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Latent *N*-methyl-D-aspartate receptors in the recurrent excitatory pathway between hippocampal CA1 pyramidal neurons: Ca^{2+}-dependent activation by blocking A_1 adenosine receptors

(local excitatory circuits/long-term potentiation)

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ABSTRACT **When performed at increased external $[Ca^{2+}]/[Mg^{2+}]$ ratio (2.5 mM/0.5 mM), temporary block of A_1 adenosine receptors in hippocampus [by 8-cyclopentyltheophylline (CPT)] leads to a dramatic and irreversible change in the excitatory postsynaptic current (EPSC) evoked by Schaffer collateral/commissural (SCC) stimulation and recorded by *in situ* patch clamp in CA1 pyramidal neurons. The duration of the EPSC becomes stimulus dependent, increasing with increase in stimulus strength. The later occurring component of the EPSC is carried through *N*-methyl-D-aspartate (NMDA) receptor-operated channels but disappears under either the NMDA antagonist 2-amino-5-phosphonovaleric acid (APV) or the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). These findings indicate that the late component of the SCC-evoked EPSC is polysynaptic: predominantly non-NMDA receptor-mediated SCC inputs excite CA1 neurons that recurrently excite each other by predominantly NMDA receptor-mediated synapses. These recurrent connections are normally silent but become active after CPT treatment, leading to enhancement of the late component of the EPSC. The activity of these connections is maintained for at least 2 hr after CPT removal. When all functional NMDA receptors are blocked by dizocilpine maleate (MK-801), subsequent application of CPT leads to a partial reappearance of NMDA receptor-mediated EPSCs evoked by SCC stimulation, indicating that latent NMDA receptors are recruited. Altogether, these findings indicate the existence of a powerful system of NMDA receptor-mediated synaptic contacts in SCC input to hippocampal CA1 pyramidal neurons and probably also in reciprocal connections between these neurons, which in the usual preparation are kept latent by activity of A_1 receptors.**

Schaffer collateral/commissural (SCC) input to the CA1 pyramidal neurons is regarded as essentially monosynaptic (1). Pharmacological dissection of the excitatory postsynaptic current (EPSC) by means of *in situ* patch clamp demonstrates additive delayed *N*-methyl-D-aspartate (NMDA) and early non-NMDA components (2). Excitatory synaptic transmission in mammalian brain is under effective control of A_1 adenosine receptors. They provide negative feedback and, when active, are able to halt excitation completely (3–5). Their blockade results in the facilitation of excitatory synaptic transmission (6, 7). Recently, we reported that the ratio of NMDA to non-NMDA receptor-mediated components of hippocampal EPSCs can be markedly increased by blocking A_1 receptors [with 80 nM 8-cyclopentyltheophylline (CPT)] at increased external Ca^{2+}/Mg^{2+} concentrations ($[Ca^{2+}]/[Mg^{2+}]$) and that this enhancement is irreversible (8). Here we report that this effect involves at least two components: (*i*) activation of latent NMDA receptors in the SCC excitatory synaptic input to CA1 neurons, and (*ii*) activation of latent NMDA receptors in reciprocal connections between these neurons.

MATERIALS AND METHODS

Experiments were carried out on transverse 300- to 500-μm-thick hippocampal slices of Wistar rats (21-day-old animals) at room temperature (22–25°C). To functionally isolate the CA1 area from CA2 and CA3 areas, "minislices" were prepared by making a cut orthogonal to the stratum pyramidale and extending it to the distal edge of the mossy fiber layer (as shown on Fig. 6*A*). During preincubation and recording, the slices were kept in oxygenated (95% O_2/5% CO_2) 138 mM NaCl/2.7 mM KCl/2.5 mM $CaCl_2$/0.5 mM $MgSO_4$/26 mM $NaHCO_3$/0.4 mM NaH_2PO_4/0.26 mM K_2HPO_4/15 mM glucose, pH 7.4 (all substances from Sigma).

