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Dependence of long-term depression on postsynaptic metabotropic glutamate receptors in visual cortex

(synaptic plasticity/homosynaptic LTD/LTP/G-protein/inositol)

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ABSTRACT **Long-term depression (LTD) is held relevant to memory and learning. Its induction is known to require postsynaptic calcium increases. However, the source of these calcium increases remains unclear. In visual cortex slices, LTD was induced by tetanization after blockade of *N*-methyl-D-aspartate (NMDA) and non-NMDA ionotropic glutamate receptors. LTD induced under this condition was prevented by an intracellular injection of each of the following drugs into the postsynaptic neuron: (*i*) guanosine 5′-[β-thio]diphosphate, which competitively inhibits the binding of GTP to GTP-binding regulatory proteins; (*ii*) heparin, which antagonizes 1,4,5-inositol trisphosphate binding; and (*iii*) calcium chelators. Moreover, LTD was induced without tetanization by applying quisqualate (10 μM), a metabotropic glutamate receptor agonist, but not another agonist, *trans*-aminocyclopentane-1,3-dicarboxylic acid (10 μM). Together, these results suggest that activation of 1,4,5-inositol trisphosphate-linked subtypes of metabotropic glutamate receptor is responsible for the increase in postsynaptic calcium concentration, which results in homosynaptic LTD.**

The efficiency of transmission at central synapses can be up- or down-regulated in use-dependent manners. The induction of these changes, called long-term potentiation (LTP) and depression (LTD), respectively, requires an increase in the concentration of calcium in the postsynaptic neuron (1–6). It is well known that the calcium increase responsible for LTP is mediated by *N*-methyl-D-aspartate (NMDA) receptors (7, 8), voltage-gated channels (9, 10), or both. However, we know little of how the calcium increase responsible for LTD originates.

LTD in the hippocampus and neocortex can be classified into two categories according to induction procedures: hetero- and homosynaptic LTD (11–15). Heterosynaptic LTD can be induced by presynaptic activities of other inputs onto the same postsynaptic neuron and requires postsynaptic activity (16). In this case, the calcium source may be quite predictable, given that postsynaptic cells are activated and depolarized during the induction. Indeed, an involvement of NMDA receptors or voltage-sensitive calcium channels has been suggested (17, 18). In contrast, homosynaptic LTD can be induced by presynaptic activities of the same inputs onto the relevant neuron and needs neither NMDA receptors nor ample postsynaptic depolarization (14, 15). If postsynaptic neurons are not depolarized well, it is unlikely that voltage-gated calcium channels are sufficiently activated to allow Ca^{2+} entry. Recently, the participation of metabotropic glutamate receptors (mGluRs) in this process has been proposed and tested by using L-2-amino-3-phosphonopropionate, an antagonist (19). However, in view of recent findings based on molecular cloning (20–24), this chemical is unlikely to block appropriately the physiological effects caused by 1,4,5-inositol trisphosphate (InsP_3)-linked mGluRs. Even in earlier reports (25, 26), it was effective at >1 mM only. In the present experiments, involvement of mGluRs was examined by blocking GTP-binding regulatory protein (G protein)-related pathways.

MATERIALS AND METHODS

Slices of visual cortex (350 μm in thickness) were prepared from young adult albino rats (110–200 g; 32–49 days old) after rapid decapitation. A submerged-type chamber was used for recording. The standard medium contained 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose and was equilibrated with 95% O_2/5% CO_2. A bipolar stimulating electrode was inserted in the white matter underneath the recording area (Fig. 1*A*). Intracellular recordings were carried out from cells in layers II–IV with a glass micropipette filled with 3 M potassium acetate. Postsynaptic potentials (PSPs) evoked by subthreshold (60–70% of threshold for a spike) stimulation of the white matter at low frequencies (0.06–0.07 Hz) were recorded with a bridge-equipped amplifier (Axoclamp-2A; Axon Instruments, Burlingame, CA). When the PSPs had been stable for ≈30 min, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 10 μM D-(−)-2-amino-5-phosphonopentanoate [D-(−)-2-amino-5-phosphonovalerate (APV)] were applied by addition to the bath medium ("bath-applied"). After 10 min or more, repetitive stimulation (2-s trains at 50 Hz repeated five times every 10 s with an intensity 1.5–2 times the threshold for the spike) was given through the stimulating electrode. Immediately after the tetanus, the drugs were washed out with normal medium at the rate of 10–18 ml/min. In some cases, LTP was subsequently elicited in the same cell with either of the two methods known to induce LTP in the cerebral cortex: 0.5 μM bicuculline hydrochloride was bath-applied or the cell was depolarized (by more than 40 mV) by intracellularly applied currents during presynaptic tetanization. The signals were digitized at a rate of 10 kH. All the sample recordings are averages of five traces. The amplitude of PSPs was measured at the peak. The Student or Welch *t* test was used for statistics.

