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D_2 DOPAMINE RECEPTORS MODULATE Gα-SUBUNIT COUPLING OF THE CB_1 CANNABINOID RECEPTOR.

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Non-standard abbreviations:
AEA: Anandamide
CB1: Brain cannabinoid receptor
CHO: Chinese hamster ovary cell line
CP: CP55,940
D2: Type 2 dopamine receptor
DMEM: Dulbecco's Modified Eagle Medium
FSK: Forskolin
HEK-293: Human embryonic kidney cell line
PTX: pertussis toxin
Quin: Quinpirole
SR: SR141716A

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Abstract

CB_1 cannabinoid (CB_1) and D_2 dopamine (D_2) receptors are known to couple to the G-protein $G\alpha_{i/o}$. It has been reported that concurrent activation of D_2 receptors and CB_1 receptors, in primary striatal neuronal culture, promotes functional CB_1 receptor coupling to $G\alpha_s$ resulting in elevations in intracellular cyclic AMP levels. We now report that in the absence of D_2 receptors, acute activation of CB_1 receptors inhibits cyclic AMP accumulation, whereas the presence of D_2 receptors promotes CB_1 stimulated cAMP accumulation, presumably through $G\alpha_s$. This $G\alpha_s$ subunit switching was not prevented by pertussis toxin treatment and occurred in the presence and absence of D_2 receptor activation. Thus, co-expression of the D_2 receptor with the CB_1 receptor was sufficient to switch the coupling of the CB_1 receptors from $G\alpha_{i/o}$ to $G\alpha_s$. Persistent activation of D_2 receptors resulted in heterologous sensitization of adenylate cyclase to subsequent stimulation by forskolin, whereas the persistent activation of CB_1 receptors did not. Additional studies in human embryonic kidney cells co-transfected with D_2 and CB_1 receptors revealed that persistent activation (18 hr) of D_2 receptors induced a switch of CB_1 receptor coupling from $G\alpha_s$ to $G\alpha_{i/o}$. This D_2 receptor-induced effect allowed for CB_1 receptor-mediated inhibition of cyclic AMP accumulation. The present studies suggest D_2 receptors may have a significant modulatory role in determining the G protein coupling specificity of CB_1 receptors.

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The CB_1 cannabinoid (CB_1) receptor is expressed primarily in the central nervous system especially in the basal ganglia and cortex (Herkenham et al., 1990; Matsuda et al., 1993; Tsou et al., 1998), with some expression occurring in peripheral tissues such as the uterus, testes, and ileum (Das et al., 1995; Pacheco et al., 1991; Pertwee et al., 1992). This distribution pattern in the brain suggests that the cannabinoid system and the ascending dopamine pathways may interact with one another. Indeed, functional links between dopaminergic and cannabinoid systems have been reported (Giuffrida et al., 1999; Glass and Felder, 1997; Mailleux and Vanderhaeghen, 1993). Microdialysis experiments have demonstrated that activation of D_2 dopamine receptors (D_2) promotes elevations in extracellular concentrations of the endocannabinoid anandamide (AEA) (Giuffrida et al., 1999). Chronic treatment with D_2 receptor antagonists causes upregulation of CB_1 mRNA in striatum (Giuffrida et al., 1999).

When expressed individually, activation of either the D_2 or CB_1 receptor inhibits cAMP accumulation. Several studies have shown that the CB_1 receptor inhibits adenylate cyclase activity through coupling with a pertussis toxin-sensitive $G\alpha_{i/o}$-protein (for review see Howlett, 1995). The D_2 receptor also inhibits adenylate cyclase via pertussis toxin-sensitive $G\alpha_{i/o}$-proteins (Sibley and Monsma, Jr., 1992). Our interest in dopamine-cannabinoid interactions stems from reports that concurrent activation of D_2 and CB_1 receptors alters CB_1 receptor coupling to signal transduction mechanisms. Specifically, in primary striatal cultured neurons, D_2 receptor activation shifts CB_1 receptor coupling from an inhibitory effect on the formation of the signaling molecule cyclic AMP (cAMP) to a stimulatory effect on cAMP formation (Glass and Felder, 1997). Glass and Felder (1997) have suggested this increase in cAMP is brought about by the CB_1 receptor

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switching from $G\alpha_{i/o}$ to $G\alpha_s$ linkage. Additional studies demonstrating the $G\alpha_s$ linkage of CB_1 receptors were carried out in Chinese hamster ovary (CHO) cells stably expressing only the human CB_1 receptors (Glass and Felder, 1997; Felder et al., 1998; Bonhaus et al., 1998). In the CB_1 receptor/CHO cells augmentation of forskolin-stimulated cAMP accumulation upon CB_1 receptor activation was observed only after pertussis toxin pre-treatment. Together, these studies described above indicate that CB_1 receptors can couple to multiple G proteins (i.e. $G\alpha_s$ and $G\alpha_{i/o}$) following acute activation.

Acute activation of $G\alpha_s$-coupled receptors enhances adenylate cyclase activity which increases cAMP levels; whereas acute activation of $G\alpha_{i/o}$-coupled receptors decreases adenylate cyclase activity which results in decreased cAMP levels. However, long-term activation of $G\alpha_{i/o}$-coupled receptors enhances subsequent stimulation of adenylate cyclase, a pharmacological phenomena known as heterologous sensitization (for review see Watts, 2002). Although the exact mechanisms are not yet fully elucidated, persistent activation of a $G\alpha_{i/o}$-coupled receptor induces heterologous sensitization via a pertussis toxin-sensitive G protein (Watts, 2002). Chronic activation of $G\alpha_{i/o}$-coupled receptors such as D_2 and CB_1 receptors has been reported to potentiate adenylate cyclase responsiveness upon subsequent drug-stimulated cAMP accumulation (Watts and Neve, 1996;Rhee et al., 2000).

Because cultured cells have a unique composition of G proteins and adenylate cyclase isoforms, our initial experiments have focused on asking the question how generalizable is the D_2 receptor effect on CB_1 receptor coupling? Do the D_2 receptors promote the CB_1 receptors to switch from $G\alpha_{i/o}$ to $G\alpha_s$ in transfected systems? Moreover, does the influence of the D_2 receptor also affect the ability of the CB_1 receptor to

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sensitize adenylate cyclase and vice versa? What are the effects of persistent activation of the D_2 and CB_1 receptor on adenylate cyclase? To address these questions, we investigated D_2 and CB_1 receptor signaling in HEK-293 cells. Our studies showed that the D_2 receptor dramatically influences CB_1 receptor coupling to Gα-subunits. We determined that co-expression of the two receptors induces the CB_1 receptor to switch to $G\alpha_s$-coupling; although, activation of the D_2 receptor is not necessary. Furthermore, overexpression of $G\alpha_{i1}$ or persistent activation of the D_2 receptor appeared to facilitate the re-establishment of $G\alpha_{i/o}$-coupling for the CB_1 receptor. We asked questions seeking to further refine the relationship between D_2 and CB_1 receptor signaling. In the present study, we provide data that demonstrate the D_2 receptor's ability to regulate the G protein coupling specificity of CB_1 receptors.

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Methods

Materials

[^{3}H]Cyclic AMP (32 Ci/mmol) was purchased from Perkin Elmer Life Science Products (Boston, MA, U.S.A). Forskolin, pertussis toxin, and (-)quinpirole were purchased from RBI/Sigma (Natick, MA, U.S.A.). CB_1 receptor cDNA was a gift from Dr. Tom Bonner. The G-protein α subunit cDNAs were purchased from the Guthrie Research Institute (Sayre, PA, U.S.A). CP55,940 was a generous gift of the Research Triangle Institute (Research Triangle Park, NC, U.S.A.). All other drugs and chemicals were of the highest grade possible and were purchased from standard commercial sources.

Cell Culture

HEK-293 wild-type cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum supplemented with 1% penicillin/streptomycin, and 1% L-glutamine. HEK-293 cells stably transfected with the D_2 receptor (HEK/D_2) were maintained in DMEM with 5% fetal bovine serum, 5% bovine calf serum supplemented with 2 mg/ml purinomycin, 1% penicillin/streptomycin, and 1% L-glutamine. The cells were grown in a 37°C humidified environment with 5% CO_2. Where indicated, cells were transfected using hCB_1pRcCMV, $G\alpha_{i1}$pcDNA3.1, $G\alpha_o$pcDNA3.1,and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions.

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Cyclic AMP Accumulation Assay

Cells were plated at a density of 50,000 cells/well in 24-well culture plates. Cells were transfected 72 hours after plating. Experiments were performed 48 hours after the transfection. The cells were washed once with warm (37°C) Krebs-Ringer-HEPES (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM HEPES, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, pH 7.4). The indicated drugs were added, and the cells were incubated in a 37°C water bath for 15 minutes. Where required, cells were treated for 18 hours with 5 ng/mL of pertussis toxin. Where indicated, cells were incubated with 1 μM SR141716A for 10 minutes prior to other drug stimulation. Following the incubation, the stimulation media was aspirated, and the reaction was terminated with 500 μL /well of ice-cold 3% trichloroacetic acid. The 24-well culture plates were stored at 4^{o}C for up to 1 week prior to analysis. Cyclic AMP accumulation was quantified using a competitive binding assay (Nordstedt and Fredholm, 1990) with minor modifications (Watts and Neve, 1996). Samples of the cell lysate (10 μL) were added to reaction tubes. [^{3}H]Cyclic AMP (~1 nM final concentration) and cyclic AMP binding protein (ca. 150 μg) were diluted in cyclic AMP assay buffer (100 mM Tris/HCl, (pH 7), 100 mM NaCl, 5 mM EDTA) and then added to each well for a total volume of 550 μL. The tubes were incubated on ice for 2-3 hours and were harvested by filtration (Packard Unifilter GF/C) using a 96-well Packard Filtermate Cell harvester (Meriden, CT). The filters were allowed to dry and Microscint O scintillation fluid was added. Radioactivity on the filters was determined using a Packard TopCount scintillation/luminescence detector.

