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Activation of cAMP-Dependent Protein Kinase Suppresses the Presynaptic Cannabinoid Inhibition of Glutamatergic Transmission at Corticostriatal Synapses

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ABSTRACT

In a previous study, we showed that type 1 cannabinoid (CB_1) receptor activation substantially depresses the corticostriatal glutamatergic transmission onto striatal neurons in the brain slice preparation. We now report that the adenylyl cyclase activator forskolin and cAMP analog (*S*)-p-8-(4-chlorophenythil) adenosine-3′,5′-monophosphorothioate (Sp-8-CPT-cAMPS) strongly suppressed the synaptic depression induced by cannabimimetic aminoalkylindole, WIN 55,212-2. Application of the cAMP-dependent protein kinase (PKA) inhibitor KT5720 alone had no consistent effect on basal synaptic transmission but the synaptic enhancement elicited by forskolin was blocked. In addition, pretreatment of striatal slices with either KT5720 or another PKA inhibitor, H89, completely abolished the attenuation by forskolin on WIN 55,212-2–induced synaptic depression. The effect of forskolin on CB_1 receptor function was still observed in a low Ca^{2+} bathing solution, suggesting that the forskolin's action is not attributable to its ability to saturate the presynaptic transmitter release processes. The possibility that forskolin acted by increasing CB_1 receptor phosphorylation was confirmed by demonstrating that the serine-phosphorylated component with CB_1 receptors was significantly increased after forskolin treatment. This forskolin effect was markedly attenuated in the presence of KT5720. Moreover, the activation of β-adrenergic receptors by isoproterenol mimics forskolin to elicit a PKA-dependent inhibition of CB_1 receptor function. Together, these observations indicate that the presynaptic inhibitory action of CB_1 receptors at corticostriatal synapses could be negatively regulated by cAMP/PKA-mediated receptor phosphorylation. This effect of PKA may play a functional role in fine-tuning glutamatergic transmission at corticostriatal synapses.

The striatum is the most important nucleus of the basal ganglia, which controls planning and execution of motor functions (Albin et al., 1989). Neurons in the striatum receive a myriad of synaptic inputs originating from different brain regions. For instance, the neocortex (McGeorge and Faull, 1989) and thalamus (Beckstead, 1984) provide the major excitatory inputs to striatal medium spiny projecting neurons (MSNs). MSNs also receive dopaminergic afferent inputs arising from the substantia nigra, which terminates near the corticostriatal inputs (Smith et al., 1994). Recently, there is increasing evidence that the abnormalities in these inputs play a crucial role in the pathophysiology of such diverse basal ganglia disorders as Parkinson's disease (Calabresi et al., 1996) and Huntington's disease (DiFiglia, 1990).

Cannabinoids (CBs), the principal psychoactive component of the marijuana plant, exert many of their effects mainly via the activation of specific G_i/G_o-protein-coupled receptors. So far, two subtypes of cannabinoid receptors, CB_1 and CB_2, have been identified. During the past couple of years, our knowledge concerning these receptors has increased substantially. The CB_1 receptor is distributed predominately in the central nervous system and testis (Gerard et al., 1991; Westlake et al., 1994), and the CB_2 receptor is restricted to the peripheral tissues, where it has been found in the marginal zone of the spleen, tonsils, and immune cells (Galiègue et al., 1995). Putative signal transduction mechanisms have also been ascribed to these receptors. Activation of CB_1 receptor has been shown to inhibit adenylyl cyclase, activate mitogen-

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ABBREVIATIONS: MSN, medium spiny neuron; CB, cannabinoid; ACSF, artificial cerebrospinal fluid; PKA, cAMP-dependent protein kinase; EPSC, excitatory postsynaptic current; Sp-8-CPT-cAMPS, (*S*)p-8-(4-chlorophenythil) adenosine-3′,5′-monophosphorothioate; H89, *N*-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; PP1, protein phosphatase 1; WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; KT5720, (9*R*,10*S*,12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-l][1,6]benzodiazocine-10-carboxylic acid; DMSO, dimethyl sulfoxide; CNQX, 6-cyano-7-notroquinoxaline-2,3-dione; D-APV, D(−)-2-aminophosphonopentanoic acid; ANOVA, analysis of variance.

activated protein kinase, reduce Ca^{2+} currents, and modulate several K^+ channels (Bouaboula et al., 1995; Howlett, 1995; Twitchell et al., 1997). Cellular consequences specifically linked to CB_2 receptor activation include inhibition of adenylyl cyclase and activation of mitogen-activated protein kinase (Bouaboula et al., 1996).

In addition to these effects, activation of CB_1 receptors in the brain has been shown to produce multiple effects on synaptic transmission. Among these, presynaptic inhibition of glutamatergic synaptic transmission has been described in several brain regions, such as the hippocampus (Sullivan, 1999), substantia nigra pars compacta (Chan and Yung, 1998), cerebellum (Lèvènés et al., 1998), prefrontal cortex (Auclair et al., 2000), and nucleus accumbens (Robbe et al., 2001). Likewise, recently, we (Huang et al., 2001) and others (Gerdeman and Lovinger, 2001) have demonstrated that the activation of CB_1 receptors can dramatically decrease the glutamatergic transmission at corticostriatal synapses through a G_i/G_o-protein-coupled inhibition of presynaptic *N*-type Ca^{2+} channel activity. Surprisingly, we have also found that application of the adenylyl cyclase activator forskolin markedly suppressed the presynaptic inhibition of CB_1 receptors at corticostriatal synapses; however, its precise molecular mechanism is not clear. Because protein phosphorylation is thought to be ubiquitous and important mechanism for controlling the function of a number of G-protein-coupled receptors and ion channels, we designed a series of experiments to examine whether a similar modulatory influence on CB_1 receptors is possibly involved in forskolin action. Using both electrophysiological and biochemical techniques, we found that the inhibition of forskolin on CB_1 receptor function is a result of the phosphorylation of receptors by activation of cAMP/PKA-coupled signaling pathway.

