Behavioral/Systems/Cognitive

Prefrontal Inositol Triphosphate Is Molecular Correlate of Working Memory in Nonhuman Primates

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Working memory (WM) is a process of actively maintaining information in the mind for a relatively short period of time, and prefrontal cortex (PFC) has been thought to play a central role in its function. However, our understanding of underlying molecular events that translate into WM behavior remains elusive. To shed light on this issue, we have used three distinct nonhuman primate models of WM where each model represents three WM conditions: normal control, WM-deficient, and recuperated to normal from WM deficiency. Based on the hypothesis that there is a common molecular substrate for the coding of WM behavior, we have studied the relationship of these animals' performance on a WM task with their PFC levels of molecular components associated with Gq-phospholipase C and cAMP pathways, with the idea of identifying the footprints of such biomolecules. We observed that in all of the primate models WM deficiency was strongly related to the reduced concentration of IP_3 in PFC, whereas recuperation of WM-deficient animals to normal condition was associated with the normalization in IP_3 level. However, this correlation was absent or weak for cAMP, active protein kinase A, dopamine D_1 receptor, and Gq protein. In addition, WM deficiency related not only to pharmacological conditions but also to aging. Thus, it is suggested that optimal IP_3 activity is essential for normal WM function and the maintenance of intracellular IP_3-mediated Ca^{2+} level in PFC may serve as biochemical substrate for the expression of WM behavior.

Introduction

Prefrontal cortex (PFC) has been thought to be instrumental in working memory (WM) processing (Baddeley, 1992), and dysfunction in this area leads to an impairment in WM function (Fuster, 1997). Damage in PFC of nonhuman primate produces profound loss in the performance of animals on WM tasks (Butters et al., 1971; Passingham, 1985). Furthermore, demonstration of almost identical cue, delay, and response-related properties in neuronal populations of PFC, together with the observation of their sustained firing during the delay period, suggest that the expression of WM behavior primarily depends on activities in this area of brain (Funahashi et al., 1989; Chafee and Goldman-Rakic, 1998). Consistent with this, imaging studies have also shown higher activity in PFC during performance on WM tasks (Wagner et al., 2001). An enhanced stimulus-specific spiking activity during the delay period has been considered to be a neuronal correlate of WM, and signaling via the Gq protein-phospholipase C (PLC) pathway is thought to be important for this activity (Runyan et al., 2005; Dash et al., 2007). However, the neurochemical substrate that serves the expression of WM behavior remains an enigma. Here, using three models with marked deficit in WM, two models after recuperation from WM loss, and vehicle-treated matching control animals (see Table 1), we have studied the correlation of animal performance on WM task with PFC IP_3, an end product of the Gq-PLC pathway, together with other biological components of Gq-PLC and cAMP pathways. The advantage of using several WM models was that it permitted us to map the footprints of biomolecules associated with WM in a stepwise manner from one WM condition to another within a model and across all models. Biomolecules showing similar levels in control normal and recovered normal animals but change in the WM-deficient condition of all models were considered highly correlated to WM. Our results show a strong correlation of the WM status of nonhuman primates with IP_3 level in PFC not only in pharmacological conditions but also in aging.

Materials and Methods

Subjects. Adult Rhesus monkeys 5–7 years of age or old monkeys 18–22 years of age were used in this study. Animals were maintained in accordance with the guidelines of local Animal Experimentation, Welfare and Protection Committee and European Union Law (Council directive 86/609/EEC adopted in 1986 and the Protocol to the Amsterdam Treaty).