To obtain access to the CA1 pyramidal neurons, the stratum oriens and alveus were removed by a saline jet from a micropipette (see Fig. 6*A*) (6). High-resistance electrical contact with the soma of a CA1 pyramidal neuron was obtained by using a standard whole-cell patch-clamp technique. The patch pipette (2–5 MΩ) was filled with the following intracellular solution: 100 mM CsF/40 mM NaH_2PO_4/10 mM Hepes-CsOH/10 mM Tris chloride, buffered to pH 7.2 with CsOH. For electrical stimulation, a bipolar Ni/Cr electrode was positioned on the surface of the slice. Stimulation of the SCC pathway at 0.2 Hz resulted in the appearance of an intracellularly recorded EPSC with a reversal potential of 5–10 mV. The current remained stable for up to 3 hr of recording.

Experiments with extracellular recording were made on hippocampal minislices with the alveus intact. The intact alveus was required to support the Ni/Cr electrode (60 μm thick) that was used for field potential measurements. Picrotoxin (50 μM) was added to block the recurrent inhibitory pathways.

The following compounds were used: CPT; dizocilpine maleate (MK-801) from Research Biochemical (Natick, MA); 2-amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and L-*trans*-pyrrolidine-2,4-dicarboxylic acid from Tocris Neuramine (Bristol, U.K.); and picrotoxin from Sigma.

RESULTS

The EPSC elicited in CA1 neurons by stimulation of the SCC pathway complies with monosynapticity criteria. In particular, its time course does not depend on the stimulus strength. Increase in external $[Ca^{2+}]$ (up to 2.5 mM, as compared with 1.5 mM in normal extracellular solution) leads to an increase in amplitude of the EPSC and to a relatively greater NMDA receptor-mediated component of the current. Some neurons in these conditions start demonstrating stimulus dependence of

Abbreviations: EPSC, excitatory postsynaptic current; NMDA, *N*-methyl-D-aspartate; MK-801, dizocilpine maleate; APV, 2-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; SCC, Schaffer collateral/commissural; CPT, 8-cyclopentyltheophylline; $[Ca^{2+}]$ and $[Mg^{2+}]$, Ca^{2+} and Mg^{2+} concentrations.

the duration of the EPSC. In view of nonisopotentiality due to the dendritic arborization of the neurons, we interpreted such experiments as due to inadequate space clamping (8). But when we measured stimulus dependence of EPSC duration on a background of inhibited A_1 receptors, a large majority of neurons (47 out of 52) displayed a monotonic increase in the duration of the EPSC with increase in stimulus strength (Fig. 1). Moreover, a temporary (4–8 min) blockade of A_1 receptors by 100 nM CPT caused a long-lasting appearance of stimulus dependence of EPSC duration and an at least partially reversible potentiation of EPSC amplitude. Neither increased concentration (up to 8 μM, $n = 8$) nor prolonged application (or reapplication, $n = 4$) of CPT produced further change in the duration of the EPSC (see also ref. 8). This change only occurred at an increased ratio of external $[Ca^{2+}]$ to $[Mg^{2+}]$ (see below). Otherwise, only the amplitude of EPSC was facilitated by CPT.

In all presented experiments, we applied CPT in the concentration range of 60–200 nM. In these concentrations it specifically blocks only A_1 receptors [K_i values for CPT binding to A_1 and A_2 receptors are 10.9 nM and 1440 nM, respectively (9)].

We applied CPT in the presence of NMDA or non-NMDA receptor antagonists to study the behavior of corresponding components of the EPSC. The application of CPT in the presence of the NMDA receptor blocker APV resulted in the usual "antiadenosine" action: the rapid component of the EPSC mediated by non-NMDA receptors was increased, but its kinetics remained unchanged at any stimulus strength (Fig. 2, trace family 3, $n = 4$). Subsequent removal of APV (after washing out CPT) revealed the appearance of a huge stimulus-dependent NMDA receptor-mediated component (Fig. 2, trace family 4). The set of traces demonstrating acquired stimulus dependence was obtained 40 min after washing out CPT. At this time the maximal amplitude of the EPSC was very close to the initial control value. This result indicates that the changes in kinetics of the EPSC were not to due to loss of voltage control.