Materials and Methods

Slice Preparation. Animal care was consistent with the guidelines set by the Laboratory Animal Center of National Cheng-Kung University. The Animal Research Committee of National Cheng-Kung University approved all experimental procedures. Corticostriatal coronal slices were obtained from 14- to 18-day-old male Sprague-Dawley rats for patch-clamp recordings made by using the procedures described previously (Huang et al., 2001). In brief, the rats were killed by decapitation under halothane anesthesia, and coronal slices (200–250 μm thick) containing the cortex and striatum were cut from a tissue block of the brain using a vibrating microtome (VT1000S; Leica, Nussloch, Germany). The slices were placed in a storage chamber of artificial cerebrospinal fluid (ACSF) oxygenated with 95% O_2/5% CO_2 and kept at room temperature for at least 1 h before recording. The composition of the ACSF solution was 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4 and 11 mM glucose at pH 7.3 to 7.4 and equilibrated with 95% O_2/5% CO_2. In some experiments, modified low Ca^{2+} solution, in which the concentration of $CaCl_2$ was reduced to 1 mM and that of $MgCl_2$ was increased to 2.7 mM, was used as described in the text (Fig. 6).

Patch-Clamp Recordings. For patch-clamp recording, slices were transferred into a recording chamber and fixed at the glass bottom of the chamber with a nylon grid on a platinum frame. The chamber consisted of a circular well of a low volume (1–2 ml) and was perfused constantly at 24 to 26°C at a speed of 2 to 3 ml/min. Visualized whole-cell patch-clamp recordings of synaptically evoked excitatory postsynaptic currents (EPSCs) were conducted using standard methods (Huang et al., 2001). Striatal neurons were visualized throughout the experiment with an upright microscope (BX50WI; Olympus, Tokyo, Japan) equipped with a water-immersion 60× objective using Nomarski-type differential interference contrast optics combined with infrared videomicroscopy. Patch pipettes were pulled from borosilicate capillary tubing and were heat-polished. The electrode resistance was typically 4 to 5 MΩ. The composition of intracellular solution was 110 mM K-gluconate, 30 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM EGTA, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 5 mM QX-314, and sucrose to bring osmolarity to 290 to 295 mOsM, pH 7.3. After a high-resistance seal (>2 GΩ before breaking into whole-cell mode) was obtained, suction was applied lightly through the pipette to break through the membrane. The cell was then maintained at −80 mV for several minutes to allow diffusion of the internal solution into the cell body and dendrites. Recordings were made using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier. Electrical signals were low-pass filtered at 2 kHz, digitized at 4 to 10 kHz using a Digidata 1200B interface, and an Intel Pentium-based computer with pCLAMP software (version 8.0; Axon Instruments) was used for on-line acquisition and off-line analysis of the data. For measurement of synaptically evoked EPSCs, a bipolar stainless steel stimulating electrode was applied to a site 1 to 2 mm dorsal to the cell under study as described previously (Huang et al., 2001). EPSCs were recorded in the presence of 20 μM bicuculline methiodide, a γ-aminobutyric $acid_A$ receptor antagonist. The capacitance of the recording cells was 10 to 25 pF. Series resistance was calculated according to the equation: series resistance = 10 mV/I, where I is the peak of transient current (filtered with 10 kHz) evoked by the 10 mV testing pulse when the pipette capacitance was compensated fully. Only cells demonstrating <25 MΩ series resistance (usually 10–20 MΩ) were used in these experiments. The input resistance was monitored continuously by applying a 10 mV (100-ms duration) hyperpolarizing current pulse, and the recording was terminated if it varied by more than 10%. Input resistances were generally between 200 and 800 MΩ.

Immunoprecipitation and Western Blotting. For immunoprecipitations, the striatal slices were lysed in ice-cold Tris-HCl buffer solution, pH 7.4, containing a cocktail of protein phosphatase and proteinase inhibitors (50 mM Tris-HCl, 100 mM NaCl, 15 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM okadaic acid, 0.5% Triton X-100, 2 mM benzamidine, 60 μg/ml aprotinin, and 60 μg/ml leupeptin) to avoid dephosphorylation and degradation of proteins, and ground with a pellet pestle (Kontes Glassware, Vineland, NJ). Samples from three slices were sonicated and spun down at 15,000*g* at 4°C for 10 min. The supernatant was then assayed for total protein concentration using Bradford Protein Assay Kit (Bio-Rad, Hercules, CA) based on the method of Bradford (Bradford, 1976), with bovine serum albumin as a standard. Samples of equal protein concentration were submitted to precipitation with polyclonal CB_1 antibody (Calbiochem, San Diego, CA) followed by incubation with protein A Sepharose. For Western immunoblotting, the precipitates were dissolved in SDS-polyacrylamide gel electrophoresis buffer and separated in 10% SDS-polyacrylamide gel gel and then transferred to nitrocellulose for immunoblot analysis. Nitrocellulose membrane was incubated in blocking buffer solution containing 5% nonfat dry milk and 0.1% Tween 20 in Tris-HCl buffer solution for 1 h and then blotted for 2 h at room temperature with monoclonal anti-phosphoserine antibody (1:500; Sigma/RBI, Natick, MA). It was then probed with horseradish peroxidase-conjugated secondary antibody for 1 h and developed using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). To determine the specificity of anti-phosphoserine antibody, in some experiments the immunoprecipitates were preincubated with 0.5 U of protein phosphatase 1 (PP1; Upstate Biotechnology Inc., Lake Placid, NY) for 20 min at 30°C before being probed with anti-phosphoserine antibody. The relative amount of CB_1 receptor serine phosphorylation was analyzed by determining the ratio of the signals detected by using

the anti-phosphoserine antibody and CB_1 receptor antibody. Immunoblots were quantified by densitometric measurement.

Drug Source and Application. All drugs were applied by dissolving them to the desired final concentrations in the ACSF and by switching the perfusion from control ACSF to drug-containing ACSF. Appropriate stock solutions of drugs were made and diluted with ACSF just before application. WIN 55,212-2, forskolin, 1,9-dideoxyforskolin, KT5720, and H89 were made up to a 20 mM stock solution in dimethyl sulfoxide (DMSO) and stored at −20°C. Aqueous dilution of these stock solutions was made daily. WIN 55,212-2, forskolin, and 1,9-dideoxyforskolin were purchased from RBI/Sigma; 6-cyano-7-notroquinoxaline-2,3-dione (CNQX), D(−)-2-aminophosphonopentanoic acid (D-APV), isoproterenol, propranolol, and bicuculline methiodide were obtained from Sigma (St Louis, MO); *S*p-8-CPT-cAMPS was purchased from Biomol (Plymouth Meeting, PA); H89 and KT5720 were obtained from Calbiochem.

