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D_5 Dopamine Receptors Enhance Zn^{2+}-Sensitive $GABA_A$ Currents in Striatal Cholinergic Interneurons through a PKA/PP1 Cascade

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Summary

Cholinergic interneurons have been implicated in striatally mediated associative learning. In classical conditioning paradigms, conditioned stimuli trigger a transient suppression of neuronal activity that is dependent upon an intact dopaminergic innervation. Our hypothesis was that this suppression reflected dopaminergic enhancement of sensory-linked GABAergic input. As a test, the impact of dopamine on interneuronal $GABA_A$ receptor function was studied by combined patch-clamp recording and single-cell reverse transcription PCR. Activation of D_5 dopamine receptors reversibly enhanced a Zn^{2+}-sensitive component of $GABA_A$ currents. Although dependent upon protein kinase A (PKA) activation, the modulation was blocked by protein phosphatase 1 (PP1) inhibition, suggesting it was dependent upon dephosphorylation. These results establish a novel mechanism by which intrastriatally released dopamine mediates changes in GABAergic signaling that could underlie the initial stages of associative learning.

Introduction

Striatal cholinergic interneurons are important regulators of striatal circuitry. These giant interneurons have widespread intranuclear connections, making striatal levels of cholinergic enzymes among the highest of any brain region (McGeer et al., 1974; Graybiel, 1990; Wilson et al., 1990). The dopaminergic innervation of these neurons originates in the ventral mesencephalon and is known to be a critical determinant of cellular behavior. The loss of this innervation is also a key factor in the emergence of motor abnormalities in neuropathologies like Parkinson's disease (Lehmann and Langer, 1983).

The dopaminergic modulation of cholinergic interneurons may also be a critical determinant of striatally mediated associative learning. In behaving monkeys, the activity of presumptive cholinergic interneurons begins to be modulated by conditioned stimuli as animals learn their association with reward (Aosaki et al., 1994; Graybiel et al., 1994). This modulation in activity is dependent upon dopaminergic input from mesencephalic neurons that initially respond to the presentation of reward and subsequently respond to presentation of the conditioned stimulus (Schultz et al., 1993). As learning progresses, the conditioned stimulus typically leads to a transient suppression in the ongoing activity of cholinergic interneurons—a signal that serves to coordinate global striatal activity. This suppression could result from dopaminergic modulation of voltage-dependent ionic conductances regulating repetitive discharge or could result from an alteration of synaptically mediated currents, such those arising from GABAergic neurons responding to the sensory cue (Alexander et al., 1986).

In spite of its recognized importance, little is actually known about how dopamine modulates the activity of cholinergic interneurons. Of the five dopamine receptors that have been cloned (Sibley, 1995), cholinergic interneurons express D_2 and D_5 receptors in abundance (LeMoine et al., 1990; Bergson et al., 1995; Yan et al., 1997). Although D_2 receptors negatively couple to N-type Ca^{2+} channels (Yan et al., 1997), they do not appear to have pronounced effects on other ionic conductances that could mediate a suppression in ongoing discharge. Similarly, activation of D_5 receptors does not have pronounced effects on Na^+, Ca^{2+}, or depolarization-activated K^+ channels (unpublished data).

Another way in which D_5 receptors could suppress ongoing activity is through modulation of GABAergic signaling (McGeer et al., 1977; Bartholini et al., 1981). Anatomical studies have shown that GABAergic recurrent axon collaterals of medium spiny neurons and interneurons make synaptic contact with cholinergic interneurons (Bolam et al., 1986; Martone et al., 1992; Kita, 1993). However, little is known about the characteristics of the postsynaptic $GABA_A$ receptors transducing this input. Nevertheless, in other cell types, $GABA_A$ receptor–mediated currents are potently modulated by protein kinases (Macdonald and Olsen, 1994; Rabow et al., 1995). Protein kinase A (PKA), in particular, commonly has been found to modulate $GABA_A$ currents (Porter et al., 1990; Cheun and Yeh, 1992; Kano and Konnerth, 1992; Moss et al., 1992a, 1992b; Browning et al., 1993; Tehrani and Barnes, 1995; Kapur and Macdonald, 1996). Activation of D_5 dopamine receptors is known to stimulate adenylyl cyclase and PKA (Grandy et al., 1991; Sunahara et al., 1991), lending plausibility to the conjecture that these receptors could couple to $GABA_A$ receptors.

The studies presented here provide molecular and physiological evidence that striatal cholinergic interneurons express a heterogeneous population of $GABA_A$ receptors that include subunits known to be phosphorylated by serine/threonine kinases. Activation of D_5 dopamine receptors results in the reversible enhancement of a Zn^{2+}-sensitive subset of these receptors. Although dependent upon PKA activation, the modulation does not appear to be directly mediated by PKA but by protein phosphatase 1 (PP1)-mediated dephosphorylation of $GABA_A$ receptors. These results establish a novel mechanism by which intrastriatally released dopamine mediates an enhancement of GABAergic signaling that could underlie the initial stages of associative learning.

Results

Cholinergic Interneurons Coexpress a Variety of $GABA_A$ Receptor Subunits

Acutely isolated cholinergic interneurons were distinguished from other striatal cell types by somal size and

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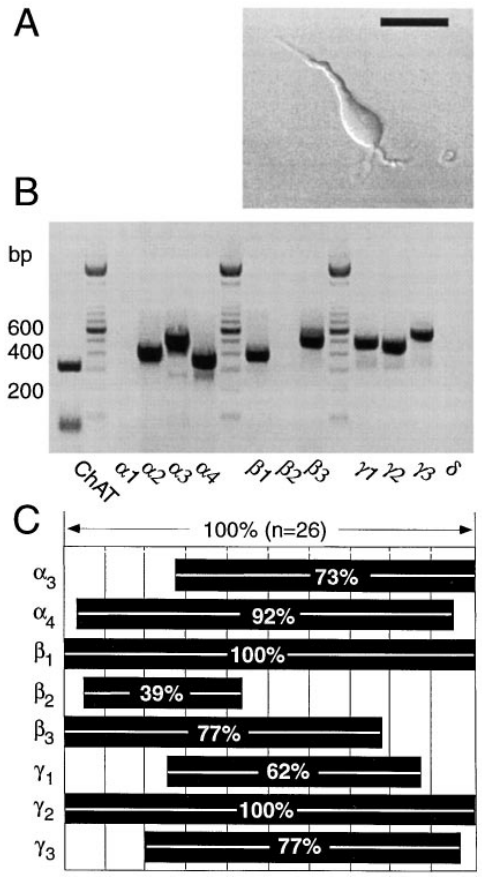

Figure 1. Multiple $GABA_A$ Receptor Subunit mRNAs Were Coexpressed in Single Cholinergic Interneurons

(A) Photomicrograph of an acutely isolated neostriatal cholinergic interneuron. Scale bar, 25 μm.

(B) Photomicrograph of an ethidium bromide–stained gel in which PCR amplicons have been separated by electrophoresis. The ChAT-positive neuron coexpressed several $GABA_A$ receptor subunit mRNAs.

(C) Bar plot showing the coordinated expression of $GABA_A$ receptor subunits (α3–4, β1–3, and γ1–3) containing PKA phosphorylation sites in a sample of 26 cholinergic interneurons. The extent of coexpression is coded by the overlap of the bars.

the expression of choline acetyltransferase (ChAT) mRNA (Figures 1A and 1B). To determine how the expression of $GABA_A$ receptor subunit isoforms was coordinated, cholinergic interneurons were analyzed using reverse transcription polymerase chain reaction (RT-PCR) techniques. To minimize problems associated with template abundance, a two-stage PCR amplification protocol was used. In the first round of amplification, each family of subunits was amplified using degenerate primer sets. In the second round, subunit-specific "nested" primers were used. Because previous studies had shown that α5–6 subunits are not found in the striatum (Wisden et al., 1992), attention was focused on the coordinated expression of α1–4, β1–3, γ1–3, and δ subunit mRNAs.

