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Protein kinase C regulates dopamine D4 receptor-mediated phospholipid methylation

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Abstract

Dopamine D4 receptors (D4 receptors) mediate dopamine-stimulated, folate-dependent phospholipid methylation. To investigate possible regulation of this multi-step D4 receptor-mediated phospholipid methylation cycle by protein kinases, specific kinase activators and inhibitors were studied in SK-N-MC human neuroblastoma cells, using [^{14}C] formate to label folate-derived single-carbon groups. Phorbol dibutyrate (PDB), an activator of protein kinase C, stimulated basal phospholipid methylation and also shifted the dose–response curve for dopamine-stimulated phospholipid methylation to the right by more than an order of magnitude. Calphostin C, an inhibitor of protein kinase C, had little effect on basal phospholipid methylation but significantly inhibited dopamine-stimulated phospholipid methylation and also blocked the stimulatory response to PDB. Chelerythrine, which inhibits protein kinase C and other kinases, strongly inhibited both basal and dopamine-stimulated phospholipid methylation. Forskolin, an activator of protein kinase A, inhibited basal and dopamine-stimulated phospholipid methylation, but only at high concentrations while Rp-cAMP, an inhibitor of protein kinase A, did not block this effect. Inhibition of protein kinase G produced a modest decrease in dopamine-stimulated phospholipid methylation, but neither sodium nitroprusside, which increases nitric oxide (NO) production and activates protein kinase G, nor the NO synthase inhibitor *N*-nitro-L-arginine had any effect on basal or dopamine-stimulated phospholipid methylation. These observations indicate that protein kinase C is an important regulator of basal and D4 receptor-mediated folate-dependent phospholipid methylation, whereas protein kinase A and protein kinase G have a lesser or minimal role.

Keywords: Dopamine; Dopamine D4 receptor; Protein kinase C; Phospholipid methylation; Folate metabolism; Chelerythrine; Calphostin C

1. Introduction

Membrane proteins, such as receptors, ion channels and transporters undergo conformational changes as an integral part of their functional cycles. Recent evidence indicates that rotational movement of transmembrane helices is a common feature of such conformational changes (Farrens et al., 1996; Perozo et al., 1999). These proteins are surrounded by a lipid environment and their function might therefore be expected to be sensitive to changes in the membrane environment such as changes in the packing density of phospholipid head groups. The potent effects of ethanol and general anesthetics (Lovinger et al., 1990; Mihic et al., 1997) as well as increased phospholipid methylation (Di Perri et al., 1983) on ligand-gated ion channels provide an example of this influence. Phospholipid methylation can reduce membrane-packing density by increasing the distance between phospholipid headgroups, permitting greater movement of fatty acid tails and increasing membrane fluidity (Casal and Mantsch 1983). Activation of phospholipid methylation can therefore exert important modulatory effects on the signaling activity of membrane proteins.

Phospholipid methylation can be carried out by a phospholipid methyltransferase utilizing *S*-adenosylmethionine as a methyl donating co-factor (Hirata and Axelrod, 1980) or by a recently described dopamine-dependent pathway involving the D4 receptor (Sharma et al., 1999). In the latter pathway, a methionine residue (Met^{313} in the D4.2 receptor) in close proximity to phospholipid head groups, appears to utilize several enzymes of the classical *S*-adenosylmethionine-dependent pathway, as outlined in Fig. 1. Upon a dopamine-induced conformational change, Met^{313} serves as a substrate for methionine adenosyltransferase, converting it to a *S*-adenosylmethionine-like side-