Statistical Analysis. The data for each experiment were normalized relative to baseline. All figures show means ± S.E.M. Paired Student's *t* test was used to determine whether responses were of different magnitude in a CB_1 receptor agonist compared with the baseline. When an additional comparison was required (such as whether a second treatment influenced the action of CB_1 receptor agonist), a two-way repeated-measures analysis of variance (ANOVA) was computed. Numbers of experiments are indicated by *n*. Probability values (*p*) of less than 0.05 were considered to represent significant differences.

Results

Forskolin and *S*p-8-CPT-cAMPS Suppress the Presynaptic Inhibitory Action of WIN 55,212-2 on EPSCs. Whole-cell patch-clamp recordings were made from the MSNs in the dorsolateral striatum, in which CB_1 receptors are highly expressed (Herkenham et al., 1991). The MSNs used in this study had a longitudinal diameter of the soma ranging from 8 to 15 μm. In all experiments, neurons were clamped at −80 mV and EPSCs were evoked by intrastriatal stimulation with a bipolar stimulating electrode every 15 s in the presence of the γ-aminobutyric $acid_A$ receptor antagonist bicuculline methiodide (20 μM). Consistent with previous reports, bath application of the selective and potent CB_1 receptor agonist WIN 55,212-2 (2 μM, dissolved in 0.01% DMSO), induced a slow and time-dependent depression of evoked EPSCs that lasted thereafter for the entire duration of experiments. Maximal inhibition of the response generally occurred within 20 to 25 min of drug application, and peak inhibition of the response was 56.4 ± 5.7% ($n = 12$; $p < 0.05$; Student's paired *t* test) with 2 μM WIN 55,212-2 (Fig. 1, A and D). Next, the effect of the selective adenylyl cyclase activator forskolin on WIN 55,212-2–induced depression of EPSCs was examined. Application of forskolin (10 μM, dissolved in 0.05% DMSO) significantly enhanced the EPSC amplitude (162.8 ± 6.2% of baseline; $n = 8$; $p < 0.05$; Student's paired *t* test) as reported previously (Colwell and Levine, 1995), which typically stabilized after 15 min (Fig. 1B). After treatment with forskolin for 20 min, the effect of WIN 55,212-2 was measured. Forskolin markedly attenuated the inhibition of WIN 55,212-2 on EPSCs; a typical example is shown in Fig. 1B. On average, the WIN 55,212-2 (2 μM)-induced depression of the amplitude of EPSCs was reduced to 16.5 ± 5.8% ($n = 8$) after the application of forskolin, which was significantly different from the inhibition produced by WIN 55,212-2 alone (56.4 ± 5.7%; $n = 12$; $p < 0.05$; repeated-measures ANOVA). Because forskolin has been reported to possess many cAMP-independent actions, including the blockade of several types of K^+ currents, it is possible that the effect of forskolin on WIN 55,212-2–induced synaptic depression is caused by its nonspecificity (Laurenza et al., 1989). To exclude this possibility, an analog of forskolin, 1,9-dideoxyforskolin, which has no effect on adenylyl cyclase but does mimic many of the cAMP-independent actions of forskolin, was used. As shown in Fig. 1, C and D, unlike forskolin, 1,9-dideoxyforskolin (10 μM, dissolved in 0.05% DMSO) neither enhanced the EPSCs (106.3 ± 2.9% of baseline; $n = 6$; $p > 0.05$; Student's paired *t* test) nor suppressed WIN 55,212-2–induced depression of EPSCs. On average, the WIN 55,212-2 (2 μM)-induced depression of the amplitude of EPSCs was 53.2 ± 5.3% ($n = 6$) after the application of 1,9-dideoxyforskolin, which was not significantly different from the inhibition produced by WIN 55,212-2 alone (56.4 ± 5.7%; $n = 12$; $p > 0.05$; repeated-measures ANOVA) (Fig. 1D). As a control, ACSF containing 0.06% DMSO was applied; the EPSCs dropped by only 4.3 ± 1.8% ($n = 3$). This inhibition is negligible because it was not statistically significant and was comparable with the decline we observed without application of any drugs. These results suggest that forskolin-induced inhibition of the response to WIN 55,212-2 is primarily mediated by activation of adenylyl cyclase-coupled signaling pathway rather than a nonspecific action of the drug.

Fig. 2 shows that the WIN 55,212-2–induced inhibition of EPSC amplitude was concentration-dependent; the threshold for the response was 0.5 μM. Forskolin (10 μM) induced

