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LTP induced by activation of voltage-dependent Ca^{2+} channels requires protein kinase activity

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WE have examined the requirement for protein kinase activity in long-term potentiation (LTP) induced by activation of voltage-dependent Ca^{2+} channels (VDCCs) in hippocampal slices. We previously demonstrated that LTP induced by application of the K^+ channel blocker tetraethylammonium (TEA-LTP) consisted of two distinct components, an NMDA receptor-dependent component and a VDCC-dependent component. The results herein demonstrate that both the NMDA and VDCC-dependent components of TEA-LTP are blocked by K-252a, a broad spectrum protein kinase inhibitor. Furthermore, VDCC-dependent TEA-LTP is attenuated by KN-62, a specific inhibitor of Ca^{2+}/calmodulin dependent protein kinase II (CaM-KII). These results demonstrate that LTP induced by VDCC activation requires protein kinase activity and suggest that different routes of postsynaptic Ca^{2+} influx activate protein kinases to trigger the induction of LTP but that these enzyme systems may be contained in different cell compartments.

Key words: Long term potentiation; NMDA receptor; Voltage-dependent calcium channel; Calcium/calmodulin-dependent protein kinase II

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Introduction

Long-term synaptic potentiation (LTP) in area CA1 of the hippocampus can be induced by high frequency tetanic stimulation (100 Hz) and requires increases in postsynaptic Ca^{2+} mediated by the activation of *N*-methyl-D-aspartate (NMDA) receptors.[1,2] However, other forms of long lasting potentiation in CA1 have recently been demonstrated that are also Ca^{2+}-dependent, but do not appear to require NMDA receptor activity. Higher frequency tetanic stimulation (200 Hz) of Schaffer collaterals induces potentiation in the presence of the NMDA receptor antagonist D,L-2-amino-5-phosphophonovalerate (APV).[3] Transient bath application of the K^+ channel blocker TEA can induce long lasting potentiation (TEA-LTP) that does not require NMDA receptor activation.[4–7] Like NMDA-dependent LTP, NMDA-independent potentiation can be blocked by postsynaptic injection of the Ca^{2+} chelator BAPTA (*N,N,N′,N′*-tetraacetic acid) and therefore is dependent on postsynaptic Ca^{2+} increases.[3,5,6] Both 200 Hz and TEA-induced LTP require postsynaptic activation of dihydropyridine sensitive or L-type voltage-dependent Ca^{2+} channels (VDCCs)[8] and are blocked by the VDCC antagonist nifedipine.[3–6] In contrast, L-type VDCC activation is not required for classic tetanus (100 Hz) induced LTP.[6,9,10]

These results suggest that two different routes of postsynaptic Ca^{2+} influx can trigger the induction of LTP. However, several important questions remain. Is the route of postsynaptic Ca^{2+} influx the only difference between these two forms of potentiation? Are similar cellular mechanisms activated by Ca^{2+} influx through NMDA receptors and VDCCs? We previously demonstrated that TEA-induced LTP consists of both NMDA receptor and VDCC-dependent components.[4] The two components of TEA-LTP have different time courses; moreover VDCC-dependent TEA-LTP does not occlude or prevent the subsequent induction of NMDA receptor-dependent TEA-LTP. Based on these results we concluded that VDCC and NMDA-dependent potentiation may not share common cellular mechanisms.

It is well established that tetanus (100 Hz) induced LTP requires protein kinase activity.[11] To determine whether NMDA receptor-dependent potentiation induced by different protocols (i.e. tetanus and TEA) is similar, we examined whether the NMDA receptor component TEA-LTP requires protein kinase activity. To further test the hypothesis that NMDA receptor and VDCC-dependent LTP do not share common cellular mechanisms, we determined whether the VDCC-dependent component of TEA-LTP requires protein kinase activity. To accomplish this, we used K-252a, a membrane permeable and broad range protein kinase inhibitor.[12] Previous studies indicated that tetanus-induced LTP is dependent on CaM-KII activity[13,14] and short-term synaptic potentiation induced by activating postsynaptic VDCCs was recently shown to be blocked by an inhibitor of CaM-KII activation.[15] We therefore tested whether VDCC-dependent TEA-LTP could be blocked by a selective inhibitor of CaM-KII. Our results demonstrate that VDCC-dependent LTP requires protein kinase activation, specifically CaM-KII, and indicate that

NMDA receptors and VDCCs both utilize protein kinase-dependent mechanisms to induce LTP.

