memory formation, the level of CaMKIINα mRNA expression increases in amygdala and hippocampus.[18] CaMKIINβ protein level has also been reported to be increased in amygdala and hippocampus during contextual memory formation and its expression is transient.[19] These findings indicate that CaMKIIN type inhibitors could function as plasticity related molecules.[20] Recent findings of Vest et al. implicated the inhibition of Ca^{2+}-independent autonomous

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CaMKII activity that is generated by Thr[286] autophosphorylation as the drug target for post-insult neuroprotection.[21]

Curcumin is a phytochemical derived from the rhizome of *Curcuma longa* commonly known as turmeric. The bioactivity of curcumin has been under intense study in the past decade and several reviews in this topic have appeared.[22,23] Natural curcumin is a mixture of three closely related linear diarylheptanoids, curcumin I–III (**1**–**3**) (Fig. 1), present in the ratio 85:11:4 approximately. All the three exist in the enol form exclusively. Extensive computational studies[24] have confirmed the predominance of the enol tautomer and have also shown that in curcumin, the middle seven-carbon chain is quite hydrophobic whereas the enolic and terminal phenolic groups provide polarity to the molecule. The bioactivity profile of curcumin includes function as an antioxidant,[25] reduction of cognitive deficits,[26] lowering of amyloid accumulation,[27] reduction of inflammation,[28] inhibition of angiogenesis,[29] prevention of tumorigenesis,[30] control of diabetes,[31] amelioration of neurological diseases,[32,33] and inhibition of protein tyrosine kinase and protein kinase C activities.[34] Neuroprotective action of curcumin has been reported in transgenic animal model for Alzheimer's disease.[35] Curcumin has been reported to prevent scopolamine–induced memory dysfunction by inducing an improvement in the memory-dependent learning in rats.[36] In addition to the reports on the neuroprotective activity of curcuminoids, a recent report[37] has identified the ability of curcumin to reduce corticosterone-induced elevated phosphorylation of CaMKII in primary hippocampal neurons. More recently, the memory stimulation effect of a phenylpyrazole-curcumin derivative called CNB-001 (**4**) (Fig. 2) has been reported[38] and this activity has been suggested to be mediated through the activation of CaMKII. The above two reports indicate that, whereas curcumin decreases the corticosterone-induced elevated phosphorylation level of CaMKII, its closely related derivative CNB-001 is an activator of CaMKII. We have now investigated, under defined set of conditions, the effect of natural curcumin I-III (**1**–**3**) and synthetic curcumin I (**1**), along with two pyrazole derivatives (**4**, **5**) and an isoxazole derivative (**6**) of curcumin I, on the kinase activity of CaMKII.

2. Materials and methods

2.1. Chemistry

TLC and dry column flash chromatography were performed using TLC grade silica gel-G, particle size 10–40 microns, 300 mesh, from Merck, India. Melting points were determined by open capillary method. FTIR spectra were recorded on a Jasco FTIR, NMR spectra were recorded on Bruker Avance DPX-300 and Avance-III 400 and FAB MS spectra were recorded on JEOL JMS 600H spectrometers. Element analysis was done on Elementar Vario EL III elemental analyzer. Natural curcumin, containing the three curcuminoids, was a gift from Synthite Industries, India.

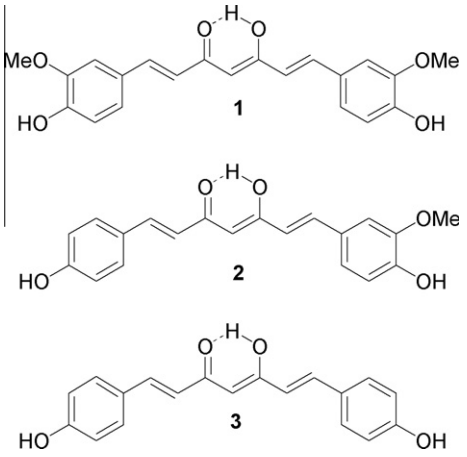


Figure 1. Chemical structure of curcumin I (**1**) curcumin II (**2**) and curcumin III (**3**).

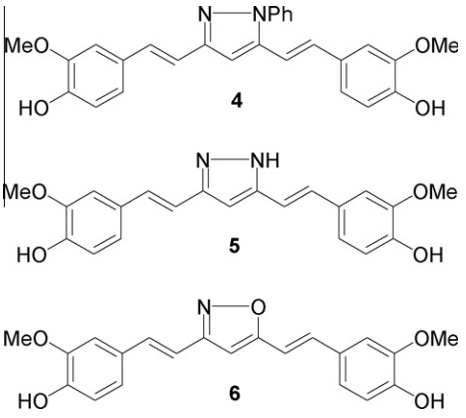


Figure 2. Chemical structure of phenylpyrazole-curcumin (**4**), pyrazole-curcumin (**5**) and isoxazole-curcumin (**6**).