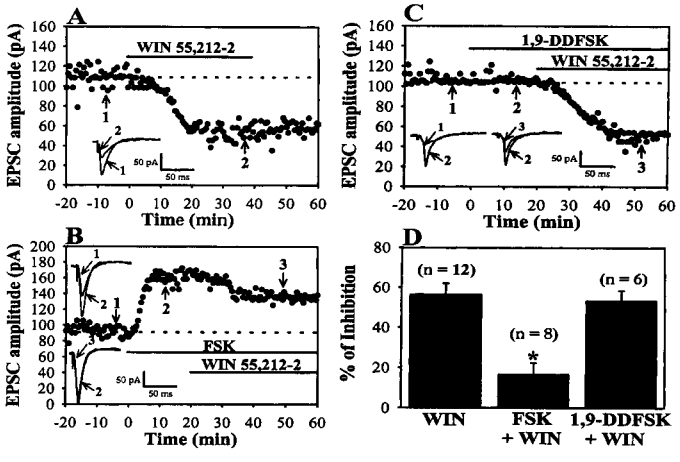

Fig. 1. The adenylyl cyclase activator forskolin (FSK), but not its inactive analog 1,9-dideoxyforskolin (1,9-DDFSK), suppresses the WIN 55,212-2–induced synaptic depression. A, representative experiment showing time course of the action of CB_1 receptor against on the peak amplitude of evoked EPSCs. EPSCs were evoked every 15 s by a single pulse and were recorded from a holding potential of −80 mV. Each data point represents a single response evoked before, during, and after the application of 2 μM WIN 55,212-2 in a striatal neuron. WIN 55,212-2 dramatically reduced EPSCs, and this effect was not reversible on washout for 20 min. B, representative experiment showing that prior application of forskolin at 10 μM significantly enhanced the basal EPSCs and prevented the inhibition of EPSCs by WIN 55,212-2 (2 μM). C, application of 1,9-dideoxyforskolin (10 μM) had no effect on basal synaptic transmission and did not affect the WIN 55,212-2–induced synaptic depression. D, summary graph of means ± S.E.M. data showing the effect of forskolin and 1,9-dideoxyforskolin on WIN 55,212-2–induced inhibition of EPSCs at corticostriatal synapses. The superimposed EPSC in the inset of each graph illustrates respective recordings from example experiments taken at the time indicated by number. Horizontal bars denote the period of delivery of WIN 55,212-2, forskolin, or 1,9-dideoxyforskolin. *, $p < 0.05$; Student's paired *t* test.

a significant reduction in the response to WIN 55,212-2 and the increase of the concentration of WIN 55,212-2 was unable to overcome this inhibition. These data suggest that the attenuation by forskolin on CB_1 receptor function is mediated in a noncompetitive manner.

If forskolin-induced inhibition of the response to WIN 55,212-2 is mediated by the formation of cAMP and then the activation of PKA, the application of membrane-permeable analogs of cAMP should mimic the modulatory effect of forskolin. We test this prediction by continuously applying the cAMP analog *S*p-8-CPT-cAMPS (100 μM) to our preparations, a manipulation that may keep PKA activity constant by clamping the cAMP concentration (Tzounopoulos et al., 1998). As illustrated in Fig. 3A, this caused the expected increase in the amplitude of EPSCs and also blocked the WIN 55,212-2–induced synaptic depression. As was the case for forskolin, application of *S*p-8-CPT-cAMPS (100 μM) alone produced a stable enhancement of EPSC amplitude (145.3 ± 5.9% of baseline; $n = 6$; $p < 0.05$; Student's t test). In the presence of *S*p-8-CPT-cAMPS, bath application of WIN 55,212-2 (2 μM) for 40 min produced only a minor inhibition of EPSC amplitude by 8.9 ± 4.6% ($n = 6$), which was significantly different from the synaptic depression produced by WIN 55,212-2 alone (56.4 ± 5.7%; $n = 12$; $p > 0.05$; repeated-measures ANOVA) (Fig. 3B). These results suggest that forskolin inhibits the WIN 55,212-2–induced synaptic depression via a mechanism that is dependent on the formation of cAMP and the activation of PKA.

PKA Inhibitors Block the Ability of Forskolin to Inhibit the WIN 55,212-2–Induced Inhibition of EPSCs. Additional evidence supporting that forskolin-induced inhibition of the response to WIN 55,212-2 is mediated through the activation of PKA came from experiments using the membrane-permeable PKA inhibitors KT5720 and H89. A 60-min application of KT5720 (5 μM, dissolved in 0.025% DMSO) had no consistent effect on the EPSC amplitude (94.5 ± 4.6% of baseline; $n = 5$; $p > 0.05$; Student's paired t test; Fig. 4A) but the forskolin (10 μM)-induced enhancement of the EPSC amplitude was prevented by the presence of KT5720 (5 μM) (106.4 ± 4.5% of those before forskolin application in KT5720 containing solution; $n = 5$; $p < 0.05$; repeated-measures ANOVA; Fig. 4B). Similar results were also obtained when H89 (10 μM, dissolved in 0.05% DMSO), a different PKA inhibitor, was applied (data not shown). As summary in Fig. 5, application of either KT5720 (5 μM) or H89 (10 μM) completely blocked the ability of forskolin to inhibit the response to WIN 55,212-2. In the presence of both

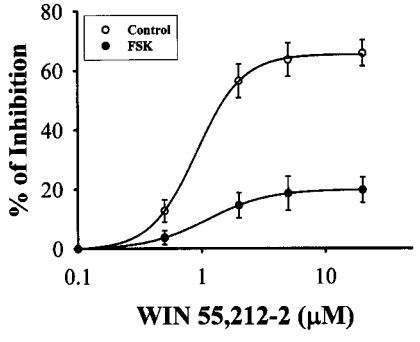

Fig. 2. Forskolin attenuates the action of WIN 55,212-2 by shifting the concentration-response curve to the right and suppresses the maximal effect of WIN 55,212-2. Concentration-response curve shows the depression of the EPSC amplitude by increasing concentration of WIN 55,212-2 in the absence (○) or in the presence (●) of forskolin (10 μM). $n = 5$ to 12 for each experiment. Data are presented as means ± S.E.M.

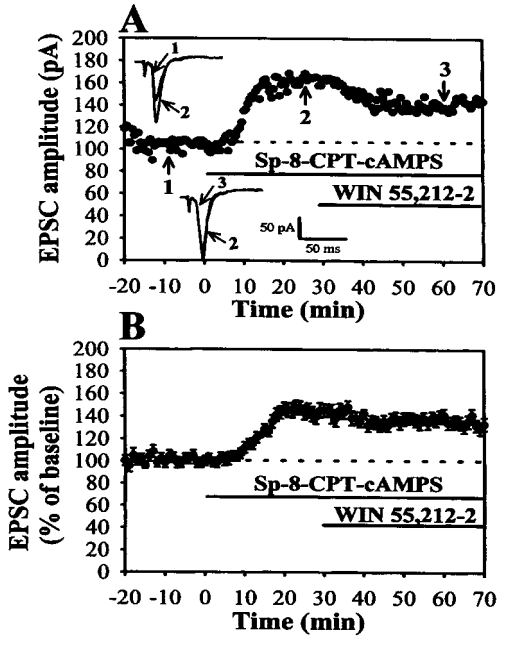

Fig. 3. Activation of PKA by cAMP analog *S*p-8-CPT-cAMPS suppresses the WIN 55,212-2–induced synaptic depression. A, representative experiment showing application of *S*p-8-CPT-cAMPS (100 μM) alone caused a stable increase of basal EPSCs and prevented WIN 55,212-2 (2 μM)-induced depression of EPSCs. B, summary of six experiments performed as in A. The superimposed EPSC in the inset of each graph illustrates respective recordings from example experiments taken at the time indicated by number. Horizontal bars denote the period of delivery of *S*p-8-CPT-cAMPS or WIN 55,212-2.

