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Presynaptic modulation of cortical synaptic activity by calcineurin

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ABSTRACT **Synaptic plasticity is modulated by Ca^{2+}-induced alterations in the balance between phosphorylation and dephosphorylation. Recent evidence suggests that calcineurin, the Ca^{2+}-calmodulin-dependent phosphatase (2B), modulates the activity of postsynaptic glutamate receptors. However, in rat cortex, calcineurin is enriched mainly in presynaptic, not postsynaptic, fractions. To determine if calcineurin modulates glutamatergic neurotransmission through a presynaptic mechanism, we used whole-cell patch clamp experiments to test effects of two specific calcineurin inhibitors, cyclosporin A (CsA) and FK506, on synaptic activity in fetal rat cortical neurons. The rate of spontaneous action-potential firing was markedly increased by either CsA or FK506 but was unaffected by rapamycin, a structural analog of FK506 which has no effect on calcineurin. In voltage-clamp experiments, CsA increased the rate but not the amplitude of glutamate receptor-mediated, excitatory postsynaptic currents, suggesting an increased rate of glutamate release. CsA had no effect on the amplitude of currents evoked by brief bath application of selective glutamate receptor agonists, providing further evidence for a pre- rather than postsynaptic site of action. In conclusion, these data indicate that calcineurin modulates glutamatergic neurotransmission in rat cortical neurons through a presynaptic mechanism.**

The Ca^{2+}–calmodulin-dependent phosphatase calcineurin, which colocalizes with the immunophilins cyclophilin and FK-binding protein (1), accounts for over 1% of the total protein in brain (2, 3). Despite this abundance, little is known about the functional role of neuronal calcineurin (4). Inhibition of the activity of glutamate receptor-operated channels in excised membrane patches by exogenously applied calcineurin recently has been demonstrated (5), and the phosphatase may also play a role in hippocampal long-term depression (6). Both of these results implicate a postsynaptic site of action. Nevertheless, calcineurin is nearly undetectable in forebrain postsynaptic densities by Western blot analysis but is highly expressed in presynaptic nerve terminals (7). Furthermore, presynaptic phosphoproteins involved in vesicle release (8) and recycling (9, 10) have been shown to be specific calcineurin substrates *in vitro.* We therefore hypothesized that calcineurin modulates glutamatergic neurotransmission via a presynaptic mechanism. To test this hypothesis, fetal rat cortical neurons, which form glutamatergic synapses in culture, were utilized to study electrical signaling with the whole-cell patch clamp technique. Calcineurin's role in synaptic neurotransmission was probed by utilizing the immunosuppressant drugs cyclosporin A (CsA) and FK506, which form drug–immunophilin complexes that are highly specific inhibitors of calcineurin (11, 12). The results herein identify a presynaptic effect of calcineurin in modulating the process of glutamatergic neurotransmission.

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MATERIALS AND METHODS

Immunophilin Ligands. CsA was provided by Sandoz Pharmaceutical, FK506 was provided by Fujisawa Pharmaceutical (Osaka), and rapamycin was provided by Wyeth–Ayerst. CsA, FK506, and rapamycin were solubilized in γ-cyclodextrin [final γ-cyclodextrin concentration was 2.25% (wt/vol)]. CsA and FK506, as their immunophilin complexes, are specific, high-affinity inhibitors of calcineurin (11, 12). Like FK506, rapamycin is a high-affinity ligand for FK-binding protein (13) and an inhibitor of the intrinsic *cis-trans* peptidylprolyl isomerase activity (14) of the immunophilin, but it has no effect on calcineurin activity (13).

Cell Culture. Cell cultures were prepared from cortices isolated from fetal Sprague–Dawley rats (16–18 days gestation) by using mechanical and enzymatic dissociation (15, 16). The dissociated cells were resuspended at a density of 1.2×10^6 cells per ml in minimal essential medium supplemented with 5.5 g of glucose per liter, 1 g of $NaHCO_3$ per liter, 2 mM glutamine, 10% (vol/vol) fetal calf serum, 10% (vol/vol) heat-inactivated horse serum, 50 units of penicillin per ml, and 0.05 mg of streptomycin per ml, plated onto poly(lysine)-coated (10 μg/ml) 35-mm culture dishes in 2 ml of medium, and placed in a 37°C CO_2 incubator.