For intracellular injections, the recording electrode was filled with each of the following chemicals dissolved in 2–3 M potassium acetate: 15 mM GDP[β-S] for experiments illustrated in Fig. 3; 2 mg of heparin sodium salt per ml for those in Fig. 4; and 200 mM EGTA or 150 mM 1,2-bis(2-

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Abbreviations: LTP, long-term potentiation; LTD, long-term depression; InsP_3, inositol 1,4,5-trisphosphate; tACPD, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid; mGluR, metabotropic glutamate receptor; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; APV, D-(−)-2-amino-5-phosphonovalerate (pentanoate); BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid; AHP, afterhyperpolarization; NMDA, *N*-methyl-D-aspartate; G protein, GTP-binding regulatory protein; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate.

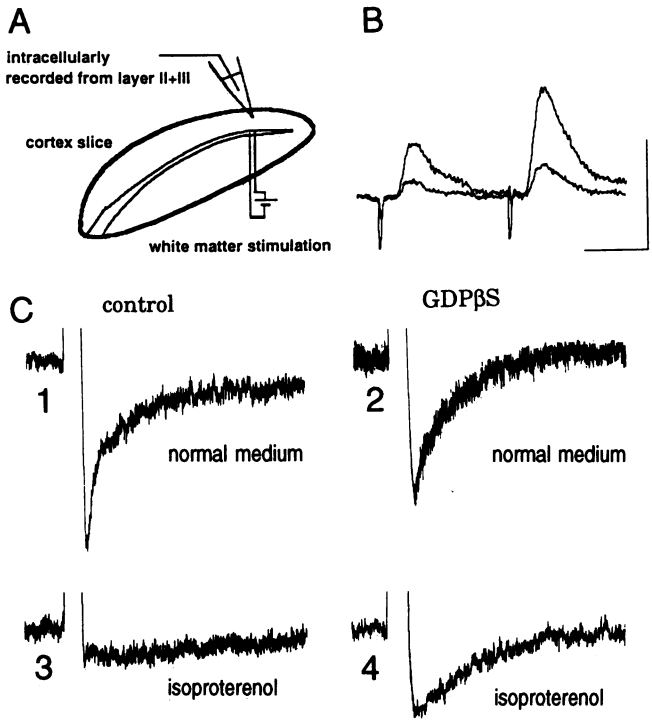

FIG. 1. (*A*) Schematic illustration of a slice containing the visual cortex. Sites of stimulation and recording electrodes are shown. (*B*) Paired-pulse facilitation of PSPs evoked in such preparations as shown in *A*. Traces are the averages of 5 successive recordings of the responses to stimulation of two different strengths. Scale bars represent 4 mV and 20 ms. (*C*) Effects of intracellularly injected guanosine 5′-[β-thio]diphosphate (GDP[β-S]) on the modulation of afterhyperpolarization (AHP) by isoproterenol. Irrespective of whether GDP[β-S] was injected or not, a large AHP was induced after application of a depolarizing current pulse (1, 2). This AHP was abolished in isoproterenol-containing medium when no GDP[β-S] was injected (3). With GDP[β-S] injected, such abolition by isoproterenol was prevented and the majority of AHP remained (4). For *C*, scale bars in *B* now represent 1.9 mV and 1.6 s.

aminophenoxy)ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid (BAPTA) for those in Fig. 5 A and B. Each chemical was injected intracellularly by repeated applications of hyperpolarizing current pulses (0.5-nA pulses of 500-ms duration at 1 Hz for 10–30 min) through the recording electrode. In five additional cells, it was tested whether the present method of GDP[β-S] application was appropriate. Use was made of the phenomenon that the β-adrenergic blocker isoproterenol abolishes or reduces the AHP that follows depolarization caused by current injection (27). This effect is known to be mediated by G-protein-coupled β-adrenoceptors (28). Induction of AHP was attempted with or without GDP[β-S] intracellularly injected, first in normal medium and then in medium containing 10 μM isoproterenol.

