Regulation of cyclin-dependent kinase 5 and casein kinase 1 by metabotropic glutamate receptors

Feng Liu*†, Xiao-Hong Ma‡, Jernej Ule*, James A. Bibb*, Akinori Nishi§, Anthony J. DeMaggio¶, Zhen Yan‡, Angus C. Nairn*, and Paul Greengard*

*Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY 10021; ‡Department of Physiology and Biophysics, State University of New York, School of Medicine and Biomedical Sciences, Buffalo, NY 14214; §Department of Physiology, Kurume University School of Medicine, Kurume, Fukuoka 830-0011, Japan; and ¶ICOS Corp., 22021 20th Avenue SE, Bothell, WA 98021

Cyclin-dependent kinase 5 (Cdk5) is a multifunctional neuronal protein kinase that is required for neurite outgrowth and cortical lamination and that plays an important role in dopaminergic signaling in the neostriatum through phosphorylation of Thr-75 of DARPP-32 (dopamine and cAMP-regulated phosphoprotein, molecular mass 32 kDa). Casein kinase 1 (CK1) has been implicated in a variety of cellular functions such as DNA repair, circadian rhythm, and intracellular trafficking. In the neostriatum, CK1 has been found to phosphorylate Ser-137 of DARPP-32. However, first messengers for the regulation of Cdk5 or CK1 have remained unknown. Here we report that both Cdk5 and CK1 are regulated by metabotropic glutamate receptors (mGluRs) in neostriatal neurons. (S)-3,5-dihydroxyphenylglycine (DHPG), an agonist for group I mGluRs, increased Cdk5 and CK1 activities in neostriatal slices, leading to the enhanced phosphorylation of Thr-75 and Ser-137 of DARPP-32, respectively. The effect of DHPG on Thr-75, but not on Ser-137, was blocked by a Cdk5-specific inhibitor, butyrolactone. In contrast, the effects of DHPG on both Thr-75 and Ser-137 were blocked by CK1–7 and IC261, specific inhibitors of CK1, suggesting that activation of Cdk5 by mGluRs requires CK1 activity. In support of this possibility, the DHPG-induced increase in Cdk5 activity, measured in extracts of neostriatal slices, was abolished by CK1–7 and IC261. Treatment of acutely dissociated neurons with DHPG enhanced voltage-dependent Ca^{2+} currents. This enhancement was eliminated by either butyrolactone or CK1–7 and was absent in DARPP-32 knockout mice. Together these results indicate that a CK1-Cdk5-DARPP-32 cascade may be involved in the regulation by mGluR agonists of Ca^{2+} channels.

Cyclin-dependent kinase 5 (Cdk5)/p35 plays a variety of roles in the developing and adult nervous system (1–3). Studies of mice in which the gene encoding either Cdk5 or p35 has been disrupted have indicated that both mutants exhibit abnormalities in the laminar structure of the cerebral cortex (4, 5). Conversion of p35 to p25 by the action of calpain causes altered localization and prolonged activation of Cdk5 (6–9). Cdk5 phosphorylates DARPP-32 (dopamine- and cAMP-regulated-phosphoprotein, molecular mass 32 kDa) on Thr-75 (10, 11). Phosphorylation at Thr-34 by cAMP-dependent protein kinase (PKA) converts DARPP-32 into an inhibitor of protein phosphatase-1 (PP-1), a process that is critical for the actions of dopamine in the neostriatum (12). Phosphorylation of Thr-75 by Cdk5 converts DARPP-32 into an inhibitor of PKA, reducing phosphorylation of DARPP-32 at Thr-34, and modulating the DARPP-32/PP-1 cascade (10).

Casein kinase 1 (CK1) was one of the first serine/threonine protein kinases to be isolated and characterized (13). It is a ubiquitous enzyme that can be found in the nucleus and the cytosol and bound to the cytoskeleton and membranes. The CK1 family consists of multiple isoforms encoded by seven distinct genes (CK1α, β, γ1, γ2, γ3, δ, and ε). These isoforms exhibit more than 50% amino acid identity within the NH_2-terminal catalytic domain, but contain divergent COOH termini. It has been reported that at least two CK1 isoforms, CK1δ and CK1ε, are regulated by autophosphorylation at their COOH termini (14–16). These studies suggest that the phosphorylated COOH terminus may act as a pseudosubstrate that inhibits enzyme activity. Elimination of autophosphorylation by either truncation of the COOH terminus or by dephosphorylation has been found to result in increased kinase activity (14–16). In the central nervous system, CK1 appears to play a role in the regulation of circadian rhythm (17, 18) and intracellular trafficking (19, 20). In the neostriatum, CK1 has been found to phosphorylate DARPP-32 at Ser-137 (21). Phospho-Thr-34 is dephosphorylated by PP-2B (also termed calcineurin) and the phosphorylation of Ser-137 impairs the ability of phospho-Thr-34 to be dephosphorylated by PP-2B, thereby modulating the DARPP-32/PP-1 cascade (22).

In view of extensive knowledge about the actions of Cdk5 and CK1 in the central nervous system, surprisingly little is known about the regulation of these two kinases by first messengers. In the present study, we have investigated the regulation of Cdk5 and CK1 by the metabotropic glutamate agonist 3,5-dihydroxyphenylglycine (DHPG). The results indicate that both Cdk5 and CK1 activity are regulated by metabotropic glutamate receptors (mGluRs) in neostriatal neurons and that activation of Cdk5 by DHPG requires CK1 activity. Moreover, our evidence indicates that DHPG treatment of acutely dissociated neurons enhances voltage-dependent Ca^{2+} channels via a CK1-Cdk5-DARPP-32 cascade.

Materials and Methods

Antibodies, Plasmids, and Chemicals. A phospho-specific antibody that recognizes phospho-Ser-137 DARPP-32 was developed by immunizing rabbits with a peptide encompassing phospho-Ser-137 of DARPP-32 conjugated with limulus hemocyanin (Sigma). Phospho-Thr-75 DARPP-32 antibody was developed as described (10). Antibodies were purified by affinity chromatography as described (23). The expression plasmid pCEP4HA-CK1ε was kindly provided by David Virshup (University of Utah, Salt Lake City). Anti-hemagglutinin (HA) was obtained from Roche Molecular Biochemicals. CK1–7 was obtained from Seikagaku Kogyo, Tokyo. IC261 was obtained from ICOS. Butyrolactone

This paper was presented at the Inaugural Arthur M. Sackler Colloquium of the National Academy of Sciences, ''Neural Signaling,'' held February 15–17, 2001, at the National Academy of Sciences in Washington, DC.

