Specificity of protein kinase inhibitor peptides and induction of long-term potentiation

(Ca²⁺/calmodulin-dependent protein kinase II/hippocampus/plasticity/protein kinase C)

ØIVIND HVALBY*, HUGH C. HEMMINGS, JR.^{†‡}, OLE PAULSEN*, ANDREW J. CZERNIK[†], ANGUS C. NAIRN[†], JEAN-MARIE GODFRAIND[§], VIDAR JENSEN*, MORTEN RAASTAD*, JOHAN F. STORM*, PER ANDERSEN*[¶], AND PAUL GREENGARD[†]

*Institute of Neurophysiology, University of Oslo, P.O. Box 1104, Blindern, N-0317 Oslo, Norway; [†]Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, New York, NY 10021; [‡]Departments of Anesthesiology and Pharmacology, Cornell University Medical College, New York, NY 10021; and [§]Department of Physiology and Pharmacology, Catholic University of Louvain, B-1200 Bruxelles, Belgium

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ABSTRACT Previous studies have used synthetic peptide analogs, corresponding to sequences within the pseudosubstrate domain of protein kinase C (PKC) or the autoregulatory domain of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), in attempts to define the contribution of each of these protein kinases to induction of long-term potentiation (LTP). However, the specificity of these inhibitor peptides is not absolute. Using intracellular delivery to rat CA1 hippocampal neurons, we have determined the relative potency of two protein kinase inhibitor peptides, PKC-(19–36) and [Ala²⁸⁶]CaMKII-(281–302), as inhibitors of the induction of LTP. Both peptides blocked the induction of LTP; however, PKC-(19–36) was 30-fold more potent than [Ala²⁸⁶]CaMKII-(281–302). The relative specificity of PKC-(19–36), [Ala²⁸⁶]CaMKII-(281–302), and several other CaMKII peptide analogs for protein kinase inhibition *in vitro* was also determined. A comparison of the potencies of PKC-(19–36) and [Ala²⁸⁶]CaMKII-(281–302) in the physiological assay with their *K_i* values for protein kinase inhibition *in vitro* indicates that the blockade of induction of LTP observed for each peptide is attributable to inhibition of PKC.

Long-term potentiation (LTP) has been widely used as a cellular model of learning (1, 2). Among the biochemical mechanisms that contribute to this phenomenon, the activity of specific protein kinases appears to play a critical role (3–19). Early studies demonstrated that extracellular application of nonselective protein kinase inhibitors, such as H-7, polymyxin B, or sphingosine, to hippocampal slice preparations could block induction of LTP (5–7). Subsequently, more compelling evidence that specific protein kinases were involved in the induction mechanism was provided by intracellular injection of various kinase inhibitors (8, 9, 11, 14). Several of these studies examined the effect of synthetic peptide analogs modeled after autoinhibitory domains, which are present in second messenger-regulated protein kinases (20, 21). While these “pseudosubstrate” peptides were developed as selective protein kinase inhibitors (8, 9, 22–27), they have been found to exhibit multiple modes of action that vary with their exact structure. For example, peptides corresponding to different residues within the autoregulatory domain of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) can inhibit catalytic activity by one or more of the following mechanisms: (i) inhibiting the binding of ATP; (ii) inhibiting the binding of peptide or protein substrates; and (iii) directly binding to Ca²⁺/calmodulin, thereby functioning as a calmodulin antagonist (23–27).

Based on the reported selectivity of several of these protein kinase inhibitor peptides, it has been suggested that protein

kinase C (PKC) (14), CaMKII (8), or both (9) are required for induction of LTP. However, these studies did not examine in detail the relative potencies of the injected peptides for the blockade of LTP. Moreover, it is now apparent that the specificity of these pseudosubstrate inhibitor peptides is not absolute (19, 22, 28). Therefore, we have now conducted a combined physiological and enzymological study with two protein kinase inhibitor peptides, PKC-(19–36) and [Ala²⁸⁶]CaMKII-(281–302), in an effort to determine the contributions of PKC and CaMKII to induction of LTP in CA1 pyramidal cells in rat hippocampal slices.

