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Statistics of quantal secretion during long trains of sympathetic nerve impulses in mouse vas deferens

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1. A statistical analysis has been made of the occurrence of excitatory junctional currents (EJCs) of similar amplitude recorded with an extracellular electrode during long trains of nerve impulses to the mouse vas deferens.

2. The number of EJCs of similar amplitude that occurred in consecutive impulses during trains of 500–1000 impulses at 0·5–2·0 Hz increased with the number of EJCs evoked during the train.

3. There was no evidence of significant dependence between consecutive EJCs of similar amplitude in sixteen out of eighteen trains in eighteen preparations.

4. The time course of clusters of EJCs of similar amplitude was examined by determining the standard deviation of different groups of EJCs within a cluster throughout their time course. Most EJCs within a cluster could be grouped with a coefficient of variation $< 0{\cdot}1$ throughout their time course.

5. The observations on EJCs of similar amplitude leave open the possibility that secretion from single varicosities is, in general, multiquantal.

In the original description of the excitatory junction potential (EJP) at autonomic neuromuscular junctions, Burnstock & Holman (1962) showed that the EJP sometimes possessed a very fast component on the rising phase which resembled the spontaneous EJPs. Blakeley & Cunnane (1979) differentiated the rising phase of the EJP in order to separate these fast components from the slower ones. They showed that the fast components consisted of intermittently occurring discrete events (DEs) which were attributed to secretion from close-contact varicosities (Bennett & Gibson, 1995). These come into such close apposition with the muscle that the basement membranes of the varicosity and muscle cell either completely fuse (50 nm distance; Richardson, 1962; Bennett & Merrillees, 1966) or the basement membrane is eliminated entirely (20 nm distance; Merrillees, 1968). As the DEs occurring with a single latency and time to peak had amplitudes that were multimodally distributed (Blakeley, Cunnane & Petersen, 1982) the possibility of multiquantal secretion from single varicosities was suggested (Blakeley, Mathie & Petersen, 1984); each varicosity may then release more than one quantum on arrival of the nerve impulse. Recordings of the EJPs from high input impedance cells in the vas deferens, which are likely to have few couplings with other cells (Bennett, 1967), also gave multimodal amplitude distributions. This confirmed the results with the DEs and supported the notion of multiquantal secretion (Blakeley, Dunn & Petersen, 1989).

In contrast to these considerations Cunnane & Stjärne (1982, 1984) showed that, during long trains of nerve impulses, DEs at a particular latency could be assigned to a class in which the entire amplitude and time course of the DEs were matched. Such matched DEs were taken as arising from a particular close-contact varicosity; in this case secretion must be monoquantal, so that each varicosity releases, at the most, one quantum on arrival of the nerve impulse (Stjärne & Åstrand, 1984). Furthermore, as matched DEs occurred at very low frequency (2–30 in 1000), the probability of monoquantal secretion from a close-contact varicosity was taken to be very low (Stjärne, 1986). Additional evidence for the idea of low probability secretion of single quanta from single varicosities came from the observation that very few DEs were observed in some recordings; in these the DEs of similar amplitude and time course tended to occur in response to consecutive or near-consecutive impulses. Such observations were interpreted in terms of a facilitatory effect of one impulse on secretion from a varicosity by a subsequent impulse in the train (Cunnane & Stjärne, 1984). These results with DEs were later confirmed with the introduction of

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extracellular recording techniques for observing the excitatory junctional currents (EJCs) that flow during the EJP (Brock & Cunnane, 1988). EJCs with the same amplitude and time course could be found at a particular latency during long trains of impulses, although these occurred infrequently. They were interpreted as arising from a single close-contact varicosity, and as spontaneous excitatory junction currents (SEJCs) of similar amplitude and time course were observed, secretion was taken as monoquantal and of low probability (Åstrand, Brock & Cunnane, 1988). In this case the EJC may be taken as a 'fingerprint' of single quantal release from a particular varicosity (Åstrand & Stjärne, 1989). More recently a technique has been introduced for recording from visualized varicosities on the surface of the vas deferens with small-diameter (~4 μm) microelectrodes (Lavidis & Bennett, 1992). In one such recording, Gaussian distributions of both spontaneous and evoked EJCs of similar mean and standard deviation were obtained, supporting the idea that for this varicosity at least, secretion was monoquantal. In the present work a statistical analysis is developed to test if EJCs of similar amplitude occurring consecutively in a long train are independent of each other or not and whether classes of EJCs of a particular amplitude and time course can be identified during long trains of impulses. These tests have been devised in order to see if there is statistical support for the idea that a single varicosity releases, at most, one quantum on arrival of the nerve impulse.

METHODS

Tissue preparation and stimulation

The vas deferens of mice (strain Balb/c), 5–6 weeks old, were used in these experiments. Animals were anaesthetized with halothane and then killed by a cervical fracture. An incision was made into the lower abdomen and the vas deferens, together with the connective tissue containing the fine hypogastric nerves, was dissected free. Careful stripping of the sheath of the epimysium from the surface of the vas deferens was then performed by gently teasing the sheath from the prostatic end for a distance of about 5 mm along the vas deferens. The tissue was then placed in a Perspex chamber of 3 ml capacity and pinned through the epididymal end to a sheet of Sylgard at the bottom of the chamber. The bathing solution had the following composition (mM): Na^+, 136·9; K^+, 4·0; Mg^{2+}, 1·0; Cl^-, 127·1–129·1; $H_2PO_4^-$, 1·5; HCO_3^-, 16·3; Ca^{2+}, 1·0–2·0 and glucose, 7·77. The solution was gassed continuously with 95% O_2–5% CO_2 so that the pH was maintained between 7·1 and 7·2. The tissue was perfused with warm Krebs solution (35–36 °C) at a rate of 2–4 ml min^{-1}.

The prostatic end of the mouse vas deferens (4 mm) was sucked up lightly into a modified bilbate which was filled with Krebs solution. Ag^+–AgCl electrodes were used to stimulate the postganglionic axons using rectangular pulses of 0·05 ms duration and 14–24 V in amplitude. Stimulus frequencies of 0·5, 1·0 and 2·0 Hz were used in this study and trains consisting of 500–1000 impulses were recorded.

