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SPECIAL REPORT

Spontaneous release of large packets of noradrenaline from sympathetic nerve terminals in rat mesenteric arteries *in vitro*

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Continuous amperometry was used to monitor noradrenaline (NA) release from sympathetic nerves supplying rat mesenteric arteries *in vitro*. During electrical stimulation the amplitude of oxidation currents evoked by successive stimuli varied over a small range, with occasional events of larger amplitude. In the absence of stimulation, spontaneous oxidation currents (s.o.cs) were recorded. The frequency of s.o.cs was increased by α-latrotoxin (1 nM). This agent also increased the frequency of spontaneous excitatory junction potentials (s.e.j.ps), which monitor the packeted release of adenosine 5′ triphosphate (ATP). The frequency of s.o.cs recorded 20–25 min after applying α-latrotoxin was about four times the control value, but that of s.e.j.ps was about 30 times the control value. The findings suggest that continuous amperometry can detect the spontaneous packeted release of NA, probably from large dense-cored vesicles. In contrast, s.e.j.ps monitor spontaneous release of neurotransmitter (ATP) from a different store, most likely the small dense-cored vesicles. *British Journal of Pharmacology* (2000) **131,** 1507–1511

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Abbreviations: ATP, adenosine 5′-triphosphate; NA, noradrenaline; NPY, neuropeptide Y; s.e.j.p., spontaneous excitatory junction potential; s.o.c., spontaneous oxidation current; s.d.v., small dense-cored vesicle; l.d.v., large dense-cored vesicle.

Introduction Historically much of the evidence for the exocytotic release of neurotransmitter comes from biochemical studies of catecholamine release from chromaffin cells and postganglionic sympathetic nerves (Stärjne *et al*., 1989). These studies demonstrated that catecholamines are stored in a membrane bound particulate store and that the soluble contents of this store are released when secretion is triggered by depolarizing stimuli. Biochemical studies also demonstrated that in sympathetic nerve terminals the particulate store of noradrenaline (NA) is heterogeneous, being composed of both 'light' and 'heavy' vesicles (Klein & Lagercrantz, 1981). These are believed to correspond, respectively, to the small (~50 nm diameter) and large (~100 nm diameter) dense-cored vesicles observed in electronmicrographs of sympathetic nerve varicosities. It is widely believed that both vesicle populations participate in neurotransmitter release, the contents of small dense-cored vesicles (s.d.vs) being preferentially released by low frequency stimuli while at higher frequencies the proportion of neurotransmitter that is released from large dense-cored vesicles (l.d.vs) increases (see Stjärne, 1989).

Evidence for the differential release of neurotransmitter from s.d.vs and l.d.vs comes primarily from studies of neuropeptide Y (NPY) release. This neuropeptide is only stored in l.d.vs and it has been reported that the amount of NPY that is released, in proportion to that of NA, increases as the frequency of stimulation is raised (Lundberg *et al*., 1989). However, more recently De Potter *et al*. (1997) have reported that, in sheep and dog spleen and rat vas deferens, changing the frequency of stimulation does not alter the proportions of NA and NPY released. In addition, these authors reported that during prolonged periods of stimulation, the amounts of NA and NPY released declined in parallel. On the basis of their observations, De Potter *et al*. (1997) have hypothesized that NA and NPY are released exclusively from l.d.vs.

In contrast to NPY, the co-transmitter adenosine 5′ triphosphate (ATP) is contained in both the 'light' and 'heavy' vesicles isolated from sympathetic nerve terminals (Klein & Lagercrantz, 1981). However, the question of whether NA and ATP are uniformly stored in the same populations of synaptic vesicles or differentially stored in vesicles of similar density remains unanswered. It is therefore possible that NA and ATP are separately stored and released. In support of this possibility there have been reports that NA and ATP release from sympathetic nerves can be differentially modulated by drugs (e.g. Todorov *et al*., 1996).

In the present study continuous amperometry was used to monitor electrically-evoked and spontaneous release of NA from sympathetic nerves supplying rat mesenteric arteries (Dunn *et al*., 1999). In the absence of stimulation, ongoing transient increases in oxidation current (termed spontaneous oxidation currents) were recorded and the possibility that these are produced by the spontaneous exocytotic release of NA was investigated. In addition, the study compared th effects of α-latrotoxin on the spontaneous oxidation currents (s.o.cs) and intracellularly recorded spontaneous excitatory junction potentials (s.e.j.ps), which monitor the ongoing exocytotic release of ATP (Brock & Van Helden, 1995). The findings of the α-latrotoxin experiments suggest that neurotransmitter is released from at least two nerve terminal stores.