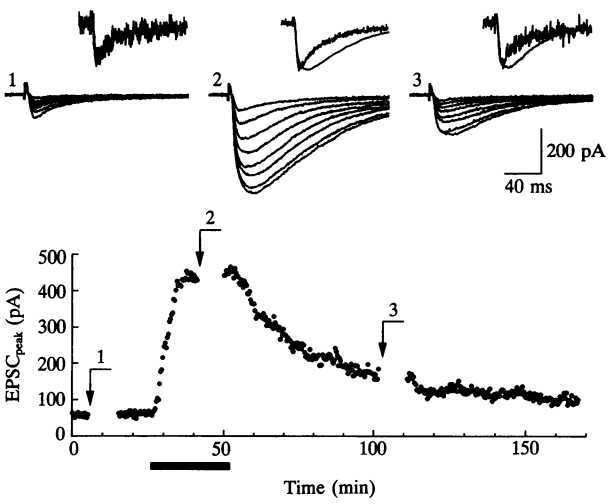

FIG. 1. The effect of bath application of CPT on the EPSC. A CA1 pyramidal neuron was held at −50 mV. (*Lower*) Time course of the amplitude of the EPSC elicited in response to the strongest stimuli (16 V). (*Upper*) Records of families of EPSCs obtained at various stimulus strengths at the moments indicated by the arrows in *Lower*. (*Upper Insets*) Traces showing normalized EPSCs corresponding to the weakest and the strongest stimuli are superimposed above each graph. The filled bar indicates when 100 nM CPT was applied. In *Upper* and *Lower*, extracellular concentrations of Ca^{2+} and Mg^{2+} are 2.5 and 0.5 mM, respectively. Hereafter each curve is the mean of four sequential traces; stimulus strength was varied from 2 to 16 V for a duration of 200 μs (if not otherwise stated).

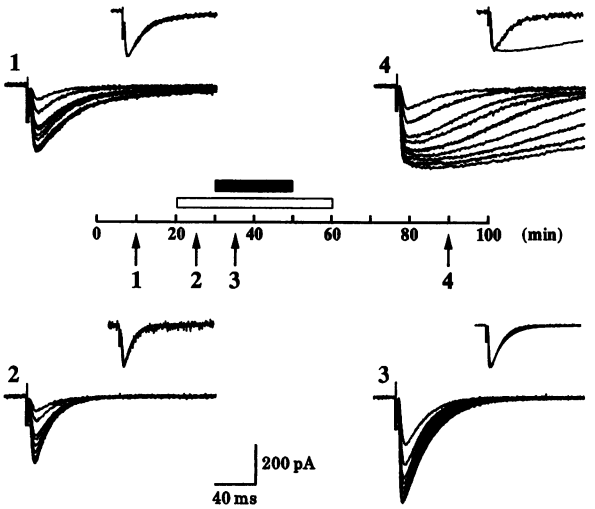

FIG. 2. Application of CPT in the presence of APV. A CA1 pyramidal neuron was held at −50 mV. Each family of traces represents the EPSCs obtained at the points indicated by the arrows on the time scale together with the sequence of drug application. The open bar indicates treatment by 100 μM APV, and the filled bar indicates when 80 nM CPT was applied. Family of traces: 1, control; 2 and 3, the non-NMDA component of the EPSC in the control (2) and under CPT treatment (3); 4, duration of the EPSC acquires stimulus-dependence only after the removal of APV. (*Insets*) Normalized EPSCs elicited in response to the weakest and the strongest stimuli are superimposed near corresponding families. A CA1 pyramidal neuron was held at −50 mV.

