Subtypes of Substantia Nigra Dopaminergic Neurons Revealed by Apamin: Autoradiographic and Electrophysiological Studies

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DOPAMINERGIC neurons in the substantia nigra pars compacta (nucleus A9), recorded in vivo, exhibit either a burst or a nonburst firing pattern (9,10,34). The burst firing pattern consists of several action potentials (approximately 3–7), with progressively decreasing amplitude and increasing duration, followed by a pause. The nonburst firing pattern consists of both regular and pacemaker firing patterns, which have a small amount or very little variation in the interspike interval distributions, respectively. The majority of cells (58%) exhibit a nonburst firing pattern, and 42% of the cells exhibit a burst firing pattern (34). That these two firing patterns belong to neurons with different structural and/or functional properties is indicated by the observation that the cells which exhibit the burst and nonburst firing patterns reside within different rostro-caudal regions of the nucleus, and the two cell types exhibit different axonal conduction velocities (34).

Midbrain dopaminergic neurons, recorded in the in vitro slice preparation, primarily exhibit nonburst firing patterns (30,35). The factors responsible for the loss of burst firing activity in vitro are not understood, but it is postulated that functional synaptic input is required for the normal expression of burst activity (31,34). In addition, there may be structural and/or functional differences among the substantia nigra dopaminergic neurons that allow only some cells to burst (21).

Burst firing patterns are thought to be regulated in many nerve cells, in part by an apamin-sensitive calcium-activated potassium channel. This channel has been localized within the substantia nigra zona compacta region of the rat using radiolabeled apamin binding (27), and several investigators have proposed that this channel plays an important role in burst firing of nucleus A9 neurons (20,22,24,31).

Apamin, a bee venom polypeptide, is the smallest neurotoxic polypeptide known [18 amino acids (13)], and it blocks one type of Ca2+-activated potassium channel. This channel (SK channel) has been shown to have a small single-channel conductance (10–12 pS, 140 K ionic (2) and is clearly distinct from the more common large conductance Ca2+-activated potassium channel (BK channel), which is blocked by charybdotoxin (26). The SK
channel has been demonstrated in many cell types—for example, in vertebrate motoneurons, cardiac Purkinje fibers, and smooth muscle (25); tissue cultured skeletal muscle (2); neuroblastoma cells (17); anterior burster neuron in the stomatogastric ganglion (14); and sympathetic ganglion cells (28).

The purpose of the present experiment was to determine whether apamin binds to cells within the nucleus A9 region, and whether it causes all dopaminergic neurons to exhibit a burst firing pattern. Preliminary results of these data were published in abstract form (8).

**METHOD**

**In Vitro Receptor Autoradiography**

Coronal sections, cut on a cryostat at 20 μm thickness from CBA/J mouse midbrain, were placed onto glass slides. The sections were incubated in buffer containing 0.18 nM $^{32}$P-apamin (New England Nuclear, 2200 Ci/mmol) for 60 min at 4°C. The buffer contained 50 mM Tris HCl, 3 mM KCl, and 0.1% BSA, pH 7.5. The sections were then rinsed twice in buffer at 4°C and dried with a cool stream of air from a hair dryer. For the determination of nonspecific binding, alternate sections were also incubated with 0.2 μM unlabeled apamin. The tissue sections, and $^{32}$P-Amersham microscale standards, were apposed to LKB Ultrofilm for 18 h at 0°C. The film was processed with Kodak D19 for 5 min at 20°C, fixed for 3 min, and washed. The grain densities were quantified using the CARP software system from BioRadics Inc. and a SUN 3/760 Workstation. Grain densities were compared with densities obtained on the same film with the 10 Amersham $^{32}$P standards. The relationships between silver grain densities and binding site concentrations were obtained with a linear curve-fitting procedure. The autoradiographic tissue sections were stained with cresyl violet to compare the autoradiographic grain densities with the Nissl-stained cellular localizations.