Methods Female outbred Wistar rats (150–200 g) were killed by an overdose of pentobarbitone sodium (100 mg kg^{-1}, i.p.). Segments of mesentery containing second order mesenteric arteries were dissected and pinned to the Sylgard (Dow-Corning) covered base of a 1 ml recording chamber. The chamber was perfused continuously at 3–5 ml min^{-1} with physiological saline of the following composition (mM): NaCl

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133.4, $NaHCO_3$ 16.3, NaH_2PO_4 1.3, $CaCl_2$ 2.0, KCl 4.7, $MgCl_2$ 1.2 and glucose 7.8. The physiological saline was gassed with a mixture of 95% O_2 and 5% CO_2 (to pH 7.4) and maintained at 35–36°C. The proximal end of the mesenteric artery was drawn into a suction electrode and the perivascular nerves excited by electrical field stimulation (0.5 ms, 20 V). In all experiments increasing the stimulation strength did not increase the amplitude of the electrically-evoked responses and it was assumed that the stimulation parameters were supramaximal for activating the sympathetic nerves. Electrochemical and electrophysiological recordings were made from the surface of the arteries at a site 3–5 mm distal of the mouth of the suction stimulating electrode.

Electrochemical recording The release of endogenous NA was measured by continuous amperometry using the technique previously described by Dunn *et al.* (1999). Briefly, an electrode constructed from a single 7 μm diameter carbon fibre and coated with Nafion (Aldrich Chemical Company, Castle Hill, NSW, Australia) was mounted so that an approximately 100 μm length of carbon fibre was in contact with the surface of the artery. The electrode was connected to an AMU130 Nano-amperometer (Radiometer – Analytical S.A., 69627 Villeurbanne Cedex, France) and a potential difference of +0.3 V applied between the recording electrode and a Ag-AgCl pellet placed in the recording chamber medium. The current required to maintain this voltage was monitored. Previous studies using this technique indicate that NA is the only substance that contributes to the electrically-evoked oxidation current (see Dunn *et al.*, 1999). Of the peptides released from the sympathetic and primary afferent innervation of the mesenteric arteries, substance P is not electroactive and NPY and calcitonin gene related peptide would not be oxidized at +0.3 V (see Bennett *et al.*, 1981). In addition, ATP is not electrochemically oxidizable.

Electrophysiological recording Intracellular recordings were made from the smooth muscle using glass microelectrodes (80–160 MΩ) filled with 0.5 M KCl and connected to an Axoclamp (Axon Instruments, Inc., Foster City, CA, U.S.A.). The criteria for accepting impalements were the same as those adopted by Brock & Van Helden (1995).

Analysis All data were digitized (sampling frequencies of 0.2–1 kHz) and analysed with a MacLab recording system (ADInstruments Pty Ltd, Castle Hill, NSW, Australia). Data are presented as mean ± s.d. Statistical comparisons were made using paired sign tests.

Drugs Capsaicin (Sigma Chemical Company, Castle Hill, NSW, Australia), textrodotoxin (Sigma) and α-latrotoxin (Alomone Laboratories, Jerusalem, Israel) were added to the solution superfusing the tissue at the required concentration.

Results *Stimulation-evoked and spontaneous oxidation currents* Activation of the perivascular nerves with an electrical stimulus evoked a transient increase in oxidation current which peaked within 100 ms and then decayed over 2–3 s. In 20 experiments the peak amplitude of oxidation currents evoked by successive stimuli at 0.2 or 0.5 Hz varied over a small range, with occasional events of larger amplitude (e.g. see Figure 1a(i–x) and b(i)). In all experiments the large amplitude events decayed more rapidly, and in 14 experiments the decay of the averaged oxidation current evoked during trains of 50 or 100 stimuli at 0.2 Hz (see Figure 1a(xii)) was best fitted by the sum of two exponentials with time constants of 180 ± 40 ms and 980 ± 340 ms. In the remaining six experiments the decay of the averaged current was best fitted by a single exponential with a time constant of 540 ± 90 ms. These findings suggest that the electrochemical technique is able to resolve impulse-by-impulse variations in the amount of NA released. Similar findings have been reported for rat tail artery (Mgshina *et al.*, 1993).

