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D_2 dopamine receptors induce mitogen-activated protein kinase and cAMP response element-binding protein phosphorylation in neurons

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ABSTRACT **Dopamine, by activating D_1- and D_2-class receptors, plays a significant role in regulating gene expression. Although much is known about D_1 receptor-regulated gene expression, there has been far less information on gene regulation mediated by D_2 receptors. In this study, we show that D_2 receptors can activate the mitogen-activated protein kinase (MAPK) and the cAMP response element-binding protein (CREB) in neurons. Treatment of brain slices with the D_2 receptor agonist quinpirole induced rapid phosphorylation of MAPK and CREB. The neuroleptic drug eticlopride, a highly selective D_2 receptor antagonist, blocked the quinpirole-induced phosphorylation of MAPK and CREB. D_2 receptor-induced MAPK phosphorylation depended on intracellular Ca^{2+} elevation, protein kinase C activation, and MAPK kinase activation, but not on the protein tyrosine kinase Pyk2, even though quinpirole stimulated Pyk2 phosphorylation. D_2 receptor-induced CREB phosphorylation was mediated by activation of protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase, but not MAPK. The dopamine and cAMP-regulated phosphoprotein DARPP-32 also was required for the regulation of MAPK and CREB phosphorylation by D_2 receptors. Our results suggest that MAPK and CREB signaling cascades are involved in the regulation of gene expression and other long-term effects of D_2 receptor activation.**

The dopamine system plays a significant role in motor function and associative learning (1, 2). Dysfunction in dopamine signaling has been implicated in many neuropsychiatric disorders, such as Parkinson's disease, schizophrenia, attention deficit hyperactivity disorder, and drug abuse. One mechanism that underlies the dopaminergic regulation of cellular physiology involves modulation of ion channel activity and associated short-term changes in cellular excitability (3–6). Another mechanism involves regulation of gene expression, which can produce long-term changes in synaptic plasticity (7, 8).

Dopamine acts through the D_1 and D_2 subfamilies of G-protein-coupled receptors. Many antipsychotic drugs, which are D_2 receptor antagonists, can induce gene expression (9), suggesting that D_2 receptors, like D_1 receptors (10–12), are important in gene regulation. Previous studies have shown that D_1 and D_2 dopamine receptors synergistically activate immediate early gene expression and locomotion in dopamine-depleted striatum (13, 14). This D_1-D_2 synergy indicates that D_2 receptors may achieve these effects through mechanisms other than that of reducing cAMP formation. One such possible mechanism is to elevate intracellular calcium (15).

Two potential targets for D_2 receptors are the mitogen-activated protein kinase (MAPK) and the cAMP response element-binding protein (CREB). MAPKs are a family of serine/threonine kinases that regulate multiple cellular responses including gene expression, and many MAPK substrates are transcription factors (16). The extracellular signal-regulated MAPK (ERK) is phosphorylated at Thr202/Tyr204 by a wide variety of stimuli, such as Ca^{2+}, growth factors, and neurotransmitters (17–19). CREB is a plasticity-associated transcription factor that regulates the expression of many downstream genes containing CRE elements, such as c-fos (20, 21). CREB is phosphorylated at Ser-133 by multiple protein kinases, including protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinases II and IV (CaMK) (22–24). In the present study, we provide evidence that D_2 receptors can regulate gene expression by coupling to the Gq/PLCβ pathway, causing an elevation of intracellular Ca^{2+} and activation of PKC, leading to the phosphorylation and activation of MAPK and CREB. Because MAPK and CREB signaling cascades are critical for neuronal plasticity and memory formation (25), our results provide a possible mechanism for long-term actions of D_2 receptors.