Cellular Electrophysiology. Electrophysiological experiments were performed on neurons which were in culture for at least 3 weeks to permit complete maturation of synapses and glutamate receptors (16). Whole-cell patch clamp recordings (17) were performed by using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) in either current-clamp or voltage-clamp mode to record excitatory postsynaptic potentials (EPSPs) or excitatory postsynaptic currents (EPSCs), respectively. In voltage-clamp experiments, the holding potential was −60 mV, unless otherwise stated. Tissue culture dishes were superfused with external solution at a rate of 1–2 ml/min of the following composition: 135 mM NaCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5.4 mM KCl/10 mM glucose/0.01 mM glycine/10 mM Hepes, pH 7.4 at room temperature (with NaOH). Patch clamp electrodes (3–6 MΩ) were filled with an internal solution containing 90 mM potassium aspartate, 50 mM KCl, 4 mM $MgCl_2$, 3 mM MgATP, 0.1 mM EGTA, 10 mM Hepes, pH 7.2 with KOH.

To test the sensitivity of postsynaptic glutamate receptors to agonists, a double-lumen pipette was placed ≈100 μm from the neuron of interest, and glutamate agonists were applied for 500 msec at 5-min intervals. The second lumen of the pipette contained bath solution alone delivered between agonist pulses to rapidly wash out agonist to minimize receptor desensitization. We used doses of α-amino-3-hydroxy-5-methylisoxazole-4-propi-

Abbreviations: CsA, cyclosporin A; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; TTX, tetrodotoxin; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; APV, 2-amino-5-phosphonovalerate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione.

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onate (AMPA) and *N*-methyl-D-aspartate (NMDA) which produced whole-cell current responses that were readily distinguishable from the spontaneous EPSCs but were not maximal. In an additional experiment, the perforated-patch technique was used instead of whole-cell recording. The pipette solution was as described above, with nystatin (100 μg/ml; prepared from a fresh stock of 5 mg/ml in methanol) (18).

To determine whether calcineurin inhibition modulates spontaneous synaptic vesicle release, 5 μM tetrodotoxin (TTX) was added to the extracellular solution to block action potential-mediated depolarizations of the nerve terminals. In the presence of TTX, baseline miniature EPSCs were recorded. TTX was then washed out of the extracellular solution to restore depolarization-evoked EPSCs and CsA was applied to confirm that the frequency of EPSCs increased in response to calcineurin inhibition. Finally, TTX was reintroduced and miniature EPSCs were recorded in the presence of CsA. Recording conditions were the same as described above, except that the intracellular solution contained 102 mM potassium gluconate, 10 mM NaCl, 0.5 mM $MgCl_2$, 5 mM MgATP, 0.1 mM EGTA and 49 mM Hepes at pH 7.2 with KOH. In these experiments, the membrane potential was held at −70 mV.

RESULTS AND DISCUSSION

Exposure to low concentrations of CsA (5 or 10 μM) resulted in a 205% increase in the action potential firing rate (control, 0.56 ± 0.10 Hz; CsA, 1.71 ± 0.55 Hz; mean ± SEM; $P < 0.05$; $n = 4$) (Fig. 1*A*), while higher concentrations (20 μM and above) further increased firing rate and led to sustained membrane depolarization (Fig. 1*A*). Equivalent amounts of solvent [γ-cyclodextrin; 2.25% (wt/vol)] in the absence of drug had no effect on firing rate (control, 0.49 ± 0.09 Hz; γ-cyclodextrin, 0.50 ± 0.12 Hz; $n = 4$). In accord with its 10- to 100-fold higher potency in inhibiting calcineurin (9, 10) compared with CsA, FK506 similarly enhanced the action potential firing rate (207% increase) but at substantially lower concentrations (0.1–1.0 μM) (control, 0.55 ± 0.20 Hz; FK506, 1.69 ± 0.62 Hz; $P < 0.05$; $n = 6$). In addition to the increase in action potentials, the frequency of EPSPs was clearly increased by CsA and FK506 (Fig. 1 *A* and *B*).

