Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5978–5982, September 1985
Neurobiology

Quantal mechanism of long-term synaptic potentiation

(synaptic memory/neuronal plasticity/crustacean neuromuscular synapses/opener muscle)

DOUGLAS A. BAXTER*, GEORGE D. BITTNER†, AND THOMAS H. BROWN*‡

*Department of Cellular Neurophysiology, Division of Neurosciences, Beckman Research Institute of the City of Hope, Duarte, CA 91010; and †Department of Zoology, University of Texas at Austin, Austin, TX 78712

Communicated by Donald Kennedy, March 11, 1985

ABSTRACT **Intracellular recordings were used to demonstrate the occurrence and to analyze the microphysiology of long-term synaptic potentiation (LTP) in the crayfish opener neuromuscular synapse. Brief stimulation of the single excitor motor axon enhanced the amplitudes of subsequent postsynaptic potentials for several hours. Three methods of quantal analysis were used to evaluate the mechanism responsible for LTP. The results of all three methods supported predictions of the hypothesis that LTP results from a presynaptic mechanism that increases the average of neurotransmitter quanta evoked by nerve impulses in the excitor axon.**

Long-term potentiation (LTP) is a use-dependent form of enhanced synaptic efficacy that can persist for hours and can be induced by activation of the synapses for only a few seconds or less (1–3). This great asymmetry between the duration of the synaptic activity and the duration of the subsequent synaptic change is the defining characteristic of LTP—a property that makes this phenomenon an interesting possible mechanism for long-term control of information transmission. Originally thought to be unique to the hippocampal formation, LTP is now known to occur in many vertebrate and invertebrate synapses (3–7).

Both pre- and postsynaptic mechanisms have been proposed to be responsible for LTP (2, 3, 7–11). In testing pre- versus postsynaptic hypotheses regarding changes in synaptic efficacy, the technique of quantal analysis has proven historically to be valuable (12–20). Unfortunately, the conventional methods of quantal analysis cannot be universally applied, because the results can be subject to interpretational errors if certain key assumptions or conditions are unsatisfied (refs. 20–23; cf. refs. 24 and 25). For our first quantal analysis of LTP, we therefore decided to select a synaptic preparation that would be most immune to such interpretational errors.

An obvious choice was the crayfish opener-excitor neuromuscular synapse. This classical preparation has well-known advantages for quantal analysis of changes in synaptic efficacy (12–14, 17) and our preliminary results (5) demonstrated that these synapses display LTP. We report here that three conventional methods of quantal analysis all supported predictions of the hypothesis that LTP in these synapses results from a presynaptic mechanism that increases the average number of neurotransmitter quanta evoked by nerve impulses in the axon.

MATERIALS AND METHODS

Experimental Preparation. All experiments were performed on cheliped opener muscles of crayfish (*Procambarus clarkii*). These muscles are innervated by a single excitor and a single inhibitor motor axon. Small animals (body length of 1–2 cm) were selected for the quantal analysis because the input resistance of their muscle fibers is sufficiently large that spontaneous miniature excitatory postsynaptic potential (MEPSP) amplitudes can be accurately measured by using intracellular recording techniques (cf. refs. 26–28). MEPSP amplitudes are known to be difficult or impossible to measure in crustacean muscle fibers, whose input resistance is much less than 200 kΩ (cf. refs. 12, 26–29). Isolated claws were maintained in physiological saline (205 mM NaCl/5.4 mM KCl/14 mM $CaCl_2$/2.6 mM $MgCl_2$, pH 7.4) at room temperature. The dorsal surface of the opener muscle was exposed in the propodite and the nerve bundle containing the single opener-excitor motor axon was isolated and stimulated in the meropodite by using conventional bipolar electrodes. This nerve bundle also contains some efferent innervation to the closer muscle as well as several afferent axons, but in the meropodite it is believed to contain only one efferent to the opener muscle—the excitor axon. Synaptic inhibition was blocked by adding 50 μM picrotoxin to the saline and severing the opener-inhibitor axon.

Electrophysiological Methods. Because of the need to maintain stable measurements for long durations, intracellular recordings seemed preferable to the focal extracellular technique (28) that is more commonly used to study the microphysiology of these synapses. Evoked excitatory postsynaptic potentials (EPSPs) and spontaneous MEPSPs were recorded differentially. The intracellular and extracellular electrode tips, which were placed within <500 μm of each other, were filled with 3 M KCl and had matching resistances of 2–5 MΩ. The filtered noise level was usually ≤20 μV peak-to-peak. Experiments were terminated and data were excluded from analysis if the electrode noise noticeably increased, if the resting potential changed by >5 mV, or if the membrane potential became less negative than −70 mV. Single suprathreshold electrical stimuli were delivered to the nerve bundle containing the opener-excitor motor axon at 0.333 Hz for 20 min to 2 hr before and for 25 min to 9 hr after a brief stimulus train was delivered to the excitor axon. The stimulus trains used in the six most carefully studied claw preparations are given in Table 1. The amplified postsynaptic potentials were recorded on magnetic tape and later digitized (5 kHz) for computer analysis.