MATERIALS AND METHODS

Preparation and Treatment of Brain Slices. Four-week-old male Sprague–Dawley rats or C57BL/6J mice were anesthetized and decapitated. Brains were quickly removed, iced, and blocked for slicing. Sagital sections (400 μm) of the brain (cerebellum removed) were cut with a Vibratome Technical Products International (St. Louis). The major brain areas in the slices included neocortex, striatum, hippocampus, thalamus, and substantia nigra. The slices were bathed in a low Ca^{2+} Hepes-buffered salt solution [in mM: 140 Na isothionate, 2 KCl, 4 $MgCl_2$, 0.1 $CaCl_2$, 23 glucose, and 15 Hepes (pH 7.4), 300–305 mosm/liter]. Slices then were preincubated for 1–2 hrs at 37°C in a $NaHCO_3$-buffered saline [in mM: 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$ 1.25 NaH_2PO_4, and 10 glucose (pH 7.4), 300–305 mosm/liter] bubbled with 95% O_2 and 5% CO_2. To avoid indirect effects caused by synaptic transmission and neuronal firing, solutions contained the glutamate receptor antagonist kynurenic acid (1 mM) and the sodium channel blocker tetrodotoxin (0.5 μM). Paired slices then were incubated for a given time in the absence or presence of test substances.

The D_2 receptor agonist quinpirole and antagonist eticlopride were obtained from Research Biochemicals. The intracellular Ca^{2+} chelator BAPTA/AM, the PKC inhibitor Go6976, the CaMK inhibitor KN-93, the MAPK kinase (MEK) inhibitor PD98059, and the protein tyrosine kinase inhibitor genistein were obtained from Calbiochem.

Western Blot Analysis. After incubation, slices were transferred to boiling 1% SDS and were homogenized immediately.

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Abbreviations: MAPK, mitogen-activated protein kinase; CREB, cAMP response element-binding protein; CaMK, Ca^{2+}/calmodulin-dependent protein kinases; MEK, MAPK kinase; PKA, protein kinase A.

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Insoluble material was removed by centrifugation (13,000 × *g* for 10 min), and protein concentration for each sample was measured. Equal amounts of protein from slice homogenates were separated on 7.5% acrylamide gels and were transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk for 1 hour at room temperature. Then the blots were incubated with phospho-p44/42 MAPK (Thr202/Tyr204) antibody (New England Biolabs, 1:1,000) or phospho-CREB (Ser133) antibody (New England Biolabs, 1:1,000) for 1 hour at room temperature. After being rinsed, the blots were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Amersham Pharmacia, 1:2,000) for 1 hour at room temperature. After three washes, the blots were exposed to the enhanced chemiluminescence substrate. Then the blots were stripped for 1 hour at 50°C followed by saturation in 5% nonfat dry milk and were incubated with antibodies recognizing total MAPK and CREB. Quantitation was obtained from densitometric measurements of immunoreactive bands on autoradiograms. Data correspond to the mean ± SEM of 3–10 samples per condition and were analyzed by paired *t* test.

Pyk2 Immunoprecipitation. After incubation, slices were immediately lysed in lysis buffer (in mM: 1% Triton X-100, 5 EDTA, 10 Tris, 50 NaCl, 30 $Na_4P_2O_7 \cdot 10H_2O$, 50 NaF, and 0.1 Na_3VO_4) on ice for 30 min. Cell lysates were centrifuged (15,000 × *g*, 20 min) and ultracentrifuged (40,000 × *g*, 1 hr) to remove insoluble material. Pyk2 antibody (Upstate Biotechnology, Lake Placid, NY, 1:1,000) was added to the homogenates and was incubated for 1 hour at 4°C. Protein A-Sepharose beads then were added and mixed for 1 hour at 4°C. The beads were pelleted by centrifugation and were washed three times with lysis buffer. After the final wash, the beads were resuspended in 50 μl of a SDS/PAGE sample buffer (50 mM Tris-HCl, pH 6.7/10% glycerol/2% SDS/10% 2-mercaptoethanol/0.01% bromophenol blue). Proteins were separated by SDS/PAGE and were subjected to Western blotting with the anti-pY antibody (Upstate Biotechnology, 1:1,000) for the detection of tyrosine phosphorylation of Pyk2.

RESULTS

Effect of D_2 Dopamine Receptor Activation on Phosphorylation of MAPK and CREB. Brain slices were incubated with the D_2 receptor agonist quinpirole and were Western blotted with phospho-MAPK (Thr202/Tyr204) or phospho-CREB (Ser133) antibody, followed by reblotting with antibodies that recognize total (phosphorylation-state independent) MAPK and CREB levels.