These experiments revealed that several $GABA_A$ receptor subunit isoforms were expressed by individual interneurons (Figure 1B). Nearly all ChAT neurons coexpressed α2, α4, β1, and γ2 mRNAs (n = 26). Both long and short isoforms of the γ2 subunit ($\gamma2_L$, $\gamma2_S$) (Whiting et al., 1990; Kofuji et al., 1991) were detected in most neurons. The majority of the sample (60%–80%) also had detectable levels of α3, β3, γ1, and γ3 mRNAs. mRNA for the α1 subunit was detected in half of the sample, whereas β2 and δ mRNAs were detected less frequently (~35%).

Many $GABA_A$ receptor subunits for which mRNA was detected contain consensus serine/threonine kinase phosphorylation sites (Macdonald and Olsen, 1994; McKernan and Whiting, 1996). To show how the expression of these subunits was coordinated within individual neurons, a plot was constructed where codetection was represented by overlap along the abscissa of bars coding the percentage of the population expressing each subunit. As shown in Figure 1C, β and γ subunit mRNAs, particularly those for β1 and γ2 subunits, were commonly detected in the same cell. This is of interest because studies of recombinant receptors have suggested that these two subunits are preferred substrates for PKA and protein kinase C (PKC) (Browning et al., 1990; Moss et al., 1992a; Krishek et al., 1994).

The coexpression of more than five $GABA_A$ subunit mRNAs in a single cell suggests that more than one type of receptor must be present. To test this hypothesis, GABA was applied to acutely dissociated cholinergic interneurons that were voltage clamped using whole-cell techniques. The application of GABA evoked a partially desensitizing, bicuculline-sensitive outward current (Figure 2A). However, the current profile alone gave no indication of receptor heterogeneity.

Pharmacological studies provided more convincing evidence in this regard. In heterologous expression systems where the receptor population is assumed to be uniform, allosteric modulators of receptor function have been shown to be dependent upon subunit composition. For example, benzodiazepines are potent modulators of receptors containing γ subunits (Macdonald and Olsen, 1994). In our cells, application of the benzodiazepine agonist diazepam (2 μM) enhanced $GABA_A$ current amplitudes (Figure 2B), in agreement with the robust expression of γ subunit mRNAs. On the other hand, low micromolar concentrations of Zn^{2+} block $GABA_A$ receptors lacking γ subunits (Draguhn et al., 1990). In our cells, application of Zn^{2+} (10 μM) reduced GABA-evoked currents by 25%–45%, suggesting that γ subunit–deficient receptors were also present. To verify that low micromolar concentrations of Zn^{2+} blocked a distinct group of receptors, dose–response experiments were performed. As shown in Figure 2D, the dose–response data were well-described by a sum of two isotherms having IC_{50}s of 6 μm and 272 μM, confirming the presence of a subset of receptors possessing a high affinity for Zn^{2+}.

Activation of D_5 Dopamine Receptors Reversibly Enhances $GABA_A$ Currents

Single-cell RT-PCR analysis of isolated cholinergic interneurons revealed that nearly all coexpress D_2 and D_5 receptor mRNAs, whereas D_1 mRNA was rarely found (Yan et al., 1997). An example of a typical dopamine receptor profile for a cholinergic interneuron is shown

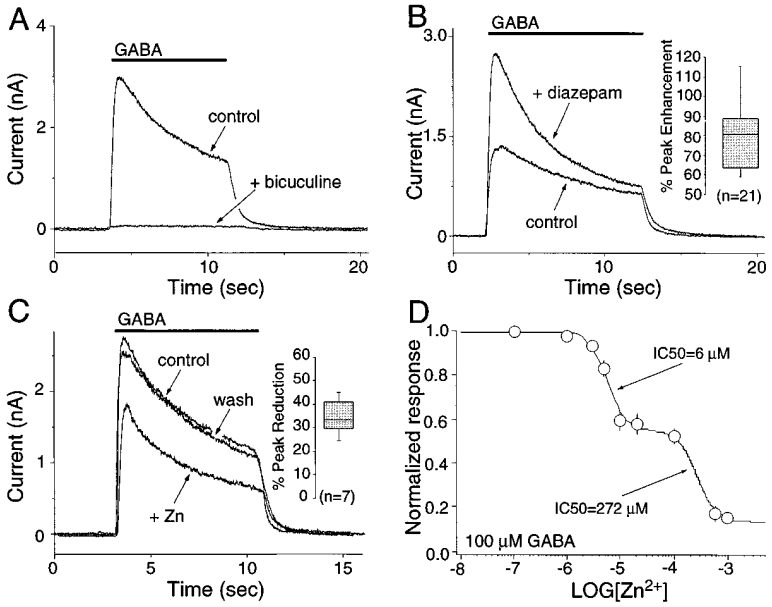

Figure 2. $GABA_A$ Currents in Cholinergic Interneurons Are Sensitive to Zn^{2+} and Benzodiazepines

(A) Currents elicited by the application of GABA (100 μM) and the coapplication of GABA and the $GABA_A$ receptor antagonist bicuculline (30 μM).

(B) Currents evoked by GABA (20 μM) in the presence and absence of the benzodiazepine agonist diazepam (2 μM). Inset: box plot showing the percent enhancement in peak $GABA_A$ current produced by diazepam (n = 21).

(C) Zn^{2+} (10 μM) reversibly blocked a portion of GABA-evoked (100 μM) currents. Inset: box plot showing the percent reduction in peak $GABA_A$ current produced by 10 μM Zn^{2+} (n = 7).

(D) Zn^{2+} dose–response relationship for the block of GABA-evoked (100 μM) currents. The data points are means derived from three to five neurons; SEMs (vertical bars) are smaller than the symbol size for many of the points. The solid line is the least square fit of a two-isotherm Hill equation with IC_{50}s of 6 μM and 272 μM.

in Figure 3A. These data strongly argue that the modulatory effects evoked by D_1 class dopamine agonists in interneurons can be attributed to the activation of D_5 receptors.

The application of dopamine or D_1 class agonists (SKF 81297, SKF 82958) reversibly enhanced whole-cell $GABA_A$ currents in nearly every cholinergic interneuron tested (90%; n = 74), whereas D_2 class agonists had no effect on $GABA_A$ currents (n = 3). Shown in Figure 3B is a plot of peak currents evoked by the repeated application of GABA (100 μM) as a function of time. GABA was applied once a minute for 3–5 s to minimize alterations in current amplitude attributable to desensitization. Experiments with a rapid agonist application system confirmed that this repetition rate was sufficiently slow to allow complete recovery of the GABA response. The dopaminergic enhancement of the response to GABA was relatively slow in onset, usually taking 1–3 min to stabilize. The modulation was not accompanied by any detectable change in current kinetics when examined with either our conventional perfusion system (Figure 3C) or with a rapid, piezoelectric perfusion system (data not shown). Removal of the agonist led to a return of currents to control amplitudes within 2–3 min. The median enhancement of peak currents evoked by 100 μM GABA was approximately 30% (Figure 3B, inset). Application of the D_1 class receptor antagonist SCH 23390 (10 μM) reversibly blocked the enhancement (Figure 3D) (n = 8; $p < 0.05$, Mann-Whitney U test), confirming mediation by the D_5 dopamine receptor. The magnitude of

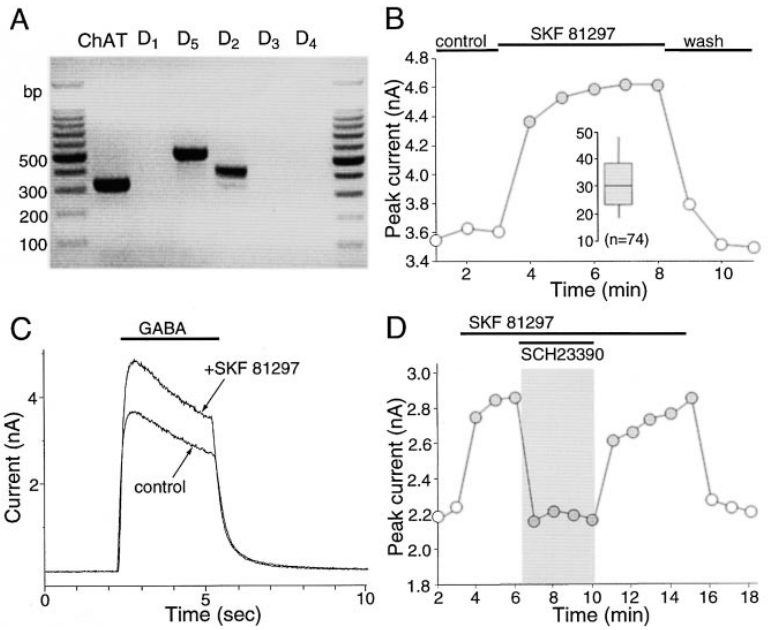

Figure 3. Activation of D_5 Dopamine Receptors Reversibly Enhanced $GABA_A$ Currents

(A) A photomicrograph of an ethidium bromide–stained gel in which dopamine receptor amplicons have been separated by electrophoresis. Note the presence of D_5 and D_2 dopamine receptor amplicons.

