Activation of Metabotropic Glutamate Receptor Subtype 1/Protein Kinase C/Mitogen-Activated Protein Kinase Pathway Is Required for Postischemic Long-Term Potentiation in the Striatum

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ABSTRACT

Excessive stimulation of glutamate receptors is believed to contribute substantially in determining neuronal vulnerability to ischemia. However, how this pathological event predisposes neurons to excitotoxic insults is still largely unknown. By using electrophysiological recordings from single striatal neurons, we demonstrate in a corticostriatal brain-slice preparation that in vitro ischemia (glucose and oxygen deprivation) activates a complex chain of intracellular events responsible for a dramatic and irreversible increase in the sensitivity of striatal neurons to synaptically released glutamate. This process follows the stimulation of both *N*-methyl-D-aspartate and metabotropic glutamate receptors and involves the activation of the mitogen-activated protein kinase ERK via protein kinase C. This pathological form of synaptic plasticity might play a role in the cell type-specific neuronal vulnerability in the striatum, because it is selectively expressed in neuronal subtypes that are highly sensitive to both acute and chronic disorders involving this brain area.

Post-tetanic long-term potentiation (LTP) is considered a physiological form of synaptic plasticity, and its occurrence in either cortical (Bliss and Collingridge, 1993) or subcortical areas (Calabresi et al., 1996) has been regarded as a cellular substrate for memory and learning. More recently, it has been reported that pathological events such as anoxia and energy deprivation may induce long-term changes of excitatory synaptic transmission in hippocampal CA1 pyramidal neurons (Crépel et al., 1993; Hsu and Huang, 1997). Long-term pathological changes of synaptic transmission induced by ischemia and energy deprivation may underlie the differential neuronal vulnerability expressed in different brain areas or even in different neuronal subtypes in the same structure. Nevertheless, the cellular and molecular mechanisms underlying brain ischemic LTP (i-LTP) have not yet been elucidated. Overstimulation of AMPA/kainate and NMDA receptors during ischemia leads to rapid sodium and calcium accumulation; the latter event is worsened by the mobilization of intracellular calcium triggered by the concomitant mGluR activation (Sheardown et al., 1990; Lee et al., 1999; Pellegrini-Giampietro et al., 1999). However, the possible interaction between ionotropic and metabotropic glutamate receptors in the formation of i-LTP has never been investigated. We addressed this issue by using electrophysiological experiments from striatal spiny neurons, which express a marked vulnerability to ischemia and energy deprivation (Ferrante et al., 1985; Pulsinelli, 1985; Chesselet et al., 1990).

We report that brief in vitro ischemia causes, in coincidence with the stimulation of NMDA and mGluR1 receptors,

ABBREVIATIONS: LTP, long-term potentiation; i-LTP, ischemic long-term potentiation; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; NMDA, *N*-methyl-D-aspartate; mGluR1, metabotropic glutamate receptor subtype 1; PKC, protein kinase C; MAP, mitogen-activated protein; ERK, extracellular signal receptor-activated kinase; EPSP, excitatory postsynaptic potential; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; EPSC, excitatory postsynaptic current; APV, 2-amino-5-phosphonovalerate; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; AIDA, (*R*,*S*)-1-aminoindan-1,5-dicarboxylic acid; LA, large aspiny; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; RMP, resting membrane potential; MEK, mitogen-activated protein kinase kinase.

the activation of protein kinase C (PKC) and mitogen-activated protein (MAP) kinase ERK. This complex cascade of biochemical events leads to the induction of i-LTP in striatal spiny neurons.

Experimental Procedures

Preparation, Maintenance of the Slices, and Electrophysiological Recordings. Corticostriatal slices 270 μm thick were prepared from adult Wistar rats and mice. The preparation and maintenance of coronal slices have been described previously (Calabresi et al., 1997, 1999). A single slice was transferred to a recording chamber and submerged in a continuously flowing Krebs' solution (35°C, 2–3 ml/min) gassed with 95% O_2/5% CO_2. To study ischemia in striatal neurons, slices were deprived of glucose by totally removing glucose from the perfusate and by adding sucrose to balance the osmolarity. This solution was gassed with a mixture of 95% N_2/5% CO_2 instead of the normal gas mixture. The composition of the control solution was 126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 11 mM glucose, and 25 mM $NaHCO_3$. In the majority of experiments, external magnesium was omitted.

Intracellular recording electrodes were filled with 2 M KCl (30–60 MΩ). Signals were recorded with the use of an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA), displayed on a separate oscilloscope, and stored on a digital system. To activate corticostriatal fibers, stimulating electrodes were located either in the cortical areas near the recording electrode or in the white matter between the cortex and the striatum. To rule out a possible contamination of the excitatory postsynaptic potentials (EPSPs) by depolarizing potentials mediated by γ-aminobutyric acid-A receptors, approximately 50% of the recordings were obtained in the presence of 50 μM picrotoxin. Because these experiments gave results that were similar to those obtained in the absence of this drug, all the data were pulled together.