Quantal Analysis. The quantum hypothesis (20, 21, 28, 30) holds that neurotransmitter substances are discharged from nerve terminals in the form of integral numbers of multimolecular packets or quanta. The mean size of an evoked EPSP $\bar{V}$ is a function of the average number of quanta released (mean quantal content m) and the mean amplitude of the postsynaptic potential produced by individual quantal releases (mean quantal size $\bar{q}$). Specifically,

$$\bar{V} = m\bar{q}. \quad [1]$$

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Abbreviations: LTP, long-term potentiation; EPSP, excitatory postsynaptic potential; MEPSP, miniature EPSP; m, mean quantal content; $\bar{q}$, mean quantal size; $\bar{V}$, mean EPSP amplitude.

‡To whom reprint requests should be addressed.

The utility of this relationship is based on two considerations. First, because the EPSP amplitudes are <0.4% of the synaptic driving force, nonlinear summation of quantal responses (31–33) is not a problem. Second, the postsynaptic voltage response to injected current is linear over the range of interest (ref. 34; unpublished data). The object of our quantal analysis was to determine whether the increase in $\bar{V}$ that occurs during LTP is due to a change in m or $\bar{q}$. If $\bar{q}$ remains constant and m changes in proportion to $\bar{V}$, then this is traditionally taken as strong evidence for a presynaptic mechanism (12–21).

We used three conventional methods to estimate the values of m and $\bar{q}$ before and after the induction of LTP (20, 21, 28, 30). Based on the *method of failures*, m was estimated from the reciprocal proportion of failures to release any quanta

$$m_0 = \ln(N/n_0), \quad [2]$$

where N is the number of stimulations (100–400 in the present experiments) and n_0 is the number of times that nerve stimulations failed to release quanta. The mean quantal size was then calculated as

$$\bar{q} = \bar{V}/m. \quad [3]$$

By using what has been termed (21) the *direct method*, $\bar{q}$ was estimated from the mean amplitude (sample of 100–300) of the spontaneous MEPSPs, which represent asynchronous quantal releases that occur in the absence of presynaptic nerve impulses, and (from Eq. **1**)

$$m_1 = \bar{V}/\bar{q}. \quad [4]$$

Finally, from the *variance method*

$$m_2 = \frac{1 + (cv)^2}{(CV)^2}, \quad [5]$$

where cv is the coefficient of variation of the single quantal events, estimated from the frequency distribution of MEPSP amplitudes, and CV is the coefficient of variation of the evoked EPSP amplitudes. Then $\bar{q}$ was determined from Eq. **3**. The parameter values reported throughout were not corrected for noise (21) because we found empirically that the correction effect was less than the standard error of the parameter estimates (see below).

The method of failures (Eq. **2**) and the variance method (Eq. **5**) assume that the number of quanta evoked by a series of nerve stimulations fluctuates in a manner that can be well approximated by a Poisson probability function. However, the direct method does not make any assumptions about the release probability function. If the evoked quantal release process is reasonably well approximated by a Poisson law, then the three separate estimates of m should agree, a prediction that we explicitly tested and confirmed. Our low-frequency testing rate (0.333 Hz) and deliberate selection of fibers in which the synapses showed a sizable proportion of quantal release failures are two factors that possibly contributed to the Poisson character of the release process in the present experiments. Our tabulated results and strongest conclusions are restricted to those six neuromuscular preparations (Table 1) that permitted application and direct comparison of the results of all three methods.

Formulae for calculating the standard errors of the three m estimates [SE(m)] are given and discussed elsewhere (35). In applying the three methods of quantal analysis, we selected sample sizes that would produce about a 10% coefficient of variation [SE(m)/m] in the m estimates, which is small relative to the stimulation-produced change.

RESULTS

The Phenomenon of LTP at the Crayfish Neuromuscular Synapse. An example of LTP in 1 of the 15 claw opener preparations whose microphysiology we studied is presented in Fig. 1. In this experiment LTP was induced by stimulating the excitor motor axon at 50 Hz for 15 sec. Twenty minutes and 2 hr after the tetanic stimulation the amplitudes of the averaged EPSP waveforms were more than twice as large as the control (pretetanic) value (Fig. 1*A*). The time course of the posttetanic changes is illustrated in Fig. 1*B*. Four hours after the tetanic stimulation, the averaged EPSP amplitude was still twice as large as the control value.

This example is typical in illustrating what are usually interpreted as two kinetically different posttetanic changes. The first component rapidly and completely decayed within the first 3–8 min, corresponding in time course to what has been termed posttetanic potentiation (PTP) (cf. refs. 18, 36, and 37). The decay of PTP was then followed by a long-term enhancement of synaptic transmission that continued for hours. The latter component satisfied the present operational definition of LTP—the enhancement could be induced by stimulating the axon for seconds and it persisted for hours. In experiments on 5 other claw preparations we were able to maintain sufficiently stable intracellular recordings to observe LTP for 7–9 hr (data not shown).