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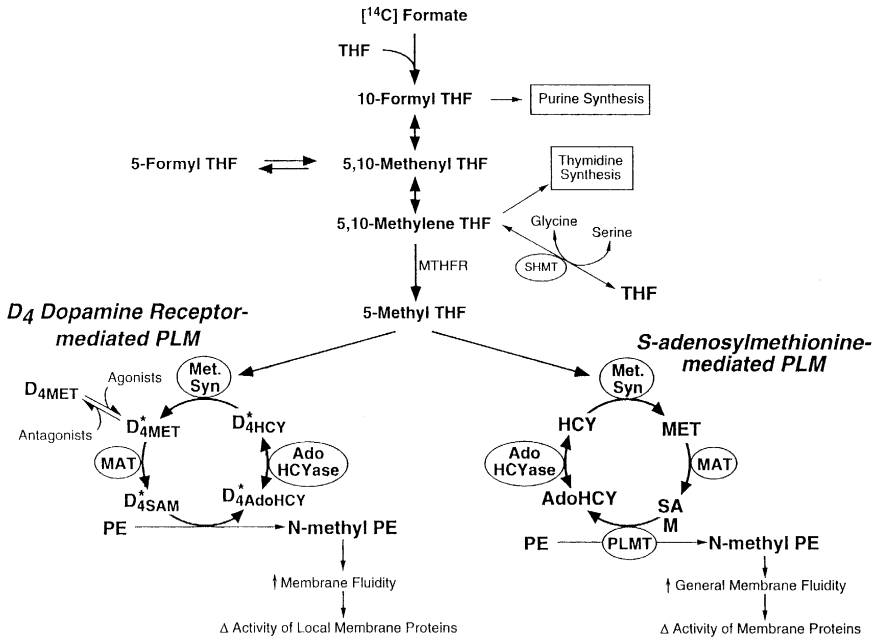

Fig. 1. Basal and D4 receptor-mediated pathways of phospholipid methylation illustrating the conversion of [^{14}C]formate to 5-methyltetrahydrofolate and its utilization by methionine synthase for remethylation of homocysteine to methionine.

chain ($D_{4\ SAM}$), facilitating subsequent transfer of its methyl group to the phospholipid phosphatidylethanolamine (PE). After methyl donation, the demethylated but *S*-adenosylated D4 receptor ($D_{4\ AdoHCY}$) is converted to its homocysteine form ($D_{4\ HCY}$) by *S*-adenosylhomocysteine hydrolase. $D_{4\ HCY}$ is converted back to the native methionine form ($D_{4\ MET}$) by methionine synthase, which utilizes 5-methyl tetrahydrofolate as a cofactor. As shown in Fig. 1, both the classical cycle of methionine metabolism and the D4 receptor utilize 5-methyltetrahydrofolate, which can be efficiently labeled via addition of [^{14}C]formate. For the purpose of these studies, phospholipid methylation mediated by the classical pathway is considered to be basal phospholipid methylation, while that stimulated by dopamine is considered to be D4 receptor-mediated phospholipid methylation, since it is completely and selectively blocked by D4 receptor-selective antagonists in SK-N-MC human neuroblastoma cells (Sharma et al., 1999).

Some of the enzymes involved in the methionine cycle and phospholipid methylation have previously been reported to be regulated by protein kinase activity. For example, hepatic methionine adenosyltransferase activity is increased upon protein kinase C activation (Pajares et al., 1992, 1994). Phospholipid methylation activity has been reported to increase following activation of either protein kinase C (Kelly, 1987; Villalba et al., 1987) or protein kinase A (Kelly and Wong, 1987). Another report has suggested that methionine synthase is regulated by protein kinase A (Kenyon et al., 1995). The current study was therefore initiated to determine whether protein kinase regulation plays a role in modulating phospholipid methylation, especially the pathway that is dependent upon 5-methyltetrahydrofolate. Using a [^{14}C]formate labeling approach, our findings provide evidence of a critical role of protein kinase C in regulating both basal and D4 receptor-mediated phospholipid methylation, but little role for protein kinase A or protein kinase G.

2. Materials and methods

2.1. Materials

Fetal bovine serum, penicillin streptomycin, Hams F-10 nutrient mixture, and Hank's balanced salt solution (HBSS) were purchased from Life Technologies (Grand Island, NY). Fungizone was obtained from Mediatech (Herndon, VA). [^{14}C] Formic acid (specific activity 50 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Drugs such as dopamine, PDB, 4-α PDB, calphostin C, chelerythrine, forskolin, Rp-cAMP, (8*R**, 9*S**, 11*S**)-(−)-9-methoxy-9-methoxycarbonyl-2,8-dimethyl-3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one (KT5823), sodium nitroprusside, *N*-nitro-L-arginine were purchased from commercial suppliers. All drugs were dissolved in distilled water except calphostin C and forskolin, which were dissolved in dimethylsulfoxide.

