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Signaling Pathways Leading to Phosphorylation of Akt and GSK-3β by Activation of Cloned Human and Rat Cerebral D_2 and D_3 Receptors

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ABSTRACT

Although dopamine (DA) regulates the serine/threonine kinase Akt and its downstream substrate glycogen synthase kinase-3β (GSK-3β), the *direct* influence of dopaminergic receptors remains poorly characterized. Short-term incubation of Chinese hamster ovary (CHO)-expressed human (h)D_{2L} and hD_3 receptors with DA (maximal effect, 5–10 min) phosphorylated Akt (Thr308 and Ser473) and GSK-3β (Ser9), actions blocked by the selective D_2 and D_3 antagonists, 3-[4-(4-chlorophenyl)-4-hydroxypiperidin-l-yl]methyl-1*H*-indole (L741,626) and (3*aR*,9*bS*)-*N*[4-(8-cyano-1,3*a*,4,9*b*-tetrahydro-3*H*-benzopyrano[3,4-*c*]pyrrole-2-yl)-butyl] (4-phenyl)benzamide (S33084), respectively. Similar findings were acquired with the specific D_2/D_3 receptor agonist quinelorane, which also enhanced (10 min after administration) levels of p-Akt and p-GSK-3β in rat nucleus accumbens, an action blocked by the D_2/D_3 receptor antagonist raclopride. Akt and GSK-3β phosphorylation mediated via CHO-expressed hD_{2L} and hD_3 receptors was prevented by pertussis toxin and by inhibitors of insulin-like growth factor-1 receptors as well as phosphatidylinositol 3-kinase and Src. Likewise, chelation of intracellular Ca^{2+} and interference with an "atypical" phorbol ester-insensitive protein kinase C (PKC) abolished recruitment of Akt and GSK-3β. Inactivation of PKCμ blocked Akt and GSK-3β phosphorylation at hD_{2L} receptors. However, blockade of conventional PKC isoforms attenuated the actions of DA at hD_3 receptors only. Furthermore, phospholipase C (PLC), calmodulin, and Akt inhibitors abolished DA-induced GSK-3β phosphorylation by hD_3 receptors, whereas phosphorylation by hD_{2L} receptors partially involved calmodulin, Akt, *and* extracellular signal-regulated kinase (ERK) 1/2. In conclusion, at both hD_{2L} and hD_3 receptors, DA elicited a $G_{i/o}$- and Ca^{2+}/calmodulin-dependent phosphorylation of Akt and GSK-3β via transactivation of insulin-like growth factor 1 receptor. However, significant differences were seen regarding the involvement of PLC, calmodulin, and ERK1/2.

Introduction

Initially identified as a regulator of glycogen synthase activity, GSK-3 has been reported to control the phosphorylation of many other substrates and to be involved in the regulation of growth and development (Rayasam et al., 2009). Two isoforms of this serine/threonine kinase, encoded by two different genes and referred to as GSK-3α and GSK-3β, have been identified in mammals and exhibit 85% sequence homology and 98% identity in the catalytic domain. Both are ubiquitous and highly abundant in cerebral tissue (Yao et al., 2002), but they exhibit functional specificity. It is noteworthy that GSK-3β is the predominant brain isoform

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ABBREVIATIONS: 10-DEBC, 10-[4′-(*N*,*N*-diethylamino)butyl]-2-chlorophenoxazine; AG1024, 3-bromo-5-*t*-butyl-4-hydroxy-benzildenemalonitrile; AG1433, 2-(3,4-dihydroxyphenyl)-6,7-dimethoxyquinoxaline; AG1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester; CaMKII, Ca^{2+}-calmodulin kinase; CHO, Chinese hamster ovary; CTX, cholera toxin; DA, dopamine; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gö6976, 12-(2-cyanoethyl)-6,7,12,13,-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]-carbazol; Gö6983, 3-[1(3-dimethylamino-propyl)-5-methoxy-1*H*-indol-3-yl]4-(1*H*-indol-3-yl)pyrrolidine-2,5-dione; GSK-3β, glycogen synthase kinase-3β; h, human; H89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor-1 receptor; KN93, 2-(*N*-[2-hydroxyethyl])-*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylamine; L741,6263-[4-(4-chlorophenyl)-4-hydroxypiperidin-l-yl]methyl-1*H*-indole; LY294,002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopiran-4-one; MAPK, mitogen-activated protein kinase; MEK, MAPK-extracellular signal-regulated kinase; mTORC, rictor-mammalian target of rapamycin complex; PAO, phenylarsine oxide; PD98059, 2′-amino-3′-methoxyflavone; PDGF, platelet-derived growth factor; PDGF-R, platelet-derived growth factor receptor; PDK1, phosphatidylinositol 3,4,5-trisphosphate-dependent protein kinase 1; PI3-K, phosphatidyl inositol-3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PTX, pertussis toxin; Ro-31-7549, 2-[1-3(aminopropyl)indol-3-yl]-3(1-methyl-1*H*-indol-3-yl-maleimide, acetate; S33084, (3*aR*,9*bS*)-*N*[4-(8-cyano-1,3*a*,4,9*b*-tetrahydro-3*H*-benzopyrano[3,4-*c*]pyrrole-2-yl)-butyl] (4-phenyl)benzamide); U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; U73122, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; Wnt, Wingless.

and is expressed in various cerebral regions such as cortical and subcortical structures, hippocampus, cerebellum, and brainstem (Leroy and Brion, 1999). GSK-3β is also a common component of three different signaling pathways: Wnt (Wingless), mitogen-activated protein kinase (MAPK) and phosphatidyl inositol-3-kinase (PI3-K). It has been implicated in processes underlying neuronal survival, development, and plasticity (Peineau et al., 2008) through receptor tyrosine kinase-mediated signaling involving activation of PI3-K/Akt cascade by growth factors. Also known as protein kinase B, the serine/threonine kinase Akt is a downstream target of PI3-K that inhibits GSK-3β kinase activity by phosphorylation at Ser9 (Vanhaesebroeck and Alessi, 2000), leading to a decrease in the phosphorylation of its substrates. Upon receptor tyrosine kinase activation, the generation of phosphatidylinositol 3,4,5-trisphosphate by PI3-K leads to the recruitment of Akt at the plasma membrane and its activation by phosphorylation at residues Thr308 and Ser473 via phosphatidylinositol 3,4,5-trisphosphate-dependent protein kinase 1 (PDK1) (Alessi et al., 1997) and rictor-mammalian target of rapamycin complex 2 (mTORC2), respectively (Sarbassov et al., 2005).

Recent work has suggested interactions between dopaminergic transmission and Akt/GSK-3 signaling (Freyberg et al., 2010), as revealed by alterations in their activity after administration of psychostimulants and dopaminergic agents (Svenningsson et al., 2003; Beaulieu et al., 2004, 2005). Furthermore,

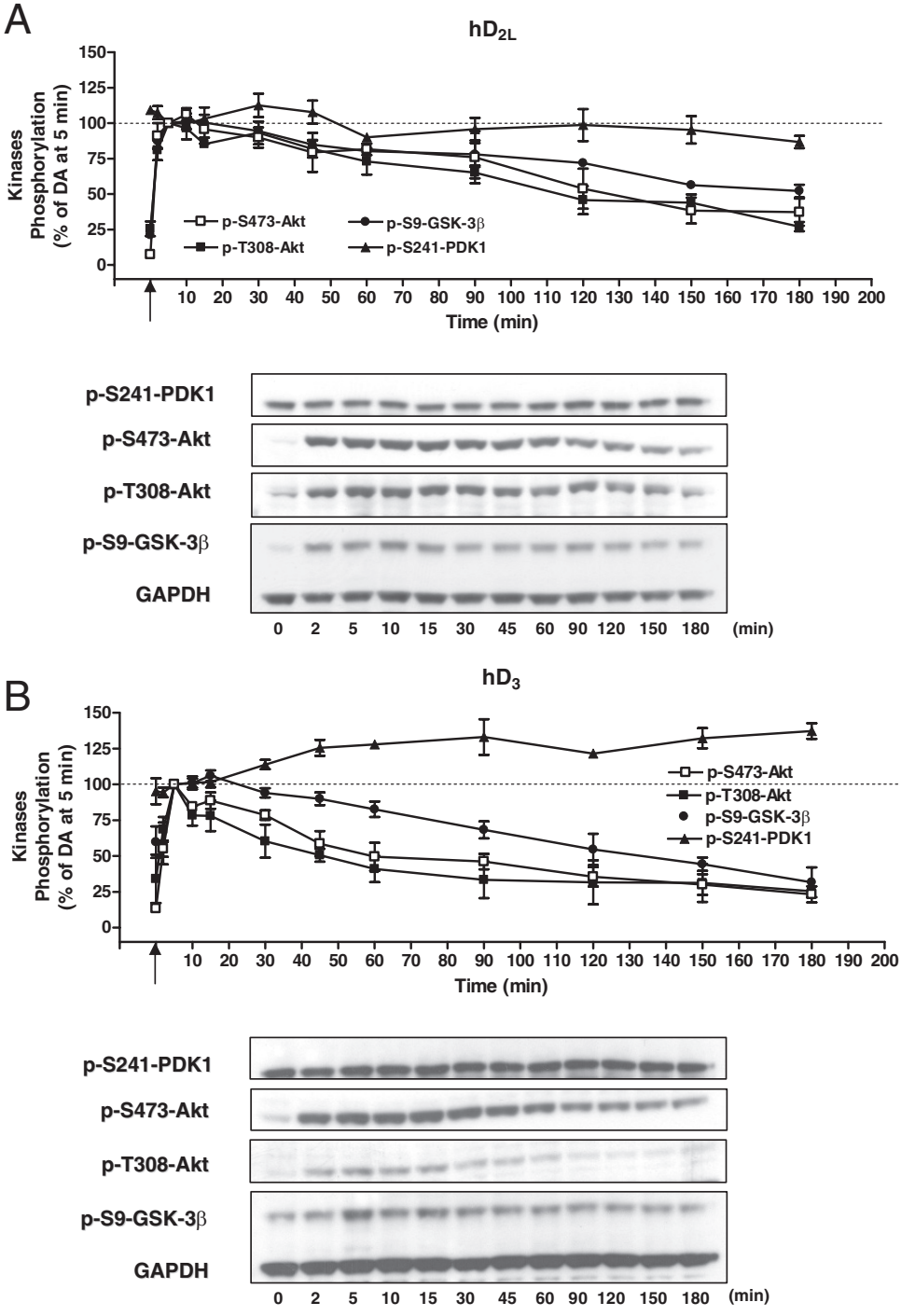

Fig. 1. Kinetics of Akt and GSK-3β phosphorylation after DA application in CHO cells expressing hD_{2L} and hD_3 receptors. Both hD_{2L} (A) and hD_3 (B) receptor-expressing cells were incubated with DA (10^{-5} M) solubilized in the culture medium for increasing stimulation time (2–180 min). At the end of each incubation period, PDK1 (Ser241), Akt (Ser473 and Thr308), and GSK-3β (Ser9) phosphorylation levels were evaluated by immunoblotting. For each graph, data are the means ± S.E.M. of at least four independent experiments. Western blots below each graph correspond to representative experiments where GAPDH was used as a control of well loading. For each time, the kinase phosphorylation levels are expressed as a percentage of the effect of DA at 10^{-5} M after 5 min of incubation.

a significant reduction of DA-dependent locomotor activity was seen upon pharmacological activation of Akt or genetic deletion of GSK-3β (Beaulieu et al., 2004, 2005, 2007). Complementary studies of D_2 and D_3 receptor knockout mice confirmed the role of these sites in the regulation of the Akt/GSK-3β pathway (Beaulieu et al., 2004, 2007). It is noteworthy that a reduction of Akt activity in response to *prolonged* elevations in extracellular levels of DA (genetic deletion of DA transporters) was preferentially mediated by D_2 receptors through formation of an Akt/β-arrestin-2/protein phosphatase 2A signaling complex (Beaulieu et al., 2004, 2005).