2.1.1. Separation of curcumin I-III (1-3)

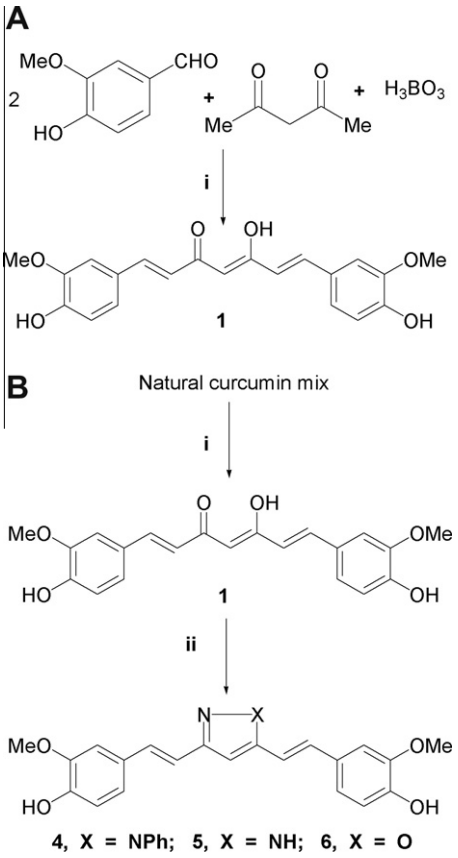
The natural curcumin supplied as a fine powder was separated into its three components by dry column flash chromatography[39] on silica gel-G (TLC grade, Merck, India) using dichloromethane - chloroform (1:1) for curcumin I, dichloromethane - chloroform (1:2) for curcumin II and chloroform for curcumin III as eluents (Scheme 1). The collected fractions were evaporated under reduced pressure and the product so obtained was crystallized from chloroform - ethyl acetate (4:1).

2.1.2. Microwave assisted rapid synthesis of curcumin I (1)

Vanillin (0.304 g, 2 mmol), boric acid (0.2 g) and acetylacetone (0.1 mL, 1 mmol) in DMF (1.0 mL) were mixed in a conical flask and were subjected to 300 W microwave irradiation in a Samsung 800 W multimode domestic microwave oven for 2 min in such a way that after each irradiation for 15 s, 15 s cooling time was given to prevent boil offs. During all microwave irradiation, a beaker containing water (50 mL) was used to absorb excess microwave energy. After microwave irradiation for 2 min, tetrahydroquinoline (0.02 mL) and glacial acetic acid (0.06 mL) were added to the above mixture and it was subjected to 300 W microwave irradiation as above. After 20 min, which involved 10 min of total heating, the reaction mixture thus obtained was worked up by adding to acetic acid (20%, 10 mL) under rapid stirring and the mixture was further stirred for 1 h. The precipitate so obtained was filtered and air dried. It was purified by dry column flash chromatography using TLC grade silica gel-G eluted with dichloromethane - chloroform (1:1) (Scheme 1) to obtain curcumin I **(1)**; yield 72%; mp 182-184 °C. Lit. mp 182 °C.[39] Anal. Calcd for $C_{21}H_{20}O_6$: C, 68.28; H, 5.47. Found: C, 68.32; H, 5.31; UV λ_{max} (methanol): 268 and 430 nm; IR (KBr): 3350, 2900, 2950, 1620, 1580, 1505, 1460, 1420, 1280, 1260, 1200, 1160, 1120, 1030, 964, 940, 910 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.91 (s, 6H, OMe), 5.82 (s, 1H, 4H), 6.56 (d, J = 16 Hz, 2H, 2,6H), 6.9–7.2 (m, 6H), 7.58 (d, J = 16 Hz, 2H, 1,7H); FAB MS spectrum, m/z: 369.78 $[M+H]^+$. Calcd. For $[C_{21}H_{20}O_6+H]$: m/z 369.14.

2.1.3. Synthesis of pyrazole-curcumins (3,5-bis[β-(4-hydroxy-3-methoxyphenyl)ethenyl]pyrazoles)

Curcumin I, separated from the natural mix, was mixed with phenylhydrazine (1 mmol each) in gl. acetic acid (2 mL) and the



Scheme 1. (**A**) Synthesis of curcumin I (**1**). Reagents and conditions: (i) 1,2,3,4-Tetrahydroquinoline and gl. HOAc/DMF, microwave 300 W, 10 min, 20% aq HOAc (**B**) Separation of curcumin I (**1**) from natural curcumin mix and its conversion to heterocyclic analogs (**4–6**). Reagents and conditions: (i) Dry column flash chromatography on TLC grade silica gel-G, eluted with dichloromethane-chloroform (1:1) (ii) $PhNHNH_2$; $H_2NNH_2.H_2O$ or $H_2NOH.HCl$, gl. HOAc, microwave, 300 W, 2 min, water.

mixture was subjected to microwave irradiation (300 W, 2 min) allowing 15 s heating-cooling cycles to prevent boil-offs. Subsequently, the mixture was diluted with water (10 mL), the precipitate was filtered and the crude product so obtained was purified by dry column flash chromatography, using TLC grade silica gel-G and chloroform - ethyl acetate (2:1) as eluent (Scheme 1), to obtain phenylpyrazole-curcumin (**4**) (3,5-bis[β-(4-hydroxy-3-methoxyphenyl)ethenyl]-1-phenylpyrazole; CNB-001) as a reddish brown amorphous solid in 87% yield; mp 88–89 °C. Lit. mp 89 °C.[40] Anal. Calcd for $C_{27}H_{24}N_2O_4$: C, 73.62; H, 5.49; N, 6.36. Found: C, 73.48; H, 5.61; N, 6.28; IR (KBr): 3510, 3030, 1595, 1508, 1273, 1029, 960, 761 and 696 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 3.89 (s, 3H), 3.92 (s, 3H), 5.68 (broad, 2H), 6.71 (d, J = 15.9 Hz, 2H), 6.82–7.14 (m, 9H), 7.33–7.52 (m, 5H). FAB MS (NBA matrix) m/z: 441.58 $[M+H]^+$. Calcd for $[C_{27}H_{24}N_2O_4+H]$: m/z 441.18.