RESULTS

Control Experiments and Interpretation. Generally, PSPs evoked by white-matter stimulation may be monosynaptic, polysynaptic, or a mixture of both. The extent to which monosynaptic inputs contribute to the present PSP was studied by monitoring paired-pulse facilitation. The second volley, which was delivered 40 ms after the first one, evoked a PSP about twice as large in amplitude as the first PSP in all six cells examined with a relatively weak strength of stimulation (Fig. 1*B*). This degree of facilitation is comparable to that reported in the hippocampal CA3-to-CA1 monosynaptic pathway on the basis of whole-cell voltage-clamped recordings (29). Facilitation was less prominent with stronger stimulation in all the cells, which is likely due to the nonlinear summation characteristic of the current-clamp recording. Thus, it is suggested that the present PSPs are attributed mostly to monosynaptic inputs. With stronger stimulation, contamination by polysynaptic components cannot be entirely excluded. However, this problem is unlikely to affect the interpretation because occurrence of LTD was checked by measuring not only the peak amplitude of PSPs but also the initial slope, which is considered to reflect mostly monosynaptic components (see below).

Effects of intracellularly injected GDP[β-S] were assessed by examining the influence on isoproterenol-induced reduction of AHP. Control experiments (Fig. 1*C*, traces 1 and 3) confirmed that application of isoproterenol to the bath medium almost abolished AHP in both cells examined as reported (27). By contrast, in all three cells with GDP[β-S] injected, isoproterenol application left the majority of the hyperpolarization preserved (Fig. 1*C*, traces 2 and 4); the peak amplitude of the negative deflection was 71.2 ± 6.6% (average ± SEM) of controls obtained before the application. The preservation was rather partial, in agreement with the competitive nature of GDP[β-S] (28). Nevertheless, it is evident that the injected chemical was effective in competition with GTP. This presumably holds true for other types of G proteins as well. Essentially the same application method was used for the heparin injection. This method again seems reasonably reliable, given the previous report (30) that heparin injected intracellularly by sharp glass microelectrodes exerted inhibitory effects on $InsP_3$-related processes.

LTD in Visual Cortex Slices. Glutamate is the major excitatory neurotransmitter in the cerebral cortex (8, 31). When both the NMDA and non-NMDA ionotropic glutamate receptors were blocked by APV and CNQX, PSPs recorded from visual cortical neurons were virtually abolished (Fig. 2*A*, traces a). Tetanic stimulation was applied to the presynaptic fibers in the presence of the blockers, which were then washed out immediately after tetanization. After removal of the blockers, the amplitude of PSPs never recovered to the pretetanic level (Figs. 2*A*, traces a and c and 2*B*). The peak amplitude of the PSP was 77.3 ± 4.3% 50 min posttetanus (n = 9), significantly smaller ($P < 0.01$) than the value obtained after the tetanus in the absence of the blockers (100.7% ± 1.2% 30 min posttetanus; n = 6). This depression in the peak amplitude was in parallel with that in the initial slope of PSPs (66.0 ± 4.3% 50 min posttetanus).

By contrast, the synaptic response recovered to the control level after washout of the glutamate receptor blockers when the presynaptic tetanization had not been applied (Fig. 2*A*, traces b; 100.6 ± 3.4% 50 min posttetanus; n = 6). Moreover, synaptic depression did not result from deterioration of the preparation because LTP could be elicited by a second tetanus applied after washing out the blockers: LTP resulted when the tetanus was delivered in medium containing 0.5 μM bicuculline, a γ-aminobutyric acid $GABA_A$ antagonist or was paired with postsynaptic depolarizing pulses in normal medium (Fig. 2*A*, traces a; 117.8 ± 2.2% 15–30 min posttetanus) (8, 15).