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Data Analysis

Experiments were performed in triplicate and repeated in three separate assays. Analyses of data were done using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.).

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Results

Acute activation of the CB_1 receptor inhibits cAMP accumulation. HEK-293 cells were transiently transfected with the CB_1 receptor (HEK-293 CB_1 cells). Forskolin (10 μM) alone increased the cAMP accumulation several fold as expected when compared with the vehicle-treated cells. The addition of CP55,940 (a CB_1 receptor agonist) inhibited forskolin-stimulated cAMP accumulation by > 95% (Figure 1). The ability of CP55,940 to inhibit cAMP accumulation was prevented by the addition of 1 μM SR141716A (a CB_1 receptor antagonist). The results of these experiments indicated that the lower levels of cAMP brought about by CP55,940 were a CB_1 receptor-mediated response. CP55,940-mediated inhibition of cAMP was also examined in cells that were pre-treated with 5 ng/mL of pertussis toxin for 18 hours. Pre-treatment with pertussis toxin which ADP-ribosylates $G\alpha_{i/o}$ and prevents the G protein heterotrimers from interacting with the receptor, blocked the effects of CP55,940 (Figure 1). The pertussis toxin pre-treatment data confirm that the CB_1 receptor is $G\alpha_{i/o}$-coupled in HEK-293 CB_1 cells. CP55,940 did not inhibit forskolin-stimulated cAMP accumulation in wild-type HEK-293 cells (data not shown), indicating that there are no functional CB_1 receptors in HEK-293 wild-type cells.

D_2 receptor expression alters CB_1 receptor signaling. These experiments were performed in a HEK-293 cell line that expressed both the D_2 and CB_1 receptors; the D_2 was stably expressed and the CB_1 receptor was transiently transfected into the cell line (HEK-293/D_2 CB_1 cells). The addition of forskolin (10 μM) induced a marked increase in cAMP; however, this increase was not inhibited by CP55,940. Surprisingly, the addition of CP55,940 to HEK-293/D2 CB_1 cells increased forskolin-stimulated cAMP

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accumulation to nearly 150% compared to forskolin alone (Figure 2A). This increase was antagonized when SR141716A was present in the incubation (Figure 2A), which indicated that the CB_1 receptor was responsible for the increase in cAMP levels. CP55,940 concentration-dependently enhanced forskolin-stimulated cAMP accumulation (Figure 2B, open circles). Additional experiments examined this concentration-dependent increase in cAMP in HEK-293/D_2 CB_1 cells that were pre-treated with pertussis toxin (5 ng/mL:18 hours). Pretreatment with pertussis toxin did not alter the elevated cAMP response (Figure 2B, closed circles), suggesting that the CP55,950-induced elevation in cAMP was not mediated *via* activation of $G\alpha_{i/o}$ proteins. These data suggest that in HEK-293 cells expressing D_2 receptors, CB_1 receptors couple to stimulatory $G\alpha_s$ and not $G\alpha_{i/o}$. Forskolin acts in concert with $G\alpha_s$ to increase adenylate cyclase activity, an action that may allow the CB_1 receptor to stimulate cAMP production. Thus, additional experiments examined the effect of CP55,940 on cAMP accumulation in the absence of forskolin stimulation. These studies demonstrate that CP55,940 stimulated cAMP accumulation in the absence of forskolin. In the absence of forskolin, 10 μM CP55,940 resulted in cAMP levels that were approximately 30% of the forskolin-stimulated cAMP accumulation (Figure 2C). This CP55,940-mediated increase in cAMP is approximately 8-fold higher than the basal response, indicating that CP55,940 is able to increase cAMP accumulation in the absence of forskolin. Co-expression of D_2 and CB_1 receptors in HEK-293 cells resulted in increased levels of cAMP after CB_1 receptor activation, suggesting that in the presence of the D_2 receptor, the CB_1 receptor can switch coupling from $G\alpha_{i/o}$ to $G\alpha_s$ proteins.

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Effect of D_2 receptor activation on CB_1 signaling. In HEK-293/D_2 CB_1 cells, 1 μM quinpirole, a potent D_2 receptor agonist, inhibited forskolin-stimulated cAMP accumulation by 90% (Figure 3A). This quinpirole-mediated inhibition was prevented in cells that were pre-treated with 5 ng/mL of pertussis toxin (Figure 3A), implicating D_2 receptor coupling to $G\alpha_{i/o}$ in HEK-293/D_2 CB_1 cells. The effect of D_2 receptor activation on CB_1 receptor signaling was also examined in these cells. Concurrent activation of the D_2 and CB_1 receptors (10 μM forskolin+ 1 μM quinpirole + 10 μM CP55,940) resulted in increased levels of cAMP when compared to the activation of the D_2 receptor alone (10 μM forskolin + 1 μM quinpirole, Figure 3B). Addition of SR141716A prevented the increase in cAMP brought about by concurrent use of quinpirole and CP55,940 (Figure 3B), indicating that the CB_1 receptor was responsible for the increase in cAMP. The CP55,940-mediated increase in cAMP accumulation was dose-dependent with an approximate EC_{50} value of 300 nM (Figure 3C).

Expression of $G\alpha_{i1}$ promotes CB_1 receptor-mediated inhibition of cAMP accumulation. We considered the possibility that the presence of the D_2 receptor along with the CB_1 receptor in the HEK-293 cells might restrict the availability of the pool of $G\alpha_{i/o}$ proteins. When an equal amount of $G\alpha_{i1}$ cDNA (1x) was co-transfected with the CB_1 receptor into the HEK-293/D_2 receptor cells, the addition of CP55,940 potentiated cAMP accumulation both in the presence and absence of D_2 receptor activation (Figure 4A,C). This effect was consistent with previous observations (Figs. 2 and 3) and comparable to control transfected cells (Figure 4A,C, CB1 only). However, co-transfection of the $G\alpha_{i1}$ cDNA with the CB_1 receptor at a mass ratio of 2:1 (2x) in the HEK-293/D_2 receptor cells, and subsequent stimulation of CB_1 receptors with CP55,940,

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in the absence of D_2 receptor activation, led to inhibition of forskolin-stimulated cAMP accumulation (Fig. 4A). Subsequent experiments revealed that in the presence of D_2 receptor activation, overexpression of $G\alpha_{i1}$ (2x cDNA) appeared to potentiate quinpirole-mediated inhibition of forskolin-stimulated cAMP accumulation (Figure 4C). Additional studies examined the ability of overexpression of $G\alpha_o$ to modulate CB_1 receptor-mediated effects on cAMP accumulation (Figure 4B,D). These results revealed that even at a co-transfection ratio of 2:1 (2x $G\alpha_o$ cDNA) $G\alpha_o$ did not alter CB_1 receptor signaling. These results suggest that overexpression of $G\alpha_{i1}$ in the HEK-293/D_2 CB_1 transfected cells promotes CB_1 receptor coupling to inhibition of cAMP accumulation.

Effect of persistent receptor activation on cAMP accumulation. HEK-293/D_2 CB_1 transfected cells were pre-treated with either 2 μM quinpirole or CP55,940 for 18 hours. When challenged with 10 μM forskolin, the cells pre-treated with the D_2 receptor agonist quinpirole exhibited a marked increase in cAMP production consistent with the development of heterologous sensitization (Figure 5). In contrast, the cells pre-treated with the CB_1 receptor agonist CP55,940 exhibited a forskolin response similar to vehicle pre-treated cells (Figure 5), i.e., they did not show heterologous sensitization to forskolin-stimulated cAMP accumulation. Persistent activation of $G\alpha_{i/o}$-coupled receptors is known to sensitize adenylate cyclase, whereas persistent activation of $G\alpha_s$-coupled receptors does not. Thus, this lack of sensitization after CP55,940 pre-treatment is consistent with the hypothesis that in the presence of D_2 receptors, the CB_1 receptors are coupled to the stimulatory $G\alpha_s$ and not $G\alpha_{i/o}$ (see Figures 2 and 3B). Pre-treatment with the CB_1 receptor agonist CP55,940 did not lead to sensitization of adenylate cyclase,

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whereas pre-treatment with the D_2 receptor agonist quinpirole did result in sensitization of adenylate cyclase (Figure 5).

Persistent activation of the D_2 receptor changes the coupling of the CB_1 receptor to $G\alpha_{i/o}$. The effects of persistent activation of the D_2 receptor on the subsequent activation of the CB_1 receptor were also examined in HEK-293/D_2 CB_1 cells. Cells were pre-treated with either vehicle or 2 μM quinpirole for 18 hours. In vehicle pre-treated cells, forskolin alone stimulated cAMP production; CP55,940 enhanced forskolin-stimulated cAMP accumulation (Figure 6), consistent with a $G\alpha_s$-coupled CB_1 receptor. Persistent D_2 receptor activation (quinpirole pre-treated cells) enhanced forskolin-stimulated cAMP accumulation by greater than 4-fold consistent with the development of heterologous sensitization; surprisingly, the addition of CP55,940 inhibited cAMP accumulation (Figure 6). That CP55,940 inhibits forskolin-stimulated cAMP accumulation in quinpirole pre-treated cells is consistent with a $G\alpha_{i/o}$-coupled receptor. These data suggest that persistent activation of the D_2 receptor re-establishes coupling of the CB_1 receptor to $G\alpha_{i/o}$.