The experiment shown in Fig. 2 indicates that the delayed component requires operation of NMDA receptors, although these receptors are not needed for the initiation of the described effect despite its critical Ca^{2+} dependence. In other similar experiments ($n = 5$), the stimulus dependence was obtained in the presence of APV and did not show any decrease over periods ranging from 50 to 120 min. CPT is quite lipophilic, and the long duration of its effect might be due to the presence of residual CPT in the membrane. However, the blocking action of adenosine on synaptic transmission was restored in <40 min, indicating that residual CPT is unlikely to account for its long-lasting action. We confirmed (but do not illustrate) our previous finding that the change in the contribution of NMDA receptors cannot be elicited at normal levels of external $[Ca^{2+}]$ and $[Mg^{2+}]$ (1.5 mM and 1.5 mM) (8). Furthermore, when $[Mg^{2+}]$ is increased from 0.5 to 1.5 mM, the effect cannot be elicited at $[Ca^{2+}] = 2.5$ mM.

In control experiments we applied CPT to hippocampal slices and measured EPSCs at various stimulus strengths in the presence of 50 μM picrotoxin or 100 μM L-*trans*-pyrrolidine-2,4-dicarboxylic acid, an inhibitor of glutamate uptake. The result was the same as in Fig. 2; neither picrotoxin ($n = 4$) nor the glutamate uptake inhibitor ($n = 3$) changed the kinetics of the EPSCs.

In view of obvious involvement of NMDA receptors in the stimulus-dependent late component of the EPSC acquired under CPT, the action of the non-NMDA receptor blocker CNQX was unexpected: CNQX inhibited not only the rapid component of EPSC but also the later part of the EPSC that demonstrated stimulus dependence (Fig. 3*A*). Seven similar experiments were made and gave basically the same result. In three (from these seven) experiments, the late component was largely but not entirely blocked: stimulus dependence of EPSC duration under CNQX appeared only at the strongest attainable stimuli.

Normally, the major role in electrical excitation of postsynaptic pyramidal neurons is played by non-NMDA receptors (10). The question arises whether the same is true in the