LTD thus induced was recorded up to 66.7 min posttetanus on average. In the case shown in Fig. 2*C*, LTD was followed up to 80 min posttetanus and was maintained stably for 35 min after complete washout. There may have been short-term potentiation or depression during the washout period, although no further investigation was carried out.

Pharmacological Blockade of LTD. From the above results it seems clear that the induction of LTD does not require activation of the ionotropic glutamate receptors, and presumably postsynaptic depolarization either. Might mGluRs be responsible for induction of LTD? Unfortunately there are as yet no blockers specific to mGluRs. Therefore, the first approach to this issue was made by testing a possible involvement of G protein-linked receptors. GDP[β-S] pre-

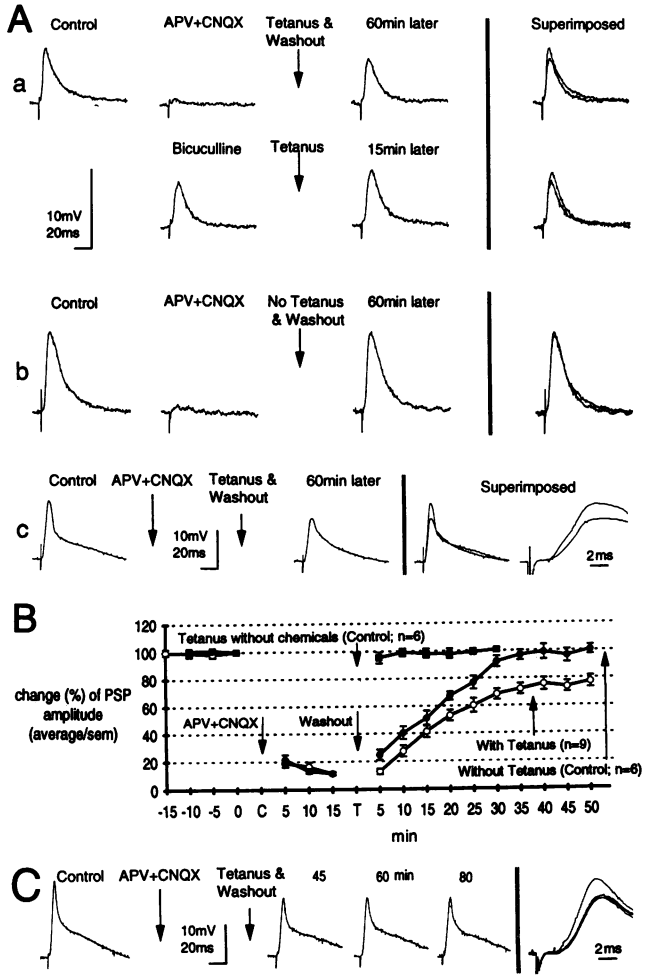

FIG. 2. Effects of ionotropic glutamate receptor blockers on induction of LTD. (*A*) Traces a show PSPs recorded in normal medium (control) and in the presence of APV and CNQX (APV+CNQX). The presynaptic fibers were tetanized in the presence of the blockers, and the PSPs were recorded 60 min after washout (60 min later). PSPs were then recorded from the same cell in the presence of bicuculline (bicuculline) and 15 min after the second tetanus (15 min later). Superimposed records on the far right show LTD (upper record) and LTP (lower record). Traces b show PSPs before (control) and after APV and CNQX were bath-applied but with no tetanus delivered. After 60 min of washout, the PSPs remained unchanged. Traces c show LTD induced as in traces a. On the far right, superimposed recordings are illustrated with a 10-times expanded time scale, showing that the initial slope of the PSP was affected. (*B*) Time courses of changes in the peak amplitude of PSPs. In the nonblocker control, tetanus was delivered in normal medium (■). In the nontetanus control, the drugs were added and then washed out without tetanization (●). In the test group, tetanization was delivered in the presence of the two blockers (○). The amplitudes are averaged and expressed as percent of the control amplitude recorded prior to the tetanus. Vertical bars indicate the SEM. The arrow indicates the time of tetanus or chemical application. (*C*) Control PSP and PSPs recorded 45, 60, and 80 min after tetanization. The induced LTD stably lasts for 35 min. On the right hand, the four recordings are superimposed on an expanded time scale. The resting membrane potential, input resistance, and control PSP amplitude were as follows: 78.9 ± 0.4 mV, 35.1 ± 6.8 MΩ, and 14.8 ± 1.1 mV (average ± SEM) for the test group; 76.8 ± 0.8 mV, 35.1 ± 2.9 MΩ, and 12.5 ± 0.9 mV for the nontetanus control; and 77.7 ± 2.3 mV, 36.7 ± 5.8 MΩ, and 20.3 ± 3.9 mV for the nonblocker control.