(B) Plot of peak current evoked by GABA (100 μM) as a function of time and agonist application. The D_1 class agonist SKF 81297 (10 μM) reversibly enhanced $GABA_A$ currents. The inset is a box plot summary of the percent enhancement of peak $GABA_A$ currents produced by D_1 class agonists in a sample of 74 cholinergic interneurons.

(C) Current traces taken from the records used to construct (B).

(D) Plot of peak $GABA_A$ current as a function of time and ligand application. The D_1 class antagonist SCH 23390 (10 μM) blocked the SKF 81297 effect on $GABA_A$ currents.

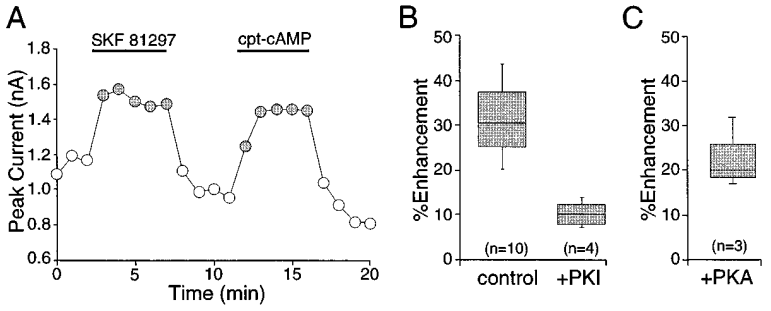

Figure 4. The Dopaminergic Modulation of $GABA_A$ Currents Was Mimicked by cAMP Analogs and PKA Dialysis but Blocked by PKA Inhibition

(A) Plot of peak $GABA_A$ current as a function of time and drug application. The membrane permeant cAMP analog cpt-cAMP (500 μM) had an effect that was similar to the D_1 class agonist SKF 81297, enhancing $GABA_A$ currents reversibly.

(B) Box plot summary of the percent enhancement on $GABA_A$ currents by SKF 81297 (10 μM) in control cells (n = 10) and in cells dialyzed with PKA inhibitory peptide PKI (50 μM; n = 4).

(C) Box plot summary of the percent enhancement of $GABA_A$ currents in cells dialyzed with PKA catalytic subunit (1 μg/ml) for 4–5 min.

the enhancement by dopamine was also sensitive to the concentration of GABA used to evoke currents. In cells where both high (1 mM) and low (100 μM) concentrations of GABA were applied after exposure to D_5 agonists, the modulation at the high concentration of GABA was only 37% (± 14%) of that at the lower GABA concentration ($n = 5$; $p < 0.05$, Mann-Whitney U test).

The D_5 Receptor Modulation Depends upon Activation of PKA and PP1

In recombinant systems, D_5 receptors positively couple to adenylyl cyclase through a G_s or G_{olf} class protein, stimulating cAMP production (Grandy et al., 1991; Sunahara et al., 1991). To test whether this cytosolic pathway mediated the effects of receptor stimulation in interneurons, the response to cAMP analogs was compared to that of receptor activation. As shown in Figure 4A, cpt-cAMP (500 μM) mimicked the effect of D_1 class agonists, reversibly enhancing $GABA_A$ currents. The median enhancement by cpt-cAMP (40%, $n = 4$) was similar to that seen with SKF 81297 (10 μM) ($p > 0.05$, Mann-Whitney U test). If the D_1 class agonist was exerting its effect through the cAMP cascade, then saturating concentrations of cAMP analogs should occlude the modulation produced by the D_1 agonist. As expected, in the presence of high concentrations of cpt-cAMP (500 μM), SKF 81297 (10 μM) failed to enhance further $GABA_A$ currents ($n = 4$; $p > 0.05$, Mann-Whitney U test).

In eukaryotic cells, the principal downstream target of cAMP is PKA. If PKA were mediating the $GABA_A$ modulation, then inhibiting PKA should block the effect of receptor activation. In agreement with this model, dialysis with the PKA inhibitory peptide PKI [5–24] (50 μM) significantly reduced the ability of SKF 81297 to enhance GABA-evoked currents (Figure 4B) ($n = 4$; $p < 0.05$, Mann-Whitney U test). In addition, the membrane-permeant PKA inhibitor H-89 (5 μM) was able to block reversibly the effects of SKF 81297 ($n = 4$; $p < 0.05$, Mann-Whitney U test). However, H-89 alone enhanced currents, suggesting that another constitutively active H-89-sensitive kinase was present and capable of reducing GABA-evoked currents. To provide an independent test of PKA involvement, neurons were dialyzed with PKA catalytic subunit (1 μg/ml). As expected, GABA-evoked currents in these cells were enhanced (Figure 4C) ($n = 3$; $p < 0.05$, Mann-Whitney U test).

If PKA directly mediated the modulation, then inhibition of protein phosphatases should augment the response. In fact, inhibition of phosphatases PP1 and PP2A dramatically reduced the modulation. The results from a typical experiment are shown in Figures 5A and 5B. In this neuron, SKF 81297 (10 μM) enhanced peak GABA-evoked currents. The application of okadaic acid (1 μM) had no effect on basal currents but significantly reduced the ability of SKF 81297 to enhance currents ($n = 10$; $p < 0.05$, Mann-Whitney U test). Washing off the okadaic acid resulted in a return of the D_5 receptor modulation. The inactive okadaic acid homolog, norokadaone (0.2–1 μM), did not significantly alter the D_5 receptor modulation (Figure 5C) ($n = 8$; $p > 0.05$, Mann-Whitney U test). Another phosphatase inhibitor, calyculin A (500 nM), mimicked the effect of okadaic acid (1 μM) ($n = 3$). To try to separate the roles of PP1 and PP2A, low nanomolar concentrations of okadaic acid were tested. PP2A-selective concentrations of okadaic acid (10 nM; Ishihara et al., 1989) failed to reduce the effects of SKF 81297 (Figure 5D) ($n = 5$; $p > 0.05$, Mann-Whitney U test), suggesting that PP1 was the obligate target in the signaling pathway. To provide additional evidence of PP1 involvement, neurons were dialyzed with the membrane-impermeant phosphatase inhibitor microcystin LR (8 μM). As shown in Figure 5E, the ability of SKF 81297 to enhance currents was significantly attenuated in microcystin-loaded neurons ($n = 5, 10$; $p < 0.05$, Mann-Whitney U test).

PKC Phosphorylation Reduced $GABA_A$ Currents but Did Not Alter the D_5 Receptor Modulation

If D_5 receptors were exerting their effect on $GABA_A$ receptors by promoting dephosphorylation, then the reversal of the modulation requires kinase activity. Phosphorylation of the receptor (or a governing protein) by this kinase should decrease GABA-evoked currents. PKC is a serine/threonine kinase that has been reported to decrease $GABA_A$ currents (Kellenberger et al., 1992; Krishek et al., 1994) and, thus, is a candidate. Application of PKC activator phorbol-12-myristate-13-acetate (PMA; 500 nM) reduced the amplitude of $GABA_A$ currents in cholinergic interneurons irreversibly (Figures 6A and 6B). The modulation was slow, usually taking 5–8 min to stabilize. The median reduction by PMA was 38% ($n = 21$; Figure 6B, inset), while the inactive 4α-phorbol