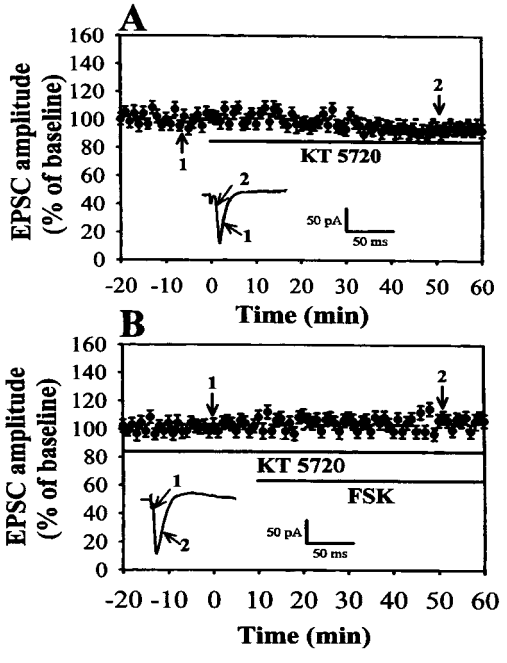

Fig. 4. Membrane-permeable PKA inhibitor KT5720 alone has no effect on basal EPSCs but suppresses the synaptic enhancement effect of forskolin. A, summary of experiments ($n = 5$) showing that prolong application of KT5720 (5 μM) had no consistent effect on EPSCs. B, summary of experiments ($n = 5$) in which KT5720 (5 μM) was applied before application of forskolin (10 μM). The forskolin-induced increase in EPSCs was completely prevented in the presence of KT5720.

forskolin (10 μM) and KT5720 (5 μM), bath application of WIN 55,212-2 (2 μM) for 30 min reduced the amplitude of EPSCs by 52.7 ± 5.1% (n = 5), a value similar to that the inhibition produced by WIN 55,212-2 alone (56.4 ± 5.7%; n = 12; $p > 0.05$; repeated-measures ANOVA). Similarly, bath application of WIN 55,212-2 (2 μM) was still able to produce a 58.6 ± 5.6% (n = 5) decrease in the amplitude of EPSCs after the application of both forskolin and H89, which was not significantly different from the inhibition produced by WIN 55,212-2 alone (56.4 ± 5.7%; n = 12; $p > 0.05$; repeated-measures ANOVA). In the presence of KT5720, the percentage inhibition of the EPSC amplitude induced by WIN 55,212-2 (62.4 ± 5.9%; n = 5; $p > 0.05$; repeated-measures ANOVA) was identical to the response obtained by WIN 55,212-2 alone. Treatment with H89 also did not significantly affect the percentage of inhibition produced by WIN 55,212-2 (57.2 ± 4.9%; n = 5; $p > 0.05$; repeated-measures ANOVA).

Forskolin Suppresses the WIN 55,212-2-Induced Synaptic Depression in Low Ca^{2+} Solution. Because forskolin has been shown to enhance the synaptic transmission by facilitating transmitter release (Chavez-Noriega and Stevens, 1994), it is possible that the saturation of transmitter release processes masks the depressive action of WIN 55,212-2 at corticostriatal synapses. To test this prediction, we examined the effect of forskolin on WIN 55,212-2–induced synaptic depression in a low-Ca^{2+} solution condition that is known to reduce the probability of transmitter release at the central synapses (Manabe et al., 1993). As shown in Fig. 6, by switching the perfusion from control ACSF (2.5 mM Ca^{2+} and 1.2 mM Mg^{2+}) to low-Ca^{2+} solution (1 mM Ca^{2+} and 2.7 mM Mg^{2+}), the EPSCs were reduced to 24.6 ± 5.5% of baseline (n = 7; $p < 0.05$; Student's paired t test), and the EPSC amplitude was still able to enhance by the subsequent application of 10 μM forskolin (213.8 ± 26.4% of those before forskolin application in low Ca^{2+} solution, n = 7; $p < 0.05$; repeated-measures ANOVA). After treatment with forskolin in low Ca^{2+} solution, 2 μM WIN 55,212-2–induced synaptic depression was reduced to 9.8 ± 5.3% (n = 7), which was not significantly different from the forskolin-induced inhibition in normal ACSF solution. These data indicate that the inhibitory effect of forskolin on WIN 55,212-2–induced synaptic depression is not mediated by the mechanism of saturation of transmitter release processes.

PKA-Mediated Serine-Phosphorylated Component of CB_1 Receptor Is Increased after Forskolin Application. The preceding results point to an involvement of cAMP-PKA-mediated mechanism in the forskolin-induced inhibition of the response to WIN 55,212-2. What is the target substrate of PKA to underlie this effect? Because PKA could inhibit the function of G-protein-coupled receptors by phosphorylation of either the receptors or G-protein (Schaffhauser et al., 2000), we subsequently evaluated whether CB_1 receptor can be directly phosphorylated by PKA after forskolin treatment. We undertook a biochemical approach to test this possibility by using the specific CB_1 receptor antibody to immunoprecipitate CB_1 receptor prepared from striatal slices. Consistent with previous report, the CB_1 receptor exhibits a pattern of two bands on Western blot analysis (Fig. 7A). CB_1 receptor antibody can recognize a ~61-kDa band and a second ~50-kDa band (Egertová and Elphick, 2000; Coutts et al., 2001). Immunoblotting with anti-phosphoserine antibody indicated a higher level of serine-phosphorylated components in the membrane prepared from forskolin (10 μM) treatment slices than in control slices of CB_1 immunoprecipates (137.6 ± 5.4% of control slices, n = 5; $p < 0.05$; unpaired Student's t test) and the response to forskolin was markedly attenuated by PKA inhibitor H89 (10 μM) (92.3 ± 4.9% of control slices, n = 5; $p > 0.05$; unpaired Student's t test). Quantitative analysis of normalized phosphorylation is shown in Fig. 7B. In parallel experiments using the same treatment schedule as for H89, another PKA inhibitor KT5720 (5 μM) also effectively blocked the forskolin-stimulated phosphorylation of CB_1 receptors (n = 2, data not shown). To determine the specificity of the anti-phosphoserine antibody, the immunoprecipitated CB_1 receptor proteins were preincubated with PP1 (0.5 U) at 30°C for 20 min before being probed with anti-phosphoserine antibody. As a typical example shown in Fig. 7C, the level of immunolabeled band probed with anti-