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Discussion

The initial studies demonstrating the $G\alpha_s$-linkage of CB_1 receptors were carried out in striatal neurons in primary culture (Glass and Felder, 1997). Both the CB_1 and D_2 receptor agonists inhibited forskolin-stimulated cAMP accumulation when applied separately. When the two receptor agonists were added concurrently, there was an enhancement of forskolin-stimulated cAMP accumulation. We obtained similar results in HEK-293/D_2 CB_1 cells. Glass and Felder (1997) also showed that in striatal neurons in primary culture, the CB_1 receptor agonist alone was only able to elicit a concentration-dependent increase in forskolin-stimulated cAMP accumulation after pertussis toxin pre-treatment. Other studies demonstrating the $G\alpha_s$-linkage of CB_1 receptors were carried out in CHO cells stably expressing human CB_1 receptors (Glass and Felder, 1997; Felder et al., 1998; Bonhaus et al., 1998). In these studies, various CB_1 cannabinoid receptor agonists inhibited forskolin-stimulated cAMP accumulation. After pertussis toxin pre-treatment, the CB_1 receptor agonists were able to concentration-dependently increase forskolin-stimulated cAMP accumulation. Pertussis toxin pre-treatment ADP-ribosylates $G\alpha_{i/o}$ and prevents the G-protein heterotrimers from interacting with the receptor. Thus, there are no $G\alpha_{i/o}$-subunits left to interact with the CB_1 cannabinoid receptor. Essentially, pertussis toxin pre-treatment promotes CB_1 cannabinoid receptor binding to the $G\alpha_s$-subunits available in the cell.

We showed that in HEK-293/D_2 CB_1 cells, CP55,940 alone elicited a concentration-dependent increase in forskolin-stimulated cAMP accumulation both with and without pertussis toxin pre-treatment. Our studies are the first to demonstrate that co-expression of the D_2 and CB_1 receptors is sufficient to alter coupling. Moreover, we

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also showed that in HEK-293/D_2 CB_1 cells, CP55,940 is able to increase cAMP levels without forskolin stimulation. Heretofore, this finding was only demonstrated in rat globus pallidus slices (Maneuf and Brotchie, 1997).

Activation of the CB_1 receptor, which is normally coupled to inhibitory G proteins ($G\alpha_{i/o}$) should result in an inhibition of adenylate cyclase and subsequent reduction in cellular levels of cAMP (for review see Howlett 1995). Under acute conditions, D_2 receptor activation is not necessary for the switch of the CB_1 receptor from $G\alpha_{i/o}$ to $G\alpha_s$ proteins. The co-expression of the D_2 receptor with the CB_1 receptor was adequate to promote the coupling of CB_1 receptors to $G\alpha_s$. Concurrent activation of the CB_1 and D_2 receptors, with or without pertussis toxin, again points to a non-$G\alpha_{i/o}$ process implicating that the CB_1 receptor is coupled to $G\alpha_s$ instead of $G\alpha_{i/o}$. We suggest that the D_2 receptor may sequester the $G\alpha_{i/o}$ pool preventing the binding of the CB_1 receptor to $G\alpha_{i/o}$, promoting interactions with $G\alpha_s$. It has been shown that the human CB_1 receptor can sequester $G\alpha_{i/o}$ protein from a common pool and prevent other pertussis toxin-sensitive $G\alpha_{i/o}$ receptors from signaling (Vasquez and Lewis, 1999). It has also been shown that cannabinoid and opioid receptors share a common pool of GTP-binding proteins in cotransfected cells (Shapira et al., 2000). It is likely that in our receptor transfected cell line, the D_2 receptor, not the CB_1 receptor sequesters the shared $G\alpha_{i/o}$ pool. Our experiments with overexpression of the $G\alpha$ subunits are consistent with this idea. Overexpression of $G\alpha_{i1}$, but not $G\alpha_o$ promoted CB_1 receptor-mediated inhibition of cAMP accumulation. Our findings suggest that if the D_2 receptor is indeed sequestering $G\alpha_i$ subunits, then this effect can be overcome by overexpressing the $G\alpha_{i1}$ subunit. The ability of CB_1 receptors to couple to $G\alpha_{i1}$ for inhibition of adenylate cyclase is consistent

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with previous findings suggesting that the effectors of $G\alpha_i$ are adenylate cyclase as well as potassium and calcium channels (Ross, 1992).

HEK-293/D_2 CB_1 cells that were pre-treated with quinpirole for 18 hours and then stimulated with forskolin exhibited an amplified cAMP response. This is the phenomena of heterologous sensitization and is an expected response following persistent activation of $G\alpha_{i/o}$-coupled receptors. HEK-293/D_2 CB_1 cells that were pre-treated with CP55,940 for 18 hours and then stimulated with forskolin did not have an amplified cAMP response. Initially, we hypothesized that this lack of sensitization was a result of the CB_1 receptor now being coupled to $G\alpha_s$. However, if the D_2 receptor has caused the CB_1 receptor to switch to $G\alpha_s$, then chronic activation of the D_2 receptor should result in a cAMP accumulation that is amplified when acutely stimulated with forskolin alone and heightened even more when stimulated with forskolin and a CB_1 receptor agonist. We predicted this because chronic activation of the D_2 receptor sensitizes adenylate cyclase, and the expression of this amplified cAMP response is thought to be a $G\alpha_s$-mediated event (for review see Watts, 2002). As expected quinpirole pre-treated cells showed an amplified cAMP response when challenged with forskolin. However, there was no heightened response for quinpirole pre-treated cells that were challenged with forskolin and CP55,940 concurrently. Not only was there no amplified cAMP response, CP55,940 markedly inhibited forskolin-stimulated cyclic AMP accumulation. If the CB_1 receptor remained $G\alpha_s$-coupled after sensitization, then we would have expected to see a sensitized response. In contrast, persistent activation of the D_2 receptor appears to revert the CB_1 receptor back to coupling with $G\alpha_{i/o}$.

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Our data led us to suggest the following model. When the CB_1 receptor is expressed alone in HEK-293 cells, it is coupled to the $G\alpha_{i/o}$ subunit. Co-expression of D_2 and CB_1 receptors in HEK-293 cells, resulted in the coupling of the CB_1 receptor to $G\alpha_s$, as a result of $G\alpha_{i/o}$ sequestration by the D_2 receptor. Overexpression of $G\alpha_{i1}$ restores coupling of the CB_1 receptor with $G\alpha_i$, consistent with our sequestration hypothesis. Persistent activation of the D_2 receptor also facilitates the re-establishment of $G\alpha_{i/o}$ coupling with the CB_1 receptor. The mechanisms for G protein switching remain unknown, however changes in membrane microdomain localization of $G\alpha$ subunits may be involved. For example, chronic activation of $G\alpha_{i/o}$-coupled receptors decreases detergent solubility of G protein subunits (i.e., $G\alpha_i$ and β) which is thought to correlate to compartmental alterations that the G protein α subunits undergo (Bayewitch et al., 2000). Alternatively, chronic drug treatment may alter the membrane microdomain localization of $G\alpha_s$ that influences receptor modulated adenylate cyclase activity (Ammer and Schulz, 1997; Ostrom et al., 2001)

Is there a physiological relevance for the coupling of the CB_1 receptor to $G\alpha_s$? A single receptor subtype can be affiliated with multiple signal transduction pathways. It is likely that different effectors may be activated by more than one G-protein subtype. It is also likely that not all signaling pathways are active all the time. These and other data in the literature suggest that certain signaling pathways can be affected by interactions with other receptors, perhaps to provide a more specific regulation of these pathways. Is there a physiological relevance for the re-establishment of $G\alpha_{i/o}$-coupling when the D_2 receptor is chronically activated? The utility of this shutdown in cAMP production is very logical when examined in the context of the "dopamine hypothesis" of schizophrenia. In this

hypothesis, schizophrenia patients have excessive dopaminergic activity that may be the underlying cause of schizophrenia (Seeman, 1987). The brains of schizophrenic patients have increased levels of adenylate cyclase activity (Kerwin and Beats, 1990; Memo et al., 1983), and there is evidence that indicates adenylate cyclase inhibitors may have a therapeutic role in the treatment of excited psychosis (Roitman et al., 1998). The switch of the CB_1 receptor back to $G\alpha_{i/o}$-coupling under persistent D_2 receptor activation may be the brain's compensatory attempt to ablate the increase in adenylate cyclase activity in the schizophrenic brain. Furthermore, the switching of the CB_1 receptor to $G\alpha_{i/o}$ coupling after persistent D_2 receptor activation may be a regulatory mode to attenuate CB_1 receptor signaling. Giuffrida et al. (1999) have shown that in the rat striatum, anandamide release is stimulated when the D_2 receptors are activated. Persistent activation of D_2 receptors could increase anandamide levels to abnormally high levels. In turn, this could result in cannabinoid hyperactivity. It is conceivable that in addition to the transport process that inactivates anandamide's actions at the synapse, switching coupling back to $G\alpha_{i/o}$ after chronic activation of the D_2 receptor is another way for the cell to modulate CB_1 receptor signaling by essentially switching off cAMP production. These present studies confirm past G protein coupling studies and provide a novel mechanism for D_2 receptor and CB_1 receptor interactions that may play an important role in central nervous disorders associated with the regulation of dopaminergic signaling such as drug abuse and schizophrenia.

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Footnotes

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Figure 1. Effect of CB_1 receptors on cAMP levels in HEK-293 CB_1 cells, under acute stimulation conditions. cAMP accumulation in the presence of 10 µM forskolin (FSK), 10 µM CP55,940 (CP), 1 µM SR141716A (SR), and 5 ng/mL pertussis toxin (PTX). cAMP accumulation assays were performed using a standard competitive cAMP binding assay as described in the methods, using HEK-293 cells transiently transfected with the CB_1 receptor. Data are mean values of three experiments performed in triplicate. Vertical lines represent SEM values. *** Significantly different than the FSK, FSK+SR+CP, and FSK+PTX+CP treated cells, with $p < 0.001$ using ANOVA with Bonferroni post-test.