Recording and analysis

Both large (18–25 μm) and small (3–5 μm) tip diameter extracellular electrodes filled with Krebs solution were used to record the terminal impulse and excitatory junctional currents (EJCs). Large-diameter electrodes (about 20 μm) were placed at random over the cleared surface of the vas deferens and sites chosen for which less than 15% of nerve impulses gave rise to an EJC in a $[Ca^{2+}]$ of 0·6–1·3 mM. Recordings which showed positive-going EJCs in more than 1% of cases were rejected.

Small-diameter electrodes were used to record from visualized varicosities. To this end the vas deferens was perfused for 40 s with 3,3 dithyloxardicarbocyanine iodide ($DiOC_2(5)$, 0·05–0·5 μM) and then washed in modified Krebs solution for 2 min (Bennett, Jones & Lavidis, 1986). $DiOC_2(5)$ fluorescence of the axons and varicosities was observed during excitation of 540 nm using an Olympus rhodamine filter set (Olympus, BH-2 microscope). Preparations were viewed using a ×50 long working distance objective (Olympus, ULWD-MS Plan 50). The adverse effects of $DiOC_2(5)$ and fluorescence on quantal secretions have previously been studied (Bennett *et al.* 1986). The concentration of $DiOC_2(5)$ was kept to less than 0·5 μM and the period of excitation to less than 2 min in order to minimize any adverse effects of $DiOC_2(5)$ excitation. The field of excitation was restricted to the region being examined and the field of fluorescing axons was hand drawn on the TV monitor screen as soon as their fluorescent profiles had been identified; excitation was then terminated and transmitted light used to view the same area. Structures such as blood vessels and connective tissue were then drawn on the TV monitor; the glass microelectrode was then moved into the field of the drawing of the varicose axons. Any shift of the varicose axons with respect to the drawing could finally be checked by refluorescing the varicose axons at the termination of the experiment. It has already been shown that a 5 μm lateral movement of the microelectrode away from a varicosity results in complete loss of the spontaneous excitatory junction potential (SEJP; Lavidis & Bennett, 1992). Electrophysiological study was only made of the fluorescing varicose axons on the surface of the muscle that were at least 10 μm distant from any other surface axons. Recordings with small-diameter microelectrodes were made in a $[Ca^{2+}]$ of 1·8 mM.

Signals were recorded using an Axoclamp 2A amplifier (10 kHz bandwith; Axon Instruments) then fed into an oscilloscope (low-pass filter cut-off 3 kHz, AC coupled; Tektronix 5111A with a 5A22 N differential amplifier module) and then finally digitized (at 20 kHz over 60 ms) and stored on hard disk (Macintosh IIVx microcomputer) using the MacLab data acquisition system in conjunction with the Scope software (version 3.3.3; AD Instruments, Australia). The Scope data files were translated into Igor data files with Translate (AD Instruments) in order to be analysed using the Igor Pro (Wavemetrics, USA) software analysis package. The software package enabled the implementation of user-defined functions. The amplitude of individual EJCs was determined by manual placement of two cursors; one of these was placed in the baseline immediately before the EJP, giving the average over sixteen sample points preceding the cursor position, covering a period of 0·8 ms; the other was placed at the peak of the EJP giving an average in which 25% of the sample points (namely 4) occurred immediately before the cursor position and 75% (12) immediately after. The EJC amplitude was determined by subtracting these two averages. In order to compare EJCs of

similar amplitude and time course, individual traces were first filtered by binomial smoothing incorporated in the Igor Pro analysis package (see Figs 1 and 6; Marchand & Marmet, 1983). Traces were smoothed by a factor of fifty. There is little alteration in the time course of the rising phase and decay phase of the EJC due to this filtering (Fig. 1). Clusters of EJCs of similar amplitude and time course in a train were determined for similarity by taking the standard deviation throughout the duration of the smoothed EJCs in the cluster (Fig. 6). For this purpose the EJCs were aligned at their maximum rate of rise. A user-defined function was written to carry out the above procedure in Igor P+ro.

RESULTS

Tests for independence of consecutive EJCs of similar amplitude in long trains

Sympathetic nerves to the mouse vas deferens were stimulated at 0·5–2·0 Hz for 500–1000 impulses and EJCs recorded from the surface of the muscle with either large-diameter (~20 μm) or small-diameter (~4 μm) microelectrodes. Up to 12% of the impulses gave rise to an EJC in an extracellular calcium concentration ($[Ca^{2+}]_o$) of 0·6–1·8 mM (Fig. 1). In some cases pairs of consecutive impulses gave rise to EJCs which were either of similar amplitude (that is within 1·5 standard deviations (S.D.) of the noise level in size) or of dissimilar amplitude.

Graphs of the amplitude of the EJC *versus* impulse number in the train were constructed like those in Fig. 2 for fifteen recordings with large-diameter electrodes from each of fifteen preparations. In addition, three recordings with small-diameter electrodes were also taken from visualized varicosities in three preparations. The occurrence of consecutive pairs of EJCs of any amplitude recorded with large-diameter electrodes increased with the percentage of EJCs observed over 500–1000 impulses (Fig. 3*A*). In the eleven preparations for which any consecutive pairs of EJCs were recorded, seven showed at least as many pairs again that were not of similar amplitude (Table 1); three of the remaining four preparations showed two or less consecutive pairs and these were all of similar amplitude (Table 1). The incidence of consecutive pairs of similar amplitude also increased with the percentage of impulses giving EJCs in the train (Fig. 3*B*).