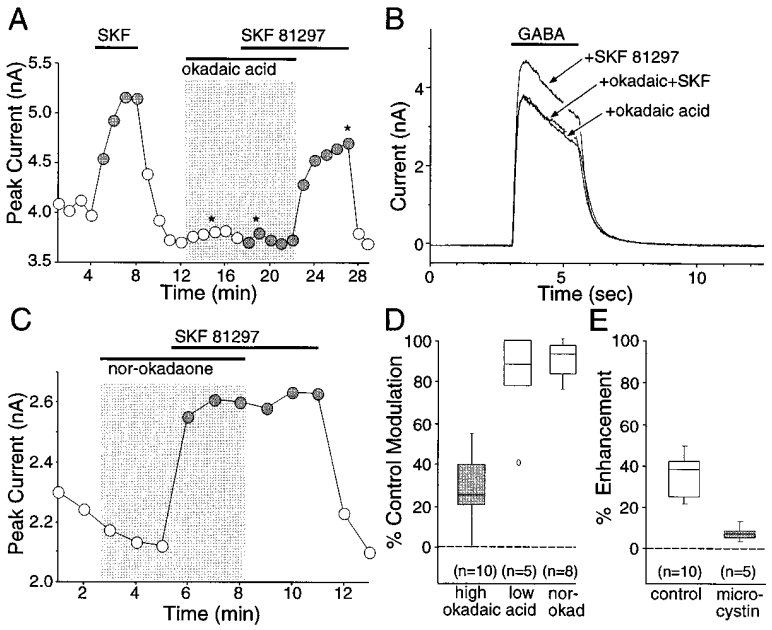

Figure 5. The D_5 Receptor-Induced Enhancement of $GABA_A$ Currents Was Blocked by Inhibition of Protein Phosphatases

(A) Plot of peak $GABA_A$ current as a function of time and drug application. The D_1 class agonist SKF 81297 (10 μM) enhanced $GABA_A$ currents reversibly. Coapplication of the phosphatase inhibitor okadaic acid (1 μM) eliminated the effect of SKF 81297. Washing off okadaic acid restored the ability of SKF 81297 to modulate $GABA_A$ currents.

(B) Representative current traces taken from the points denoted by asterisks in (A).

(C) Plot of peak $GABA_A$ current as a function of time and drug application. The inactive analog of okadaic acid, nor-okadaone (0.2 μM) did not affect the SKF 81297–induced enhancement of $GABA_A$ currents.

(D) Box plot summary of the modulation of $GABA_A$ currents by SKF 81297 in the presence of high (1 μM) and low (10 nM) concentrations of okadaic acid and nor-okadaone (0.2–1 μM; n = 8), expressed as a percentage of the modulation in the absence of these reagents. Note that the low concentration of okadaic acid did not affect the SKF 81297 modulation, suggesting the involvement of PP1 in the modulation.

(E) Box plot summary of the percent modulation of $GABA_A$ currents by SKF 81297 (10 μM) in control neurons (n = 10) and in neurons dialyzed with microcystin LR (8 μM; n = 5).

(500 nM) did not affect current amplitudes (Figure 6A) ($n = 3$; $p > 0.05$, Mann-Whitney U test). Dialysis with PKC inhibitory peptide $PKC_{19–31}$ (30 μM) blocked the effect of PMA (Figure 6D) ($n = 3$; $p < 0.05$, Mann-Whitney U test), suggesting that its effect was mediated by PKC activation.

Following the modulation of $GABA_A$ currents by PKC, D_5 receptor activation or application of cAMP analogs led to an enhancement of currents and an apparent reversal of the PKC effects (Figure 6C). However, the magnitude and kinetics of the dopaminergic modulation before and after PKC activation were not significantly different ($n = 5$; $p > 0.05$, Mann-Whitney U test). Therefore, it was possible that convergence of the PKC and PP1 pathways was more apparent than real. If dephosphorylation of a PKC site was responsible for the PP1-mediated enhancement of currents, and rephosphorylation was required for reversal of the modulation, then inhibition of PKC should attenuate the enhancement brought about by D_5 receptors and prevent its reversal. To test this hypothesis, PKC inhibitory peptide was dialyzed into interneurons and the modulation by D_5 receptors reexamined. As shown in Figure 6D, dialysis with the inhibitory peptide had little or no effect on the D_5 modulation ($n = 5$; $p > 0.05$, Mann-Whitney U test) in spite of its effective inhibition of PMA's modulation. These results argue that constitutively active PKC is not responsible for phosphorylation of the site targeted by D_5 receptor–activated PP1.

D_5 Receptors Target a Zn^{2+}-Sensitive Subpopulation of $GABA_A$ Receptors

Because PKC failed to alter the PP1-mediated modulation, it was possible that these enzymes target distinct populations of $GABA_A$ receptors. In heterologous expression systems, the reduction in GABA-evoked currents depends critically upon a PKC phosphorylation site on γ2 subunits (Krishek et al., 1994). In addition to providing a substrate for PKC, the presence of γ subunits (in the absence of α6 and δ subunits) renders receptors insensitive to exogenous Zn^{2+} (Draguhn et al., 1990; Saxena and Macdonald, 1994). Hence, Zn^{2+} provided a means of fractionating the $GABA_A$ receptors in a way that might be meaningful to the D_5 receptor modulation.

In fact, low micromolar concentrations of exogenous Zn^{2+} (10 μM) that selectively blocked high affinity $GABA_A$ sites in interneurons (Figure 2D) almost completely eliminated the effects of D_5 receptor activation ($n = 7$; $p < 0.01$, Mann-Whitney U test). A typical time course showing the enhancement of $GABA_A$ currents in the absence of Zn^{2+}, the loss of the modulation in the presence of Zn^{2+} (10 μM), and the recovery of the modulation with washing is shown in Figure 7A. In our sample of cells, the median modulation in the presence of Zn^{2+} was less than 10% of the control modulation (Figure 7A, inset). To verify that Zn^{2+} was acting at the $GABA_A$ receptor and not at the dopamine receptor, its interaction with cAMP analogs was studied. Zn^{2+} effectively blocked the modulation produced by cAMP analogs as well ($n = 4$; $p < 0.05$, Mann-Whitney U test). It also should be noted that as it stimulates PP1 activity (Chu et al., 1996), it is unlikely that Zn^{2+} was acting at an intracellular site.

As shown earlier (Figure 2C), Zn^{2+} did not change (or increase) the apparent rate of desensitization measured with our sewer pipe perfusion system, suggesting that the Zn^{2+}-sensitive currents desensitized slowly. As shown in Figure 7B, isolation of each current component by subtraction confirmed this inference. Isolation of the SKF 81297–enhanced currents by subtraction yielded a current with kinetics very similar to those blocked by Zn^{2+} (Figure 7C), as expected from the ability of Zn^{2+} to block dopamine's effects. Although Zn^{2+} was able to block the effects of D_5 receptor stimulation, it had little

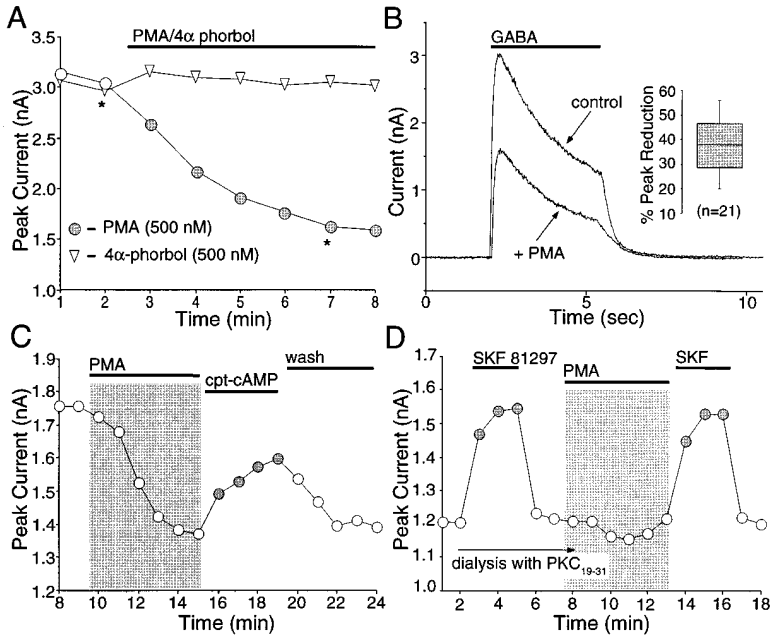

Figure 6. PKC Phosphorylation Reduced $GABA_A$ Currents but Did Not Prevent the Recovery of D_5 Effect on $GABA_A$ Currents

(A) Plot of peak $GABA_A$ current as a function of time and the application of PKC activator phorbol-12-myristate-13-acetate (PMA) (circles). PMA (500 nM) caused an irreversible reduction in the amplitude of $GABA_A$ currents. The inactive phorbol 4α-phorbol (500 nM) did not induce the reduction of $GABA_A$ currents (triangles).