MATERIALS AND METHODS

Peptides. Substrate and inhibitor peptides used in the physiological and enzymological studies were synthesized and purified to >95% purity by reversed-phase HPLC by the Keck Foundation Biotechnology Resource Laboratory at Yale University, except where noted. The identity of each peptide was confirmed by amino acid analysis and mass spectroscopy. The protein kinase inhibitor peptides [Ala²⁸⁶]CaMKII-(281–302) (MHRQEAVDCLKKFNARRK-LKGA) and PKC-(19–36) (RFARKGALRQKNVHEVKN-amide) were used in both studies; the enzymological studies also used the following analogs of CaMKII inhibitor peptides: [Ala²⁸⁶]CaMKII-(273–302) (HRSTVASCMMHRQEAVDCL-KKFNARRKLKGA; one sample prepared at Yale University and another generously provided by H. Schulman, Stanford University), CaMKII-(273–302) (HRSTVASCMMHR-QETVDCLKKFNARRKLKGA; generously provided by H. Schulman), and acetyl-CaMKII-(273–302) (generously provided by A. Silva, Cold Spring Harbor Laboratory). Synapsin I-(3–13) (YLRRRLSDSNF-amide) was used as the substrate for PKC and bovine [Tyr⁵⁸⁶]synapsin I-(586–609) (YRQGP-QKPPGPAGPTRQASQAGP-amide) was used as the substrate for CaMKII. Peptides were dissolved in H₂O and cleared by centrifugation; their concentrations were determined by quantitative amino acid analysis.

Electrophysiology. Male Wistar rats (150–300 g) were anesthetized with diethyl ether and sacrificed by decapitation. The brain was removed and cooled in artificial cerebrospinal fluid (124 mM NaCl/2 mM KCl/1.25 mM KH₂PO₄/2 mM CaCl₂/2 mM MgSO₄/26 mM NaHCO₃/10 mM glucose, pH 7.4, saturated with 95% O₂/5% CO₂ and kept at 2–4°C) and the hippocampi were dissected out. Transverse slices (0.4 mm thick) were cut and placed in an interface chamber exposed to 95% O₂/5% CO₂ and maintained at a temperature

FIG. 1. Electrophysiological recording from hippocampal slices. (A) Schematic diagram of the electrode arrangement. Two extracellular stimulation electrodes, one in stratum radiatum (rad) and one in stratum oriens (or), were stimulated alternately. One electrode served as the tetanizing input; the other served as the control input. The responses elicited from a large population of CA1 neurons were monitored by an extracellular electrode placed in the stratum radiatum. The response of a single neuron was recorded by an intracellular electrode located in the cell body. The stippled region shown within the intracellular electrode and the impaled cell symbolizes the peptide. (B) Representative intracellular and extracellular recordings. Six superimposed responses recorded intra- and extracellularly (Upper and Lower, respectively) after stimulation of the stratum radiatum input and the stratum oriens input before injection of the inhibitor peptide. Note that the extracellular stratum oriens responses are well reflected in the recording electrode placed in the stratum radiatum.

of $32^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Intracellular recordings were obtained from CA1 pyramidal cells with micropipettes (filled with 2.0 M KOAc; 40–90 M Ω), whose tips were filled with various concentrations of PKC-(19–36) or [Ala²⁸⁶]CaMKII-(281–302), dissolved in 2.0 M KOAc (pH 7.0), with the micropipette shafts backfilled with 2.0 M KOAc. After impalement, a steady hyperpolarizing current was often injected during the first minutes of recording to stabilize the responses. An extracellular microelectrode (filled with 1.0 M NaCl; 5–30 M Ω) was placed in the stratum radiatum in order to record field responses signaling synaptic activity of a large number of cells surrounding the impaled neuron (Fig. 1A). Orthodromic synaptic stimulation was delivered alternately to two independent pathways, each at 0.25 Hz. Intra- and extracellular synaptic responses were assessed by measuring the slope of the middle one-third of the excitatory postsynaptic potential (EPSP) rising phase. After a stable baseline for >15 min was obtained, tetanic stimulation was delivered to one of the pathways (16 stimuli at 100 Hz, in 15 series at intervals of 5 s), while activation of the other served as a control. Sixty-nine CA1 pyramidal cells met the selection criteria (membrane potential more negative than -60 mV, spike amplitude >85 mV, recording time after tetanization >45 min). The mean values ($\pm$ SD and range) were as follows: membrane potential, -70 ± 7 mV (-60 to -80 mV); action potential amplitude, 102 ± 9 mV (85 – 120 mV). The experiments included in the analysis were those in which the increase of the extracellular field EPSP slope surpassed 120% of the control value 30 min after the tetanization procedure (85% of the experiments).

