STATISTICAL ANALYSIS OF CALCIUM ACCUMULATION IN THE NERVE ENDING DURING REPETITIVE STIMULATION

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Several successive stages can be distinguished in evoked mediator secretion: depolarization of the presynaptic membrane during the action potential, opening of potential-dependent calcium channels, entry of Ca^{++} ions into the cytoplasm of the nerve ending, activation of the secretion mechanism, and liberation of the transmitter from its release point [2, 5]. Transmitter release depends mainly on the intracellular Ca^{++} ion concentration. Special structures which utilize active calcium, thereby reducing its intracellular concentration, are present in the nerve ending. They include mitochondria, the endoplasmic reticulum, the presynaptic plasma membrane, etc. [3]. The fall in the Ca^{++} ion concentration in the cytoplasm after the action potential lasts several tens of milliseconds. If a second stimulus is applied during this time, the new portion of Ca^{++} ions, on entering the nerve ending, undergoes summation with the calcium remaining after the previous stimulus, thus causing increased transmitter secretion [6]. This mechanism lies at the basis of facilitation of transmitter release during paired and repetitive stimulation. Consequently increased transmitter secretion during repetitive stimulation ought to be dependent only on the number and frequency of stimulating pulses.

This paper describes a statistical analysis of transmitter secretion using the conditional probability method. It was found that during paired and repetitive stimulation, quantum transmitter release in response to the first (previous) stimulus leads to inhibition of secretion in response to the next stimulus.

EXPERIMENTAL METHOD

Experiments were carried out on nerve-muscle preparations of the sartorius muscle of Rana ridibunda. The nerve-muscle preparation was kept in a bath containing continuously flowing Ringer's solution of the following composition (in mM): NaCl – 115.0, KCl – 2.0, $CaCl_2$ – 0.2-0.6, $MgCl_2$ – 1-4, $NaHCO_3$ – 2.4, pH 7.2-7.4. End-plate potentials (EPP) were recorded extracellularly by means of glass microelectrodes filled with 2 M NaCl solution with a resistance of 10-40 MΩ. All experiments were carried out at a very low (m = 0.1-0.5) EPP quantum composition. Analysis of transmitter secretion in these experiments is indicated schematically in Fig. 1 for the case of stimulation by series of two or three pulses. The total number of series (N_T), the number of series in which quantum release took place in response to the i-th (1st, 2nd, 3rd) stimulus (n_1, n_2, n_3), and the number of series in which a response to the first stimulus was absent [N(0)] and present [N(1)], the number in which there was no response to either stimulus [N(00)], a response only to the first stimulus [N(10)], or a response to both stimuli [N(11)], and so on, were determined. The mean probability of quantum transmitter release ($\bar{p}$) in response to the 1st, 2nd, and 3rd stimulus and the number of functioning release points ($\bar{n}$) were determined on the basis of the rules of binomial statistics [4]: $\bar{p} = 1 - S/\overline{m}$; $\bar{n} = \overline{m}/\bar{p}$, where N stands for variations. The motor nerve was stimulated by 100-200 series of pulses, the interval between series being 15-30 sec. The number of pulses in the burst varied from two to four and their frequency was 50-100 Hz.

EXPERIMENTAL RESULTS

Determination of the statistical parameters showed that during repetitive stimulation the mean probability of quantum transmitter release increased to each successive stimulus. Data showing an increase in the parameter p during stimulation by three pulses with a frequency of 100 Hz are given in Table 1. At low initial

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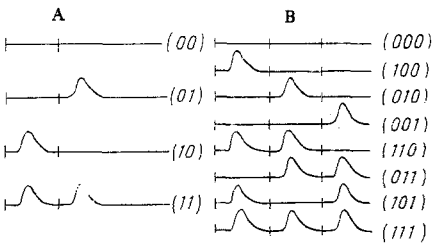

Fig. 1. Scheme of analysis of EPPs recorded during four series, each of two stimuli (A), and eight series each of three stimuli (B). Numbers in parentheses indicate presence (1) or absence (0) of response to 1st, 2nd, and 3rd stimuli. Time of stimulation indicated by short vertical line. Calculated values of parameters for A: $N_T = 4$, $n_1 = 2$, $n_2 = 2$, N(00) = 1, N(01) = 1, N(10) = 1, N(11) = 1.

