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Presynaptic adenosine A_{2A} receptors enhance GABAergic synaptic transmission *via* a cyclic AMP dependent mechanism in the rat globus pallidus

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1 We previously reported a presynaptic facilitatory action of A_{2A} receptors on GABAergic synaptic transmission in the rat globus pallidus (GP). In the present study we identify the intracellular signalling mechanisms responsible for this facilitatory action of A_{2A} receptors, using biochemical and patch-clamp methods in rat GP slices.

2 The adenosine A_{2A} receptor selective agonist CGS21680 (1, 10 μM) and the adenylyl cyclase activator forskolin (1, 10 μM) both significantly increased cyclic AMP accumulation in GP slices. The CGS21680 (1 μM)-mediated increase in cyclic AMP was inhibited by the A_{2A} receptor selective antagonist KF17837 (10 μM).

3 In an analysis of miniature inhibitory postsynaptic currents (mIPSCs), forskolin (10 μM) increased the mIPSC frequency without affecting their amplitude distribution, a result similar to that previously reported with CGS21680.

4 The adenylyl cyclase inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22,536, 300 μM) abolished the CGS21680-induced enhancement in the frequency of mIPSCs.

5 H-89 (10 μM), a selective inhibitor for cyclic AMP-dependent protein kinase (PKA), blocked the CGS21680-induced enhancement of the mIPSC frequency.

6 The calcium channel blocker $CdCl_2$ (100 μM) did not prevent CGS21680 from increasing the frequency of mIPSCs.

7 These results indicate that A_{2A} receptor-mediated potentiation of mIPSCs in the GP involves the sequential activation of the A_{2A} receptor, adenylyl cyclase, and then PKA, and that this facilitatory modulation could occur independently of presynaptic Ca^{2+} influx.
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Abbreviations: APV, D-2-amino-5-phosphonovaleric acid; cyclic AMP, cyclic adenosine monophosphate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; GABA, γ-aminobutyric acid; H-89, N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulphonamide; KF17837, (*E*)-8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxanthine; mIPSCs, miniature inhibitory postsynaptic currents; NMDA, N-methyl-D-aspartate; PKA, cyclic AMP-dependent protein kinase; SQ22,536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine; TTX, tetrodotoxin

Introduction

The globus pallidus (GP), one of the main projection areas of the striatum, is the principal component of the basal ganglia circuitry. Converging evidence indicates that the GP not only controls the activity of the subthalamic nucleus in the striatopallidal indirect pathway, but exerts widespread effects on the output of the basal ganglia as a whole (Albin *et al*., 1989; Delong, 1990; Smith *et al*., 1993; 1998; Parent & Hazrati, 1995). Changes in the pattern of firing activity and a resulting increase of GABAergic synaptic transmission in the GP output neurons have been suggested to be a consequence of the loss of nigrostriatal neurons, a cardinal feature of Parkinson's disease (Chesselet & Delfs, 1996).

There is growing evidence that the adenosine A_{2A} receptor serves to play a crucial role in basal ganglia function, particularly in the control of motor behavior (for review, see Richardson *et al*., 1997). Indeed, adenosine A_{2A} receptor antagonists have been shown to exhibit a prominent anti-Parkinsonian activity in several animal models (Kanda *et al*., 1994; 1998; Shimada *et al*., 1997; Grondin *et al*., 1999). In the striatum, adenosine A_{2A} receptors have been indicated to presynaptically suppress GABAergic inhibitory synaptic transmission onto striatal medium spiny neurons, on the basis of both electrophysiological (Mori *et al*., 1996; Chergui *et al*., 2000) and neurochemical data (Kirk & Richardson, 1994; Kurokawa *et al*., 1994). It has been suggested that the striatal A_{2A} receptor-mediated disinhibition could cause an overall increase in the activity of the striatopallidal projection

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neurons, resulting in suppression of GP neuron activity (Richardson *et al*., 1997).