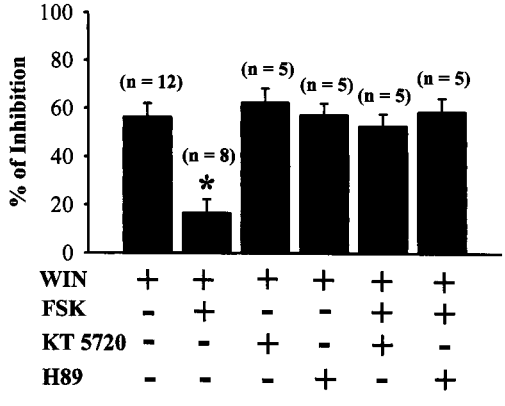

Fig. 5. PKA inhibitors KT5720 and H89 block the ability of forskolin to inhibit the WIN 55,212-2–induced synaptic depression. Summary bar plots represent the percentage inhibition of EPSCs by WIN 55,212-2 (2 μM) in the presence and absence of forskolin or forskolin plus the PKA inhibitor KT5720 (5 μM) or H89 (10 μM). Note that forskolin significantly reduced WIN 55,212-2–induced depression of EPSCs in the absence of either KT5720 or H89 but was without effect in the presence of KT5720 or H89. In addition, neither KT5720 nor H89 alone affected the percentage inhibition of EPSCs induced by WIN 55,212-2. Number of experiments is indicated by n. Data are presented as means ± S.E.M. *, $p < 0.05$; repeated-measures ANOVA.

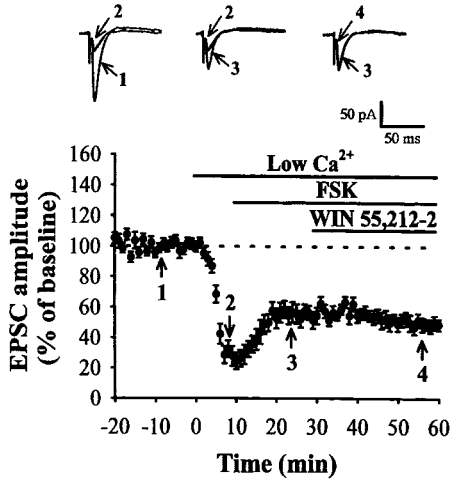

Fig. 6. The WIN 55,212-2–induced depression of EPSCs is also suppressed by forskolin in low-Ca^{2+} solution. Summary of seven experiments in which WIN 55,212-2 was applied after 10 μM forskolin application. The standard ACSF was replaced with low Ca^{2+} solution containing 1 mM Ca^{2+} and 2.7 mM Mg^{2+}. The superimposed EPSC in the inset of each graph illustrates respective recordings from example experiments taken at the time indicated by *number*. Horizontal bars denote the period of delivery of low-Ca^{2+} solution, forskolin, and WIN 55,212-2.

phosphoserine antibody was expectedly decreased after PP1 treatment, indicating that the anti-phosphoserine antibody, used in our study, is suitable to detect the serine phosphorylation of proteins. This phenomenon was observed in all three separated experiments tested in this study. These results suggest that the activation of PKA leading to an increase in serine-phosphorylated component of CB_1 receptors is possibly involved in forskolin-induced inhibition.

β-Adrenergic Receptor Agonist Isoproterenol Reduces the Win 55,212-2–Induced Inhibition of EPSCs. The final test was to determine whether activation of receptors that are positively coupled to cAMP-PKA-dependent signaling pathways can mimic forskolin to elicit an inhibition of presynaptic CB_1 receptor function at corticostriatal synapses. In the striatum, functional β-adrenergic receptors are present in the excitatory nerve terminals (Aoki et al., 1987) and the action of β-adrenergic receptors results in a long-lasting increase in synaptic transmission via a PKA-mediated mechanism (Nittykoski et al., 1999). Thus, we conducted a series of experiments to test the hypothesis that activation of β-adrenergic receptors would induce a PKA-mediated reduction of CB_1 receptor function. Consistent with previous report (Nittykoski et al., 1999), application of selective β-adrenergic receptor agonist isoproterenol (30 μM) to the striatal neurons induced a long-lasting enhancement of synaptic transmission (138.9 ± 4.8% of baseline, $n = 6$; $p < 0.05$; paired Student's t test). After treatment with isoproterenol for 20 min, the effect of WIN 55,212-2 was examined. Isoproterenol significantly reduced the inhibition of WIN 55,212-2 on EPSCs; a typical example is shown in Fig. 8A. On average, 2 μM WIN 55,212-2–induced depression of the amplitude of EPSCs was reduced to 26.2 ± 4.9% ($n = 6$) after the application of isoproterenol, which was significantly different from the inhibition produced by WIN 55,212-2 alone (56.4 ± 5.7%; $n = 12$; $p < 0.05$; repeated-measures ANOVA). In addition, the response to isoproterenol was blocked completely by propranolol (30 μM), a selective β-adrenergic receptor antagonist, suggesting that this effect is indeed mediated by activation of β-adrenergic receptors. As shown in Fig. 8B, in the presence of both isoproterenol (30 μM) and propranolol (30 μM), bath application of WIN 55,212-2 for 30 min reduce the amplitude of EPSCs by 53.8 ± 5.3% ($n = 4$), a value similar to that the inhibition produced by WIN 55,212-2 alone ($p > 0.05$; repeated-measures ANOVA). Moreover, application of KT5720 (5 μM) also completely blocked the ability of isoproterenol to inhibit the response to WIN 55,212-2. The inhibitory effect of WIN 55,212-2 on EPSC amplitude in the presence of both isoproterenol (30 μM) and KT5720 (5 μM) was not significantly from the response to WIN 55,212-2 alone (59.8 ± 5.1% versus 56.4 ± 5.7%; $n = 4$; $p > 0.05$; repeated-measures ANOVA). These data are consistent with the hypothesis that the inhibitory action of β-adrenergic receptor