Figure 2. Effect of co-expression of D_2 and CB_1 receptors on cAMP levels, under acute stimulation conditions. cAMP accumulation in the presence of 10 µM forskolin (FSK), 10 µM CP55,940 (CP), and 1 µM SR141716A (SR) (**A**), or 10 µM FSK, 1 nM to 10 µM CP, and 5 ng/mL pertussis toxin (PTX) (**B**), or 1 nM to 10 µM CP alone (**C**). cAMP accumulation assays were performed using a standard competitive cAMP binding assay as described in the methods, using HEK-293/D_2 cells transiently transfected with the CB_1 receptor. Data are mean values of three experiments performed in triplicate. Vertical lines represent SEM values. ***Significantly different from the FSK and FSK+SR+CP treated cells, with $p < 0.001$ using ANOVA with Bonferroni post-test.

Figure 3. Effect of concurrent activation of D_2 and CB_1 receptors on cAMP levels, under acute stimulation conditions. cAMP accumulation in the presence of 10 µM forskolin (FSK), 1 µM quinpirole (Quin), and 5 ng/mL pertussis toxin (PTX) **(A),** or 10

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μM FSK, 1 μM Quin, 10 μM CP55,940 (CP), and 1 μM SR141716A (SR) **(B)**, or 10 μM FSK, 1 μM Quin, 1 nM to 10 μM CP **(C)**. cAMP accumulation assays were performed using a standard competitive cAMP binding assay as described in the methods, using HEK-293/D_2 cells transiently transfected with the CB_1 receptor. Data are mean values of three experiments performed in triplicate. Vertical lines represent SEM values. **A**:***Significantly different from the FSK and FSK+PTX+Quin treated cells, with $p < 0.001$ using ANOVA with Bonferroni post-test. **B**:***Significantly different than the FSK-treated cells, with $p < 0.001$ using ANOVA with Bonferroni post-test. †††Significantly different than the FSK+Quin and FSK+Quin+SR+CP treated cells, with $p < 0.001$ using ANOVA with Bonferroni post-test.

Figure 4. Effect of overexpression of $G\alpha_{i1}$ and $G\alpha_o$ on cAMP levels in HEK-293/D_2 CB_1 cells, under acute stimulation conditions. cAMP accumulation in the presence of 10 μM forskolin (FSK) and 10 μM CP55,940 (CP) in HEK-293/D_2 CB_1 cells expressing $G\alpha_{i1}$ (**A**), or $G\alpha_o$ (**B**). cAMP accumulation in the presence of 10 μM forskolin (FSK), 1 μM quinpirole (Quin), and 10 μM CP55,940 (CP) in HEK-293/D_2 CB_1 cells expressing $G\alpha_{i1}$ (**C**), or $G\alpha_o$ (**D**). cAMP accumulation assays were performed using a standard competitive cAMP binding assay as described in the methods, using HEK-293/D_2 cells transiently transfected with the CB_1 receptor and an equal amount (1X, 0.9 μg) or twice the amount (2X, 1.8 μg) of the indicated Gα subunit. Data are mean of three experiments performed in triplicate. Vertical lines represent SEM values.

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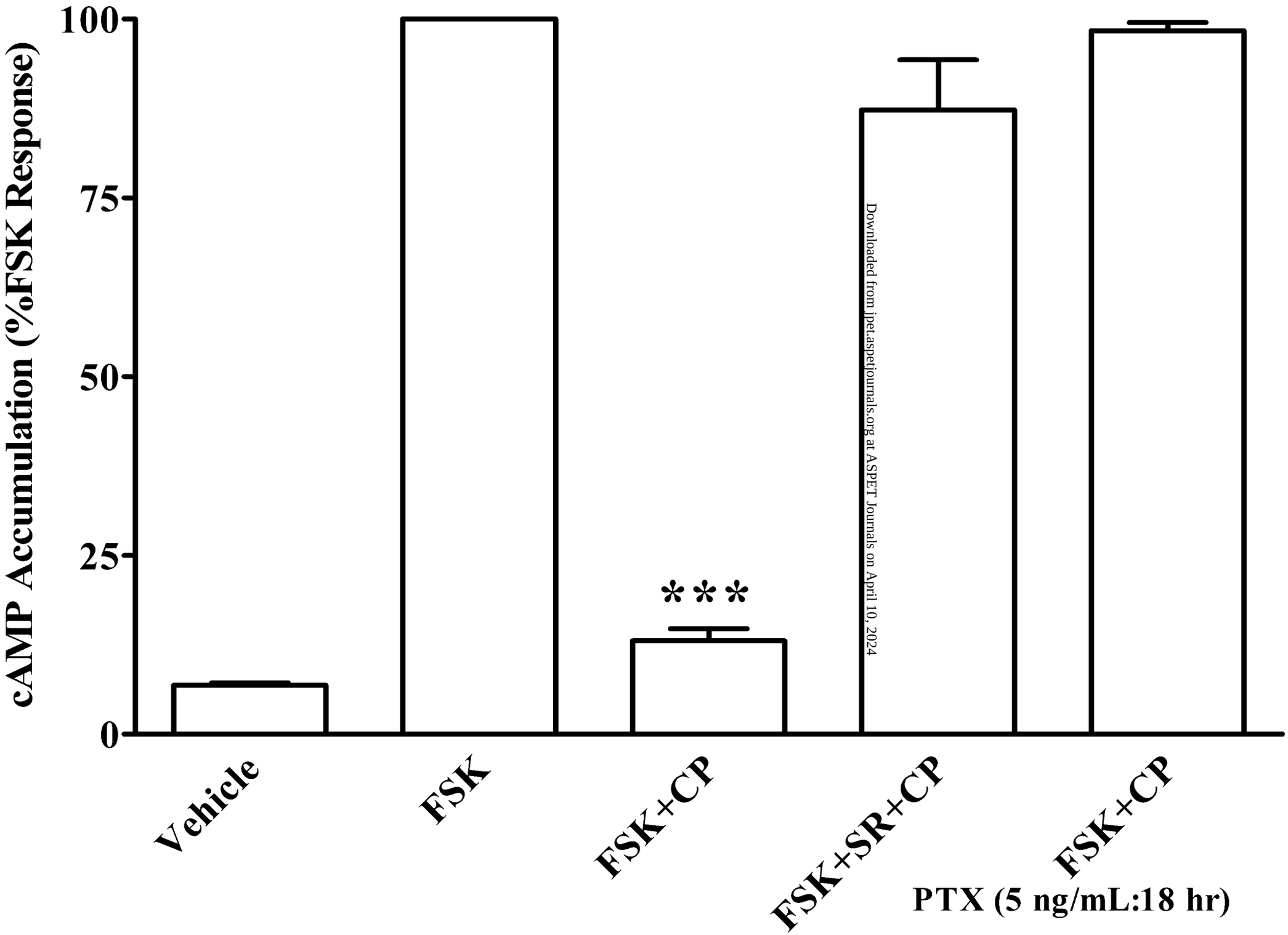
Figure 5. Effect of forskolin on cAMP levels after chronic pretreatment with CP55,940 and quinpirole in HEK-293/D_2 CB_1 cells. cAMP accumulation in the presence of 10 μM forskolin (FSK). Cells were pretreated for 18 hrs. with either 2 μM CP55,940 (CP) or 2 μM quinpirole (Quin). Basal levels of the vehicle, Quin, and CP pretreatments were 6.3 ± 1.1, 5.6 ± 0.7, and 13.3 ± 3.2 pmol/well respectively. cAMP accumulation assays were performed using a standard competitive cAMP binding assay as described in the methods, using HEK-293/D_2 cells transiently transfected with the CB_1 receptor. Data are mean values of three experiments performed in triplicate. Vertical lines represent SEM values. ***Significantly different from the vehicle pretreatment, with $p < 0.001$, using ANOVA with Bonferroni post-test; ††Significantly different from the quinpirole pretreatment, with $p < 0.01$ using ANOVA with Bonferroni post-test.

Figure 6. Effect of forskolin and CP55,940 on cAMP levels after chronic pretreatment with quinpirole in HEK-293/D_2 CB_1 cells. cAMP accumulation in the presence of 10 μM forskolin (FSK) and 10 μM CP55,940 (CP). Cells were pretreated for 18 hours with 2 μM quinpirole. cAMP accumulation assays were performed using a standard competitive cAMP binding assay as described in the methods, using HEK-293/D_2 cells transiently transfected with the CB_1 receptor. Data are mean values of three experiments performed in triplicate. Vertical lines represent SEM values.