**Tyrosine Hydroxylase Immunocytochemistry**

To examine the locations of the midbrain dopaminergic neurons, immunocytochemical staining for the catecholaminergic cell marker, tyrosine hydroxylase, was performed. Sections through the mouse midbrain were cut in the coronal plane at 30 μm thickness on a freezing microtome. Free-floating sections were stained with an antibody against tyrosine hydroxylase (Eugene Tech, 1:2000) using methods previously described (7). This procedure clearly labels dopaminergic somata within the substantia nigra and ventral tegmental area.

**In Vitro Brain Slice Preparation**

Mice (Swiss, CBA/J, BALB/c, C3H/He, 15–20 g) were decapitated, and their brains were removed rapidly. The midbrain was sectioned into 400-μm coronal slices with a McIlwain tissue chopper. Slices containing the midbrain were incubated in an artificial cerebrospinal fluid (ACSF) solution that contained (mM) NaCl, 124; KCl, 5; NaHCO$_3$, 26; glucose, 10; CaCl$_2$, 2.4; KH$_2$PO$_4$, 1.25; and MgCl$_2$, 3.35. The slice was placed in a superfusion chamber that was humidified with a 95% O$_2$/5% CO$_2$ gas mixture, for at least 1 h prior to recording.

Midbrain slices were continuously superfused in a chamber that had a volume of 0.5 ml, either with normal ACSF solution or ACSF solution into which drugs were added. Single strands of gauze were placed on top of the slice to aid in the wicking of the superfusate. The solutions were maintained at pH 7.2–7.4 and temperature of 34–35°C. All solutions were bubbled with the 95% O$_2$/5% CO$_2$ gas mixture. The slices were superfused by gravity feed at a flow rate of 0.5–1.5 ml/min.

**In Vitro Single Unit Recording**

For extracellular recordings, glass micropipettes were filled with 2 M NaCl saturated with Fast Green dye. The impedance of the electrode was 2–3 MΩ as measured at 135 Hz (Winston Impedance Meter). Action potentials were stored on an FM tape recorder. Substantia nigra dopaminergic neurons were identified according to the following criteria: The cells exhibited slow firing rates (1–6 spikes/s), regular firing patterns, and long-duration action potentials (>2 ms). A stereomicroscope was used to position the recording microelectrode within the substantia nigra pars compacta region.

**Chemicals**

Dopamine HCl, gamma-aminobutyric acid, and apamin were obtained from Sigma, and quinpirole HCl (a D2 dopamine receptor agonist) was obtained from Research Biochemicals Inc.

**Data Analysis**

The firing rates and firing patterns of dopaminergic neurons were stored, displayed, and analyzed on an IBM-compatible 386 computer. Firing rate was analyzed using a rate histogram program (10 s bin width), and firing pattern was analyzed using an interspike interval program (10 ms bin width). Interspike interval histograms were constructed with approximately 1000 spikes (988–1083 spikes) before and after apamin superfusion.

**RESULTS**

** Autoradiography/Immunocytochemistry**

Four mouse brains were used for the autoradiographic experiments. Coronal sections were taken through three animals and sagittal sections through the fourth. The binding densities were comparable in all animals. Fig. 1A illustrates the pattern of $^{32}$P-apamin binding in a section through the rostral portion of the mouse midbrain. Distinct binding occurred within the substantia nigra pars compacta and the adjacent ventral tegmental area. Sections stained for tyrosine hydroxylase (Fig. 1B) illustrate that the dopaminergic somata within the substantia nigra pars compacta and ventral tegmental area overlap in location with the apamin binding (Fig. 1A).

Table 1 illustrates the density of binding sites within three representative sections, from rostral to caudal, through the midbrain. There was from 3.6 to 4.7 fmol/mg tissue-specific binding within the nucleus A9 from rostral to caudal. The binding was always higher (47–103%) in the substantia nigra pars compacta (dopaminergic cell body region) than in the underlying substantia nigra pars reticulata (nondopaminergic cell body region). Non-specific binding, as determined by the grain densities over sections incubated in the presence of 0.2 μM unlabeled apamin, was relatively low (0.22 fmol/mg tissue).