In preliminary experiments, an attempt was made to retain the inhibitor peptides (positively charged at pH 7) in the intracellular recording electrode during the pretetanic period

by applying a steady negative holding current and to inject the peptides with depolarizing current pulses. However, the results indicated that the peptides diffused into the cell in spite of the retaining current. In the main series of experiments, the inhibitor peptides entered the cells by diffusion from the electrodes.

Phosphorylation Assays. PKC was purified from rat brain (29) and its activity was determined by using synapsin I-(3–13) as a substrate [20 – 200 μM ; $K_m = 100$ μM ; $V_{max} = 3.4$ $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ($n = 5$)] in a final reaction vol of 100 μl containing 50 mM Hepes (pH 7.6), 10 mM MgOAc, 1 mM EGTA, 1.5 mM CaCl₂, 50 μg of L- γ -phosphatidyl-L-serine per ml, 4 μg of 1,2-dioleoylglycerol per ml, 100 μM [γ -³²P]ATP (500–1000 cpm/pmol), in the absence or presence of protein kinase inhibitor peptides at 30°C . Reactions were terminated after 5 min by the addition of 10 μl of glacial acetic acid, and peptide phosphorylation was determined by phosphocellulose paper assays as described (30). CaMKII was purified from rat brain (31) and its activity was determined by using bovine [Tyr⁵⁸⁶]synapsin I-(586–609) as substrate [20 – 200 μM ; $K_m = 76$ μM ; $V_{max} = 6.8$ $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ($n = 6$)] in a final reaction vol of 100 μl containing 50 mM Hepes (pH 7.6), 10 mM MgOAc, 1 mM EGTA, 1.5 mM CaCl₂, 30 μg of bovine brain calmodulin per ml, 30 μg of bovine serum albumin per ml, and 100 μM [γ -³²P]ATP (500–1000 cpm/pmol) in the absence or presence of protein kinase inhibitor peptides at 30°C . Reactions were terminated after 1 min with 10 μl of glacial acetic acid and peptide phosphorylation was analyzed by phosphocellulose paper assays. Kinetic parameters were determined by linear regression analysis of double-reciprocal plots at each inhibitor concentration. K_i values were determined by linear regression analysis of secondary plots of K_m/V_{max} vs. inhibitor concentration. Neither

FIG. 2. LTP induction in control experiments. (A) Extracellular EPSP slopes evoked in the tetanized pathway (solid symbols) and in the untetanized control pathway (open symbols) are shown as the average of 10 experiments. (B) Corresponding intracellular EPSP measurements are shown for the experiments described in A. Each point represents 20 extracellular (circles) or intracellular (triangles) EPSP slope measurements within a time block of 80 s. The data were averaged and normalized with respect to the values obtained for the three last blocks (4-min period) before tetanic stimulation. Vertical bars indicate SEM. Arrows indicate the time of tetanic stimulation.

FIG. 3. Effect of PKC-(19-36) on LTP induction. Extracellular (A, C, and E) and intracellular (B, D, and F) normalized EPSP slope measurements given as averages from n experiments. Concentrations of PKC-(19-36) present in the tip of the intracellular recording electrodes are indicated in boxes. Experiments with 500 μM PKC-(19-36) in the tip ($n = 10$) gave results similar to those with 100 μM and are not shown. Solid symbols show tetanized pathway; open symbols show control pathway. Vertical bars indicate SEM. Arrows indicate the time of tetanic stimulation. Normalization and pooling procedures were as described in the legend to Fig. 2.