Using hydrazine hydrate in the above reaction instead of phenylhydrazine, pyrazole-curcumin (**5**) (3,5-bis[β-(4-hydroxy-3-methoxyphenyl)ethenyl]pyrazole) was obtained as a pale yellow amorphous solid in 84% yield, after purification by dry column flash chromatography using TLC grade silica gel-G and chloroform - ethyl acetate (2:1) as eluent (Scheme 1); mp 208-10 °C. Lit. mp 211-4 °C[40,41]; 215 °C.[42] Anal. Calcd for $C_{21}H_{20}N_2O_4$: C, 69.22; H, 5.53; N, 7.69. Found: C, 69.39; H, 5.38, N, 7.74; IR (KBr): 1595, 1512, 1274, 1031 and 817 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 3.93 (s, 6H), 6.57 (s, 1H), 6.89 (d, J = 16 Hz, 2H), 6.80–7.20 (m, 8H). FAB MS (NBA matrix) m/z: 365.71 $[M+H]^+$. Calcd for $[C_{21}H_{20}N_2O_4+H]$: m/z 365.15.

2.1.4. Synthesis of isoxazole-curcumin (3,5-bis[β-(4-hydroxy-3-methoxyphenyl)ethenyl]isoxazole) (6)

Curcumin I, separated from the natural mix, was mixed with hydroxylamine hydrochloride (1 mmol each) in gl. acetic acid (2 mL) and the mixture was subjected to microwave irradiation (300 W, 2 min) allowing 15 s heating-cooling cycles to prevent boil-offs. Subsequently, the mixture was diluted with water (10 mL), the precipitate was filtered and the crude product so obtained was purified by dry column flash chromatography, using TLC grade silica gel-G and chloroform - ethyl acetate (1:1) as eluent (Scheme 1), to obtain isoxazole-curcumin (3,5-bis[β-(4-hydroxy-3-methoxyphenyl)ethenyl]isoxazole) (**6**) as a reddish brown amorphous solid in 83% yield; mp 158–159 °C, Lit. mp 162 °C;[40] 172 °C.[43] Anal. Calcd for $C_{21}H_{19}NO_5$: C, 69.03; H, 5.24; N, 3.83. Found: C, 68.83; H, 5.04; N, 3.66; ^1H NMR (300 MHz, $CDC1_3$): δ 3.95 (s, 6H); 6.48 (d, J = 15.6 Hz, 1H); 6.51 (s, lH), 6.78–7.11 (m, 8H), 7.59 (d, J = 15.9 Hz,1H). FAB MS (NBA matrix) m/z: 366.53 $[M+H]^+$. Calcd for $[C_{21}H_{19}NO_5+H]$: m/z 366.14.

2.2. Biological experiments and assays

Insect cell culture medium (IPL-41) was from Sigma Chemicals, USA. Bac-to-bac baculovirus expression kit was from GIBCO-BRL/Invitrogen, USA. [γ-^{32}P] ATP was from Bhabha Atomic Research Centre, India. pGEX-2T vector was from Amersham (GE).

2.2.1. Expression of GST-fusion protein of NR2A substrate sequence in *Escherichia coli*

Using the construct in pGEX-2T vector, the fusion protein, GST-NR2A (NR2A amino acid residues 1265-1301), was expressed in *Escherichia coli* (BL21 (DE3) pLys strain) and was purified as described previously.[44] Glutathione was removed from the purified GST-NR2A by PD-10 column. The purified fusion protein was used for the phosphorylation assay. The fusion protein was run on SDS-PAGE[45] and the amount of protein was determined as described previously.[44]

2.2.2. Expression and purification of α-CaMKII using baculovirus and Sf21 or High Five cell system

α-CaMKII was expressed using baculovirus expression system, in monolayer cultures of Sf21 or High Five cells. The α-CaMKII protein was purified by ion-exchange chromatography followed by affinity chromatography as described previously.[44,46] The purified enzyme preparation showed a single major band of the expected size on 10% SDS PAGE (Fig. 3A) and Western blot (Fig. 3B).[47] The final purified sample was concentrated using Amicon ultracentrifugal devices with 100 kDa molecular weight cut-off by centrifuging at 5000 $\times g$ to get the required concentration.

2.2.3. Preparation of postsynaptic density (PSD) fraction from rat brain

The PSD fraction was prepared from five male Albino Wistar male rats aged 40–45 days weighing an average of about 100 g. The animals were maintained at the animal house of Rajiv Gandhi Centre for Biotechnology and experiments conducted were in accordance with guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India and Institutional Animal Ethics Committee. Rats were sacrificed by cervical dislocation. Brains were gathered immediately and were homogenized in solution A (0.32 M sucrose, 1 mM sodium bicarbonate, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM PMSF, 1 μg/mL leupeptin) in a Teflon glass homogenizer by 12 up and down strokes. 4 mL of solution A was used per gram weight of the brain tissue. The homogenate was diluted to 10% (weight/volume) in solution A and was centrifuged at 1400 $\times g$ for 10 min at 4 °C. The supernatant was collected and stored. The pellets were resuspended in solution