Whole-cell patch-clamp recordings were made using borosilicate glass pipettes (1.8 mm o.d.; 3–5 MΩ) containing 125 mM K^+-gluconate, 10 mM NaCl, 1.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 0.5 mM BAPTA,19 mM HEPES, 0.3 mM GTP, and 1.0 mM Mg-ATP, adjusted to pH 7.3 with KOH. Membrane currents were monitored using an Axopatch 1D patch clamp amplifier (Axon Instruments). Whole-cell access resistances measured in voltage clamp were in the range of 5 to 30 MΩ before electronic compensation (60–80% was routinely used).

Data Analysis and Drug Applications. Quantitative data on postischemic modifications of EPSP and excitatory postsynaptic currents (EPSC) are expressed as a percentage of the controls, which represent the mean of responses recorded during a stable period (15–30 min) before the ischemic episode. Values given in the text and in the figures are mean ± S.E.M. of changes in the respective cell populations. Wilcoxon's test or Student's t test (for paired and unpaired observations) were used to compare the means, and analysis of variance was used when multiple comparisons were made against a single control group. Drugs were applied by dissolving them to the desired final concentration in the saline or in the KCl intraelectrode solution. 2-Amino-5-phosphonovalerate (APV) and staurosporine were from Sigma (St. Louis, MO). BAPTA, PD98059, and Ro 32–0432 were from Sigma/RBI (Natick, MA). Calphostin C, CPCCOEt, LY367385, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), (*R*,*S*)-1-aminoindan-1,5-dicarboxylic acid (AIDA), and picrotoxin were from Tocris Cookson (St. Louis, MO). UO126 was from Alexis Corporation (Läufelfingen, Switzerland).

Extract Preparation and Immunoblotting. Corticostriatal slices (270 μm) were prepared as described above. Slices were preincubated for 20 min in magnesium-free control solution alone or containing one of the following compounds: 10 μM PD98059, 30 μM LY367385, 30 μM MPEP, or 1 μM calphostin C. Slices were then subjected to 5-min ischemia as described above in the presence of the appropriate inhibitor, followed by either increasing wash time in magnesium-free control solution or a set 15-min wash in magnesium-free control solution plus the appropriate inhibitor. Striata were microdissected and rapidly homogenized using a Microfuge tube pestle in 40 μl of extraction buffer (10 mM Tris-HCl, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 5 μM $ZnCl_2$, 30 mM sodium pyrophosphate, 1 mM dithiothreitol, titrated to pH 7.05, with freshly added protease and phosphatase inhibitors) (Hipskind et al., 1994). Insoluble proteins were removed by centrifugation at 10,000g for 15 min at 4°C, and supernatant proteins were immediately denatured in 50 mM Tris-HCl, pH 6.8, 2% SDS, 2% glycerol, and 1% 2-mercaptoethanol. Western blots containing 5 μg of extract protein per lane were immunodetected as described (Bowler et al., 1999) using antisera specific for ERKs 1 and 2 activated by phosphorylation on Thr 202 and Tyr 204 (New England Biolabs, Beverly, MA), as well as control antisera directed against ERKs 1 and 2 (New England Biolabs). After incubation with horseradish peroxidase-coupled goat anti-rabbit antibodies (Sigma), immune complexes were visualized with the use of Renaissance ECL (PerkinElmer Life Sciences, Boston, MA) and exposure to Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

Results

Electrophysiological Characterization of the Recorded Neurons. Intracellular and whole-cell patch-clamp recordings were performed from striatal spiny neurons and large aspiny (LA) cholinergic interneurons in corticostriatal slices. These neurons were easily distinguished by their typical electrophysiological properties, as shown previously in intracellular or whole-cell patch-clamp experiments (Kita et al., 1984; Kawaguchi, 1993; Calabresi et al., 1997, 1998a,b). Striatal spiny neurons represented the majority ($n = 122$; resting membrane potential, -85 ± 4 mV; input resistance, 40 ± 8 MΩ) of the recorded cells, whereas 24 cells had electrophysiological characteristics of LA interneurons (resting membrane potential, -60 ± 4 mV; input resistance, 162 ± 12 MΩ). Also, the physiological and pharmacological properties of cortically evoked EPSPs significantly differ in the two groups of neurons. In fact, in the presence of a physiological concentration of external magnesium (1.2 mM), spiny neurons responded to the stimulation of corticostriatal fibers by producing an EPSP mediated completely by the activation of glutamate AMPA receptors, whereas LA interneurons responded by generating synaptic potentials mediated in part by NMDA receptors. The AMPA glutamate receptor antagonist CNQX (10 μM), in fact, fully suppressed the EPSPs recorded intracellularly from spiny neurons, whereas the subsequent application of the NMDA receptor antagonist APV (50 μM) was required to abolish EPSPs evoked in LA interneurons ($n = 6$). In contrast, in spiny neurons, omission of external magnesium, a procedure that removes the voltage-dependent block of NMDA receptors, was necessary to reveal the NMDA component of the EPSP that could be blocked by APV (50 μM) ($n = 20$).