(B) Representative current traces taken before and after treatment of PMA. The inset is a box plot summary of the percent reduction in peak $GABA_A$ currents produced by PMA in a sample of 21 cholinergic interneurons.

(C) Plot of peak $GABA_A$ current as a function of time and drug application. $GABA_A$ currents were reduced by PMA treatment and increased by subsequent application of cpt-cAMP.

(D) Dialysis with PKC inhibitor PKC_{19-31} (30 μM) did not prevent the recovery of SKF 81297 effect, though it blocked the PMA effect.

or no effect on the ability of PMA (and PKC) to reduce evoked currents (Figure 7D).

Discussion

Striatal Cholinergic Interneurons Express a Heterogeneous Population of $GABA_A$ Receptors

Although cloning work has driven home the potential heterogeneity of pentameric $GABA_A$ receptors, it has not been clear whether this heterogeneity would be found at the single-cell level. For example, many of the features of $GABA_A$ receptor–mediated responses in hippocampal neurons can be accounted for by a single population of receptors (Jones and Westbrook, 1995; cf., Schonrock and Bormann, 1993). However, in those cells where it has been examined, single-cell expression profiling of $GABA_A$ receptor subunit mRNAs has failed to support the view that neurons express a single type of $GABA_A$ receptor (Grigorenko and Yeh, 1994). Our results are

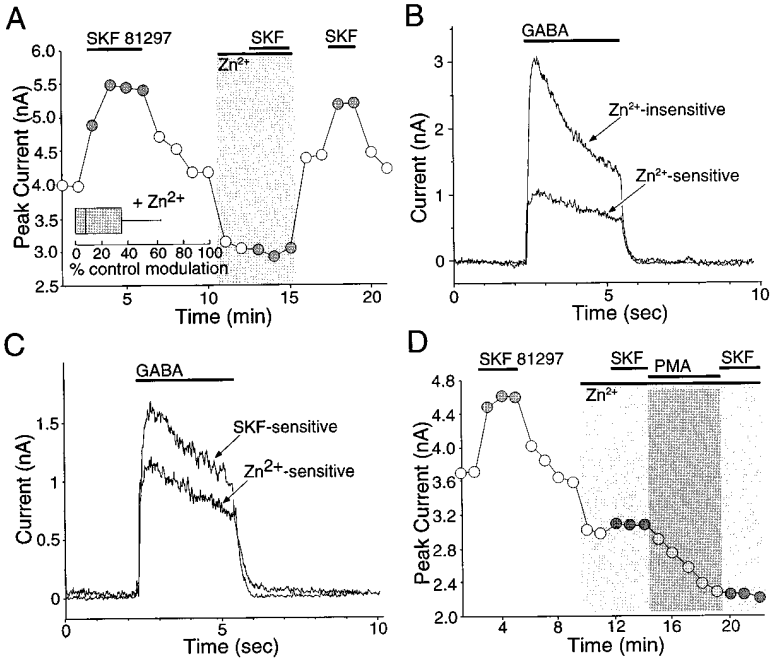

Figure 7. The Principal Target of the D_5 Receptor Pathway Was a Zn^{2+}-Sensitive Subpopulation of $GABA_A$ Receptors

(A) Plot of peak $GABA_A$ current as a function of time and drug application. The D_1 class agonist SKF 81297 (10 μM) enhanced $GABA_A$ currents reversibly. In the presence of Zn^{2+} (10 μM), $GABA_A$ currents were reduced; SKF 81297 had no effect on remaining currents. Washing the Zn^{2+} restored currents to control levels and restored the ability of SKF 81297 to modulate $GABA_A$ currents. The inset is a box plot summary of the percent modulation (relative to control) of peak $GABA_A$ currents by SKF 81297 in the presence of Zn^{2+} (10 μM; $n = 7$).

(B) Representative current traces taken from the same cell showing the Zn^{2+}-insensitive part of currents and Zn^{2+}-sensitive part of currents. Note that these two currents have ostensibly different desensitization rates.

(C) Representative current traces taken from the same cell showing the SKF 81297–enhanced part of currents and Zn^{2+}-sensitive part of currents (determined by subtraction). Note that these two currents are similar in time course.

(D) Plot of peak $GABA_A$ current as a function of time and drug application. Zn^{2+} (10 μM) blocked the SKF 81297 effect but not the PMA effect, further suggesting that D_5 and PKC were targeting different subpopulations of $GABA_A$ receptors.

similar on this score. In individual striatal cholinergic interneurons, the mRNAs for α1–4 subunits, β1,3 subunits, and γ1–3 subunits were consistently coexpressed. The less consistently detected mRNAs for other subunits are presumably present in all interneurons but at lower levels of abundance (Surmeier et al., 1996). Although it is possible that more than one type of α, β, or γ subunit is incorporated into an individual receptor, studies in recombinant systems suggest that this is unlikely (McKernan and Whiting, 1996).

The presence of a heterogeneous receptor population in cholinergic interneurons is also consistent with the pharmacological properties of the GABA-evoked currents. As expected from the robust expression of γ subunit mRNAs, GABA-evoked currents were potentiated by the benzodiazepine agonist diazepam. On the other hand, low micromolar concentrations of Zn^{2+} blocked a significant portion of the GABA-evoked current. Recombinant studies of receptors containing subunits found in interneurons (α1–4) have shown that a noncompetitive block by low micromolar concentrations of Zn^{2+} is a hallmark of receptors lacking γ subunits (Draguhn et al., 1990; Saxena and Macdonald, 1994; cf., White and Gurley, 1995). The Zn^{2+} sensitivity of receptors containing $\gamma 2_L$ subunits can be moderately increased by the addition of δ subunits (Saxena and Macdonald, 1994), but the IC_{50} of these receptors ($\sim$100 μM) is an order of magnitude greater than that of the high-affinity receptor site identified in interneurons (IC_{50} = 6 μM). Taken together with the effects of benzodiazepines, these findings suggest that cholinergic interneurons possess $GABA_A$ receptors containing γ subunits and receptors lacking γ subunits.

D_5 Dopamine Receptors Enhanced $GABA_A$ Currents through a PKA-Dependent Activation of PP1

Single-cell RT-PCR analysis also revealed that cholinergic interneurons coexpressed $GABA_A$ subunits possessing consensus serine/threonine phosphorylation sites. Single neurons coexpressed α3–4, β1–3, and γ1–3 subunits—all of which are known to be potential substrates for PKA or PKC phosphorylation (McKernan and Whiting, 1996). Based upon in vitro assays, the most important of these sites are on the β1 and γ2 subunits (Browning et al., 1990; Moss et al., 1992a; Krishek et al., 1994), both of which were robustly expressed by every neuron profiled.

The dopaminergic signaling pathway necessary to regulate these subunits in interneurons was also in place. In agreement with previous studies (LeMoine et al., 1990; Bergson et al., 1995; Yan et al., 1997), interneurons coexpressed D_2 and D_5 dopamine receptors. D_5 dopamine receptor agonists reversibly enhanced $GABA_A$ currents in the majority of these cells, while D_2 receptor agonists had no effect. The sensitivity of the modulation to the D_5 receptor antagonist SCH 23390 argues that the modulation was mediated by activation of D_5 dopamine receptors. In heterologous expression systems, D_5 receptors are positively coupled to adenylyl cyclase and cAMP production (Grandy et al., 1991; Sunahara et al., 1991). Several lines of evidence suggest that a similar pathway mediated the D_5 receptor effect on $GABA_A$ receptors. First, the effect of D_5 receptor stimulation was mimicked by membrane-permeant cAMP analogs and occluded by coincident application of cAMP analogs at saturating concentrations. Secondly, the D_5 receptor–induced enhancement of $GABA_A$ currents was mimicked by dialysis with PKA catalytic subunit and blocked by peptide and isoquinoline PKA inhibitors. This pattern of response clearly implicates PKA in the modulation (cf., Leidenheimer et al., 1990). The modulation did not appear to be directly mediated by PKA, however.