Methods

Hippocampal slices (400 μm) were prepared from Harlan–Sprague–Dawley rats as described.[4] Hippocampi were dissected in ice-cold medium containing 10 mM $MgCl_2$ and no added $CaCl_2$ (see below). $CaCl_2$ was added to the slice incubation buffer to 2 mM and the temperature of the medium was gradually warmed to 30°C over 30 min. Slices were then transferred to standard medium and incubated for at least 30–45 min before transfer to a submersion recording chamber (31°C) and constantly perfused at 2 ml min^{-1}. Standard medium for electrophysiological recordings consisted of 124 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM dextrose and 10 mM Hepes (pH 7.35); media were continuously gassed with 95% O_2/5% CO_2. Changes in the standard medium are noted in the text and figure legends. Standard reagents were purchased from Sigma. K-252a was purchased from Kamiya Biochemical Co. (Thousand Oaks, CA) and KN-62 was purchased from Seikagaku America, Inc. (Rockville, MD). Stock solutions of KN-62 (10 mM) and K-252a (2 mM) were prepared in DMSO and stored at −20°C prior to dilution (1:1000) in standard medium.

Field potential recordings from stratum radiatum in area CA1 of hippocampal slices were obtained using 1–3 MΩ recording electrodes filled with standard medium. Schaffer collaterals were stimulated at a rate of 0.05–0.1 Hz with tungsten monopolar (20–50 μm) stimulating electrodes (Frederick Haer & Co., Brunswick, ME). Data were digitized on a Nicolet 410 oscilloscope and analyzed on a computer with custom software which computed EPSP slopes and amplitudes. Initial EPSP slopes were normalized to baseline values obtained from 5 or 10 min prior to TEA application. Each data point represents a 1 min average of EPSP slopes. Values of potentiation ± s.e.m. reported in the text and figures were computed from average values between 55 and 60 min following TEA washout. Our criteria for TEA-LTP was a ⩾20% increase in EPSP slope (relative to baseline) that lasted 60 min after the washout of TEA. All data represent averaged results from all slices studied under a given experimental condition. Independent *t*-tests were conducted on data utilizing a critical *p* value of 0.05.

Results

NMDA receptor-dependent TEA-LTP requires protein kinase activity: Previous results indicated that TEA-LTP induced in hippocampal slices with severed CA3-CA1 connections (isolated CA1) was strictly dependent on NMDA receptor activity.[4] Isolated CA1 slices were therefore used to determine whether the

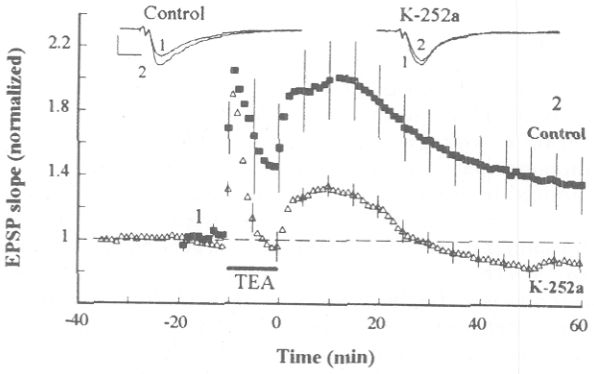

FIG. 1. NMDA receptor-dependent TEA-LTP requires protein kinase activity. Isolated CA1 slices incubated in 2 μM K-252a (△; n = 10) 20 min before, during and 20 min after TEA applications did not exhibit TEA-LTP. Control slices (0.1% DMSO; ■; n = 8) from the same hippocampi were interleaved in these experiments and exhibited TEA-LTP 60 min after TEA washout. Representative EPSPs are shown from each group at the times indicated (calibration 5 ms 0.5 mV).