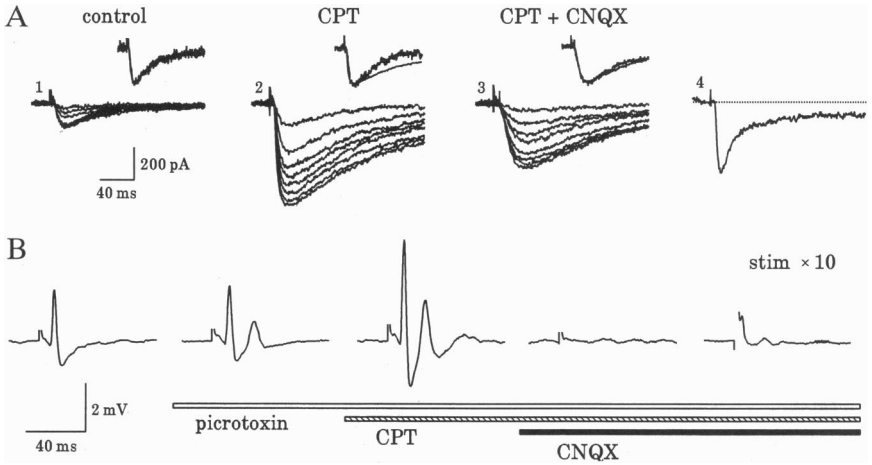

FIG. 3. (*A*) CNQX blocks the late component of the EPSC. Family of traces: 1, EPSCs obtained in response to various stimuli in the control; 2, 10 min after application of 100 nM CPT; 3, after addition of 10 μM CNQX; 4, subtraction of currents from families 2 and 3 at the strongest stimulus. The early part represents current through the CNQX blockable receptors; the later part represents the NMDA-mediated polysynaptic component. (*A Insets*) Normalized EPSCs elicited in response to the weakest and the strongest stimuli are superimposed near corresponding families. (*B*) Field potentials recorded in stratum pyramidale of the area CA1 during stimulation of SCC pathway (0.1 Hz, 2 V, 150 μs). Blockade of recurrent inhibition by bath application of 50 μM picrotoxin (open bar) led to repetitive firing of pyramidal neurons. Addition of 100 nM CPT (hatched bar) facilitated the postsynaptic spike and enhanced cell firing. In these conditions 10 μM CNQX (filled bar) was still capable of blocking electrical excitation of CA1 pyramidal neurons. However, a small postsynaptic spike appeared after a 10-fold increase in stimulus strength (20 V, 150 μs). This spike was delayed consistent with the slower rise time of NMDA receptor-mediated postsynaptic potentials.

conditions when the NMDA component has been enhanced—first by an increased external $[Ca^{2+}]/[Mg^{2+}]$ ratio and then by application of CPT. The lack of recurrent inhibition due to the removal of the alveus could further increase the role of NMDA receptors in cell firing. We tested this possibility, measuring field potentials (in slices with an intact alveus) in the presence of 50 μM picrotoxin. Fig. 3*B* shows that current through NMDA receptors initiates little if any impulse activity even after CPT: the enhanced population spike field potential after CPT treatment is almost completely blocked by 10 μM CNQX. Subsequent 10-fold increase in the strength of the stimulus caused a slight reappearance of excitation of CA1 neurons. This observation could well account for incomplete blockade by CNQX of the stimulus-dependent late component of the EPSC at high stimulus strengths.

Simultaneous application of APV and CNQX in the presence of CPT led to a complete inhibition of EPSCs (not illustrated, but see Fig. 5) indicating that, although normal additivity of early CNQX-sensitive and late APV-sensitive components appears to be impaired, the overall pharmacology of the hippocampal EPSC remains unchanged.

The sensitivity of the late component of the EPSC to non-NMDA antagonists and the results in Fig. 3*B* suggest that the late component is polysynaptic. Its abolition by APV (Fig. 2) when excitation of CA1 neurons by SCC inputs is unaffected suggests that it is entirely NMDA receptor-mediated. The experiment illustrated in Fig. 4 confirms this suggestion. We measured the EPSC at various stimuli at a holding voltage of −40 mV, at which the NMDA component is maximum, and at −108 mV, where the NMDA component is largely blocked (Fig. 4*A*; ref. 11). The stimulus dependence of duration was only present when there was an NMDA component.

Fig. 5 shows an experiment in which the synaptic inputs to CA1 neurons were totally inhibited by application of CNQX and stimulation in the presence of MK-801 (open bar). Maximal stimulation led to the use-dependent and practically irreversible block of NMDA receptors. To ensure that all NMDA receptors belonging to functional synapses were blocked, 20 mM (in other trials, 50 mM) KCl was applied for 10–15 min (filled bar). Subsequent application of CPT led to a transient reappearance of NMDA receptor-mediated current. The accelerated decay of the EPSC that appeared in CPT and its rapid decline from stimulus to stimulus were evidently due to residual MK-801 (12), which is quite lipophilic. Altogether, seven complete experiments of this kind were performed. The current reappeared under CPT in all cases, independent of whether CPT was applied in the presence of MK-801 or after its removal from the bath. In all trials with 50 mM KCl, the reappearing current was smaller than with 20 mM KCl, suggesting that KCl can slowly activate some of the latent synapses. (In two cases, omitting the high-KCl treatment was indistinguishable from the 20 mM KCl treatment.)