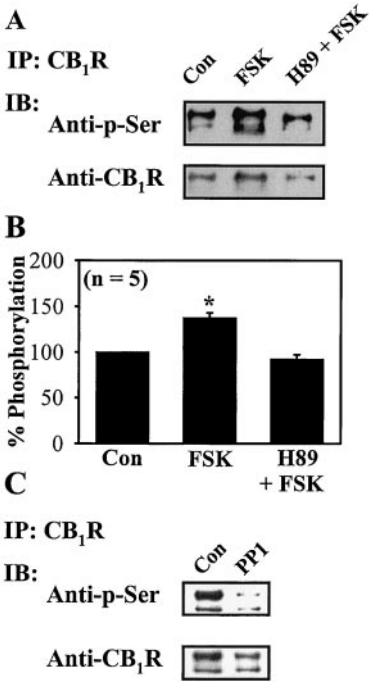

Fig. 7. The increased phosphorylation of CB_1 receptor induced by forskolin is prevented by prior application of KT5720. A, antiphosphoserine immunoblot (IB) from CB_1 receptor immunoprecipitates (IP) showing enhanced serine-phosphorylated component of CB_1 receptor 20 min after the forskolin (10 μM) treatment in striatal slices (top gel). The effect of forskolin was markedly attenuated by H89 (10 μM). The bottom gel is the reprobing of the same membrane with anti-CB_1 receptor antibody to normalize the phosphorylation of CB_1 receptor. B, normalized CB_1 receptor phosphoserine immunoreactivity from control, forskolin and forskolin plus H89 slices. C, preincubation of immunoprecipitates with PP1 (0.5 U) for 20 min at 30°C significantly decreased the level of immunolabeled band. Data are presented as means ± S.E.M. Number of experiments is indicated by n. *, $p < 0.05$; Student's unpaired t test.

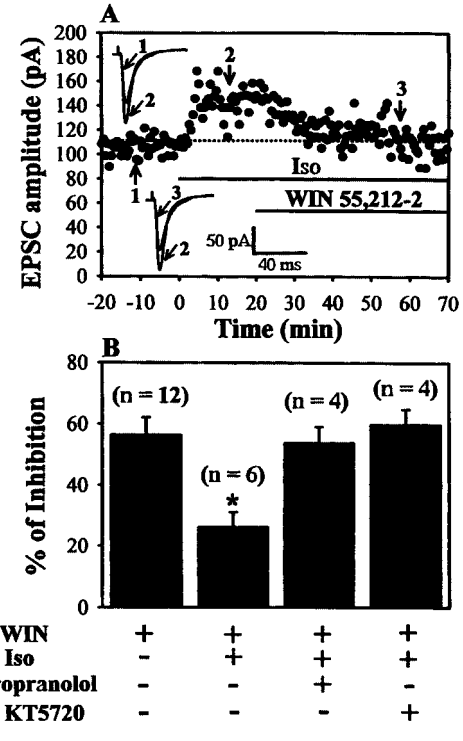

Fig. 8. β-Adrenergic receptor agonist isoproterenol reduces WIN 55,212-2–induced synaptic depression. A, time course showing the effect of isoproterenol (30 μM) on WIN 55,212-2 (2 μM)-induced inhibition of EPSCs. Application of isoproterenol for 20 min before WIN 55,212-2 significantly enhanced the basal EPSCs and reduced the inhibition of EPSCs by WIN 55,212-2. B, summary bar plots represent the percentage inhibition of EPSCs by WIN 55,212-2 (2 μM) in the presence and absence of isoproterenol (30 μM) or isoproterenol plus the β-adrenergic receptor antagonist propranolol (30 μM) or the PKA inhibitor KT5720 (5 μM). Note that propranolol and KT5720 block the ability of isoproterenol to inhibit the WIN 55,212-2–induced synaptic depression. Number of experiments is indicated by n. Data are presented as means ± S.E.M. *, $p < 0.05$; repeated-measures ANOVA.

activation on CB_1 receptor function is mediated by a PKA-dependent mechanism.

Discussion

In the present study, we have extended our previous observations (Huang et al., 2001) showing that the presynaptic inhibitory action of CB_1 receptor agonists at corticostriatal synapses could be negatively regulated by cAMP/PKA systems. Here, we show that the adenylyl cyclase activator forskolin and cAMP analog *S*p-8-CPT-cAMPS treatment markedly attenuated the WIN 55,212-2–induced inhibition of the evoked EPSC amplitude. This inhibition is not caused by saturation of transmitter release process by forskolin but is largely attributable to the phosphorylation of the CB_1 receptors through a cAMP/PKA-dependent signaling pathway.

As mentioned above, the CB_1 receptors are negatively coupled to adenylyl cyclase through G_i/G_o proteins in many different cell types (Howlett, 1995). If the inhibition of adenylyl cyclase is the mechanism underlying the inhibition of CB_1 receptors on glutamatergic transmission at corticostriatal synapses, one possible mechanism by which forskolin and *S*p-8-CTP-cAMPS could inhibit WIN 55,212-2-mediated response is by simply overcoming the ability of WIN 55,212-2 to reduce the cAMP accumulation. There are two reasons to believe that forskolin- and *S*p-8-CPT-cAMPS–induced inhibition of the response to WIN 55,212-2 does not rely upon this mechanism. First, application of the PKA inhibitor KT5720 (5 μM) did not mimic the action of WIN 55,212-2 to inhibit the neurotransmission at corticostriatal synapses at a concentration that completely inhibits the synaptic enhancement elicited by forskolin (Fig. 4). These findings confirm and extend our earlier observations that WIN 55,212-2 inhibits corticostriatal synaptic transmission by a mechanism that is unrelated to its ability to inhibit adenylyl cyclase. Second, more importantly, the higher concentration of WIN 55,212-2 can not overcome the forskolin-induced inhibition of the response to WIN 55,212-2. Taken together, these observations point to an alternative mechanism involving the forskolin-induced inhibition.