To determine whether the enhancement of synaptic activity was mediated by the ability of the cyclophilin ligands to inhibit calcineurin, we compared the effects of CsA and FK506 with those of rapamycin. Like FK506, rapamycin is a high-affinity ligand for FK-binding protein (13) and an inhibitor of the intrinsic *cis-trans* peptidylprolyl isomerase activity (14) of the

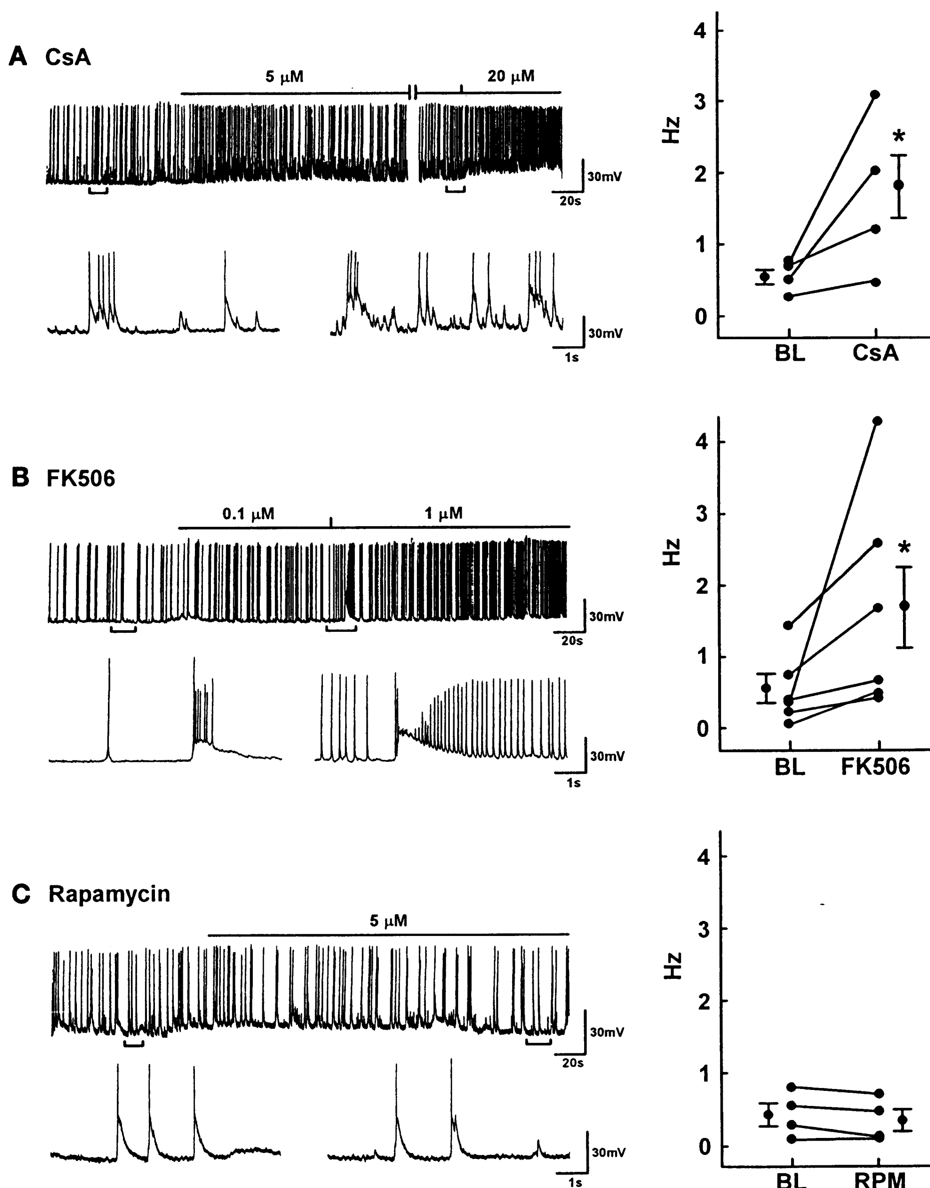


FIG. 1. Effects of immunophilin ligands on spontaneous cortical synaptic activity. (*A*) Concentration-dependent increases in action potential discharge rate with 5 μM and 20 μM CsA. Bracketed segments of the original records are reproduced at an expanded time scale below to better illustrate the EPSPs, the largest of which gave rise to action potential spikes. The individual values of action potential discharge rates (Hz) and summary data (mean ± SEM; $n = 4$) at baseline (BL) and with CsA (5 μM) are displayed on the right. (*B*) Concentration-dependent increases in action potential discharge rate with FK506 (0.1 and 1 μM). Summary data show the responses to 1 μM FK506 ($n = 6$). (*C*) Unchanged action potential discharge with rapamycin (RPM; 5 μM; $n = 4$). The asterisks indicate $P < 0.05$.

immunophilin, but it has no effect on calcineurin activity (13). Rapamycin did not increase the rate of action potential firing (Fig. 1*C*), even at higher concentrations than were effective for FK506 (control, 0.43 ± 0.16 Hz; 5 μM rapamycin, 0.35 ± 0.35 Hz; $n = 4$).

We then performed experiments to characterize the membrane currents underlying the spontaneous synaptic activity in the absence and presence of the calcineurin inhibitors. The spontaneous activity recorded in the cortical neurons could be accounted for primarily by three types of postsynaptic currents: rapidly inactivating, voltage-gated Na^+ current; rapidly inactivating, glutamate-activated current; and a slowly inactivating, glutamate-activated current. Postsynaptic