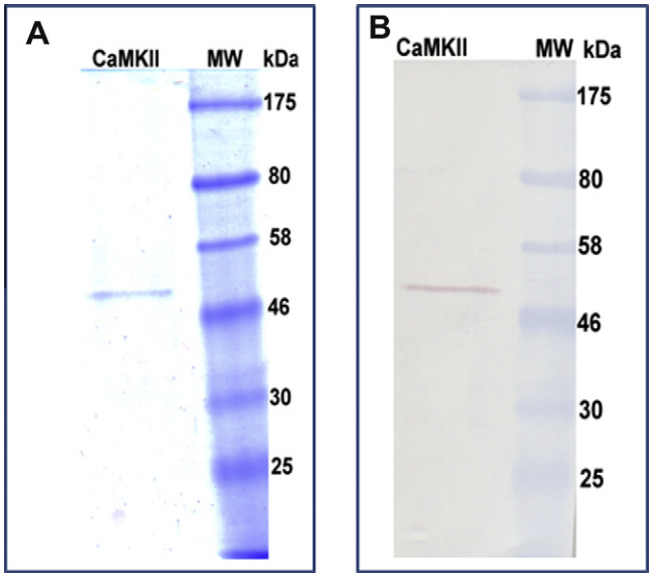


Figure 3. A. 10% SDSPAGE of purified α-CaMKII (4 μg) after staining with Coomassie Brilliant Blue and B. Western blot of purified α-CaMKII (2 μg) using anti-αCaMKII monoclonal antibody. MW represents molecular weight marker.

A (10% weight/volume) by homogenization by three strokes and were centrifuged at 710 ×*g* for 10 min. The two supernatants were pooled and were centrifuged at 13,800 ×*g* for 10 min. The pellet obtained was suspended in solution B (0.32 M sucrose, 1 mM sodium bicarbonate) in a ratio of 2.4 mL per gram weight of the brain tissue. Sucrose gradient was prepared in a 40 ml tube by layering 10 mL each of 1.2 M sucrose, 1.0 M sucrose, and 0.85 M sucrose containing 1 mM sodium bicarbonate. The gradient was constituted with highest concentration at the bottom and the lowest at the top. The pellet suspended in solution B was layered on top of the sucrose gradient and was centrifuged at 82,500 ×*g* for 2 h in a swinging bucket rotor. After centrifugation the band between 1.2 M and 1.0 M concentrations was collected and was diluted with solution B (6 mL per gram weight of the tissue). An equal volume of 1% Triton X-100 in 0.32 M sucrose, 12 mM Tris–HCl (pH 8.1) was added and the mixture was stirred in the cold for 15 min. The mixture was centrifuged at 36,800 ×*g* for 45 min. The pellet was suspended in 3 mL of 40 mM Tris pH 8.0, and was used as the PSD fraction.

2.2.4. Preparation of cytosolic fraction from rat brain

Albino Wistar male rat was sacrificed by cervical dislocation. Forebrain was collected immediately and was homogenized in 20 mM Tris, pH 7.0, 0.1 mM DTT, 0.1 mM PMSF and 1X protease inhibitor cocktail. The homogenate was clarified by centrifugation at 100,000 ×*g* for 30 min. The supernatant was used as the cytosolic fraction.

2.2.5. Fusion protein phosphorylation

The purified GST-NR2A (NR2A amino acid residues 1265–1301) was used as the substrate for CaMKII in an in vitro assay. The reaction mix containing a total volume of 20 μL including 50 mM Tris pH 8.0, 10 mM $MgCl_2$, 12 μM CaM or 2500 Units/mL of CaM, 0.2 mg/mL BSA, 1.3 mM $CaCl_2$, 10 mM DTT and varying concentrations of curcumin or its analogues was incubated with 0.02 μg of WT-α-CaMKII for 1 min at 30 °C. Curcumin was dissolved in DMSO before addition to the reaction mix. Controls received the same amount of DMSO. The reaction was initiated by the addition of GST-NR2A and 50 μM [γ-^{32}P] ATP (200–1500 cpm/pmol). The reaction mix was incubated for 1 min at 30 °C. The phosphorylation was terminated by the addition of 5 μL of 5× SDS sample buffer. The samples were subjected to 10% SDS–PAGE. The dried gel was exposed to phosphor screen. Single band corresponding to the phosphorylated fusion protein could be visualized on scanning the phosphor screen using the Bio-Rad Personal Molecular Imager FX and the band intensities were quantitated using Quantity One software (Bio Rad). Activity was calculated from the band intensity values as described earlier.[44] The data from at least three independent experiments were plotted using Origin software to calculate IC_{50} values.

2.2.6. Autophosphorylation of CaMKII

Thr^{286}-autophosphorylation of CaMKII was carried out in a reaction mix containing 50 mM Tris pH 8.0, 10 mM $MgCl_2$, 1.3 mM $CaCl_2$, 4000 Units/mL of CaM, 0.2 mg/mL BSA and 50 μM [γ-^{32}P] ATP (1000–2000 cpm/pmol), with or without different concentrations of curcumin or its analogues in a total volume of 20 μL. Autophosphorylation assay was performed by pre-incubating the purified WT-α-CaMKII enzyme containing assay mixture without ATP for 30 s at 30 °C followed by a 30 s phosphorylation reaction, initiated by the addition of [γ-^{32}P] ATP. The reaction was stopped by the addition of 5× sample buffer and the samples were run on SDS–PAGE and were processed as described in the previous section. The 50 kDa band corresponding to α-CaMKII was quantitated. Autophosphorylation at Thr^{286} was confirmed by the generation of Ca^{2+}-independent activity.

2.2.7. Ca^{2+}-independent activity

Autophosphorylation was carried out as described in the previous section using nonradioactive ATP. Reaction was stopped by adding EGTA to a final concentration of 3 mM. The Ca^{2+}-independent activity of the autophosphorylated enzyme for substrate phosphorylation was carried out as described previously, but keeping EGTA concentration to 5 mM in excess of Ca^{2+}.

3. Results and discussion

3.1. Chemical synthesis

Several synthetic approaches to curcumins, their analogs and derivatives, especially for investigating the biological activities, have appeared.[48] These methods generally involve prolonged heating for several hours. We have now shown that curcumin I (**1**) could be synthesized (Scheme 1A) from vanillin, acetylacetone and boric acid in a double Knoevenagel condensation under microwave conditions in a shorter reaction duration.