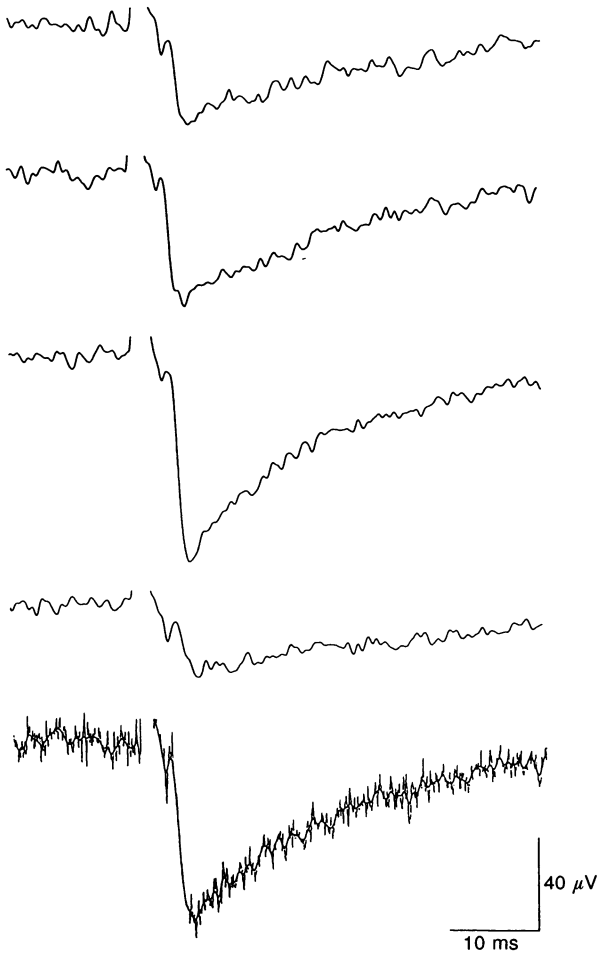

Figure 1. EJCs recorded during trains of 500 impulses
EJCs, which are smoothed, are shown for impulse numbers 4, 5, 46, 183 and 437 in the train of Fig. 2*A*. The stimulus frequency was 0·5 Hz in a $[Ca^{2+}]_o$ of 0·6–1·8 mM. The last trace shows both the recorded EJC and its binomial smoothed version superposed. Large-diameter electrode.

Table 1

Prepn number	τ	Observations (%)	(c_1) P_1	(c_2) P_2	(c_3) P_3	(c_4) P_4	(c_5) P_5	$[Ca^{2+}]$	Freq. (Hz)	Consec. pairs	$\bar{y}$ (μV)	$\hat{\lambda}$	$\hat{\theta}$
1	3·2	21/500	(1)	(1)	(1)	(1)	(1)	0·6	0·5	1	23·3	0·073	0·261
			0·23	0·41	0·54	0·65	0·73						
2	3·7	18/500	(2)	(3)	(3)	(3)	(3)	0·6	0·5	2	21·6	0·096	0·268
			0·03	0·02	0·05	0·09	0·15						
3	4·6	36/500	(0)	(1)	(2)	(2)	(3)	0·6	0·5	4	37·3	0·043	0·650
			1·0	0·74	0·60	0·75	0·65						
4	3·6	15/500	(0)	(1)	(1)	(1)	(1)	0·6	0·5	0	23·3	0·080	0·158
			1·0	0·27	0·37	0·46	0·54						
5	6·3	40/1000	(0)	(1)	(1)	(2)	(3)	0·6	0·5	0	34·3	0·065	0·734
			1·0	0·77	0·89	0·79	0·71						
6	3·3	13/500	(0)	(0)	(0)	(0)	(1)	0·9	0·5	0	23·4	0·074	0·104
			1·0	1·0	1·0	1·0	0·40						
7	4·0	58/500	(2)	(3)	(4)	(5)	(6)	0·6	0·5	6	27·9	0·063	2·118
			0·63	0·79	0·88	0·93	0·95						
8	2·9	40/500	(2)	(3)	(4)	(5)	(6)	0·6	0·5	4	17·6	0·112	1·234
			0·35	0·45	0·51	0·55	0·58						
9	3·3	84/1000	(4)	(5)	(5)	(6)	(7)	1·3	0·5	9	28·1	0·055	1·682
			0·09	0·25	0·57	0·66	0·73						
10	4·8	141/1000	(8)	(15)	(19)	(21)	(27)	1·3	1·0	27	32·6	0·055	6·501
			0·33	0·33	0·58	0·86	0·86						
11	3·2	72/1000	(1)	(6)	(7)	(7)	(7)	1·3	1·0	3	22·4	0·078	1·619
			0·80	0·11	0·22	0·47	0·70						
12	2·8	16/1000	(0)	(0)	(0)	(0)	(0)	1·3	4·0	0	16·2	0·129	0·107
			1·0	1·0	1·0	1·0	1·0						
13	3·8	60/1000	(2)	(3)	(3)	(3)	(3)	1·3	1·0	2	23·2	0·085	1·382
			0·40	0·52	0·78	0·91	0·97						
14	3·6	118/1000	(2)	(6)	(13)	(20)	(24)	1·3	0·5	5	21·0	0·098	5·722
			0·98	0·97	0·87	0·76	0·83						
15	5·8	79/1000	(6)	(9)	(10)	(13)	(15)	1·3	2·0	6	19·7	0·445	6·111
			0·57	0·86	0·99	0·99	1·0						
16	7·5	190/500	(28)	(41)	(51)	(55)	(58)	18	0·5	189	49·3	0·037	24·583
			0·27	0·89	0·99	1·0	1·0						
17	7·5	70/500	(6)	(10)	(13)	(17)	(20)	18	0·5	69	40·9	0·054	4·462
			0·29	0·40	0·57	0·60	0·71						
18	8·2	38/500	(2)	(2)	(3)	(5)	(5)	18	0·5	37	35·6	0·092	1·957
			0·58	0·90	0·93	0·89	0·97						

Prepn, preparation; τ, noise S.D.; c_{1-5}, number of pairs separated by fewer than 1–5 impulses; Freq., frequency; Consec., consecutive; $\bar{y}$, mean amplitude; $\hat{\lambda}$, estimate of parameter in exponential model for the amplitudes; $\hat{\theta}$, expected number of consecutive pairs assuming independence; P_{1-5}, P values for independence of pairs separated by fewer than 1–5 impulses.

One explanation for the existence of consecutive pairs of similar amplitude is that secretion from a given varicosity is characterized by an EJC of a particular amplitude; consecutive pairs of similar amplitude then arise because the occurrence of one EJC indicates secretory conditions in a varicosity which facilitate the occurrence of a second EJC from that varicosity in the next few impulses. A statistic has therefore been devised (see Appendix) to ascertain if the occurrence of such pairs of EJCs is independent or dependent as a consequence of a facilitatory interaction. The P value testing independence of consecutive pairs of similar amplitude EJCs was greater than 0·20 in all but two out of ten recordings with large-diameter electrodes that had such pairs (Fig. 4 and Table 1). There was then no evidence in over 80% of observations for an EJC of a particular amplitude in a pair to occur other than at random. It is possible that the facilitatory effects in obtaining a second EJC from a varicosity last beyond that of adjacent impulses during trains of impulses at 0·5–2·0 Hz. In order to test for this, the test statistic was extended to see if independence occurs between pairs of similar amplitude separated by one or more impulses up to four. All but one of the ten recordings with large-diameter electrodes showed independence between the pairs when

separated by up to four impulses (Table 1). The recording from preparation 2 gave a significant failure of the independence test for separation between EJCs of similar amplitude by one up to four impulses (Table 1). Graphs of the amplitude of the EJC *versus* impulse number in the train were also constructed for three recordings with small-diameter electrodes placed over visualized varicosities. Table 1 shows that EJCs of a particular amplitude occur at random for the varicosities in these preparations (numbers 16–18), according to the statistical test.