Na^+ current could be selectively eliminated in the neuron under study without affecting other neurons in the culture dish by including the membrane-impermeant Na^+ channel blocker QX314 (3 mM) in the intracellular solution. The enhancement of synaptic activity in response to CsA or FK506 was similar with postsynaptic Na^+ currents blocked but was manifest as an increase in the frequency of EPSPs in current-clamp experiments (data not shown) or EPSCs in voltage-clamp experiments. EPSCs had a reversal potential of approximately 0 mV, characteristic of glutamate receptor-mediated currents (20) (Fig. 2*A*). At a holding potential of −60 mV, in physiological extracellular [Mg^{2+}], the spontaneous EPSCs were almost completely eliminated by 40 μM CNQX, a high-affinity antagonist of non-NMDA glutamate receptors which also has antagonist activity against NMDA receptors (20) (Fig. 2*B*). In the presence of 50 μM APV, a highly specific NMDA receptor antagonist (20), the slow component of the EPSCs could be selectively removed (Fig. 2*B*), indicating that both non-NMDA and NMDA subtypes of glutamate receptor-operated channels were present on the postsynaptic neuron.

The fast and slow components were quantified by analyzing the duration of the currents. EPSCs with durations greater than 1 sec were attributed to mainly NMDA receptor-activated (APV-sensitive) currents, whereas EPSCs less than 1 sec were attributed to mainly non-NMDA glutamate receptor-activated currents (APV-insensitive) (Fig. 2*B*). Importantly, CsA still increased the frequency of EPSCs when all postsynaptic excitatory currents other than those mediated by non-NMDA glutamate receptors were blocked (control, 0.47 ± 0.12 Hz; CsA in the presence of QX314 in the internal solution and APV in the external solution, 0.82 ± 0.20 Hz; $P < 0.05$; $n = 8$) (Fig. 2*C*). Therefore, the activation of postsynaptic Na^+ currents or NMDA currents (pre- or postsynaptic) during the