We have further demonstrated that the A_{2A} receptor enhances GABAergic synaptic transmission in the GP by a presynaptic mechanism (Shindou *et al*., 2001). This new finding is consistent with a previous neurochemical study that A_{2A} receptors increase electrical field stimulation-induced GABA release from rat pallidal slices (Mayfield *et al*., 1993). In fact, in *in vivo* rat microdialysis experiments, the intrapallidal infusion of the A_{2A} receptor agonist increased GABA levels in the GP (Ochi *et al*., 2000). This presynaptic A_{2A} receptor-mediated increase in GABA transmission in the GP could cause suppression of GP output neurons, and therefore significantly affect GP output neuron activity and, therefore, downstream basal ganglia activity. Thus, the GP neuron activity could be efficiently controlled by A_{2A} receptors in both striatum and GP presynaptic terminals in the anatomical convergence or funneling structure of the striatum-GP pathway (for review see: Kase, 2001).

In this work we aimed to identify the intracellular presynaptic mechanisms responsible for the facilitatory action of A_{2A} receptors on GABA release in the GP. Although an increase in intracellular Ca^{2+} concentration is the primary signal for neurotransmitter release, cyclic AMP is also well known as a regulator of this process. Since A_{2A} receptor stimulation is reported to be positively coupled to adenylyl cyclase to produce cyclic AMP (van Calker *et al*., 1979), we examined the relationship between A_{2A} receptor stimulation, cyclic AMP accumulation, and GABA release in GP slices. Transmitter release was assessed through the analysis of spontaneous miniature synaptic currents in a single GP neuron by use of patch-clamp recording. An analysis of the frequency of such spontaneous miniature events, and their amplitude distribution, can provide useful indications of changes in the sensitivity of postsynaptic receptors and/or of changes in the presynaptic release process. This, in turn, leads to accurate identification of the GABA release mechanism in the A_{2A} receptor-located presynaptic terminals in the GP. In the present study, we describe the intracellular signalling mechanisms underlying the facilitatory effect of A_{2A} receptors in the GP neurons by electrophysiological and biochemical methods in rat slice preparations.

Methods

Slice preparation

Slices were prepared and recordings were obtained as described (Shindou *et al*., 2001). Male Sprague Dawley rats (12–16 day postnatal) were anaesthetized with ether and decapitated. The brain was removed rapidly. Slices containing the globus pallidus were cut on a DTK-1000 microslicer (Dosaka, Japan) at a thickness of 250 μm in a plane oblique, about 30° rostral-up to the horizontal. Slices were then incubated in oxygenated Ringer solution at a temperature of 29–30°C for 1 h. The standard Ringer solution had the following composition (mM): NaCl 124, KCl 3.0, $CaCl_2$ 2.4, $MgCl_2$ 1.2, $NaHCO_3$ 26.0, NaH_2PO_4 1.0, and glucose 10.0, continuously bubbled with a mixture of 95% O_2 and 5% CO_2. After incubation, a single slice was transferred to a recording chamber placed on the stage of an upright microscope, and was continuously perfused (3–4 ml min^{-1}) with oxygenated Ringer solution at 30°C. The remaining slices were kept in a holding chamber containing oxygenated Ringer solution at room temperature.

Whole-cell recording and data analysis

Neurons in the globus pallidus were visualized using infrared differential interference videomicroscopy (IR-DIC) with a 40× water immersion objective (Nikon, Tokyo, Japan). Micropipettes for whole-cell recordings were pulled with a P-97 Flaming-Brown electrode puller (Sutter Instrument Company, Novato, CA, U.S.A.) from borosilicate glass (1.5 mm outer diameter, Clark Electrical Instruments, Reading, U.K.). These had a final resistance of 2–5 MΩ when filled with intracellular solution.

For whole-cell voltage-clamp recordings, the pipette solution containing (mM): cesium methanesulphonate 120, KCl 5.0, EGTA 10.0, $CaCl_2$ 1.0, $MgCl_2$ 2.0, ATP 4.0, GTP 0.3, HEPES 8.0, and QX314 5.0, pH 7.2–7.4; osmolarity, 295 mOsm. The quaternary lidocaine derivative QX314 was included to suppress fast sodium currents. Voltage-clamp recordings were made with an Axopatch-1D amplifier (Axon Instruments). Throughout voltage recordings, series resistance was monitored *via* a voltage step (−5 mV, 10 msec), and typical values for the series resistance were 10–20 MΩ. Experiments were discarded if changes over 20% of the series resistance were seen. The experiments showing no recovery of IPSC amplitude in washout of the drug were omitted. Liquid junction potentials were not corrected.