Tests for EJCs of similar amplitude and time course

EJCs of a given amplitude may be due to secretion from a particular varicosity and may, in addition, have the same time course. It might be possible, if this is the case, to show that relatively large-amplitude EJCs in a long train can be grouped according to their amplitude and time course. The amplitude–frequency distribution of EJCs collected during a long train often shows clusters of EJCs of similar amplitude (Fig. 5). The time course of EJCs within each

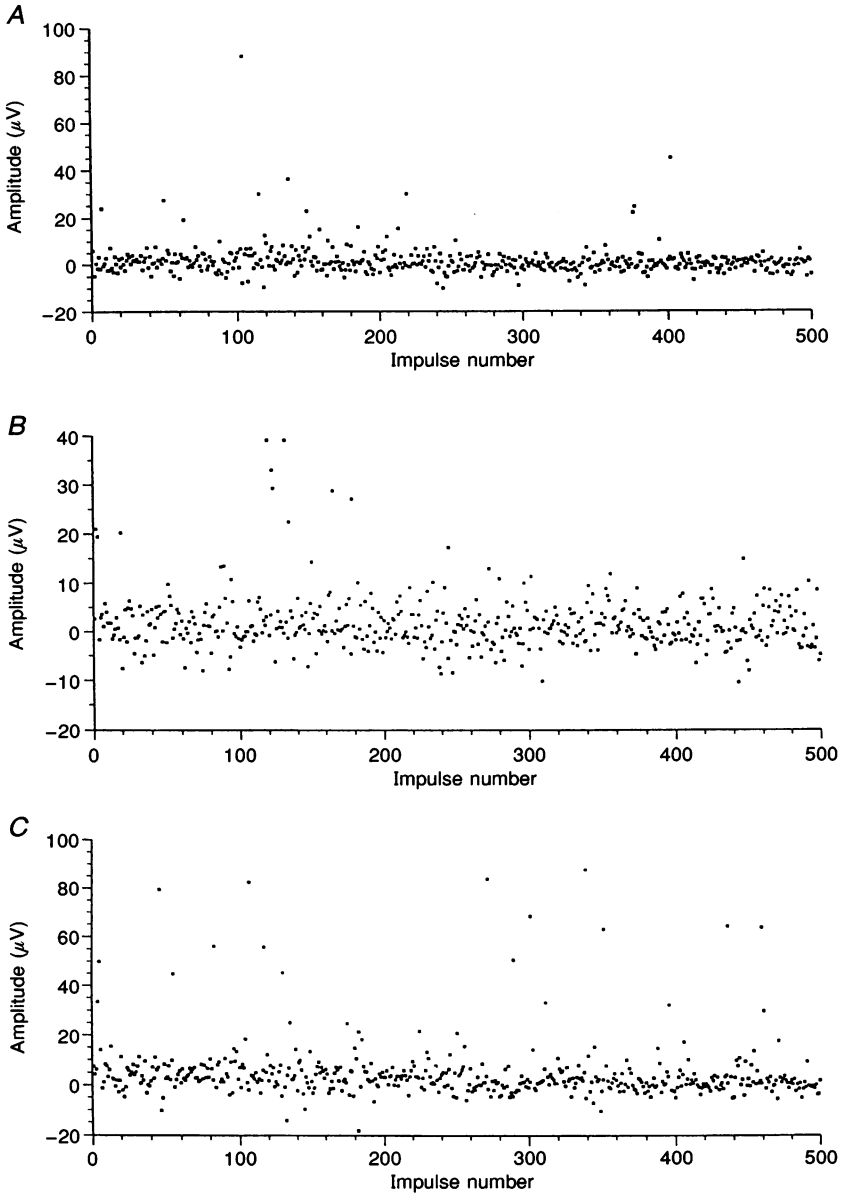

Figure 2. Amplitude of responses to each impulse in a long train

EJC amplitudes are shown for different impulses in a long train of 500, together with failures to elicit an EJC so that only the noise level is indicated. In *A*, consecutive EJCs occurred at 376/377 and these were of similar amplitude. In *B*, consecutive EJCs occurred at impulses 2/3 and 122/123 and both of these pairs were of similar amplitude. In *C*, consecutive EJCs occurred at impulses 4/5; these were not of similar amplitude. Large-diameter electrodes only. *A*, *B* and *C* correspond to the first three preparations in Table 1.

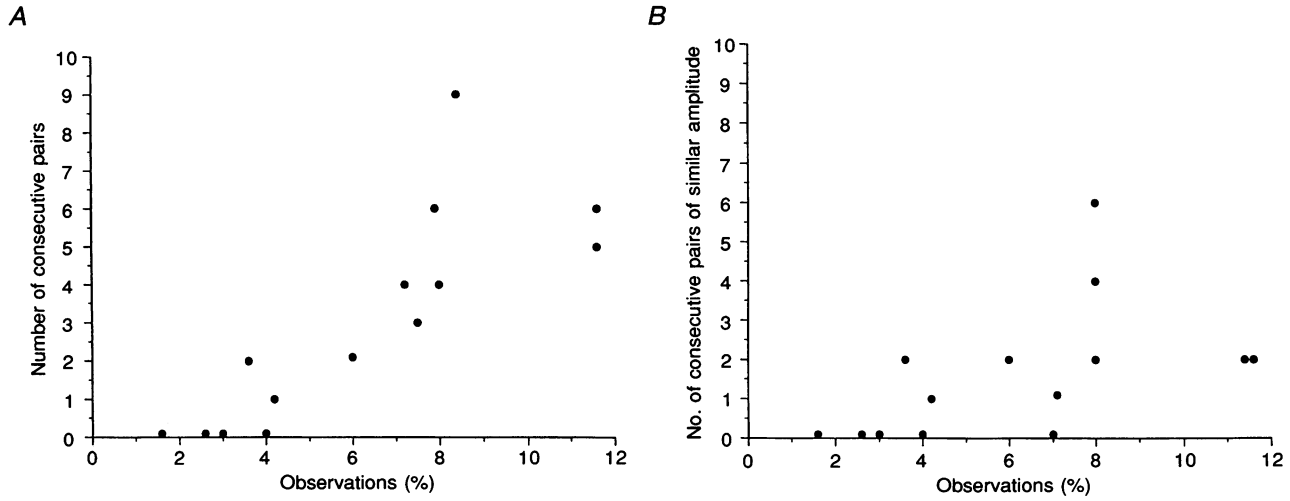

Figure 3. Consecutive pairs of EJCs occur more frequently with line number of EJCs in a train