We have further shown that the microwave assisted synthetic approach also provided a rapid method for the synthesis of three analogs of curcumin I: phenylpyrazole-curcumin (**4**) (CNB-001; 3,5-bis[β-(4-hydroxy-3-methoxyphenyl)ethenyl]-1-phenylpyrazole), pyrazole-curcumin (**5**) (3,5-bis[β-(4-hydroxy-3-methoxyphenyl)ethenyl]pyrazole) and isoxazole-curcumin (**6**) (3,5-bis[β-(4-hydroxy-3-methoxyphenyl)ethenyl]isoxazole), starting from curcumin I separated from natural curcumin mix (Scheme 1B), in good yield in few minutes, instead of heating for 6–24 h as reported in literature.[40,42,43]

3.2. Biological assay

3.2.1. Curcumin inhibits CaMKII activity

In our attempt to identify modulators of CaMKII from natural products, we observed natural curcumin to be an inhibitor of the kinase activity of CaMKII. We then separated the three components of natural curcumin mix (Fig. 1). Activity of CaMKII was measured using a protein substrate, GST-NR2A, which harbors the sequence

around the CaMKII phosphorylation site, Ser^{1291}, in the sequence of the NR2A subunit of NMDAR.[49] The Ca^{2+}-dependent activity of purified CaMKII (Fig. 3) for the phosphorylation of a protein substrate GST-NR2A, is inhibited by the components of natural curcumin, curcumin I (**1**) (Fig. 4), II (**2**) and III (**3**) (Table 1). The inhibitory potency of the three components did not differ markedly, curcumin I being the most potent inhibitor among the three. Hence, detailed investigations were carried out using curcumin I. To rule out the possibility that curcumin I, II and III chromatographically separated from the natural curcuminoid mixture, may contain minor amounts of any coeluted compounds which could be inhibiting CaMKII, the inhibitory activity of synthetically prepared curcumin I was checked. Curcumin I made synthetically (Scheme 1A) also inhibited Ca^{2+}-dependent activity of CaMKII (Table 1). Synthetic curcumin I however showed only a slightly higher inhibitory potency than that of curcumin I isolated from the natural mix. The inhibition of CaMKII by curcumin I was also found to be partially reversible (data not shown).

3.2.2. Effect of curcumin I (1) on autophosphorylation

Some of the inhibitors currently available inhibit only Ca^{2+}-stimulated activity of CaMKII, but do not inhibit autonomous activity of CaMKII.[21] Curcumin is now seen to be effective as an inhibitor of autophosphorylation of CaMKII as well. In spite of the fact that autophosphorylation of CaMKII is essential for the enzyme to function as a memory molecule, autophosphorylation also occurs during restrained stress[50] and after traumatic brain injury[51] in hippocampal neurons.

It was found that inhibition of CaMKII autophosphorylation required a higher concentration of curcumin than that was required for substrate phosphorylation. Further, both the Ca^{2+}-dependent and Ca^{2+}-independent activities of autophosphorylated CaMKII were inhibited by curcumin I (**1**) (Table 2). The Ca^{2+}/CaM-independent activitiy of T286D-α-CaMKII mutant, which is an autonomously active, autophosphorylation mimicking mutant form, was also inhibited by curcumin I (**1**) (Table 2). Since calmodulin is not required for Ca^{2+}-independent activity, we concluded that curcumin I (**1**) must be binding directly to CaMKII, and subsequently inferred that the inhibition of curcumin on CaMKII is independent of calmodulin binding. It was also seen that the inhibition of the Ca^{2+}-dependent activity of CaMKII by curcumin prevailed even at higher concentrations of calmodulin (data not shown).

3.2.3. Effect of curcumin I (1) on CaMKII in the cytosol and postsynaptic density

CaMKII, being one of the most abundant proteins in the brain, is found to be distributed both in the cytosol and localized at the postsynaptic density (PSD) (Fig. 5A). In order to understand the effect of curcumin on CaMKII in a more physiologically relevant state, we have investigated the effect of curcumin on PSD and cytosolic fractions of CaMKII. Both the cytosolic and PSD fractions have autophosphorylated and non-autophosphorylated forms of CaMKII (Fig. 5), among which the autophosphorylated forms can exhibit both Ca^{2+}/CaM-dependent and Ca^{2+}/CaM-independent activity. The presence of autonomous CaMKII in cytosolic and PSD fractions were confirmed by immunoblot probed with anti-phospho-Thr^{286}-CaMKII antibody (Fig. 5B). Curcumin I inhibited both Ca^{2+}/CaM-dependent and Ca^{2+}/CaM-independent activities of CaMKII in PSD (Table 3) and cytosol (Table 4). Autophosphorylation of CaMKII in cytosolic and PSD fractions was also inhibited, but with slightly higher IC_{50} values (Table 3 and 4).

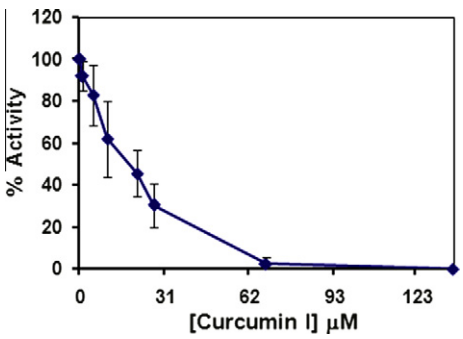


Figure 4. Effect of curcumin I on Ca^{2+}/CaM-dependent activitiy of α-CaMKII. Dose response plot as function of the concentration of curcumin I, is shown. Data from four experiments is used to calculate the mean and standard deviation shown the figure. The IC_{50} value for each experiment was calculated separately and the mean and standard deviation were calculated and are shown in the tables.