**Electrophysiology**

Action potentials were recorded from 31 single cells located within the substantia nigra pars compacta in the mouse midbrain slice preparation. All neurons exhibited characteristic electrophysiological and pharmacological response properties of dopaminergic neurons. Fig. 2 illustrates the action potential characteristics of dopaminergic neurons and the burst firing pattern sometimes observed following apamin administration.
TABLE I

<table>
<thead>
<tr>
<th>Brain</th>
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<th>Region</th>
<th>Binding Density</th>
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<tr>
<td>1</td>
<td>1</td>
<td>SNC</td>
<td>4.71 ± 0.75</td>
</tr>
<tr>
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<td>SNR</td>
<td>2.41 ± 0.19</td>
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<td>VTA</td>
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<td></td>
<td>SUM</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>VTA</td>
<td>2.67 ± 0.22</td>
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Mean ± SD binding density (fmol/mg tissue) from regions on one side of the brain from two representative animals. The mean represents the average density of the 40-60 pixels within the region analyzed and the SD represents the variability in the pixel densities. These densities represent specific binding and reflect the subtraction of nonspecific binding (0.22 fmol/mg tissue). The first tissue section is from the rostral portion of the midbrain dopaminergic cell complex, at the level of the mammillary and adjacent telencephalic regions, and the last section is from the most rostral portion of the midbrain dopaminergic cell complex at the level of the rostral pons. Abbreviations: Cr, crus cerebri; CW, cortical white matter; SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; SUM, supramammillary region; VTA, ventral tegmental area.

**High-Dose Apamin**

The effects of 1 μM apamin on the firing rates and patterns of dopaminergic neurons were varied (n = 21). The firing rates of most cells were increased and the firing patterns of some cells changed from pacemaker to burst (n = 9) (Figs. 3 and 4). The average firing rate of these nine cells before apamin was 3.0 ± 0.4 (mean ± SEM) impulses/s and after apamin 4.9 ± 0.6 impulses/s (63% increase, paired t test, p = 0.013). Six of the nine cells showed a gradual increase in firing rate following apamin superfusion; two of the cells were first decreased in rate and subsequently increased in rate; one cell first stopped firing altogether and then began bursting while firing at an increased rate.

In two cells, the firing rates and patterns were not changed by apamin (Fig. 5). These cells exhibited regular firing patterns before the drug and regular firing patterns after the drug. After high-dose apamin, the cells were still responsive to the inhibitory effects of superfused dopamine on firing rate.

The firing rates of some cells were decreased by apamin (n = 5). Three of the cells quickly ceased firing when apamin was perfused and regained their firing rates gradually after perfusion with ACSF. Two of the cells decreased their firing rates gradually after apamin.

Six dopaminergic cells exhibited a marked cyclical increase and decrease in firing rate following apamin superfusion (Fig. 6). The cycle period in four of the cells was 12-15 min. The firing rates varied from a low of 0-4.0 spikes/s up to a high of 2.5-55.0 spikes/s within one cycle.

In 11 of the 21 cells tested with 1 μM apamin, the subsequent administration of dopamine (20-200 μM) produced an inhibi-

**FIG. 2.** Midbrain dopaminergic neurons recorded in vitro exhibited long-duration action potentials (A) and slow, pacemaker-like firing patterns (B). Some cells, following superfusion with apamin (1 μM), exhibited a burst firing pattern (C). Comparable burst firing was also induced by 1 nM apamin. Time marker for A = 2 ms, and for B and C = 1 s.
The firing rates and patterns of some dopaminergic cells were markedly changed following apamin superfusion. This cell exhibited a 49% increase in firing rate and changed from a pacemaker to a burst firing pattern following apamin superfusion. Interspike interval histograms (top two panels) and a rate histogram (bottom panel) are illustrated. The firing of this cell was also inhibited 85% by dopamine (DA; 50 μM concentration). The interspike interval histograms measure the changes in the cell's firing pattern before (1) and after (2) apamin. The two interspike interval histograms were generated from portions of the rate histogram (lower panel) indicated by the numbers 1 and 2. Before apamin, the interspike interval histogram was characteristic of a pacemaker firing pattern (narrow, symmetrical interspike interval distribution), and after apamin it was characteristic of a burst firing pattern (positively skewed interspike interval distribution).