A, the number of consecutive pairs of impulses that gave rise to EJCs of any amplitude, recorded with large-diameter electrodes, is plotted against the percentage of all impulses that gave EJCs of any amplitude. *B*, the number of consecutive pairs of impulses that gave rise to EJCs of similar amplitude (within 1·5 S.D. of the noise) is plotted against the percentage of all impulses that gave EJCs of any amplitude. The results are for 15 different recordings in 15 preparations. $[Ca^{2+}]_o$ ranged from 0·6 to 1·3 mM; microelectrode diameters ranged from 18 to 22 μm.

cluster has been compared for four different recordings in different preparations.

The standard deviation (S.D.) throughout the time course of EJCs within a cluster was determined by adding the EJCs together in such an order that the S.D. increased from its smallest to largest value. In this way the S.D. could be used to give a quantitative measure of the extent to which EJCs in a similar-amplitude cluster could be said to have the same time course. This was, in general, the case if the S.D. was less than about 3 μV throughout most of the time course of the EJCs. Figure 6 gives examples of EJCs from one recording that showed the greatest extent of EJCs of similar amplitude and time course during a train of 1000 impulses; the amplitude–frequency distribution is given in Fig. 5. The two largest EJCs of size about 86 μV were numbers 44 and 349; these had very similar peak amplitude and time course of decline; after the peak was reached the S.D. did not vary by more than 1 μV, except near the peaks of the EJCs where it reached 5 μV (Fig. 6*A*). The next cluster of EJCs were in the size range 74–76 μV (numbers 694, 808 and 844); of these two (694 and 844) had similar time courses, with the S.D. remaining elevated at about 3 μV throughout most of the EJP and only transiently reaching as high as 5 μV during the decline (Fig. 6*Ba*); addition of the third EJC (808) substantially increased the S.D. to over 5 μV for most of the time course of the EJC (Fig. 6*Bb*), indicating that this additional EJC was very different to the other two. The third cluster of EJCs were in

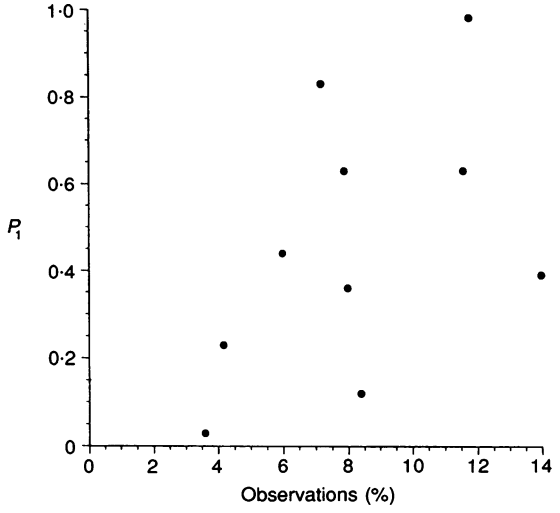

Figure 4. The probability of independence of successive EJCs of similar amplitude

The P value (P_1) for the test that successive EJCs of similar amplitude are independent of each other during a long train of impulses (500–1000) is plotted against the percentage of EJCs of any amplitude evoked during the train. Results are given for the 12 experiments, with large-diameter electrodes, that showed any successive pairs of similar amplitude; of these 10 possessed a P_1 greater than 0·15, indicating that the pairs were probably independent whereas 2 had a P_1 less than 0·15 and so showed some dependence.

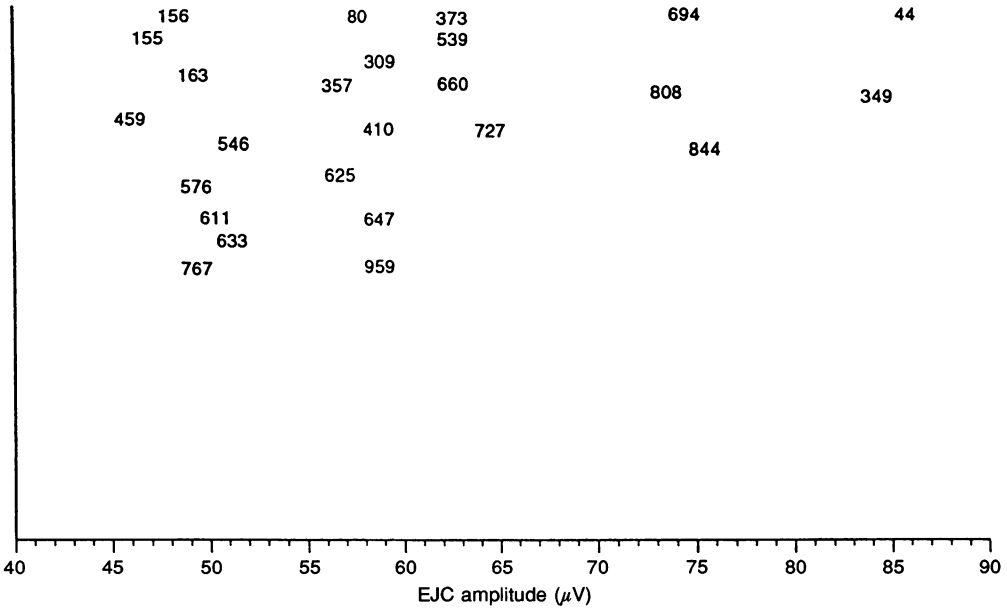

Figure 5. Amplitude–frequency histogram of EJCs in a long train

The amplitudes of the largest 25 EJCs out of 141 recorded during a train of 1000 impulses at 0·5 Hz is given. Each EJC has a number which indicates its position in the train. Clusters of EJC amplitudes can be discerned about 86, 75, 63 and possibly 58 and 50 μV. There were 116 EJCs smaller than 45 μV and EJCs smaller than this did not separate into clusters. The ordinate indicates the arbitrary positioning of the impulse number to best display this number. Large-diameter electrode.

the size range 62–65 μV (numbers 373, 539, 660 and 727); all of these had very similar time courses with a maximum S.D. of about 3·5 μV occurring only transiently during the declining phase (Fig. 6*C*); a fast transient of unknown origin occurred on the declining phase of 660. The fourth cluster of EJCs were in the size range 57–59 μV (occurring at impulses 80, 309, 357, 410, 625, 647 and 959); all of these except 357 and 410 had very similar time courses with a S.D. that only transiently reached about 2·5 μV (Fig. 6*Da*); the addition of 357 and 410 substantially increased the S.D. to over 5 μV for most of the EJC time course (Fig. 6*Db*). The smallest amplitude cluster analysed was in the size range 46–52 μV; all of these had a very similar time course, so that the S.D. only increased very transiently to 4 μV (Fig. 6*E*). In the four recordings analysed in this way, most of the EJCs within any size category had a similar time course, as indicated by the relatively small changes in the S.D. as individual EJCs were added together as described above, although within some categories there were EJCs of very different time course.