Spontaneous mIPSCs were observed in the presence of D-2-amino-5-phosphonovaleric acid (APV, 50 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and tetrodotoxin (TTX, 0.5 μM, a concentration that blocked sodium channel-dependent action potentials and evoked-postsynaptic currents) at a holding potential of 0 mV. Under these conditions, whole-cell voltage recordings were reversibly and completely abolished by bicuculline (10 μM), demonstrating that they were mediated by $GABA_A$ receptors (Shindou *et al*., 2001).

Signals were filtered at 2 kHz and digitized at 5 kHz using an ITC-16 interface (Instrutech, Great Neck, NY, U.S.A.) connected to a Power Macintosh computer running Pulse (HEKA Elektronik, Lambrecht, Germany) software.

Spontaneous synaptic currents were automatically analysed using the Mini Analysis Program (Synaptosoft Inc., Leonia, NJ, U.S.A.). Events were ranked by amplitude and inter-event interval for preparation of cumulative probability distributions within 200–300 s epochs for control and drug conditions. The cumulative probability distributions were compared by the Kolmogorov–Smirnov test; $P<0.05$ was taken as indicating statistical significance.

Measurement of cyclic AMP level in GP slices

Cyclic AMP levels in rat GP slices were measured as follows: oblique GP slices (300 μm thickness) were prepared from male Sprague Dawley rats (35–40 day postnatal). Great care was taken to dissect the GP area free of the other areas (including the striatum), the dissection being performed under a binocular dissection microscope. The slices were preincubated for 60 min in standard Ringer solution continuously bubbled with a mixture of 95% O_2 and 5% CO_2 at 32°C.

After preincubation, the slices were then incubated in buffer containing 50 μM rolipram as a phosphodiesterase inhibitor. The slices were perfused with buffer alone (control) or with buffer containing various concentration of CGS21680 or forskolin for 10 min. The slices were then withdrawn and inactivated by the addition of 2.4% perchloric acid. Cyclic AMP levels in the supernatants obtained after sonication and centrifugation (30,000 $\times g$, 15 min, 4°C) were neutralized with $CaCO_3$ and then measured by radioimmunoassay (Yamasa, Tokyo, Japan). Pellets were dissolved in 0.1 N NaOH and saved for subsequent protein determination. Proteins were measured with a protein assay (Bio-Rad, Richmond CA, U.S.A.) with bovine serum albumin as the standard.

Statistical analysis

All data were given as mean $\pm$ s.e.mean, unless stated otherwise. Responses to CGS21680 or forskolin in cyclic AMP experiments were compared to the effects of agonist-free solution using Steel's test. The effects of inhibitors or antagonists were compared using Wilcoxon's rank sum test. Paired *t*-tests were performed to compare the raw values of the control with the responses in the presence of drugs applied on the same cell for analysis of spontaneous synaptic currents.

Drugs

Drugs were applied by replacing the solution superfusing the slice with one containing a set concentration. D-2-amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and CGS21680 were obtained from Tocris (Bristol, U.K.); tetrodotoxin (TTX) was from Wako (Tokyo, Japan); forskolin, 1,9-dideoxyforskolin, 9-[tetrahydro-2-furanyl]-9H-purin-6-amine (SQ22,536) and N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H-89) were from Sigma (St. Louis, MO, U.S.A.). (*E*)-8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxanthine (KF17837) was synthesized at the Medicinal Chemistry Department of the Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co. Ltd. (Shizuoka, Japan). KF17837 was used as KF17837S (Nonaka *et al*., 1994a, b).