Application of quinpirole induced the phosphorylation of MAPK (Fig. 1 *A* and *B*) in brain slices. This effect was blocked by the D_2 antagonist eticlopride, confirming mediation by D_2 dopamine receptors. The D_2-induced phosphorylation of MAPK showed rapid and transient kinetics, reaching a peak within 5 to 15 min and returning to basal level within 35 min (Fig. 1*C*). The dose-dependence of quinpirole-induced MAPK phosphorylation is shown in Fig. 1*D*. A saturating effect was seen at 10 μM. Total MAPK showed no change with any drug treatment (data not shown).

Treatment of brain slices with quinpirole also induced phosphorylation of CREB, and this effect was eliminated in the presence of the D_2 antagonist eticlopride (Fig. 1 *E* and *F*). As with MAPK phosphorylation, the D_2-induced phosphory-

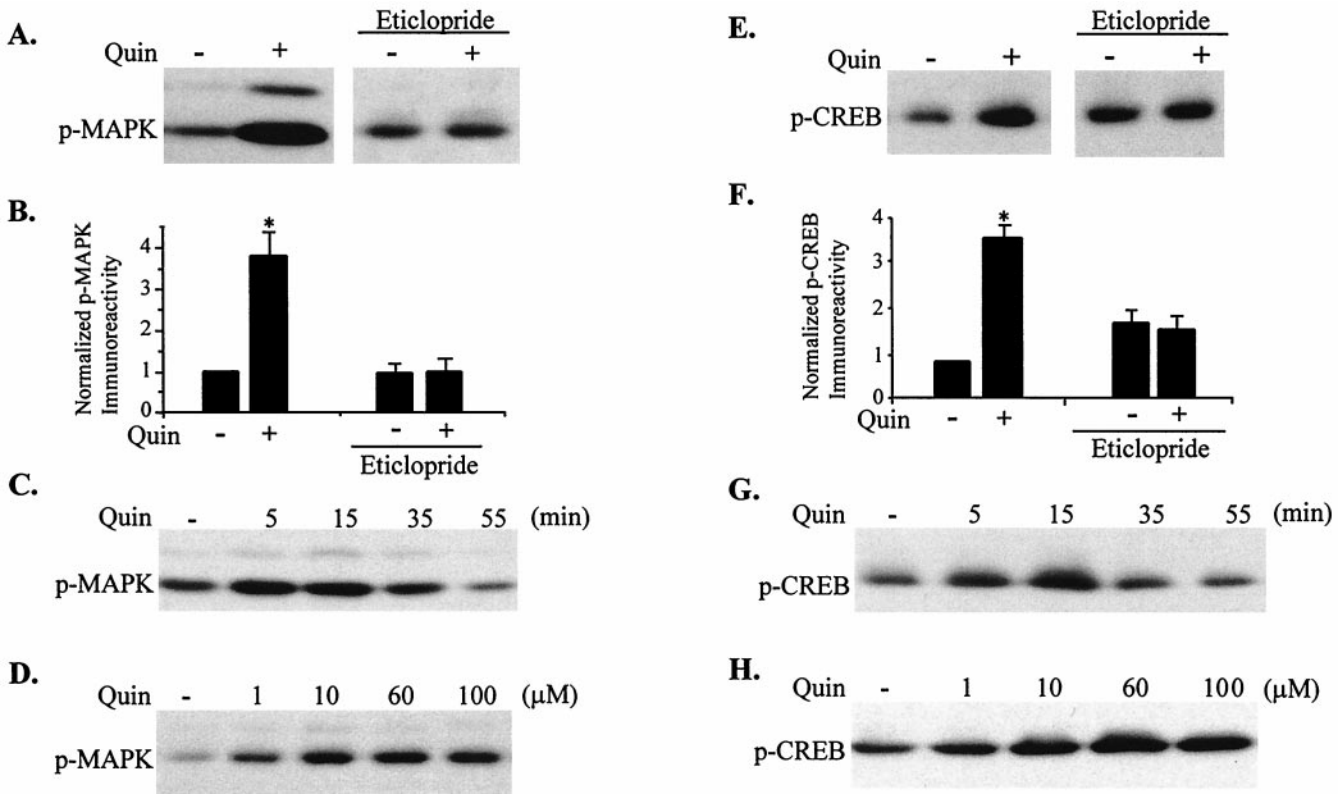

FIG. 1. Stimulation of phosphorylation of MAPK and CREB by the D_2 dopamine receptor agonist quinpirole. (*A* and *E*) Immunoblots of phospho-MAPK (*A*) and phospho-CREB (*E*). Brain slices were preincubated in the absence or presence of the D_2 antagonist eticlopride (40 μM) for 20 min, followed by incubation with or without the D_2 agonist quinpirole (60 μM) for 15 min. Extracts of slices were immunoblotted with anti-phospho-MAPK or anti-phospho-CREB antibody. (*B* and *F*) Quantitation of p42 MAPK phosphorylation (*B*) and CREB phosphorylation (*F*). *, $P < 0.01$, compared with minus quinpirole. (*C* and *G*) Time courses of the D_2 receptor-induced phosphorylation of MAPK (*C*) and CREB (*G*). Slices were treated with quinpirole (60 μM) for the indicated times, and phospho-MAPK and phospho-CREB were detected by immunoblotting of slice extracts. (*D* and *H*) Dose-dependence of the D_2 receptor-induced phosphorylation of MAPK (*D*) and CREB (*H*). Slices were treated with quinpirole for 15 min at the indicated concentrations, and phospho-MAPK and phospho-CREB were detected by immunoblotting of slice extracts.

lation of CREB showed rapid and transient kinetics, reaching a peak at 15 min and returning to basal level within 35 min (Fig. 1*G*). The dose-dependence of CREB phosphorylation (Fig. 1*H*) was similar to that of MAPK. Total CREB was not affected by any of these drug treatments (data not shown).