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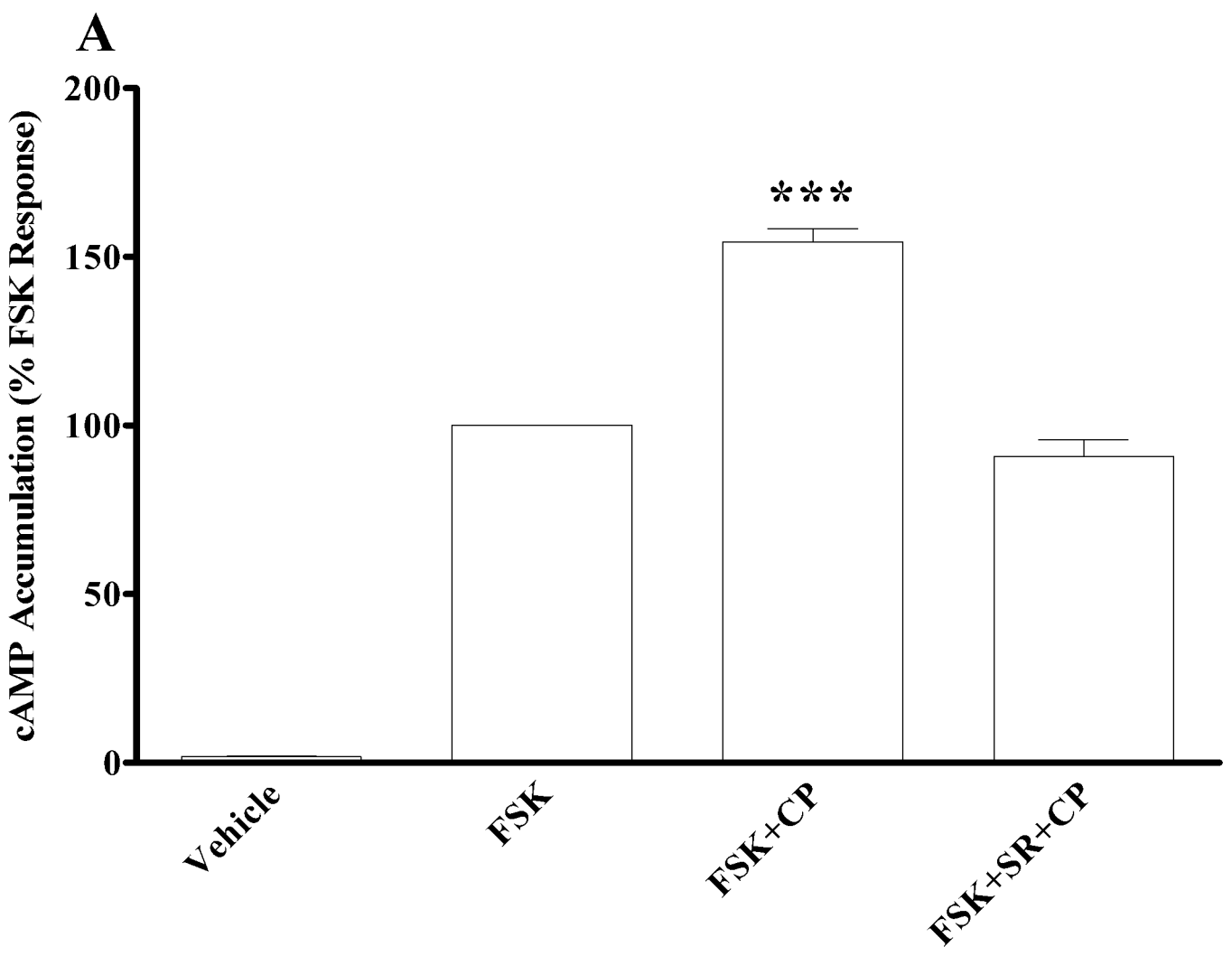