2.2. Cell culture

SK-N-MC cells, from which the D4 receptor was first cloned (VanTol et al., 1991) were cultured in F-10 medium with glutamine containing 12% fetal bovine serum and 1%

of penicillin, streptomycin and fungizone. Cells were cultured in 100-mm^2 plates containing 10 ml of the culture medium and were maintained in a controlled humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were then plated into six-well plates at a density of 200,000 cells per well in 2 ml of culture medium, 24 h prior to the experiment.

2.3. Formate labeling assay

In order to study 5-methyltetrahydrofolate-dependent basal and D4 receptor-mediated phospholipid methylation, a radiolabeling technique was employed using [^{14}C] sodium formate (Fig. 1). Confluent cells in six-well plates were incubated with 1 μCi/ml of [^{14}C]formate in HBSS with or without kinase stimulators or inhibitors for 30 min. When kinase inhibitors were studied, a half an hour preincubation with inhibitors in non-labeled HBSS preceded the study in order to abolish ongoing kinase activity. To study the D4 receptor component, dopamine was added in the presence of the radiolabel. The ongoing reaction was stopped by the addition of 10% trichloroacetic acid. Cells were scraped and the contents transferred to micro-centrifuge tubes. After homogenization to attain a uniform dispersion, 100-μl samples were removed for estimation of protein. The remaining contents of the micro-centrifuge tubes were centrifuged at 10 K RPM for 15 min in a benchtop micro-centrifuge. The supernatant was removed and the phospholipids were extracted from the pellet by 1.5 ml of a 6:3:1 mixture of chloroform, methanol and 2 *N* hydrochloric acid after which the samples were vortexed and supernatant removed. The chloroform layer was washed twice with 400 μl of 0.1 M KCl in 50% methanol. Aliquots of chloroform were placed in scintillation vials and evaporated to dryness. Scintillation fluid was added and the samples counted for radioactive content, normalized to protein values by expressing the radiolabeled phospholipids as cpm/μg protein.

2.4. Data analysis and statistics

Data was expressed as mean cpm/μg protein ± S.E.M. of at least five observations. Statistical analyses were conducted by using Student's *t*-test and differences with $P < 0.05$ were considered to be significant.

3. Results

3.1. Effect of protein kinase C stimulation on basal and dopamine-stimulated phospholipid methylation

PDB, a phorbol ester known to activate protein kinase C, stimulated basal phospholipid methylation at a concentration of 1 μM, while the inactive form, 4-α PDB, was ineffective at the same concentration (Fig. 2A). Calphostin C, an inhibitor of protein kinase C, abolished the stimulatory effect of PDB at a concentration of 1 μM (Fig. 2A). These results indicate that PDB stimulates basal phospholipid methylation specifically via activation of protein kinase C.