Table 1
Inhibition of Ca^{2+}-dependent activity of purified CaMKII[a]

Compound	IC_{50} μM
Curcumin I[b]	21 ± 3.5
Curcumin II	24 ± 7.0
Curcumin III	37 ± 19.6
Synthetic Curcumin I	13 ± 4.5

[a] The IC_{50} value for each experiment was analyzed separately and the mean and standard deviation from a minimum of three experiments were calculated and are shown in the table.
[b] Indicates that the same data is shown in Table 2 also.

Table 2
Inhibition of activity of purified CaMKII by natural curcumin I[a]

Type of CaMKII activity studied	IC_{50} μM
Ca^{2+}-dependent activity of CaMKII[b]	21 ± 3.5
Ca^{2+}-dependent activity of autophosphorylated CaMKII	45 ± 6.7
Ca^{2+}-independent activity of autophosphorylated CaMKII	34 ± 4.5
CaMKII autophosphorylation	33 ± 9.0
Ca^{2+}-independent activity of T286D-α-CaMKII	37 ± 6.3

[a] The IC_{50} value for each experiment was analyzed separately and the mean and standard deviation from a minimum of three experiments were calculated and are shown in the table.
[b] Indicates that the data shown is the same as in Table 1.

3.3. Effect of phenylpyrazole-curcumin (4) on CaMKII

A heterocyclic analog of curcumin, named CNB-001, was shown to facilitate the induction of LTP in rat hippocampal slices, to enhance memory in rat in an object recognition test and also to enhance CaMKII activity in vitro of primary cortical neurons and of CaMKII activity in a commercial assay kit.[38] CNB-001 has also been reported to show broad neuroprotective activity based on cell culture studies where the cells were subjected to diverse toxic insults.[52] The ability of CNB-001 to cross blood brain barrier and its bioavailability upon oral administration have also been established.[52] Another report indicated that curcumin blocked the corticosterone induced elevation of phospho-CaMKII and NR2B levels in hippocampal neurons.[37] In the light of these studies, we investigated the effect of phenylpyrazole-curcumin (**4**) on CaMKII. The phenylpyrazole-curcumin (**4**) that we synthesized is structurally identical to CNB-001, reported before.[38,52] Contrary to the above report, which indicates that CNB-001 activates CaMKII, we now find that Ca^{2+}-stimulated activity of CaMKII is inhibited by phenylpyrazole-curcumin (**4**), based on our in vitro biochemical assay (Table 5). Autophosphorylation, as well as Ca^{2+}-dependent and Ca^{2+}-independent activities of autophosphorylated CaMKII, are inhibited by phenylpyrazole-curcumin (**4**) (Table 5). Autophosphoryltion and Ca^{2+}-dependent and Ca^{2+}-independent activities of CaMKII in cytosolic and in PSD fractions are also inhibited by phenylpyrazole-curcumin (**4**) (Table 5).

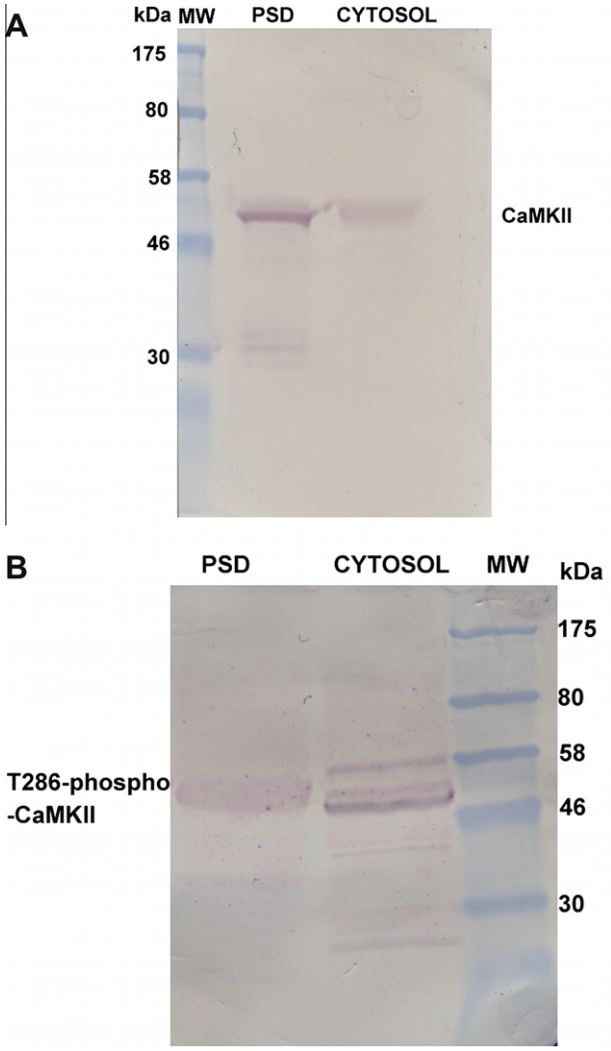


Figure 5. Western blot showing the presence of A: CaMKII in PSD (40 μg) and cytosolic fractions (30 μg) of rat brain and B: Thr^{286}-phospho-CaMKII in PSD (40 μg) and cytosolic fractions (30 μg) of rat brain. MW represents molecular weight marker.