[Ala²⁸⁶]CaMKII-(281-302) nor PKC-(19-36) was phosphorylated by either PKC or CaMKII under the conditions described.

RESULTS AND DISCUSSION

Effects of Protein Kinase Inhibitor Peptides on Induction of LTP. The experimental protocol was to induce LTP in a population of hippocampal CA1 neurons, while protein kinase inhibitor peptides were delivered into single cells (Fig. 1). The synaptic activation of the impaled cell was compared to that of its neighboring cells. The responses of the cell population were monitored by an extracellular electrode, while the impaled cell was monitored by an intracellular electrode, through which the delivery of each inhibitor peptide was made.

In a total of 10 control experiments, dispersed throughout the experimental series, the intracellular recording electrode contained recording solution without peptide. Tetanic stimulation of afferent fibers, in either stratum radiatum or stratum oriens, elicited a persistent potentiation of the synaptic responses characteristic of LTP, both in the impaled cell and in the surrounding population of cells (Fig. 2). After high-frequency stimulation (arrows), there was a significant increase in the EPSP slope of the impaled cells (Fig. 2B, solid triangles) as well as in the neighboring population of cells (Fig. 2A, solid circles). Responses to the untetanized pathway were virtually unchanged (Fig. 2, open circles and triangles).

The effects of PKC-(19-36) and [Ala²⁸⁶]CaMKII-(281-302) on the induction of LTP were determined by using several concentrations of each peptide (Figs. 3 and 4). Grouped data

FIG. 4. Effect of [Ala²⁸⁶]CaMKII-(281-302) on LTP induction. Extracellular (A, C, and E) and intracellular (B, D, and F) normalized EPSP slope measurements given as averages from n experiments. Concentrations of [Ala²⁸⁶]CaMKII-(281-302) present in the tip of the intracellular recording electrodes are indicated in boxes. Solid symbols show tetanized pathway; open symbols show control pathway. Vertical bars indicate SEM. Arrows indicate the time of tetanic stimulation. Normalization and pooling procedures were as described in the legend to Fig. 2.

FIG. 5. Summary plot of the effects produced by protein kinase inhibitor peptides on induction of LTP. Concentration of peptide in the tip of the intracellular electrode is plotted on a logarithmic scale along the abscissa. The ratio between the increase of the intracellular and extracellular EPSP slope relative to the control values (potentiation index) is given on the ordinate. *, $P < 0.05$ (one-tailed Student's t test). The value for PKC-(19–36) at 500 μM did not reach the 5% significance level ($P = 0.06$) because induction of LTP was blocked in 7 of 10 cells only. Vertical bars indicate SEM.

from experiments in which the tip of the intracellular recording electrode contained a concentration of 10, 50, or 100 μM PKC-(19–36) are shown in Fig. 3. In all cases, the cell population exhibited a synaptic enhancement (Fig. 3 A, C, and E). With a concentration of 10 μM in the electrode tip, LTP induction was blocked in one of six impaled cells (Fig. 3B). When the electrode contained 50 μM PKC-(19–36), the induction of LTP was blocked in six of seven impaled cells (Fig. 3D). Nearly total blockade of LTP induction was also observed for PKC-(19–36) concentrations of 100 μM (Fig. 3F) or 500 μM (data not shown). The failure to elicit LTP in the impaled cells cannot be ascribed to a nonspecific synaptic depression or to deterioration of the intracellular recordings since transmission in the control pathway (without tetanus) was unchanged (Fig. 3 B, D, and F; open triangles).

The results from experiments in which cells were impaled with electrodes that contained 100, 500, or 3000 μM [Ala²⁸⁶]CaMKII-(281–302) are shown in Fig. 4. LTP was observed in the impaled cells when the intracellular electrode contained 100 or 500 μM [Ala²⁸⁶]CaMKII-(281–302). When the electrode contained 3000 μM [Ala²⁸⁶]CaMKII-(281–302), LTP was completely blocked. Again, in the control pathway, synaptic transmission was virtually unchanged (Fig. 4 B, D, and F; open triangles).