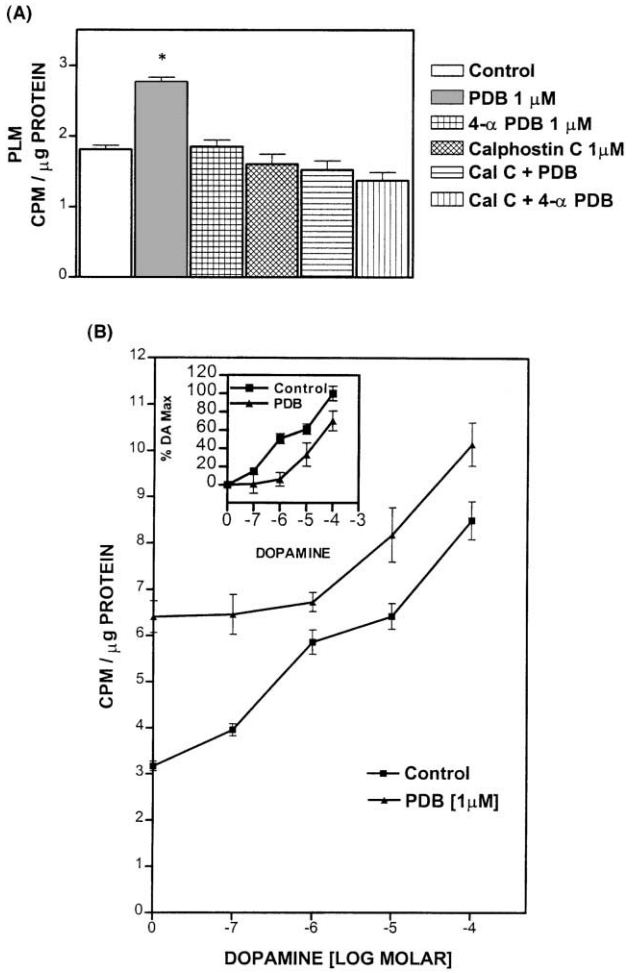

Fig. 2. (A) Protein kinase C activation increases basal phospholipid methylation. SK-N-MC cells were treated with PDB or 4α-PDB (1 μM) in the presence or absence of calphostin C during a 30-min incubation with [^{14}C]formate. * Indicates $P < 0.01$ as compared to control group. (B) Protein kinase C activation inhibits dopamine-stimulated phospholipid methylation. SK-N-MC cells were incubated with or without PDB (1 μM) in the presence of increasing concentrations of dopamine (0.1–100 μM). Inset: Data expressed as % maximal dopamine-stimulated phospholipid methylation.

Dopamine caused a dose-dependent increase of phospholipid methylation with an EC_{50} of 1–2 μM (Fig. 2B). However, as previously observed in SH-SY5Y neuroblastoma cells (Zhao et al., 2001), the control dose–response relationship for dopamine-stimulated phospholipid methylation showed evidence of two components, with a broad range and little change between 1 and 10 μM. At a concentration of 1 μM, PDB not only elevated basal phospholipid methylation, but also shifted the dose–response curve for dopamine-stimulated phospholipid methylation to the right by nearly 20-fold (Fig. 2B). A replot of

the data as a percentage of control dopamine-stimulated phospholipid methylation (Fig. 2B, inset) suggests that protein kinase C activation results in a selective loss of the more sensitive component of D4 receptor-mediated phospholipid methylation.

3.2. Effect of protein kinase C inhibitors on basal and dopamine-stimulated phospholipid methylation

In order to further clarify the role of protein kinase C, protein kinase C inhibitors were examined for their effect on basal and dopamine-stimulated phospholipid methylation. Calphostin C, which acts on the regulatory site of protein kinase C, had no effect on basal phospholipid methylation at concentrations of 0.1 and 1 μM (Fig. 3A). However, the stimulatory effect of dopamine was significantly inhibited by calphostin C at 0.1 μM and completely inhibited at 1 μM (Fig. 3A).

Chelerythrine, a putative protein kinase C inhibitor acting at the catalytic site, had no effect on basal or dopamine-stimulated phospholipid methylation at a concentration of 1 μM (Fig. 3B). Although initially reported to inhibit protein kinase C with an IC_{50} of 0.7 μM, (Herbert et al., 1990), chelerythrine was subsequently shown to only weakly block protein kinase C with an IC_{50} of 0.2 mM (Lee et al., 1998). Indeed, at 0.1 mM, chelerythrine caused a dramatic decrease in both basal and dopamine-stimulated phospholipid methylation (Fig. 3B). The fact that two protein kinase C inhibitors, each with a distinct site of action, both abolished dopamine-stimulated phospholipid methylation, supports a role of protein kinase C basal and D4 receptor-mediated phospholipid methylation. However, the inhibitory effect of chelerythrine at such a high concentration could reflect an action at a site other than protein kinase C.