All monkeys were trained on a spatial working memory task in the Wisconsin General Testing Apparatus (WGTA) according to methods described previously (Goldman et al., 1970). Animals were always tested at the same time of day immediately before feeding. Highly palatable food rewards (e.g., a piece of peanut or miniature chocolate chips) were used during testing to minimize the need for dietary regulation. Animals performing 75–85% correctly (in the case of young animals) and 65–

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Table 1. Monkeys' WM condition on behavioral test before killing

Animal groups and their treatments	WM status[a]
Amphetamine young animal group	
Vehicle control	Normal
Amphetamine treatment	Deficit
Amphetamine-treated animals 6 mo after withdrawal	Recuperated to normal
Haloperidol young animal group	
Vehicle control	Normal
Haloperidol treatment	Deficit
Haloperdol-treated animals after D_1 agonist administration	Recuperated to normal
Haloperidol aged animal group	
Vehicle control	Normal
Haloperidol treatment	Deficit

[a]The WM status was determined by the results of animal performance on spatial delayed response shown in Figure 1*A*. Animals with significant reduction in the percentage of correct choices were considered to be WM deficient.

70% correctly (in the case of old animals) with a 5 s delay period were used for the treatments and further studies.

WM behavioral test. Performance on a spatial delayed response task was used to assess the WM status of all the animals throughout the study. To execute the spatial delayed response task, one of the two food wells in the WGTA was baited in view of the monkey, then both wells were covered with identical plaques and an opaque screen was lowered for 5 s. During the delay period, the monkey had to hold in mind the spatial location of the baited well to respond appropriately and be rewarded. After the delay, the animal had to move the correct plaque to obtain the reward. Animals were required to perform 30 trials per test session. Initial values were obtained from a mean of three consecutive test sessions corresponding to each monkey, and then these values were used for the calculation of the percentage of correct choice, as shown in Figure 1*A* and Table 1 for each group.

WM models. Using a recipe from published articles, three WM models of both young and old nonhuman primates were prepared. A summary of the treatment of animals and their corresponding WM status is described in Table 1. Animals that showed significant reduction in performance on spatial delayed response task after treatment with either haloperidol or amphetamine were considered WM deficient (Table 1). All of the animals included in this study responded to the drug treatment.

Amphetamine-based WM models. Ten adult monkeys received intermittent, escalating doses (0.1–1.0 mg/kg body weight) of amphetamine (from Sigma-RBI) via intramuscular injections for 6 weeks (Castner et al., 2005; Selemon et al., 2007). Forty-five days after withdrawal, when a significant loss in WM was observed (see Fig. 1*A*), a group of five animals was killed. Another group of five animals was killed 6 months after withdrawal, at which time animal performance on WM tasks returned to normal (see Fig. 1*A*).

Haloperidol-based WM models. A powder form of haloperidol (Sigma-RBI) in fruit was administered daily to nine adult monkeys at a dose of 0.07 mg/kg body weight during the first month, which was increased to 0.14 mg/kg body weight in the second month (Castner et al., 2000). Haloperidol-mediated WM deficits emerged between 3 and 4 months. A group of five animals was killed 4 months after the initiation of drug treatment when a substantial WM loss was observed (see Fig. 1*A*). The other group of four monkeys was continued with the treatment and was coadministered a selective D_1 agonist ABT 431 (Lilly) at a dose of 0.0001 mg/kg body weight for five consecutive days. This D_1 agonist treatment has been shown to produce a progressive and long-lasting reversal of haloperidol-induced WM deficits (Castner et al., 2000). Once the animals showed normal WM status (see Fig. 1*A*), they were killed.

In another set of four aged monkeys, haloperidol was started by administration of daily doses of 0.20 mg/kg body weight and was increased to 0.27 and 0.35 mg/kg body weight at 2 week intervals. The latter dose was maintained until the time of killing. Once the WM deficit was observed (see Fig. 1*A*), animals were killed.

Brain tissues. After 2–6 h of determination of behavioral status (Table 1; see Fig. 1*A*), animal brains were removed and small blocks of the dorsolateral prefrontal cortex (area 46) and striatum (caudate–putamen) were frozen in liquid N_2 and stored at −80°C.