Results

cyclic AMP accumulation by CGS21680 and forskolin in rat GP slices

We first examined whether cyclic AMP accumulation was a consequence of A_{2A} receptor activation in rat GP slice preparations. Basal levels of cyclic AMP in GP slices were 44.8 $\pm$ 10.5 pmol mg protein^{-1} (n = 11, Figure 1). An A_{2A} receptor selective agonist, CGS21680, significantly increased cyclic AMP accumulation in GP slices (1 μM: 114.8 $\pm$ 15.3 pmol mg protein^{-1}, n = 11, P < 0.05, 10 μM: 134.9 $\pm$ 16.4 pmol mg protein^{-1}, n = 7, P < 0.01 by Steel's test, Figure 1). In the presence of the A_{2A} receptor selective antagonist, KF17837 (10 μM), CGS21680 had little effect on cyclic AMP accumulation in GP slices (58.3 $\pm$ 10.9 pmol mg protein^{-1}, n = 4, Figure 1). These results indicate that an increase in cyclic AMP is caused by A_{2A} receptor stimulation.

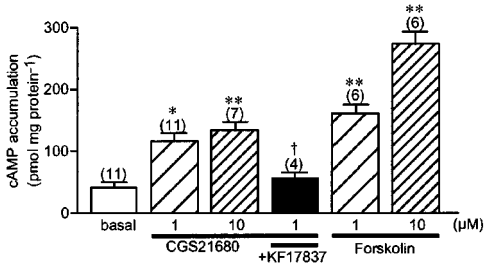

Figure 1 Cyclic AMP accumulation in GP slices induced by adenosine A_{2A} receptors and forskolin. CGS21680-induced cyclic AMP accumulation was blocked by KF17837. The error bars represent s.e.mean. Statistical significance was *P < 0.05, **P < 0.01 *vs* basal by Steel's test, †P < 0.05 *vs* 1 μM CGS21680 by Wilcoxon's rank sum test.

In addition, forskolin also significantly increased cyclic AMP accumulation in GP slices (1 μM: 160.4 $\pm$ 16.9 pmol mg protein^{-1}, n = 6, P < 0.01, 10 μM: 274.0 $\pm$ 22.1 pmol mg protein^{-1}, n = 6, P < 0.01 by Steel's test, Figure 1).

Effects of forskolin on GABAergic synaptic transmission in the GP

We next examined the effect of the adenylyl cyclase activator forskolin on IPSCs in the GP (Figure 2). Data from a typical experiment, in which the effect of forskolin (1 μM) on mIPSC frequency and amplitude were examined, are shown in Figure 2a–d. Figure 2a displays the representative consecutive current traces taken before and during forskolin application, showing that forskolin caused an increase in synaptic activity. Forskolin greatly increased the frequency of mIPSCs (Figure 2b). Application of forskolin did not change the cumulative distribution of mIPSC amplitudes (Figure 2c), but shifted the cumulative distribution of intervals between successive mIPSCs toward shorter intervals (Figure 2d). On average, the mean mIPSC frequency was significantly increased from 5.6 $\pm$ 1.3 to 10.0 $\pm$ 1.9 Hz (188 $\pm$ 21% of control, P < 0.01 by paired *t*-test; n = 5), whereas the mean amplitude in these experiments was not changed (mean amplitude in the presence of forskolin: 29.7 $\pm$ 2.5 pA, 102 $\pm$ 2% of control, n = 5) (Figure 3). Application of 1,9-dideoxyforskolin (1 μM), an inactive forskolin analogue, had no effect on the frequency and the mean amplitude of mIPSCs (mean frequency: 101 $\pm$ 3% of control, mean amplitude: 98 $\pm$ 1% of control, n = 4). These results indicate that the enhancement of mIPSC frequency seen on application of forskolin was due to activation of adenylyl cyclase. Thus, adenylyl cyclase activation at presynaptic terminals in the GP was found to enhance GABA release.

Effects of adenylyl cyclase inhibitor on CGS21680-induced enhancement of the mIPSC frequency

To investigate the involvement of adenylyl cyclase in the potentiating action of A_{2A} receptor agonists, we examined effects of an inhibitor of adenylyl cyclase on CGS21680-induced enhancement of the mIPSC frequency. In control experiments, CGS21680 (1 μM) increased the mIPSC frequency from 7.3 $\pm$ 1.2 to 9.6 $\pm$ 1.4 Hz (135 $\pm$ 6% of control,

