

Blockade of IP₃-mediated SK channel signaling in the rat medial prefrontal cortex improves spatial working memory

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Planning and directing thought and behavior require the working memory (WM) functions of prefrontal cortex. WM is compromised by stress, which activates phosphatidylinositol (PI)-mediated IP₃-PKC intracellular signaling. PKC overactivation impairs WM operations and *in vitro* studies indicate that IP₃ receptor (IP₃R)-evoked calcium release results in SK channel-dependent hyperpolarization of prefrontal neurons. However, the effects of IP₃R signaling on prefrontal function have not been investigated. The present findings demonstrate that blockade of IP₃R or SK channels in the prefrontal cortex enhances WM performance in rats, suggesting that both arms of the PI cascade influence prefrontal cognitive function.

The prefrontal cortex coordinates and controls cognitive and emotional processes utilizing appropriate judgment, flexibility, and attention (Goldman-Rakic 1995a,b; Fuster 2000). A hallmark operation of the prefrontal cortex is working memory (WM)—the ability to internally maintain information in the absence of external representation. Debilitating deficits in WM and prefrontal function are associated with numerous conditions including schizophrenia, attention deficit hyperactivity disorder, aging, and stress (Goldman-Rakic 1992; Arnsten 1993; Goldman-Rakic and Selemon 1997; Ramos et al. 2003; Birnbaum et al. 2004). Elucidating mechanisms of cognitive impairment will contribute to identification and development of novel therapeutic targets for treatment of prefrontal decline.

Local excitatory circuits are believed to underlie WM operations (Goldman-Rakic 1995a,b) and disruption in prefrontal neuronal activity is associated with WM impairment (Goldman-Rakic 1995a,b). Disruptions can arise from altered catecholamine release within the prefrontal cortex. High levels of norepinephrine release, which typically follow a stressor (Rossetti et al. 1990; Finlay et al. 1995), impair WM performance by binding G_q-coupled α 1-adrenoceptors, leading to activation of the phosphatidylinositol (PI) cascade (Garcia-Sainz 1993; Birnbaum et al. 1999, 2004). In the PI pathway, phospholipase C cleaves the phospholipid phosphatidylinositol bisphosphate (PIP₂) to generate membrane-bound diacylglycerol (DAG) and diffusible inositol 1, 4, 5-trisphosphate (IP₃). IP₃ interacts with receptors (IP₃R) on the endoplasmic reticulum to mediate the release of calcium (Ca²⁺) from intracellular stores. Rises in intracellular Ca²⁺ serve a variety of regulatory and signaling functions and may activate kinases and open ion channels to influence processes relevant to prefrontal network integrity, including synaptic plasticity and neuronal excitability (for review, see Berridge 1993).

Recent studies have established that Ca²⁺-dependent kinases negatively influence cognitive operations of the prefrontal cortex. Activation of Ca²⁺-calmodulin-dependent kinase II (CaMKII) and inhibition of the Ca²⁺-activated phosphatase, calcineurin, impair WM performance in the rat (Runyan et al. 2005; Dash et

al. 2007). Protein kinase C (PKC), which is cooperatively activated by Ca²⁺ and DAG following PI signaling, suppresses delay-related firing of prefrontal neurons in monkeys performing a WM task (Birnbaum et al. 2004) and impairs spatial WM in rats (Birnbaum et al. 2004; Runyan et al. 2005). Another candidate target for the impairing effects of intracellular Ca²⁺ is the Ca²⁺-activated small conductance potassium (SK) channel (Kohler et al. 1996). SK channels influence firing frequency by modulating after-hyperpolarization (Pedarzani et al. 2005; Sah 1996) and are therefore positioned to impact the network functions of the prefrontal cortex, which rely on highly synchronized sequences of firing. A recent study by Hagenston et al. (2007) indicates that IP₃R-SK channel signaling regulates prefrontal neuron firing. IP₃-mediated release of Ca²⁺ resulted in hyperpolarization of layer V pyramidal neurons in rat medial prefrontal slices (Hagenston et al. 2007). This hyperpolarization was reversed by application of the SK channel blocker, apamin (Hagenston et al. 2007), suggesting a mechanistic role of SK channels in prefrontal dysfunction.

This study sought to clarify whether IP₃R signaling influences prefrontal cognitive function, and whether SK channel activation may underlie these effects. We identified doses of IP₃R and SK channel pharmacological blockers capable of enhancing basal WM performance in rats when delivered directly to the medial prefrontal cortex. Our results indicate that both arms of the PI pathway (IP₃, PKC) regulate prefrontal cognitive function and IP₃R-mediated signaling is likely central to previous reports of Ca²⁺-activated enzymes impairing WM (Birnbaum et al. 2004; Runyan et al. 2005). These findings also suggest that suppression of IP₃R signaling may be useful in treating prefrontal cognitive impairment.