Despite these studies, very little is known concerning the *direct* influence of D_2 and D_3 receptors upon Akt/GSK-3β signaling. Although the PI3-K/Akt pathway is known to be activated by native D_2 and D_3 receptors in primary cultures of striatal neurons (Brami-Cherrier et al., 2002), by D_{2L} and D_3 receptors stably expressed in PC12 cells (Nair and Sealfon, 2003; Nair and Olanow, 2008; Chen et al., 2009), and by GFP-tagged D_3 receptors expressed in EM4 cells (Zapata et al., 2007), the intracellular cascades leading to recruitment of Akt remain to be characterized. Furthermore, although activation of D_2 receptors expressed in PC12 and CHO cells (Welsh et al., 1998; Nair and Olanow, 2008) enhanced phosphorylation of GSK-3β and p70S6 kinase, there is otherwise little information about D_2 receptor-recruited pathways that interact with Akt signaling, such as Wnt and β-catenin. Information is even sparser for D_3 receptors, because there is only fragmentary evidence that stimulation leads to the phosphorylation of Akt and GSK-3β (Zapata et al., 2007; Chen et al., 2008). Finally, mechanisms underlying the engagement of Akt/GSK-3β signaling by D_{2L} and D_3 receptors remain to be elucidated.

In light of the above observations, the present study evaluated the roles of various molecular substrates in the DA-induced phosphorylation of Akt and GSK-3β by hD_{2L} compared with hD_3 receptors stably expressed in CHO cells. In addition, complementary experiments were conducted to deter-

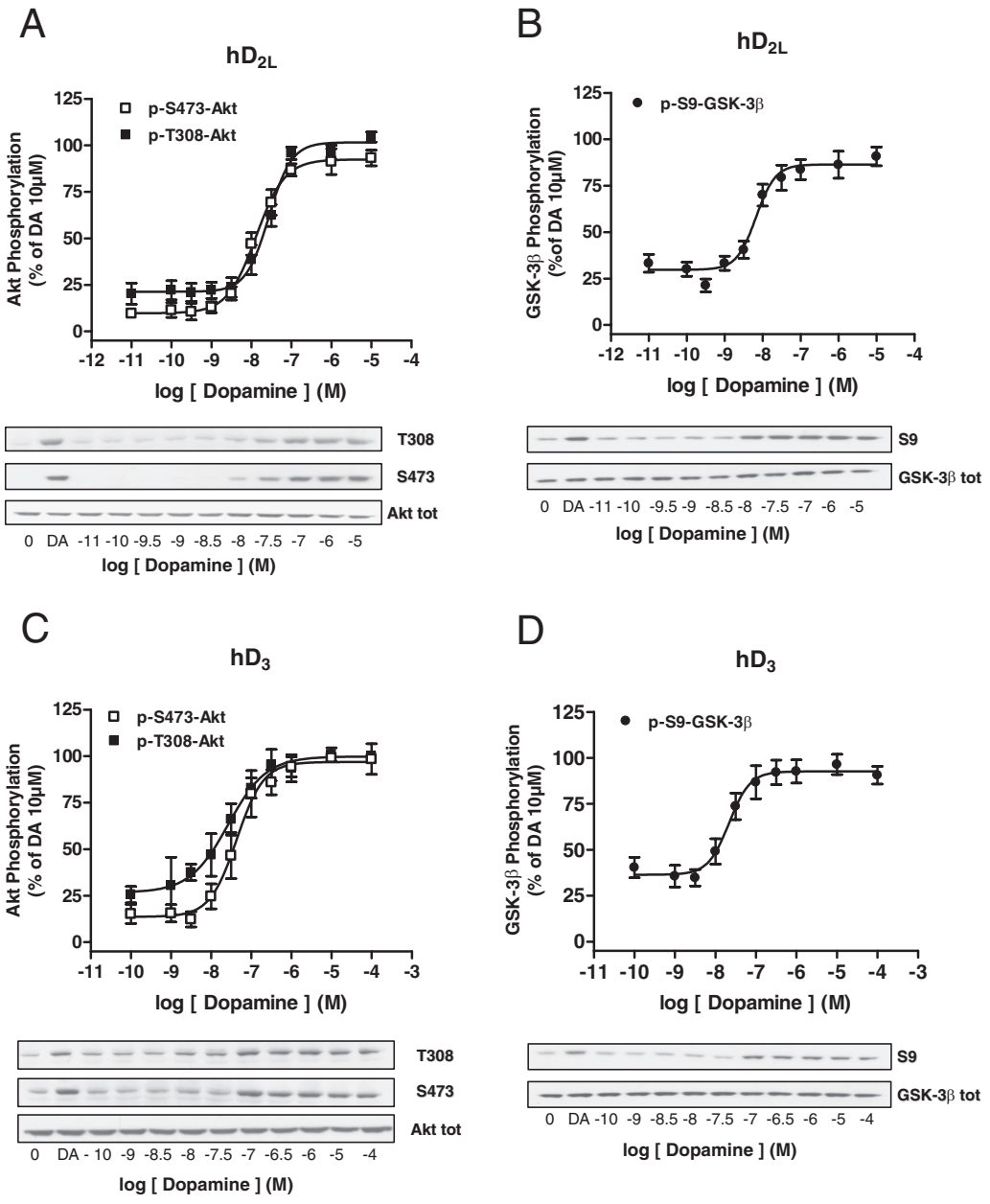

Fig. 2. Concentration-dependent increase of Akt and GSK-3β phosphorylation by DA in CHO cells expressing hD_{2L} and hD_3 receptors. Increase of Akt phosphorylation levels at Ser473 and Thr308 residues induced by hD_{2L} (A) and hD_3 (C) receptor after 5-min incubation with increasing concentration of DA. Concentration-dependent increase of GSK-3β phosphorylation level (at Ser9) after stimulation of hD_{2L} (B) and hD_3 (D) receptors by DA. For each curve, data are the means ± S.E.M. of at least three independent experiments. Western blots below each curve correspond to representative experiments in which GAPDH was used as a control of well loading. Lane 0, no DA; lane DA, 10^{-5} M DA; DA concentration is indicated for all other lanes.

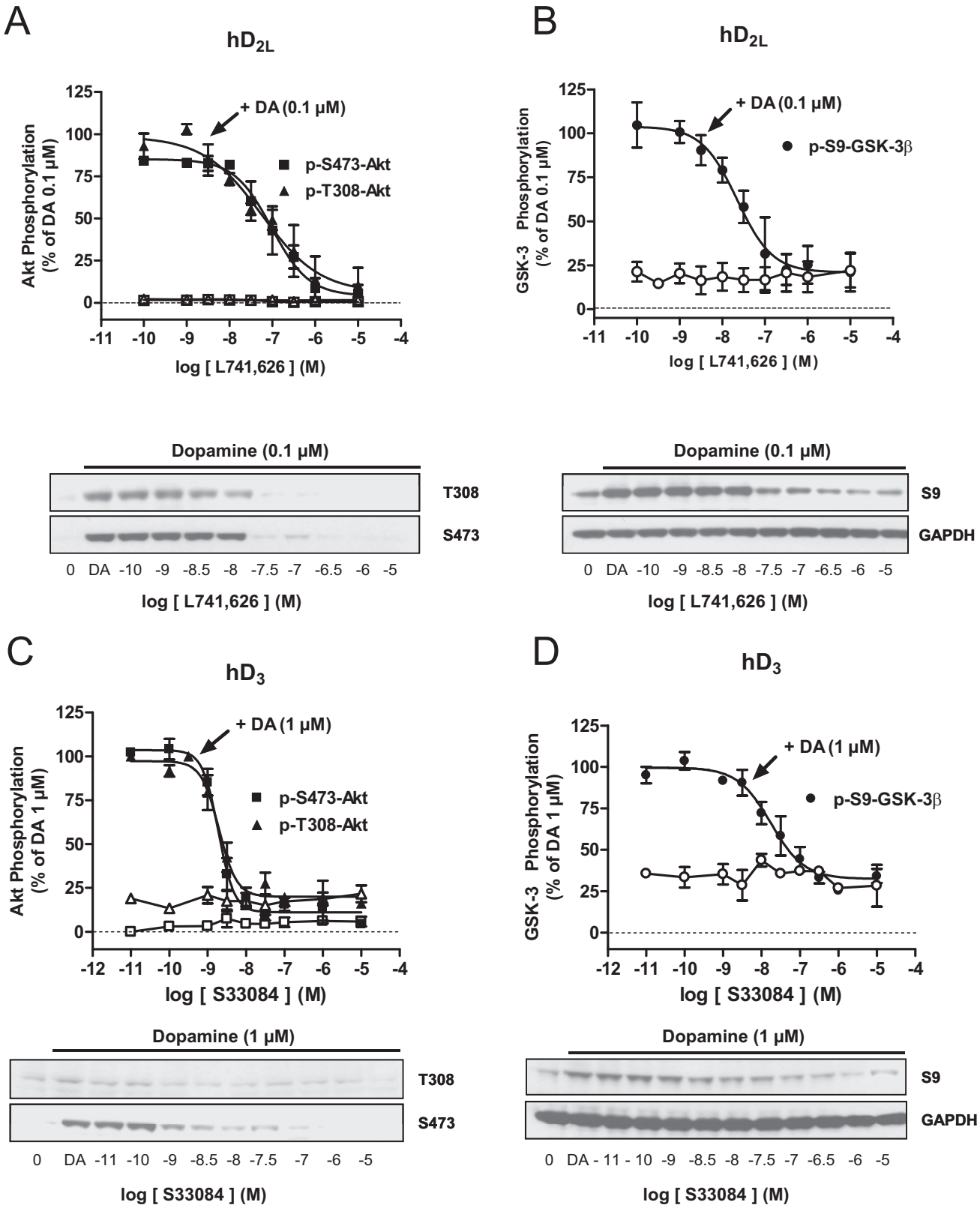

Fig. 3. Concentration-dependent inhibition of DA-induced Akt and GSK-3β phosphorylation in CHO cells expressing hD_{2L} and hD_3 receptors by selective antagonists. Inhibitory effects of increasing concentrations of L741,626, a hD_{2L} receptor antagonist, on DA-mediated (0.1 μM) increase of p-Akt (at Ser473 and Thr308) (A) and p-GSK-3β (B) levels in CHO-hD_{2L} cells. Concentration-dependent inhibition of DA-induced (1 μM) phosphorylation of Akt (C) and GSK-3β (D) in CHO-hD_3 cells. For each curve, data are the means ± S.E.M. of at least three independent experiments. Western blots below each curve correspond to representative experiments where GAPDH was used as a control of well loading. Lane 0, no DA and no antagonist; lane DA, DA (concentration indicated) and no antagonist; DA + antagonist concentration indicated in all other lanes. Closed and open symbols correspond to each of the three kinase phosphorylation levels induced by increasing concentrations of antagonist in the absence and in the presence of DA, respectively.

mine whether native, cerebral populations of D_2/D_3 receptors might also recruit Akt and GSK-3β. To this end, we examined the influence of the highly selective D_2/D_3 receptor agonist quinelorane upon phosphorylated Akt and GSK-3β, both at CHO-expressed D_2 and D_3 receptors. In addition, we evaluated its actions in rats, focusing upon the nucleus accumbens, a region expressing high levels of both D_{2L} and D_3 receptors (Bouthenet et al., 1991).