Effects of Ischemia on Spiny Neurons and Cholinergic Interneurons. To test the effect of energy deprivation on glutamatergic corticostriatal transmission in both the physiological condition (1.2 mM Mg^{2+}) and magnesium-free medium, both spiny and LA neurons were deprived of oxygen and glucose for 3 min and their responses were recorded intracellularly. According to previous reports (Calabresi et al., 1999; Pisani et al., 1999), in vitro ischemia in striatal spiny neurons caused a membrane depolarization associated

with a decreased input resistance ($n = 101$), whereas the same insult resulted in a membrane hyperpolarization of LA interneurons ($n = 15$). In the two neuronal subtypes, the ischemia-induced membrane potential and input resistance changes were unaffected by the omission of magnesium ions, and in all neurons used for data analysis, these electrophysiological parameters recovered to the control values within 3 to 6 min of wash (Figs. 1, A and B, and 2A). In both experimental conditions (1.2 mM and free Mg^{2+} medium), a marked blockade of synaptic transmission was observed within 2 min from the onset of the ischemic episode in the two neuronal subtypes. Upon return to control solution, the EPSP amplitude recorded from spiny neurons in the magnesium-free medium were potentiated. This i-LTP lasted throughout the period of observation (usually longer than 30 min) and reached a plateau after 15 to 20 min of wash ($n = 45$) (Fig. 1). On the contrary, in striatal neurons recorded in 1.2 mM Mg^{2+} medium (data not shown), as well as in LA interneurons recorded either in 1.2 mM or free magnesium medium, cortically evoked EPSPs returned to control value after the brief ischemic episode without showing any significant long-term change (Fig. 2A). Similar results were obtained from LA interneurons in whole-cell patch-clamp experiments. In the presence of 1.2 mM magnesium, in fact, in vitro ischemia (4 min) produced outward currents in all of the cells that were voltage-clamped at -50 mV ($+35 \pm 18$ pA; $n = 9$), and although cortically evoked EPSCs were partially sensitive to 50 μM APV, they did not exhibit any long-term

enhancement at the wash of the ischemic episode (Fig. 2B). Similar results were obtained in magnesium-free medium ($n = 3$) (data not shown).

Critical Role of NMDA Receptors in i-LTP Induction. Bath application of the NMDA receptor antagonist APV (50 μM) fully prevented the induction of i-LTP in striatal spiny neurons recorded in magnesium-free medium, supporting the conclusion that this experimental procedure allowed the induction of this form of synaptic plasticity by facilitating NMDA-mediated neurotransmission ($n = 6$) (Fig. 1B). In physiological conditions, the Mg^{2+}-dependent blockade of NMDA receptors is typically relieved by membrane depolarization. Use of the whole-cell patch-clamp technique, therefore, even in the presence of physiological concentrations of Mg^{2+} ions (1.2 mM), an NMDA-mediated component of corticostriatal EPSCs could be unmasked by clamping the membrane potential of the recorded striatal cells at membrane values significantly positive to their resting membrane potential (RMP). Accordingly, when recorded at holding potentials of -50 mV but not at -80 mV, striatal spiny neurons responded to a single stimulation of corticostriatal fibers by producing an EPSC that was partially blocked by 50 μM APV ($19 \pm 4\%$, $n = 6$, $p < 0.01$).

Combined oxygen and glucose deprivation (4 min) invariably produced inward currents in striatal neurons clamped at -80 mV (-180 ± 38 pA; $n = 6$), whereas it caused inward currents (-40 ± 10 pA; $n = 5$), outward currents ($+20 \pm 13$ pA; $n = 3$), or no effect ($n = 2$) in striatal neurons clamped at
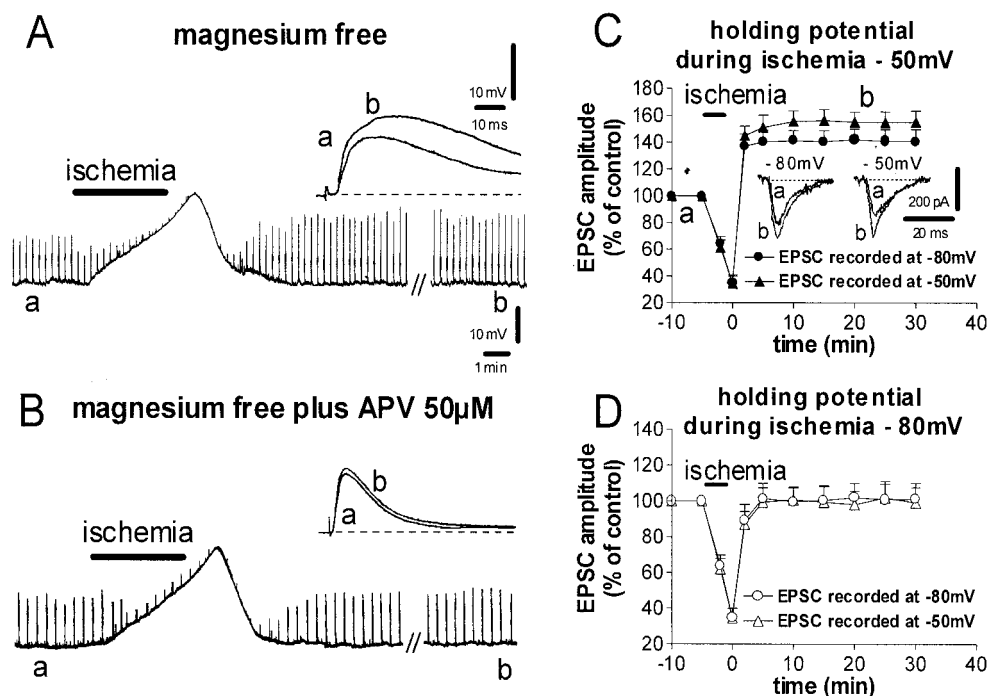