TABLE 1. Binomial Parameters ($\bar{m}$, $\bar{n}$, $\bar{p}$) of Transmitter Secretion during Repetitive Stimulation (three pulses with frequency of 100 Hz)

Experiment No.	No. of stimulating pulse								
	1			2			3		
	$\bar{m}$	$\bar{n}$	$\bar{p}$	$\bar{m}$	$\bar{n}$	$\bar{p}$	$\bar{m}$	$\bar{n}$	$\bar{p}$
1	0,058	1,16	0,050	0,117	1,07	0,109	0,150	1,05	0,143
2	0,125	1,10	0,114	0,275	1,03	0,266	0,413	1,02	0,405
3	0,100	1,10	0,091	0,170	1,05	0,162	0,300	1,02	0,293
4	0,167	1,04	0,159	0,258	1,02	0,252	0,300	1,02	0,294
5	0,125	1,04	0,120	0,261	1,02	0,257	0,190	1,02	0,186
6	0,042	1,24	0,034	0,092	1,09	0,084	0,142	1,05	0,134
7	0,030	1,19	0,025	0,035	1,16	0,030	0,075	1,07	0,070
8	0,020	1,19	0,010	0,070	1,12	0,061	0,160	1,06	0,152

values of $\bar{p}$ repetitive stimulation caused an increase in $\bar{p}$ without any appreciable changes in the parameter $\bar{n}$, which remained equal to 1. In this case facilitation of transmitter secretion can be represented as follows:

$$p(\varepsilon_1 = 1) < p(\varepsilon_2 = 1) < p(\varepsilon_3 = 1), \tag{1}$$

where ε is a random value equal to 1, if in the response to the i-th pulse transmitter was released, and equal to 0 in the opposite case. The value of $P(\varepsilon = 1)$ can be estimated by the relationship:

$$n_1/N_T, \quad p(\varepsilon_2 = 1) - n_2/N_1, \quad p\varepsilon_3 = 1) - n_3/N_T.$$

If facilitation during repetitive stimulation was due to accumulation of Ca^{++} ions in the nerve ending on account of addition of Ca^{++} ions entering the ending during the action potential to calcium remaining after the previous stimulation [2, 6], in series of two stimuli the following equation ought to hold good:

$$p(\varepsilon_2 = 1/\varepsilon_1 = 0) = p(\varepsilon_2 = 1/\varepsilon_1 = 1, \tag{2}$$

where $P(\varepsilon_2 = 1/\varepsilon_1 = 0)$ is the probability of quantum appearance to the second stimulus provided that there was no response to the first stimulus, and $p(\varepsilon_2 = 1/\varepsilon_1 = 1)$ is the probability of appearance of a response to the second stimulus provided that secretion was present to the first stimulus. An estimate of the conditional probability $p(\varepsilon_2 = 1/\varepsilon_1 = 0)$ is the ratio N(01)/N(0), and an estimate of the conditional probability $p(\varepsilon_2 = 1/\varepsilon_1 = 1)$ is the ratio N(11)/N(1). These ratios will subsequently be described as N(01/0) and N(11/1). In the same way, for series of three stimuli the following equations ought to be satisfied:

$$\begin{aligned} p(\varepsilon_3 = 1/\varepsilon_1 = 0, \quad \varepsilon_2 = 0) = p(\varepsilon_3 = 1/\varepsilon_1 = 0, \\ \varepsilon_2 = 1) = p(\varepsilon_3 = 1/\varepsilon_1 = 1, \quad \varepsilon_2 = 1) \end{aligned} \tag{3}$$