Materials and Methods

Cell Culture. CHO cells expressing human (h) D_{2L} or D_3 receptors (4.6 and 12 pmol/mg protein, respectively) were grown in Ham's F12 medium or Dulbecco's modified Eagle's medium as described previously (Cussac et al., 1999). For determination of ERK1/2, Akt, and GSK-3β phosphorylation, cells were grown in 12-well plates until they were 90% confluent. The cells were then washed with serum-free medium and starved overnight in this medium. All drugs used were diluted in the serum-free medium at the appropriate final concentration. In antagonist experiments, cells were preincubated for 30 min at 37°C with antagonists before stimulation by dopamine (DA) (10 μM) for 5 min. Kinase inhibitors were also preincubated 30 min before addition of DA for 5 min. The cell viability has been tested and was not affected by pretreatment with any inhibitors used in our experiments. To evaluate the desensitization of the receptors and the role of this desensitization on the recruitment of the Akt/GSK-3β pathway, cells were incubated with DA for 1 h. Inactivation of $G_{s/olf}$ or $G_{i/o}$ proteins was achieved by an overnight treatment of the cells with serum-free medium containing cholera toxin (CTX; 1 ng/ml) or pertussis toxin (PTX; 100 ng/ml). At the end of the incubation period, 250 μl of Laemmli sample buffer containing 200 mM dithiothreitol was added. Whole-cell lysates were then boiled for 3 min at 95°C, sonicated, and stored at −80°C.

In Vivo Experiments. Male Wistar Han rats (226–250 g; Charles River Laboratories Inc., L'arbresle, France) received a single injection of quinelorane (0.16 mg/kg s.c.) 10 min before rapid removal of brains, which were immerged for 15 s in liquid nitrogen then laid over a dry ice slab. Brains were stored at −80°C until use. Mounted on a Peltier refrigerated sliding plate, sections were cut and the nucleus accumbens isolated using a cooled 1.8-mm diameter punch. Frozen samples were sonicated in a warmed Laemmli sample buffer containing SDS (1%) to reach a concentration of ~2 μg/μl of protein. Samples were then boiled for 3 min at 95°C and stored at −80°C. For antagonist experiments, raclopride (0.16 mg/kg s.c.) or vehicle (1 ml/kg s.c.) was administrated 30 min before quinelorane.

Immunoblotting. Cell extracts from CHO cells (10 μl) or homogenates from rat nucleus accumbens (12 μl) were loaded on 15-well 12% polyacrylamide gels (NuPAGE Novex Bis-Tris Gels; Invitrogen, Cergy-Pontoise, France). After migration (200 V, 150 mA) for 75 min, proteins were transferred onto nitrocellulose membranes for 1 h (30 V, 150 mA). At the end of the transfer, membranes were washed with a buffer containing 150 mM NaCl, 500 mM Tris-HCl, pH 7.5, and Tween 20 (1%) and incubated for 1 h in blocking buffer (BLOT-QuickBlocker; Millipore, Saint Quentin-en-Yvelines, France) to saturate nonspecific sites. The phosphorylation of Akt, GSK-3β, and PDK-1 was revealed using rabbit polyclonal antibodies raised against the phosphorylated forms of Akt (on Ser473 and Thr308 residues), GSK-3β (on Ser9 residue), and PDK-1 (on Ser241 residue) (Cell Signaling Technology, Inc. Danvers, MA). Activated MAPKs were detected with a mouse monoclonal antibody specifically recognizing the phosphorylated $pp42^{mapk}$ (ERK2) and $pp44^{mapk}$ (ERK1) forms on both threonine and tyrosine residues (Cell Signaling Technology) as described previously by Cussac et al. (1999). Phosphorylation of Src (on Tyr416 residue) and β-catenin (on Ser33, Ser37, and Thr41) were quantified using specific rabbit polyclonal antibodies (Cell Signaling Technology) as well as total forms of both Src and β-catenin. Nonphosphorylated β-catenin was detected with a mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) specifically recognizing the active form of the protein. After an overnight incubation at 4°C (or 2 h at room temperature) with the primary antibody, nitrocellulose membranes were then washed three times and incubated 1 h with the secondary anti-rabbit (polyclonal) or anti-mouse (monoclonal) antibody coupled to horseradish peroxidase conjugate (GE Healthcare, Les Ulis, France) and submitted to an enhanced chemiluminescence detection using ECL Western Blotting detection reagents (GE Healthcare). The signal was quantified by the measurement of optical density and expressed as a percentage of the maximal effect induced by DA (10 μM). Total immunoreactivity of Akt and GSK-3β was determined with antibodies recognizing both phosphorylated and unphosphorylated forms of Akt and GSK-3β (Cell Signaling Technology). In each experiment, the same immunoblot was probed sequentially for phosphorylated and total form of each kinase by de-blotting of the nitrocellulose membrane using Re-Blott 10× solution (Millipore) and reblotting with another primary antibody. GAPDH was used as a control of the quantity of cell lysate loading in each well and was detected with anti-GAPDH (Cell Signaling Technology). The percentage values reported in bar graphs were obtained by calculating the ratio between optical densities measured for each phosphokinase and those of the loading control (GAPDH) of the corresponding well. Immunoblots shown are from representative experiments repeated at least three times.

Drugs and Chemicals. Dopamine chlorhydrate was purchased from Sigma (Saint Quentin Fallavier, France), 3-[4-(4-chlorophenyl)-4-hydroxypiperidin-l-yl]methyl-1*H*-indole (L741,626) is available from Tocris Bioscience (Bristol, UK) and (3*aR*,9*bS*)-*N*[4-(8-Cyano-1,3*a*,4,9*b*-tetrahydro-3*H*-benzopyrano[3,4-*c*]pyrrole-2-yl)-butyl] (4-phenyl)benzamide (S33084) was synthetized by G. Lavielle (Servier, Paris, France). For experiments using kinase inhibitors, all the following compounds were commercialized by EMD Chemicals, Inc. (Gibbstown, NJ): wortmannin; 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopiran-4-one (LY294,002); 12-(2-cyanoethyl)-6,7,12,13,-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]-carbazol (Gö6976); 3-[1-(3-dimethylamino-propyl)-5-methoxy-1*H*-indol-3-yl]4-(1*H*-indol-3-yl)pyrrolidine-2,5-dione (Gö6983); 2′-amino-3′-methoxyflavone (PD98059); *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89); 3-bromo-5-*t*-butyl-4-hydroxy-benzildenemalonitrile (AG1024); 2-(3,4-dihydroxyphenyl)-6,7-dimethoxyquinoxaline (AG1433); 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478); 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2); 2-[1-3(aminopropyl)indol-3-yl]-3(1-methyl-1*H*-indol-3-yl-maleimide, acetate (Ro-31-7549); 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126); 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester (BAPTA-AM); phorbol 12-myristate 13-acetate (PMA); thapsigargin; 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino) hexyl]-1*H*-pyrrole-2,5-dione (U73122). CTX and PTX were purchased from Sigma (Saint Quentin Fallavier, France). Phenylarsine oxide (PAO) and methyl-β-cyclodextrin are available from Sigma. The selective inhibitor of Akt 10-[4′-(*N*,*N*-diethylamino)butyl]-2-chlorophenoxazine (10-DEBC) is available from Tocris Bioscience.

Results

Dopamine-Induced Phosphorylation of Akt and GSK-3β by Activation of Human D_{2L} and D_3 Receptors in CHO Cells. In CHO cells stably expressing human (h) D_{2L} receptors, DA (10 μM) induced phosphorylation of Akt at both Ser473 and Thr308 residues, with maximal activation observed 5 min after stimulation (Fig. 1A). The level of p-Akt (Ser473 and Thr308) slowly returned to basal by 3 h after exposure to DA (Fig. 1A). A similar profile of activation was observed for GSK-3β, with a maximal level of phosphorylation at Ser9 between 5 and 10 min after exposure of hD_{2L} receptors to DA. In CHO cells expressing hD_3 receptors, the kinetics of Akt and GSK-3β phosphorylation were compara-

ble with those at hD_{2L} receptors, with maximal stimulation 5 (for Akt) and 10 (for GSK-3β) min after application of DA (Fig. 1B). Compared with hD_{2L} receptor-expressing cells, the decrease of phosphorylation was more rapid, especially for p-Ser473-Akt and p-Thr308-Akt, which returned to basal level by 2 h of stimulation. By contrast, short-term treatment with DA exerted no significant influence upon the basal level of PDK1 phosphorylation, which was high in both hD_{2L} and hD_3 cell lines, suggesting a constitutive activation of this kinase (Alessi et al., 1997). In line with these kinetic observations, Akt and GSK-3β phosphorylation was systematically evaluated after 5 min of incubation with DA. Under these experimental conditions, the stimulation of hD_{2L} receptors by DA induced a concentration-dependent increase of p-Akt at both Ser473 and Thr308 residues with pEC_{50} values of 7.87 $\pm$ 0.08 and 7.56 $\pm$ 0.09, respectively (Fig. 2A). A similar increase of phosphorylation was shown after activation of hD_3 receptors by DA, with pEC_{50} values of 7.40 $\pm$ 0.15 and 7.52 $\pm$ 0.19 at Ser473 and Thr308 phosphorylation sites, respectively (Fig. 2C). Five minutes of exposure to increasing concentrations of DA induced a concentration-dependent increase of p-GSK-3β (Ser9) in both hD_{2L} and hD_3 receptor-expressing cells (pEC_{50}, 7.94 $\pm$ 0.13 and 7.56 $\pm$ 0.17, respectively) (Fig. 2, B and D). In both cell lines, DA did not significantly modify levels of total Akt and GSK-3β (Fig. 2). Preincubation of hD_{2L} receptors with the selective D_2 receptor antagonist L741,626 concentration-dependently and fully inhibited the phosphorylation of Akt (Ser473 and Thr308) and GSK-3β by DA (Fig. 3, A and B): pK_B values were 7.95 $\pm$ 0.12 and 7.85 $\pm$ 0.30 for Akt (Ser473 and Thr308, respectively) and 8.95 $\pm$ 0.18 for GSK-3β. Furthermore, the selective D_3 receptor antagonist S33084 abolished the DA-elicited phosphorylation of