Homogenate and membrane preparation. Collected brain tissues were homogenized in Tris-HCl, pH 7.4, containing a mixture of protease inhibitors (Sigma-Aldrich) and were processed for the membrane preparation, as described previously (Khan et al., 1994, 1998; Khan and Gutierrez, 2004). In brief, homogenates were centrifuged at 3000 rpm (1075 × *g*) for 10 min in an RC5C centrifuge (Sorvall). The resultant supernatant was centrifuged at 48,100 rpm (105,000 × *g*) for 1 h in an XL-90 Ultracentrifuge from Beckman to collect the membrane fraction as a pellet. Membrane fractions were washed twice in 50 mM Tris-HCl, pH 7.4, and were finally suspended in the same buffer and aliquoted. Aliquots were stored at −80°C until used. The protein concentration was determined by the Lowry method.

IP_3 assay. A 0.5 ml aliquot of homogenate from above in membrane preparation was mixed with the same amount of cold 15% TCA. After 10 min on ice, it was centrifuged at 5000 rpm for 15 min in a Microfuge. The collected supernatant was extracted three times with 10 volumes of diethyl ether saturated with water. The supernatant was then neutralized to pH 7.5 by titration with 1 M NaHCO3. IP_3 was separated by a solid-phase procedure using Amprep SAX minicolumns (GE Healthcare). Briefly, columns were washed with 1 M KHCO3 followed by 15 ml of water. After the samples were applied to the column, the column was washed with water and then with 0.1 ml of KHCO3. The IP_3 fraction was eluted with 5 ml of 0.17 M KHCO3. The determination of IP_3 concentration in collected fractions was performed by D-myoinositol 1,4,5-triphosphate [^{3}H] assay system from GE Healthcare.

cAMP immunoassay. In 0.5 ml of prepared homogenate from homogenate and membrane preparation, 0.5 ml of Hank's buffer was added, followed by the addition of 1.0 ml of acetonitrile. The samples were mixed, incubated on ice for 10 min, and centrifuged at 6000 rpm for 10 min. The collected supernatants were loaded on conditioned Amprep SAX minicolumns. Samples were eluted by 3.0 ml of methanol with 0.1 M HCl and dried under nitrogen. The cAMP assay was performed as described by the manufacturer using the cAMP enzyme immunoassay system from GE Healthcare.

Immunoblots. Homogenates (for active PKA levels) and prepared membrane fractions (for Gαq protein levels) were treated with SDS buffer and processed for Western blot analysis similar to as described previously (Khan et al., 2000, 2001; López-Aranda et al., 2006). Homogenate proteins (395 μg/lane) and membrane proteins (100 μg/lane) were separated by 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (GE Healthcare). The membranes were blocked with 5% milk in 10 mM Tris-HCl, pH 7.5 containing 0.05% Tween-20 and 0.2.9% NaCl (TTBS) for 15 min and washed with TTBS, followed by incubation with either mouse monoclonal antibody to active PKA (1:1000 dilution in TTBS with 2% BSA; Transduction Laboratories) or affinity-purified rabbit polyclonal antibodies to Gαq (1:500 dilution in TTBS with 2% BSA; Santa Cruz Biotechnology). Corresponding rabbit and mouse secondary antibodies coupled to HRP (GE Healthcare) were diluted to 1:2500 in TTBS with 2% BSA and 5% nonfat dry milk, and incubated for 1 h. Development of the immunoreactivity was performed with ECL Western Blotting Detection Reagents (GE Healthcare) according to the manufacturer protocol. For the analysis of immunoreactive bands, we used ScanMaker 9800XL (Microtek) to scan the film and program Visilog for the determination of optical density. Relative gray values were obtained from the scale of 0–255 preset in the program. Vehicle control of amphetamine group was considered 100%.