Table 3
Inhibiton of activity of CaMKII in PSD by natural curcumin I[a]

Type of CaMKII activity studied	IC_{50} μM
Ca^{2+}-dependent activity of CaMKII in PSD	80 ± 15.6
Ca^{2+}-independent activity of CaMKII in PSD	33 ± 6.3
Autophosphorylation of CaMKII in PSD	69 ± 3.3

[a] The IC_{50} value for each experiment was analyzed separately and the mean and standard deviation from a minimum of three experiments were calculated and are shown in the table.

Table 4
Inhibiton of activity of CaMKII in cytosol by natural curcumin I[a]

Type of CaMKII activity studied	IC_{50} μM
Ca^{2+}-dependent activity of CaMKII in cytosol	112 ± 22.8
Ca^{2+}-independent activity of CaMKII in cytosol	64 ± 15.9
Autophosphorylation of CaMKII in cytosol	143 ± 27.9

[a] The IC_{50} value for each experiment was analyzed separately and the mean and standard deviation from a minimum of three experiments were calculated and are shown in the table.

Table 5
Inhibition of activity of CaMKII by phenylpyrazole-curcumin, pyrazole- curcumin and isoxazole-curcumin

Type of CaMKII activity studied	IC_{50} μM
Phenylpyrazole-curcumin	
Ca^{2+}-dependent activity of CaMKII	37 ± 6.40
Ca^{2+}-dependent activity of autophosphorylated CaMKII	79 ± 9.37
Ca^{2+}-independent activity of autophosphorylated CaMKII	54 ± 8.5
CaMKII autophosphorylation	111 ± 11.49
Cytosol CaMKII Ca^{2+}-dependent activity	94 ± 27.78
Cytosol CaMKII Ca^{2+}-independent activity	96 ± 22.16
Cytosol CaMKII autophosphorylation	80 ± 34.2
PSD CaMKII Ca^{2+}-dependent activity	103 ± 6.98
PSD CaMKII Ca^{2+}-independent activity	62 ± 12.95
PSD CaMKII autophosphorylation	68 ± 26.93
Pyrazole-curcumin	
Ca^{2+}-dependent activity of CaMKII	1.49 ± 0.46
Ca^{2+}-dependent activity of autophosphorylated CaMKII	6.3 ± 2.80
Ca^{2+}-independent activity of autophosphorylated CaMKII	0.29 ± 0.05
CaMKII autophosphorylation	6.5 ± 3.38
Cytosol CaMKII Ca^{2+}-dependent activity	4.4 ± 1.90
Cytosol CaMKII Ca^{2+}-independent activity	0.44 ± 0.023
Cytosol CaMKII autophosphorylation	16.32 ± 1.63
PSD CaMKII Ca^{2+}-dependent activity	3.6 ± 1.08
PSD CaMKII Ca^{2+}-independent activity	0.34 ± 0.33
PSD CaMKII autophosphorylation	7.58 ± 2.43
Isoxazole-curcumin	
Ca^{2+}-dependent activity of CaMKII	11.37 ± 4.08
Ca^{2+}-dependent activity of autophosphorylated CaMKII	24.44 ± 0.96
Ca^{2+}-independent activity of autophosphorylated CaMKII	0.68 ± 0.04
CaMKII autophosphorylation	18.01 ± 1.61
Cytosol CaMKII Ca^{2+}-dependent activity	28.9 ± 1.79
Cytosol CaMKII Ca^{2+}-independent activity	13.68 ± 2.75
Cytosol CaMKII autophosphorylation	17.10 ± 4.84
PSD CaMKII Ca^{2+}-dependent activity	22.87 ± 6.06
PSD CaMKII Ca^{2+}-independent activity	18.88 ± 4.59
PSD CaMKII autophosphorylation	22.79 ± 4.23

The IC_{50} value for each experiment was analyzed separately and the mean and standard deviation from a minimum of three experiments were calculated and are shown in the table.

3.4. Effect of pyrazole-curcumin (5) on CaMKII

Since phenylpyrazole-curcumin (**4**) inhibited CaMKII activity, we next decided to investigate whether the simpler, unsubstituted, pyrazole-curcumin (**5**) would have any effect on CaMKII. This was investigated because the effect of pyrazole-curcumin (**5**) on CaMKII has not been hitherto studied. Further, compared to phenylpyrazole-curcumin (**4**), pyrazole-curcumin (**5**) would be more similar in structure to natural curcumin-1 (**1**), as pyrazole-curcumin (**5**) lacks the bulky phenyl group. In addition, pyrazole-curcumin (**5**) is a hydrogen bond donor through its NH group and would be less hydrophobic (Fig. 6). We find that the Ca^{2+}-dependent activity of CaMKII is inhibited by pyrazole-curcumin (**5**) with a much lower IC_{50} (Table 5) compared to phenylpyrazole-curcumin. Autophosphorylation of CaMKII is also inhibited by pyrazole-curcumin (**5**), but with a higher IC_{50} value compared to that of the Ca^{2+} stimulated substrate phosphorylation activity (Table 5) of the non-autophosphorylated enzyme. Both Ca^{2+}-dependent and Ca^{2+}-independent activities of autophosphorylated CaMKII are inhibited by pyrazole-curcumin (**5**). Interestingly, pyrazole-curcumin (**5**) is a more potent inhibitor of the Ca^{2+}-independent activity than that of the Ca^{2+}-dependent activity (Table 5). Autophosphorylation and Ca^{2+}-dependent substrate phosphorylation activity are both dependent on the presence of Ca^{2+}/calcmodulin and thus are likely to arise from a similar conformational state of the enzyme. The Ca^{2+}-independent activity arises from the enzyme that is not having significant amounts of bound Ca^{2+}/calmodulin and hence, may be in a different conformational state, which could be more sensitive to pyrazole-curcumin. Pyrazole-curcumin inhibited