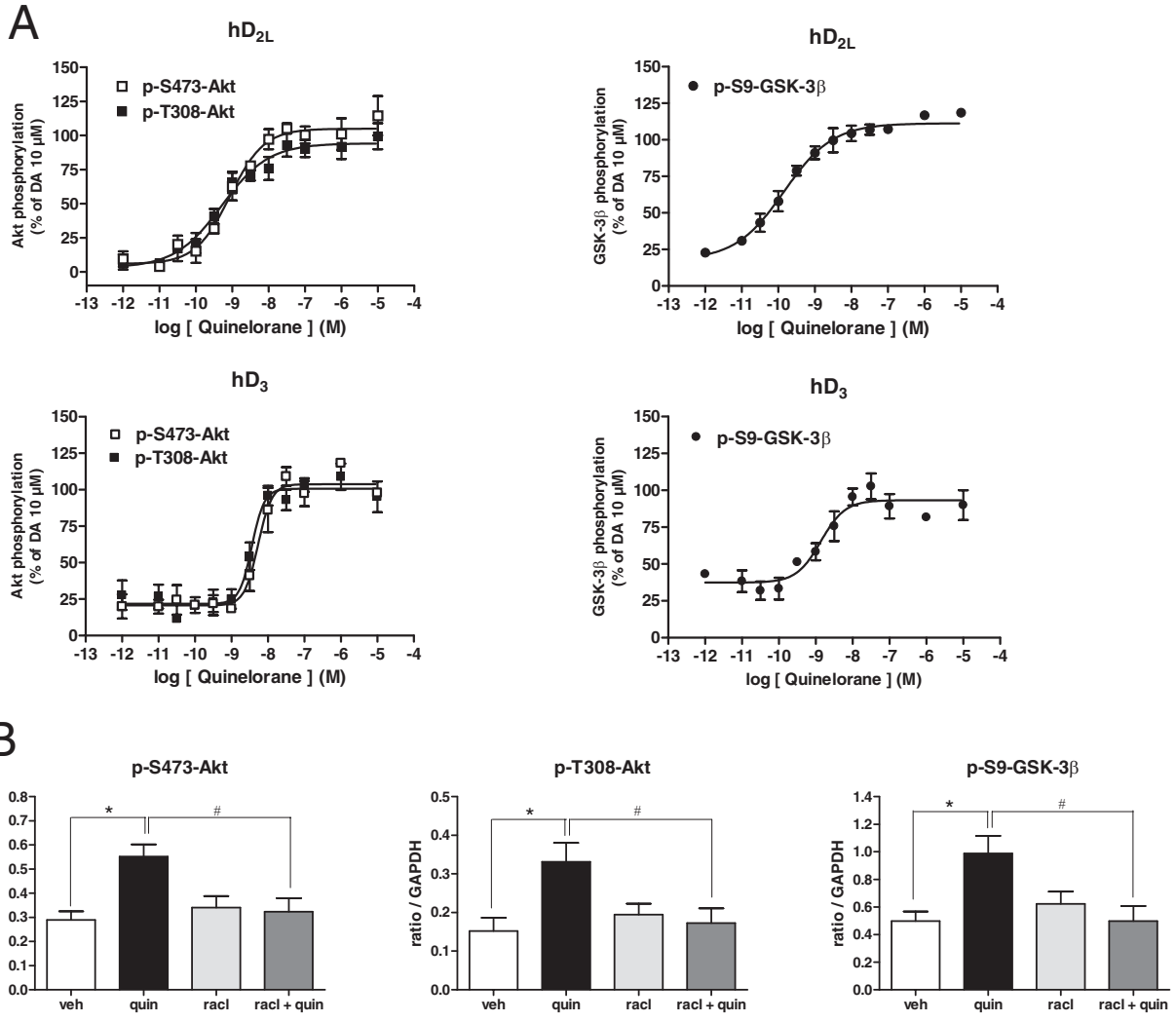

Fig. 4. Quinelorane-induced increase of Akt and GSK-3β phosphorylation in CHO cells expressing hD_{2L} and hD_3 receptors and in the rat nucleus accumbens. A, concentration-dependent increase of Akt (at Ser473 and Thr308) and GSK-3β (at Ser9) phosphorylation levels after stimulation of hD_{2L} and hD_3 receptors by increasing concentration of quinelorane. Phosphorylation levels of Akt and GSK-3β have been evaluated by Western blot where GAPDH was used as a control of well loading. For each curve, data are the mean $\pm$ S.E.M. of four independent experiments. B, increase of p-Akt (at both Ser473 and Thr308) and p-GSK-3β (at Ser9) levels induced by quinelorane (0.16 mg/kg s.c.) 10 min after injection, and the inhibition of these effects by preincubation of the selective D_2/D_3 antagonist, raclopride, (0.16 mg/kg s.c.) for 40 min before quinelorane administration. Kinase phosphorylation levels were measured by Western blotting, and data are expressed as the mean $\pm$ S.E.M. of the ratio calculated between optical densities measured for each phospho-kinase and those of the loading control (GAPDH) of the corresponding well. Histograms represent the compilation of data from three independent experiments. One-way analysis of variance data are as follows: p-473-Akt $F(3,29) = 5.0$, $p < 0.05$; p-Thr308-Akt $F(3,34) = 4.5$, $p < 0.05$; p-Ser9-GSK-3β $F(3,38) = 5.1$, $p < 0.05$. $*$, $p < 0.05$ (significance of quinelorane-treated versus vehicle-treated values); #, $p < 0.05$ (significance of quinelorane-treated versus raclopride + quinelorane-treated values).

Akt and GSK-3β at hD_3 receptors (Fig. 3, C and D): pK_B values for p-Akt on Ser473 and Thr308 residues were 10.21 ± 0.07 and 10.25 ± 0.08, respectively, and 9.16 ± 0.16 for p-GSK-3β. L741,626 and S 33084 were inactive alone (Fig. 3).

Quinelorane Induced Increases of p-Akt and p-GSK-3β Levels in CHO Cells Expressing hD_{2L} and hD_3 Receptors and in Rat Nucleus Accumbens. As seen with DA, the highly selective D_2/D_3 receptor agonist, quinelorane, induced a concentration-dependent increase of p-Ser473-Akt, p-Thr308-Akt and p-Ser9-GSK-3β in hD_{2L} and hD_3 receptor expressing cells after 5 min of incubation (Fig. 4A). As for DA, the potency of the quinelorane was higher at hD_{2L} receptors than at D_3 sites for all kinases (pEC_{50} values = 8.97 ± 0.11, 9.31 ± 0.22, and 9.97 ± 0.26 for p-Ser473-Akt, p-Thr308-Akt, and p-Ser9-GSK-3β, respectively, at hD_{2L}, and pEC_{50} = 8.25 ± 0.12, 8.50 ± 0.06 and 8.74 ± 0.15 p-Ser473-Akt, p-Thr308-Akt, and p-Ser9-GSK-3β, respectively, at hD_3). Systemic injection of quinelorane to rats induced a time-dependent (5–40 min) increase of the phosphorylation of both Akt (Ser473 and Thr308) and GSK-3β in the nucleus accumbens, with a maximal effect 10 min after administration (time course not shown). In line with this kinetic study, the quinelorane-induced increase of p-Ser473-Akt, p-Thr308-Akt, and p-Ser9-GSK-3β levels was confirmed in a second set of experiments that also demonstrated that the selective D_2/D_3 receptor antagonist, raclopride—inactive alone—fully antagonized the actions of the agonist (Fig. 4B).

Involvement of a PTX-Sensitive G-Protein in hD_{2L} and hD_3 Receptor-Mediated Phosphorylation of Akt and GSK-3β. Because hD_{2L} and hD_3 receptors are known to recruit $G_{i/o}$ proteins, the two cell lines were pretreated over-

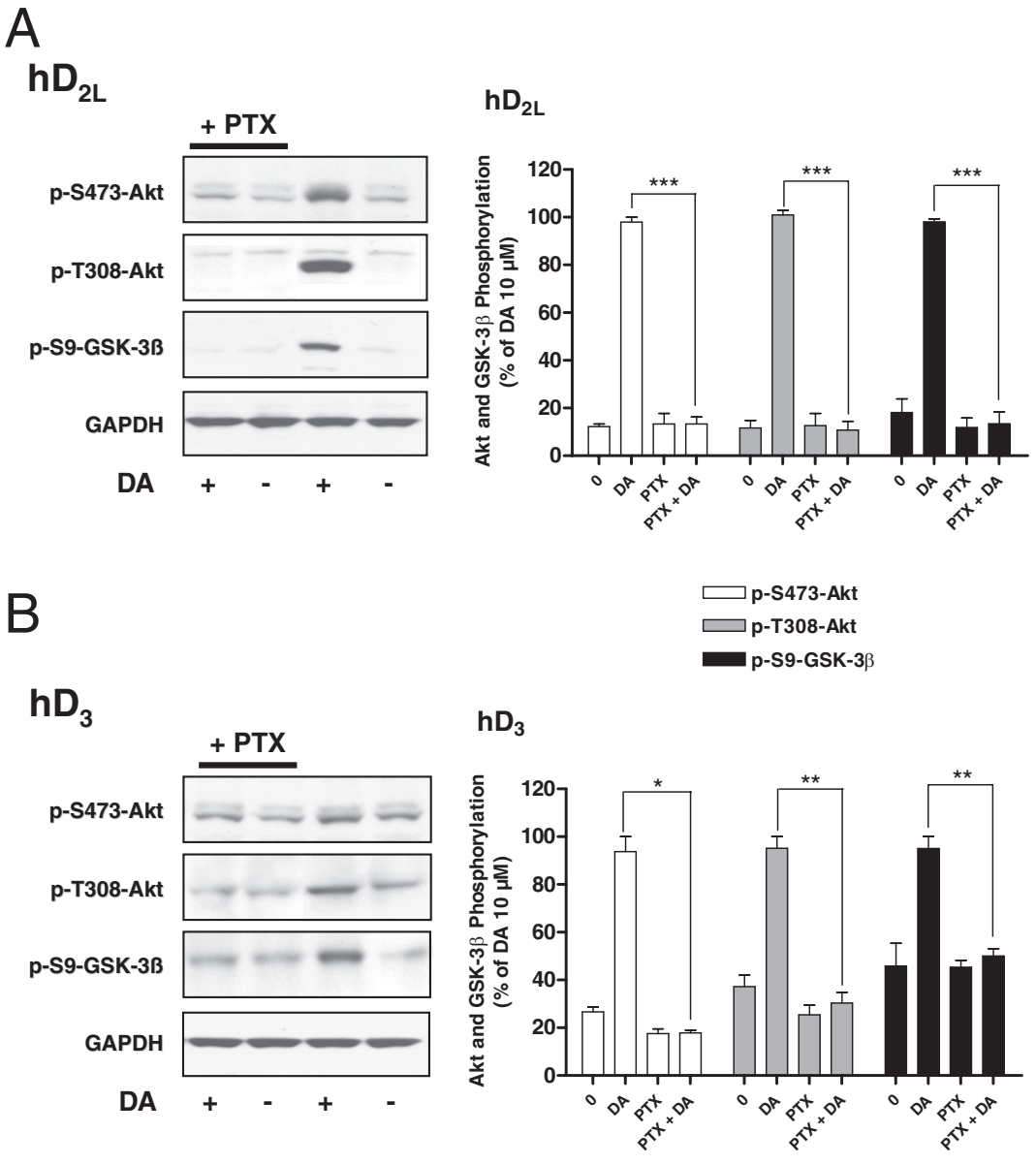

Fig. 5. Identification of G-protein family involved in hD_{2L} and hD_3 receptor-mediated Akt activation in CHO cells. Effects of PTX and CTX pretreatment of CHO cells stably expressing hD_{2L} (A) or hD_3 (B) receptors on DA-induced increase of p-Akt (Ser473 and Thr308) and p-GSK-3β levels. Cells were pretreated overnight with PTX (100 ng/ml) before being incubated 5 min with DA (10^{-5} M). Western blots correspond to representative experiments, each of which have been performed at least three times. Histograms represent the compilation of data provided by at least three independent experiments. The unpaired Student's *t* test was used for the statistical comparison between control (DA) and PTX pretreated cells (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

night with the PTX, which catalyzes the ADP-ribosylation of the Gα subunits of $G_{i/o}$, thereby inhibiting their interaction with G-protein-coupled receptors. As shown in Fig. 5, PTX (100 ng/ml) abolished the increase in levels of p-Akt (Ser473 and Thr308) and p-GSK-3β elicited by DA at both hD_{2L} and hD_3 receptors.

Inhibition of hD_2 and hD_3 Receptor Internalization Does Not Influence the Recruitment of Akt/GSK-3β Pathway by DA. PAO, known to block clathrin-dependent endocytosis, has recently been used to study the regulation of cell-surface expression of D_2 receptors expressed in CHO cells. As shown in Fig. 6, preincubation in CHO cells expressing hD_{2L} and hD_3 receptors with PAO for 30 min significantly attenuated the effects of short-term application of DA upon Akt (Ser473 and Thr308) and GSK-3β phosphorylation levels. The decrease of p-Akt and p-GSK-3β levels has been also observed after prolonged exposure of the cells to methyl-β-cyclodextrin (10 mM), a cell-permeant inhibitor of dynamin that alters the structure of cholesterol-rich domains in the cell membranes and then blocks lipid raft/caveolae-mediated endocytosis as well as invagination of clathrin-coated pits required for G-protein-coupled receptor internalization (not shown).