3.3. Effect of protein kinase A stimulators and inhibitors on basal and dopamine-stimulated phospholipid methylation

The role of protein kinase A was investigated using forskolin, a stimulator of protein kinase A with an EC_{50} value of 5 μM (Seamon et al., 1981). Forskolin had no effect on basal or dopamine-stimulated phospholipid methylation at concentrations up to 10 μM, but at a concentration of 0.1 mM, it partially inhibited both basal and dopamine-stimulated phospholipid methylation (Fig. 4A). This effect at such a high concentration might reflect a non-specific mechanism, especially since forskolin is highly lipophilic. This conclusion was supported by the observation that Rp-cAMP, a specific inhibitor of protein kinase A, failed to reverse the inhibitory effect of forskolin observed at this concentration (Fig. 4B). Rp-cAMP alone did not have any effect on basal or dopamine-stimulated phospholipid methylation at a concentration of 0.1 mM (Fig. 4B). These observations suggest that protein kinase A

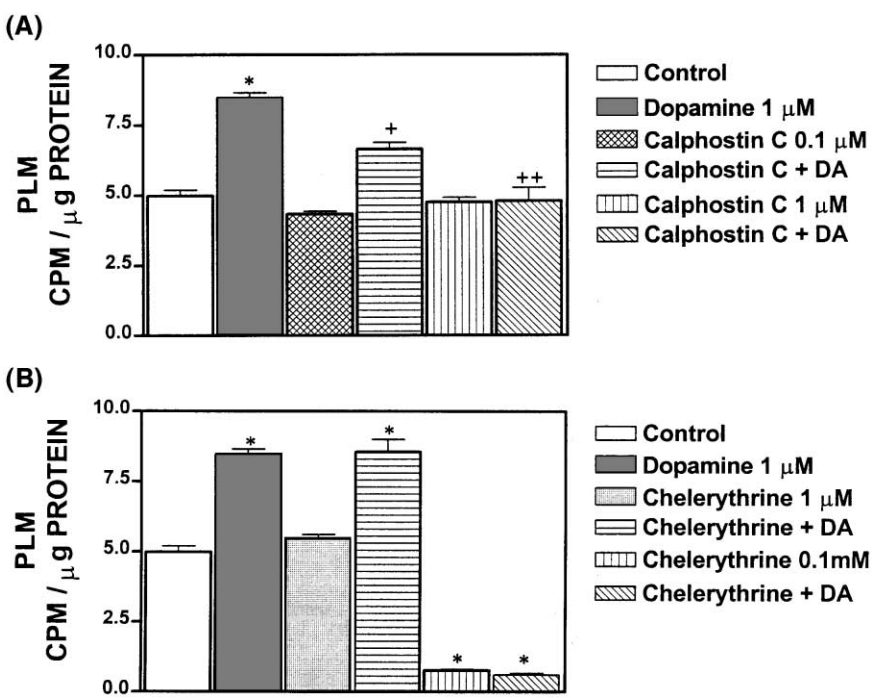

Fig. 3. (A) Calphostin C inhibits D4 receptor-mediated phospholipid methylation. SK-N-MC cells were incubated with or without dopamine (1 μM) in the presence or absence of 0.1- or 1-μM calphostin C. * Indicates $P < 0.01$ as compared to control group. + and ++ indicate $P < 0.05$ and $P < 0.01$, respectively, as compared to dopamine-stimulated control group. (B) Chelerythrine inhibits basal and dopamine-stimulated phospholipid methylation. SK-N-MC cells were incubated with or without dopamine (1 μM) in the presence or absence of 1- or 100-μM chelerythrine. * Indicates $P < 0.01$ as compared to untreated control group.