Signaling Pathways Underlying D_2 Receptor Regulation of MAPK Phosphorylation. In previous studies, it was found that Ca^{2+} and PKC can activate the Ras/Raf/MEK/MAPK signaling pathway in cell lines and neuronal cultures (17, 26). We tested the possibility that D_2 receptors phosphorylate MAPK by coupling to the Gq/PLCβ pathway, which leads to the elevation of intracellular Ca^{2+} and activation of PKC. For this purpose, the selective PKC inhibitor Go6976 and the intracellular Ca^{2+} chelator BAPTA/AM were used. Pretreatment of brain slices with Go6976 alone or BAPTA/AM alone reduced the MAPK phosphorylation induced by quinpirole. Combined application of Go6976 and BAPTA/AM abolished the ability of quinpirole to induce MAPK phosphorylation (Fig. 2 *A* and *B*). These results suggest that increases in intracellular Ca^{2+} and activation of PKC both contribute to the D_2 induction of MAPK phosphorylation.

One possible mechanism by which D_2 receptors regulate MAPK phosphorylation in central nervous system neurons is by causing phosphorylation of the protein tyrosine kinase Pyk2, secondary to the elevation of intracellular Ca^{2+} and activation of PKC, leading to activation of the MAPK signaling pathway (27). Indeed, application of the D_2 agonist quinpirole induced the tyrosine phosphorylation of Pyk2, and this effect was blocked by the D_2 antagonist eticlopride (Fig. 2*C*). To test whether the ability of D_2 receptors to induce MAPK phosphorylation was regulated through Pyk2, the broad-spectrum tyrosine kinase inhibitor genistein was used. If MAPK were the downstream target of Pyk2, pretreatment of brain slices with genistein should prevent the D_2 induction of MAPK phosphorylation. However, quinpirole still induced phosphorylation of MAPK in the presence of genistein (Fig. 2*D*), indicating that D_2 receptor signaling can bypass Pyk2 to activate MAPK. Inhibiting the MAPK kinase (MEK) with PD98059 abolished the D_2 receptor-induced phosphorylation of MAPK (Fig. 2*D*), indicating the involvement of MEK in this process.

Signaling Pathways Underlying D_2 Receptor Regulation of CREB Phosphorylation. In previous studies, it was found that CREB can be phosphorylated by multiple kinases *in vitro*, including PKA, PKC, CaMK, and the MAPK-activated Rsk family of protein kinases (22–24, 28). To test which signaling molecules are responsible for the D_2 receptor-induced phosphorylation of CREB, selective protein kinase inhibitors were used. Inhibiting PKC with Go6976 or inhibiting CaMK with KN-93 did not block CREB phosphorylation induced by quinpirole (Fig. 3). However, combined application of Go6976 and KN-93 eliminated the ability of quinpirole to induce CREB phosphorylation (Fig. 3). These results suggest that the D_2 receptor-induced phosphorylation of CREB is caused by the combined activation of CaMK and PKC.