Role of PI3-K in DA-Induced Akt and GSK-3β Phosphorylation via hD_{2L} and hD_3 Receptors. Generation of phosphatidylinositol bis- and trisphosphate by PI3-K is essential for the recruitment and activation of PDK1 and Akt (Alessi et al., 1997). The selective PI3-K inhibitors wortmannin and LY294,002 (1 and 30 μM, respectively) have been reported to attenuate (but not abolish) the activation of ERK1/2 at hD_{2L} and hD_3 receptors (Welsh et al., 1998; Cussac et al., 1999; Oak et al., 2001). These observations were corroborated herein, with wortmannin partially suppressing p-ERK1/2 activation both at hD_{2L} and at hD_3 receptors (Fig. 7C). At a concentration of 30 μM, LY294,002 abolished the increase of p-Akt (Ser473 and Thr308) and p-GSK-3β induced by DA at both hD_{2L} and hD_3 receptors (Fig. 7, A and B, respectively). As shown in Fig. 7C, wortmannin concentration-dependently decreased DA-induced phosphorylation of Akt and GSK-3β and full inhibition was obtained at concentrations of 1 and 10 μM. In both cell lines, a slight decrease of basal levels of p-Akt (Ser473 and Thr308) and p-GSK-3β was observed in the presence of PI3-K inhibitors alone. Although this tendency to decrease basal levels of phospho-kinases was not statistically significant for LY294,002 (Fig. 7, A and B), differences in basal levels of p-GSK-3β reached significance in both cell lines pretreated by wortmannin (Fig. 7C).

hD_{2L} and hD_3 Receptor-Mediated Akt and GSK-3β Phosphorylation Involves Src Kinase and IGF-1 Receptor Transactivation. In view of the implication of PI3-K in the phosphorylation of Akt and GSK-3β, the role of receptor tyrosine kinases was evaluated and, in particular, the potential involvement of insulin-like growth factor-1 receptors (IGF-1R), which are known to regulate the activity of Akt and GSK-3β. As expected from the endogenous expression of IGF-1R in CHO cells, incubation of cells with IGF-1 (10^{-8} M, 5 min) resulted in an increase of Akt (on Ser473 and Thr308) and GSK-3β (Ser9) phosphorylation levels (data not shown). In addition, pretreatment of cells with the selective inhibitor of IGF-1R tyrosine kinase activity AG1024 (10 μM, 30 min) abolished hD_{2L} and hD_3 receptor-mediated phosphorylation of Akt and GSK-3β (Fig. 8, A and B; Table 1). In contrast, AG1433 (10 μM), a selective inhibitor of platelet-derived growth factor receptors (PDGF-Rs)—which are expressed in CHO cells and participate in the activation of ERK1/2 by D_{2L} receptors (Oak et al., 2001)—did not affect the increase of p-GSK-3β and p-Akt elicited by DA at hD_{2L} or hD_3 receptors (Fig. 8A; Table 1). As expected from the absence of epidermal growth factor receptors (EGF-Rs) in CHO cells, AG1478 (300 nM), an inhibitor of EGF-Rs, was likewise inactive (Fig. 8A; Table 1). The cytosolic tyrosine kinase c-Src is recruited by receptor-tyrosine kinases via

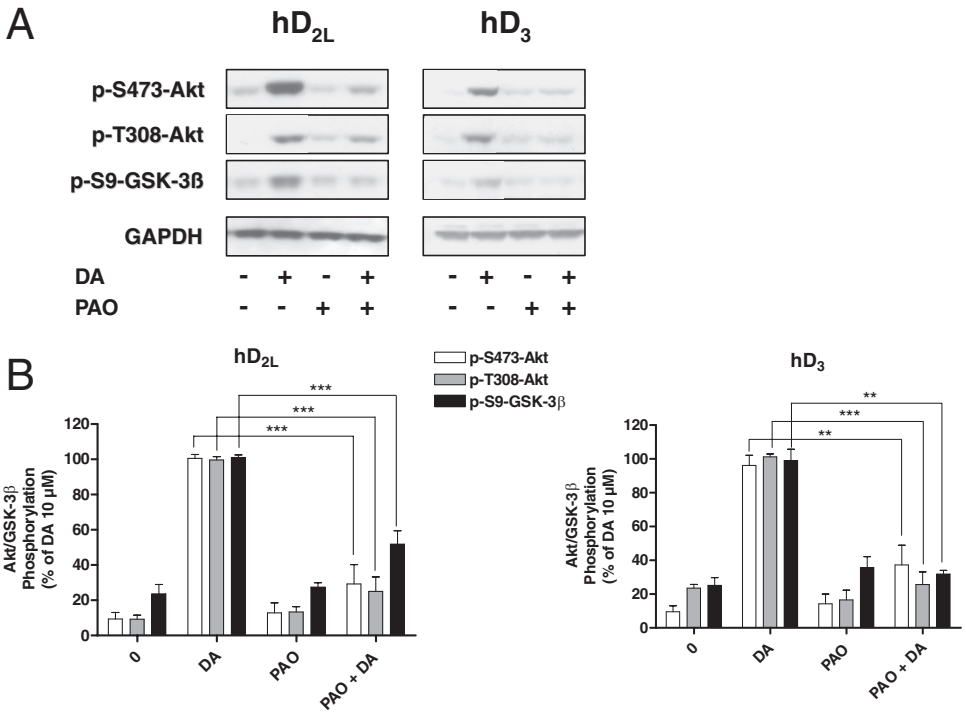

Fig. 6. Effects of the blockade of receptor internalization upon DA-induced phosphorylation of Akt and GSK-3β in hD_{2L}- and hD_3-CHO cells. Each cell line was pretreated for 1 h with PAO (10 μM), an inhibitor of clathrin-dependent endocytosis, before application of DA (10^{-5} M) for 5 min. A, Western blots corresponding to representative experiments performed at least three times. B, histograms representing the compilation of data provided by at least three independent experiments. The unpaired Student's *t* test was used for the statistical comparison between control (DA) and PAO pretreated cells (**, $p < 0.01$; ***, $p < 0.001$).

an interaction with the proline-rich tyrosine kinase 2. Preincubation with the Src inhibitor PP2 (10 μM) did not modify basal levels of p-Akt and p-GSK-3β (Fig. 8, A and B) but eliminated the DA-induced increase of p-Akt (Ser473 and Thr308 residues) and p-GSK-3β levels at both hD_{2L} and hD_3 receptors (Fig. 8, A and B; Table 1). Consistent with this finding, DA significantly increased levels of p-Thr416-Src by 19 and 43% at hD_{2L} and hD_3 receptors, respectively (Fig. 8C).

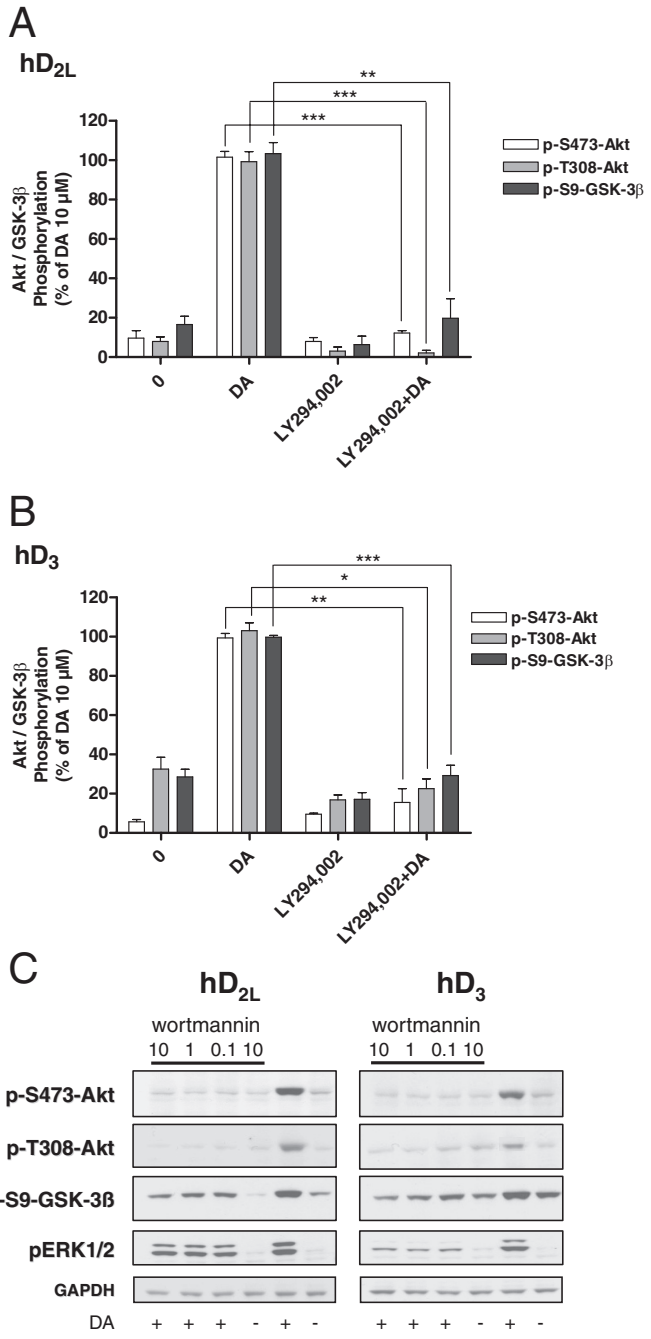

Fig. 7. Influence of PI3-K inhibitors on DA-induced increase of p-Akt and p-GSK-3β levels in CHO cells expressing hD_{2L} and hD_3 receptors. CHO cells stably expressing hD_{2L} (A) or hD_3 (B) were pretreated for 30 min with the PI3-K inhibitor LY294,002 (30 μM) and then incubated with DA (10^{-5} M) for 5 min. Histograms correspond to the compilation of data provided by at least three independent experiments. C, both cell lines were preincubated with increasing concentrations of wortmannin (0.1, 1, and 10 μM) before application of DA. Western blots are representative of three independent experiments. The unpaired Student's *t* test was used for the statistical comparison between control (DA) and LY294,002 pretreated cells (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Recruitment of Akt and GSK-3β Is Independent of PKA but Requires Mobilization of Protein Kinase C by hD_{2L} and hD_3 receptors. Preincubation with the PKA inhibitor, H89 (1 μM) exerted no effect upon hD_{2L} and hD_3 receptor-mediated (or basal) phosphorylation of Akt and GSK-3β (Fig. 9A, Table 1). However, Gö6983 (1 μM), which inhibits all isoforms of protein kinase C (PKC) [including PKC_γ, PKC_δ, and PKC_ξ but not PKC_μ (Gschwendt et al., 1996)], strongly attenuated the influence of DA on Akt and GSK-3β phosphorylation levels in CHO cells expressing hD_3 receptors (Fig. 9B; Table 1). By contrast, Gö6983 did not modify hD_{2L} receptor-mediated phosphorylation of Akt (Ser473 and Thr308) and GSK-3β (Fig. 9B; Table 1). Gö6976 (5 μM), an inhibitor of conventional Ca^{2+}-dependent isoforms of PKC (PKC_α, PKC_β, and PKCγ) (Martiny-Baron et al., 1993), as well as PKC_μ, reduced DA-induced phosphorylation of Akt and GSK-3β in both cell lines (Fig. 9B; Table 1). Ro-31-7549, which specifically inhibits PKC_α at 100 nM (Wilkinson et al., 1993), did not significantly alter DA-induced increase of p-Akt and p-GSK-3β levels at hD_{2L} and hD_3 receptors (Fig. 9B; Table 1). However, used at a higher concentration (1 μM), which also inhibits PKCβI, βII and ε, Ro-31-7549 (Wilkinson et al., 1993) abolished D_3 receptor-mediated phosphorylation of Akt and GSK-3β (not shown) whereas it was still ineffective at hD_{2L} receptors. Finally, depletion of classic—but not atypical—PKCs by overnight pretreatment with PMA (1 μM) suppressed (desensitized) the phosphorylation of Akt and GSK-3β by PMA (30 min) but did not modify hD_{2L} and hD_3 receptor-mediated increase of p-Akt and p-GSK-3β levels (Fig. 9C).