NMDA receptor component of TEA-LTP required protein kinase activity. A 10 min application of TEA (25 mM) to control slices induced sustained potentiation (35 ± 18%) for at least 1 h after TEA washout (n = 8; Fig. 1). Preincubation of slices in vehicle alone (0.1% DMSO) did not alter the magnitude of TEA-LTP. Incubation of isolated CA1 slices in K-252a (2 μM in 0.1% DMSO) for 20 min prior until 20 min after TEA applications blocked potentiation in all slices tested (− 15 ± 7%; $p < 0.05$; Fig. 1). K-252a inhibits protein kinase activity by competing with ATP and inhibits both tyrosine and serine/threonine kinases including Ca^{2+}-dependent and independent activities of PKC and CaM-KII with IC_{50}s in the low nM range.[12,16] The concentrations of K-252a used in these experiments do not affect basal synaptic transmission.[17] These results indicate that NMDA receptor-dependent TEA-LTP, like tetanus-induced LTP, requires protein kinase activity and suggest that different protocols which induce NMDA receptor-dependent LTP utilize protein kinase activity as a common mechanism.

VDCC-dependent TEA-LTP requires protein kinase activity: Although TEA-induced LTP in isolated CA1 slices is strictly dependent on NMDA receptor activation, TEA-LTP in slices with intact CA3-CA1 connections consists of both NMDA receptor-dependent and VDCC-dependent components.[4] TEA-LTP in intact slices is blocked by the combined application of the NMDA receptor antagonist APV (50 μM) and the L-type VDCC antagonist nifedipine (10 μM).[4] To ensure that only the VDCC-dependent component of TEA-LTP was induced, intact slices were used and APV (50 μM) was present for 30 min before until 30 min after TEA application. The efficacy of APV applications was verified by delivery of a 100 Hz tetanus to