Abbreviations: Cdk5, cyclin-dependent kinase 5; DARPP-32, dopamine and cAMP-regulated phosphoprotein, molecular mass 32 kDa; CK1, casein kinase 1; mGluR, metabotropic glutamate receptor; DHPG, 3,5-dihydroxyphenylglycine; PKA, cAMP-dependent protein kinase; PP-1, protein phosphatase-1; HA, hemagglutinin.

†To whom reprint requests should be addressed. E-mail: liuf@rockvax.rockefeller.edu.

www.pnas.org/cgi/doi/10.1073/pnas.191353898

was obtained from Biomol, Plymouth Meeting, PA. (S)-3,5-DHPG and L-AP3 were obtained from Tocris Neuramin, Bristol, U.K. Protease inhibitor cocktail tablet was obtained from Roche Molecular Biochemicals.

Preparation and Treatment of Striatal Slices. Neostriatal slices were prepared from male C57/BL6 mice (6–8 weeks old) as described (24). Slices were treated with drugs as specified in the text and figure legends. After drug treatment, slices were immediately frozen in liquid nitrogen and stored at −80°C until assayed.

Immunoblotting. Frozen slices were sonicated in hot homogenization buffer containing 1% SDS and 50 mM NaF, and samples were boiled for 10 min. SDS/PAGE sample buffer was then added and samples were boiled for 5 min. Samples (≈120 μg protein) were separated by SDS/PAGE (10% polyacrylamide) and transferred to nitrocellulose. Immunoblots were first probed with anti-phospho-Ser-137 DARPP-32 antibody. The blots were stripped and probed with anti-phospho-Thr-75 DARPP-32 antibody. Blots were stripped again and probed with anti-total DARPP-32 antibody. Antibody binding was detected by enhanced chemiluminescence (ECL) using x-ray film. Results were quantified by analysis by laser scanning densitometry using National Institutes of Health IMAGE 1.52 software. Data were statistically analyzed by Student's *t* test in Microsoft EXCEL software as indicated.

Transfection, Immunoprecipitation, and Assay of CK1. Neuroblastoma N2a cells were cultured in DMEM containing 5% FBS to 50–60% confluency. Four micrograms of the expression plasmid for HA-CK1ε was transfected into N2a cells in 100-mm dishes using FuGENE 6. Twenty-four hours after transfection, cells were incubated at room temperature in phosphate-buffered Kreb's-Henseleit solution (Sigma) for 10 min and then with or without CK1 inhibitor for 30 min before treating with a group I mGluR agonist, (S)-3,5-DHPG (100 μM), for 2 min. Cells were then lysed in 1 ml of lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 50 mM Tris (pH 8.0), 5 mM Na_3VO_4, 20 mM NaF, 20 mM β-glycerol-phosphate, and protease inhibitors. Lysates were centrifuged at 10,000 × *g*, and supernatants were used for immunoprecipitation and kinase assay.

For immunoprecipitation of CK1ε from N2a cells, lysates (1 mg of total protein) were precleared with 5 μl of mouse IgG (ICN) and 50 μl of protein A agarose for 30 min. Five microliters of anti-HA antibody was added and samples were incubated for 1 h at 4°C. Five microliters of anti-mouse rabbit IgG and 50 μl of protein A agarose were then added for 45 min. Immunocomplexes were washed three times in lysis buffer and two times in kinase buffer (30 mM Hepes, pH 7.5/7 mM $MgCl_2$/0.5 mM DTT). CK1 assays were performed in a 30-μl assay volume with 2 μg of purified DARPP-32, 500 μM ATP, and 5 μCi γ-[^{32}P]ATP. Samples were incubated at 30°C for 20 min and reactions were stopped by addition of SDS-sample buffer and boiled for 5 min. Samples were separated by SDS/PAGE (12% polyacrylamide). SDS/PAGE gels were dried and exposed to Kodak film for autoradiography. Results were quantified by using a Molecular Dynamics PhosphorImager.

Immunoprecipitation and Assay of Cdk5. For Cdk5 immunoprecipitation, striatal tissue slices (≈150 μg total protein) were lysed by using a Dounce homogenizer in 1 ml of lysis buffer (150 mM NaCl/20 mM Tris·HCl pH 7.4/1 mM EDTA/0.5% Nonidet P-40/5 mM NaF/5 mM Na_3VO_4/protease inhibitors). Lysates were centrifuged at 10,000 × *g* for 10 min at 4°C. Supernatants were precleared by adding rabbit IgG-conjugated agarose and incubated for 40 min at 4°C. Ten microliters of Cdk5 antibody conjugated to agarose was added and samples were incubated for 1 h at 4°C with mixing. Agarose pellets were collected and washed three times with 1 ml of lysis buffer and two times with kinase assay buffer (50 mM Hepes, pH 7.5/10 mM $MgCl_2$/1 mM DTT). Cdk5 assays were performed in 30-μl reaction mixtures with 2 μg histone H-1, 500 μM ATP, and 5 μCi of γ-[^{32}P]ATP. Samples were incubated at 30°C for 10 min, reactions were stopped by addition of SDS-sample buffer, and samples were boiled for 5 min. Proteins were separated by SDS/PAGE and analyzed as described above for CK1 assays.

***In Vitro* Phosphorylation of Cdk5 and p35 by CK1.** Cdk5 and p35-His_6 were purified from insect Sf9 cultures after expression of protein using baculovirus vectors. To examine phosphorylation of Cdk5 or p35 by CK1α (New England Biolabs), 1 μM Cdk5 or 1.5 μM p35 was incubated with 2 units/μl of CK1 in 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT, 200 μM ATP, and 0.6 mCi/ml [γ-^{32}P]ATP. Samples were analyzed by SDS/PAGE and autoradiography.