DA-Induced Akt and GSK-3β Phosphorylation is Ca^{2+}/Calmodulin Dependent but CaMKII-Independent. Concentration-dependent depletion of intracellular Ca^{2+} by BAPTA-AM (5–100 μM) decreased the generation of p-Akt (Ser473 and Thr308) and p-GSK-3β by hD_{2L} and hD_3 receptor activation (Fig. 10A; Table 1). Furthermore, preincubation with the calmodulin inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7; 30 μM) likewise decreased the actions of DA at hD_{2L} receptors by approximately 50%, whereas its effect was more pronounced at D_3 receptors leading to complete extinction of the signal (Fig. 10B). However, despite this dependence on intracellular Ca^{2+} and calmodulin, the Ca^{2+}-calmodulin kinase (CaMKII) inhibitor 2-(*N*-[2-hydroxyethyl])-*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylamine (KN93; 1 and 20 μM) exerted no significant effect upon DA-induced phosphorylation of Akt and GSK-3β at hD_{2L} or hD_3 receptors (Fig. 10C, Table 1). Finally, the PLC inhibitor U73122 (10 μM) suppressed recruitment of Akt/GSK-3β signaling by DA in CHO cells expressing hD_3 receptors but did not modulate hD_{2L} receptor-mediated Akt and GSK-3β phosphorylation (Fig. 10D, Table 1).

The hD_{2L} and hD_3 Receptor-Mediated Increases of p-GSK-3β Are Partially and Fully Akt-Dependent, Respectively. Inhibition of Akt by incubation with the specific inhibitor 10-DEBC (10 μM, 1 h) before application of DA concentration-dependently decreased phosphorylation of GSK-3β by DA at both hD_{2L} and hD_3 receptors (Fig. 11A). However, although the action of DA was abolished by 10 μM 10-DEBC at the latter, it was only submaximally

inhibited at hD_{2L} sites (~50%) (Fig. 11A). In addition, certain studies suggest a role for ERK1/2 in the recruitment of Akt by D_2 receptors (Brami-Cherrier et al., 2002). Herein, preincubation with the selective MEK (MAPK-extracellular signal-regulated kinase) inhibitors PD98059 (30 μM) or U0126 (10 μM) did not influence D_3 receptor-mediated phosphorylation of Akt (not shown) or GSK-3β (Fig. 11B) despite abolition of DA-induced phosphorylation of ERK1/2 (not shown). However, these two inhibitors strongly attenuated the increase of both p-Akt (not shown) and p-GSK-3β (Fig. 11B) levels elicited by DA in CHO cells expressing hD_{2L} receptors. Hence, GSK-3β phosphorylation was entirely Akt-dependent for hD_3 receptors where both Akt and ERK1/2 were involved in the phosphorylation of GSK-3β by hD_{2L} receptors.

***β*-Catenin And the Canonical Wnt Pathway Are Not Affected in hD_2 and hD_3-Mediated Phosphorylation of Akt and GSK-3*β*.** As a major component of the canonical Wnt pathway, GSK-3β is involved in the control of phosphorylation and degradation of β-catenin through the activation of Frizzled receptors by Wnt ligands. Inactivation of GSK-3β by D_2 and D_3 receptor activation may then reduce the level of phosphorylated β-catenin and increase the accumulation of its active (nonphosphorylated) form. Preincubation of hD_{2L} and hD_3 receptor-expressing CHO cells with conditioned medium containing Wnt (5 h) increased active-β-catenin compared with nontreated CHO cells. No significant change was observed in levels of β-catenin phosphorylated on its Ser33 and Thr41 residues. Incubation with DA did not significantly modify levels of phospho- and active-β-catenin in either hD_{2L} or hD_3 receptor-expressing CHO cells (Fig. 12).

Discussion

Transient Activation of hD_{2L} and hD_3 Receptors Elicits Phosphorylation of Akt (Ser473 and Thr308) and GSK-3*β* (Ser9). Short-term exposure of CHO cells stably expressing hD_{2L} and hD_3 receptors to DA increased Akt phosphorylation (at Ser473 and Thr308) and enhanced GSK-3β phosphorylation. These effects reflected direct stimulation of hD_2 and hD_3 receptors, because the actions of DA

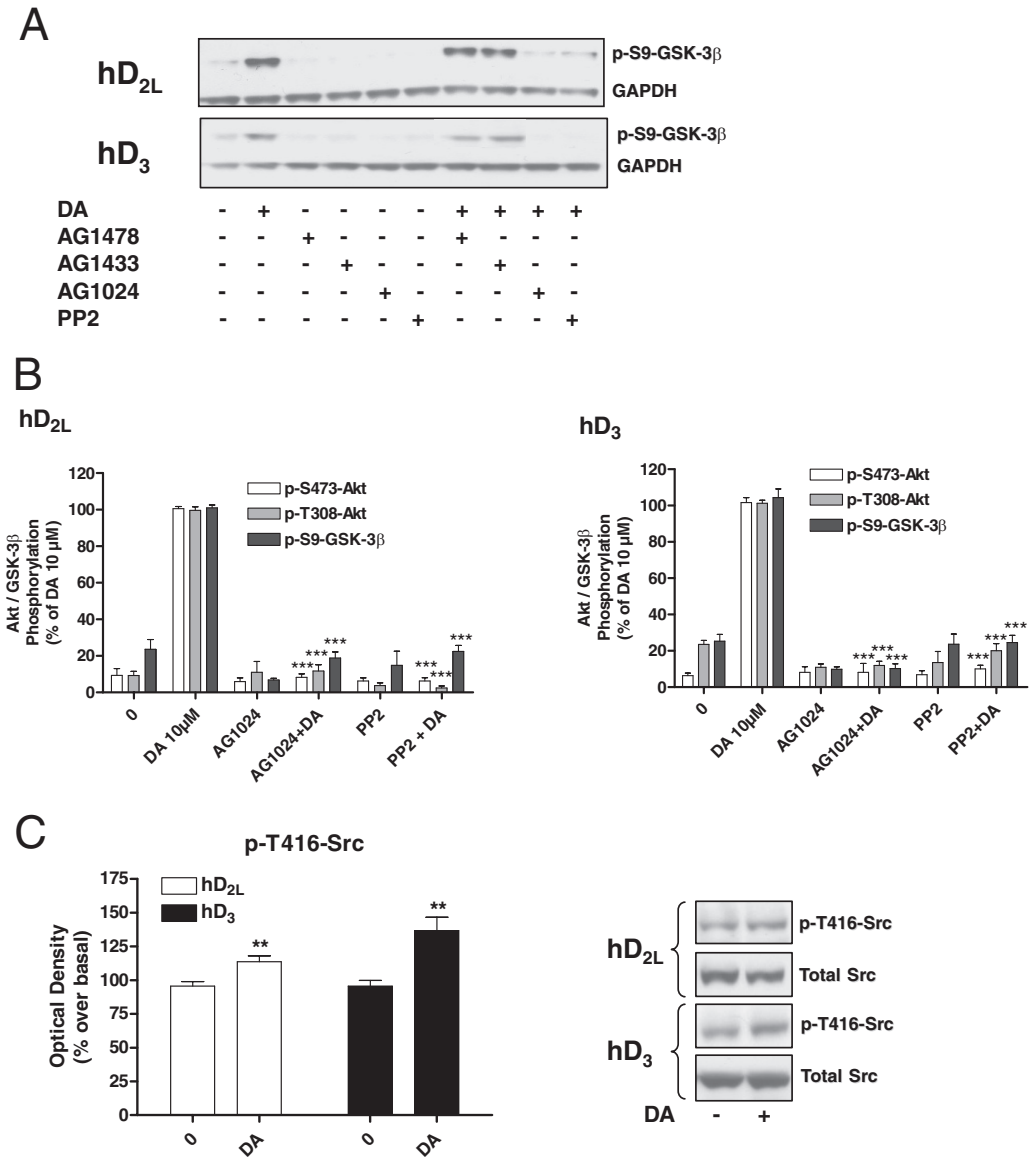

Fig. 8. Role of IGF-1 receptors and Src in hD_{2L} and hD_3 receptor-mediated Akt and GSK-3β phosphorylation in CHO cells. A, both cell lines were pretreated with inhibitors of PDGF, EGF, and IGF-1 receptors (AG1433, AG1478, and AG1024, respectively) and with the Src inhibitor PP2 before being exposed to DA (10^{-5} M) for 5 min. Western blots correspond to representative experiments in which GAPDH was used as a control of well loading. B, effects of PP2 and AG1024 inhibitors upon DA-induced phosphorylation of both Akt and GSK-3β. C, activation of Src was evaluated by quantification of total and phospho-Src (p-T416-Src) in cell lysates of both cell lines. Data of histograms shown in B are the means ± S.E.M. of at least three independent experiments. For each phosphokinase, data obtained in the presence of inhibitor were compared with their respective control (***, $p < 0.001$, significantly different from cells treated by DA alone, unpaired Student's t test). Western blots correspond to representative experiments, each of which was performed at least three times.

TABLE 1

Effects of inhibitors upon DA-induced increase of p-Akt (Ser473), p-Akt (Thr308), and p-GSK-3β (Ser9) levels in CHO cells expressing hD_{2L} and hD_3 receptors.

Data are expressed as a percentage of Akt (Ser473 and Thr308) and GSK-3β phosphorylation induced by DA at 10^{-5} M in the absence of inhibitors. Values are means ± S.E.M. of at least three independent determinations.