Because D_2 receptors can induce MAPK phosphorylation (shown above) and phospho-MAPK can indirectly activate CREB via Rsk family kinases (28), one potential pathway for the D_2 induction of CREB phosphorylation is through activated MAPK. To test this possibility, brain slices were pretreated with the MAPK kinase (MEK) inhibitor PD98059. As shown in Fig. 3, the D_2 receptor-induced phosphorylation of CREB was not blocked by PD98059, although this compound blocked D_2 receptor-induced MAPK phosphorylation (Fig. 2*D*). Moreover, a combination of PD98059 with either Go6976 or KN-93 failed to block D_2 receptor-regulated phosphorylation of CREB (data not shown). These results suggest that the D_2-induced phosphorylation of CREB does not depend on MAPK activation.

Requirement for DARPP-32 in the D_2-Induced Phosphorylation of MAPK and CREB. DARPP-32, a dopamine and cAMP-regulated phosphoprotein (M_r 32) that is highly enriched in the basal ganglia, plays a key role in many biological responses to dopamine (29, 30). DARPP-32 is phosphorylated by PKA in response to D_1 receptor activation and is dephosphorylated by calcineurin in response to D_2 receptor activation

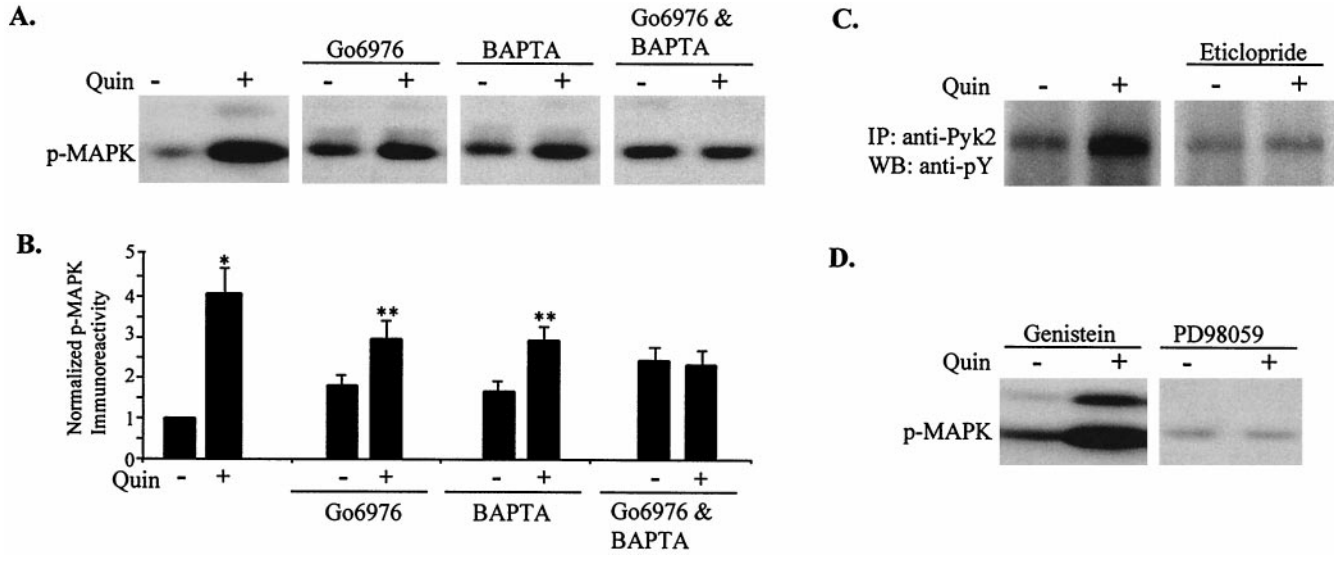

FIG. 2. D_2 receptor-induced phosphorylation of MAPK: evaluation of the roles of intracellular Ca^{2+}, PKC, and the protein tyrosine kinase Pyk2. (*A*) Immunoblots of phospho-MAPK. Brain slices were preincubated in the absence or presence of various inhibitors for 20 min, followed by incubation with or without quinpirole (60 μM) for 15 min. Extracts of slices were immunoblotted with anti-phospho-MAPK antibody. MAPK phosphorylation induced by quinpirole was reduced in the presence of the PKC inhibitor Go6976 (1 μM) alone or the intracellular Ca^{2+} chelator BAPTA/AM (20 μM) alone and was abolished in the presence of Go6976 plus BAPTA/AM. (*B*) Quantitation of p42 MAPK phosphorylation. *, $P < 0.01$; **, $P < 0.05$, compared with minus quinpirole. (*C*) Immunoblots of phospho-Pyk2. Slices were preincubated in the absence or presence eticlopride (40 μM) for 20 min, followed by incubation with or without