Fig. 1. Induction of i-LTP requires NMDA glutamate receptor activation in striatal spiny neurons. A, the electrophysiological trace shows a chart record of the membrane potential changes induced in a striatal spiny neuron by a brief period of ischemia (black bar) in the absence of magnesium ions. The upward deflections reflect EPSPs evoked by cortical stimulation. Note that after a 5-min wash, the recording of the membrane was interrupted for approximately 15 min. The inset shows superimposed EPSPs (averages of four single sweeps) at higher sweep speed before ischemia (a) and 20 min after (b). The RMP of the neuron was -86 mV. B, the electrophysiological trace shows a chart record of the membrane potential changes induced in a striatal spiny neuron by a brief period of ischemia (black bar) in magnesium-free medium plus 50 μM APV. The inset shows superimposed EPSPs (averages of four single sweeps) at higher sweep speed before (a) and 20 min after ischemia (b). The RMP of the neuron was -84 mV. Scale bars in A also apply to B. C, cumulative data obtained by whole-cell patch-clamp recordings in 1.2 mM magnesium. Note that i-LTP was evident either at -80 mV (●) or at -50 mV (▲) when the ischemic insult was applied, holding the cell at -50 mV. Superimposed traces in the inset represent single experiments showing synaptic currents before ischemia (a) and 20-min washout (b) recorded at holding potential of -80 mV (left) and at -50 mV (right). D, i-LTP was evident neither at -80 mV (○) nor at -50 mV (△) when the ischemic insult was applied, holding the cell at -80 mV.

-50 mV. These data are consistent with previous reports in which the reversal potential of the ischemic current was estimated at approximately -40 mV (Kawaguchi, 1993).

When spiny neurons were held at -50 mV during the application of the ischemic insult, we detected an i-LTP of time course and amplitude that was similar to the one observed in Mg^{2+}-free solution (see above). This experimental condition led to the enhancement not only of EPSCs recorded at -50 mV but also of those recorded at -80 mV ($n = 10$; $p < 0.01$ for both holding potentials) (Fig. 1C). EPSCs evoked at -80 mV remained insensitive to APV even after their potentiation ($n = 4$; $p > 0.05$) (data not shown). Conversely, either EPSCs recorded at -50 mV or EPSCs recorded at -80 mV were unchanged after the application of the ischemic challenge at holding potential of -80 mV ($n = 6$) (Fig. 1D). Taken together, these data indicate that in the presence of physiological concentrations of Mg^{2+}, inactivation of NMDA receptors by membrane depolarization is a crucial requirement to

induce i-LTP and that, after its induction, this form of synaptic plasticity is dependent, at least in part, on the potentiation of AMPA currents.

Role of mGluR1 in i-LTP. Antagonists of group I mGluRs (mGluR1 and mGluR5) have been found to be consistently neuroprotective in a variety of experimental conditions, including brain ischemia (Nicoletti et al., 1999). To investigate the possibility that group I mGluR antagonists exert their protective effects by interfering with the excitotoxic damage that follows the formation of i-LTP, we studied the effects of selective antagonists of these receptors (Schoepp et al., 1999) on this pathological form of synaptic plasticity. We found that the selective mGluR5 antagonist MPEP (30 μM, $n = 4$, 10-min bath application) failed to affect i-LTP ($p > 0.05$), whereas LY367385 (30 μM, $n = 4$), AIDA (300 μM, $n = 4$), and CPCCOEt (100 μM, $n = 4$), blockers of mGluR1, fully prevented its induction ($p < 0.01$ for both experimental conditions) (Fig. 3A). The involvement of mGluR1 in the generation of i-LTP was also confirmed by using transgenic mice that selectively lacked this receptor subtype (Conquet et al., 1994). Striatal spiny neurons recorded intracellularly from these mice ($n = 10$) were indistinguishable electrophysiologically from their wild-type counterparts ($n = 9$) and from rat striatal neurons, but they did not exhibit i-LTP after the ischemic challenge ($n = 10$, $p > 0.05$). Wild-type mice showed robust i-LTP under the same conditions ($n = 9$, $p < 0.01$) (Fig. 3B). This difference could not be attributed to different membrane potential changes induced by in vitro ischemia, because mGluR1 knockout mice and wild-type mice responded to 3 min of energy deprivation with a membrane
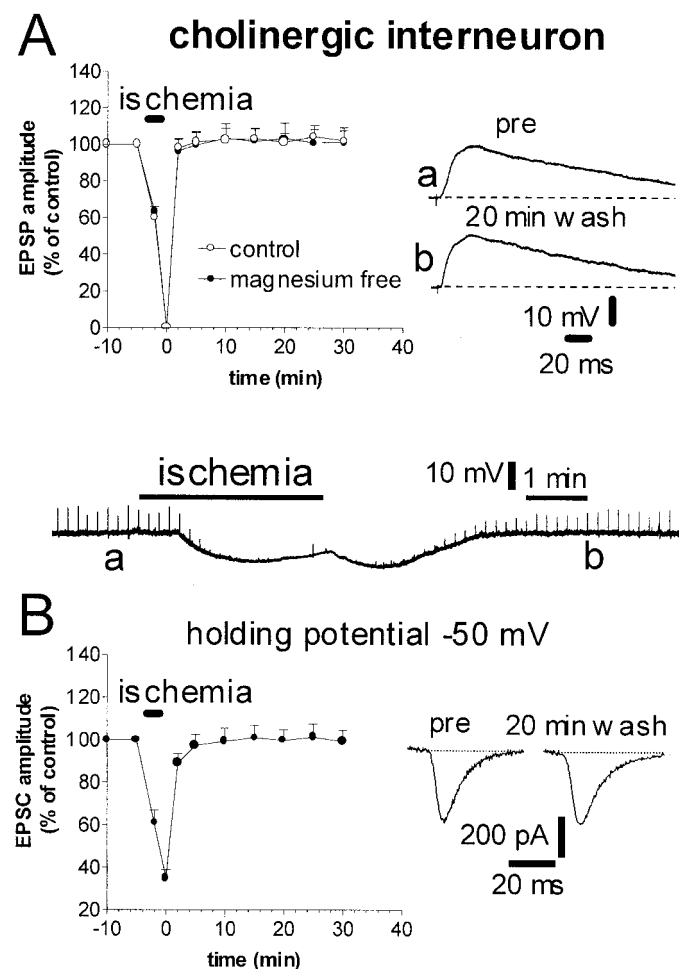