Whole-Cell Recordings of Voltage-Dependent Ca^{2+} Channels. Neostriatal neurons from 3- to 4-week-old mice were acutely dissociated by using procedures similar to those described (25). Whole-cell current recordings used standard voltage-clamp techniques. The internal solution consisted of 180 mM *N*-methyl-D-glucamine, 40 mM Hepes, 4 mM $MgCl_2$, 0.5 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate, 12 mM phosphocreatine, 2 mM Na_2ATP, 0.2 mM Na_3GTP, 0.1 mM leupeptin (pH 7.2–3), 265–270 mosm/liter. The external solution consisted of 135 mM NaCl, 20 mM CsCl, 1 mM $MgCl_2$, 10 mM Hepes, 0.001 mM tetrodotoxin, 5 mM $BaCl_2$, 10 mM glucose (pH 7.3), 300–305 mosm/liter. Recordings were obtained with an Axon Instruments 200B patch clamp amplifier that was controlled and monitored with an IBM PC running PCLAMP (v. 8) with a DigiData 1320 series interface (Axon Instruments, Foster City, CA). Electrode resistances were typically 2–4 MΩ in the bath. After seal rupture, series resistance (4–10 MΩ) was compensated (70–90%) and periodically monitored. Drugs were applied with a gravity-fed "sewer pipe" system. The array of application capillaries was positioned 250 μm from the cell under study. Solution changes were effected by altering the position of the array with a DC drive system controlled by a microprocessor-based controller (Newport-Klinger, Irvine, CA). Data analyses were performed with AXOGRAPH (Axon Instruments), KALEIDAGRAPH (Albeck Software, Reading, PA), and STATVIEW (Abacus Concepts, Berkeley, CA).

Results

Effect of Group I mGluR Agonist on Phosphorylation of DARPP-32 at Thr-75 and Ser-137. The effects of an mGluR group I agonist, DHPG, on phosphorylation of DARPP-32 at Thr-75 (Cdk5 site) and Ser-137 (CK1 site) were assessed in mouse neostriatal slices by using phosphorylation state-specific antibodies. Treatment of slices with DHPG (100 μM) resulted in a rapid, but transient, increase in phosphorylation of both Thr-75 and Ser-137 (Fig. 1*a*). The level of phosphorylation of Thr-75 reached a peak at 2 min and declined to the basal level by 5 min. Similar results were observed for the effect of DHPG on phosphorylation of Ser-137. Pretreatment of slices with the mGluR group I antagonist L-AP3 (100 μM) for 20 min blocked the effects of DHPG at both Thr-75 and Ser-137 (Fig. 1*b*). Previous studies have shown that the basal stoichiometry of phosphorylation of Thr-75 is ≈0.26 mol/mol (10). The basal stoichiometry of Ser-137 phosphorylation in mouse neostriatal slices was determined to be 0.4 mol/mol. Therefore, the 2- to 3-fold increase in phosphorylation of both Thr-75 and Ser-137 in response to group I mGluR activation resulted in a high stoichiometry of phosphorylation of both sites.

Effect of Group I mGluR Agonist on Cdk5 and CK1 Activities. Neostriatal slices were incubated in the absence or presence of

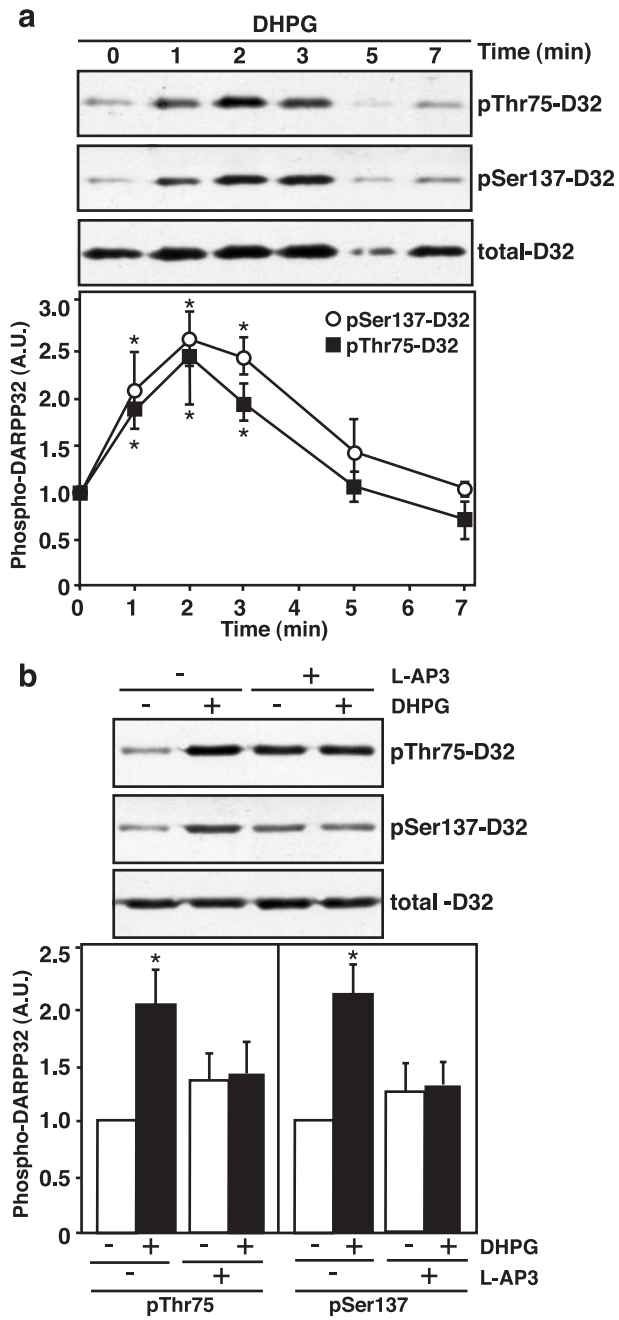

Fig. 1. Activation by DHPG of group I mGluRs increases phosphorylation of Thr-75 and Ser-137 on DARPP-32. Mouse neostriatal slices were treated with DHPG for various periods of time as indicated. Slice homogenates were analyzed by SDS/PAGE and immunoblotting with phospho-Thr-75, phospho-Ser-137, and total DARPP-32 antibodies. (*a*) Time course of treatment with DPHG (100 μM). (*b*) Slices were preincubated without or with the group I mGluR antagonist L-AP3 (100 μM) for 20 min before treatment with DHPG for 2 min. Cumulative data (means ± SEM) obtained from five experiments are shown in *a* and *b Lower*. Data were normalized to values for untreated slices. *, $P < 0.05$, Student's *t* test, compared with untreated slices.