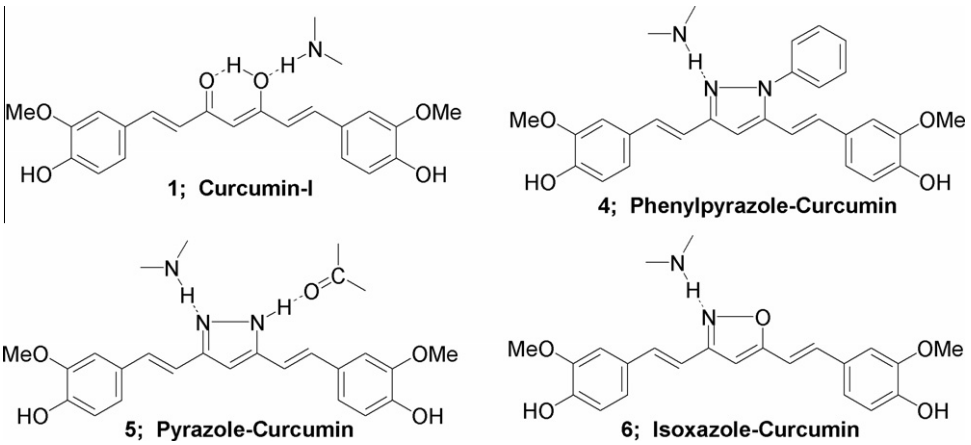


Figure 6. Representation of hydrogen bonding possible in curcumin I (**1**) and its heterocyclic derivatives (**4**–**6**).

autophosphorylation of CaMKII in PSD and cytosolic fractions. Both Ca^{2+}-dependent and Ca^{2+}-independent activities of CaMKII in PSD and cytosolic fractions are inhibited by pyrazole-curcumin (Table 5).

3.5. Effect of isoxazole-curcumin (6) on CaMKII

Subsequent to the investigations described above, we next examined the effect of isoxazole-curcumin (**6**) on CaMKII as isoxazole ring is isosteric with pyrazole ring. This study showed that Ca^{2+}-dependent activity of CaMKII is inhibited by isoxazole-curcumin (**6**) with an IC_{50} value which is higher than that is observed for pyrazole-curcumin (**5**) (Table 5). Autophosphorylation, Ca^{2+}-dependent and Ca^{2+}-independent activities of autophosphorylated CaMKII are inhibited by isoxazole-curcumin (**6**) (Table 5). We find that, even in the case of isoxazole-curcumin, the Ca^{2+}-independent activity of autophosphorylated CaMKII is more sensitive compared to its Ca^{2+}-dependent activities similar to pyrazole-curcumin. Isoxazole-curcumin (**6**) inhibited the autophosphorylation of CaMKII in PSD and cytosolic fractions and also the Ca^{2+}-dependent and Ca^{2+}-independent activities as well (Table 5).

The structure of curcumin is characterized by two *ortho*-methoxyphenolic rings connected by linear C7 carbon chain bearing three *trans* carbon–carbon double bonds, an oxo function, an enol moiety and an enone group. It has been suggested that the termini are hydrophilic whereas the middle carbon chain portion is very hydrophobic, an observation that would substantiate the extreme water insolubility of curcuminoids. Spectroscopic and crystallographic evidence have shown the central 1,3-diketone unit exist exclusively as an intramolecularly hydrogen bonded 1,3-keto-enol unit which would thus provide much rigidity to the overall linear structure of the molecule. Such a rigidity can further be bolstered by converting the central 1,3-diketone ⇆ 1,3-keto-enol unit into a pyrazole or an isoxazole ring. This heterocyclization of curcumin I to pyrazole-curcumin would also alter the hydrogen bonding ability of this central part of the curcumin structure. Thus, in curcumin, the central intramolecularly hydrogen bonded 1,3-keto-enol could form one hydrogen bond through the enol oxygen, acting as an hydrogen bond acceptor, to an external protein molecule (Fig. 6). Such would be the case in phenylpyrazole-curcumin, and in isoxazole-curcumin. In contrast, pyrazole-curcumin is capable of forming two hydrogen bonds externally. These possibilities are schematically shown in Figure 6, which imply that, other parameters that affect molecular recognition being the same, the pyrazole-curcumin could form a better hydrogen bonding. The higher inhibitory effect of pyrazole-curcumin may partly be ascribed to its ability to form better binding to CaMKII.

4. Concluding remarks

CaMKII is a downstream component of the neuronal glutamate signaling pathway and is deemed to be involved in the route to glutamate-induced excitotoxicity. Hence acute inhibition of CaMKII may be a potential strategy for providing neuroprotection as shown by two independent groups using the CaMKII inhibitor tat-CN21 peptide.[21,53] We now report that curcumin and its analogues show inhibition of the Ca^{2+}-dependent and Ca^{2+}-independent activities of CaMKII in vitro. Among the three cyclic derivatives, pyrazole-curcumin (**5**) is most effective as an inhibitor of CaMKII, even better than the parent curcumin I. Further, pyrazole-curcumin (**5**) is more effective on Ca^{2+}-independent "autonomous" activity in the case of purified CaMKII and also in PSD and cytosolic CaMKII fractions. Thus, pyrazole-curcumin (**5**) as well as natural curcumin I (**1**) could offer neuroprotective action in the excitotoxic cascade by inhibiting CaMKII activity. On the basis of the above observations, it will be interesting to study further the CaMKII signaling pathways that are altered by curcumin.

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