The firing rate and firing pattern of some dopaminergic cells were not changed by apamin. The firing pattern of this cell was characteristic of a regular firing cell both before and after drug (i.e., the interspike interval histograms were normally distributed before and after apamin; top two panels). This cell's firing rate was completely inhibited by dopamine (DA; 200 μM).

to dopamine superfusion following apamin administration, although normally a 50% inhibition in firing rate occurs after 100 μM dopamine (1). When dopamine (50 μM) was superfused before and after apamin, it produced a complete cessation of cell firing both times in one cell. In another cell, dopamine (200 μM) was superfused before, during, and after apamin superfusion; the first time it caused a 100% inhibition in cell firing, the second time an 85% inhibition in cell firing, and the third time a 75% inhibition in cell firing.

Low-Dose Apamin

From 1 to 50 nM apamin was tested on 10 nucleus A9 dopaminergic neurons. As with the higher toxin dose, some cells exhibited a cyclical firing rate after apamin superfusion. The firing rate of this dopaminergic neuron was completely inhibited by apamin. Superfused dopamine (DA; 200 μM) also inhibited (>80%) the neuronal firing rate. This cell exhibited a cyclical firing rate after apamin treatment. This slice was also treated with 1 μM apamin 2 h before this recording.
were unchanged in rate or pattern following apamin (n = 2). Some cells were decreased in rate (n = 5), and some increased in rate (n = 3) following apamin. The firing rate of one cell, following 1 nM apamin, was rapidly increased and then became completely inhibited—it apparently went into depolarization block (11). Before apamin, this cell exhibited a pacemaker firing pattern, and after the discontinuation of apamin superfusion it exhibited a pronounced burst firing pattern. Two cells were tested with quinpirole (1 and 10 nM), and their firing rates were inhibited by 80–100%, respectively, even following apamin superfusion. Normally, the 10-nM dose of quinpirole produces approximately 50% inhibition in the firing rates of midbrain dopaminergic neurons (1).

**DISCUSSION**

The autoradiographic and immunocytochemical data indicate that there is specific apamin binding in mouse midbrain dopaminergic cell regions, both within the substantia nigra pars compacta and ventral tegmental area. The density of binding sites varied somewhat from rostral to caudal within the nucleus A9 region with a range of 3.6 to 4.7 fmol/mg tissue. The binding was from 47 to 103% higher in the dopaminergic cell body region of the substantia nigra pars compacta, compared to that in the adjacent substantia nigra pars reticulata. In the rat, apamin has been reported to bind with high affinity (Kd = 23 pM) to a single class of sites, and the specific binding was saturated with concentrations of 100–400 pM (27). In the rat, the binding was also about twice as high in the pars compacta as compared to the pars reticulata region of the substantia nigra (8.0 vs. 3.6 fmol/mg protein).

The present results indicate that although apamin can cause some nucleus A9 neurons to change from a pacemaker firing pattern to a burst firing pattern, not all cells are affected in the same manner. Similar results with 1 μM apamin were reported for nucleus A9 cells in the in vitro slice preparation from the rat (31). In some rat dopaminergic cells the firing rate increased, whereas others decreased, and in others it did not change at all. Thus, SK channel blockade does not comparably influence all substantia nigra dopaminergic neurons in the rodent.