The present study used adult (4–8 mo), male Sprague-Dawley rats (Taconic). All procedures were approved by the Yale Animal Care and Use Committee and were in accordance with the National Institutes of Health's *Guide for Care and Use of Laboratory Animals*. Rats were single-housed in filter frame cages, maintained on a 12 h light/dark cycle, and fed a restricted diet of Purina rat chow (~12 g/d per rat) following behavioral testing. Food rewards during cognitive testing were miniature chocolate chips. Water was available *ad libitum*.

WM was evaluated using the medial prefrontal cortex-dependent spatial delayed alternation task (Wortwein et al.

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Article is online at <http://www.learnmem.org/cgi/doi/10.1101/lm.767408>.

1994). Training, testing, surgery, and drug administration have been described previously (Birnbaum et al. 1999). Briefly, testing was conducted within the same time period (e.g., 9:00 a.m.–11:00 a.m.) 5 d/week by a single experimenter who remained blind to treatment conditions throughout the study. Rats were habituated to a T maze (dimensions, 90 × 65 cm) until taking rewards from the hand of an experimenter and then trained to alternate. On the first trial, animals were rewarded for entering either arm. Thereafter, for a total of 10 trials per session, rats were rewarded only if they entered the maze arm that was not previously chosen. Between trials, the choice point was wiped with alcohol to remove any olfactory clues.

Upon achievement of a 2-d average of at least 70% correct at a 0-sec delay, animals underwent surgery to implant indwelling, guide cannulae (9.0 mm, 23 gauge, Plastics One). Cannulae were directed to deliver infusions into the prelimbic and infralimbic cortices (stereotaxic coordinates: anteroposterior, +3.2 mm; mediolateral, ±0.75 mm; dorsoventral, –3.0 mm). Surgery was performed under ketamine (80 mg/kg) and xylazine (10 mg/kg) anesthesia using aseptic methods. Sterile stylets were inserted to prevent occlusion. Testing recommenced 10 d after surgery. Inter-trial delays were raised in 5-sec intervals to maintain performance between 65% and 75% correct, allowing for detection of improvement or impairment following drug administration. If inter-trial delays exceeded 20 sec, rats were tested on a 12-trial task with four quasi-randomly distributed delay lengths (e.g., 0, 10, 20, and 30 sec; mean delay = 15 sec/trial). Variable delay testing enabled maintenance of the desired baseline without requiring mean delays in excess of 40 sec/trial. Two animals received treatment while being tested with the variable delay paradigm. As expected, ceiling effects were observed for the two shortest delays. Drug-related improvements were observed in the two longer delays. However, analysis by delay was not performed due to the small subject number.

Animals were adapted to the infusion procedure, and infusions of drug or vehicle were administered only when performance was stable ($\geq 65\%$ correct) following a “mock” infusion. For infusions and mocks, 30-gauge sterile infusion probes extending 4.5 mm ventral to the skull were inserted. Bilateral infusions (0.5 μ L/hemisphere) were delivered by a syringe pump (Harvard Apparatus) at a rate of 0.25 μ L/min. Testing commenced 15 or 20 min following the infusion, depending on the study. Run times were calculated (total delay time minus the total time required for the animals to complete the task) for test sessions following a drug or vehicle treatment. Infusions that failed to deliver at least 75% of the intended volume for each hemisphere were excluded from the analyses (Birnbaum et al. 1999, 2004; Ramos et al. 2006).

A range for drug dose and latency from infusion to cognitive testing were informed by previous infusion studies of channel blockers and cell permeable agents (Birnbaum et al. 1999, 2004; Ramos et al. 2006; Wang et al. 2007). For the present study, optimal doses and latencies were determined by pilot studies. Rats in Experiment 1 ($n = 13$) received 0.01 μ g/0.5 μ L 2-aminoethoxydiphenyl borate (2-APB, Tocris) in sterile PBS or an equal volume of vehicle 15 min prior to cognitive assessment. For Experiment 2 ($n = 11$), Xestospongine C (XeC, Sigma-Aldrich) was dissolved at a concentration of 1 μ g/1 μ L in dimethyl sulfoxide

(DMSO) and diluted in sterile PBS to a concentration of 0.1 ng/0.5 μ L. XeC and vehicle treatments were infused 20 min prior to cognitive assessment. In Experiment 3, rats ($n = 8$) received 0.1 μ g/0.5 μ L apamin (Alexis) dissolved in PBS, or vehicle, 15 min prior to cognitive assessment. Doses of apamin exceeding 0.1 μ g/0.5 μ L resulted in seizures in three animals; thus, these doses were avoided in all subsequent experiments. Doses of apamin lower than 0.1 μ g/0.5 μ L were found to have no effect on WM performance. At the completion of each experiment, rats were anesthetized with isoflurane and rapidly decapitated. Brains were stored in formalin, sectioned, and analyzed for histological verification of drug delivery. The ventral extent of all infusion probes reached the prelimbic or dorsal infralimbic cortices, indicating that all infusions were delivered to the medial prefrontal cortex (Fig. 1).