Receptor-ligand binding assay. Membrane-bound dopamine D_1 receptors were assayed by [^{3}H]SCH 23390 binding, as described previously (Khan et al., 1998, 2000). Briefly, 200 μg of membrane protein was incubated with 0–20 nM radioligand for 1 h at 22°C in a total volume of 0.5 ml of 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl2, 4 mM MgCl2, and 120 mM NaCl. The reaction was stopped by rapid filtration through 0.3% polyethylenimine-soaked Whatman GF/B filters and washed three times with 50 mM Tris-HCl, pH 7.4. Filters were dried and counted for retained radioactivity. Nonspecific binding was determined by performing the assay in the presence of 1000× of either (+) butaclamol-HCl or fluphenazine. Background activity was subtracted from total activity, which was 5–7%.

Data analysis. Digital images of immunoblots were analyzed by densitometry using Visilog 6. Values are presented as the mean ± SEM of four

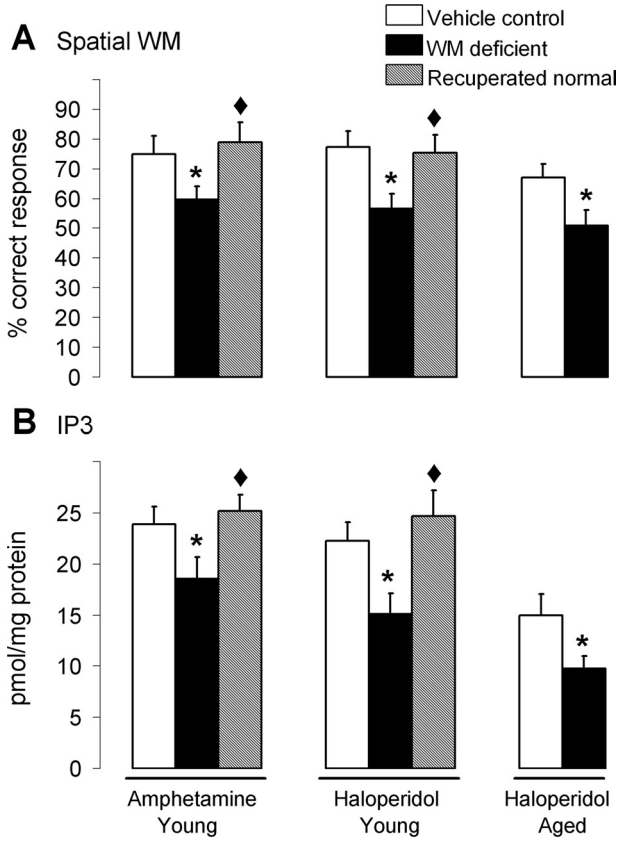

Figure 1. WM status of nonhuman primate models before they were killed and their PFC IP_3 concentration. ***A***, As shown previously (Castner et al., 2000, 2005), both amphetamine (black bars in amphetamine young group) and haloperidol (black bars in haloperidol young and haloperidol aged groups) treatments produced significant loss in WM. Six months after withdrawal in amphetamine-treated monkeys (Castner et al., 2005) (hatched bars in amphetamine young group) and after dopamine D_1 agonist administration to haloperidol-treated monkeys (Castner et al., 2000) (hatched bars in haloperidol young group) the WM deficit observed in animals recovered to a normal level. ***B***, A significant reduction in the amount of IP_3 was observed in animals with WM deficiency. Recuperation in WM was associated with the normalization in IP_3 concentration. * results are significantly different from the control group; ♦ results are significantly different from the WM-deficient group.