	EGF-R AG1478 (0.3 μM)	PDGF-R AG1433 (10 μM)	IGF-R AG1024 (10 μM)	Src PP2 (10 μM)	PKA H89 (1 μM)	PKC			Ca^{2+} Chelator BAPTA-AM (100 μM)	PLC U73122 (10 μM)	CaMKII KN93 (20 μM)
						Gö6976 (5 μM)	Gö6983 (1 μM)	Ro-31-7549 (0.1 μM)			
						%					
hD_{2L}											
p-Akt (Ser473)	94.6 ± 4.8	91.4 ± 7.9	−1.0 ± 0.8	−3.8 ± 3.1	99.3 ± 2.7	15.1 ± 2.1	78.8 ± 3.6	93.7 ± 6.5	3.8 ± 1.5	81.8 ± 9.7	76.2 ± 4.4
p-Akt (Thr308)	107.8 ± 5.5	100.2 ± 1.1	−0.7 ± 0.4	−12.7 ± 1.5	102.1 ± 3.2	15.1 ± 1.9	87.5 ± 2.4	94.7 ± 4.1	8.4 ± 3.2	89.5 ± 1.0	80.8 ± 6.9
p-GSK-3b	103.2 ± 3.8	80.3 ± 8.4	−6.3 ± 6.1	1.6 ± 1.7	97.3 ± 13.1	15.1 ± 3.5	83.5 ± 8.2	91.1 ± 10.8	6.7 ± 2.3	92.7 ± 10.9	68.0 ± 7.3
hD_3											
p-Akt (Ser473)	90.2 ± 10.1	80.6 ± 14.9	−1.9 ± 0.5	1.7 ± 0.4	94.4 ± 10.4	13.1 ± 5.2	14.2 ± 5.2	103.3 ± 5.9	2.6 ± 0.2	5.9 ± 2.4	76.2 ± 1.8
p-Akt (Thr308)	84.9 ± 7.8	75.7 ± 12.5	−15.4 ± 3.0	4.6 ± 1.2	109.2 + 5.1	16.4 ± 7.6	8.5 ± 4.9	93.9 ± 3.1	1.3 ± 1.1	2.4 ± 2.1	82.3 ± 4.1
p-GSK-3b	77.7 ± 9.1	86.6 ± 15.0	−16.8 ± 3.4	1.8 ± 0.6	89.4 + 10.6	15.1 ± 6.4	10.4 ± 7.5	101.7 ± 3.0	6.5 ± 2.3	−5.5 ± 1.1	70.7 ± 6.0

were abolished by L741,626, a preferential D_2 receptor antagonist, and S33084, a selective D_3 receptor antagonist, respectively. The phosphorylation of Akt at both Ser473 *and* Thr308 is of note because the D_2/D_3 agonist ropinirole induced Akt phosphorylation solely at Ser473 in D_2 receptor-transfected PC12 cells (Nair and Sealfon, 2003; Nair and Olanow, 2008). Contrariwise, in striatal neurons, whereas IGF phosphorylated both Ser473 *and* Thr308, quinpirole elevated levels of p-Akt at Thr308 only (Brami-Cherrier et al., 2002). It is unclear how selective phosphorylation of Ser473 *or* Thr308 can occur. One explanation may be that activation of D_2 receptors in striatal neurons leads to PDK1-mediated phosphorylation of Akt at Thr308 (Alessi et al., 1997), whereas actions at D_2 receptors in PC12 cells principally involves mTORC2 that phosphorylates Akt at Ser473 (Sarbassov et al., 2005). In the CHO cells studied herein, both PDK-1 and mTORC2 are presumably mobilized by hD_{2L} and hD_3 receptors to enhance phosphorylation of Akt at Ser473 and Thr308, an interpretation consistent with key roles for IGF1 receptors and PI3-kinase (see *Results*). As for DA, the

Fig. 9. Influence of PKA and PKC inhibition upon DA-induced phosphorylation of Akt and GSK-3β in CHO cells expressing hD_{2L} and hD_3 receptors. A, cells expressing hD_{2L} and hD_3 receptors were treated with H89 (1 μM) before addition of DA (10^{-5} M). B, hD_{2L} and hD_3-CHO cells were preincubated with PKC inhibitors, each of them acting specifically on different PKC subtypes: Gö6976 (5 μM), Gö6983 (5 μM), and Ro31.7549 (0.1 μM). C, effect of PMA-induced PKC depletion on hD_{2L} and hD_3 receptor-mediated GSK-3β phosphorylation. CHO cells were treated with PMA for 30 min or overnight before incubation with DA (10^{-5} M). Western blots are representative of three independent experiments.

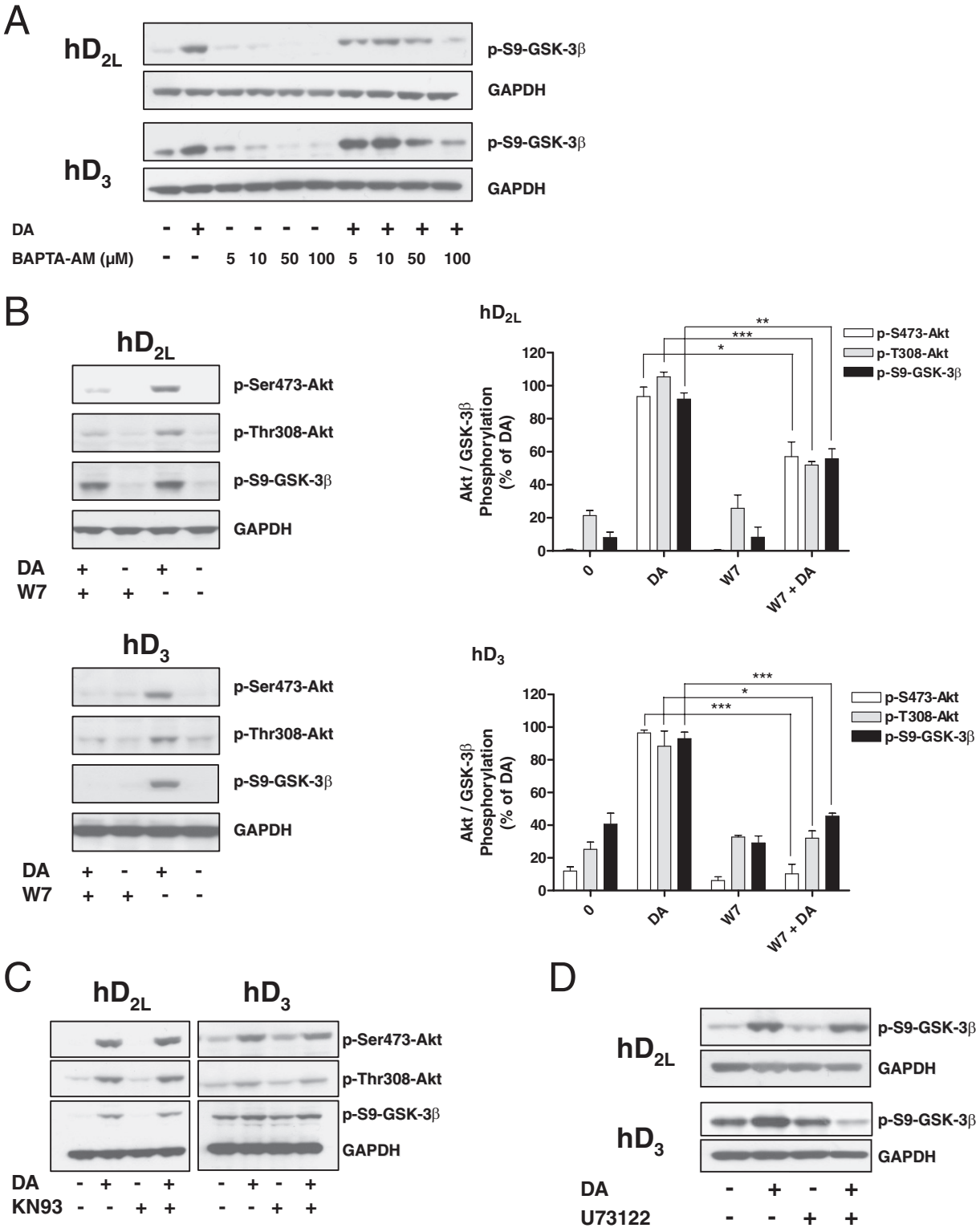

Fig. 10. Role of intracellular calcium and effect of PLC inhibition on hD_{2L} and hD_3 receptor-mediated phosphorylation of Akt and GSK-3β in CHO cells. A, reduction of intracellular calcium concentration by pretreatment of CHO cells with increasing concentration of BAPTA-AM (5–100 μM) for 30 min before stimulation by DA (10^{-5} M). B, effect of calmodulin inhibition on hD_{2L} and hD_3 receptor-mediated Akt and GSK-3β phosphorylation by preincubation of cells with W7 (30 μM) 30 min before application of DA. Histograms correspond to the compilation of data provided by at least three independent experiments. The unpaired Student's t test was used for the statistical comparison between control and pretreated cells (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). C, influence of the CaMKII inhibitor KN93 (20 μM) on DA-mediated phosphorylation of Akt and GSK-3β in hD_{2L} and hD_3 receptors expressing cells. D, consequences of PLC inactivation by U73122 (10 μM) on DA-induced increase of p-GSK-3β level. Western blots correspond to representative experiments performed at least three times.

D_2/D_3 receptor agonist quinelorane elicited a significant increase of p-Ser473-Akt, p-Thr308-Akt, and p-S9-GSK-3β in CHO cells.

By analogy, a rapid increase of phosphorylation was observed in the rat nucleus accumbens, a region expressing both D_2 and D_3 receptors (Bouthenet et al., 1991), 10 min after injection of quinelorane. These results corroborate observations of Svenningsson et al. (2003), who reported an increase of p-GSK-3β in rat frontal cortex and striatum 15 min after D-amphetamine. In addition, in the present study, the actions of quinelorane were antagonized by the D_2/D_3 receptor antagonist raclopride, confirming the implication of D_2 and/or D_3 receptors and underpinning the relevance of findings obtained in cell lines to events in the central nervous system. Further studies with selective D_3 versus D_2 antagonists (and in knockout mice) will be necessary to determine the respective roles of D_3 and D_2 sites. It is noteworthy that the quinelorane-induced increase of p-Akt and p-GSK-3β returned to basal levels by 40 min, providing a possible explanation of the contrasting observations of Beaulieu et al. (2004) who reported a *decrease* of Akt and GSK-3β phosphorylation after amphetamine and apomorphine 30 to 90 min after administration. These differential findings strongly suggest that mechanisms involved in the short versus long-term influence of DA upon the Akt/GSK-3β cascade are likely to differ, a complex and interesting issue justifying further study.

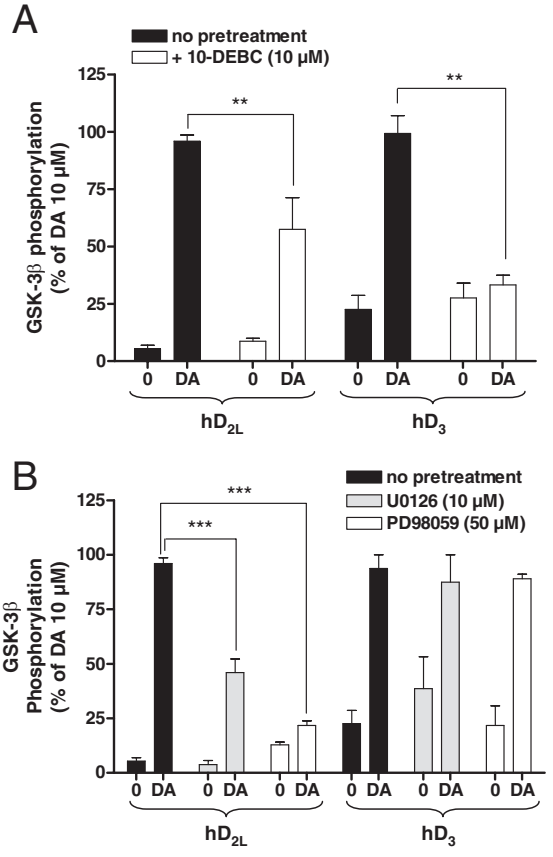

Fig. 11. Inhibitory effects of the inhibition of Akt kinase activity on DA-induced increase of p-GSK-3β in CHO-hD_{2L} and CHO-hD_3 cells. A, cells were treated for 1 h with the Akt inhibitor 10-DEBC before application of DA (10^{-5} M). 10-DEBC (10 μM) induced a partial and a full inhibition of D_{2L} and D_3 receptor-mediated increase of GSK-3β phosphorylation, respectively. B, effects of preincubation of both cell lines with two MEK inhibitors, PD98059 (30 μM) and U0126 (10 μM), upon p-GSK-3β levels. Histograms represent the means $\pm$ S.E.M. of three independent experiments performed in duplicate. The unpaired Student's t test was used for the statistical comparison between control and pretreated cells (**, $p < 0.01$; ***, $p < 0.001$).