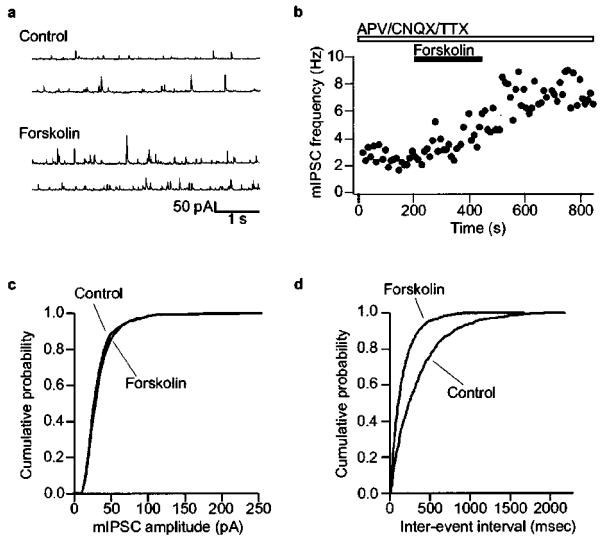

Figure 2 Effects of forskolin on GABAergic synaptic transmission in the GP. The data are taken from the same neuron. (a) Two consecutive traces of mIPSCs before (control) and during application of forskolin (1 μM). (b) Time course of the frequency of mIPSCs during application of forskolin (1 μM). (c,d) Cumulative probability distribution of mIPSC amplitude (c) and inter-event interval (d) before (572 events) and during application of forskolin (1228 events). Forskolin had no effect on the amplitude distribution ($P>0.1$, Kolmogorov–Smirnov test for control-forskolin), but shifted the frequency distribution to shorter inter-event internals ($P<0.001$, Kolmogorov–Smirnov test for control-forskolin). The frequencies and amplitudes (mean$\pm$s.d.) of mIPSCs were 2.88 Hz and 32.90$\pm$24.80 pA in control and 6.19 Hz and 33.97$\pm$22.69 pA in forskolin application, respectively.

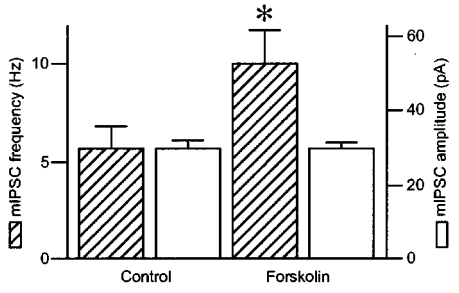

Figure 3 Summary graph of the experiments which tested the effect of forskolin on mIPSC frequency and amplitude. Pooled data of five neurons show that forskolin (1 μM) increased the mean frequency without affecting the mean amplitude of mIPSCs. Mean frequency (hatched bar) and mean amplitude (open bar) of mIPSC were 5.6$\pm$1.3 Hz and 29.4$\pm$2.9 pA before (control) and 10.0$\pm$1.9 Hz and 29.7$\pm$2.5 pA during application of forskolin, respectively. Forskolin increased the frequency of mIPSCs by 188$\pm$21%. The error bars represent s.e.m. Statistical significance was $^*P<0.01$ *vs* control by paired *t*-test.

$n=8$, Figure 4), without any change in the mean amplitude (26.0$\pm$1.8 pA and 26.3$\pm$1.6 pA, $n=8$). In slices preincubated (>30 min) with 9-[tetrahydro-2-furanyl]-9H-purin-6-amine (SQ22,536, 300 μM), an agent which is known to inhibit the activity of adenylyl cyclase (Madison & Nicoll, 1986; Kondo & Marty, 1997), the mIPSC frequency increase by CGS21680 was 106$\pm$4% of control ($n=7$, mean frequency in the presence of CGS21680: 4.6$\pm$0.5 Hz, Figure

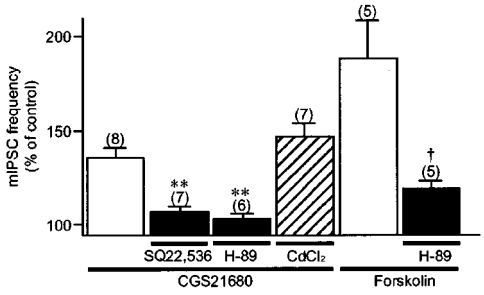

Figure 4 Summary of pharmacological characterization of the mIPSCs frequency. Data are normalized as a percentage of control values. The error bars represent s.e.m. The numbers of cells examined are given in parentheses. Statistical significance was $^{**}P<0.01$ *vs* CGS21680 by Wilcoxon's rank sum test, $\dagger P<0.05$ *vs* forskolin by Wilcoxon's rank sum test.