Fig. 2. Large cholinergic aspiny interneurons do not express i-LTP. A, top left, striatal cholinergic interneurons express ischemic LTP neither in control medium (○) nor in the absence of external magnesium (●). The single traces in the top right are EPSPs evoked by cortical stimulation before (a) and 20 min after (b) ischemia. The trace (bottom) shows a chart record of the membrane potential and EPSP amplitude changes (upward deflections) induced in a striatal aspiny cholinergic interneuron by a brief period of ischemia (black bar) in magnesium-free medium (RMP, 60 mV). B, during whole-cell patch clamp recordings (left), striatal cholinergic interneurons do not express i-LTP even when voltage is clamped at -50 mV. The single traces are EPSCs evoked by cortical stimulation before (a) and 20 min after ischemia (b), obtained from a single experiment.
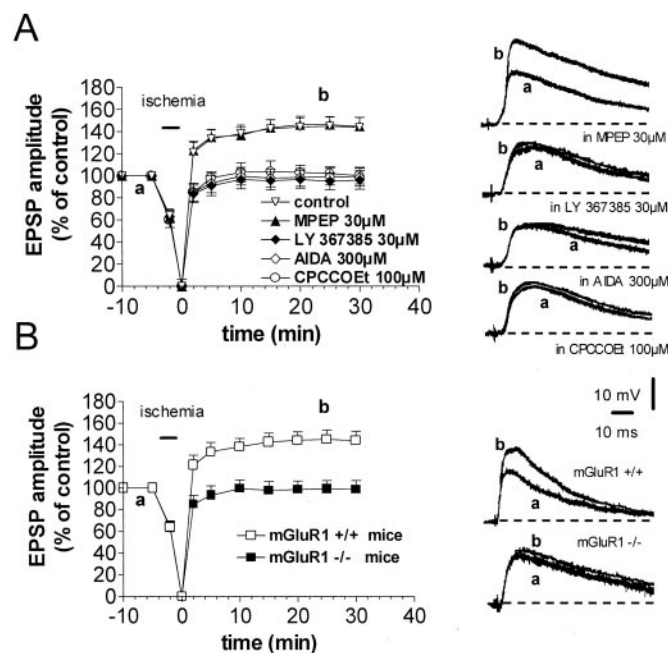

Fig. 3. Activation of mGluR1s is required for i-LTP in striatal spiny neurons. A, i-LTP is blocked by LY367385, AIDA, and CPCCOEt but not by MPEP. The superimposed voltage traces represent single experiments using these mGluR antagonists. RMPs were -85 mV (in MPEP), -84 mV (in LY367385), -85 mV (in AIDA), and -86 mV (in CPCCOEt). B, i-LTP is absent in mice lacking mGluR1 but not in normal mice. The superimposed voltage traces represent single experiments obtained from normal (mGluR1 $+/+$) and mGluR1-lacking mice (mGluR1 $-/-$). RMPs were -85 mV (mGluR1 $+/+$) and -85 mV (mGluR1 $-/-$). In each experimental condition, the averages of single traces were obtained before (a) and 20 min after ischemia (b).

depolarization of comparable amplitude (26 ± 3 mV and 28 ± 4 mV, respectively; $p > 0.05$).