DHPG. Cdk5 was immunoprecipitated from homogenates obtained from these slices and assayed by using histone H-1 as substrate. Treatment of slices with DHPG caused a rapid, but transient, increase in Cdk5 activity. Cdk5 activity was increased ≈4-fold at 2 min and declined back to basal levels after 5 min (Fig. 2).

We found that CK1α, CK1δ, and CK1ε are expressed in the striatum (data not shown), suggesting that CK1δ or CK1ε, both

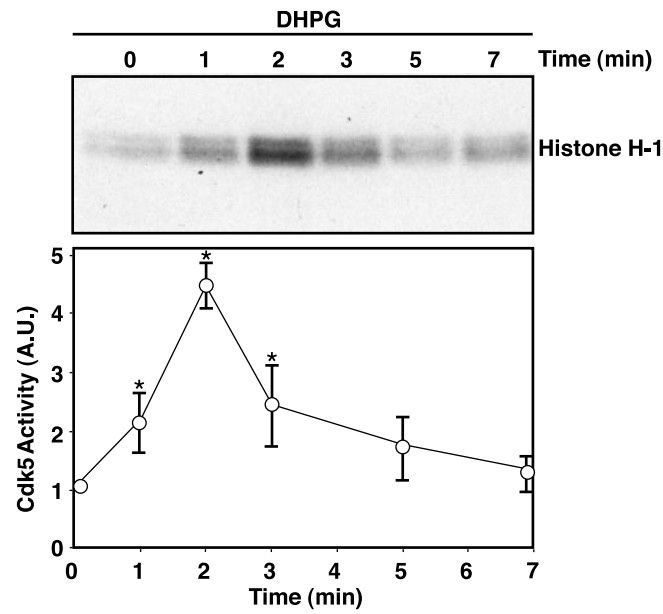

Fig. 2. Activation of mGluR1s transiently increases Cdk5 activity. Mouse neostriatal slices were treated with DHPG for various periods as indicated. Slices were homogenized and Cdk5 was immunoprecipitated by using anti-Cdk5 (C-8) antibody. Cdk5 activity was assayed by using histone H-1 as substrate, and samples were analyzed by SDS/PAGE and autoradiography. (*Upper*) An autoradiogram of histone H-1 phosphorylation. (*Lower*) Cumulative data. The autoradiograms were analyzed by using a PhosphoImager. Data for three experiments (means ± SEM) were normalized to the values obtained at 0 min. *, $P < 0.05$, Student's *t* test, compared with 0 min.

of which have a COOH-terminal regulatory domain, might be subject to regulation by mGluR activation. Because none of the commercially available CK1 antibodies are useful for immunoprecipitation, and because a screen for different subtypes of group I mGluRs indicated that mGluR1 is expressed in N2a cells (data not shown), we used a transient transfection system using N2a cells. HA-tagged CK1ε was transiently transfected into neuroblastoma N2a cells. Cells were treated with DHPG for 2 min, CK1ε was immunoprecipitated by using anti-HA antibody, and CK1 activity was assayed by using DARPP-32 as substrate (Fig. 3). Treatment of cells with DHPG resulted in a ≈2-fold increase in CK1 activity compared with the basal level. Addition of CK1–7 (100 μM) blocked the ability of DHPG to increase CK1 activity (Fig. 3).

Effect of Cdk5 and CK1 Inhibitors on Phosphorylation of DARPP-32 at Thr-75 and Ser-137. To further evaluate the possible role of Cdk5 and CK1 in mediating the actions of DHPG on DARPP-32 phosphorylation, we measured the effects of the Cdk5 inhibitor, butyrolactone, and the CK1 inhibitors, CK1–7 and IC261, on DARPP-32 phosphorylation. The crystal structure of the catalytic domain of CK1 complexed with CK1–7 has provided evidence that CK1–7 achieves its selectivity through hydrophobic contacts in the ATP binding site (26). The structure of CK1 complexed with IC261 revealed that IC261 stabilizes CK1 in an inactive conformation midway between nucleotide-liganded and nonliganded conformations (27). Before studying these inhibitors in neostriatal slices, we measured their inhibitory potencies (IC_{50}) toward Cdk5/p35 and CK1 *in vitro*. The IC_{50} of butyrolactone for Cdk5 was 0.49 μM and for CK1 was 58.5 mM. The IC_{50} of CK1–7 for CK1 was 15 μM and for Cdk5 was 200 μM. The IC_{50} of IC261 for CK1 was 16 μM and for Cdk5 was 4.5 mM. These values suggest that butyrolactone, CK1–7, and IC261 can be used to distinguish between Cdk5 and CK1 activities in intact cells.

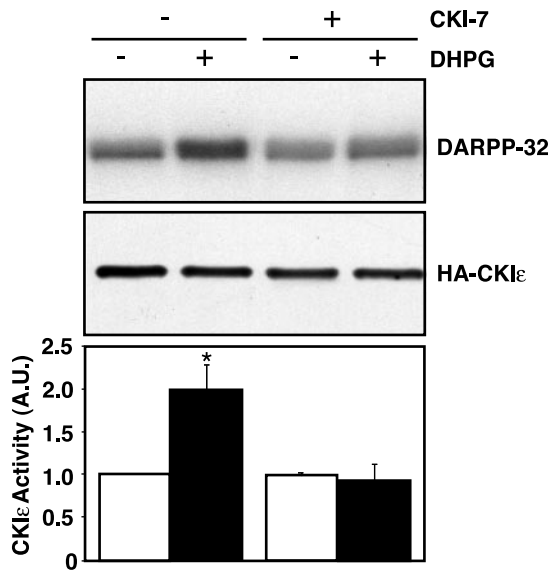

Fig. 3. Activation of mGluR1s stimulates CK1 activity. N2a cells were transiently transfected with HA-tagged CK1ε. Cells were preincubated without or with the CK1 inhibitor CK1–7 (100 μM) for 30 min before treatment with DHPG (100 μM) for an additional 2 min. HA-CK1ε was immunoprecipitated and CK1 was assayed by using DARPP-32 as a substrate. Samples were analyzed by SDS/PAGE and autoradiography. (*Top*) Autoradiogram of DARPP-32 phosphorylation. (*Middle*) Immunoblot of the level of expression of HA-CK1ε. (*Bottom*) Cumulative data of kinase activity obtained from five experiments (means ± SEM). Data were normalized to values obtained for untreated cells. *, $P < 0.05$, Student's *t* test, compared with untreated cells.