In the absence of stimulation there were occasional transient increases in oxidation current with durations of about 300 ms (see Figure 1b). These s.o.cs normally occurred at low frequencies and in the 13 experiments that were fully analysed the frequency was 0.9 ± 0.21 events min^{-1}. No

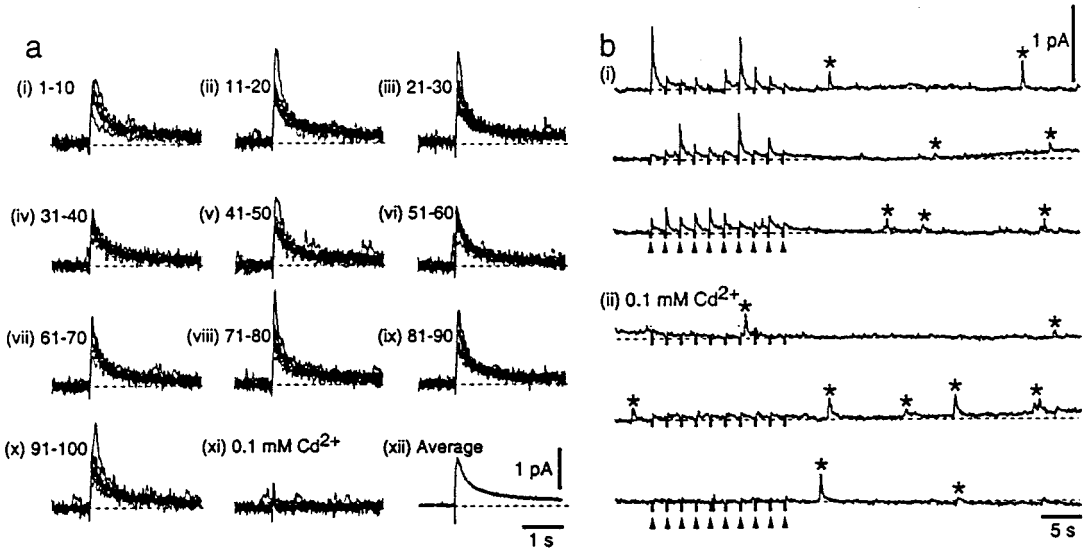

Figure 1 Noradrenaline-induced oxidation currents recorded amperometrically with a carbon fibre electrode. (a) Each panel (i–x) shows 10 overlaid traces recorded during a train of 100 pulses at 0.2 Hz. Panel (xi) shows 10 overlaid traces recorded 10 min after application of 0.1 mM Cd^{2+} to abolish neurotransmitter release and panel (xii) is the averaged oxidation current evoked by the train of 100 pulses. (b) Single traces showing responses to 10 pulses at 0.5 Hz (indicated by the arrowheads) before (i) and during application of 0.1 mM Cd^{2+} (ii). In this recording spontaneous oxidation currents (s.o.cs; indicated by *) occurred at a relatively high frequency. Application of Cd^{2+} abolished the electrically-evoked oxidation currents but the s.o.cs remained.

spontaneous or electrically-evoked oxidation currents were recorded when the electrode potential was held at 0 V, a value below the oxidation potential for NA ($n=9$ experiments, see Dunn *et al*., 1999). In addition, oxidation currents evoked by electrical stimulation were abolished by tetrodotoxin (0.3 μM, $n=6$ experiments, see Dunn *et al*., 1999) and by the non-selective Ca^{2+} channel blocker, Cd^{2+} (0.1 mM, $n=18$ experiments, Figure 1a(xi) and b(ii)), but these agents did not prevent the occurrence of the s.o.cs (e.g. Figure 1b(ii)). These findings suggest the s.o.cs may be produced by spontaneous packeted release of NA.