This study used a within-subjects design. All rats received one of the three drug treatments (2-APB, XeC, or apamin) and the corresponding vehicle. Vehicle and drug administration were counterbalanced with at least 1 wk washout between each treatment. Scores following a specific treatment were averaged. Mean scores reflect, on average, two treatments (each, drug and vehicle) per animal per experiment. For each study, animals received an average of four infusions (minimum, two; maximum, nine). Higher numbers of infusions were required when a previous infusion failed to deliver an adequate volume of drug. For each experiment, group comparisons were evaluated using paired two tailed *t*-tests, and values were represented as the means \pm SEM. An α level below 0.05 was considered statistically significant.

Infusions of IP₃R and SK channel blockers directly into the medial prefrontal cortex improved spatial WM performance. IP₃R blockade with 2-APB or XeC significantly improved performance relative to vehicle (2-APB = 80.9 \pm 2.9% correct; vehicle_{2-APB} = 69.8 \pm 1.7% correct; $t_{12} = 3.317$, $P = 0.006$; XeC = 85.5 \pm 3.4% correct; vehicle_{XeC} = 73.9 \pm 2.7% correct; $t_{10} = 3.082$, $P = 0.012$; Fig. 2A,B). Run-time was determined by subtracting the total delay time from the total time to completion. Run-time was not significantly affected by IP₃R blockade (2-APB = 135.6 \pm 26.4 sec; vehicle_{2-APB} = 117.8 \pm 13.8 sec; $t_{12} = -0.966$, $P = 0.353$; XeC = 142.9 \pm 18.0 sec; vehicle_{XeC} = 160.4 \pm 24.2 sec; $t_9 = 0.586$, $P = 0.572$; Fig. 2C), indicating that performance enhancement was unlikely attributed to nonspecific sensorimotor or motivational effects. SK channel blockade also significantly improved performance (apamin = 78.4 \pm 2.2% correct; vehicle_{apamin} = 71.9 \pm 1.5% correct;

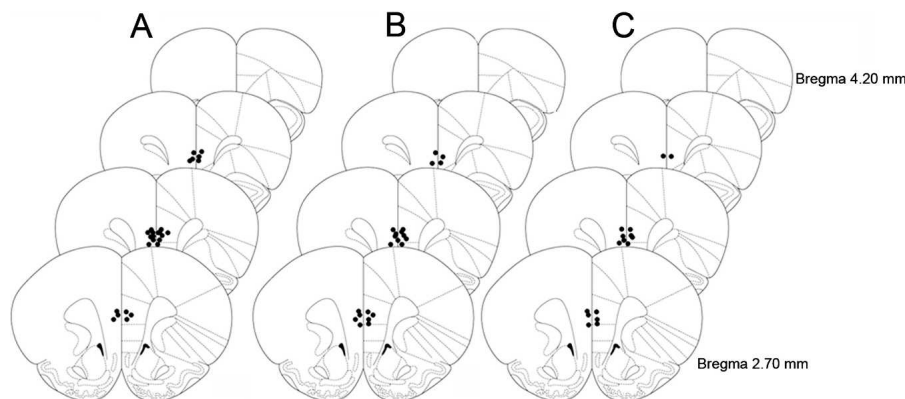


Figure 1. Brains were histologically examined for verification of cannulae placement. All rats had correctly placed cannulae. The ventral-most extent of tracks from probes delivering drug to the prelimbic and infralimbic cortex are illustrated. (A) 2-APB; (B) XeC; (C) apamin. Reprinted with permission from Elsevier © 1998, Paxinos and Watson 1998.