Estimates for $p(\varepsilon_3 = 1/\varepsilon_1 = 0, \varepsilon_2 = 0)$, $p\varepsilon_3 = 1(\varepsilon_1 = 0, \varepsilon_2 = 1)$, $p(\varepsilon_3 = 1)\varepsilon_3 = 1)\varepsilon_1 = 1$ are N(001/1), N(011/1), and N(111/1). Conditional probabilities of quantum transmitter secretion during paired stimulation are given

TABLE 2. Conditional Probabilities of Quantum Transmitter Release in Response to Paired Stimulation

Experiment No.	$\bar{p}_1$	$N(01\|0)$	$N(11\|1)$	Experiment No.	$\bar{p}_1$	$N(01\|0)$	$N(11\|1)$
1	0,55	0,65	0,78	15	0,14	0,23	0,20
2	0,54	0,60	0,54	16	0,10	0,13	0
3	0,04	0,06	0	17	0,07	0,12	0
4	0,08	0,11	0,13	18	0,17	0,16	0,23
5	0,38	0,31	0,40	19	0,16	0,16	0,26
6	0,21	0,25	0,24	20	0,21	0,19	0,14
7	0,24	0,28	0,16	21	0,22	0,18	0,09
8	0,22	0,28	0,18	22	0,11	0,11	0,09
9	0,13	0,24	0,08	23	0,32	0,47	0,47
10	0,18	0,20	0,17	24	0,12	0,27	0,17
11	0,24	0,25	0,37	25	0,10	0,14	0,07
12	0,21	0,22	0,30	26	0,30	0,38	0,68
13	0,21	0,23	0,16	27	0,40	0,48	0,6
14	0,21	0,21	0,10				

Legend. $\bar{p}_1$) Probability of quantum transmitter release to first stimulus; No. (01/0) probability of quantum release to second stimulus, under the condition that secretion is absent to the first stimulus; No. (11/1) probability of secretion to second stimulus provided that response to first stimulus was present.

in Table 2. In 17 of the 27 experiments values of N(01/0) were found to be higher than those of N(11/1), in 9 they were lower, and in one experiment there was no difference. Since the probabilities of the corresponding events differed in different experiments, to test the null hypothesis (equation 2) Wilcoxon's sign test was used. The principle of this test is that if the null hypothesis is valid the number of experiments in which the inequalities $N(01/0) > N(11/1)$ and $N(01/0) < N(11/1)$ are satisfied ought to be about equal. We found from special tables that, at a 0.05 level of significance, the null hypothesis must be rejected. Similar results also were obtained when conditional probabilities were studied in series consisting of three stimuli. In this case values of N(001/00) were greater than the values of N(111/11) in 6 experiments, and smaller in only two experiments. Analysis also showed that values of conditional probabilties N(111/11) during stimulation by three pulses were higher in 7 of 8 experiments than the mean probability of quantum transmitter release to the first stimulus ($\bar{p}_1$).

The results indicate that during repetitive stimulation of the motor nerve the mean probability ($\bar{p}$) of quantum transmitter release is increased (inequality 1 is satisfied). This increase took place at values of the binomial parameter $\bar{n}$ close to 1 (Table 1). Since, in the modern view, the parameter $\bar{n}$ reflects the number of functioning transmitter release points [1, 7, 8], it can be tentatively suggested that in these experiments activity of only one release point was recorded. The increase in $\bar{p}$ was due to an increase in the Ca^{++} ion concentration in the nerve ending at the quantum transmitter release point.

However, calculation of conditional probabilities with paired and repetitive stimulation showed a decrease in the probability of release in the case when the previous stimulus evoked transmitter secretion (equations 2 and 3 not satisfied). This fact is evidence of a fall in the intracellular Ca^{++} ion concentration at the moment of quantum transmitter secretion or immediately after secretion. Two hypotheses can be put forward to explain the results: 1) at the time of secretion of a portion of transmitter, a certain number of Ca^{++} ions are discharged from the axoplasm of the nerve ending; 2) quantum transmitter release and emptying of a vesicle leads to the entry of Ca^{++} ions into the synaptic vesicle. In those cases the effective Ca^{++} ion concentration falls at the release point, and this leads to a decrease in the probability of quantum transmitter release to subsequent stimulation.