Dose-response curves for the blockade of LTP induction by the inhibitor peptides are presented in Fig. 5. Inhibition of LTP induction was expressed as the ratio between the increase in the intracellular and extracellular EPSP slopes measured 30 min after tetanization. PKC-(19–36) blocked LTP with an IC_{50} value ≈ 30 -fold lower than that observed with [Ala²⁸⁶]CaMKII-(281–302) (Fig. 5 and Table 1).

Inhibition of PKC and CaMKII by Protein Kinase Inhibitor Peptides. The potency and specificity of PKC-(19–36) and [Ala²⁸⁶]CaMKII-(281–302) as inhibitors of PKC and CaMKII were determined and these values were compared with the efficacy of the peptides as inhibitors of the induction of LTP

(Table 1). PKC-(19–36) was a potent inhibitor of PKC, with a K_i value of 0.28 μM , and exhibited a 20-fold selectivity for inhibition of PKC over CaMKII. [Ala²⁸⁶]CaMKII-(281–302) was less potent as an inhibitor of PKC and exhibited a selectivity of only 5- to 6-fold for inhibition of CaMKII over PKC.

Since neither peptide inhibitor exhibited absolute specificity *in vitro*, the K_i values for kinase inhibition and the IC_{50} values for LTP induction must be considered together to interpret the effects of the peptides on LTP. We have made the assumption that several sources of potential variation, such as delivery from the pipette, and the intracellular diffusion and degradation of the peptides, together with possible substrate-dependent variations in the efficacy of the inhibitors (28), did not significantly affect the results of the intracellular injection studies. If CaMKII, but not PKC, were involved in the induction of LTP, [Ala²⁸⁶]CaMKII-(281–302) would be expected, based on the ratio of K_i values (Table 1), to be ≈ 4 -fold more potent than PKC-(19–36) for blockade. However, PKC-(19–36) was estimated to be 33-fold more potent than [Ala²⁸⁶]CaMKII-(281–302) for inhibition of induction of LTP, a value in agreement with the PKC-(19–36)/[Ala²⁸⁶]CaMKII-(281–302) potency ratio of 32 calculated for inhibition of PKC *in vitro* (Table 1). Based on this comparison, we conclude that the effect of both peptides on LTP induction may be ascribed to inhibition of PKC activity.

Malinow *et al.* (9) concluded that both PKC and CaMKII activities were required for induction of LTP. Their argument for the involvement of CaMKII was based on the ability of a synthetic peptide, CaMKII-(273–302), to exhibit an apparent selectivity of >200 -fold for *in vitro* inhibition of CaMKII over PKC and to block the induction of LTP when injected intracellularly. Since our results with a related peptide, [Ala²⁸⁶]CaMKII-(281–302), had demonstrated only a 5-fold selectivity for CaMKII over PKC (Table 1), we tested a series of structurally related peptide analogs, including CaMKII-(273–302), for their ability to inhibit CaMKII and PKC. Under the conditions used in our assays, none of these peptides was selective for CaMKII (Table 2). The cause for the wide disparity in the reported selectivity of CaMKII-(273–302) (9) with the results presented in Table 2 is not evident. One factor that could provide an explanation was our observation that [Ala²⁸⁶]CaMKII-(273–302), as well as other peptides in the 273–302 series, are substrates for PKC. The ability of these peptides to be phosphorylated was not readily apparent from their amino acid sequences, as there are no definitive consensus sites for PKC phosphorylation present in these peptides. Since phosphocellulose filter assays were used to measure protein kinase activity, the level of incorporation of radioactivity into CaMKII-(273–302) could have been sufficiently high to have masked an inhibitory effect on the incorporation of radioactivity into the substrate peptide. In our studies, additional control assays were included that measured ³²P incorporation into the inhibitor peptide alone, and these values were used to correct the level of ³²P incorporation in those assays that contained both substrate peptide and an inhibitor.