Dopaminergic cell firing rates can change without a change in firing pattern which indicates that firing rate and firing pattern are under independent control. In some cells, apamin increased the firing rate but did not alter the firing pattern. In other cells, apamin altered the firing pattern which usually occurs along with an increase in net firing rate.

If the SK channel is normally involved in the generation of burst firing, why should its blockade with apamin produce bursting? Normally with greater cell depolarization there would be a concomitant increase in firing rate up to the point at which the SK channels open (in response to increased intracellular Ca²⁺), resulting, in part, from the activation of voltage sensitive Ca²⁺ channels. Once the SK channels open, the membrane would become hyperpolarized and cell firing would be reduced or cease for a brief period of time. Such a repetitive depolarization, and the SK channel opening sequence, could produce a burst firing pattern. Because apamin has been demonstrated to reduce the normal slow after-hyperpolarization observed in substantia nigra pars compacta neurons (33) and in other neurons (3, 17, 18, 36), it seemed that blockade of SK conductance would merely increase the firing rate of the cell but not change the pattern, as observed in some cells in the present study. Because some cells do go into burst firing pattern following apamin, this suggests that these cells possess other mechanisms for the production of prolonged hyperpolarization and regulation of repetitive firing.

Anatomical, neurochemical, and electrophysiological data indicate that there are different types of substantia nigra dopaminergic neurons. For example, there are different types of dopaminergic somata (4), striatal dopaminergic axon terminals (12, 19, 29) and dopaminergic axon terminal innervation of striatal patch/matrix compartments (6). Some midbrain dopaminergic neurons co-contain calbindin-D²⁸k (5), cholecystokinin (16, 31), and/or neurotensin (15, 31). Because not all A9 cells were induced to burst following blockade of SK conductance, this represents electrophysiological evidence for heterogeneity among A9 neurons, to go along with other electrophysiological evidence suggesting more than one type of substantia nigra dopaminergic neuron (34). Kubota et al. (21) presented electrophysiological evidence for the existence of two types of substantia nigra pars compacta neurons. Type I neurons were found to change firing patterns from rhythmic firing to a burst mode and these cells had low threshold calcium spikes. The Type II neurons did not exhibit bursting and did not exhibit low threshold calcium spikes. Perhaps the neurons examined in the present experiment, which changed firing pattern from regular to burst, are equivalent to the Type I neurons, and those that did not change pattern are equivalent to the Type II neurons.

Rhythmic patterns of firing were induced in several A9 neurons by 1 μM apamin. The cells sometimes fired action potentials at rates as high as 55 spikes/s. These unusually high firing rates have not been previously reported for dopaminergic neurons. Such rhythmic bursting has been observed in the quiescent anterior burst neuron of the lobster stomatogastric ganglion after superfusion with apamin (14). These neurons fired impulses at a rate of approximately 0.3 spikes/s and were silent for about 2 s.

Dopamine has been demonstrated to inhibit the firing of midbrain dopaminergic neurons via activation of a D2 type receptor and a potassium-induced membrane hyperpolarization (22, 23). Although the specific potassium channel that is activated via D2 receptor stimulation has not been identified, the present data indicate that the SK channel does not appear to be a significant contributor to the potassium conductance resulting from D2 receptor stimulation. Thus, other potassium channels must be responsible for the dopamine autoreceptor-induced hyperpolarization.

In conclusion, a) apamin binds to midbrain sections in the same regions where the substantia nigra and ventral tegmental area dopaminergic neurons reside in the mouse; b) following the administration of a drug that blocks one type of Ca²⁺-activated K⁺ channel, some nucleus A9 neurons can be induced to exhibit a burst firing pattern in the in vitro slice preparation, suggesting that SK channels may play a role in dopaminergic cell burst generation; c) not all dopaminergic neurons were induced to burst following the administration of apamin, suggesting that these cells may either lack SK channels or that SK channels in these cells are not responsible for the regulation of repetitive firing; and d) SK channel activation is not specifically responsible for the dopamine-induced inhibition of dopaminergic neuronal impulse flow.

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