DISCUSSION

The amplitude and time course of EJCs recorded with an extracellular electrode are determined by at least two factors other than the amount of transmitter secreted in a quantum. One of these relates to the position of a transmitting varicosity in the electrode: varicosities towards the edge of an electrode give rise to larger and faster-rising EJCs in response to a quantum than do varicosities at the middle of the electrode (Bennett, Gibson & Poznanski, 1993). The other factor is responsible for fluctuations in the amplitude and time course of EJCs and SEJCs that arise from the stochastic nature of transmitter action on receptors together with the subsequent hydrolysis and diffusion of transmitter from the junctional cleft (Bennett, Farnell, Gibson & Karunanithi, 1995). The first of these factors will help in establishing the EJC finger print in relation to monoquantal secretion from a particular varicosity within the electrode. The second factor will militate against such an identification between EJCs arising from different close-contact varicosities within the electrode. The present work showing that EJCs in the same amplitude category may occur with different time courses is consistent with the stochastic nature of transmitter action on receptors. Because of these considerations it is not possible to tell whether EJCs of a particular amplitude category and time course originate from a single varicosity or not. It is not clear then whether monoquantal secretion occurs at single varicosities, if the criteria of EJC fingerprinting is used, at least in the mouse vas deferens.

Another reason for supporting the notion that EJCs of a particular amplitude category and time course originate from a specific varicosity is that such EJCs sometimes occur for consecutive impulses in a long train during which less than 14% of the impulses give rise to an EJC at all (see Fig. 13 in Cunnane & Stjärne, 1984; Cunnane & Stjärne,

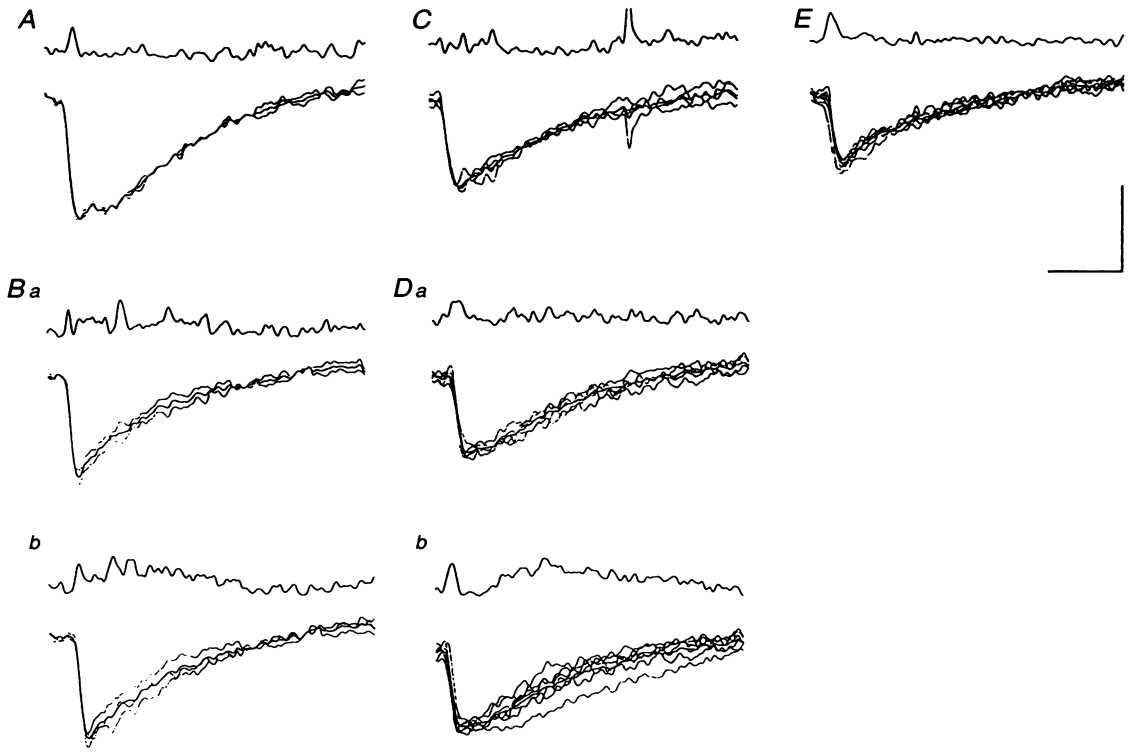

Figure 6. Time course of EJCs in a similar amplitude cluster

Use of the S.D. determined throughout the duration of EJCs in a similar amplitude cluster to ascertain the similarity of their time course. Results for data in Fig. 5. Each panel consists of a lower component in which certain EJCs are superimposed and the average given by a thick line: the upper component gives the S.D. of the EJCs over their time course. *A*, largest amplitude cluster (44 and 349 in Fig. 5). *B*, next largest cluster showing 694 and 844 (*Ba*) and 694, 844 and 808 (*Bb*). *C*, next largest cluster showing (373, 539, 660 and 727). *D*, next largest cluster showing 80, 309, 625, 647 and 959 (*Da*) and 80, 309, 357, 410, 625, 647 and 959 (*Db*). *E*, smallest amplitude cluster analysed (155, 156, 163, 459, 611, 633 and 767). Vertical calibration is 40 μV for the EJCs and 10 μV for the S.D.; horizontal calibration is 10 ms.