4). Application of SQ22,536 significantly reduced the action of CGS21680 on the mIPSC frequency ($P<0.01$ by Wilcoxon's rank sum test). These results show that inhibiting adenylyl cyclase markedly diminishes the CGS21680-induced increase in mIPSC frequency. SQ22,536 itself had no significant effect on the frequency and amplitude of mIPSCs compared to those observed under control conditions ($P>0.1$ in both cases, Wilcoxon's rank sum test). This indicated that the endogenous activity of adenylyl cyclase made little significant contribution to basal GABAergic synaptic transmission in the GP.

Effects of PKA inhibitor on CGS21680-induced enhancement of the mIPSC frequency

Regulation of neurotransmitter release by cyclic AMP is known to be mediated thorough cyclic AMP-dependent protein kinase (PKA) activation or by PKA-independent mechanisms (Beaumont & Zucker, 2000; Ozaki *et al.*, 2000; Sakaba & Neher, 2001). Hence, in order to determine whether PKA pathway was responsible for the action of CGS21680 on mIPSC frequency, we investigated the effect of a PKA selective inhibitor, H-89 (Chijiwa *et al.*, 1990). We incubated slices for at least 2 h in saline containing H-89 (10 μM) prior to and during the challenge with CGS21680. In buffers incubated with H-89 (10 μM), CGS21680 (1 μM) failed to increase the mIPSC frequency (mean frequency in the presence of CGS21680: 6.8$\pm$1.6 Hz, 102$\pm$4% of control, $n=6$) (Figure 4). Under H-89-treated conditions, the action of forskolin on the mIPSC frequency was also significantly reduced to 118$\pm$5% of control ($n=5$, $P<0.05$ by Wilcoxon's rank sum test) (Figure 4).

Independence of Ca^{2+} conductance blockade on CGS21680-induced enhancement of the mIPSC frequency

An increase in a presynaptic voltage-dependent Ca^{2+} conductance might be responsible for the CGS21680-induced enhancement of the mIPSC frequency. To determine the contribution of voltage-dependent Ca^{2+} channels at GABAergic presynaptic terminals to the CGS21680-induced augmentation of mIPSC frequency, we examined the effectiveness of CGS21680 on mIPSCs after adding $CdCl_2$

(100 μM), a non-selective voltage-dependent Ca^{2+} channel blocker, to the external solution. The mean frequency and amplitude of the mIPSCs in the presence of Cd^{2+} were 4.2 ± 1.0 Hz and 24.3 ± 0.7 pA, respectively ($n=7$). Thus, mIPSCs still occurred in the external solution containing $CdCl_2$, and Cd^{2+} did not exert a significant effect on the frequency and amplitude of mIPSCs. Figure 5a shows the representative consecutive current traces taken before and during CGS21680 application in Cd^{2+}-containing saline. Application of CGS21680 in the presence of Cd^{2+} increased the frequency of mIPSCs (Figure 5b), and there was no significant change in these mIPSC-amplitude distributions (Figure 5c). On average, application of CGS21680 in the presence of Cd^{2+} significantly increased the mean mIPSC frequency to $146\pm8\%$ of control (mean frequency in the presence of CGS21680: 6.0 ± 1.3 Hz, $P<0.01$ by paired t-test; $n=7$) (Figure 5d), not different from the 135% increase observed in the absence of Cd^{2+}. On the other hand, the mean amplitude in the presence of Cd^{2+} was not changed before and after application of CGS21680 ($103\pm4\%$ of control, $n=7$). These results indicate that the action of CGS21680 on mIPSCs is independent of Ca^{2+} influx into the presynaptic terminal through voltage-dependent Ca^{2+} channels.