DISCUSSION

Local excitatory circuits are generally accepted in hippocampal CA3 area (13–15) with a few reports of their presence in

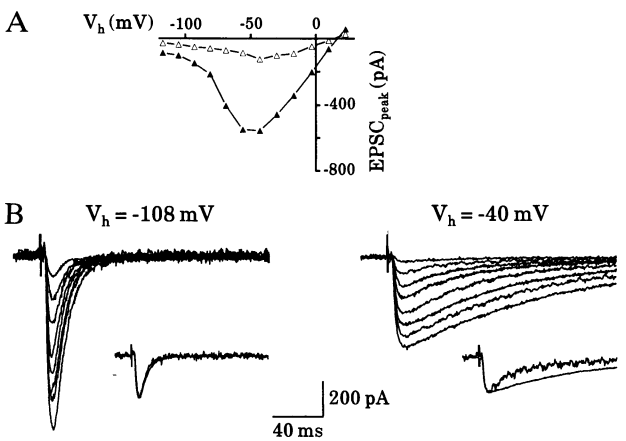

FIG. 4. The late component of the EPSC is carried via NMDA receptor-operated channels. (*A*) Current–voltage relationship for the NMDA component of the EPSC (all measurements were in the presence of 10 μM CNQX). △, Control; ▲, with 100 nM CPT. (*B*) Families of EPSCs measured at holding voltages of −108 and −40 mV. (*B Insets*) Normalized EPSCs corresponding to the weakest and the strongest stimuli are superimposed. The EPSC lacks a late component at the more negative holding voltage. *A* and *B* are neurons from different slices.

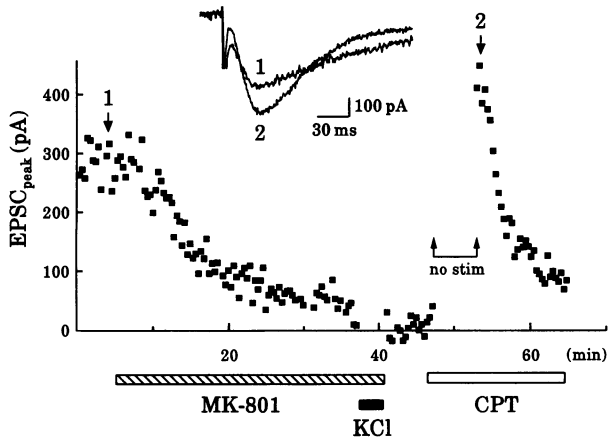

FIG. 5. Application of CPT causes the reappearance of NMDA receptor-mediated EPSCs after block by MK-801. A CA1 pyramidal neuron was held at −50 mV in the presence of 10 μM CNQX throughout the experiment. Gradual inhibition of the EPSC during repetitive stimulation was due to the use-dependent nearly irreversible action of 100 μM MK-801 (hatched bar). Only open NMDA receptor-mediated channels were blocked during the period of stimulation. To block any remaining functional NMDA receptors with MK-801, the presynaptic terminals were depolarized by addition of 20 mM KCl (filled bar). Consequently, reappearance of current under 200 nM CPT treatment (open bar) was due to the recruitment of latent synapses or previously inactive channels in functional synapses. There was a gap in the stimulation at the beginning of the application of CPT to prevent immediate use-dependent blockade of newly activated channels by MK-801. (*Inset*) Two superimposed EPSCs obtained at the times indicated on the graph.

CA1 as well (16, 17). Thomson and Radpour (16) have demonstrated local circuit excitatory connections in CA1 that are partially mediated by NMDA receptors. We have created experimental conditions that facilitate measurement of NMDA receptors and increase their activity: (*i*) removal of the alveus removes feedback inhibition (18, 19) (this is probably the reason why picrotoxin did not affect the EPSC in our experiments), and (*ii*) elevated external $[Ca^{2+}]/[Mg^{2+}]$ results in an increase in the contribution of the NMDA component. These conditions were already sufficient to record the appearance of the late component in some neurons (8), but really dramatic manifestation of this phenomenon came with the blockade of A_1 receptors. As to the origin of the late component of the EPSC, our data lead to the following picture.