1982; for a review see Brock & Cunnane, 1993; Stjärne, Bao, Ganon, Msghina & Stjärne, 1993). The statistical technique devised in the Appendix failed to show dependence between consecutive EJCs of similar amplitude. Furthermore, there was no significant dependence between EJCs of similar amplitude even when these were separated by up to four impulses. It may be that the existence of consecutive pairs of similar amplitude EJCs in long trains observed in previous work (see for example, Cunnane & Stjärne, 1984, their Fig. 12) would also fail statistical tests for dependence. Cunnane & Stjärne (1982) showed in one experiment (their Fig. 3a) that only three DEs occurred in 500 impulses, and these had a peak amplitude coefficient of variation of about 0·15. Such DEs would not have been treated as being of similar amplitude in the present statistical tests as the difference in their amplitudes was greater than 1·5 times the S.D. of the noise level. If they were treated as having similar amplitude they would still not have reached a significant level of dependence as they did not occur consecutively. The failure in the present work to show that consecutive EJCs of similar amplitude are dependent suggests that further research is required to support the hypothesis that EJCs of similar amplitude originate from the same varicosity.

Recordings with small-diameter electrodes from visualized varicosities in general give rise to gamma-like amplitude–frequency distributions of spontaneous EJCs (MacLeod, Lavidis & Bennett, 1994) although occasional Gaussian distributions have been observed (Lavidis & Bennett, 1992). Statistical tests of the gamma-like distributions have shown that they most probably arise from a Poisson mixture of Gaussians (Bennett, 1994). There is evidence for amplitude–frequency distributions of spontaneous EJCs consistent with multiquantal secretion in both guinea-pig arterioles (Hirst & Neild, 1980) as well as mouse vas deferens (Blakeley, Dunn & Petersen, 1989). These results, together with earlier ones using DEs (Blakeley *et al.* 1982) and intracellular recordings of EJCs from relatively uncoupled cells in the vas deferens (Blakeley, Dunn & Petersen, 1989), support the concept of multiquantal secretion from some varicosities on the surface of the mouse vas deferens. Varicosities, partly or almost entirely bare of Schwann cell

sheath, are found on the surface of the mouse vas deferens (Lavidis & Bennett, 1993). Ultrastructural analysis of serial sections through these varicosities show that all of those belonging to single axons come within 50 nm of muscle cells, whereas those in axon bundles do not, possessing regions of close apposition to muscle cells that are at least 100 nm away (Cottee, Lavidis & Bennett, 1995). If all these varicosities have the capacity to secrete a quantum of transmitter on arrival of the nerve impulse, then there will be considerable variability in the size and time course of EJCs and DEs due to both differences in the apposition of varicosities to muscle cells and to the stochastic effects of transmitter–receptor interaction at a given varicosity. Such effects will make it difficult to identify EJCs as arising from a particular varicosity. The problem of monoquantal *versus* multiquantal secretion from varicosities can only be resolved by reliable recording from single visualized varicosities.

APPENDIX

Independence test for consecutive EJCs of similar amplitude in a long train of nerve impulses (with a generalization)

For each of the trains the data consist of N recordings of the amplitude of a current at successive impulses with serial numbers 1 to N. Each recording is either an observation of electrical noise or a measurement of the amplitude of EJC.

Under independence and assuming a constant probability, p, of observing a measurement of EJC at each impulse, p is estimated by n/N, the proportion of impulses which result in a measurement of EJC. The appearance of two successive measurements of EJC which have consecutive serial numbers and which are of similar amplitude is an unusual event, and this fact is used to construct appropriate test statistics. (When successive EJCs have consecutive serial numbers we shall call them consecutive pairs of EJCs.)

Notation. We use (K, Y_K) to represent the serial number and the amplitude, respectively, of an EJC measurement recorded with a large-diameter extracellular electrode, denoting the observed values by $(k_1, y_{k_1}), \ldots, (k_n, y_{k_n})$. Thus, the ith EJC measurement has serial number k_i and amplitude y_{k_i}. Consecutive pairs of EJCs occur whenever $k_{i+1} - k_i = 1$. The noise standard deviation is represented by τ. Whenever independent measurements of noise were available they were used to estimate τ, but for some of the preparations, no independent measurements of noise were available, so that τ was estimated from the $N - n$ impulses which did not result in an EJC. We use X_M to represent the number of consecutive pairs of EJC measurements. Under independence, X_M is approximately Poisson (Np^2) provided p is small. Finally, we use X_C to represent the number of consecutive pairs which are of *similar amplitude* and we denote its observed value by c.

For illustration, preparation 1 consists of 500 impulses, with the recordings displayed in Fig. 2*A*. A lower limit, denoted T, is set (somewhat arbitrarily) at $T = 3\tau$ for the EJC recordings, as this effectively ensures that no noise measurements are mistaken for EJC recordings. The 500 recordings are by this means reduced to 21 bivariate observations (7, 23·917), (50, 27·278), ..., (402, 44·694) and this reduced set is used to judge the strength of the evidence against independence of recordings in the light of the alternative proposal.

From the data displayed in Fig. 2*A* we note that there is only one pair of successive serial numbers (k_i, k_{i+1}) with $k_{i+1} - k_i = 1$, which means that there is only one consecutive pair of EJCs (serial numbers 376 and 377). They also appear on closer inspection to be of *similar amplitude* or *close enough* to have come from the same site given the presence of noise, since their amplitudes are 21·8 and 24·2 μV. The problem of defining what is meant by similar amplitude is now addressed by considering two successive EJC measurements y_{k_i} and $y_{k_{i+1}}$ to have come from the same site if they are of 'similar amplitude', i.e. if $|y_{k_{i+1}} - y_{k_i}| < T/2$. The choice of $T/2 = 1{\cdot}5\tau$ is somewhat arbitrary (other multiples of τ were also considered) but it is a practical attempt to allow for noise. Thus the observed value of X_C is:

$$c = \sum_i I(k_{i+1} - k_i = 1).I(|y_{k_{i+1}} - y_{k_i}| < T/2),$$

where I is the indicator function. For preparation 1, $\tau = 3{\cdot}2$ μV, $T/2 = 4{\cdot}8$ μV and the only consecutive pair of EJCs differ in amplitude by 2·4 μV so that $c = 1$.

Test for independence of recordings at consecutive episodes

A test is considered for the hypothesis, H_0, of independence of recordings against the alternative, H_1, of an enhanced conditional probability of a recording from a site given that there was a recording from *that site* at the immediately preceding episode. The test is based on counting X_C, the number of consecutive pairs of measurements judged to be from the same site (using the above definition of like amplitude), and it assumes that the EJC measurements, Y, follow an exponential distribution when recorded with a large-diameter extracellular electrode, with lower bound $T = 3\tau$ and with density

$$f_Y(y) = \lambda \exp(-\lambda(y - T)), \quad y > T.$$

Experience with similar sets of data indicates that this is not an unreasonable assumption, and, in fact, the similarity of the P values to those based on permutation tests and bootstrap tests lends further credence to this assumption.