In heterologous recombinant systems, receptor phosphorylation by PKA has consistently been found to reduce $GABA_A$ currents (Moss et al., 1992a, 1992b). Studies of PKA effects on $GABA_A$ currents in native neurons have been less harmonious—some studies have reported decrements (Heuschneider and Schwartz, 1989; Porter et al., 1990; Schwartz et al., 1991), while many others have found enhancements (Cheun and Yeh, 1992; Kano and Konnerth, 1992; Feigenspan and Bormann, 1994). Our results suggest that some of the discrepancies between these studies may turn on differences in receptor subunit composition, as Zn^{2+}-sensitive receptors alone were modulated by the PKA cascade. However, it is also clear that the role of protein phosphatases must be considered. In heterologous expression systems, the access of kinases or phosphatases to exogenously originating proteins seems not to be tightly controlled. As a consequence, the effects of PKA on GABA-evoked currents are most parsimoniously interpreted in terms of direct phosphorylation of receptor subunits (Kellenberger, et al., 1992; Moss et al., 1992a). In native cell types, however, the access of these enzymes to substrates is tightly controlled by anchoring proteins (Faux and Scott, 1996; Klauck et al., 1996). For example, A-kinase anchoring proteins (AKAP) are instrumental in the PKA-mediated modulation of AMPA/KA receptors in hippocampal neurons (Rosenmund et al., 1994). The existence of subcellular networks of anchoring proteins endows neurons with the capacity to control tightly the access of broad specificity enzymes like PKA to substrates. It also provides a means by which PKA can control the activity of other enzymes, like protein phosphatases.

A case in point appears to be present in cholinergic interneurons. Here, the effects of PKA on $GABA_A$ currents were blocked by three different inhibitors of protein phosphatases (okadaic acid, calyculin A, microcystin LR). In addition, low micromolar concentrations of okadaic acid, but not nanomolar concentrations, effectively blocked the modulation, implicating PP1 (Bialojan and Takai, 1988; Ishihara et al., 1989). In accord with this conclusion, preliminary studies have found that "knocking out" an inhibitor of PP1 (DARPP-32) dramatically enhances D_1 receptor–mediated enhancement of GABA-evoked currents in striatal medium spiny neurons (Flores et al., unpublished data). It is possible that PP1 activity was necessary to maintain a low level of $GABA_A$ receptor phosphorylation and that inhibition of PP1 elevated basal phosphorylation, effectively occluding the direct effects of PKA. However, inhibition of PP1 had no obvious effect on basal GABA-evoked currents, arguing

against this possibility. Taken together, these observations clearly argue against the observed enhancement in $GABA_A$ currents being mediated by receptor phosphorylation. Rather, the enhancement can most readily be explained by PP1-mediated dephosphorylation of a $GABA_A$ subunit or a closely associated regulatory protein.

How might this occur? There are several examples of potentially similar cascades. In muscle, glycogen metabolism is tightly controlled by a PKA-regulated anchoring protein for PP1 that positions the phosphatase close to glycogen, allowing it to dephosphorylate other glycogen-bound enzymes (Hubbard and Cohen, 1993). PKA phosphorylation of the anchoring protein changes this substrate relationship by altering its affinity for PP1. In striatal medium spiny neurons, D_1 receptor stimulation triggers a PKA-dependent redirection of PP1, resulting in the dephosphorylation of Ca^{2+} channels (Surmeier et al., 1995), presumably through control of a recently cloned phosphatase anchoring protein referred to as spinophilin (Allen et al., 1997). It is possible that like in muscle and medium spiny neurons, PKA phosphorylates a PP1 anchoring protein in interneurons. Phosphorylation of this anchoring protein could enable PP1 to dephosphorylate nearby $GABA_A$ receptor subunits (or associated proteins), resulting in an enhancement of GABA-evoked currents. The absence of PKA-regulated cytosolic inhibitors (inhibitor-1, DARPP-32) of PP1 should create a particularly favorable situation for this type of signaling cascade in interneurons (Gustafson et al., 1991; Walaas and Greengard, 1991).

PKC and PKA Target Different Populations of $GABA_A$ Receptor in Cholinergic Interneurons

If D_5 receptor activation results in the enhancement of GABA-evoked currents by promoting PP1-mediated dephosphorylation of $GABA_A$ receptors (or a closely associated regulatory protein), what controls phosphorylation of this site(s)? The relatively rapid reversal of the D_5 receptor–mediated modulation suggests that the kinase responsible must be constitutively active or coactivated by the PKA pathway. PKC is a candidate, since recombinant and native cell studies have shown that $GABA_A$-evoked currents can be down-regulated by PKC phosphorylation (Browning et al., 1990; Kellenberger et al., 1992; Leidenheimer et al., 1992; Krishek et al., 1994; cf., Lin et al., 1996). Although PKC was capable of reducing GABA-evoked currents in interneurons, the reversal of the dopaminergic modulation appeared to rely upon other mechanisms. For example, if D_5 receptor–activated PP1 enhanced currents by reversing a PKC-mediated phosphorylation of a $GABA_A$ subunit, then D_5 receptor stimulation should increase currents after PKC modulation. This was in fact the case, but the magnitude of the modulation was not enhanced by prior PKC activation, as one might expect. More importantly, if PP1 was acting at a PKC site, then reversal of the dopaminergic modulation requires PKC activity. Yet, inhibition of PKC had no effect on the reversal of the PP1-mediated modulation, arguing against a role for PKC.

Another argument against a role for PKC is that the D_5 receptor cascade and PKC targeted distinguishable populations of $GABA_A$ receptors. As noted above, low micromolar concentrations of Zn^{2+} blocked a subset of $GABA_A$ receptors that presumably lack γ subunits. In the presence of Zn^{2+}, D_5 receptor agonists and cAMP analogs failed to enhance GABA-evoked currents, suggesting that the Zn^{2+}-sensitive subset of $GABA_A$ receptors was selectively targeted by the D_5 cascade. Although tempered by the limitations in our perfusion system, the observation that the apparent desensitization kinetics of the Zn^{2+}-sensitive currents and the currents enhanced by D_5 receptor stimulation were very similar strengthened this inference. In contrast, the PKC modulation of GABA-evoked currents was not blocked by exogenous Zn^{2+}. This was not surprising given that recombinant studies have suggested that the $\gamma 2$ subunit (which confers Zn^{2+} insensitivity) is a principal target of PKC (Krishek et al., 1994). Taken together, these results argue that the receptors targeted by the D_5/PKA/PP1 pathway were γ-deficient, whereas those targeted by PKC contained γ subunits.

If PKC does not regulate the site targeted by the D_5 receptor cascade, then what kinase does? It is unclear at this point in time. Ca^{2+}-dependent kinases can be excluded because chelation of intracellular Ca^{2+} to low nanomolar levels did not affect the dopaminergic modulation. Protein kinase G also seems to be excluded by the observation that certain subunit combinations are potentiated, rather than inhibited, by phosphorylation with this kinase (Leidenheimer, 1996). Another interesting possibility is a receptor-associated kinase described by Sweetnam et al. (1988). They found kinase activity copurified with the $GABA_A$ receptor. The kinase constitutively phosphorylated α subunit serine residues and was independent of activators and inhibitors of second messenger-dependent kinases. Although these features are consistent with a role in our modulation, little more is known about this kinase or its physiological consequences for $GABA_A$ receptor function, making any linkage speculative.

The Dopaminergic Modulation of $GABA_A$ Receptors Provides a Novel Means of Regulating the Activity of Cholinergic Interneurons

Dopamine has long been known to be an important regulator of striatal cholinergic interneurons, an importance that is evident in Parkinson's disease, where the loss of dopamine leads to elevated acetylcholine (ACh) release (Lehmann and Langer, 1983). The potentiation of postsynaptic $GABA_A$ receptor currents by D_5 receptors suggests that dopamine release should lead to an augmentation of intrastriatally derived GABAergic inhibition of cholinergic interneurons. This augmented inhibition should result in a suppression of activity and diminished ACh release. This pattern of effects is consistent with the recently described D_2 receptor suppression of Ca^{2+} receptors linked to ACh release in cholinergic interneurons (Yan et al., 1997). That is, both receptors act in ways that should diminish ACh release.