Role of Intracellular Calcium and PKC in i-LTP. Stimulation of mGluR1 results in the activation of PKC and in the elevation of intracellular levels of calcium ions (Nicoletti et al., 1999; Schoepp et al., 1999). This latter event also follows NMDA receptor activation and ischemic membrane depolarization and is recognized, along with PKC activation, as an important determinant for the generation of many forms of synaptic plasticity (Bliss and Collingridge, 1993; Calabresi et al., 1996). Therefore, we tested the dependence of i-LTP on intracellular calcium accumulation and PKC stimulation. Intraneuronal injection of high concentrations of the calcium-chelating agent BAPTA (100 mM), which did not affect per se the membrane depolarization produced by ischemia (30 ± 2 mV, $p > 0.05$, $n = 6$), fully prevented i-LTP ($p > 0.05$) (Fig. 4A). Similar results were obtained using the PKC blockers calphostin C (1 μM, 7 min, $n = 7$) or staurosporine (100 nM, 7 min, $n = 6$, $p > 0.05$) and after intracellular administration of Ro 32–0432 (100 μM, $n = 6$, $p > 0.05$), another highly selective antagonist of PKC (Fig. 4B). This

Fig. 4. Intracellular calcium elevation, as well as PKC and MAP kinase activation, is required for i-LTP in striatal spiny neurons. A, buffering of intracellular calcium by BAPTA prevents i-LTP. B, i-LTP is blocked by either bath application of calphostin C and staurosporin or intracellular application of Ro 32–0432. C, the i-LTP is also prevented by PD98059 and UO126.

also demonstrates that the induction of PKC by energy deprivation that is crucial for i-LTP occurs at the postsynaptic site of corticostriatal synapses.

Inhibitors of the ERK Cascade Suppress i-LTP. Mitogen-activated protein kinases are involved in the generation of NMDA-mediated neurotoxicity (Ghosh and Greenberg, 1995) and ischemic neuronal damage (Alessandrini et al., 1999; Sugino et al., 2000). Thus, we tested the induction and expression of i-LTP in the presence of PD98059 and UO126, two specific inhibitors of MAP kinase kinase (MEK) and thereby of p42/44 MAP kinase activation (Alessi et al., 1995). As shown in Fig. 4C, long-term incubation (2 h) of the slices in 10 μM PD98059 ($n = 8$) or 30 μM UO126 ($n = 5$) prevented the induction of striatal i-LTP ($p > 0.05$). Neither drug affected the intrinsic membrane properties of the striatal spiny neurons, nor did the inhibitors alter the physiological and pharmacological characteristics of corticostriatal EPSPs and the amplitude of ischemia-induced membrane depolarization (30 ± 2 mV in PD98059 and 27 ± 3 mV in UO126; $p > 0.05$ for each experimental condition).

To further address the possible interaction between NMDA receptors, ischemia, and MAP kinase pathway, we performed experiments in magnesium-free medium plus 10 μM CNQX. The pharmacological isolation of NMDA receptors by this treatment did not prevent the ischemia (3 min)-induced potentiation of corticostriatal EPSPs ($135 \pm 6\%$ 20 min after the ischemic episode; $p < 0.01$, $n = 5$, data not shown). Moreover, the incubation in the presence of PD98059 (10 μM) fully prevented i-LTP even in this experimental condition ($102 \pm 3\%$ 20 min after the ischemic episode; $p > 0.05$, $n = 5$, data not shown). Ischemia-Induced ERK Phosphorylation Is Blocked by mGluR1 and PKC Antagonists. The ability of the MEK inhibitors to block i-LTP suggests that in vitro ischemia activated MAP kinase in striatal neurons. We used immunoblotting to confirm this and to investigate the possible interplay between mGluR1, PKC, and NMDA stimulation with MAP kinase activation. Striatal slices were incubated in vitro under various conditions, lysed, and analyzed by immunoblotting of protein gels with an antiserum against activated ERK that specifically recognizes ERK phosphorylated on Thr 202 and Tyr204 (Fig. 5). In vitro ischemia led to increased ERK phosphorylation, particularly that of p44[ERK1], an effect that became stronger upon washing with Mg^{2+}-free buffer (Fig. 5A). Based on these kinetics, we then tested the effects of different pharmacological agents on ERK activation in vitro using 5-min ischemia and a 20-min wash (Fig. 5B). As expected, the MEK inhibitor PD98059 fully blocked ERK activation. MPEP did not block ERK activation, whereas calphostin C had an intermediate inhibitory effect and LY367385 a strong effect. Control blots using several different ERK-specific antisera (Fig. 5 and data not shown) showed equal loading in all lanes, although all sera tested recognized rat ERK1 very weakly. The effects of the different pharmacological agents on ERK activation in striatal slices in vitro corroborate those obtained by electrophysiology and thereby strongly implicate ERK as an important intermediate in i-LTP via mGluR1 and PKC activation.
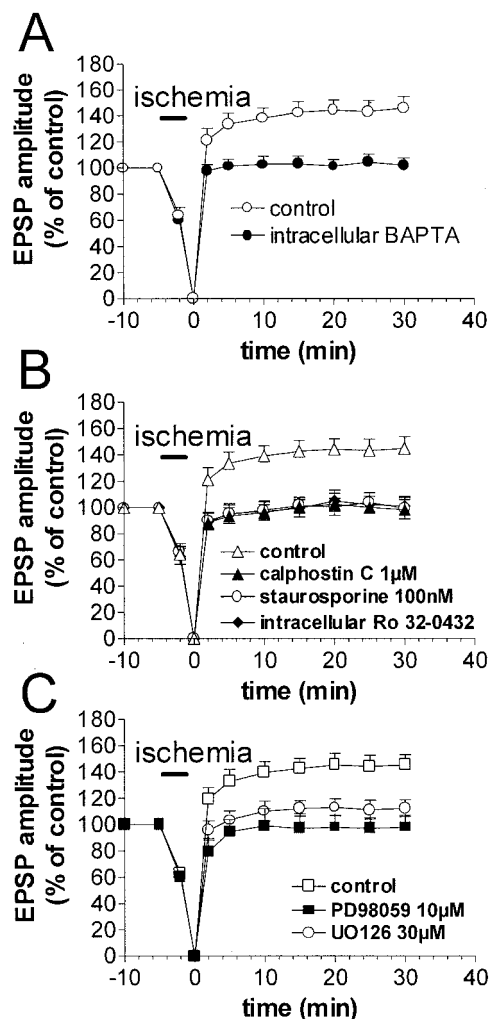
Discussion