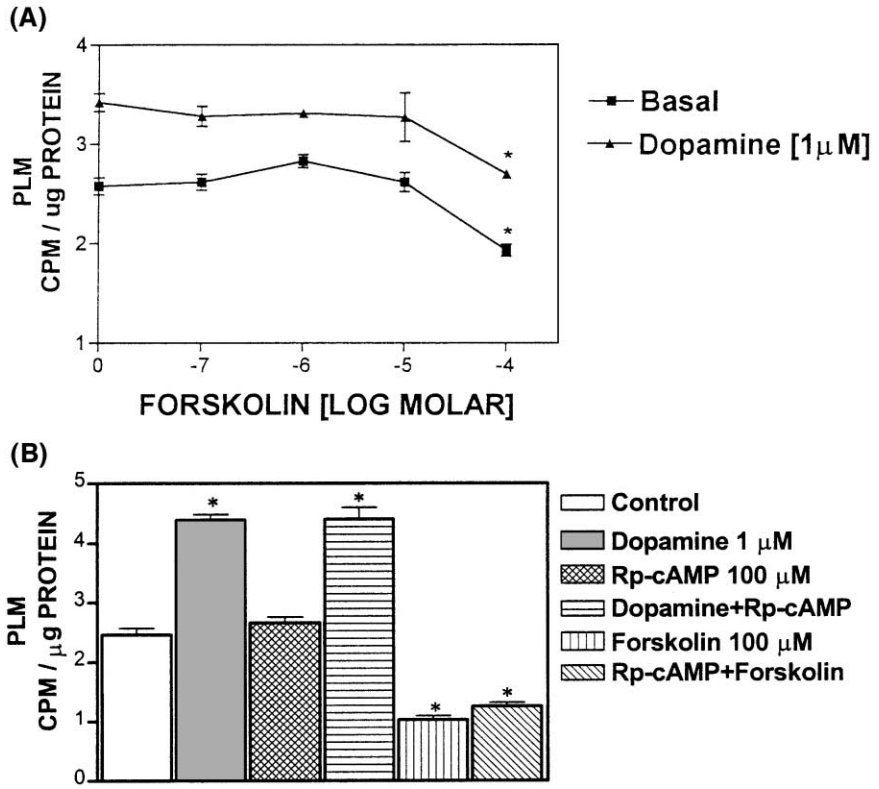

Fig. 4. (A) Effect of forskolin on basal and dopamine-stimulated phospholipid methylation. SK-N-MC cells were incubated with or without dopamine in the presence of forskolin (0.1–100 μM). * Indicates $P < 0.01$ as compared to untreated control or dopamine-only groups. (B) Rp-cAMP does not affect forskolin inhibition of basal or dopamine-stimulated phospholipid methylation. SK-N-MC cells were incubated with or without dopamine (1 μM) in the presence or absence of Rp-cAMP (100 μM) or forskolin (100 μM). * Indicates $P < 0.001$ as compared to untreated control group.