The targeting of Zn^{2+}-sensitive receptors could provide a novel means of regulating dopaminergic signaling. Histochemical studies have shown high levels of vesicular Zn^{2+} in the neostriatum (Vincent and Semba,

1989; Mengual et al., 1995). The distribution of Zn^{2+} is not uniform and is often associated with striosomal compartments, particularly in the rostral striatum. If the vesicular Zn^{2+} is coreleased from glutamatergic terminals as it is in other regions (Assaf and Chung, 1984), corticostriatal activity could effectively gate the dopaminergic effects on intrastriatal GABAergic inhibition of interneurons.

The dopaminergic modulation also has important implications for models of associative learning involving the striatum. Cholinergic interneurons have been hypothesized to act as coordinators of striatal activity during associative learning paradigms (Aosaki et al., 1994). In behaving monkeys, the activity of interneurons begins to be modulated by conditioned stimuli as animals learn their association with reward (Aosaki et al., 1994; Graybiel et al., 1994). This modulation in activity is dependent upon dopaminergic input from mesencephalic neurons that initially respond to the presentation of reward and subsequently to presentation of the conditioned stimulus (Schultz et al., 1993). In most cases, the burst in dopaminergic activity following presentation of the conditioned stimulus leads to a transient suppression in the background activity of cholinergic interneurons. This suppression could result from the D_5 receptor–mediated enhancement of the response to input from spiny or aspiny GABAergic neurons responding to the sensory cue (Alexander et al., 1986). Repeated pairing of the reward-dependent dopaminergic input and the sensory-linked GABAergic input may lead to a lasting potentiation of GABAergic responses (Kano and Konnerth, 1992), serving as the cellular substrate for associative learning in the striatum.

Experimental Procedures

Acute Dissociation Procedure

Neostriatal neurons from adult (>4 weeks) rats were acutely dissociated using procedures similar to those we have previously described (Surmeier et al., 1995). In brief, rats were anesthetized with methoxyflurane and decapitated; brains were quickly removed, iced, and then blocked for slicing. The blocked tissue was cut in 400 μm slices with a Microslicer (Dosaka, Kyoto, Japan) while bathed in a low Ca^{2+} (100 μM) N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (HEPES) buffered salt solution (in mM): 140 Na isethionate, 2 KCl, 4 $MgCl_2$, 0.1 $CaCl_2$, 23 glucose, 15 HEPES (pH = 7.4), 300–305 mosm/l. Slices were then incubated for 1–6 hrs at room temperature (20°C–22°C) in a $NaHCO_3$-buffered saline bubbled with 95% O_2, 5% CO_2 (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 1 pyruvic acid, 0.2 ascorbic acid, 0.1 N^G-nitro-L-arginine, 1 kynurenic acid, 10 glucose (pH = 7.4 with NaOH), 300–305 mosm/l. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Slices were then removed into the low Ca^{2+} buffer and, with the aid of a dissecting microscope, regions of the dorsal neostriatum were dissected and placed in an oxygenated Cell-Stir chamber (Wheaton, Inc., Millville, NJ) containing pronase (Sigma protease type XIV, 1–3 mg/ml) in HEPES-buffered Hank's balanced salt solution (HBSS, Sigma Chemical Co.) at 35°C. Dissections were limited to tissue rostral to the anterior commissure to reduce the possibility of contamination from the globus pallidus. After 20–40 min of enzyme digestion, tissue was rinsed three times in the low Ca^{2+}, HEPES-buffered saline and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35 mm Lux Petri dish mounted on the stage of an inverted microscope containing HEPES-buffered HBSS saline.

Whole-Cell Recordings

Whole-cell recordings of GABA-activated currents employed standard techniques (Bargas et al., 1994; Surmeier et al., 1995). Electrodes were pulled from Corning 7052 glass and fire-polished prior to use. The internal solution consisted of (in mM): 180 N-methyl-d-glucamine (NMG), 40 HEPES, 4 $MgCl_2$, 5 1,2 bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA), 12 phosphocreatine, 2 Na_2ATP, 0.2 Na_3GTP, 0.1 leupeptin (pH = 7.2–7.3 with H_2SO_4), 265–270 mosm/l. The external solution consisted of (in mM): 135 NaCl, 20 CsCl, 1 $MgCl_2$, 10 HEPES, 0.001 TTX, 0.5 $BaCl_2$, 10 glucose (pH = 7.3 with NaOH), 300–305 mosm/l.

Recordings were obtained with an Axon Instruments 200 patch-clamp amplifier that was controlled and monitored with a PC 486 clone running pCLAMP (v. 6.0) with a DigiData 1200 series interface (Axon instruments, Foster City, CA). Electrode resistances were typically 2–4 MΩ in the bath. After seal rupture, series resistance (4–10 MΩ) was compensated (70%–90%) and periodically monitored. Recordings were made only from large neurons (>10 pF) that had short (<75 μm) proximal dendrites. The cell membrane potential was held at 0 mV. GABA (100 μM) was applied briefly (3–5 s) every minute.

In most of the experiments, drugs were applied with a gravity-fed "sewer pipe" system. The array of application capillaries (ca. 150 μm i.d.) was positioned a few hundred microns from the cell under study. Solution changes were effected by altering the position of the array with a DC drive system controlled by a microprocessor-based controller (Newport-Klinger, Inc., Irvine, CA). The time constant of the change in solutions was approximately 450 ms, as judged by alterations in junctional potentials. In an additional series of experiments aimed at determining the faster components of desensitization, neurons were perfused from a theta tube connected to a piezoelectric positioning system (Burleigh Instruments, Fishers, NY). The time constant of the change in solution was approximately 500 μs for this system.

Dopamine receptor ligands—dopamine, SKF 81297, SKF 82958, R(+)-SCH 23390, and R(−)-propylnorapomorphine (NPA) (RBI, Inc., Natick, MA)—were made up as concentrated stocks in deoxygenated water containing 0.1% sodium metabisulfite. Solutions were protected from ambient light. Second messenger reagents 8-(4-chlorophenolthio)-adenosine 3′:5′-cyclic monophosphate (cpt-cAMP) (RBI, Inc., Natick, MA), PKA catalytic subunit (Sigma), PKI [5–24], PMA, 4α-phorbol, PKC_{19-31}, okadaic acid, nor-okadaone, calyculin A, and microcystin LR (Alexis Biochemical Co., San Diego, CA) were made up as concentrated stocks in water or dimethyl sulfoxide and stored at −70°C. Stocks were thawed and diluted immediately prior to use.

Statistical Methods

Data analyses were performed with AxoGraph (Axon Instr., Inc., ver. 2.0), Kaleidagraph (Albeck Software, Reading, PA, ver. 3.0.4) and SYSTAT (SPSS, Inc., Chicago, IL, ver. 5.2). Box plots were used for graphic presentation of the data because of the small sample sizes (Tukey, 1977). The box plot represents the distribution as a box with the median as a central line and the hinges as the edges of the box (the hinges divide the upper and lower halves of the distributions in half). The inner fences (shown as a line originating from the edges of the box) run to the limits of the distribution excluding outliers (defined as points that are more than 1.5 times the interquartile range beyond the interquartiles; Tukey 1977); outliers are shown as asterisks or circles.

The Zn^{2+} dose–response curve was drawn by fitting the data to the following equation, using a nonlinear least squares method: $I = I_H/(1 + \{[C]/IC_{50H}\}^n) + I_L/(1 + \{[C]/IC_{50L}\}^n) + I_R$, where I_H and I_L were the amplitudes of high- and low-affinity components of the $GABA_A$ currents, [C] was the concentration of Zn^{2+}, IC_{50H} and IC_{50L} were the Zn^{2+} concentrations at half-maximal inhibition, n was the Hill coefficient, and I_R was the amplitude of Zn^{2+}-insensitive component. Nonparametric statistics were computed with SYSTAT. Mann-Whitney U tests were performed to compare groups.

Single-Neuron RNA Harvest and RT-PCR Analysis

After recording, cells were lifted up into a stream of control solution and aspirated into the electrode by negative pressure. Electrodes

contained ~5 μl of sterile recording solution (see above). The capillary glass used for making electrodes had been autoclaved and heated to 150°C for 2 hr. Sterile gloves were worn during the procedure to minimize RNase contamination.