Forskolin has been reported to possess many cAMP-independent actions, including the blockade of several types of potassium currents, which could result in prolongation of presynaptic action potentials and consequent increase in transmitter release (Hoshi et al., 1988). However, the cAMP-independent action of forskolin could be mimicked by its analog 1,9-dideoxyforskolin, which is unable to activate adenylyl cyclase. In our experiments, 1,9-dideoxyforskolin neither enhanced EPSCs nor suppressed WIN 55,212-2's action. Thus, the effect of forskolin is not caused by its nonspecificity. This idea was also supported by the finding that the PKA inhibitors KT5720 and H89 could successfully antagonize the synaptic enhancement of forskolin (Figs. 4 and 5). Consistent with this idea, we have found that the activation of β-adrenergic receptors that are coupled to Gs-proteins and activation of cAMP/PKA-dependent signaling pathways also mimic forskolin to elicit a PKA-mediated reduction of CB_1 receptor function (Fig. 8).

Activation of cAMP/PKA-dependent cascade has been shown to enhance transmitter release at glutamatergic synapses in a variety of brain regions, including the hippocampus, amygdala, cerebellum, and striatum (Chavez-Noriega and Stevens, 1994; Colwell and Levine, 1995; Huang et al., 1996; Chen and Regehr, 1997). It is suggested that modulation of these synapses by PKA occurs via presynaptic mechanisms that do not affect presynaptic Ca^{2+} influx or resting Ca^{2+} levels; PKA may directly modulate the probability of vesicular release and the number of release sites (Chen and Regehr, 1997). It is possible that forskolin-induced inhibition of CB_1 receptor function is a result of the saturation of presynaptic transmitter release process by forskolin, which in turn masks the depression action of CB_1 receptor on synaptic transmission. This mechanism, however, was ruled out by our findings that the effect of forskolin on the WIN 55,212-2 action in low Ca^{2+} solution was not significantly altered (Fig. 6). In the striatum, cAMP/PKA-dependent pathway can also act postsynaptically by modifying glutamate receptors (Colwell and Levine, 1995). It is also likely that forskolin-induced inhibition of CB_1 receptor function is mediated via a postsynaptic modification of glutamate receptor function by forskolin. In our previous experiments, we have demonstrated that the blockade of glutamatergic synaptic transmission by CB_1 receptor in the striatal neurons is not caused by a change in postsynaptic sensitivity to glutamate (Huang et al., 2001). Therefore, the postsynaptic regulation of glutamate receptor function by forskolin can not account for the mechanism underlying the inhibitory action of forskolin on CB_1 receptor function.

There is considerable evidence revealing that protein phosphorylation and dephosphorylation provide an important regulation of the function of a number of G-protein-coupled receptors and ion channels. For example, activation of protein kinase C inhibits the function of multiple presynaptic metabotropic glutamate receptor subtypes by direct phosphorylation of the receptor at various central glutamatergic synapses. These include group II mGluRs at medial perforant path-dentate gyrus (Macek et al., 1998), mossy fiber-CA3 (Kamiya and Yamamoto, 1997), and corticostriatal (Swartz et al., 1993) synapses, as well as group III mGluRs at the lateral perforant path-dentate gyrus and Schaffer collateral-CA1 synapses (Macek et al., 1998). In addition, at mossy fiber-CA3 synapses, activation of adenylyl cyclase by forskolin induces a PKA-mediated inhibition of mGluR2 signaling by direct phosphorylation of a single serine residue (Ser843) on the C-terminal tail region of the receptor (Schaffhauser et al., 2000). Results from AtT-20 cells transfected with CB_1 receptor also demonstrated that protein kinase C acted by phosphorylating CB_1 receptor to inhibit the activation of an inward rectifying potassium current and depression of P/Q-type calcium channels by CB_1 receptor agonists (Garcia et al., 1998). The results of present study provide a further demonstration that the phosphorylation of a G-protein-coupled receptor by PKA can disrupt its activation and signaling. However, it is unlikely that this mechanism could explain fully the effects of PKA on CB_1 receptors. We cannot rule out the possibility that PKA could phosphorylate downstream proteins, such as Ca^{2+} channels and proteins involved in exocytosis, to inhibit CB_1 receptor effects on these effector proteins. Why does PKA-mediated phosphorylation of CB_1 receptor lead to an inhibition of CB_1 receptor function? Although at present the precise molecular mechanisms by which receptor phosphorylation inhibits CB_1 receptor function remain unknown, one potentially possible mechanism is that phosphorylation of CB_1 receptors by PKA inhibits coupling of the receptors to G-proteins. This idea is strengthened by recent experiments demonstrating that PKA-induced inhibition of mGluR2 function is mediated by the inhibition of

coupling of receptor to G-proteins at mossy fiber-CA3 synapses (Schaffhauser et al., 2000).

What is the physiological significance of PKA-induced inhibition of CB_1 receptor function at corticostriatal synapses? Although the functional role of PKA-induced inhibition of CB_1 receptor function explored here is not clear, this effect of PKA may play a critical role in fine-tuning glutamatergic transmission at corticostriatal synapses. The present finding that β-adrenergic receptor activation disrupts CB_1 receptor function provides a major advance in establishing a role for more physiologically relevant stimuli in eliciting this effect. Therefore, selective agonists or antagonists of presynaptic receptors that activate PKA could provide novel therapeutic targets for the development of drugs that could be used to regulate transmission at glutamatergic synapses. Because the corticostriatal projections represent the major excitatory input to the striatum (Buchwald et al., 1973) and these afferents converge on the medium spiny neurons, which are γ-aminobutyratergic inhibitory cells projecting to the output structure of basal ganglia (e.g., pallidus and substantia nigra reticular), a reduction of this excitatory synaptic transmission by CB_1 receptor activation will cause a decreased inhibitory influence on the output structure of basal ganglia from the striatum and produce motor inhibition. Therefore, it is expected that PKA may play a critical role in regulation of the motor inhibition produced by endogenous or exogenous cannabimimetic compounds in both normal physiological conditions and in various pathological conditions.

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