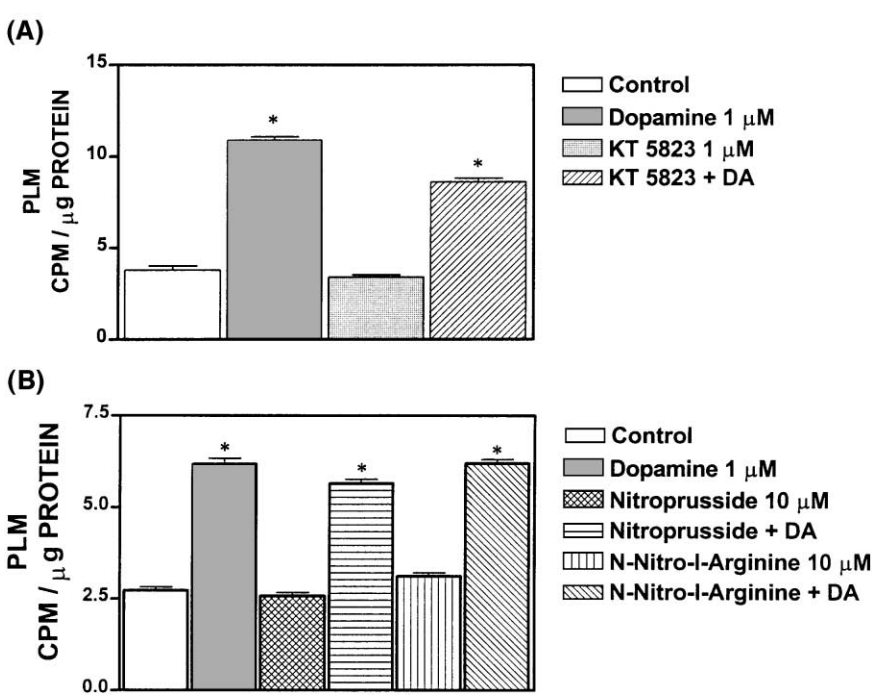

Fig. 5. (A) The protein kinase G inhibitor KT5823 causes a partial inhibition of basal and dopamine-stimulated phospholipid methylation. SK-N-MC cells were incubated with or without dopamine (1 μM) in the presence or absence of KT5823 (1 μM). * Indicates $P < 0.01$ as compared to untreated control or dopamine-treated group. (B) Sodium nitroprusside and *N*-nitro-L-arginine do not affect basal or dopamine-stimulated phospholipid methylation. SK-N-MC cells were incubated with or without dopamine (1 μM) * $P < 0.001$ as compared to respective untreated control group.

activity does not modulate either basal or dopamine-stimulated phospholipid methylation.

3.4. Effect of protein kinase G inhibition or nitric oxide stimulators and inhibitors on basal and dopamine-stimulated phospholipid methylation

KT5823 inhibits protein kinase G with an IC_{50} of 0.2 μM and protein kinase C with an IC_{50} of 4.0 μM (Kase et al., 1987). At a concentration of 1.0 μM, KT5823 reduced both basal phospholipid methylation by 9% and dopamine-stimulated phospholipid methylation by 27% (Fig. 5A), which may reflect either a modest role for protein kinase G or partial inhibition of protein kinase C. Neither sodium nitroprusside, which releases nitric oxide and activates cGMP formation, nor *N*-nitro-L-arginine, which inhibits nitric oxide synthase, had any effect on basal or dopamine-stimulated phospholipid methylation at concentrations of 10 μM (Fig. 5B). Together, these results suggest that protein kinase G and nitric oxide do not play an important role in regulating folate-dependent phospholipid methylation.

4. Discussion

The D4 receptor possesses several features that suggest that it may provide unique signaling activity. For example, the D4 receptor is potently activated by adrenaline and noradrenaline as well as by dopamine (Lanau et al., 1997; Sharma et al., 1999), indicating that it can mediate responses to multiple biogenic amines. D4 receptors in humans and other primates express proline-rich hypervariable repeat segments (VanTol et al., 1992), which allow SH3 domain interactions with other proteins such as Grb2, Nck and Ras GAP (Oldenhof et al., 1998). More relevant to the present studies, our laboratory recently described the direct involvement of D4 receptors in mediating phospholipid methylation (Sharma et al., 1999) and its dependence upon the folate pathway as a source of methyl groups (Zhao et al., 2001).

Our current results support an important role for protein kinase C, but not protein kinase A or protein kinase G in regulating basal and D4 receptor-mediated phospholipid methylation. The stimulatory effect of PDB and the failure of the inactive isomer 4-α PDB to affect basal phospholipid methylation as well as blockade of the stimulatory effect of PDB by calphostin C (Fig. 2A) indicate that higher protein kinase C activity can increase basal phospholipid methylation. Although protein kinase C stimulation increased basal phospholipid methylation, its inhibition with calphostin C did not reduce basal phospholipid methylation (Fig. 3A), indicating that protein kinase C activity is not essential to basal phospholipid methylation under control conditions. The ability of calphostin C to block dopamine-stimulated phospholipid methylation (Fig. 3A), however, indicates that some degree of basal protein kinase C activity is essential for D4 receptor-mediated phospholipid methylation, a distinction between basal and receptor-initiated pathways.