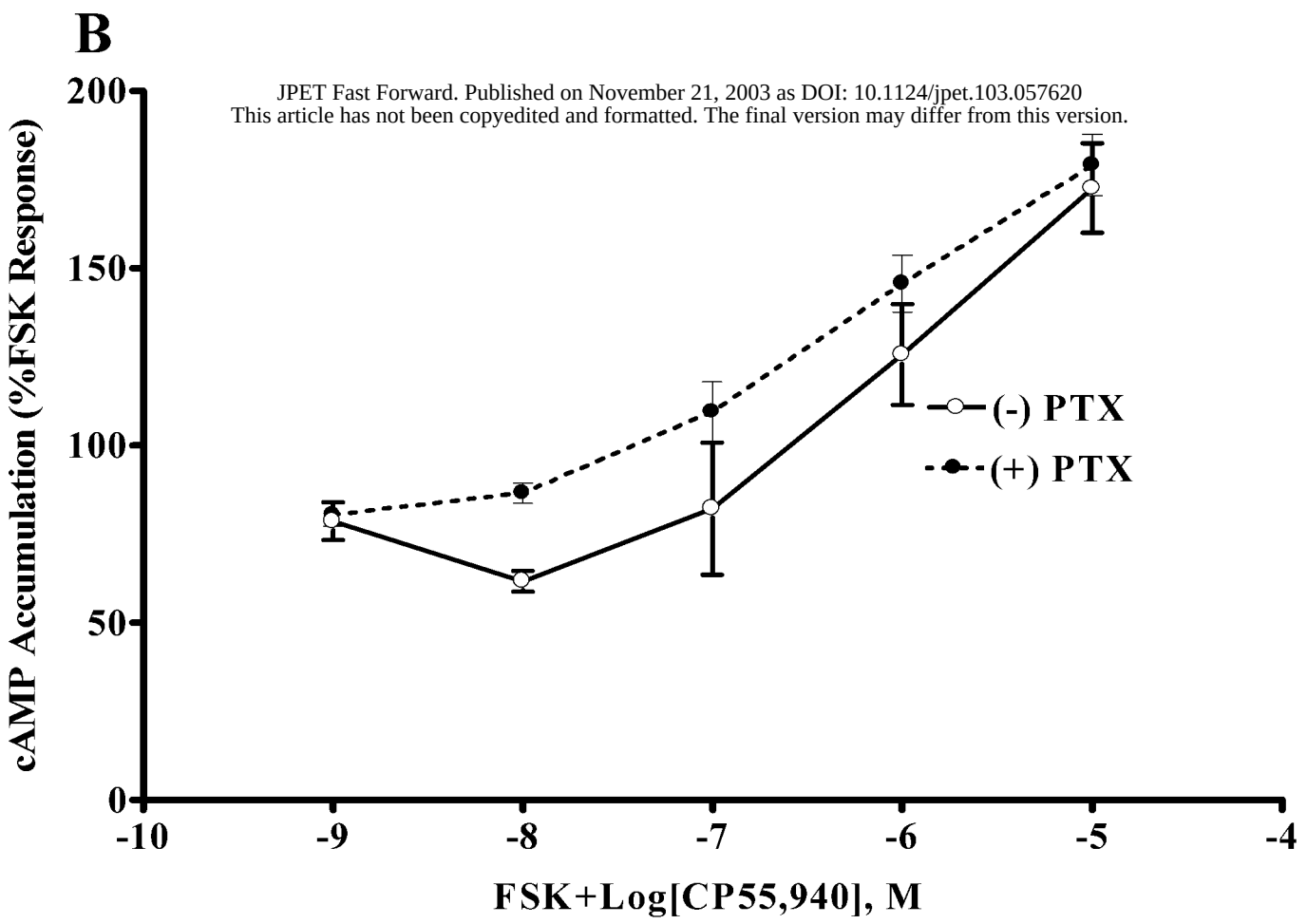

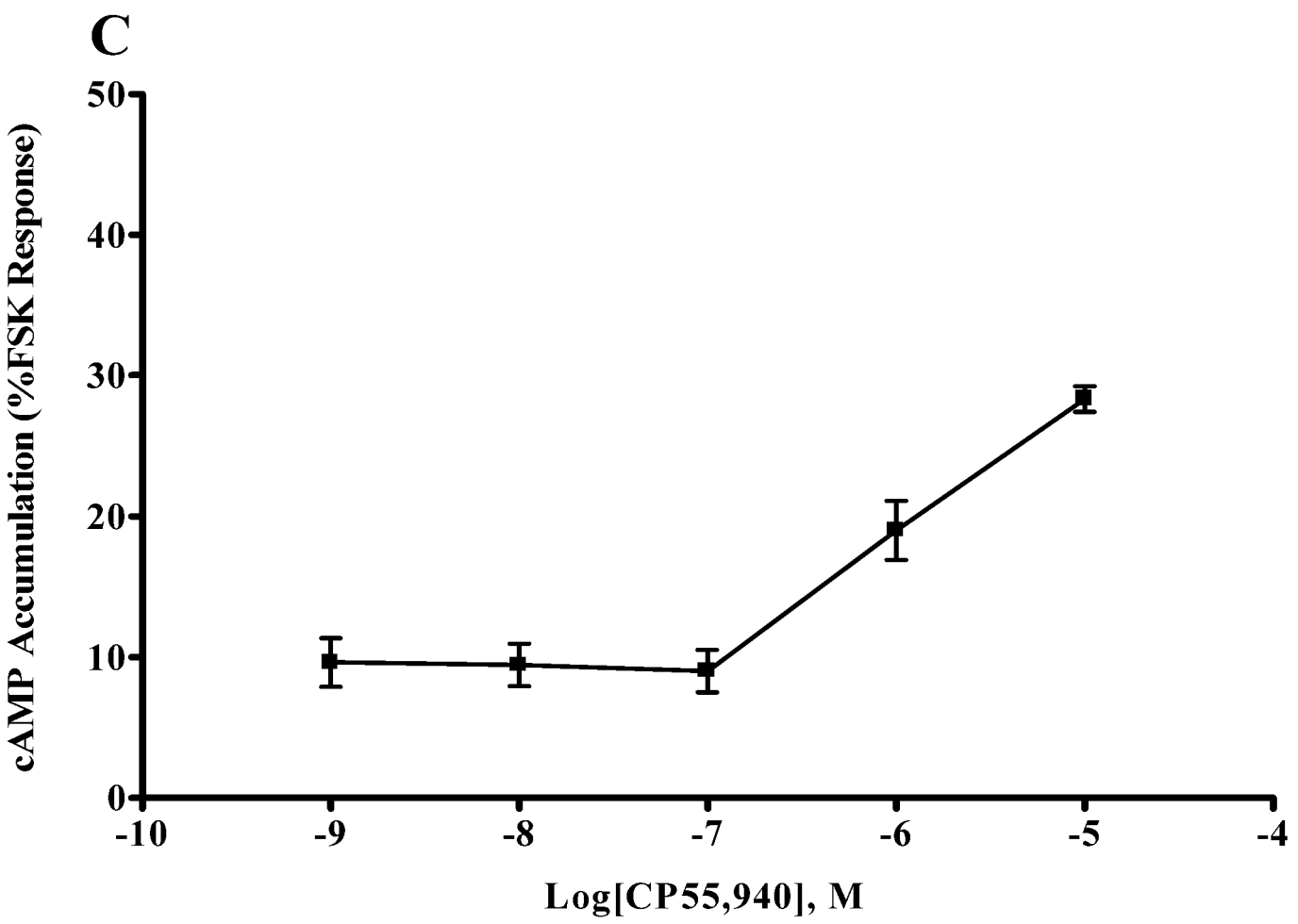

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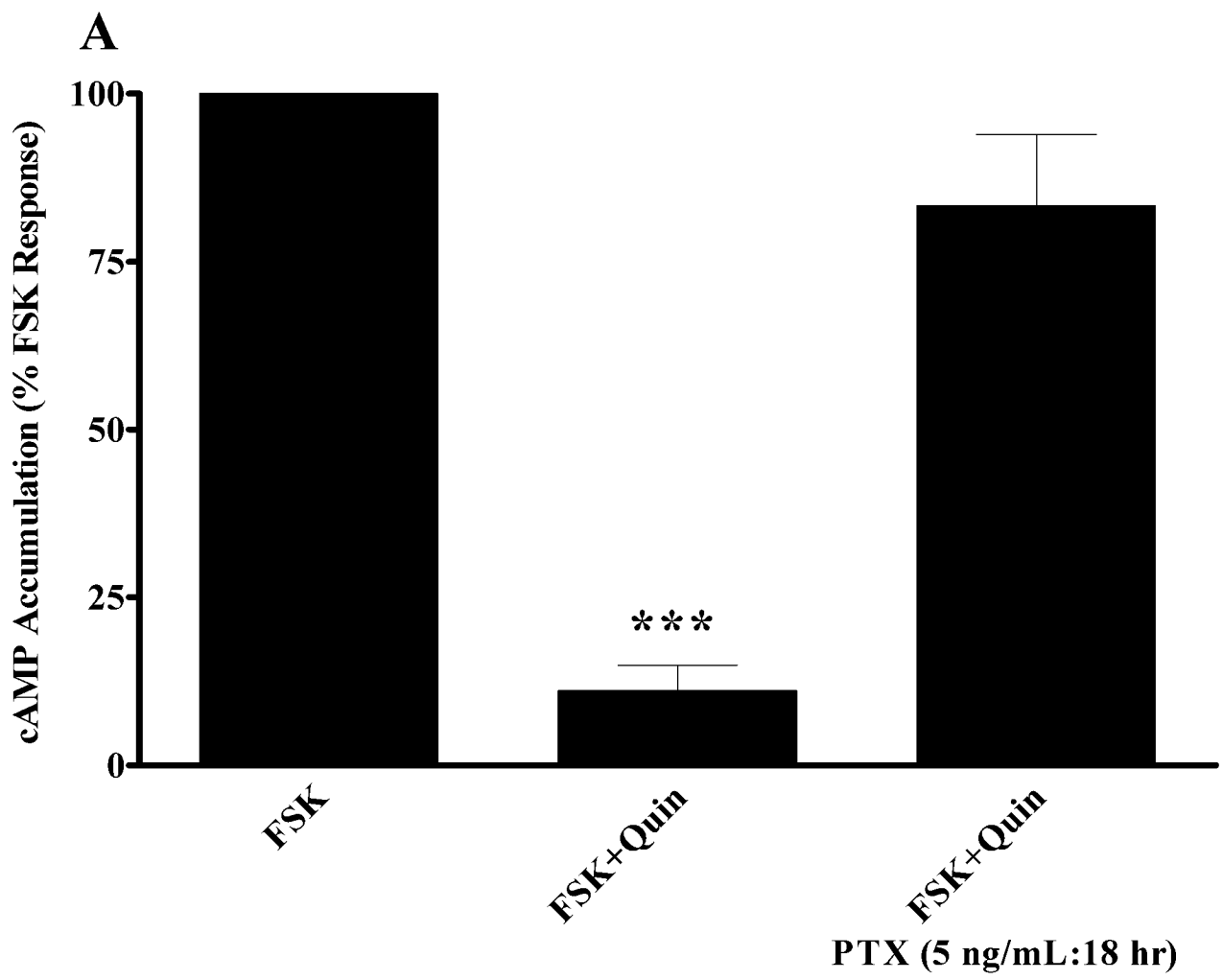