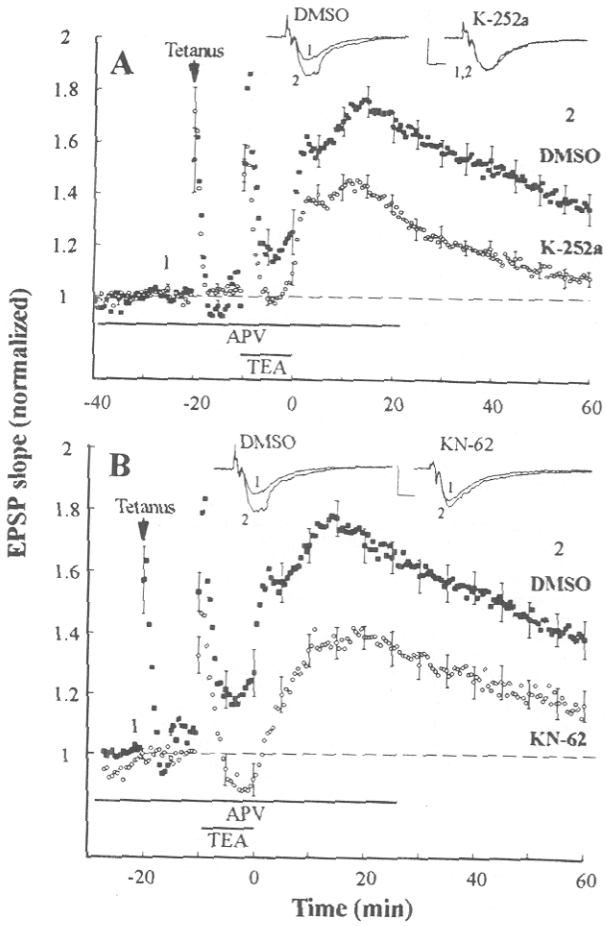

FIG. 2. VDCC-dependent TEA-LTP requires protein kinase activity. (**A**) VDCC-dependent TEA-LTP was induced in intact control slices treated with 50 μM APV plus 0.1% DMSO (■; $n = 10$). A tetanus (100 Hz; arrow) was given prior to TEA application to verify the efficacy of APV. K-252a (0.6–2.0 μM) application 20 min before until 20 min after TEA treatment blocked the induction of VDCC-dependent TEA-LTP (○; $n = 10$). (**B**) VDCC-dependent TEA-LTP was significantly reduced ($p < 0.01$) by KN-62, a specific inhibitor of CaM-KII (○; $n = 6$) compared with control slices (0.1% DMSO; ■; $n = 12$). Slices were preincubated (45–60 min) in KN-62 (10 μM) before being placed in the recording chamber. Representative EPSPs from each experimental group are shown at the times indicated (calibration 5 ms; 0.5 mV).

Schaffer collaterals prior to TEA application and only transient (1–5 min) post-tetanic potentiation was observed (arrow; Fig. 2). TEA application to control slices treated with vehicle alone (0.1% DMSO) produced robust TEA-LTP (38 ± 4%; $n = 10$; Fig. 2A). Preincubation of slices in K-252a (0.6–2.0 μM) for 20 min prior to until 20 min after TEA application significantly reduced potentiation to 8 ± 3% ($n = 10$; $p < 0.01$; Fig. 2A). These results suggest that VDCC-dependent LTP, like NMDA receptor-dependent LTP, requires protein kinase activity.

To determine whether a particular protein kinase was important for VDCC-dependent TEA-LTP, such as CaM-KII, slices were treated with the specific CaM-KII inhibitor KN-62.[18] KN-62 is membrane permeable and is more specific for CaM-KII than other kinase inhibitors because it is competitive with calmodulin binding and inhibits CaM-KII activation.[18] Therefore, KN-62 inhibits only the Ca^{2+}/calmodulin-dependent activation of CaM-KII and not Ca^{2+} independent activity. Additionally, KN-62 does not affect basal synaptic transmission[19] or voltage activated Ca^{2+} currents in hippocampal pyramidal neurons.[15] Slices were preincubated in KN-62 (10 μM; 0.1% DMSO) for 45–60 min before being placed in the recording chamber. VDCC-dependent TEA-LTP (i.e. intact slices in 50 μM APV) was significantly reduced by KN-62 (18 ± 4%; $n = 6$; $p < 0.01$; Fig. 2B) when compared with DMSO (0.1%)-treated controls (41 ± 4%; $n = 12$). Even though TEA-LTP was significantly reduced by KN-62, it was not completely blocked when measured 60 min after TEA washout. This result, combined with the observation that the broad range protein kinase inhibitor K-252a completely blocked VDCC-dependent TEA-LTP, suggests that other protein kinases, possibly PKC, may also contribute to TEA-LTP. This notion is consistent with a recent report that TEA-LTP is associated with an increase in Ca^{2+}-independent PKC activity.[20] However, our results indicate that the induction of VDCC-dependent TEA-LTP, like tetanus induced LTP,[13] requires CaM-KII activation.