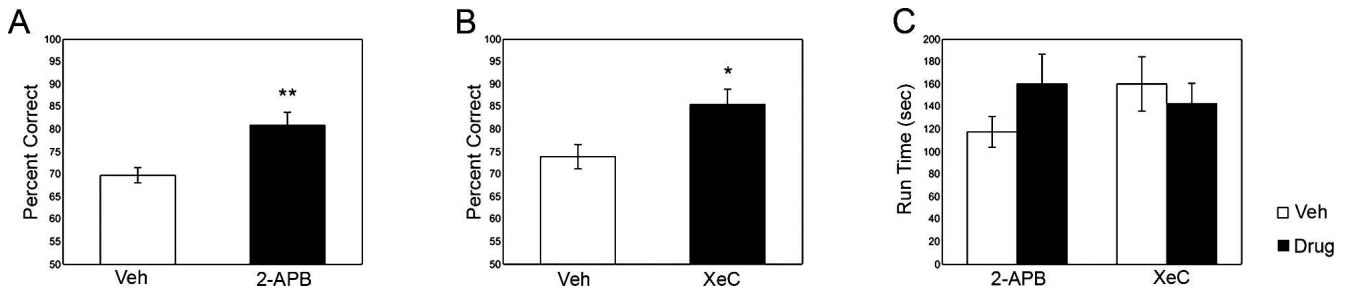


Figure 2. Experiments 1 and 2 examined WM performance following intra-prefrontal cortical infusions of the IP₃R blockers, 2-APB and XeC. (A) A total of 0.01 μ g/0.5 μ L 2-APB significantly improved performance relative to vehicle; (B) 0.1 ng/0.05 μ L XeC also significantly improved performance. (C) Run-time following treatment with 2-APB and XeC did not significantly differ from the respective vehicle treatments, indicating that enhanced WM performance was not due to nonspecific motor or motivational effects. Means \pm SEM are shown. (**) $P < 0.01$; (*) $P < 0.05$.

$t_7 = 3.945$; $P = 0.006$; Fig. 3A), and no significant effects of apamin on run-time were observed (apamin = 111.1 ± 6.5 sec; vehicle_{apamin} = 124.9 ± 13.0 sec; $t_9 = 1.172$, $P = 0.280$; Fig. 3B).

The present study is the first to demonstrate that IP₃R activation negatively regulates prefrontal cognitive function. More specifically, blocking IP₃-mediated intracellular Ca²⁺ release within the medial prefrontal cortex enhanced WM performance by ~10% relative to vehicle. This is consistent with previous reports of Ca²⁺-dependent enzymes impairing WM performance in rats (Birnbaum et al. 2004; Runyan et al. 2005). We found a similar magnitude of WM enhancement following treatment with apamin, suggesting that IP₃R effects may be mediated, in part, by activation of Ca²⁺-dependent SK channels. Pharmacological blockade was most effective in very low doses. This may be explained by the regulatory functions served by Ca²⁺ and SK channels. For instance, high doses of blockers may interfere with Ca²⁺-regulated cellular homeostatic mechanisms (e.g., Berridge 1993) or activation of calcineurin, a phosphatase activated by small rises in intracellular Ca²⁺ concentration that is required for optimal WM performance (Runyan et al. 2005; Dash et al. 2007).

IP₃R signaling research has been greatly limited by the dearth of reliable IP₃R agonists and antagonists. Furthermore, the specificity and mechanism of action of available IP₃R antagonists have not been fully elucidated, and there are no previous reports of IP₃R blockers being used in vivo. In an effort to avoid misinterpretation of our results due to a nonspecific effect of a membrane permeant IP₃R blocker, we tested two compounds, 2-APB and XeC, which are known to block IP₃R-mediated intracellular Ca²⁺ release, but have unrelated nonspecific effects (Gafni et al. 1997; Bootman et al. 2002). For example, in addition to blocking IP₃Rs, 2-APB has been reported to block store-operated channels in non-neuronal cells, thereby preventing replenishment of the endoplasmic reticulum Ca²⁺ (Bootman et al. 2002). Little is known, however, about whether store-operated channels play a role, or even exist, in neurons. The other IP₃R blocker that we tested, XeC, is a bis-1-oxaquinolizidine isolated from the marine sponge *Xestospongia*. XeC has been reported to block IP₃R more specifically than 2-APB (Gafni et al. 1997; Bootman et al. 2002). The slower action of XeC was not an impediment in the present in vivo experiments, as cognitive function was measured 20 min following drug infusion. Our observation of similar effects of XeC and 2-APB indicate that effects of these drugs are due to their common mechanism of reduced intracellular Ca²⁺ release.