Activation of NMDA receptors is critical for the induction of i-LTP. This evidence suggests that i-LTP occurs in vivo during the "up state" of striatal neurons. In fact, although

striatal spiny neurons are highly polarized in vitro and require manipulations to reveal an NMDA-mediated component of excitatory transmission, they show a characteristic membrane oscillatory behavior when recorded in vivo (Calabresi et al., 1996; Wilson and Kawaguchi, 1996). During the depolarized "up state," neurons reach membrane potentials of approximately −50 mV, thereby enabling the inactivation of NMDA receptors and possibly the induction of i-LTP (Wilson and Kawaguchi, 1996). In our in vitro experiments, we were able to mimic this in vivo condition either by removing external magnesium or by holding the membrane potential of the cells at depolarized levels. Although cholinergic interneurons possess a more positive resting membrane potential and show tonic firing activity (Graybiel et al., 1994; Aosaki et al., 1995; Wilson and Kawaguchi, 1996), they do not express i-LTP. This finding is in agreement with those of previous studies showing that this neuronal subtype is resistant to ischemia and energy deprivation (Pulsinelli, 1985; Chesselet et al., 1990).

The observation that i-LTP is blocked by mGluR1 antagonists and is absent in mice selectively lacking this receptor subtype provides a new synaptic mechanism explaining the neuroprotective effects of group I mGluR antagonists in isch-

emic and excitotoxic neuronal damage (Nicoletti et al., 1999; Pellegrini-Giampietro et al., 1999). The evidence that intracellular application of Ro 32–0432, as well as bath application of staurosporin and calphostin C, prevented i-LTP demonstrates that i-LTP requires the activation of PKC expressed postsynaptically in spiny neurons. PKC activity is critically regulated by intracellular calcium levels (Calabresi et al., 1998a). Accordingly, intracellular application of the calcium chelator BAPTA also blocks i-LTP. Because intracellular calcium elevation and PKC stimulation represent critical biochemical events resulting from the activation of mGluR1 (Nicoletti et al., 1999), we favor the hypothesis that mGluR1s required for i-LTP are postsynaptically located on striatal spiny neurons.

The convergent action of energy deprivation and mGluR1 and PKC stimulation allows the induction of i-LTP through ERK stimulation, because we found that the activation of this enzyme by brief ischemia is significantly reduced by mGluR1 and PKC inhibitors. We also observed that the blockade of the ERK cascade with specific inhibitors (Alessi et al., 1995) prevented i-LTP. In addition, the finding that the inhibition of MAP kinase pathway was able to prevent i-LTP even in the presence of the AMPA receptor antagonist CNQX favors the idea of a close interaction among ischemia, NMDA receptors, and MAP kinase pathway stimulation in the induction phase of i-LTP.