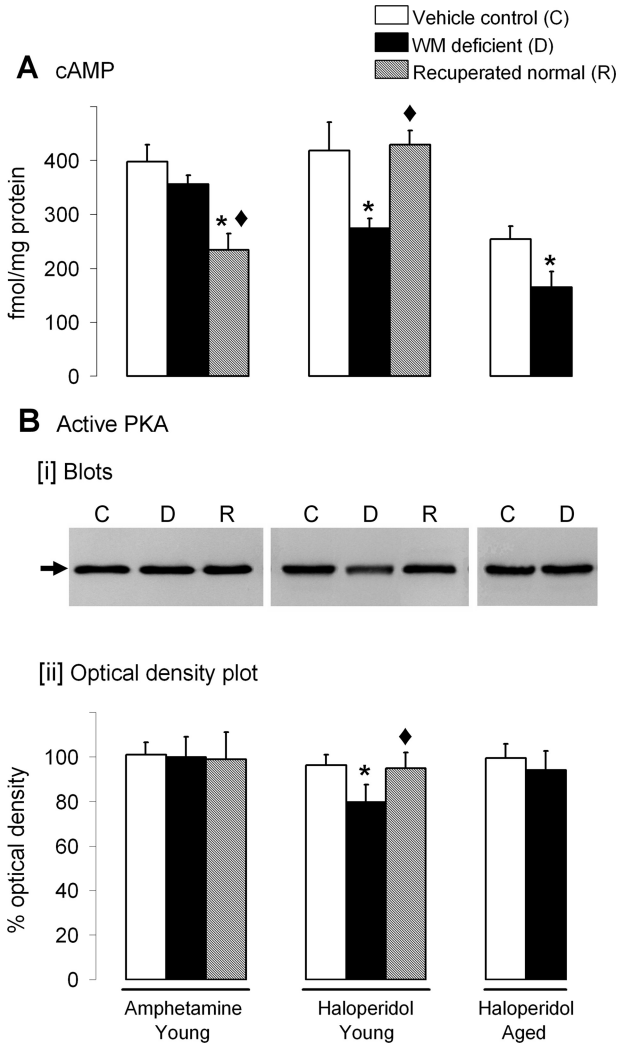

Figure 2. Levels of cAMP and active PKA in PFC. ***A***, The cAMP level in amphetamine young group did not coincide with the behavioral performance seen in these animals; however, there were coincidence in cases of haloperidol young and haloperidol aged animal groups where WM-deficient animals showed reduced cAMP levels. ***Bi***,***ii***, Representative blots of active PKA (***i***) and their optical density plot (***ii***) are shown. The amphetamine young and haloperidol aged animal groups did not show any change in active PKA level. However, a reduction in WM-deficient animals and an increase in recuperated normal animals of the haloperidol young group were observed. The arrow in the blots in ***i*** indicates the immunoreactive band of 39 kDa corresponding to a catalytically active subunit of PKA protein size. * results are significantly different from control group; ♦ results are significantly different from WM-deficient animals.

to five animals. Statistical analysis of the physiologic variables was performed using Student's *t* test. A probability value <0.05 was considered statistically significant.

Results

Three models with marked deficit in WM, two models after recuperation from WM loss and vehicle-treated matching control animals were included in this study (Table 1). Similar to previous reports, the treatment of young animals with amphetamine produced a significant loss in WM; however, 6 months after withdrawal these animals returned to normal condition (Castner et al., 2005) (Fig. 1*A*). In another experiment, exposure of both young and aged animals with haloperidol also resulted in a marked reduction in WM (Castner et al., 2000) (Fig. 1*A*). Further administration of D_1 agonist ABT 431 to haloperidol-induced, WM-deficient young animals resulted in the reversal of WM loss observed in these animals (Castner et al., 2000) (Fig. 1*A*). After confirming WM status (Table 1), all the animals were killed and their PFCs were processed for neurochemical study. Animals with WM deficiency developed after treatment with either amphetamine in young animals or with haloperidol in young and aged animals had significantly lower IP_3 levels (Fig. 1*B*). A lower IP_3 level was also observed in normal aged animals compared with normal young animals (Fig. 1*B*). Most importantly, we found that the recovery in WM after either D_1 agonist administration to haloperidol-treated monkeys or 6 months after withdrawal to amphetamine-treated young animals was associated with an increase in IP_3 level (Fig. 1*B*). In all the WM models, there was strong correlation between the level of animal performance on the WM task and the IP_3 concentration in PFC. However, this strong correlation was absent or weak for other components associated with cAMP (Fig. 2*A*,*B*) and Gq-PLC pathways (Fig. 3*A*,*B*). In contrast to PFC, IP_3 level in the striatum showed no correlation with WM (Fig. 4). cAMP and active PKA, main components of the cAMP pathway, showed no relation with WM in amphetamine young and haloperidol aged animals (Fig. 2*A*,*B*). However, a correlation was observed in the haloperidol young group of animals. An inverse relation of WM with both cAMP