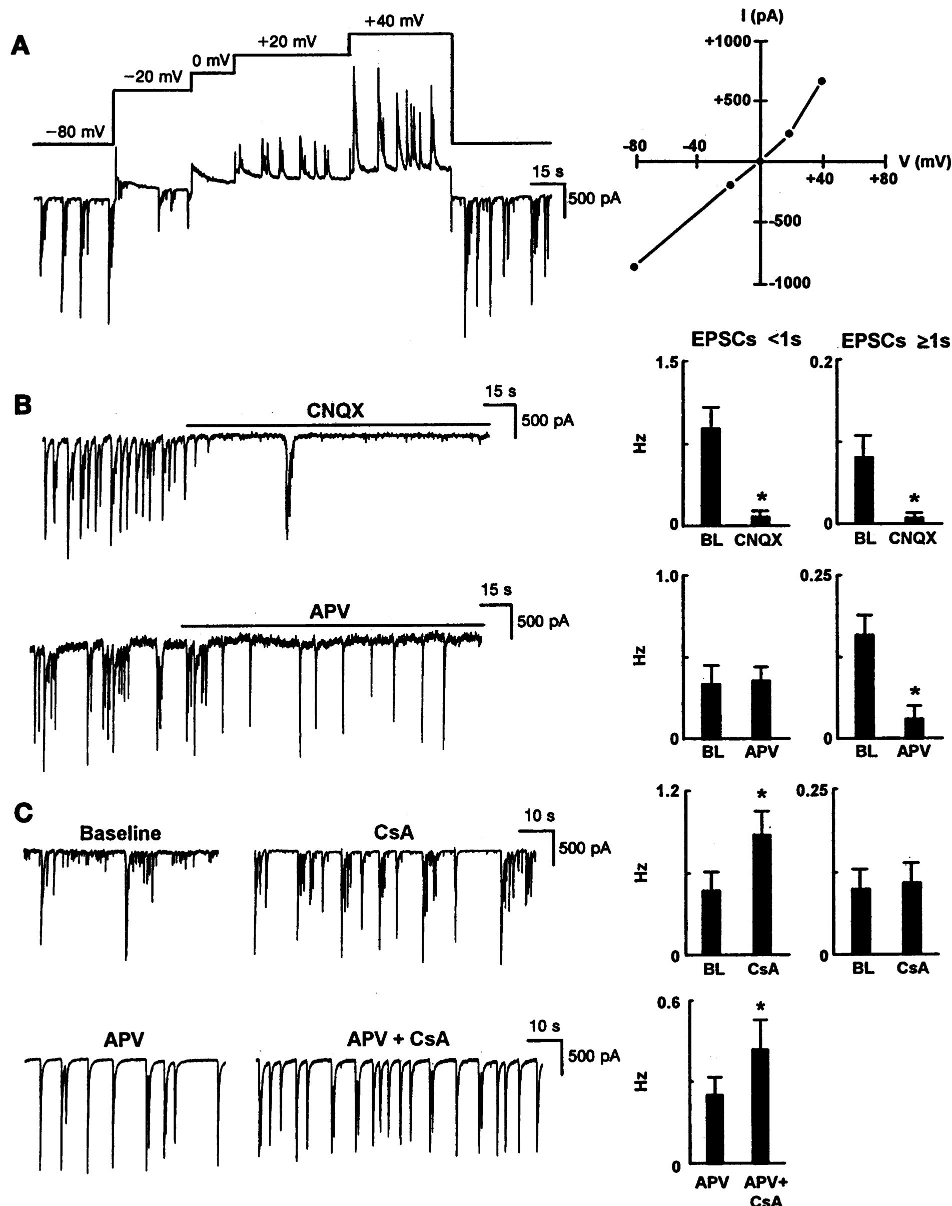


FIG. 2. Characterization of the EPSCs. (*A*) Spontaneous EPSCs recorded in one neuron as the holding potential is stepped sequentially from −80 to +40 mV are shown on the left. Plot of current–voltage (I–V) relation in this neuron showing a reversal potential of 0 mV, characteristic of glutamate receptor currents, is shown on the right. (*B*) Differential effects of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2-amino-5-phosphonovalerate (APV) on EPSCs (recorded at a holding potential of −60 mV) in the absence of CsA. Segments of two illustrative experiments are shown on the left and the summary data for these experiments are shown on the right. Blocking non-NMDA and NMDA subtypes of glutamate receptors with 40 μM CNQX ($n = 4$) eliminated almost all EPSCs, whereas blocking only NMDA receptors with 50 μM APV ($n = 6$) eliminated EPSCs ≥1 s in duration without affecting the frequency of EPSCs <1 s in duration. (*C*) CsA-mediated increase in the frequency of EPSCs in the absence and presence of APV. Segments of two experiments showing effects of CsA on EPSCs ($V_h = -60$ mV) in the absence (top) or presence (bottom) of APV are shown on the left. Summary data showing the mean response to 5 μM CsA ($n = 4$) in the absence or presence ($n = 5$) of APV are shown on the right. CsA led to a significant increase in the frequency of EPSCs <1 s, in both the absence and presence of APV. While the summary data do not reveal an increase in the mean frequency of EPSCs ≥1 s after CsA, in two of the five neurons studied, increases in EPSCs ≥1 s were observed after CsA (0.02 to 0.16 Hz and 0.05 to 0.19 Hz). The asterisks indicate $P < 0.05$.