A possible model to explain the interaction between NMDA receptors, mGluR1, PKC, and MAP kinase pathway in the formation of striatal postischemic LTP is shown in Fig. 6. We suggest that during a brief ischemic episode, an abnormal amount of glutamate is released from corticostriatal terminals, causing a membrane depolarization. The ischemia-induced membrane depolarization leads to the activation of NMDA glutamate receptors which, in turn, causes an elevation of intracellular calcium levels. A further increase of intracellular calcium levels is also induced by the activation of mGluR1. The augmented intracellular calcium concentration is critical for the activation of PKC. The activated PKC triggers a cascade of events resulting in the activation of MEK and MAP kinase. Activated MAP kinase probably has
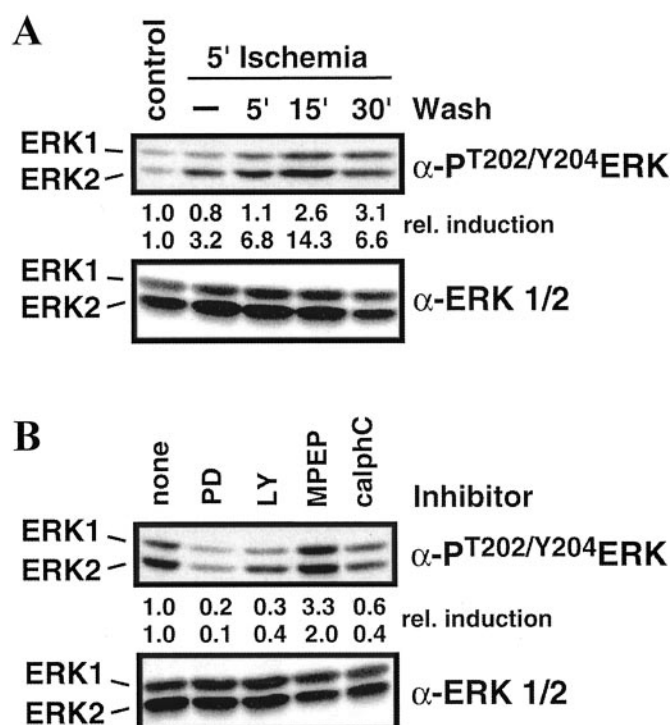
Fig. 5. ERK is activated by ischemia and is modulated by drugs interfering with i-LTP expression. A, ERK is activated by ischemia in striatal slices. Striatal slices were incubated in Mg-free solution (control), subjected to 5-min ischemia, and followed by increasing wash time in Mg-free solution. Proteins in whole-tissue lysates were separated by SDS-polyacrylamide gel electrophoresis, blotted onto polyvinylidene difluoride, and immunodetected using activated ERK-specific antiserum or control ERK antiserum as indicated. The two species of ERK recognized by these sera, p44[ERK1] and p42[ERK2], are indicated to the left of each panel. B, striatal slices were subjected to in vitro ischemia and 20-min Mg-free wash in the presence of the following inhibitors: 10 μM PD98059, 30 μM LY367385, 30 μM MPEP, or 1 μM calphostin C. ERK activation was determined by immunoblotting, as described above. In both A and B, the data have been quantified and the phospho ERK1 and ERK2 signals corrected for the amount of the kinase revealed by the control signal. The corresponding numbers are presented in the appropriate panels as relative ERK induction.
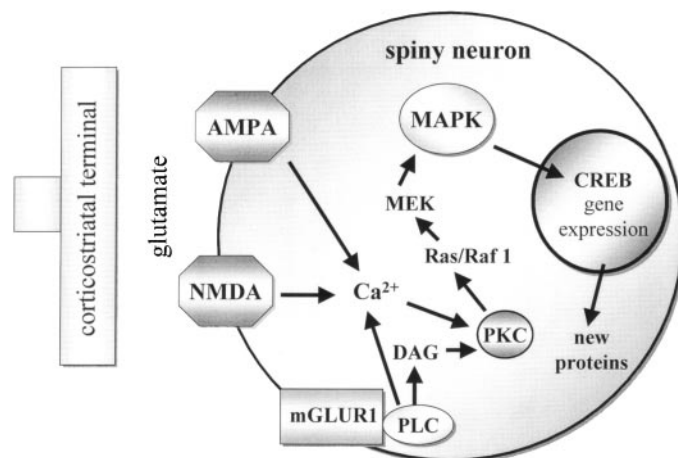
Fig. 6. Putative signal transduction pathways operating in striatal postischemic LTP. See description under *Discussion*. AMPA, AMPA subtype of glutamate receptor; NMDA, NMDA subtype of glutamate receptor; mGluR1, metabotropic glutamate receptor subtype 1; PLC, phospholipase C; DAG, diacylglycerole; MAPK, mitogen-activated protein kinase; CREB, cAMP response element binding protein.

multiple targets, including cAMP-response element-binding protein, which mediates its ability to induce long-term adaptive changes in neurons. The resulting synthesis of new proteins mediates the long-term remodeling of the synapse believed to underlie postischemic LTP.

ERK cascade inhibition has been shown to alter hippocampal LTP (English and Sweatt, 1997) and to block long-term facilitation in the mollusk *Aplysia californica* (Martin et al., 1997). At present, however, very little is known about the role of the ERK pathway in the synaptic processes after ischemia. It has been hypothesized that cerebral ischemia leads to the activation of this signal transduction cascade via glutamate release and activation of NMDA receptors, which, in turn, causes calcium entry (Wieloch et al., 1996; Xia et al., 1996; Alessandrini et al., 1999).

One would expect that the ERK cascade exerts its effect selectively on late phases of synaptic plasticity by altering the pattern of gene expression. However, we found that the pharmacological inhibitors of the ERK cascade also blocked early stages of i-LTP, thereby suggesting a role early in this phenomenon. In agreement with this, PD98059 completely prevents LTP in the dentate gyrus and long-term depression in the prefrontal cortex, indicating that the activation of the MAP kinase ERK cascade can play a role in the induction phases of different forms of synaptic plasticity in several brain areas (Coogan et al., 1999; Otani et al., 1999; Sweatt, 2001).

Studies dealing with the role of ERK activity in ischemia have proven contradictory. Sustained ERK activation after ischemia was suggested to mediate selective resistance to ischemia in adult (Hu and Wieloch, 1994) and neonatal brains (Hee Han and Holtzman, 2000). Conversely, it has been supposed that ERK activity might favor neuronal death through inappropriate protein phosphorylation in CA3 pyramidal cells and disruption of the cytoskeleton in CA1 neurons (Runden et al., 1998). We provide the first evidence that activation of the mGluR1/PKC/MAP kinase pathway is required for the generation of the ischemia-induced long-term enhancement of excitatory transmission in the brain.

Protein kinase A is involved in several forms of synaptic plasticity in various brain areas (Greengard et al., 1999; Otani et al., 1999; Calabresi et al., 2000) and interacts with both PKC and MAP kinase activity (Bowler et al., 1999; Otani et al., 1999; Calabresi et al., 2000; Sweatt, 2001). Thus, it would be interesting to address the role of this kinase in striatal i-LTP. Experiments are in progress in our laboratory to investigate this important issue.

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