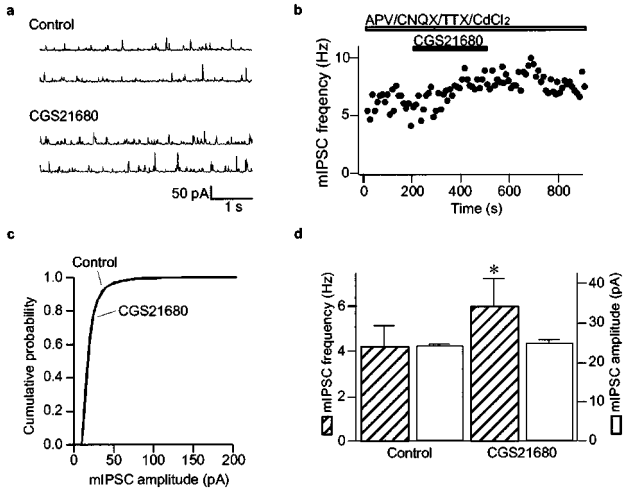

Figure 5 Effects of CGS21680 on mIPSCs in the presence of voltage-gated Ca^{2+} channel blocker. Spontaneous mIPSCs were recorded in a solution containing $CdCl_2$ (100 μM). (a–c) the data are taken from the same neuron. (a) Two consecutive traces of mIPSCs before (control) and during application of CGS21680 (1 μM). (b) Time course of the frequency of mIPSCs during application of CGS21680 (1 μM). (c) Cumulative probability distribution of mIPSC amplitude before (1225 events) and during application of CGS21680 (1670 events). CGS21680 had no effect on the amplitude distribution ($P>0.2$, Kolmogorov–Smirnov test for control-CGS21680). (d) Summary graph of the experiments which tested the effect of CGS21680 on mIPSC frequency and amplitude. Pooled data of seven neurons show that CGS21680 (1 μM) increased the mean frequency without affecting the mean amplitude of mIPSCs. Mean frequency (hatched bar) and mean amplitude (open bar) of mIPSC were 4.2 ± 1.0 Hz and 24.3 ± 0.7 pA before (control) and 6.0 ± 1.3 Hz and 25.0 ± 1.4 pA during application of CGS21680, respectively. CGS21680 increased the frequency of mIPSCs by $146\pm8\%$. The error bars represent s.e.mean. Statistical significance was $*P<0.01$ *vs* control by paired t-test.

Discussion

Involvement of the adenylyl cyclase-PKA pathway in GABAergic synaptic transmission in the GP

The purpose of this work was to elucidate the cellular mechanisms underlying presynaptic A_{2A} receptor-mediated regulation of GABAergic synaptic transmission in the GP (Shindou *et al*., 2001). In our previous study, CGS21680 (1 μM)-induced enhancement of GABAergic synaptic transmission in the GP was caused *via* adenosine A_{2A} receptors (Shindou *et al*., 2001). Other studies reported that CGS21680 (0.1–1 μM) caused a net inhibition of electrical field stimulation-evoked GABA release from pallidal slices, perhaps *via* adenosine A_1 receptors (Mayfield *et al*., 1993). However, we did not detect any CGS21680-induced inhibition of IPSCs in the GP. In addition, in our previous report using rat striatal slices, the actions of CGS21680 (1 μM) on striatal IPSCs were blocked by the selective adenosine A_{2A} antagonist KF17837 but not by a selective A_1 antagonist (Mori *et al*., 1996). From these data, the action of CGS21680 (1 μM) on GABAergic synaptic transmission in the GP is caused *via* A_{2A} receptors, but not A_1 receptors. We thus used this concentration of CGS21680 (1 μM) to selectively activate A_{2A} receptors in the GP.

CGS21680 increased cyclic AMP accumulation in GP slices (Figure 1). This is in line with the general concept that the A_{2A} receptor is positively coupled to adenylyl cyclase (van Calker *et al*., 1979). We next examined whether the A_{2A} receptor-mediated regulation of the adenylyl cyclase-cyclic AMP signal transduction pathway is involved in regulating GABA release in the GP, using whole-cell patch-clamp recording. Forskolin, which also caused an increase in cyclic AMP accumulation in GP slices, increased the frequency of mIPSCs without affecting their amplitude distribution (Figures 2–4), thus showing that it was acting presynaptically. Forskolin therefore mimicked the previously reported effects of CGS21680 on mIPSCs (Shindou *et al*., 2001). In addition, the CGS21680-induced enhancement of mIPSC frequency was inhibited by an inhibitor of adenylyl cyclase, SQ22,536 (Figure 4). These results suggest that A_{2A} receptors activate adenylyl cyclase at presynaptic terminals in the GP, resulting in cyclic AMP accumulation.