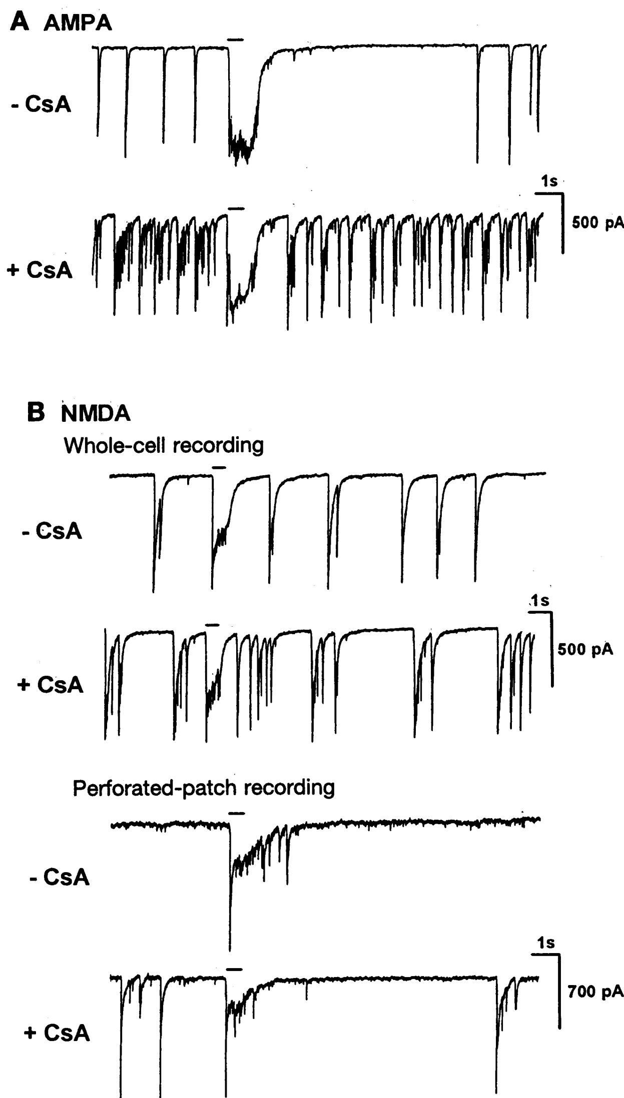
CsA exposure was not required to observe the increase in spontaneous neuronal activity. These observations and the lack of effect of CsA on the amplitude of the EPSCs suggested that a calcineurin-sensitive process was modulating the frequency of presynaptic glutamate release.

Additional evidence against a postsynaptic site of CsA action was obtained by applying exogenous glutamate-receptor agonists directly to the neurons. Other investigators have reported enhanced agonist sensitivity of AMPA receptors in the presence of phosphatase 1 or 2A inhibitors (21). CsA, a phosphatase 2B (calcineurin) inhibitor, did not increase the postsynaptic current response to AMPA (n = 3) (Fig. 3*A*), even though CsA markedly increased the frequency of the spontaneous EPSCs in the same neurons.

Recent studies suggest that NMDA receptors also may be modulated by phosphatases 1, 2A, and 2B, which act to downregulate channel activity (5, 22). With regard to phosphatase 2B in particular, the duration of single NMDA channel openings was shortened by activated calcineurin applied to the internal face of excised neuronal membrane patches and was prolonged by FK506 in cell-attached patches (5). In the present study, CsA had no effect on the amplitude of whole-cell current responses evoked by externally applied NMDA (Fig. 3*B*). In addition, CsA did not affect current amplitudes when the Mg^{2+} block of NMDA receptor-operated channels was eliminated by removing extracellular Mg^{2+} (Fig. 3*B*).