quinpirole (60 μM) for 15 min. Lysates of the slices were immunoprecipitated with anti-Pyk2 and were blotted with anti-pY. (*D*) Immunoblots of phospho-MAPK. Brain slices were preincubated in the absence or presence of the tyrosine kinase inhibitor genistein (50 μM) or the MAPK kinase inhibitor PD98059 (20 μM) for 20 min, followed by incubation with or without the D_2 agonist quinpirole (60 μM) for 15 min. Extracts of slices were immunoblotted with anti-phospho-MAPK antibody.

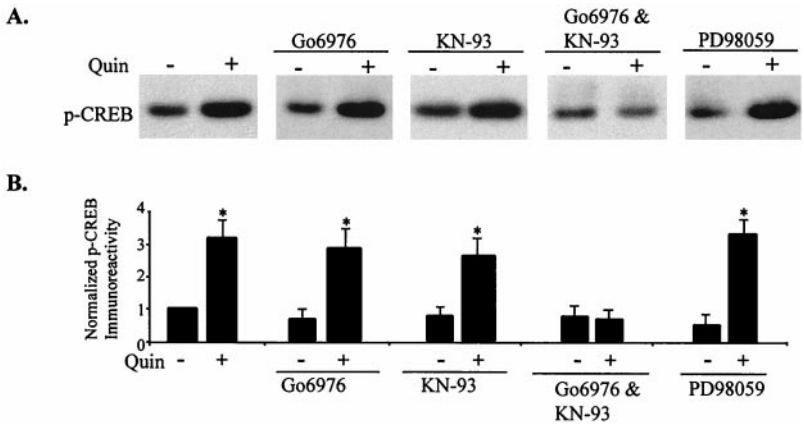

FIG. 3. D_2 receptor-induced phosphorylation of CREB: evaluation of the roles of CaMK, PKC, and MAPK. (*A*) Immunoblots of phospho-CREB. Brain slices were preincubated in the absence or presence of various inhibitors for 20 min, followed by incubation with or without the D_2 agonist quinpirole (60 μM) for 15 min. Extracts of slices were immunoblotted with anti-phospho-CREB antibody. CREB phosphorylation induced by quinpirole was observed in the presence of the PKC inhibitor Go6976 (1 μM) alone or the CaMK inhibitor KN-93 (10 μM) alone but not in the presence of Go6976 plus KN-93. CREB phosphorylation induced by quinpirole also was observed in the presence of the MAPK kinase inhibitor PD98059 (20 μM). (*B*) Quantitation of CREB phosphorylation. *, $P < 0.01$, compared with minus quinpirole.

(15). Phosphorylation of DARPP-32 at Thr-34 converts it into a potent inhibitor of protein phosphatase 1 (31), thereby affecting the phosphorylation state and physiological activity of many downstream targets of protein phosphatase 1 (29). To investigate whether DARPP-32 is involved in the mechanism by which D_2 receptors regulate MAPK and CREB phosphorylation, DARPP-32 knockout mice (30) were examined. The basal MAPK and CREB phosphorylation levels were elevated in slices from DARPP-$32^{-/-}$ mice. The D_2-induced phosphorylation of MAPK and CREB was virtually abolished in DARPP-$32^{-/-}$ mice, compared with that in wild-type mice (Fig. 4). The results indicate that DARPP-32 is an important component of the signaling cascades that mediate the D_2 receptor-induced phosphorylation of MAPK and CREB.