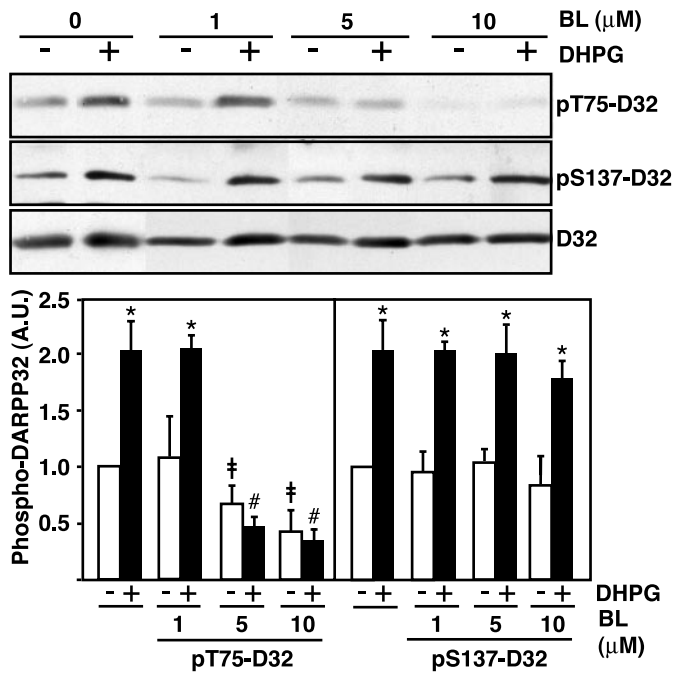

Fig. 4. A Cdk5 inhibitor, butyrolactone, blocks the effect of DHPG on phosphorylation of DARPP-32 on Thr-75 (Cdk5 site) but not on Ser-137 (CK1 site). Mouse neostriatal slices were preincubated with 0, 1, 5, or 10 μM butyrolactone (BL) for 30 min, and then DHPG (100 μM) was added for an additional 2 min. Slice homogenates were analyzed by SDS/PAGE and immunoblotting with phospho-Thr-75, phospho-Ser-137, and total DARPP-32 antibodies. (*Upper*) Immunoblots. (*Lower*) Cumulative data (means ± SEM) obtained from three experiments. Data were normalized to values obtained for untreated slices. *, $P < 0.05$, Student's *t* test, compared with slices in the absence of DHPG; ‡, $P < 0.05$, Student's *t* test, compared with untreated slices; #, $P < 0.05$, Student's *t* test, compared with slices treated with DHPG alone.

Slices were preincubated with various concentrations of butyrolactone for 30 min followed by addition of DHPG (100 μM) for 2 min (Fig. 4). Butyrolactone, at 5 and 10 μM, reduced the basal level and abolished DHPG-stimulated phosphorylation of Thr-75. In contrast, basal and DHPG-stimulated phosphorylation of Ser-137 were unaffected by butyrolactone at the concentrations tested.

Slices were preincubated with different concentrations of CK1–7 or IC261 for 30 min followed by addition of DHPG for 2 min (Fig. 5). At higher concentrations, CK1–7 reduced the basal phosphorylation level of Ser-137 and reduced or abolished DHPG-stimulated phosphorylation of Ser-137. IC261 did not reduce the basal phosphorylation level of Ser-137, but at 5 and 10 μM, abolished DHPG-stimulated phosphorylation of Ser-137. Of particular interest, both CK1–7 and IC261 also abolished DHPG-stimulated phosphorylation of Thr-75.

The ability of CK1–7 and IC261 to reduce phosphorylation, not only of Ser-137 but also of Thr-75, suggested the possibility that CK1 might affect Thr-75 phosphorylation through an effect on Cdk5. To test this possibility, we studied the ability of CK1 inhibitors to affect DHPG-enhanced Cdk5 activity. Both CK1–7 and IC261 abolished the effect of DHPG on Cdk5 activity, as assayed by measuring histone H-1 phosphorylation in cell-free extracts after immunoprecipitation of Cdk5 (Fig. 6).

***In Vitro*, CK1 Phosphorylates p35.** To investigate a potential mechanism by which CK1 might regulate Cdk5, we examined whether CK1 could phosphorylate either the Cdk5 catalytic subunit or its regulatory subunit, p35. *In vitro*, purified CK1 was able to efficiently phosphorylate purified p35, but not the purified Cdk5 catalytic subunit (Fig. 7). Incubation with high concentrations of CK1 for prolonged times resulted in phosphorylation of p35 to ≈2 mol/mol (data not shown). Under similar conditions, Cdk5 was never phosphorylated to more than 0.05 mol/mol by CK1. To test the possible effect of CK1 phosphorylation of p35 on the catalytic activity of Cdk5/p35, p35 that was incubated without or with CK1 was mixed with an equimolar amount of Cdk5. Under conditions in which p35 was phosphorylated by CK1 to stoichiometries of ≈0.2 to 2 mol/mol, there was no obvious effect of phosphorylation on Cdk5/p35 activity measured in a secondary assay using inhibitor-1 as substrate (28) (data not shown).