Discussion

Our results demonstrate that LTP induced by VDCC activation requires protein kinase activity, specifically CaM-KII. These results are consistent with a recent study which demonstrated that short-term potentiation (20–30 min) induced by postsynaptic depolarization and VDCC activation was dependent CaM-KII activity.[15] This short-term potentiation could be converted into long-lasting potentiation (1 h) if depolarizing pulses were given in the presence of the protein phosphatase inhibitor calyculin A.[15] This short-term potentiation is believed to be expressed postsynaptically, since it is associated with increased α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor sensitivity and miniature EPSP amplitudes.[21] A model of VDCC-dependent LTP was proposed in which increases in postsynaptic Ca^{2+} activate CaM-KII which in turn phosphorylates AMPA receptors[22] and increases postsynaptic AMPA responses.[15] There is evidence that the locus of TEA-LTP induction is postsynaptic.[5] However, it is not known whether VDCC-dependent TEA-LTP is expressed postsynaptically, or whether the action of the protein kinase inhibitors used in this study is exclusively postsynaptic. However, K-252a and KN-62 do not affect basal synaptic transmission in hippocampal slices[17,19] and KN-62 does not attenuate VDCC activity in hippocampal neurons.[15] In the context of these previous findings, our results suggest that protein kinase activation occurs downstream of VDCC activation to

phosphorylate substrates important for the expression of LTP.

Our results also indicate that NMDA receptor-dependent TEA-LTP requires protein kinase activity, which supports earlier work indicating that NMDA receptor-dependent TEA-LTP and tetanus induced LTP rely on similar mechanisms.[4] These similarities suggest that knowledge gained from studies of TEA-LTP will be useful in understanding classic tetanus-induced LTP.

Previous occlusion experiments indicated that NMDA receptor-dependent and VDCC-dependent TEA-LTP do not share a common saturable cellular mechanism.[4] However, the results herein indicate that Ca^{2+} influx through both NMDA receptors and VDCCs activate protein kinases to induce TEA-LTP. This apparent paradox may be explained by the possibility that similar protein kinase-dependent mechanisms may exist in different subcellular locations. L-type VDCCs have been localized to the soma and proximal dendrites of hippocampal neurons[23] and mediate Ca^{2+} influx in proximal dendrites and dendritic shafts.[24,25] In contrast, NMDA receptor-mediated Ca^{2+} influx is localized to dendritic spines and VDCC-mediated Ca^{2+} influx is believed to be limited to dendritic shafts.[25,26] VDCCs and NMDA receptors may activate similar LTP induction machinery, but VDCCs may potentiate non-spine synapses proximal to the cell body and NMDA receptor-dependent LTP may be restricted to distal and/or spine synapses. Protein kinases present in the dendritic shaft may be preferentially activated by Ca^{2+} entering through VDCCs, whereas NMDA receptor-mediated Ca^{2+} may primarily activate protein kinases in the spine head. Information regarding Ca^{2+} dependent pathways regulated by VDCCs is instrumental in determining the enzyme cascades activated by NMDA receptors and VDCCs and how multiple LTP induction mechanisms interact.

Conclusions

The present study demonstrates that both NMDA receptor and VDCC-dependent components of TEA-LTP are dependent on protein kinase activity. Furthermore, VDCC-dependent TEA-LTP, like tetanus-induced LTP, requires CaM-KII activation. Since previous occlusion experiments indicated that VDCC and NMDA-dependent LTP do not share common cellular mechanisms, we conclude that NMDA receptors and VDCCs activate similar protein phosphorylation pathways to produce LTP, however these mechanisms may be located in different compartments in the postsynaptic cell.

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General Summary

This study examined molecular mechanisms involved in regulating synaptic strength in brain. Brain cells or neurons are interconnected by synapses which underlie neuron–neuron communication. Increases in synaptic strength are believed to occur during learning. Long lasting changes in synaptic strength are believed to be one of the brain's mechanisms for storing memories. Calcium influx and increases in the level of calcium in neurons are known to trigger the induction of long lasting increases in synaptic strength. Calcium can enter neurons by many different routes, and our studies examined the hypothesis that calcium entry through different routes can activate similar cellular mechanisms to increase synaptic strength. In this study we observed that increases in synaptic strength induced by calcium influx through different routes both utilize a common family of intracellular enzymes called protein kinases. These results indicate that different routes of calcium activate common cellular mechanisms to increase synaptic strength.