As shown in Fig. 4, the late component of the EPSC after CPT treatment flows through NMDA receptors. In addition, it disappears when either NMDA or non-NMDA antagonist is applied (Figs. 2 and 3*A*). After CPT, the stimulation of CA1 neurons via SCC input continues to depend on the activity of non-NMDA receptors (Fig. 3*B*). For the minislice preparation, we see only one explanation: these neurons are reciprocally excited by NMDA receptor-mediated synaptic connections. These connections are normally completely suppressed by the action of A_1 receptors. The inferred CA1 circuitry is schematically represented in Fig. 6. Normally the majority of A_1 receptors are activated by basal adenosine present in the extracellular space (20). Correspondingly, the EPSC measured in one of pyramidal neurons, P3, is monosynaptic. When A_1 receptors are blocked, the EPSC in P3 will demonstrate late components resulting from activation of neighboring neurons. This scheme accounts for the observations presented: the late component disappears when either NMDA receptors are blocked (the recurrent CA1 inputs are NMDA receptor-mediated) or when non-NMDA receptors are blocked [NMDA receptor-mediated current is usually insufficient to initiate action potentials in the pyramidal neurons (10)]. However, as diagrammed in Fig. 6, NMDA current may cause repetitive firing of CA1 neurons in the presence of non-NMDA input from SCC. Obviously, this firing (of neighboring neurons) would increase the duration of the late (polysynaptic) component of the EPSC. However, they do not affect the EPSC duration in neurons with blocked NMDA receptors (see Fig. 4*B*).

In our major point—i.e., the existence of NMDA receptor-mediated recurrent excitation in CA1—our data are in agree-

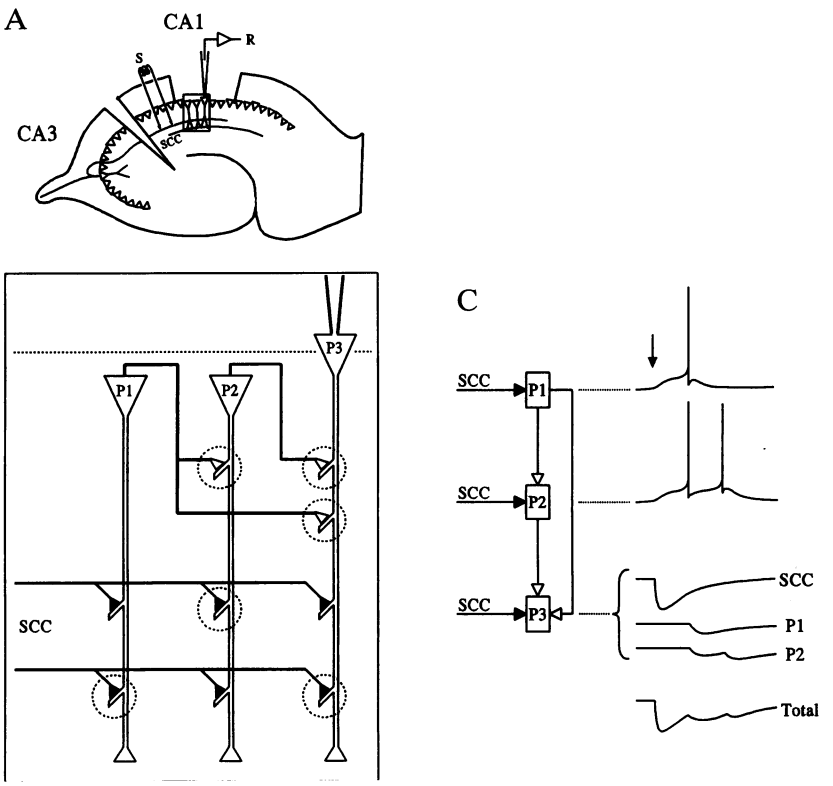

FIG. 6. Simplified model of CA1 synaptic circuitry that accounts for the activation of latent interneuronal connections. (*A*) Schematic representation of the experiment: S, stimulating electrode; R, recording electrode; SCC, SCC pathway. The area of hippocampal slice enclosed in the box is enlarged in *B*. (*B*) Filled synaptic terminals represent SCC excitatory input with both NMDA and non-NMDA components to CA1 pyramidal neurons (P1–P3), and open synaptic terminals represent local NMDA receptor-mediated excitatory connections between CA1 neurons. Broken circles indicate initially silent synapses that are recruited by blocking A_1 receptors. (*C*) The equivalent circuit of the interneuronal connections after the action of CPT and the expected response of the neurons to the stimulation of SCC, resulting in the prolonged stimulus-dependent duration of the EPSC in P3.