vents GTP binding to the G protein, thereby halting the second-messenger process induced by the G protein (28, 32). The intracellular application of GDP[β-S] had no effect on the resting membrane potential or the PSP amplitude (Fig. 3*A*, traces a). However, tetanization of the presynaptic fibers in the presence of APV and CNQX now failed to induce LTD (Fig. 3*A*, traces b; 96.8 ± 2.2% 50 min posttetanus). In the absence of APV and CNQX, presynaptic tetanization induced LTP in the neuron injected with GDP[β-S] (Fig. 3*A*, traces c; 122.7 ± 7.2% 15–30 min posttetanus). This suggests that a G protein-mediated process may be involved in the induction of LTD but not in LTP.

Molecular cloning has revealed the existence of multiple types of mGluRs (20–24); some of them mediate inositol signal transduction, whereas others regulate cAMP levels. Which mGluRs might be involved in LTD? This was tested by heparin injection. Heparin blocks the binding of $InsP_3$ to its specific receptors on cytosolic calcium stores (33–36). Intracellular injections of heparin did not alter the resting membrane potential or the PSP amplitude. However, presynaptic tetanization in the presence of APV and CNQX again failed to induce LTD (Fig. 4*A*, traces a, and Fig. 4*B*). The mean PSP amplitude measured 50 min after washing out the ionotropic receptor blockers (97.4 ± 7.2%; $n = 4$) was not significantly different from the pretetanic level. In three heparin-injected cells, LTP could be induced by pairing presynaptic tetanization with postsynaptic depolarization (Fig. 4*A*, traces b; 118.0 ± 7.7% 15–30 min posttetanus). Thus, heparin blocked the induction of LTD but did not affect the LTP induction. This suggests that LTD requires an