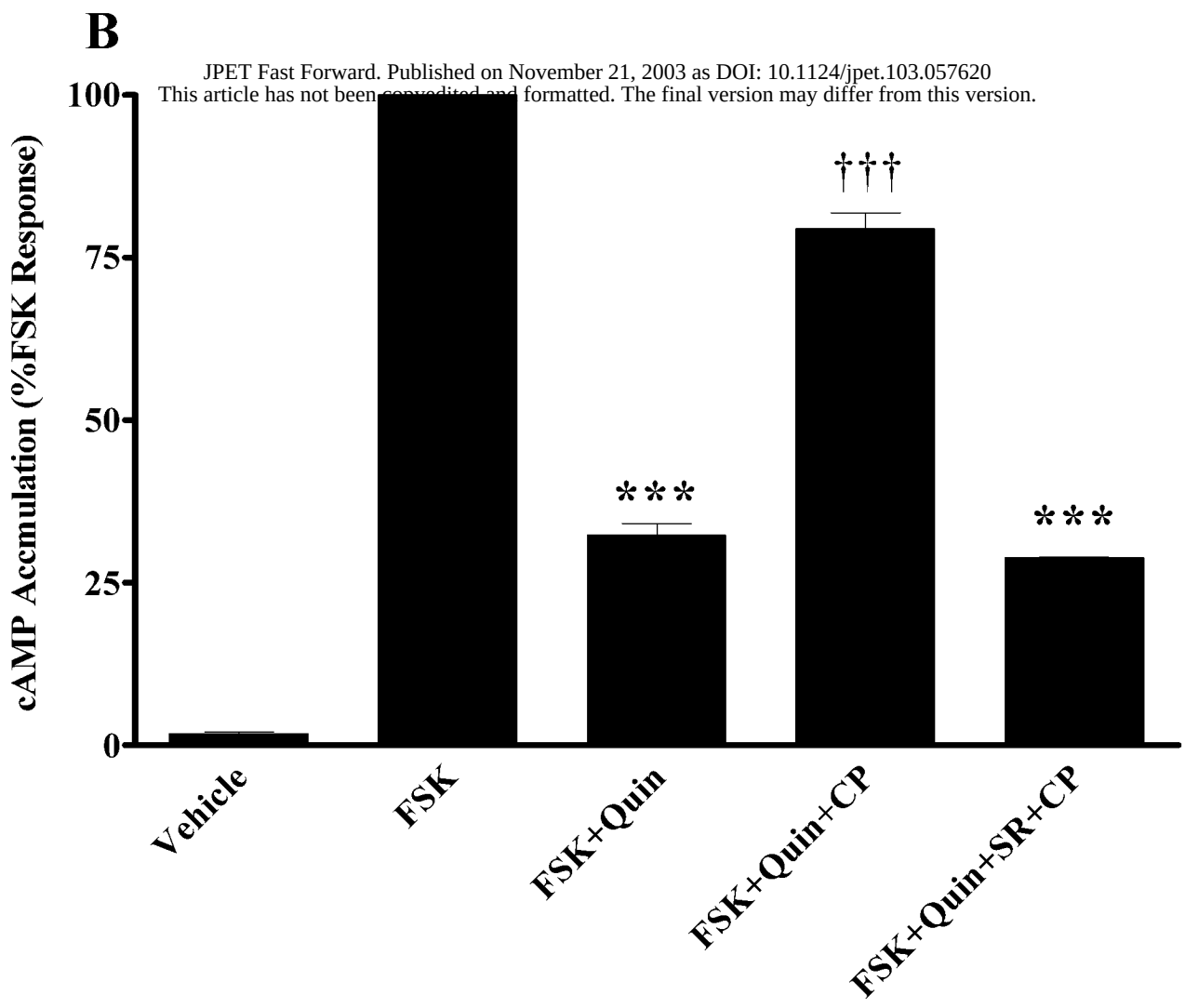

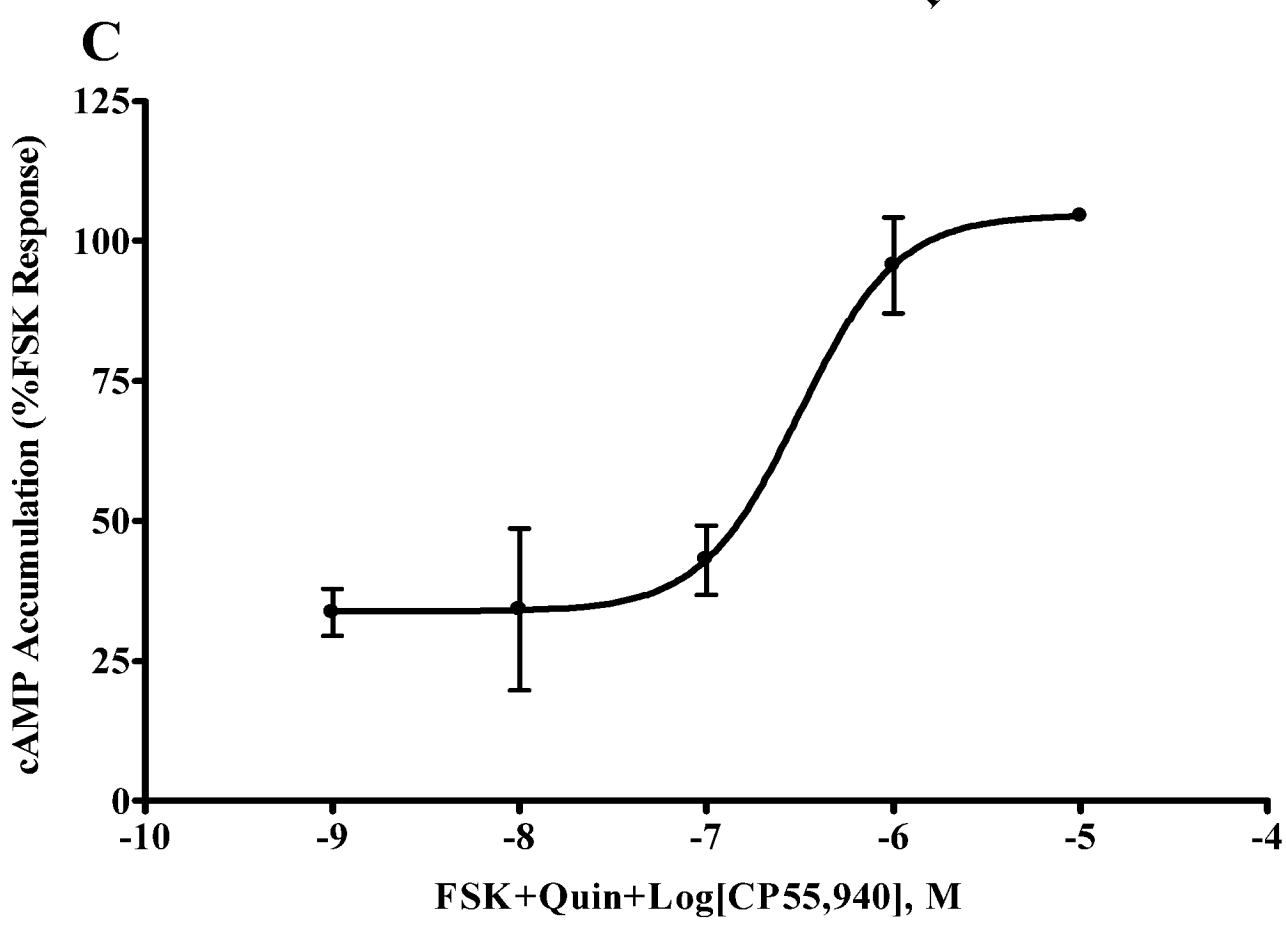

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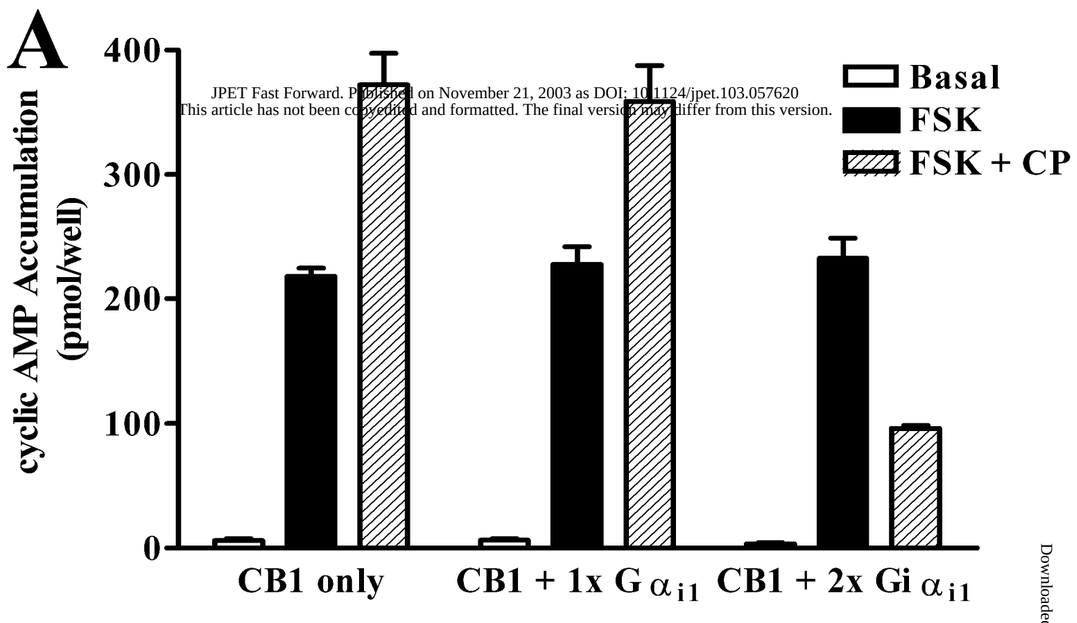

