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Short communication

Electrochemical monitoring of bursting exocytotic events from the giant dopamine neuron of *Planorbis corneus*

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Abstract

We have discovered a neuronal system that fires bursting exocytotic events. In the giant dopamine neuron of the fresh water snail *Planorbis corneus*, bursting exocytotic events are evoked following in situ stimulation with elevated potassium. Amperometric detection using carbon fiber microelectrodes, which provides high temporal resolution, has been used to record exocytotic events released from the neuron. Evaluation of the time interval between consecutive exocytotic events (inter-spike interval) recorded from about 80% of the neurons reveals the occurrence of distinct bursting patterns defined by transients having an equal interval among them. Statistical analysis of these bursting exocytotic events shows three distinct distributions of inter-spike intervals with mid points occurring at 5, 22 and 45 ms. This bursting release behavior is not observed from cultured pheochromocytoma cells although they show calcium-dependent exocytosis following in situ stimulation with elevated potassium. Our data appear to indicate that the *Planorbis* dopamine neuron in vivo is actively involved in specific modes of neural communication and may represent an important phenomenon in understanding single cell activities.

Keywords: Dopamine exocytosis; Inter-spike interval; Bursting pattern; Burst; Distribution; Cell body; Giant dopamine neuron; *Planorbis corneus*

According to the classical model of neural communication, neurons are generally classified as bursting and non-bursting neurons based on their characteristics in firing action potentials. Bursting neurons are capable of firing bursts of action potentials spontaneously or under stimulation. Nonbursting neurons can only generate single action potentials. It has been shown extensively that bursting neurons are actively involved in neural communication which leads to various macroscopic activities, such as locomotion [10], respiration [22], feeding [3] and defense reaction [2]. The *Aplysia* R15 neuron has been shown to gradually develop endogenous bursting with the adult species having stronger bursting activity than the juvenile [18]. A three-neuron network, including the giant dopamine neuron in the right pedal ganglion, capable of generating the respiratory rhythm of air-breathing in the fresh water snail *Lymnaea stagnalis* has been investigated and reconstructed in vitro [22]. Numerous small neurons of the fresh water snail *Planorbis corneus* have also been shown to control feeding movement [3] or defense reaction [2]. All these studies have shown that bursting neurons participating in various macroscopic activities fire rhythmic action potentials. Little is known as to whether bursting neurons generate bursting exocytosis when they are stimulated. There has been no report to our knowledge about recording bursting exocytotic events from any neurons.

Electrochemical techniques have previously been used to study individual exocytotic events from single adrenal cells [9,14], mast cells [1], pheochromocytoma cells [7], pancreatic β cells [13], the leech serotonin-containing neuron [4] and the cell body of the giant dopamine neuron of *Planorbis corneus* [8]. These techniques involve the use of small carbon fiber electrodes to monitor and quantitate electroactive neurotransmitters (catecholamines, serotonin etc.) released from individual exocytotic events at the cell membrane. Due to the small structure, high sensitivity, and high spatial and temporal resolution of the microelectrode, dynamic measurements of fast exocytotic events can be obtained.

In this paper, we use amperometry with carbon fiber electrodes to study dopamine exocytosis and the bursting release behavior of the cell body of the giant dopamine neuron of the fresh water snail *Planorbis corneus*. This neuron has previously been shown to fire bursting action

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potentials when it is electrically or chemically stimulated [15–17]. Now we demonstrate that this neuron can evoke bursting exocytotic events after it is stimulated with in situ application of elevated potassium. Three distinct bursting patterns with inter-spike interval mid points at 5, 22 and 45 ms are observed. These bursting patterns of exocytotic events are absent from data obtained from the cultured pheochromocytoma (PC12) cells. Our present data indicate that the *Planorbis* dopamine neuron is actively involved in bursting exocytosis which may play an important role in neural communication.

Working electrodes were prepared from 10-μm-diameter carbon fibers sealed in glass capillaries [12]. The sensing tip of the electrode was exposed by cleaving with a surgical blade. The reference electrode was a Ag/AgCl pellet electrode (RC1, World Precision Instruments, Sarasota, FL). Constant-potential amperometry was performed with a commercial potentiostat (EI-400, Ensman Instrumentation, Bloomington, IN). The applied potential was 700 mV. The output was connected to an A/D converter (Labmaster, Scientific Solutions, Solon, OH) interfaced with a personal computer (486, 33 MHz, Gateway2000, S. Dakota).