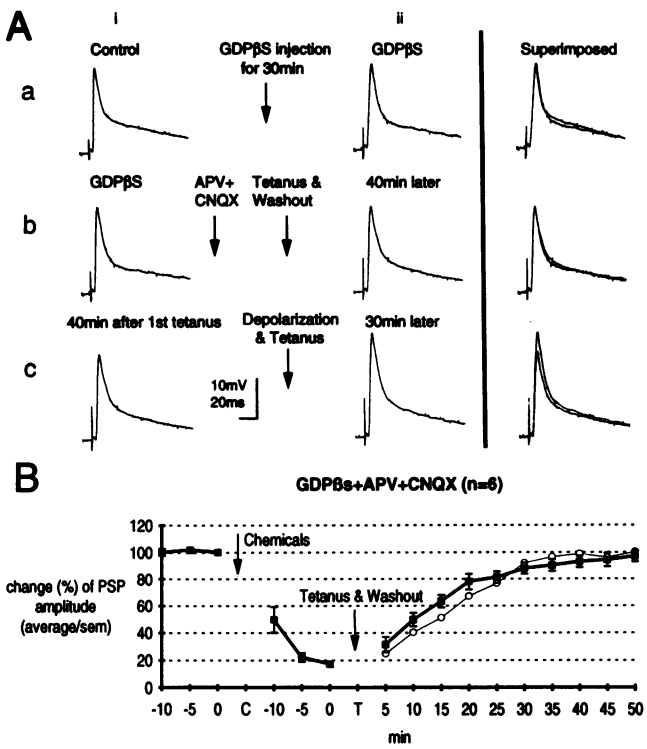

FIG. 3. Effects of intracellularly injected GDP[β-S] on LTD. (*A*) Traces a compare PSPs elicited before (i) and after (ii) iontophoretic application of GDP[β-S], and traces b show that intracellularly applied GDP[β-S] prevented LTD induction. The control PSP trace bi is the same as trace aii; the PSP was then recorded 40 min after washout of APV and CNQX, which had been added before tetanization of the presynaptic fibers (ii). In traces c, the same cell was then depolarized by an intracellularly applied current pulse in conjunction with presynaptic tetanization. Superimposition of the "ci" record (same as the bii record) and the cii record shows the induction of LTP. (*B*) Time course of changes in the peak amplitude of the PSPs observed in six different neurons injected with GDP[β-S]. The plot for nontetanus control (Fig. 2*B*) is superimposed (open circles). The resting membrane potential, input resistance, and control PSP amplitude were: 78.4 ± 1.6 mV, 33.9 ± 5.1 MΩ, and 15.3 ± 1.7 mV (average ± SEM).

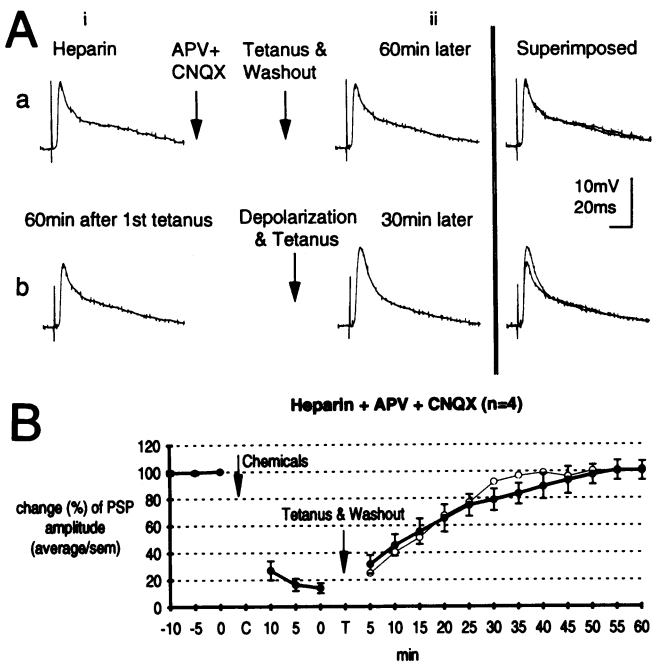

FIG. 4. Effects of intracellularly injected heparin on LTD. (*A*) In traces a, intracellularly applied heparin prevented LTD induction. The control PSP was recorded after heparin injection (trace ai); the PSP was then recorded 60 min after washout of APV and CNQX, which had been added before the tetanus (trace aii). In traces b, the same cell was then depolarized with a depolarizing current pulse in conjunction with presynaptic tetanization. Superimposition of the "bi" record (same as the aii record) and the bii record shows the induction of LTP. (*B*) Time course of changes in the peak amplitude of the PSPs observed in four different cells. The plot for nontetanus control (Fig. 2*B*) is superimposed (open circles). The difference between the two plots may reflect a short-term depression or nonspecific effect by heparin. The resting membrane potential, input resistance, and control PSP amplitude were: 75.8 ± 0.9 mV, 37.5 ± 9.9 MΩ, and 13.8 ± 1.7 mV (average ± SEM).

increase in the intracellular calcium concentration via an InsP_3-mediated pathway in the postsynaptic neuron.

To address the above possibility further, EGTA or BAPTA was loaded intracellularly to chelate intracellular calcium ions (1–6). Effective chelation of intracellular calcium was shown by suppression of the AHP after a train of spikes induced by a depolarizing pulse (27). Usually an almost complete suppression of AHP was observed 5 min after beginning injections of EGTA or BAPTA (Fig. 5*A*). After a 10- to 30-min loading, control PSPs were recorded; this was followed by a tetanus applied to the presynaptic fibers in the presence of APV and CNQX. The results were essentially the same as those observed with GDP[β-S] or heparin (Fig. 5*B*): chelation of intracellular calcium had no effects on the control PSPs but prevented the induction of LTD (98.3 ± 5.7%; n = 4).