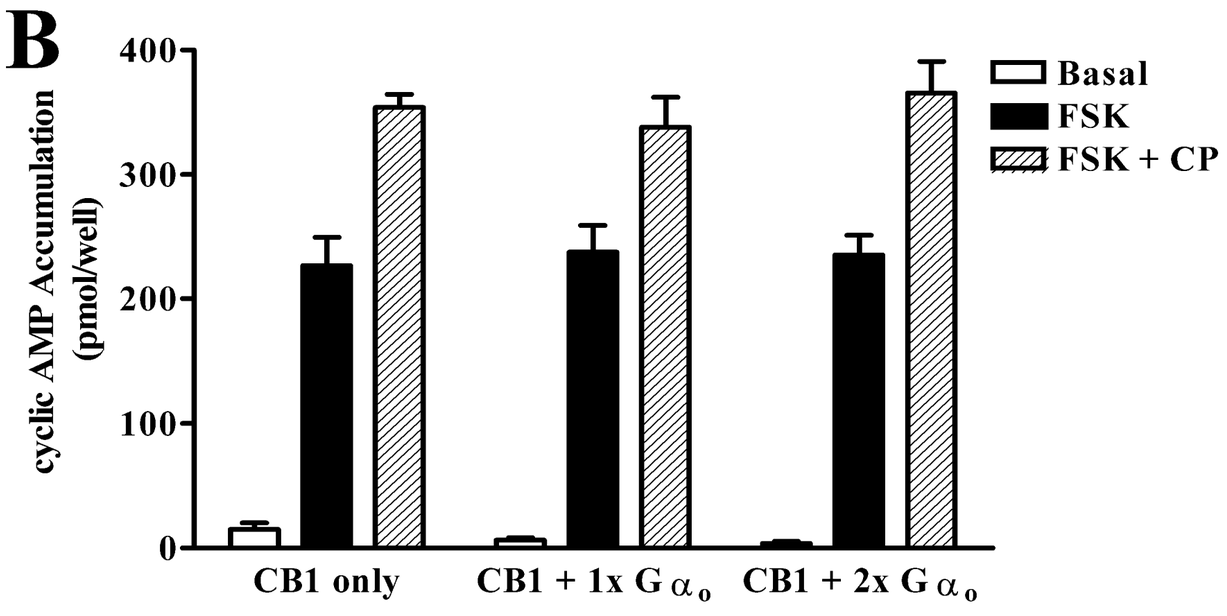

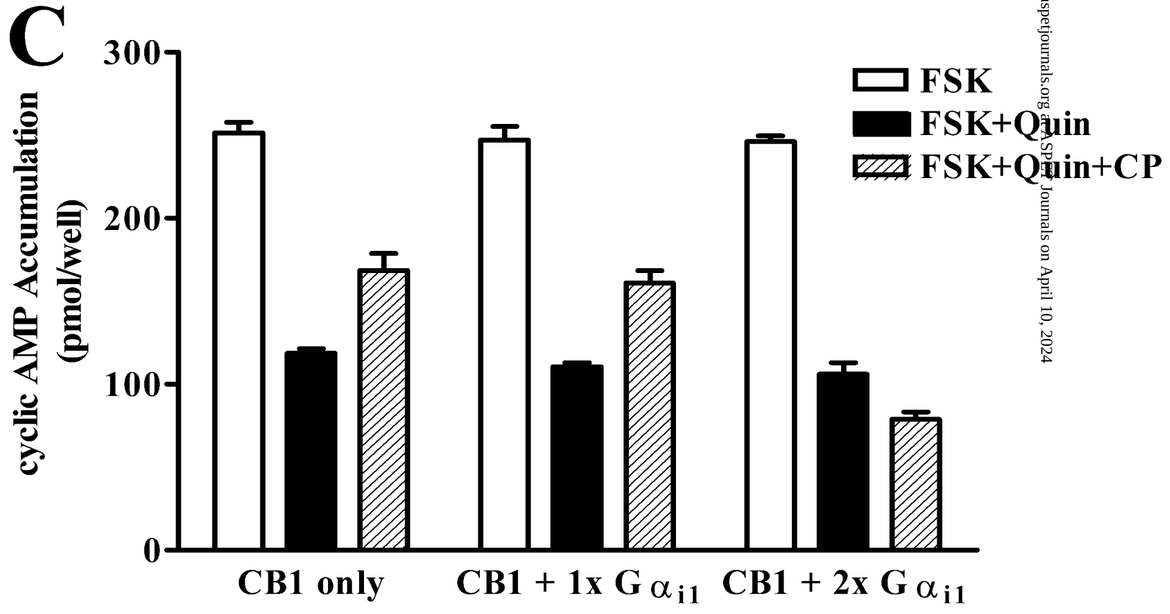

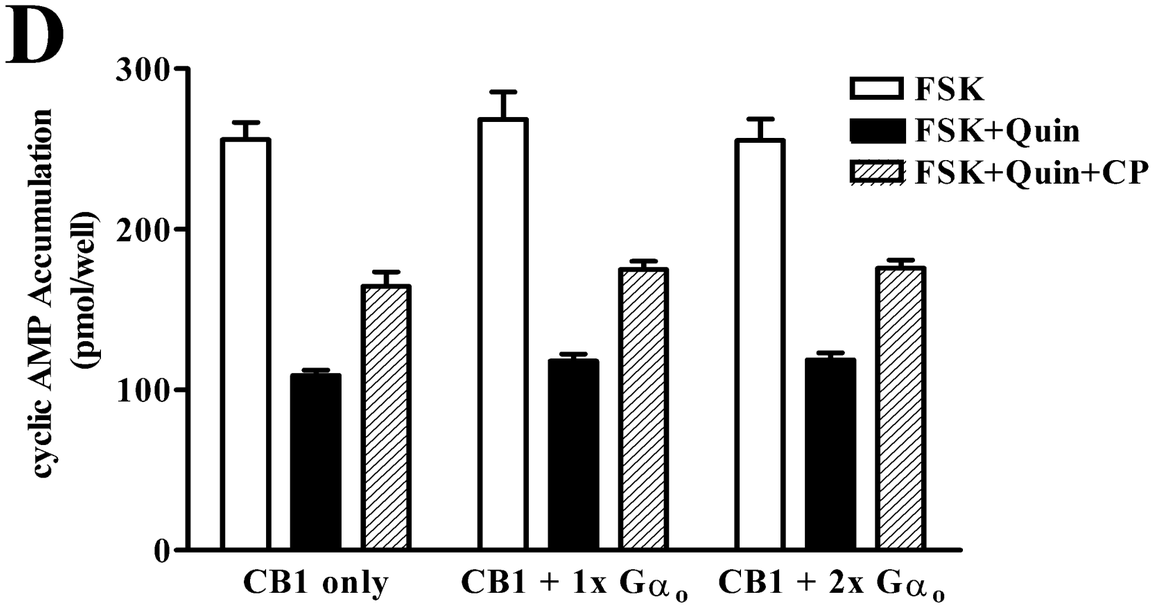

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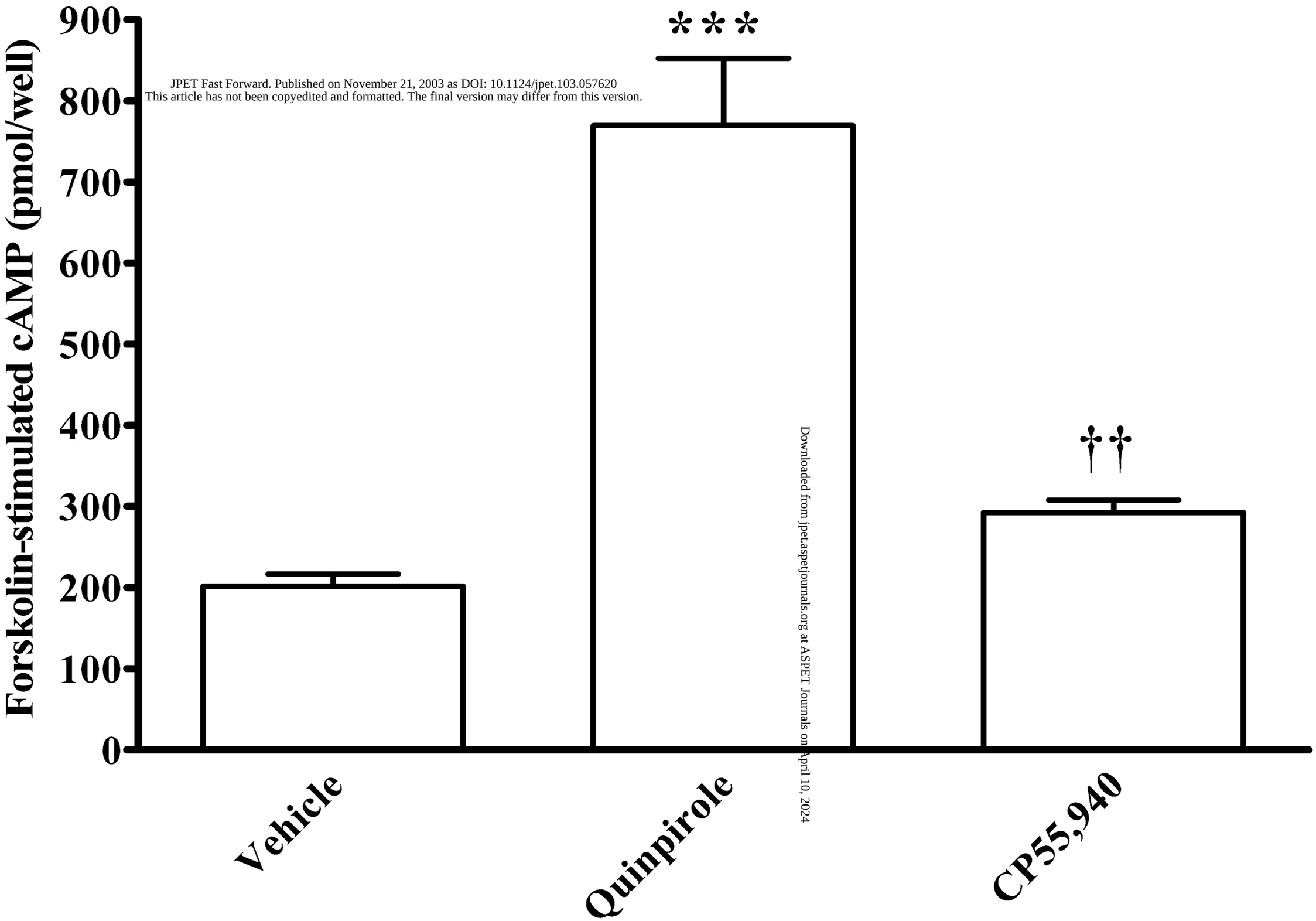
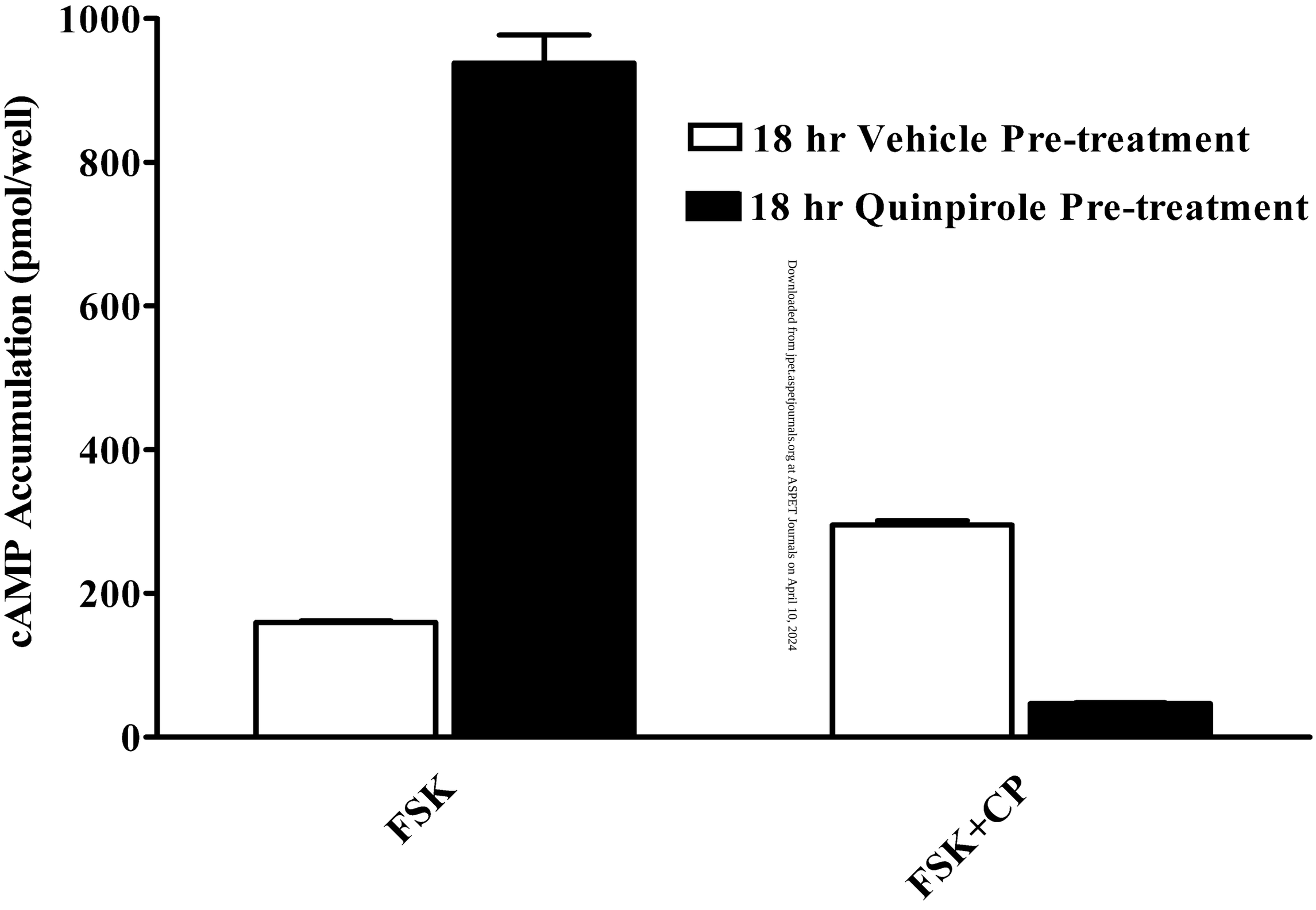


18 hr. Pre-treatment

Jarrahian et al., Figure 5



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