Planorbis corneus were obtained from NASCO (Fort Atkinson, WI) and were maintained in aquaria at room temperature until used. The snails were dissected under a snail Ringer solution (39.5 mM NaCl, 1.3 mM KCl, 4.5 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 6.9 mM $NaHCO_3$) adjusted to pH 7.4. The dissection procedure and the identification of the dopamine containing neuron were described previously [12,15–17]. All data were digitized at a rate of 2 kHz and filtered with a 500 Hz two-pole low pass filter. Experiments with PC12 cells used a procedure described previously [18]. Experiments were carried out at room temperature. Nicotine (1 mM final concentration) was added to a potassium-elevated balanced salt solution containing 105 mM KCl and 50 mM NaCl, 2 mM $CaCl_2$, 0.7 mM $MgCl_2$, 1 mM NaH_2PO_4, and 10 mM Hepes for single cell experiments as chemical stimulant.

Current transients obtained in the amperometric mode were evaluated by a locally developed peak-detection-integration algorithm. Only transients with peak widths less than 10 ms and larger than 2 ms and peak heights larger than twice the peak-to-peak noise were considered to be significant. For Fig. 2C, bursting patterns (correlations of consecutive spike intervals) were identified in the data by an algorithm written in house. Briefly, the difference in time between consecutive exocytosis events (spikes) was called the inter-spike interval. Histograms of the frequency of exocytosis events vs. the inter-spike intervals were obtained. A tolerance was set to compare the consecutive inter-spike intervals. If the difference in time between the consecutive inter-spike intervals (e.g. time between spikes 1 and 2 compared to spikes 2 and 3 and then spikes 3 and 4 in a 4-spike sequence) was less than the tolerance (± 1 ms), the exocytosis events involved in computing those inter-spike intervals were considered to be part of a correlated burst. Thus, only bursts of 4 or more consecutive spikes with equivalent inter-spike intervals were included in Fig. 2C.