Activation of mGluRs Regulates Ca^{2+} Channels Through a Mechanism Involving CK1, Cdk5, and DARPP-32. To test the potential physiological consequence of the mGluR activation of CK1 and Cdk5 in neostriatal neurons, we examined the effect of CK1 and Cdk5 inhibitors on voltage-dependent Ca^{2+} channels. In acutely dissociated neurons, application of DHPG enhanced Ca^{2+} current, and the enhancement was greatly reduced in the presence of CK1–7 (Fig. 8 *a* and *b*) or butyrolactone (Fig. 8 *c* and *d*). These results suggested that the mGluR-mediated up-regulation of Ca^{2+} current occurs via activation of CK1 and Cdk5. Treatment with CK1–7 (Fig. 8*a*) or butyrolactone (Fig. 8*c*) also caused a significant reduction in basal Ca^{2+} current, suggesting that constitutively active CK1 and Cdk5 are involved in maintaining Ca^{2+} channel activity under basal conditions. To test the potential involvement of DARPP-32 in this process, we examined mGluR modulation of Ca^{2+} current in acutely dissociated neurons from the neostriatum of DARPP-$32^{-/-}$ mice. In nine of 10 DARPP-$32^{-/-}$ neurons tested, DHPG had no detectable effect on Ca^{2+} current. A representative experiment is shown in Fig. 8*e*.

Discussion

The present studies have provided information concerning the intracellular signal transduction machinery used by group I mGluRs. The results indicate that activation of these receptors leads to the activation of CK1, and through CK1, to the

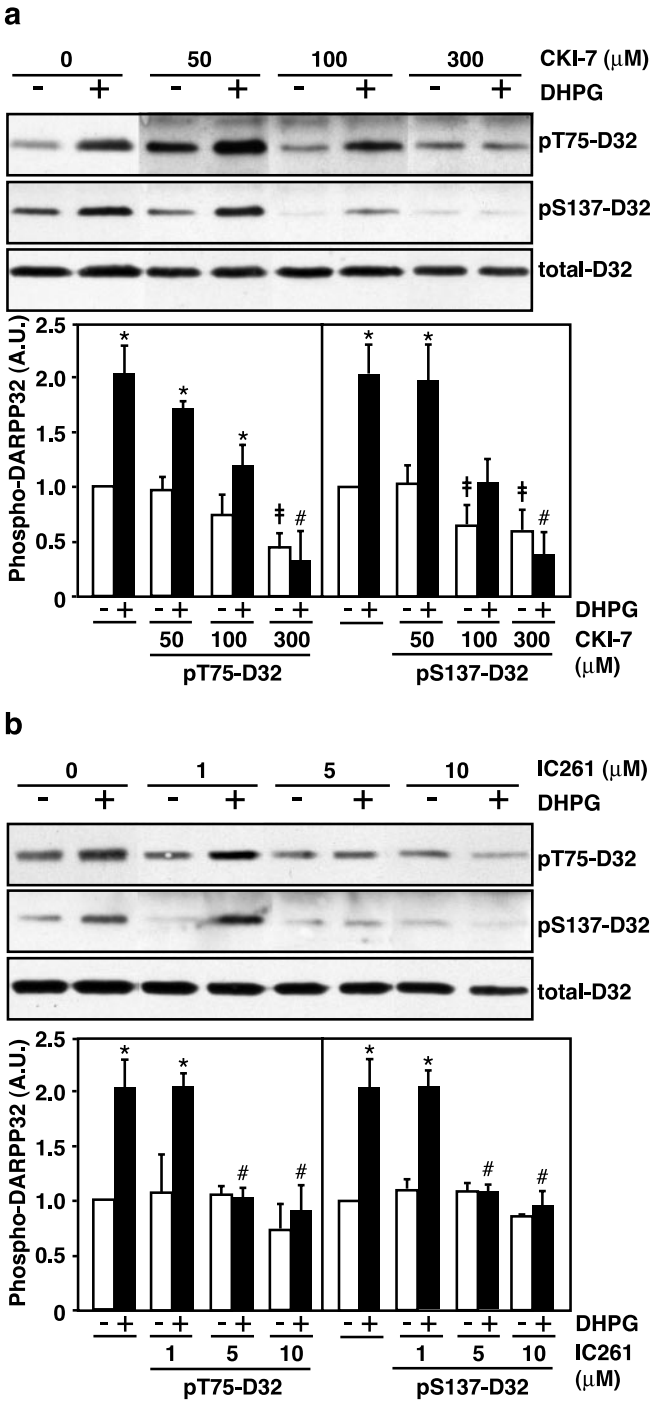

Fig. 5. CK1 inhibitors, CK1–7 and IC261, block the effects of DHPG on phosphorylation of DARPP-32 on both Ser-137 (CK1 site) and Thr-75 (Cdk5 site). Mouse neostriatal slices were preincubated for 30 min with (*a*) 0, 50, 100, or 300 μM CK1–7 or (*b*) 0, 1, 5, or 10 μM IC261, and then DHPG (100 μM) was added for an additional 2 min. Slice homogenates were analyzed by SDS/PAGE and immunobloting with phospho-Ser-137, phospho-Thr-75, and total DARPP-32 antibodies. (*Upper*) Immunoblots. (*Lower*) Cumulative data (means ± SEM) obtained from three experiments. Data were normalized to values obtained for untreated slices. *, $P < 0.05$, Student's *t* test, compared with slices in the absence of DHPG; ‡, $P < 0.05$, Student's *t* test, compared with untreated slices; #, $P < 0.05$, Student's *t* test, compared with slices treated with DHPG alone.

activation of Cdk5. Despite extensive knowledge about Cdk5 and CK1, regulation of these two kinases by first messengers has remained unknown. However, recent work has demonstrated that two long isoforms of CK1, CK1δ and CK1ε, are inhibited by