DISCUSSION

In this study, we demonstrated that the D_2 receptor agonist quinpirole can stimulate the phosphorylation and activation of MAPK and CREB, two molecules that are crucial for the induction of many immediate-early and late response genes. Signal transduction pathways involved in mediating these D_2 effects are illustrated in Fig. 5. Elevated intracellular Ca^{2+}, CaMK, PKC, and DARPP-32 are important components in these cascades.

MAPK is highly expressed in the nervous system (32) and is localized primarily in neuronal cell bodies and dendrites (33), consistent with a postsynaptic function in neuronal signaling. In addition to being involved in gene expression, MAPK also regulates cytoskeleton dynamics and cytoplasmic signaling (34, 35). Given the wide variety of downstream targets for MAPK, the D_2 activation of MAPK signaling potentially has a diverse array of physiological functions.

MAPK can be activated by a complex set of extracellular stimuli and intracellular molecules. In this study, we obtained evidence that D_2 receptor agonists, by increasing intracellular Ca^{2+} and PKC activity, lead to activation of the MEK/MAPK cascade. Though the protein tyrosine kinase Pyk2 also was activated by D_2 receptors, it is not required for the D_2-induced MAPK activation. Because Pyk2 has multiple targets besides MAPK, it seems likely that the D_2-induced activation of Pyk2 affects other Pyk2 targets in neurons.

CREB is another target found to be activated by D_2 receptors in this study. By regulating new gene expression and

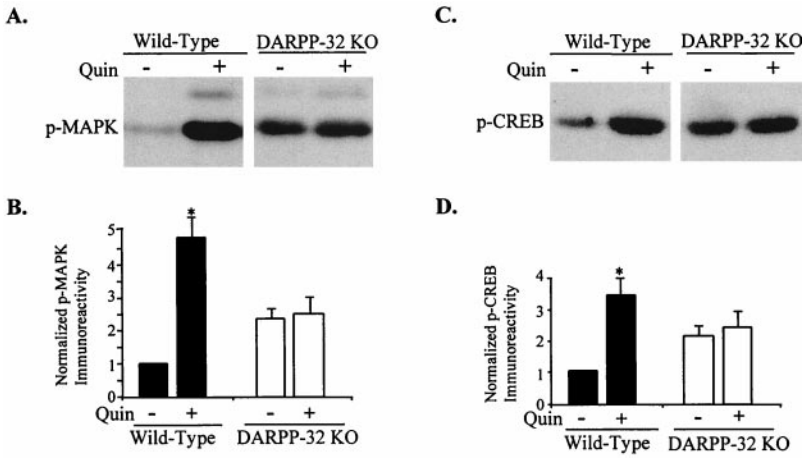

FIG. 4. D_2 receptor-induced phosphorylation of MAPK and CREB in wild-type and DARPP-32-deficient mice. (*A*) Immunoblots of phospho-MAPK in brain slices from wild-type and DARPP-32-deficient mice. Brain slices were incubated with or without quinpirole (60 μM) for 15 min. Extracts of slices were immunoblotted with anti-phospho-MAPK antibody. (*B*) Quantitation of quinpirole-induced phosphorylation of p42 MAPK. *, $P < 0.01$, compared with minus quinpirole. (*C*) Immunoblots of phospho-CREB in brain slices from wild-type and DARPP-32-deficient mice. Brain slices were incubated with or without quinpirole (60 μM) for 15 min. Extracts of slices were immunoblotted with anti-phospho-CREB antibody. (*D*) Quantitation of quinpirole-induced phosphorylation of CREB. *, $P < 0.01$, compared with minus quinpirole.