Next, we tested the involvement of PKA in the facilitatory action of cyclic AMP on GABA release. The forskolin- and CGS21680-induced enhancement of the mIPSC frequency was suppressed by H-89, a PKA inhibitor (Figure 4). These results imply that cyclic AMP accumulation produced by A_{2A} receptor or adenylyl cyclase-activation enhances GABA release by PKA activation. Taken together, we can conclude that the A_{2A} receptor-mediated regulation of GABA release in the GP synaptic terminals involves sequentially the activation of A_{2A} receptors, adenylyl cyclase, cyclic AMP production, and then PKA. We demonstrated for the first time that the presynaptic control of GABAergic synaptic transmission in the GP could be exerted through the adenylyl cyclase-PKA pathway. With regard to the facilitating action in other inhibitory synapses, an elevation of intracellular cyclic AMP level has been reported to facilitate transmitter release in a number of other systems, including adrenergic enhancement in cerebellar Purkinje neurons (Llano & Gerschenfeld, 1993; Kondo & Marty, 1997), dopaminergic

facilitation in the substantia nigra pars reticular (Radnikow & Misgeld, 1998) and the potentiation of hippocampal GABAergic transmission (Sciancalepore *et al*., 1995; Wang *et al*., 1997).

No direct involvement of Ca^{2+} channels in enhancement of GABAergic synaptic transmission via *an A_{2A} receptor*

In the present study, the presynaptic facilitation of mIPSCs by CGS21680 remained unaffected by blockade of presynaptic Ca^{2+} entry with Cd^{2+} (Figure 5), indicating that an increase of Cd^{2+} -sensitive voltage-dependent Ca^{2+} conductance at presynaptic terminals is not required for the potentiating actions of CGS21680. This finding suggests that CGS21680 produces its facilitatory effect on the release of GABA subsequent to calcium entry *via* voltage-dependent Ca^{2+} channels. Similar calcium-independent mechanisms of presynaptic facilitation in inhibitory synapses have been observed in other regions, such as the hippocampus (Wang *et al*., 1997), the substantia nigra pars reticular (Radnikow & Misgeld, 1998) and the cerebellum (Kondo & Marty, 1997). In addition, forskolin-induced potentiation was shown to be preserved in the presence of Cd^{2+} in hippocampal inhibitory synapses (Capogna *et al*., 1995). Trudeau *et al*. (1996) reported that presynaptic activation of PKA directly facilitates the secretory process at a step downstream from Ca^{2+} influx. Thus, A_{2A} receptors at the GABAergic presynaptic terminals in the GP could serve to act on the GABA release machinery through the adenylyl cyclase-PKA pathway.

In our previous study, the amplitude of evoked IPSCs was also enhanced by the A_{2A} receptor activation (Shindou *et al*., 2001). The present result that CGS21680 enhanced the Ca^{2+}-independent release of GABA does not exclude an additional interaction with the Ca^{2+} channels underlying evoked synaptic release. Indeed, in the striatum, a study using rat synaptosomes showed the A_{2A} receptor activation increased the potassium-evoked release of acetylcholine from striatal nerve terminals through a pathway involving cyclic AMP and PKA activation for P-type Ca^{2+} channels (Gubitz *et al*., 1996).

In conclusion, we have performed a series of electrophysiological and biochemical studies demonstrating that the adenosine A_{2A} receptor-mediated potentiation of mIPSCs in the GP is mediated through the sequential activation of adenylyl cyclase and PKA. We also suggest that activated PKA phosphorylates substrate proteins on synaptic vesicles or the presynaptic membrane and this phosphorylation then facilitates the vesicle release mechanism downstream from Ca^{2+} entry.

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