Agonist-Induced LTD. To confirm the involvement of mGluRs, application of agonists was attempted without tetanization. The type of mGluR involved in LTD was also examined by determining its agonist selectivity. Quisqualate is an agonist for the mGluR linked to inositol phosphate turnover, whereas *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (tACPD) most potently stimulates the receptor coupled to the adenylate cyclase cascade (20–24). CNQX and APV were first bath-applied to prevent quisqualate from activating α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors and also to simulate the experimental protocol except for tetanization. Quisqualate was then added, and 10 min later all of the drugs were washed out without tetanization. LTD was induced under this condition (75.8 ± 2.6% 50 min posttetanus; n = 9; $P < 0.01$) (Fig. 5 *C* and *D*). In contrast,

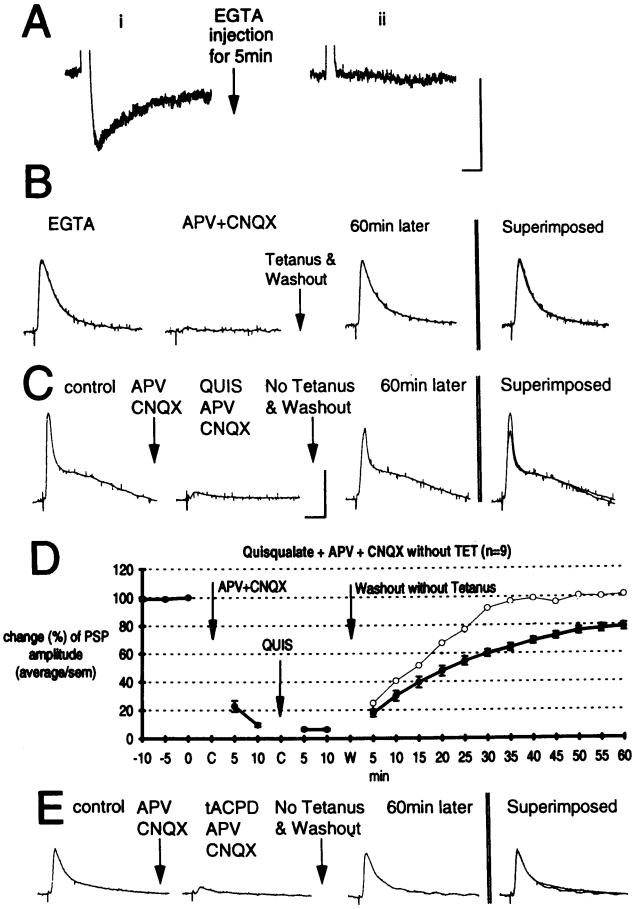

FIG. 5. (*A*) Effects of intracellularly injected EGTA on AHP. Before EGTA injection, depolarizing current pulses (+0.5 nA for 500 ms) were applied to elicit AHP (trace i). EGTA application for 5 min abolished AHP, indicating an effective chelation (trace ii). Scale bars represent 3.2 mV and 1.3 sec. (*B*) Intracellularly applied EGTA prevented LTD induction. Control PSPs were recorded after EGTA injection (trace EGTA); the PSP was then recorded 60 min after washout of APV and CNQX (60 min later). The scale bars in *A* now represent 10 mV and 20 ms for *B*. (*C*) Quisqualate mimics tetanization. Quisqualate (QUIS; 10 μM) was bath-applied 10 min after APV and CNQX had been added, and recordings were made with electrodes filled with potassium acetate alone; 10 min after the quisqualate application, all of the chemicals were washed out without tetanization. The control PSPs are shown (control trace); then the PSP was recorded 60 min after washout of all the chemicals (60 min later). Scale bars represent 10 mV and 20 ms. (*D*) Time course of changes in the peak amplitude of the PSPs in nine different cells. The prior application of APV and CNQX abolished the PSP. Quisqualate added at this stage stimulates mGluRs but not AMPA receptors. The plot for nontetanus control (Fig. 2*B*) is superimposed (open circles). (*E*) Failure of tACPD to mimic tetanization. Here 10 μM tACPD was bath-applied instead of quisqualate. The control PSPs are shown (control trace); then the PSP was recorded 60 min after simple washout without tetanization (60 min later). The same scale bars in *C* apply for *E*. The resting membrane potential, input resistance, and control PSP amplitude were: 76.3 ± 3.0 mV, 38.5 ± 6.2 MΩ, and 13.5 ± 2.5 mV (average ± SEM) for chelator experiments; 79.5 ± 1.0 mV, 45.1 ± 3.0 MΩ, and 14.8 ± 0.8 mV for quisqualate experiments; and 72.5 mV, 39.0 MΩ, and 12.5 mV for tACPD experiments.