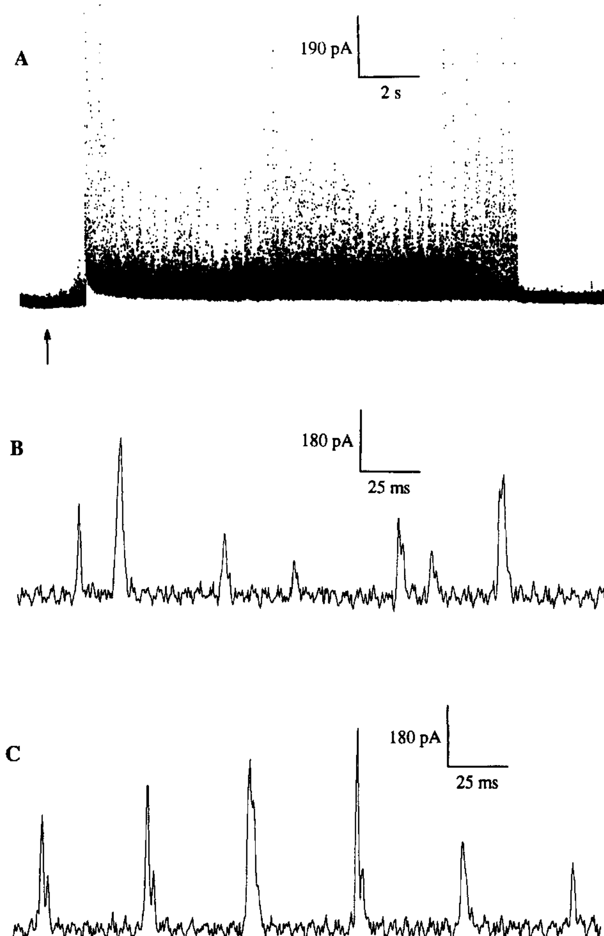
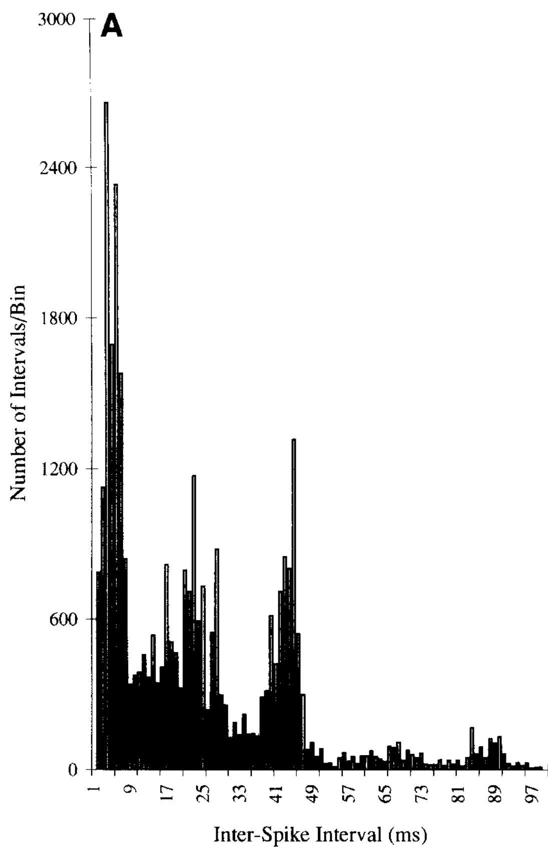
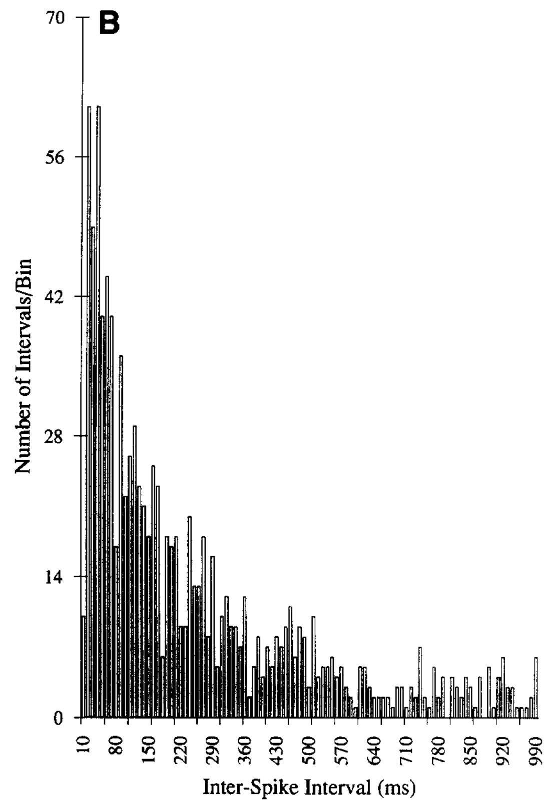
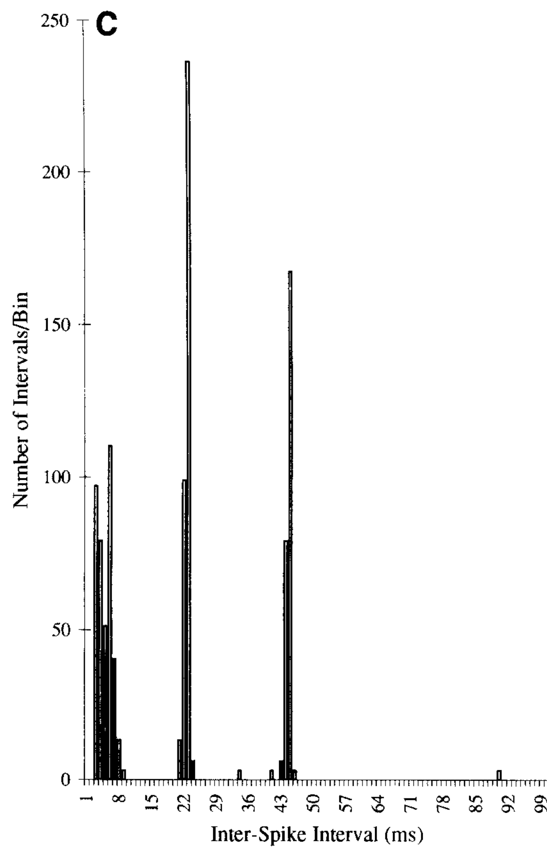


Fig. 1. Dopamine exocytotic events detected by carbon fiber electrodes following cellular stimulation. A: representative trace recorded from a 130-μm *Planorbis* dopamine neuron following in situ stimulation (6 s, 530 nl) with 1 M potassium chloride. A 10-μm-diameter pipette placed about 15 μm away from the cell body was used to stimulate the neuron. The stimulation time is denoted by the arrow under the trace. B,C: representative single and bursting exocytotic events selected from the expansion of (A).