Effects of α-latrotoxin To examine the possibility that exocytotic release of NA generates the s.o.cs, the effects of α-latrotoxin was investigated. This peptide evokes the exocytotic release of neurotransmitters from a variety of nerve terminals, including those of postganglionic sympathetic neurones (Zhou & Mizler, 1995; De Potter *et al*., 1997). In eight experiments, α-latrotoxin (1 nM) produced a marked increase in the frequency of the ongoing activity (Figure 2) which peaked about 10 min after its addition to the superfusing solution (Figure 2b) and then waned so that 20–25 min after its addition the frequency of the ongoing activity was about four times that observed under control conditions (Figure 2c; control 1.5 ± 1.4 events min^{-1}, α-latrotoxin 6.2 ± 6.0 events min^{-1}; $P<0.01$).

The effects of α-latrotoxin (1 nM, six experiments) on intracellularly recorded s.e.j.ps were similar to those on the s.o.cs, with activity peaking 5–10 min after its addition to the superfusing solution and then waning (Figure 3a,b). However, when measured 20–25 min after adding α-latrotoxin the frequency of s.e.j.ps was still markedly increased compared to pretreatment control values (control 1.4 ± 1.2 events min^{-1}; α-latrotoxin 44.2 ± 16.8 events min^{-1}; $P<0.05$).

In four experiments application of capsaicin (0.1 μM) to selectively activate the peptidergic primary afferent nerves had no effect on the frequency of s.o.cs (control 1.7 ± 1.2 events min^{-1}; 5–10 min in capsaicin 1.7 ± 1.0 events min^{-1}).

Characteristics of s.o.cs To obtain sufficient numbers of s.o.cs to determine their characteristics, data recorded under control conditions were pooled from 13 experiments. In each of these experiments only events with amplitudes >0.25 pA (background noise level 0.1–0.15 pA peak-to-peak) were analysed (e.g. see Figure 4a). The amplitude distribution of the s.o.cs (Figure 4b) was positively skewed, most events having amplitudes <0.5 pA (median 0.39 pA, mean 0.51 ± 0.34 pA,

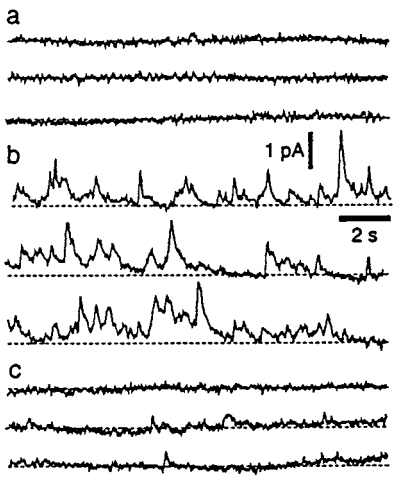

Figure 2 Effects of α-latrotoxin (1 nM) on ongoing electrochemical activity. Three traces recorded before (a) and 10 min (b) and 25 min (c) after the addition of α-latrotoxin.

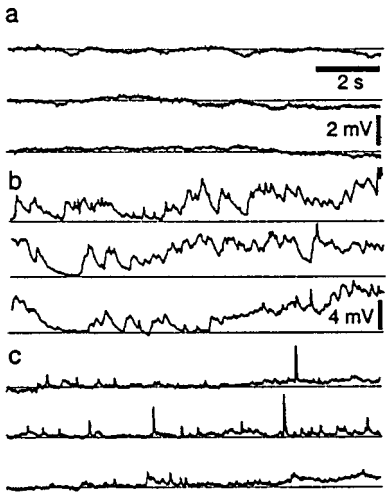

Figure 3 Effects of α-latrotoxin (1 nM) on ongoing electrical activity. Three traces recorded before (a) and 10 min (b) and 25 min (c) after the addition of α-latrotoxin. The voltage scale bar in (a) also applies in (c).