The beneficial effects of IP₃R blockade likely arise, at least in part, from reduced activation of SK channels, which are capable of transducing fluctuations in intracellular Ca²⁺ concentration into changes in membrane potential (Xia et al. 1998). We observed that SK channels play a similar role to XeC and 2-APB in WM function. Since SK channels require a rise in cytosolic Ca²⁺ (and are unlikely to be effected by store-operated channels), this

is further support that XeC and 2-APB acted by inhibiting IP₃R-mediated internal Ca²⁺ release. Previous research indicates a role for apamin-sensitive SK channels in behaviorally relevant neuronal activity in the hippocampus, amygdala, and sensory neurons (Stackman et al. 2002; Faber et al. 2005; Hammond et al. 2006; Ellis et al. 2007). Cortical pyramidal neurons also express SK channels (Stocker and Pedarzani 2000; Hagenston et al. 2007). Since these channels play a role in various aspects of neuronal dynamics including synaptic excitability (Stackman et al. 2002) and spike frequency adaptation (Pedarzani et al. 2005), it is likely that they influence the reverberating, excitatory prefrontal networks that maintain information over the delay period of a WM task (Goldman-Rakic 1995a,b). Consistent with in vitro studies showing SK channel activation underlies IP₃-mediated prefrontal cortical pyramidal cell hyperpolarization (Hagenston et al. 2007), we found that blockade of SK channels within the medial prefrontal cortex improved WM performance. It is unlikely that these enhancements reflect nonspecific motor effects, as run-times were similar following drug and vehicle treatments.

The beneficial effects of IP₃R blockade may arise from inhibition of Ca²⁺-dependent enzymes such as PKC and CamKII, which impair basal WM performance (Birnbaum et al. 2004; Runyan et al. 2005). This is consistent with the Ca²⁺ phosphatase calcineurin improving WM performance (Runyan et al. 2005; Dash et al. 2007). The mechanism through which Ca²⁺-activated enzymes effect WM performance may include SK channel activation; however, it is important to note that they can also influence the kinetics of a variety of other channels including K_v 1.4, K_v 2.1, and L-type Ca²⁺ channels (Roeper et al. 1997; Norris et al. 2002; Misonou et al. 2004).

Increased intracellular Ca²⁺ may also stimulate adenylyl cyclase (Litvin et al. 2003), resulting in increased cAMP-hyperpolarization-activated cyclic nucleotide-gated cation

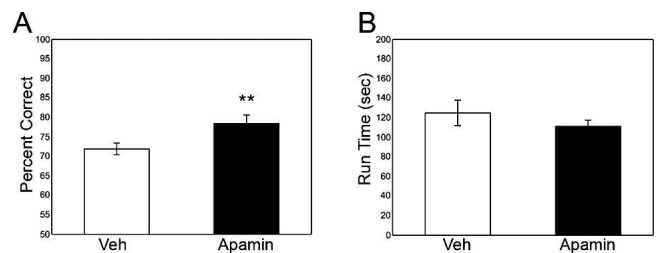


Figure 3. Experiment 3 examined WM performance following intra-prefrontal cortical infusion of the SK2 channel blocker, apamin. (A) Performance following 0.1 μ g/0.5 μ L apamin was significantly improved relative to vehicle. (B) Run-time was not significantly influenced by drug treatment, indicating that enhanced WM performance was not due to nonspecific motor or motivational effects. Means \pm SEM are shown. (**) $P < 0.01$.

(HCN) channel signaling (for review, see Dash et al. 2007; Wang et al. 2007). Our lab has recently shown that HCN channels are in close conjunction with both glutamatergic and adrenergic synapses and act to shunt local current. cAMP-HCN channel signaling profoundly impacts prefrontal network operations, and ultimately, impairs WM performance (Wang et al. 2007). Similar studies examining how the spatial and temporal properties of IP₃R and SK channels might contribute to prefrontal cortex network function underlying WM have yet to be performed.

The work presented here indicates that WM is impaired by IP₃R-mediated signaling within the prefrontal cortex. Inhibiting this arm of the PI cascade may enhance or protect prefrontal cognitive function by (1) blocking Ca²⁺-activated enzymes that have been previously shown to impair WM, (2) inhibiting the activation of SK channels, and thereby protecting the excitatory networks that underlie WM, or (3) some combination thereof. Research delineating the contributions of specific ion channels and kinases to prefrontal neuronal and cognitive function will provide important information about the therapeutic potential of the agents investigated in this study. Such studies will benefit from in vivo reversal studies requiring IP₃R activation. Until a reliable IP₃R agonist becomes available, the evidence for a link between IP₃R and SK channel activity in prefrontal cognitive function will remain indirect.

Acknowledgments

This research was supported by NIH Conte Center grant P50MH068789 to A.F.T.A and M.Y. and The National Science Foundation Graduate Research Fellowship to A.R.B.

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Received October 18, 2007; accepted in revised form January 8, 2008.