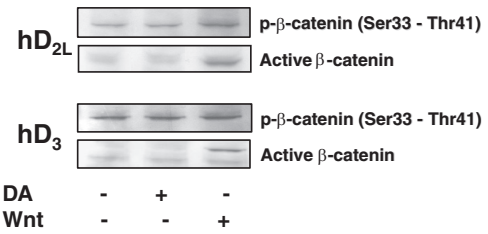

Fig. 12. The recruitment of Akt/GSK-3β signaling by hD_{2L} and hD_3 receptors has no influence on β-catenin stability. In CHO cell lines, β-catenin phosphorylation levels (on Ser33 and Thr41) have been evaluated in basal conditions and after exposure of CHO cells expressing hD_{2L} and hD_3 receptors to DA (10^{-5} M) for 5 min. The stimulation of endogenous Frizzled receptors by preincubation of CHO cells with serum containing the Wnt ligand for 5 h was used as a positive control. Western blots are representative of three independent experiments.

Akt and GSK-3β Phosphorylation is G-Protein-Dependent and Involves IGF-1 Receptor Transactivation, PI3-K, and Src. DA-induced phosphorylation of Akt and GSK-3β at hD_{2L} and hD_3 receptors was abolished by PTX, suggesting a crucial role for $G_{i/o}$ proteins, as reported for induction of MAP-kinase (Cussac et al., 1999; Oak et al., 2001; Wang et al., 2005). In PC12 cells, the PTX-insensitive activation of Akt by bromocriptine suggested a $G_{i/o}$ protein-independent mechanism requiring EGF-R transactivation via Src and PI3-K (Nair and Sealfon, 2003). In the present study, the blockade of DA-induced phosphorylation of Akt and GSK-3β by selective inhibitors of PI3-K and Src and the increased phosphorylation of Src at Tyr416 after exposure to DA supported a common role for PI3-K and Src as reported in PC12 cells expressing D_2 receptors (Nair and Sealfon, 2003) but not in striatal neurons (Brami-Cherrier et al., 2002). The implication of PI3-K in the recruitment of Akt/GSK-3β parallels its role in the stimulation of MAP-Kinase after activation of D_2 and D_3 receptors. However, contrasting with the *partial* inhibitory effects of wortmannin and LY294002 upon ERK1/2 phosphorylation (Welsh et al., 1998; Cussac et al., 1999; Oak et al., 2001; Kim et al., 2004), these PI3-K inhibitors abolished hD_{2L} and hD_3 receptor-mediated Akt/GSK-3β signaling.

The actions of DA were blocked by AG1024, a selective inhibitor of IGF-1R, whereas AG1478 or AG1433 were inactive, showing that neither EGF-R nor PDGF-R was involved in these effects. A role of IGF-1R is unsurprising in view of the participation of PI3-K in insulin/IGF-1 signaling, which is known to regulate Akt and GSK-3β activity both in vitro (Welsh et al., 1994) and in native tissue (Bondy and Cheng, 2004). Furthermore, the implication of Src and PI3-K in signaling is consistent with transactivation of IGF-1R by G$\beta\gamma$, as for D_2 and D_3 receptor-mediated MAP-kinase activation through EGF-R and PDGF-R (Oak et al., 2001; Beom et al., 2004; Kim et al., 2004; Wang et al., 2005).

The Role of Diverse PKC Isoforms, PLC, and ERK1/2: Differences between D_2 and D_3 Receptors. At hD_{2L} receptors, the effects of DA upon Akt and GSK-3β phosphorylation were blocked by the PKCμ inhibitor Gö6976 yet unaffected by the unselective inhibitor of all PKC isoforms, Gö6983, which is inactive at PKCμ (Martiny-Baron et al., 1993). A role for PKCμ, which has been related to activation of the PI3-K/Akt pathway via IGF-1 (Qiang et al., 2004), is then consistent with the involvement of an IGF-1R/PI3-K

cascade in hD_{2L} receptor-mediated Akt and GSK-3β phosphorylation. However, the insensitivity to overnight treatment with PMA suggests that an "atypical" PKC may also be implicated, the exact nature of which remains to be clarified.

By contrast, DA-induced phosphorylation of Akt and GSK-3β at hD_3 receptors was abolished by both Gö6976 and Gö6983, suggesting roles for both conventional and novel PKC isoforms. Nonetheless, the inhibition of other PKC subtypes by Gö6983 could mask a potential implication of PKCμ. The inactivity of Ro-31-7549, which specifically inhibits PKC_α at low concentration, discounts a role for this isoenzyme but the decrease of D_3 receptor-mediated recruitment of Akt and GSK-3β observed at higher concentrations suggests a role for conventional PKCs (PKCβI/βII, PKCε) (Wilkinson et al., 1993). In addition, the insensitivity of D_3 receptor-mediated increase of p-Akt and p-GSK3β to PMA supports the idea that Akt/GSK-3β signaling at hD_3 receptors may then involve an atypical PKC such as PKCζ directly activated by PI3-K and mirroring the role of PKCζ in the hD_3 receptor-mediated phosphorylation of ERK1/2 (Cussac et al., 1999).

The PLC inhibitor U73122 abolished the hD_3 receptor-mediated increase of p-Akt and p-GSK-3β, probably through the inactivation of PLCγ recruited by IGF-1R/PI3-K (Bae et al., 1998) secondarily inhibiting the recruitment of PKCα/βI/βII/γ (but not PKCε) (Goode et al., 1992). However, alternative routes to PLC activation could also involve $G_{i/o}\beta\gamma$ (Marinissen and Gutkind, 2001) or $G\alpha_q$ (Newman-Tancredi et al., 1999). Concerning hD_{2L} receptors, the independence of Akt/GSK-3β signaling from PLC implies recruitment of PKC via a contrasting pathway possibly involving direct activation through Gαo (van Biesen et al., 1996), or an IGF-1 receptor-activated Ca^{2+}-permeable channel sensitive to chelation of extracellular Ca^{2+} (Kanzaki et al., 1997). PKCμ can also be activated through a mechanism involving "novel" PKCs isoforms and Src (Wang, 2006).

The Akt inhibitor 10-DEBC reduced and abolished the hD_{2L} and hD_3 receptor-mediated phosphorylation of GSK-3β, respectively, suggesting that this effect was entirely Akt-dependent at hD_3 sites. On the other hand, two MEK inhibitors attenuated the DA-induced increase of p-GSK-3β in hD_{2L} cells, whereas they were inactive at hD_3 receptors. These results are consistent with the role of the ras/ERK1/2 signaling cascade in D_2 receptor-mediated Akt (Thr308) phosphorylation in striatal neurons (Brami-Cherrier et al., 2002), and suggest that ERK1/2 is involved in the DA-induced phosphorylation of GSK-3β at hD_{2L} but not hD_3 receptors. How ERK1/2 leads to generation of p-GSK-3β remains to be clarified, but one possibility is via activation of p90 ribosomal S6 kinase (Carriere et al., 2008).

The Ca^{2+}/Calmodulin Dependence of hD_{2L} and hD_3 Receptor-Mediated Phosphorylation of Akt and GSK-3β. The implication of PKCs and the decrease of basal and DA-induced Akt/GSK-3β phosphorylation after Ca^{2+} chelation by BAPTA-AM supports the idea that Ca^{2+} may influence this signaling. However, release of Ca^{2+} from intracellular stores did not significantly potentiate the increase of p-Akt and p-GSK-3β levels elicited by DA, suggesting that resting levels of Ca^{2+} gate the recruitment of the Akt/GSK-3β in both the absence and the presence of DA. Accordingly, the calmodulin inhibitor W7 fully and partially inhibited phosphorylation of Akt/GSK-3β mediated by hD_3 and hD_{2L} receptors, respectively. This dependence on basal levels of Ca^{2+} and calmodulin corroborates studies showing that activation of PI3-K/Akt signaling by growth and neurotrophic factors is regulated by free intracellular Ca^{2+} and calmodulin (Cheng et al., 2003; Dong et al., 2007; Zheng et al., 2008). The coimmunoprecipitation of Akt with calmodulin in an EGF-dependent manner confirms that calmodulin plays a crucial role in the regulation of Akt, possibly through control of its localization at the plasma membrane (Deb et al., 2004). Although blockade of CaMKII exerted no effect upon hD_{2L} and hD_3 receptor-mediated Akt and GSK-3β phosphorylation, contrasting with the inhibitory effects of CaMKII itself upon D_3 receptor signaling reported by Liu et al. (2009), it should be noted that we did not specifically investigate a possible potentiation of the effects of a submaximal concentration of DA by interference with the activity of CaMKII.

Influence of DA-Induced Inhibition of GSK-3β Activity upon β-Catenin. Because inactivation of GSK-3β after the binding of Wnt ligands to Frizzled receptors prevents the phosphorylation and degradation of β-catenin, the increase of GSK-3β phosphorylation mediated by hD_{2L} and hD_3 receptors in CHO cells could in theory decrease phospho-β-catenin levels. However, no alteration in levels of either p-β-catenin or nonphosphorylated β-catenin was observed, suggesting that the pool of GSK-3β regulated by hD_{2L} and hD_3 receptors may be different, in time and/or in space, from that recruited by the Wnt signaling cascade as reported by Ding et al. (2000). This lack of cross-talk between PI3-K and Wnt pathways is consistent with the compartmentalization of GSK-3β upon association with Axin and with the inability of PI3-K and Akt to modulate transcriptional activity of the Wnt cascade in HEK293T cells (Ng et al., 2009).

Perspectives. In the present study, inhibitors of clathrin-mediated endocytosis partially decreased both hD_{2L} and hD_3 receptor-mediated phosphorylation of Akt and GSK-3β. Thus, short-term regulation of Akt/GSK-3β signaling by hD_{2L} or hD_3 receptors is partially dependent on the formation of clathrin-coated pits. A potential role for β-arrestins in the DA-induced phosphorylation of Akt and GSK-3β should not be excluded inasmuch as the influence of dopaminergic agents upon Akt signaling was blunted in mice genetically deprived of β-arrestin 2 (Beaulieu et al., 2005). Herein, the *slow* decrease of p-Akt and p-GSK-3β levels observed in CHO-hD_{2L} and -hD_3 cells after prolonged exposure to DA is consistent with a β-arrestin-dependent phenomenon (Ahn et al., 2004), but further investigations are needed to evaluate a potential role for β-arrestins in this signaling pathway.

Finally, the D_{2L} (long) isoform of D_2 receptors is located mainly postsynaptically to dopaminergic neurons and fulfills a major role in the influence of DA upon motor behavior and mood. In view of subtle differences in the coupling and intracellular cycling of D_{2L} receptors versus their D_{2S} (short isoform) counterparts (Kim et al., 2004; De Mei et al., 2009), it would be interesting to extend the present studies to the latter, which, as inhibitory autoreceptors on dopaminergic perikarya, regulate their activity and, possibly, integrity (Joyce and Millan, 2007; De Mei et al., 2009).

Conclusions

Activation of CHO-expressed hD_{2L} and hD_3 receptors by DA increased phosphorylation of Akt and GSK-3β without affecting the canonical Wnt pathway. These short-term and transient effects of DA were $G_{i/o}$- and Ca^{2+}/calmodulin-dependent but required transactivation of IGF-1R through PI3-K and Src. None-

theless, despite similar cellular backgrounds, there were some interesting differences between cascades recruited by hD_{2L} versus hD_3 receptors, notably regarding roles of ERK1/2 and PLC, respectively. Extension of such comparative investigations to other cellular signals involved in the control of mood and neuronal integrity, such as mTOR/p70S6-kinase and p38/Jun N-terminal kinases, would be of considerable interest.

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Authorship Contributions

Participated in research design: Mannoury la Cour and Salles.
Conducted experiments: Salles and Pasteau.
Performed data analysis: Mannoury la Cour, Salles, and Pasteau.
Wrote or contributed to the writing of the manuscript: Mannoury la Cour and Millan.

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