Fig. 2. Histograms of the frequency of exocytosis events vs inter-spike interval between consecutive spikes. A: data collected from 11 *Planorbis* giant dopamine neurons (35370 spikes). Experimental conditions the same as in Fig. 1. B: data were collected from 41 PC12 cells (1333 total spikes). C: only spikes that occur in bursts containing 4 spikes or greater with a difference between consecutive inter-spike intervals (tolerance) less than 1 ms are displayed. Data selected from nine *Planorbis* giant dopamine neurons. 1005 spikes met the bursting criteria (3% of the 35370 total spikes observed).


A
Number of Intervals/Bin
3000
2400
1800
1200
600
0
1
9
17
25
33
41
49
57
65
73
81
89
97
Inter-Spike Interval (ms)


B
Number of Intervals/Bin
70
56
42
28
14
0
10
80
150
220
290
360
430
500
570
640
710
780
850
920
990
Inter-Spike Interval (ms)


C
Number of Intervals/Bin
250
200
150
100
50
0
1
8
15
22
29
36
43
50
57
64
71
78
85
92
99
Inter-Spike Interval (ms)

Dopamine (DA) was obtained from Sigma (St. Louis, MO) and was used as received. Dopamine solutions were deoxygenated for 20 min prior to experiments and a blanket of nitrogen was then maintained over the solution. All solutions were prepared with doubly distilled water.

It has been shown previously that current spikes corresponding to individual dopamine exocytotic events can be obtained with microelectrode amperometry at the cell body of the giant dopamine neuron of *Planorbis* [8]. Exocytosis is observed following stimulation of the cell body with either elevated external potassium ion or intracellular sodium ion. In the work reported here this dopamine containing neuron was stimulated with elevated extracellular potassium ion. Fig. 1A shows the response obtained from a neuron after a 6-s stimulation with elevated potassium. Expanded plots of the time axis for part of data in Fig. 1A are shown in Fig. 1B,C. These spikes have rapid rise times (1 to 2 ms), that correspond to exocytotic events which are expected to occur on the ms time scale. Qualitative examination of Fig. 1B,C reveals that some single exocytosis events occur at random times (Fig. 1B) while a greater number occur in groups with a fixed inter-spike interval (Fig. 1C). Here, we consider trains of events with fixed inter-spike interval to be bursts (correlated events). To quantitatively evaluate the bursting exocytotic events detected from the *Planorbis* giant dopamine neuron, we have applied a burst detection algorithm described above. Nine out of 11 cells showed bursting release characteristics when bursts having at least four events and an internal tolerance of 0.5 ms were used. The percentage of those bursting events out of the total events obtained in each cell was in the range of 7.3 to 34.9% with an average of 17.8%.

To examine if specific bursting firing patterns (bursting exocytotic events) exist in the current-time responses as shown in Fig. 1, we have investigated all the exocytotic events obtained from 11 cells. The histogram of all these transients having inter-spike intervals varying from 1 ms to 100 ms is shown in Fig. 2A. An overall decay profile can be seen from these data although the majority of events occur at short inter-spike intervals. More importantly, three distinct areas with inter-spike intervals centered at 5, 22 and 45 ms are evident.

In order to examine if a similar histogram can be obtained from other cells, we have carried out experiments in cultured PC12 cells. Fig. 2B shows the histogram of inter-spike intervals of transients obtained from 41 PC12 cells. In contrast to the data in Fig. 2A for *Planorbis*, data from PC12 cells does not show regions with elevated numbers of events at specific inter-spike interval. The PC12 cell data clearly has the largest number of events with small inter-spike intervals with an exponential drop off at larger intervals.

To explore the unique bursting patterns for dopamine exocytosis from the *Planorbis* dopamine neuron, we have studied the distribution of bursts detected in the data. Fig. 2C shows the histogram of the distribution of inter-spike intervals for detected bursts (1005 total transients) that have four events or greater and a tolerance of 1 ms in the difference between consecutive inter-spike intervals. Intervals that do not satisfy these requirements are excluded from the figure. Here, three well-separated distributions with inter-spike intervals centered at 5, 22 and 45 ms are observed. The percentages of transients calculated from Fig. 2C that have bursting patterns centered at 5, 22 and 45 ms are 39.1, 35.2 and 25.7%, respectively.

The high temporal resolution of the amperometric method reported here provides accurate measurements of exocytotic events occurring on the millisecond time scale. Individual exocytotic events released from single vesicles from the cell body of the giant dopamine neuron of *Planorbis corneus* are observed following stimulation with in situ application of elevated potassium. The data show that exocytotic events recorded from this neuron reveal three distinct distributions of inter-spike interval (Fig. 2C). 39.1% of these exocytosis events show inter-spike intervals centered around 5 ms and 35.2 and 25.7% of bursting events have inter-spike intervals centered at 22 and 45 ms. These three distributions of exocytotic events probably represent three independent bursting patterns occurring at this neuron while participating in a complicated neuronal communication feedback network.