We also considered the possibility that the whole-cell recording technique might have masked a postsynaptic enhancement of NMDA-evoked currents due to diffusional loss of intracellular signaling components (23). The following observations make this possibility unlikely. First, prior to administration of CsA, we observed no rundown of either the spontaneous EPSCs or of the whole-cell current responses to application of NMDA. Second, we repeated the NMDA experiment by using the nystatin perforated-patch technique (18). With diffusional exchange between the pipette and cytoplasm limited to monovalent cations, CsA still had no effect on NMDA-evoked current amplitudes (Fig. 3*B*). Because we were unable to detect postsynaptic modulation of glutamatergic currents by calcineurin inhibition, we turned our attention to a further examination of the presynaptic role of calcineurin in glutamatergic neurotransmission.

The CsA-induced increase in synaptic activity could result from an alteration in one or more of the steps involved in glutamate release, from activation of presynaptic, voltage-gated Ca^{2+} channels to the docking and release of neurosecretory vesicles. We tested whether calcineurin modulates the rate of spontaneous vesicular release by recording miniature EPSCs, which represent postsynaptic responses to the spontaneous release of individual quanta of neurotransmitter (19) (Fig. 4*A*). While the frequency of action potential-mediated synaptic activity clearly was increased by CsA (EPSCs recorded in the absence of TTX increased from 0.68 ± 0.10 Hz



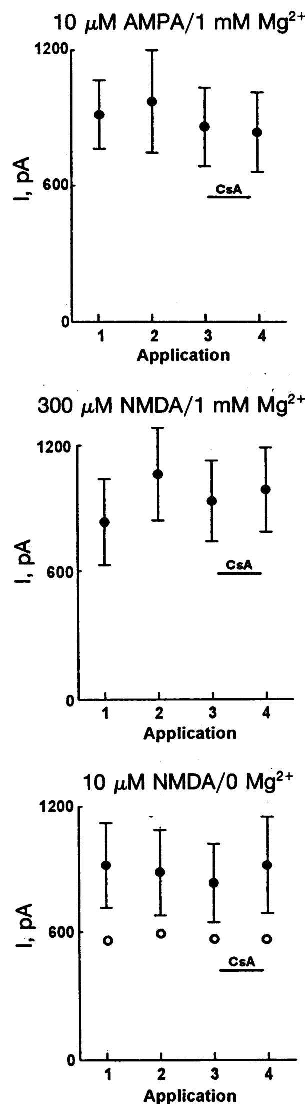


FIG. 3. Whole-cell current responses evoked by brief bath application of exogenous glutamate-receptor agonists in the absence or presence of CsA. (*A*) Lack of effect of CsA on current responses to AMPA. Current response to application of 10 μM AMPA (horizontal bar) in the absence or presence of CsA in one neuron is shown at the left. Summary data showing peak current responses to repeated applications of AMPA before and during CsA addition (horizontal bar; 5–10 μM CsA; n = 3) are shown at the right. (*B*) Lack of effect of CsA (5–10 μM) on current responses to NMDA in either the presence or absence of external Mg^{2+}. Current traces showing responses to NMDA as measured by standard whole-cell recording (300 μM NMDA in 1 mM Mg^{2+}) and by a perforated-patch recording (10 μM NMDA in 0 mM Mg^{2+}) are shown at the left. Summary data showing peak current responses to repeated applications of NMDA in the presence (n = 4) or absence (n = 5) of external Mg^{2+} (closed circles, data from whole-cell recordings; open circles, data from perforated-patch recording) before and during CsA (5–10 μM) addition are shown at the right.