From the observed values $y_{k_1}, \ldots, y_{k_n}$ of Y, the maximum likelihood estimate of λ is $\hat{\lambda} = 1/(\bar{y} - T)$ and the probability, P, that the recordings at any two given episodes (with serial numbers j and k say) are *both*

Table 2. A comparison of P values

	c	P_{exp}	$\hat{P}_{perm}$	$\hat{P}_{boot}$
Set 1	1	0·230	0·244	0·246
Set 2	2	0·030	0·021	0·024
Set 3	0	1·000	1·000	1·000

The P values refer to three different tests of the hypothesis of independence of EJC recordings against the alternative of an enhanced conditional probability of a recording from a site given there was a recording from that site at the immediately preceding episode. P_{exp} is the P value under the assumption of an exponential distribution of EJCs, while $\hat{P}_{perm}$ and $\hat{P}_{boot}$ are the permutation and bootstrap P values, respectively, based on 500 replicates each time. The agreement of the P values from the two non-parametric tests with the P value from the test using the assumed model is seen to be particularly good.

measurements of EJC and are also of similar amplitude is, under the hypothesis of independence,

$$P = P_0 = p^2 P(|Y_k - Y_j| < T/2), \qquad k \neq j.$$

In particular, this is the case for all *consecutive pairs* of episodes, and we use as test statistic X_C, the number of consecutive pairs of EJCs of similar amplitude. The hypotheses can be written formally

$$H_0 : P = P_0,\ H_1 : P > P_0.$$

Thus if the observed value of X_C is

$$c = \sum_i I(k_{i+1} - k_i = 1).I(|y_{k_{i+1}} - y_{k_i}| < T/2)$$

the P value is $P_{exp} = P(X_C \geqslant c)$.

Under H_0, and since N is very large relative to n, X_C is approximately binomial (N, P_0) by an elementary argument. Writing $\theta = NP_0$, it follows that X_C is approximately Poisson with parameter θ since N is large and P_0 is small with NP_0 fixed. It remains to estimate θ, the expected value of X_C. If $Y_{K_{i+1}}$ and Y_{K_i} are independent measurements with density f_Y as given above, it can be shown, writing $W_i = |Y_{K_{i+1}} - Y_{K_i}|$, that W_i has density $f_W(w) = \lambda e^{-\lambda w}$, $w > 0$ and hence

$$P(|Y_{K_{i+1}} - Y_{K_i}| < T/2) = 1 - \exp(-\lambda T/2).$$

Thus $\theta = NP_0 = Np^2(1 - e^{-\lambda T/2})$ and is estimated from the data by

$$\hat{\theta} = \frac{n^2}{N}(1 - \exp(-\hat{\lambda} T/2)).$$

An estimate of the P value under this model is then

$$P_{exp} \approx \sum_{x=c}^{\infty} \exp(-\hat{\theta})\hat{\theta}^x / x!.$$

For preparation 1 for example, $\hat{\tau} = 3{\cdot}2$, $T = 9{\cdot}6$, $\bar{y} = 23{\cdot}3$, $\hat{\lambda} = 0{\cdot}073$, $\hat{\theta} = 0{\cdot}261$ and $c = 1$, since there is only one consecutive pair of EJCs of like amplitude. Thus, the P value is approximately 0·23, and the data are consistent with independence of release.

Discussion. An obvious drawback of the test outlined above is the assumption of an exponential distribution for the amplitudes of measurements in the presence of noise. This assumption can be avoided by using a permutation test or a bootstrap test. Accordingly, 500 permutation replicates $\{c^{(p)}(k),\ k = 1, \ldots, 500\}$ and 500 bootstrap replicates $\{c^*(b),\ b = 1, \ldots, 500\}$ of c were produced, and permutation P values (P_{perm}) and bootstrap P values (P_{boot}) were estimated by calculating the proportion of the replicates which are greater than c,

$$\hat{P}_{perm} = \#\{c^{(p)}(k) \geqslant c\}/500,$$

$$\hat{P}_{boot} = \#\{c^*(b) \geqslant c\}/500.$$

The results for the first three preparations are found in Table 2 as an illustration. (Of course no calculations were necessary for set 3 since $c = 0$.)

The agreement of the P values from the two non-parametric tests with the P value from the test using the assumed model is particularly good, so that with hindsight, the assumed exponential model for the measurements was appropriate.

Tests of independence against a more general alternative

It is possible that the enhancing effect of a release may be prolonged at some synapses to the next j episodes where j is small (e.g. $\leqslant 5$) provided there is no intervening EJC recording from a different site. The tests discussed to this stage can be extended naturally to cover this more general alternative. The procedure is to count the number of pairs X_{C_j} of successive measurements of similar amplitude for which the serial numbers differ by at most j. The variable X_{C_j} is approximately Poisson with parameter θ_j, by analogy with the case for $j = 1$, and, as a first approximation, we use $\hat{\theta}_j = j\hat{\theta}$ to estimate θ_j. (Of course, $\hat{\theta}_1$ is simply $\hat{\theta}$.) The observed value c_j of X_{C_j} is given by:

$$c_j = \sum_i I(k_{i+1} - k_i \leqslant j).I(|y_{k_{i+1}} - y_{k_i}| < T/2).$$

For example, if we take $j = 5$, then for preparation 1, the observed value of the count c_5 is 1, since there is only one pair of successive measurements (y_{k_i} and $y_{k_{i+1}}$) with serial

numbers (k_i and k_{i+1}) differing by at most 5 and for which the amplitudes differ by less than $T/2$ (= 4·799). The P value is calculated as before by $P(X_{C_j} \geqslant c_j)$. For this example, $\hat{\theta}_5 = 5\,\hat{\theta} = 1{\cdot}304$ and the P value is $P_5 = 0{\cdot}73$.

The P values are found in columns P_1–P_5 of Table 1, the values in parentheses being the observed values of c_1–c_5. Of course c_1 is simply the value c in the previous test. As before, bootstrap and permutation tests were performed as a check on the use of the exponential model. Although the P values are not reported here they were in general very close to the P values recorded in Table 1 using the above test.

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