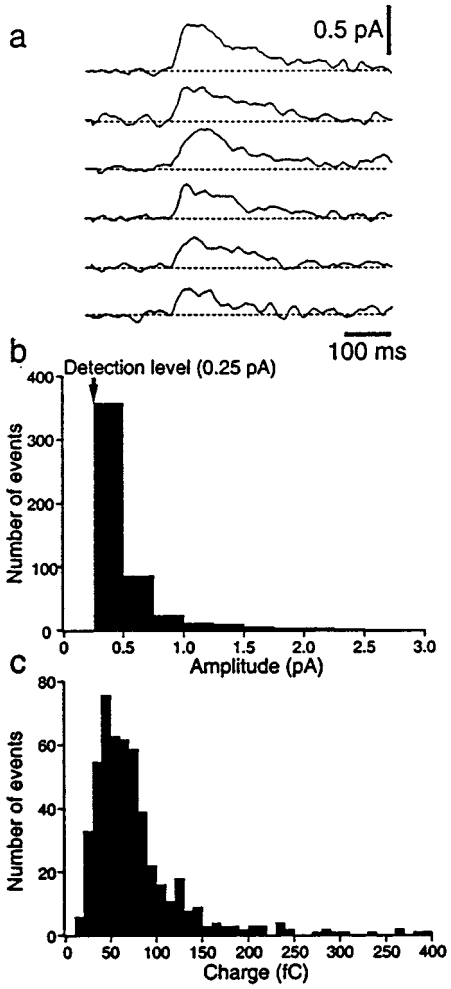

Figure 4 Characteristics of the spontaneous oxidation currents (s.o.cs). (a) Examples of s.o.cs recorded in a single experiment. (b) Amplitude frequency distribution of the s.o.cs. (c) Frequency distribution of the charge generating the s.o.cs. In (b) and (c) the s.o.cs were recorded in 13 experiments ($n=511$).

$n=511$). For a sample of 200 s.o.cs the 10–90% rise time was 39 ± 31 ms and the duration (10% of the rise to 90% of the decay) was 292 ± 130 ms. The decay of the averaged s.o.c. recorded in each tissue (individual events aligned by their maximum rate of rise) was best fitted with a single exponential with a time constant of 110 ± 40 ms ($n=13$). Figure 4c shows the distribution of the total charge (i.e. the integral of the current over time) underlying the s.o.cs. This distribution was positively skewed with a mode between 40–50 fC and median of 53 fC (mean 66 ± 52 fC, $n=511$). Assuming that NA is the only constituent released from synaptic vesicles that undergoes oxidation at +0.3 V and that its electro-oxidation requires two electrons (see Zhou & Misler, 1995), the median amount of NA generating a s.o.c. is 165,000 molelcules.

Discussion The present study provides the first direct evidence for the packeted release of NA from sympathetic nerves in an intact tissue. During trains of stimuli the electrically-evoked oxidation currents fluctuated in amplitude and, in the absence of stimulation, s.o.cs were recorded. Previous studies using both electrochemical and pharmacological techniques have confirmed that the electrically-evoked oxidation currents detected at the adventitial surface of arteries are due to neuronally released NA (see Dunn *et al*., 1999). However, because of their small amplitude it has not been possible to confirm that the s.o.cs are generated by NA. Attempts to slow the time course of the s.o.cs by blocking neuronal uptake of NA were unsuccessful (results not presented). However, the time course of oxidation currents evoked by single electrical stimuli are similarly unaffected by blocking neuronal uptake of NA, presumably because clearance of NA from its site of release is primarily by diffusion and not by uptake (see Dunn *et al*., 1999.

The most convincing evidence that the s.o.cs are produced by the vesicular discharge of NA is provided by the effects of α-latrotoxin. This agent produced a marked increased in the frequency of ongoing activity recorded amperometrically. The frequency of occurrence of intracellularly recorded s.e.j.ps, which monitor the spontaneous packeted release of ATP from the sympathetic nerve terminals, was also greatly increased by α-latrotoxin. When measured 20–25 min after adding α-latrotoxin, the frequency of s.ej.ps was about 30 times the control value but that of the s.o.cs was about four times the control value. This finding suggests that s.o.cs and s.e.j.ps monitor neurotransmitter release from different nerve terminal stores.

The l.d.vs, which constitute about 5% of the vesicle population present in the varicosities in rat mesenteric arteries (Tranzer, 1973), are manufactured in the cell body and transported to the nerve terminal where they are believed to undergo only a single cycle of exocytosis/endocytosis and are not regenerated (see De Potter *et al*., 1997). It might therefore be expected that the number of l.d.vs would be readily depleted if their rate of exocytosis was markedly increased by agents like α-latrotoxin. In contrast, s.e.j.ps have been suggested to monitor primarily the release of ATP from s.d.vs (Stjärne, 1989), which in rat mesenteric arteries constitute about 90% of the vesicle population (Tranzer, 1973). The s.d.vs are formed locally within the varicosity and can therefore be replenished during prolonged periods of increased exocytotic activity. Thus the simplest explanation for our observations is that the s.o.cs monitor release of NA from the l.d.vs, while, as previously suggested, the s.e.j.ps monitor neurotransmitter release from s.d.vs.