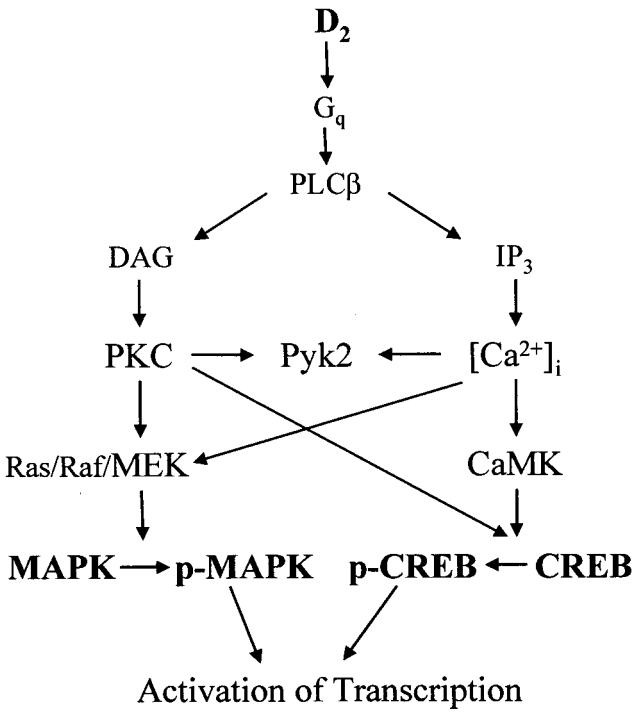

FIG. 5. Model of signaling cascades involved in D_2 receptor-induced phosphorylation of MAPK and CREB. In this model, D_2 receptors couple to the Gq protein and activate PLCβ, which promotes the hydrolysis of phosphatidlyinositide biphosphate (PIP_2) to form 1,2-diacylglycerol and 1,4,5-inositol trisphosphate. 1,2-diacylglycerol activates PKC, and 1,4,5-inositol trisphosphate causes the release of Ca^{2+} from intracellular stores. PKC and Ca^{2+} together activate Ras/Raf/MEK/MAPK signaling through mechanisms independent of Pyk2. CaMK activated by elevation of intracellular Ca^{2+}, together with activated PKC, phosphorylate CREB. DARPP-32 is involved in the D_2 receptor regulation of phosphorylation of both MAPK and CREB. The mechanism by which this occurs most likely involves regulation by D_2 receptors of the phosphorylation state of DARPP-32, and therefore of the activity of protein phosphatase 1 (29), which dephosphorylates CREB.

protein synthesis, CREB can mediate the long-term remodeling of synapses, which is believed to underlie memory consolidation and neuronal plasticity. The D_2 receptor-induced activation of CREB signaling provides a potential mechanism for the effect of antipsychotic drugs on immediate early gene expression (12) and neural circuit rewiring.

It is known that CREB can be activated through multiple pathways. These include PKA, PKC, CaMK, and the MAPK-activated Rsk family of protein kinases (22–24, 28). In some systems, MAPK activation was necessary for increased CREB phosphorylation (36, 37). In the neurons studied here, activation of PKC and CaMK, but not MAPK, was responsible for the D_2 receptor-induced CREB phosphorylation.

The different subcellular localizations of MAPK and CREB signaling would be expected to result in activation of different downstream targets and physiological responses to D_2 receptors. MAPK phosphorylation occurs primarily in dendrites, regulating protein synthesis, cytoskeletal dynamics, and ion channel activities at synapses. On the other hand, CREB phosphorylation occurs in cell bodies, regulating gene expression in the nucleus.

One signaling component that seems to be critically involved in the D_2 receptor-induced phosphorylation of MAPK and CREB is DARPP-32. In DARPP-32 knockout mice, the basal phosphorylation levels of MAPK and CREB were elevated, and the ability of D_2 receptors to induce phosphorylation of MAPK and CREB was lost. DARPP-32 plays a central role in signal transduction in dopaminoceptive neurons (29). Numerous first messengers regulate the phosphorylation state of DARPP-32. DARPP-32, in turn, regulates the phosphorylation and activity of many downstream effectors. Further studies will be necessary to determine the precise molecular mechanisms by which DARPP-32 regulates the phosphorylation of MAPK and CREB.

In conclusion, this study indicates that activation of D_2 receptors can lead to the phosphorylation and activation of MAPK and CREB in neurons. The results provide a potential molecular mechanism for some long-term effects of D_2 receptors, such as alterations of gene expression. Because D_1 receptors also can phosphorylate and activate CREB (10–12) and MAPK (38), the present results provide a possible explanation for the synergistic effects of D_1 and D_2 dopamine receptors on immediate early gene induction in striatum (13, 14). It remains for future studies to identify downstream targets that are regulated by these signaling cascades. The insight gained from these studies may be helpful for understanding the mechanisms underlying dopamine-related neurological disorders.

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