In contrast to calphostin C, chelerythrine did inhibit basal phospholipid methylation at the high concentrations which are reportedly required for protein kinase C inhibition (Lee et al., 1998). However, at these supramicromolar concentrations, chelerythrine also blocks the activity of other kinases (Herbert et al., 1990) and inhibits a number of phosphodiesterases (Eckly-Michel et al., 1997). One or more of these additional actions of chelerythrine might be responsible for its strong inhibitory effect on both basal and dopamine-stimulated phospholipid methylation.

Our studies do not directly address the molecular basis for protein kinase C regulation of phospholipid methylation, and there is considerable complexity within both the protein kinase C signaling system and the folate-dependent phospholipid methylation pathway (Fig. 1). PDB treatment has reported to regulate hepatic methionine adenosyltransferase activity, associated with the production of active monomer species from the native tetrameric form (Pajares et al., 1994). Treatment of adipocytes with PDB caused an increase in the activity of phospholipid methyltransferase when measured with [^{3}H]-*S*-adenosylmethionine (Kelly, 1987). Either or both of these actions could account for the stimulatory effect of PDB on basal phospholipid methylation. The fact that calphostin C inhibits dopamine-stimulated but not basal phospholipid methylation suggests the presence of a protein kinase C control point which is critical for receptor participation. It is possible to speculate that protein kinase C control over methionine adenosyltransferase oligomerization might play such a role.

In addition to its stimulatory action on basal phospholipid methylation, PDB treatment had an inhibitory effect on dopamine-stimulated phospholipid methylation, shifting the dose–response curve for dopamine to the right by almost 20-fold (Fig. 2B). Moreover, the reduced dopamine response appeared to particularly reflect the loss of a discrete higher sensitivity component (Fig. 2B, inset), consistent with previous observations of a biphasic phospholipid methylation response to dopamine (Zhao et al., 2001). This inhibition of dopamine-stimulated phospholipid methylation could reflect either a distinct inhibitory action of protein kinase C on the D4 receptor pathway, or an effect of the higher level of basal phospholipid methylation on the receptor pathway. Adenosylation of the D4 receptor by methionine adenosyltransferase appears to be a conformation-dependent event (Sharma et al., 1999), and changes in basal membrane fluidity could alter the equilibrium between R and R^* states, with the latter being required for adenosylation. Thus, increased basal membrane fluidity might decrease the potency of dopamine in stimulating phospholipid methylation. Alternatively, the increase in basal phospholipid methylation could negatively affect D4 receptor-mediated phospholipid methylation by diverting

more 5-methyltetrahydrofolate to the non-receptor pathway (Fig. 1). Further studies are needed to clarify the precise role of protein kinase C in regulating dopamine-stimulated, folate-dependent phospholipid methylation.

SK-N-MC cells express both dopamine D1 and D4 receptor subtypes (Sidhu and Fishman, 1990; VanTol et al., 1991), and D1 receptor stimulation has been shown to cause activation of PKC (Nowicki et al., 2000). In the context of our findings, this raises the possibility that dopamine co-stimulation of D1 receptors could serve to support and/or augment the efficiency of D4 receptor-mediated phospholipid methylation. However, previously we showed that treatment with the selective dopamine D1 receptor antagonist SCH23390 does not inhibit dopamine-stimulated phospholipid methylation in SK-N-MC cells (Sharma et al., 1999). Thus, D1 receptor co-activation does not appear to be a critical regulator of D4 receptor-mediated phospholipid methylation.

Recently, we found preliminary evidence suggesting that D4 receptor receptors are co-localized with SAP-97 (Deth et al., 1999), a postsynaptic density scaffolding protein which also co-localizes with both AKAP (a kinase-anchoring protein) and protein kinase Cγ (Colledge et al., 2000). This raises the interesting possibility that D4 receptors and protein kinase Cγ may be co-localized at excitatory postsynaptic densities, and that protein kinase Cγ in particular might modulate D4 receptor-mediated phospholipid methylation at this locus. Protein kinase Cγ has been proposed to play an important role in memory formation and LTP induction (Abeliovich et al., 1993).

In summary, protein kinase C appears to play a critical role in modulating folate-dependent, D4 receptor-mediate phospholipid methylation. However, the mechanism by which it exerts its regulation remains to be elucidated.

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