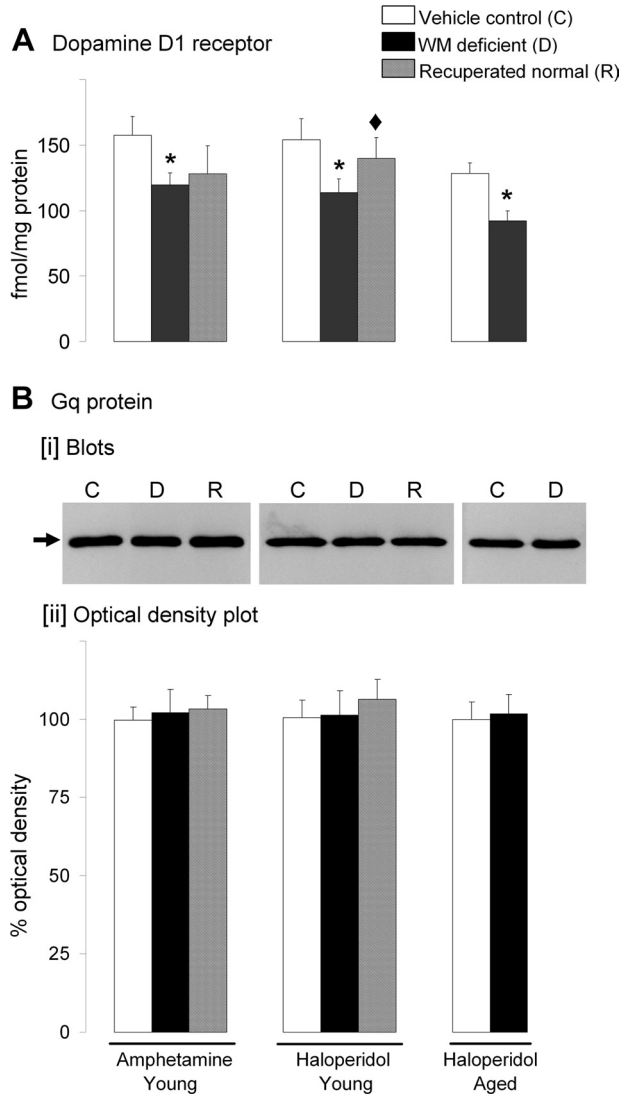

Figure 3. Amount of dopamine D_1 receptor and Gq protein in PFC. ***A***, A correlation of D_1 receptor level with WM status was seen in WM-deficient animals of all the groups but this correlation was lacking in the recuperated normal animals of amphetamine young group. ***Bi***, ***ii***, Representative blots of Gq protein (***i***) and their optical density plot (***ii***) are shown. Gq protein level was not changed in any of the groups. The arrow in the blots in ***i*** indicates the immunoreactive polypeptide band of 42 kDA corresponding to Gq protein size. * results are significantly different from control group; ◆ results are significantly different from WM-deficient animals.