ment with the findings of Thomson and Radpour (16). However, there are some differences. These authors dispute the existence of monosynaptic NMDA receptor-mediated input via SCC. In our experiments (Fig. 5), NMDA receptor-mediated current was detected when polysynaptic interactions were largely excluded by CNQX and reappeared under CPT after complete block of functional NMDA receptors by MK-801. These data also indicate that a population of NMDA receptors in the SCC input stays latent under the guard of A_1 receptors.

Our data do not distinguish whether reciprocal NMDA connection between CA1 neurons are revealed or potentiated—i.e., facilitated from a probability of zero or from a probability somewhat greater than zero. However, by analogy to SCC input, we propose revelation rather then potentiation of reciprocal connections. The rare presence of stimulus-dependence of the duration of the EPSC in the control (without CPT) could well be due to a partial revelation of such connections during ontogenesis (or during preparation of slices, see below about anoxia). According to Radpour and Thomson (21), local circuits are formed by collaterals of axons descending from alveus to stratum oriens. In our preparation, the alveus was destroyed, so that only the neurons in stratum pyramidale closer to stratum radiatum could retain basal dendrites and some parts of axons. Branching of these axons before entering the alveus could account for the local interneuronal connections. Another hypothetical alternative is the existence of dendrodendritic interactions. To our knowledge, the anatomical correlates for excitatory local circuitry in CA1 are still missing.

We see only one principal alternative to the presented interpretation. A rapidly diffusing factor released by the stimulated axons and capable of dramatically modulating the properties of NMDA receptors could produce similar effects. We tried to examine a possible candidate for this role: 10 μM quinacrine or 10 μM 4-bromophenacyl bromide (compounds that inhibit the production of arachidonic acid) together with bovine serum albumin at 1 mg/ml (a free fatty acid scavenger) failed to prevent the appearance of stimulus-dependence of EPSC duration after treatment by CPT. The effect of CPT was also totally preserved in the presence of 10 μM hemoglobin, which cleaves another diffusible modulator, NO. Despite these negative results, this somewhat exotic alternative cannot be ruled out.

As to the existence of exclusively (or predominantly) NMDA receptor-operated synaptic terminals, application of agonists to cultured hippocampal neurons revealed colocalized NMDA and non-NMDA receptors on the majority of terminals; however, 10% of them demonstrated only NMDA receptors (22).

Our preliminary data indicate that brief periods of anoxia are to some extent equivalent to the inhibition of A_1 receptors (see also ref. 23). We consistently observed that the duration of EPSCs in our preparation became abnormally long and stimulus-dependent within 1–2 hr of a brief period (5–10 min) of anoxia. In natural conditions, decoupling A_1 receptors from their downstream actions may substitute for pharmacological blockade of these receptors (24). Superposition of excitatory signals from afferent inputs and from other CA1 neurons (21, 25) may create sufficient local Ca^{2+} signals to substitute for the overall increase in external Ca^{2+} concentration employed in the presented experiments. The powerful system of latent excitatory synaptic connections in the CA1 area has to be considered in the analysis of rhythmicity, epileptogenesis, and long-term potentiation. According to a recent report, synapses transmitting exclusively with NMDA receptors trigger long-term potentiation in CA1 (26). Specifically responsible for the activity of such synapses, the A_1 receptors could play a major role in regulation of susceptibility of the CA1 area to the induction of long-term potentiation.

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