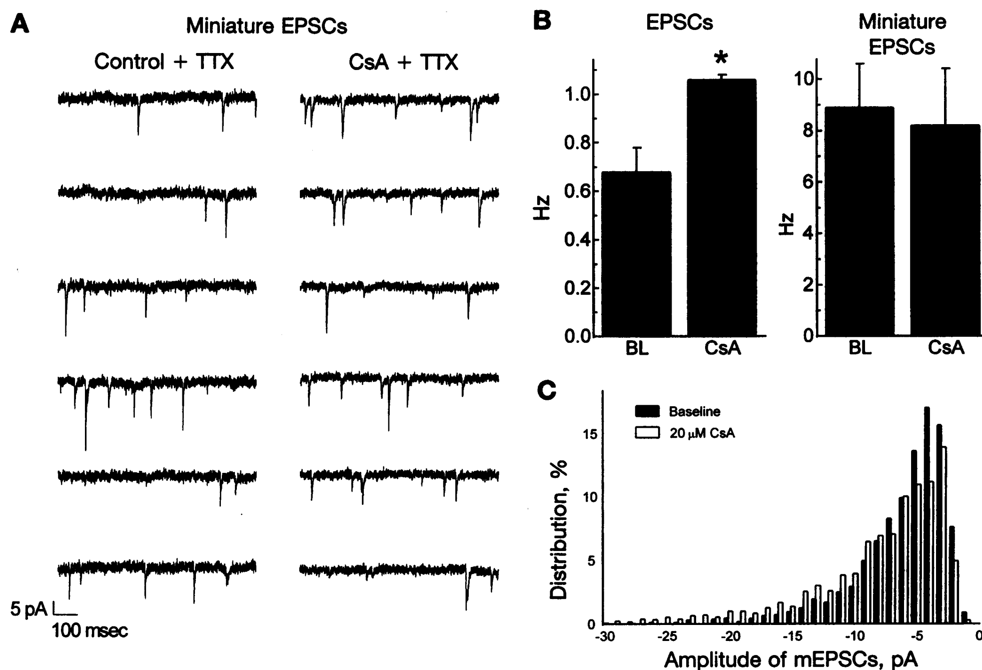


FIG. 4. Differential effects of CsA on EPSCs and miniature EPSCs. (*A*) Miniature EPSCs recorded in one neuron in the absence (Control + TTX) or presence (CsA + TTX) of 20 μM CsA with neuronal activity blocked by 5 μM TTX. (*B*) Summary data (n = 3) showing that CsA increased the frequency of EPSCs (in the absence of TTX) but had no effect on the frequency of miniature EPSCs (in the presence of TTX) in the same neurons. Asterisk indicates $P < 0.05$. (*C*) Histogram showing the amplitude distribution of miniature EPSCs (mEPSCs) at baseline and after administration of CsA. The bars represent the mean data from three neurons. The amplitude distribution was not significantly altered by CsA.

to 1.06 ± 0.02 Hz; n = 3), the frequency of miniature EPSCs recorded in the same cells in the presence of TTX was unaffected by CsA (8.9 ± 1.7 Hz vs. 8.2 ± 2.2 Hz for control and CsA, respectively; n = 3) (Fig. 4*B*). Miniature EPSC amplitudes also were unaffected by CsA (mean amplitudes were −5.46 ± 1.4 pA vs. −5.19 ± 0.8 pA for control and CsA, respectively; n = 3) (Fig. 4*C*), lending further support to our conclusion that calcineurin inhibition does not significantly modulate postsynaptic glutamatergic responses. These results suggest that calcineurin acts presynaptically to influence excitation–secretion coupling, rather than directly affecting the vesicle-release process. Thus, calcineurin may serve as the Ca^{2+}-sensitive component of a negative-feedback mechanism which limits the probability of vesicle release in response to increased internal [Ca^{2+}] during nerve-terminal depolarization.

These findings do not preclude the possibility that calcineurin modulates postsynaptic mechanisms under other experimental conditions. Indeed, the pre- and postsynaptic phosphatase actions may be complementary. Long-term depression is a form of synaptic plasticity mediated by both reduced glutamate release (24) and decreased postsynaptic responsiveness (6). An important role for phosphatases, including calcineurin, has been implicated in the latter effect (6). Because the spontaneous firing rate of the cultured fetal rat cortical neurons (≈0.5–1 Hz) is similar to the stimulation frequency used to elicit long-term depression, the rapid increases in spontaneous synaptic activity in response to calcineurin inhibitors in our experiments may involve relief of endogenous long-term depression. The present findings may explain why clinical immunosuppressive treatment with calcineurin inhibitors, such as CsA and FK506, can lead to central nervous system excitotoxicity (25, 26) and neurogenic hypertension (27–29) and suggest a specific role for calcineurin in synaptic neurotransmission.

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