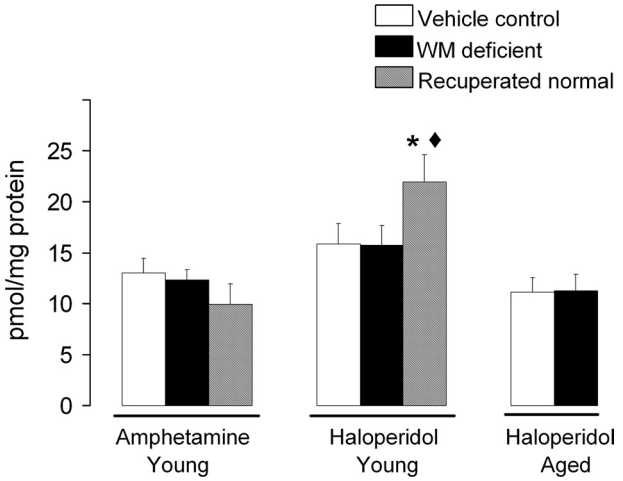

Figure 4. IP_3 concentration in the striatum. IP_3 level in this area did not correlate with the WM status seen in the animals. An increase in IP_3 was observed in recuperated normal animals of the haloperidol young group. However, this may be due to the activation of dopamine D_1 receptor while D_1 agonist is on board in these animals. Values are expressed as the mean ± SEM of four to five animals. * results are significantly different from control; ◆ results are significantly different from WM-deficient animals.

(Dash et al., 2007; Wang et al., 2007) and higher PKA activity (Taylor et al., 1999) has been shown previously. Thus, the changes observed in components related to the cAMP pathway of the haloperidol group of animals do not actually reflect an association with WM. In contrast to the cAMP pathway, activation of dopamine receptor is known to enhance the WM in nonhuman primates (Castner et al., 2000; Castner and Goldman-Rakic, 2004). Our results show that the level of dopamine D_1 receptor in WM-deficient animals in both the amphetamine young and haloperidol young and old groups were significantly reduced (Fig. 3*A*). The recovery in WM of haloperidol young animals was seen with the increase in dopamine D_1 receptor concentration. However, this correlation was lacking in amphetamine young animals, where no change was observed (Fig. 3*A*). In contrast to the dopamine D_1 receptor, Gq protein, another component of the Gq-PLC pathway, was unchanged in all the animal groups (Fig. 3*B*).

Discussion

The activation of cAMP–PKA pathway has an inverse relation with WM (Dash et al., 2007; Wang et al., 2007). An increase in cAMP concentration or activation of PKA is expected to reduce the level of animal performance on a WM task. Therefore, the absence of correlation of cAMP and active PKA with WM suggests that the cAMP-PKA pathway may not be responsible for the expression of WM behaviors seen in nonhuman primates. However, on the other hand, the results of the activation of Gq protein pathway during performance on WM task and the finding that the inhibition of PLC impairs WM (Runyan et al., 2005) suggest a directly proportional relation of Gq-PLC pathway with WM. Therefore, lower WM performance in WM-deficient animals was perhaps due to lower activity in this pathway. Activation of Gq protein-coupled receptors, such as dopamine D_1 receptor, which are known to influence WM functions (Dash et al., 2007), leads to the production of IP_3 and diacylglycerol through the Gq-PLC pathway. The IP_3, then, releases Ca^{2+} from intracellular stores. It has been shown that stimulation of Gq-coupled dopamine D_1 receptor depresses recurrent excitatory transmission and generates a substantial and prolonged attenuation of IP_3 signaling (Abdul-Ghani et al., 1996), a process shown to be necessary for the mediation of WM functions (Goldman-Rakic, 1995). Therefore, IP_3 deficit in WM-deficient monkeys may reflect a chronic state of depressed neuronal transmission. A requirement of Ca^{2+} release from intracellular stores via IP_3 for a persistent recurrent excitation in prefrontal neurons (Gao and Goldman-Rakic, 2006) further indicates the importance of optimal IP_3 signaling for normal WM function. In addition, schizophrenics, patients with dominant WM deficit, exhibit low levels of PFC RGS 4, an inhibitor of Gq protein-induced intracellular Ca^{2+} release (Mirnics et al., 2001). It is proposed that IP_3-mediated intracellular Ca^{2+} in PFC may provide a biochemical environment that is crucial for the maintenance of information during the delay period, a period when the animal has to hold the information on stimulus for few seconds in the mind before responding. In summary, our results show a strong link between the IP_3 concentrations in PFC and WM performance in nonhuman primates.

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