We consider that a decrease in the probability of release, associated with secretion of a quantum of transmitter in response to a preceding stimulus, can be detected only by recording from a single release point. This view is confirmed by experiments Nos. 1, 5, 23, 26, and 27 (Table 2), in which the values of N(01/0) were smaller than those of N(11/1), i.e., secretion of a quantum to the first stimulus did not inhibit release

to the second stimulus. Values of the parameter $\overline{m}$ in these experiments were found to be considerably greater than 1. Consequently, activity of several release points was recorded.

At the same time, it was found that the inequalities $N(11/1) > \overline{p}_1$ and $N(111/11) > \overline{p}_1$ were satisfied, where $\overline{p}_1$ stands for the probability of quantum transmitter release to the first stimulus. This is evidence that the number of Ca^{++} ions leaving the cytoplasm of the nerve ending in response to secretion of a portion to acetylcholine is smaller than the quantity of calcium entering the terminal during depolarization of the presynaptic membrane. Hence it follows that, despite the continuous loss of calcium as a result of transmitter secretion, during repetitive stimulation the intracellular calcium concentration will rise.

The presence of two mechanisms reducing the calcium concentration in a nerve ending can thus be postulated. The first mechanism is independent of transmitter secretion and is due to the functioning of intracellular structures which utilize Ca^{++} ions from the axoplasm of the nerve ending (mitochondria, endoplasmic reticulum, etc.) [3]. The second mechanism is directly connected with quantum transmitter secretion or with processes lying at its basis.

LITERATURE CITED

1. A. L. Zefirov and E. L. Stolov, Neirofiziologiya, 14, 326 (1982).
2. M. A. Kamenskaya, Usp. Fiziol. Nauk, 3, 23 (1972).
3. M. P. Blaustein, R. W. Ratzlaff, and N. K. Kendrick, Ann. N. Y. Acad. Sci., 307, 195 (1978).
4. E. W. Johnson and A. Wernig, J. Physiol. (London), 218, 757 (1971).
5. B. Katz and R. Miledi, J. Physiol. (London), 189, 535 (1967).
6. A. Mallart and A. R. Martin, J. Physiol. (London), 193, 679 (1967).
7. A. Wernig, J. Physiol. (London), 244, 207 (1975).
8. R. S. Zucker, J. Physiol. (London), 229, 787 (1973).

INACTIVATION OF THE FAST SODIUM CURRENT ACROSS THE MEMBRANE OF SINGLE HEART CELLS

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Many investigations have been devoted to the study of inactivation of sodium channels in electrically excitable membranes. Recent investigations [2, 3, 7, 9] have revealed deviations in the development of inactivation from the monoexponential course predicted by the Hodgkin–Huxley model [6]. Contradictory information on this subject has been obtained by experiments on single heart cells [2, 4].

In the present investigation inactivation of the sodium current was studied in the membrane of single heart cells by the microrecording technique described in [1]. The preliminary results were described previously.

EXPERIMENTAL METHOD

Cells were isolated by the method in [8]. The experimental procedure was described previously. A cell selected under the microscope was transferred to a working chamber containing solution of the following composition (in mM): NaCl – 130, KCl – 5.4, $MgSO_4$ – 1.2, $CaCl_2$ – 0.9, glucose – 11, MOPS buffer – 20, pH 7.4. The experiments were carried out at room temperature (20-22°C). A V-shaped polyethylene suction cap, with a pore 5-7 μ in diameter, was applied to a small area of cell membrane. The solution in the cap had the composition described above, with the addition of 1 mM $MnCl_2$ and 1 mM 4-aminopyridine to block the calcium and potassium channels respectively.

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