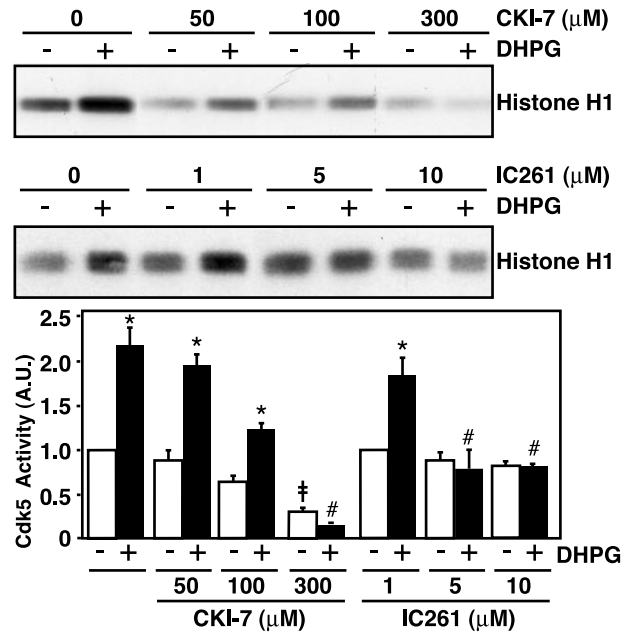

Fig. 6. CK1 inhibitors block the effect of DHPG on Cdk5 activity. Mouse neostriatal slices were preincubated with 0, 50, 100, or 300 μM CK1–7 or 0, 1, 5, or 10 μM IC261 for 30 min, and then DHPG (100 μM) was added for an additional 2 min. Slices were homogenized and Cdk5 was immunoprecipitated with anti-Cdk5 (C-8) antibody. Cdk5 activity was assayed by using histone H-1 as substrate and samples were analyzed by SDS/PAGE and autoradiography. (*Upper*) Autoradiograms indicating histone H-1 phosphorylation. (*Lower*) Cumulative data (means ± SEM) from three experiments. The autoradiograms were analyzed by using a PhosphorImager, and the data were normalized to values obtained for untreated slices. *, $P < 0.05$, Student's *t* test, compared with slices in the absence of DHPG; ‡, $P < 0.05$, Student's *t* test, compared with untreated slices; #, $P < 0.05$, Student's *t* test, compared with slices treated with DHPG alone.

autophosphorylation at their COOH termini (14, 15), suggesting a potential mechanism for regulation of CK1 activity by first messengers. Recent work has shown that Cdk5 is regulated by Ca^{2+} via activation of the Ca^{2+}-dependent protease calpain and cleavage of p35 to p25 (6–9). In the present study, we have shown that the activity of CK1 and Cdk5 both are regulated by a common first messenger in intact neuronal cells.

mGluRs belong to the superfamily of seven transmembrane segment G protein-coupled receptors that exert their effects via direct modulation of ion channels or formation of second messengers (29, 30). The mGluRs have been classified in three subgroups: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8), on the basis of pharmacological profiles, sequence homology, and coupling to second messenger formation. Activation of group I mGluRs results in the stimulation of phosphoinositide hydrolysis, whereas activation of group II and group III mGluRs results in inhibition of cAMP formation (31, 32). Like other groups of mGluRs, group I mGluRs exhibit a heterogeneous distribution in the central nervous system (33, 34). In the striatum, an area richly innervated with glutamatergic afferents from widespread regions of the forebrain, morphological studies show a dense distribution of group I mGluRs (35).

In the present study, we examined the effect of activation of group I receptors by using DHPG, a selective group I receptor agonist that activates both mGluR1 and mGluR5. Some interesting questions arise from the demonstration that group I mGluRs activate CK1, and thereby also activate Cdk5. Does activation of phosphoinositide hydrolysis result in activation of CK1, and if so by what mechanism? One possible mechanism involves mGluR-dependent activation of phospholipase Cβ,

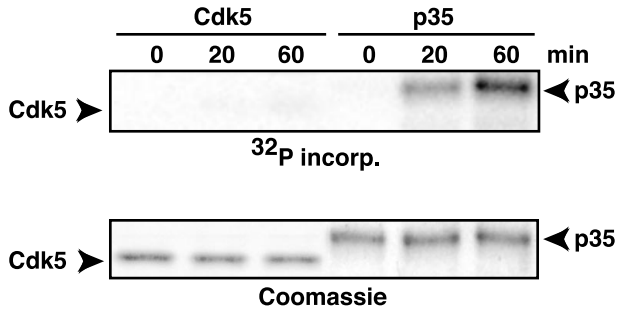

Fig. 7. CK1 phosphorylates p35 *in vitro*. Cdk5 (*Left*) or p35 (*Right*) was incubated *in vitro* with CK1 and [^{32}P]ATP for various times as indicated. Samples were analyzed by SDS/PAGE and autoradiography. ^{32}P incorporation is shown in the autoradiogram (*Upper*). Total amounts of Cdk5 and p35 are shown in the Coomassie-stained gel (*Lower*).

production of inositol trisphosphate, and release of Ca^{2+} from the endoplasmic reticulum. Increased intracellular Ca^{2+} might activate either a Ca^{2+}-dependent protein phosphatase that dephosphorylates inhibitory autophosphorylation sites on CK1δ or CK1ε, or a Ca^{2+}-dependent protease that would remove the inhibitory COOH-terminal domain. Notably, it has been reported that exposure of neurons to excitotoxins, hypoxic stress, or calcium influx for relatively long periods of time causes activation of calpain, increases levels of p25, and thereby upregulates Cdk5 activity (8). In contrast to previous studies of Cdk5, the effect of DHPG on CK1 and Cdk5 found in the present study was rapid and transient. In support of a role for Ca^{2+} in the actions of DHPG, we have found that the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate/acetoxymethyl blocks the effects of DHPG on both CKI and Cdk5 (unpublished results).

Another question raised by this study is the relationship between CK1 and Cdk5. Our studies in intact neostriatal cells indicate that CK1 activates Cdk5. Previous studies reported that the catalytic subunit of Cdk5 could be phosphorylated by CK1 and that this was associated with activation of Cdk5 activity (36). However, our studies using Cdk5 and p35 expressed in, and purified from, Sf9 cells, indicate that CK1 efficiently phosphorylates p35, but only very poorly phosphorylates the catalytic subunit of Cdk5. Although p35 phosphorylation was not associated with an increase in Cdk5/p35 enzymatic activity in our reconstituted system, the ability of CK1 to phosphorylate the Cdk5 cofactor p35 might contribute to stabilizing the formation of an active Cdk5 complex *in vivo*. Alternatively, some additional factor, present in neostriatal neurons and missing from the purified system, may play a role in the activation by CK1 of Cdk5.