Other experimental evidence for a possible role of CaMKII in the induction of LTP has also been presented. Malenka *et*

Table 1. Comparison of inhibition of PKC and CaMKII and induction of LTP by protein kinase inhibitor peptides

	PKC K_i , μM	CaMKII K_i , μM	LTP IC_{50} , μM
PKC-(19–36)	0.28 (0.30, 0.27)	5.8 (5.9, 5.6)	≈ 30
[Ala ²⁸⁶]CaMKII-(281–302)	9.1 (8.4, 9.8)	1.6 (2.0, 1.2)	≈ 1000
Potency ratio	32	0.28	33

K_i values given are means of two independent experiments, with the value for each experiment shown in parentheses. Potency ratio indicates ability of PKC-(19–36) relative to that of [Ala²⁸⁶]CaMKII-(281–302) to inhibit PKC, CaMKII, or LTP.

Table 2. Specificity of CaMKII-(273–302) peptide analogs for inhibition of PKC and CaMKII

CaMKII probe	PKC IC ₅₀ , μ M	CaMKII IC ₅₀ , μ M
[Ala ²⁸⁶]CaMKII-(273–302)	1.1	1.7
[Ala ²⁸⁶]CaMKII-(273–302)*	0.77	2.3
CaMKII-(273–302)*	<1.0	3.8
Acetyl-CaMKII-(273–302) [†]	<1.5	5.7

IC₅₀ values were determined in two or three independent trials in which the PKC and CaMKII substrate concentrations were 20 and 10 μ M, respectively. We observed that [Ala²⁸⁶]CaMKII-(273–302) could serve as a substrate for PKC (K_m = 490 μ M; V_{max} = 4.4 μ mol·min⁻¹·mg⁻¹), with phosphorylation occurring at both Ser²⁷⁵ and Ser²⁷⁹. The other peptides in the CaMKII-(273–302) series were also phosphorylated by PKC. Therefore, the level of ³²P incorporation into the inhibitor peptides alone was subtracted from the total ³²P incorporation into the substrate and inhibitor peptide together, and this value was used to determine the IC₅₀ values in the PKC assays.

*Peptide sample obtained from H. Schulman.

[†]Peptide sample obtained from A. Silva.

al. (8) suggested, based in part on results of intracellular injection of peptides that act as calmodulin antagonists [CBP and CBP₋₃, corresponding to CaMKII-(281–309) and CaMKII-(284–309), respectively], that induction of LTP requires calmodulin-dependent activation of CaMKII. However, the potential involvement of other calmodulin-dependent processes could not be ruled out. For example, several reports have indicated that nitric oxide, acting as a retrograde messenger from the postsynaptic neuron, may play a critical role in the induction of LTP (32–34). Thus, inhibition of nitric oxide synthase, a Ca²⁺/calmodulin-dependent enzyme, could provide an alternative explanation for the effects of the calmodulin antagonists on LTP induction. It was also suggested (8) that PKC alone cannot be responsible for the induction of LTP, since CBP and CBP₋₃ did not inhibit PKC activity *in vitro*. However, other reports have shown that CBP inhibits PKC activity (19, 27), and our current studies demonstrated that a homologous peptide, [Ala²⁸⁶]CaMKII-(281–302), is an effective inhibitor of PKC (Table 1).

Our results support the hypothesis that the activation of PKC in postsynaptic neurons is essential for induction of LTP in hippocampal CA1 cells. Furthermore, we conclude that intracellular application of either PKC-(19–36) or [Ala²⁸⁶]CaMKII-(281–302) blocks the induction of LTP by inhibition of PKC. However, we emphasize that our results do not exclude a role for CaMKII in either induction or maintenance of LTP, since the lower specificity and potency of the CaMKII inhibitor peptides preclude their use to reveal the precise contribution of CaMKII. In fact, mutant mice that lack the α subunit of CaMKII exhibit deficient hippocampal LTP (16), and CaMKII activity was reported to be increased in extracts of hippocampal slices and organotypic cultures after induction of LTP (19). The present study also demonstrates the need to use caution when interpreting the results of whole cell experiments with pseudosubstrate inhibitor peptides. The elucidation of the precise roles of PKC, CaMKII, and other protein kinases in LTP, as well as in other cellular processes, would be greatly aided by the development of more selective inhibitors.

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