Other alternatives to the feedback hypothesis of bursting exist. One possibility is that the three distributions in inter-spike intervals observed here might be generated from one or two independent bursting patterns. Since the carbon fiber electrodes used here for detecting exocytotic events do not discriminate single events from individual bursts, it is possible that events occurring at 5-ms inter-spike intervals are from multiple bursts of exocytosis events occurring at 22- or 45-ms intervals. Assuming that bursts occurring at 22- or 45-ms intervals are not synchronized, summation of three of these bursts occurring at 5-ms delay from one another will generate bursts occurring at 5-ms intervals. The generation of bursts occurring at 22-ms intervals from non-synchronized bursts with 45-ms intervals is also possible. However, the possibility of summation of one or two independent bursting patterns to form three bursting patterns as shown in Fig. 2C is considered remote for the following reason. If the three bursting patterns are virtually from one independent bursting pattern, namely bursts with 45-ms intervals, with a non-synchronized mechanism, it should be possible to generate bursts with 10- to 20-ms inter-spike intervals. The lack of a distribution centered around 10- to 20-ms inter-spike interval in Fig. 2C does not support the summation mechanism.

It is therefore reasonable to suggest that the three bursting patterns observed from this neuron are independent. The mechanism of generating these three distinct bursting patterns is at present unclear. One hypothesis is that this neuron receives different types of synchronized

inputs from other cells in this system. It has been reported that the activation of the statocyst receptor cells leads to excitation of this dopamine neuron [2]. Also supporting this hypothesis, experimental results from the giant dopamine neuron of *Lymnaea* that participate in the generation of the respiratory rhythm of air-breathing are found to receive different inputs from two neural networks [22]. It is possible that the *Planorbis* giant dopamine neuron might be involved in a three-neuron network as shown in the *Lymnaea* giant dopamine neuron since the physiological functions of these two neurons are very similar. The lack of a bursting pattern from exocytotic events recorded from isolated PC12 cells in culture suggests that bursting release is not a universal phenomenon. The hypothesis that this neuron receives synchronized inputs from other cells is further supported by a recent experiment that synchronized evoked and spontaneous bursting action potentials can be obtained from a subset of interneurons in an inhibitory network to the goldfish Mauthner cell [6]. Although all the above evidence is from measurements of presynaptic or postsynaptic potentials, it appears reasonable to compare the results of exocytotic measurements with those of synaptic potential measurements. It has been shown extensively that presynaptic action potentials lead to exocytosis and postsynaptic miniature end-plate potentials [11].

An alternative hypothesis to explain the present findings is that the three bursting patterns observed from the *Planorbis* giant dopamine neuron might result from oscillations in intracellular calcium during neural activation. Oscillations in the intracellular calcium concentration which occur over longer time scales have been observed for other neurons and glial cells [5,21,23]. Since exocytosis takes place after the intracellular calcium level increases, oscillations in intracellular calcium might lead to bursts of exocytotic events. In addition, different oscillating modes of intracellular calcium signalling responsible for generating different bursting patterns might exist from a neuron participating in active transmission. First, intracellular calcium waves may be generated from different sources after the cell is stimulated, such as calcium-influx from the external medium, and calcium release from internal mitochondria and endoplasmic reticulum. Dynamic calcium fluctuation in different modes is possible. Second, calcium oscillation may be obtained from intercellular interactions such as astrocyte-neuron signalling. Recent evidence has shown that astrocytes can regulate neuronal calcium levels through intercellular communication [19]. Since the *Planorbis* giant dopamine neuron is surrounded by a number of small glial cells [20], direct calcium signalling from those glial cells is possible.

Our present data show that three distinct bursting patterns exist in exocytotic events recorded from the giant dopamine neuron of *Planorbis corneus*. However, these data might underlie a much more complicated bursting pattern as the current algorithm only includes bursts with no intervening spikes. Here, bursts with large inter-spike intervals would be precluded from the data summary. Indeed only 3% of the events observed are involved in bursting patterns described here. The remaining 97% might be involved in bursting patterns with longer inter-spike intervals. To our knowledge, this is the first demonstration of distinct bursting of exocytotic events. Although the mechanism causing the bursting pattern is far from elucidated, bursting exocytosis at a neuron may represent an important phenomenon in understanding single cell activities in neural communication.

Acknowledgements

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