The relatively large amount of NA required to generate s.o.cs also suggests that they may be generated by the discharge of the NA content of l.d.vs. However, it is not possible to exclude the possibility that s.o.cs result from the near synchronous release of the contents of several vesicles. It is usually assumed that spontaneous exocytotic events occur at random and that the distribution of intervals between events is that expected for a Poisson process. If this is correct, it can be predicted that as the frequency of spontaneous activity is increased, the number of events that occur within a short interval of each other would also increase. Importantly, during the first 30 min period of α-latrotoxin application when the frequency of spontaneous activity was increased, the amplitudes of the clearly defined single s.o.cs recorded in all experiments (mean $=0.54\pm0.40$ pA; median $=0.42$ pA, $n=523$) was not greatly different from those recorded under control conditions (see Results section). This finding indicates that s.o.cs are unlikely to arise from the random summation of NA released from several s.d.vs or l.d.vs. Therefore, if the majority of s.o.cs are 'multiquantal', they would have to result from some process that transiently elevates the probability of release at single or groups of closely related release sites (see Bornstein, 1978).

Previous estimates of the NA content of l.d.vs isolated on density gradients was about 15,000 molecules (Klein & Lagercrantz, 1981). However, more recent studies using amperometry to monitor NA release from the nerve terminals of cultured rat superior cervical ganglion neurones have estimated the median content of spontaneously released packets to be 35,000 molecules (Zhou & Misler, 1995). Using their estimate of the catecholamine concentration in chromaffin granules (~0.5 M), Zhou & Misler (1995) calculated that the 35,000 molecules could be accommodated in spherical vesicles with diameters of ~60 nm. This estimate of vesicle size is in the mid range of vesicle diameters present presynaptically in cultures of rat superior cervical ganglion neurones (Rees & Bunge, 1974). In the present study where the recordings are made at some distance from the release sites (see below), the release of 35,000 molecules would not be readily detected. However, if it is assumed that the NA concentration in l.d.vs (100 nm diameter) is also 0.5 M, then the NA content would be about 160,000 molecules. This value is very close to that determined in the present study and is similar to the estimated dopamine content of l.d.vs in carotid body glomus cells (mean $=138{,}000\pm84{,}000$ molecules) which have diameters of about 100 nm (Urena *et al*., 1994).

The time courses of the s.o.cs recorded in the present study are relatively slow when compared to those of the 'quantal' oxidation currents recorded close to the visualized terminals of sympathetic nerves in culture (half widths of 1–5 ms; Zhou & Misler, 1995). This can be explained if they are recorded at some distance from the sites of NA release. To provide a rough estimate of this distance the rise time of the s.o.cs was matched using Green's function (see Crank, 1967) and the estimated diffusion coefficient for NA in the adventitia of rabbit aorta (4×10^{-1} cm^2 s^{-1}; Bevan & Su, 1973). This analysis suggested that s.o.cs are recorded at a distance of about 8 μm from the site of NA release. This value is similar to the measured thickness of the adventitia in second order rat mesenteric arteries (~10 μm).

Because the events are recorded at some distance from the sites of release it would be expected, if NA diffuses freely in all directions, that the NA content of the 'quanta' would be substantially underestimated. However, NA diffuses much less readily in the media than in the adventitia (Bevan & Su, 1973). Thus it would be predicted that NA would preferentially diffuse towards the surface of the adventitia. Furthermore, the

negatively charged Nafion coating on the carbon fibre surface will attract NA ions to the electrode.

The present findings do not resolve the question of whether NA and ATP are released together or separately from the sympathetic nerve terminals because: (1) the electrochemical technique is not sensitive enough to resolve NA released from single s.d.vs; (2) while the s.e.j.ps probably monitor ATP release primarily from s.d.vs (see Stjärne, 1989), ATP released from l.d.vs may also generate s.e.j.ps, albeit at a very low frequency. It is also possible that exocytosis of l.d.vs releases both NA and ATP, but at sites where the released ATP does not generate s.e.j.ps.

In conclusion, the findings suggest that continuous amperometry can detect the spontaneous packeted release of NA, probably from l.d.vs. In contrast, s.e.j.ps monitor spontaneous release of neurotransmitter (ATP) from a different nerve terminal store, most likely the s.d.vs.

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