Group I mGluRs have been implicated in several aspects of neostriatal function. Unilateral intracaudate injection of DHPG induces contralateral rotation (37), whereas bilateral injection of DHPG causes a long-lasting increase in locomotion and stereotypical behavior (38, 39). mGluR activity is linked to the regulation of expression of several sets of genes in striatal neurons (40). Moreover, group I mGluRs have been suggested as useful therapeutic targets for the treatment of Parkinson's disease (41, 42). However, the molecular and cellular mechanisms underlying mGluR regulation of striatal physiological and behavioral functions are far from understood. To begin to evaluate the possible functional consequences of mGluR activation of CK1 and Cdk5, we investigated the impact of the mGluR/CK1/Cdk5 pathway on voltage-gated Ca^{2+} channels (VDCCs). VDCCs are involved in a wide range of cellular functions, including synaptic integration, neurotransmitter release, and gene expression (43), and modulation of VDCCs is an important factor in the regulation of neuronal function. Previous studies have shown that VDCCs can be regulated by a G

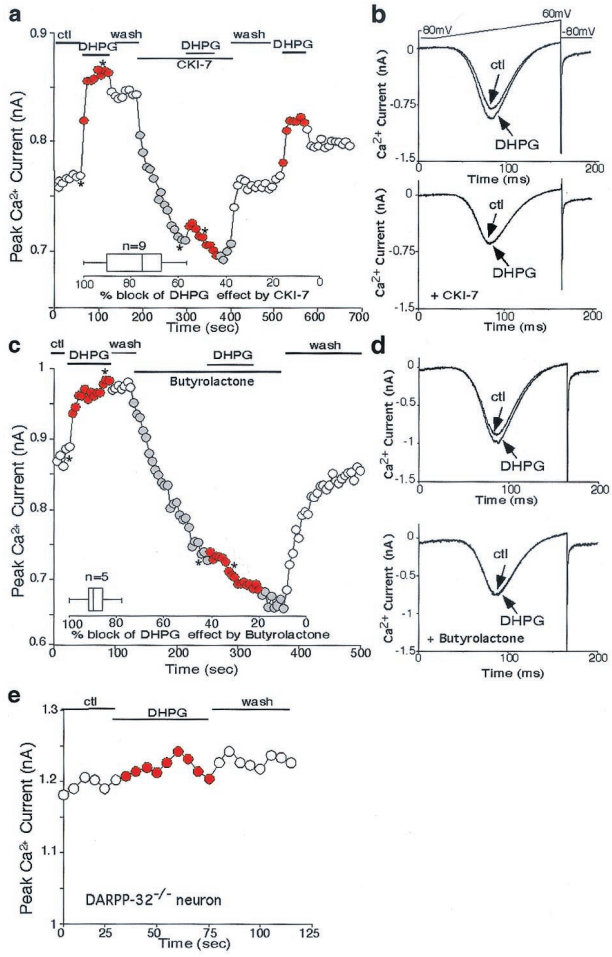

Fig. 8. Activation by DHPG of group I mGluRs enhances Ca^{2+} current, and this enhancement is blocked by inhibiting CK1 or cdk5 and is absent in DARPP-32 knockout mice. (*a* and *c*) Peak Ca^{2+} current evoked by a voltage ramp protocol as a function of time and drug application. (*a*) DHPG (100 μM) increased Ca^{2+} current. In the presence of CK1–7 (100 μM), basal Ca^{2+} currents were reduced and the DHPG effect was attenuated. Washing out CK1–7 led to recovery of the DHPG effect. (*c*) DHPG (100 μM) increased Ca^{2+} current. In the presence of butyrolactone (25 μM), basal Ca^{2+} currents were reduced and the DHPG effect was eliminated. (*b* and *d*) Representative current traces showing the modulation by DHPG before and after the application of (*b*) CK1–7 or (*d*) butyrolactone (at time points denoted by ⋆). (*Insets*) Box plot summaries showing the percentage block of the DHPG effect on Ca^{2+} currents by (*a*) CK1–7 and (*c*) butyrolactone. (*e*) Plot of peak Ca^{2+} current as a function of time and agonist application in a representative neostriatal neuron from a DARPP-32 knockout mouse. DHPG (100 μM) had no effect on Ca^{2+} current in neurons from DARPP-32$^{-/-}$ mice (n = 9).

protein-mediated membrane-delimited pathway (44) or by serine/threonine kinases such as PKA, protein kinase C, and Ca^{2+}/calmodulin-dependent protein kinase II (45). In this study, we have shown that both CK1 and Cdk5 are involved in maintaining basal Ca^{2+} channel activity, and that group I mGluR activation enhances Ca^{2+} currents through activation of CK1 and Cdk5. The adaptor protein, Homer, has been suggested to bridge the interaction between mGluRs and ryanodine or inositol 1,4,5-triphosphate receptors (46). It has recently been demonstrated that functional interactions occur between mGluRs, ryanodine receptors, and Ca^{2+} channels in cultured cerebellar granule neurons (47). Our results raise the possibility that a similar interaction between mGluRs and Ca^{2+} channels may exist in striatal neurons.

The present studies have implicated not only CK1 and Cdk5, but also DARPP-32 in the mechanism by which activation of

group I mGluR increases Ca^{2+} current. DARPP-32 in its Thr-34-phosphorylated form is an inhibitor of PP-1. DARPP-32 in its Thr-75-phosphorylated form is an inhibitor of PKA, and thereby inhibits phosphorylation by PKA of Thr-34. DARPP-32 in its Ser-137-phosphorylated form inhibits the dephosphorylation by PP-2B of Thr34. Thus, one would predict opposing effects of phospho-Thr-75 and phospho-Ser-137 on signaling through the dopamine/D1 receptor/cAMP/PKA/phospho-DARPP-32/PP-1 pathway. The relative importance of the phospho-Thr-75 and phospho-Ser-137 pathways to the regulation of Ca^{2+} channel activity will require further investigation. Transgenic mice with point mutations of various phosphorylation sites on DARPP-32 should help answer outstanding questions on the precise mechanisms by which CK1, Cdk5, and DARPP-32 mediate mGluR control of neostriatal neurons.

We are grateful to Dr. David Virshup (University of Utah, Salt Lake City) for providing HA-tagged CK1ε expression plasmid. We thank Drs. Gretchen Snyder and Andrew Czernik for providing valuable advice and Yong Kim for preparing purified Cdk5 and p35. This work was supported by National Institutes of Health Grants MH40899, DA10044, and AG09464.

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