After aspiration, the electrode was broken and contents ejected into a 0.5 ml Eppendorf tube containing 5 μl diethyl pyrocarbonate (DEPC)-treated water, 1 μl RNAsin (28 U/μl), 1 μl dithiothreitol (DTT) (0.1 M), and 1 μl of oligo(dT) (0.5 μg/μl) primer. The mixture was heated to 70°C for 10 min and incubated on ice for 1 min. Single-strand cDNA was synthesized from the cellular mRNA by adding SuperScript II RT (1 μl, 200 U/μl) and buffer (4 μl, 5× first strand buffer: 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), DTT (1 μl, 0.1 M), and mixed dNTPs (1 μl, 10 mM). The mixture (20 μl) was incubated for 50 min on a 42°C water bath. The reaction was terminated by heating the mixture to 70°C for 15 min and then icing. The RNA strand in the RNA–DNA hybrid was then removed by adding 1 μl RNase H (2 U/μl) and incubated for 20 min at 37°C. All reagents except for RNAsin (Promega, Madison, WI) were obtained from GIBCO-BRL (Grand Island, NY). The cDNA from the reverse transcription of RNA in single neostriatal neurons was subjected to PCR to detect the expression of various mRNAs.

PCR amplification was carried out with a thermal cycler (MJ Research, Inc., Watertown, MA) with thin-walled plastic tubes. Conventional 45 cycle PCR amplification was used for the detection of ChAT mRNA. Reaction mixtures contained 2–2.5 mM $MgCl_2$, 0.5 mM of each of the dNTPs, 0.8–1 μM primers, 2.5 U Taq DNA polymerase (Promega), 5 μl 10× buffer (Promega), and 1–2 μl cDNA template made from the single-cell RT reaction (see above). The thermal cycling program for these PCR amplifications was: 94°C for 1 min, 58°C for 1 min, 74°C for 1.5 min. For the detection of D_1–D_5 dopamine receptors, a two-stage multiplex amplification was performed to maximize the probability of detecting low-abundance mRNAs (Surmeier et al., 1996). In the first step, the dopamine receptor cDNAs were selectively amplified using almost all the remaining single-cell cDNA (18 μl) as a template in a multiplex PCR reaction. All five dopamine receptor primers were added to a reaction mixture containing the same reagents as with conventional PCR except for slightly elevated $MgCl_2$ (4.0 mM) and dNTPs (1.0 mM). Thirty cycles were performed using the parameters given above. An aliquot of this PCR product (2 μl) was then used as a template for a second round of conventional PCR amplification (40 cycle) with each pair of subtype-specific primers.

To detect simultaneously all the $GABA_A$ receptor subunit mRNAs (α1–6, β1–3, γ1–3, δ) in single cells, degenerate and nested primers were employed. In the first step, degenerate primers targeting the conserved regions of all the α subunits were mixed with one-fourth of the single-cell cDNA template, and 30 rounds of amplification were performed. The same procedure was used to amplify β and γ subunit cDNA. In the second step, an aliquot (2 μl) of diluted (1:10) first-round PCR product was used as the template for a 40 cycle of PCR with subunit-specific nested primers. These primers were positioned inside the region spanned by the degenerate primers. Since the δ subunit has only a single isoform, detection was carried out using conventional PCR (45 cycle) with one-fourth of the single-cell cDNA template.

The PCR primers for dopamine receptors and $GABA_A$ receptor subunits were developed from GenBank sequences or PIR (Protein Information Resource). Primers for ChAT mRNA and D_1–D_5 dopamine receptor mRNAs were the same as described before (Yan et al., 1997).

The degenerate primers used to amplify α subunits were: 5′-TGY CCM CTR AAR TTT GGV AG-3′, 5′-AM ATA YGT DGC CCA RTA RAC-3′, 5′-TGC CCT TTG AAA TTT GGG AG-3′, and 5′-AA ATA AAC CAC CCA GTA GAC-3′. The degenerate primers used to amplify β subunits were: 5′-TGG AGA TCG AAA GYT ATG GCT-3′, 5′-CAC RTC AGT CAA GTC RGG GAT-3′, 5′-CC TGG AAA TTG AAA GCT ATG G-3′, and 5′-CAC ATC GGT TAG ATC AGG GAT-3′. The degenerate primers used to amplify γ subunits were: 5′-CAC TGG ATM ACV ACD CCC AA-3′ and 5′-C CCA RTA RAC MAG RTT GAA C-3′.

The specific primers for the α1 subunit (L08490) were: 5′-GTT GAC TCT GGA ATT GTT CAG TCC-3′ and 5′-GAG AGG ATC CTT CAC TTT CTT TGG-3′. The size of α1 amplicon was 410 bp. The specific primers for the α2 subunit (P23576) were: 5′-CCA GTC AAT TGG GAA GGA GAC AAT-3′ and 5′-TAG GCG TTG TTC TGT ATC ATG ACG-3′. The size of α2 amplicon was 434 bp. The specific primers for the α3 subunit (X51991) were: 5′-T GTT GTT GGG ACA GAG ATA ATC CG-3′ and 5′-CAC TGT TGG AGT TGA AGA AGC ACT-3′. The size of α3 amplicon was 549 bp. The specific primers for the α4 subunit (P28471) were: 5′-AGC TGC CCC AGT ACT GAA GGA AAA-3′ and 5′-ACT GTT GTC TTA ATG CGC CCA AGT-3′. The size of α4 amplicon was 374 bp. The specific primers for the α5 subunit (X51992) were: 5′-ACA GTA GGC ACT GAG AAC ATC AGC-3′ and 5′-AGG ATG GGT CAA CTT CCC AGT TGT-3′. The size of α5 amplicon was 467 bp. PCR analysis of whole striatal cDNA failed to detect α6 subunit mRNA, so it was not included in our screens.

The specific primers for the β1 subunit (P15431) were: 5′-CGA CTA CAA GAT GGT GTC CAA GAA-3′ and 5′-TC TGG TCT TGT TTG CTC GCT CCT T-3′. The size of β1 amplicon was 397 bp. The specific primers for the β2 subunit (P15432) were: 5′-AGC AGC TGA GAA AGC TGC TAA TGC-3′ and 5′-TTT TGT GCC ACA TGT CGT TCC AGG-3′. The size of β2 amplicon was 254 bp. The specific primers for the β3 subunit (P15433) were: 5′-TCT GGT CTC CAG GAA TGT TGT CTT-3′ and 5′-ATT GCT GAA TTC CTG GTG TCA CCA-3′. The size of β3 amplicon was 527 bp.

The specific primers for the γ1 subunit (P23574) were: 5′-GAG TAC AAG TGG AAA AAG CCC TCA-3′ and 5′-AAT CAG AGT AGA TCC AGC ATG GAG-3′. The size of γ1 amplicon was 515 bp. The specific primers for γ2 subunit (L08497) were: 5′-T GAA GTG GGA GAC ACA AGG TCA TG-3′ and 5′-GT TGC TGA TCT GGG ACG GAT ATC A-3′. The size of γ2 amplicon was 482 bp. The specific primers for the γ3 subunit (M81142) were: 5′-GAG GCA GCT GAT CAG AAA TCA TGG-3′ and 5′-ACA GGT GTC CTC AAA CTC CTG CCA-3′. The size of γ3 amplicon was 587 bp.

The specific primers for the δ subunit (M35162) were: 5′-GGT GTC TAC ATC ATC CAG TCT TAC-3′ and 5′-AGA CCT ATA GGA ACC CAT GAG GTT-3′. The size of δ amplicon was 427 bp.

Products were visualized by staining with ethidium bromide and analyzed by electrophoresis in 2% agarose gels. Representative products were sequenced using a dye termination procedure by St. Jude Children's Research Hospital Molecular Resource Center and found to match the published sequences.

Care was taken to ensure that the PCR signal arose from cellular mRNA. In addition to the controls noted above (e.g., primers that span splice sites), negative controls for contamination from extraneous and genomic DNA were run for every batch of neurons. To ensure that genomic DNA did not contribute to the PCR products, neurons were aspirated and processed in the normal manner except that the reverse transcriptase was omitted. Contamination from extraneous sources was checked by replacing the cellular template with water. Both controls were consistently negative in these experiments.

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