when tACPD was applied together with CNQX and APV, the PSP amplitude returned to the control level (Fig. 5*E*; n = 2). These results again point to the involvement of mGluRs linked to inositol phosphate turnover.

DISCUSSION

The obvious implication of the present results is that the induction of LTD requires activation of InsP_3-linked

mGluRs, presumably the subtype(s) mGluR1 or mGluR5 or both (20–24). An involvement of the other known subtypes, linked mainly to cAMP cascades, seems less likely because addition of 10 μM tACPD to the bath medium failed to mimic the effect of tetanization. This concentration of tACPD was shown to activate cAMP-linked subtypes but not those linked to InsP_3 (20–24). To activate the latter subtypes of cloned receptor, more than 100 μM is required. Obviously there is a possibility that activation of native receptors may need a higher concentration of tACPD than that of artificially expressed receptors and, if so, 10 μM may be too low to stimulate any subtypes.

In cerebellar Purkinje neurons, the following three conditions must be fulfilled for LTD: activation of mGluRs, activation of AMPA receptors, and depolarization of postsynaptic neurons (37). Given that AMPA-receptor activation can initiate activation of voltage-gated calcium channels and that all of these three conditions are able to increase intracellular calcium, it is impossible to single out the calcium source responsible. In contrast, for the homosynaptic LTD observed in the present experiments, the latter two of the above three conditions seem unnecessary; both AMPA and NMDA receptors were blocked, preventing the depolarization that would normally be elicited by tetanization. Thus, mGluR activation alone appears to be the prerequisite for homosynaptic LTD in the visual cortex; hence, the responsible calcium source may be cytosolic stores.

NMDA receptors or voltage-gated calcium channels are required for heterosynaptic LTD (17, 18), whereas neither of them seem involved directly in the induction of homosynaptic LTD (14, 15). Activation of mGluRs, which eventually releases calcium from cytosolic stores, has been suggested to be necessary for homosynaptic LTD (19). In the present experiments, this suggestion was substantiated by pharmacological blockade of the pathway involved, which is initiated by certain subtypes of mGluRs and ends with calcium release from internal stores. Induction of homosynaptic LTD in the dentate gyrus has been reported to consist of two phases: the priming phase and the subsequent tetanization phase (38). The priming, but not tetanization, phase involves NMDA receptor activation. This activation of NMDA receptors may fulfil the requirement of postsynaptic calcium increase in the dentate gyrus, but it is not required for homosynaptic LTD in the visual cortex.

The present study shows that LTP and LTD can be induced in the same neuron of the visual cortex but require different sources of calcium increase. G-protein activation and subsequent cytosolic-calcium release are necessary for LTD but not for LTP. On the other hand, NMDA-receptor activation is required for LTP but not for LTD (8, 39). This difference in the calcium source may be manifest in multiple aspects of the intracellular calcium increase, such as absolute concentration, regional compartmentalization, and temporal pattern. Differences in multidimensional calcium increases may relate to differential activation of second messengers, which I propose could be the basis for differential induction of LTP and LTD. This proposal, although awaiting further investigation, seems to have relevance to recent findings. Regarding the temporal aspect, LTP induction has been shown to require calcium increases lasting for at least a few seconds (40). As for the compartmentalization, associative interactions during LTP- or LTD-inducing tetanus can be restricted to a local dendritic domain (41). Taking the absolute concentration into account, one theory (42) suggests that large and moderate increases of calcium lead to LTP and LTD, respectively. It is, however, unclear whether activities of second messengers alone can determine the "watershed" between LTP and LTD.

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