In the hippocampus and superior cervical sympathetic ganglion a variety of different patterns of stimulus frequency and duration are known to be capable of inducing LTP (3, 7). Similar stimulus patterns were effective in the crayfish opener-excitor neuromuscular synapse (see Table 1 for examples of stimulus patterns that were used). In the 15 claw preparations that we examined, the magnitude of LTP (the percent increase above the control value) ranged from 55% to 280%, with a mean value of 136% (a 2.4-fold increase). Unless otherwise indicated, these and all other measurements (Table 1) reported here were made beginning 20 min posttetanus, so that the results could be directly compared with intracellular recordings of LTP in hippocampus (cf. refs. 25, 37, and 38), where long-term stability poses a greater problem.

Quantal Analysis of LTP. What follows (Figs. 2 and 3) illustrates the application of all three conventional methods of quantal analysis before and during LTP (starting 20 min

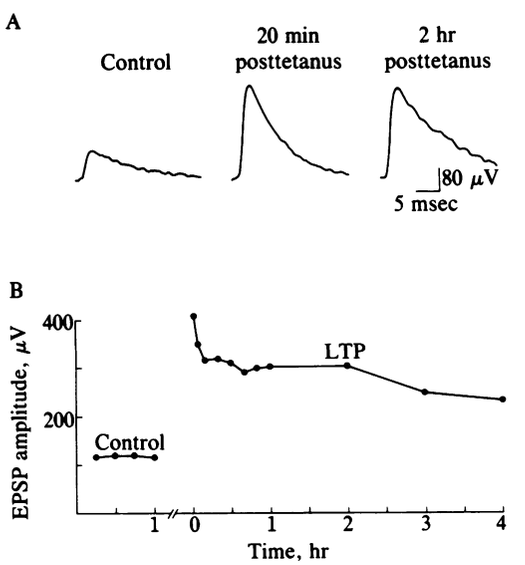

FIG. 1. LTP in the crayfish opener nerve-muscle preparation. (*A*) Averages of 32 successive EPSP waveforms obtained at the indicated portions of the experiment. (*B*) Plot of data from the same cell. Each datum point represents the average of 32 EPSP waveforms.

posttetanus). Repetitive stimulation of the opener-excitor axon on 0.333 Hz sometimes fails on randomly distributed trials to evoke the release of any quanta of neurotransmitter (Fig. 2*A*, control traces). When this occurred, the method of failures (Eqs. **2** and **3**) was used to estimate the values of m and $\bar{q}$. In this (Figs. 2 and 3) and every other claw preparation in which failures could be detected confidently, we found that LTP is accompanied by a decrease in the proportion of transmission failures. In the present example (Fig. 2), we obtained parameter values of $\bar{V}$ = 262 μV, m = 2.53, and $\bar{q}$ = 104 μV during the control period. The axon was then stimulated at 40 Hz for 30 sec. During LTP the parameter values changed to $\bar{V}$ = 432 μV, m = 4.20, and $\bar{q}$ = 103 μV. The change in $\bar{V}$ was accompanied by a corresponding change in m (Eq. **1**) and there was no detectable change in $\bar{q}$ (standard errors of the estimates are discussed below).

By using the direct method, $\bar{q}$ was determined from the amplitude of the spontaneous MEPSPs and m was obtained from Eq. **4**. Examples of spontaneous MEPSPs, before and during LTP, are illustrated in Fig. 2*B*. The frequency distributions of the MEPSP amplitudes, before and during LTP, are shown in Fig. 3. In this fiber the frequency–amplitude distributions were bimodal. Whenever this occurred, we excluded from the reported $\bar{q}$ estimate the minor mode at the right, which probably represents the nearly synchronous release of two quanta (39, 40). In this and every other claw preparation in which MEPSP amplitudes were accurately measured, we found that the mean MEPSP amplitude was unchanged during LTP—a result that held whether we examined only the major mode or the entire frequency distribution. By using the direct method, in the present example m = 2.73 and $\bar{q}$ = 96 μV during the control period, whereas m = 4.15 and $\bar{q}$ = 104 μV during LTP. These values agree well with those obtained from the method of failures and indicate again that of the two quantal parameters only m increases during LTP.

The method of variance was applied to fluctuations in the amplitudes of the evoked EPSPs (see Fig. 2*A*). The coefficient of variation of these fluctuations provided the third estimate of m (Eq. **5**), and $\bar{q}$ was then determined from Eq. **3**. In this and every other claw preparation to which we have applied the method of variance, the coefficient of variation of EPSP amplitudes decreased during LTP. In the present example, the parameter values obtained by using this method were m = 2.71 and $\bar{q}$ = 97 μV during the control period,

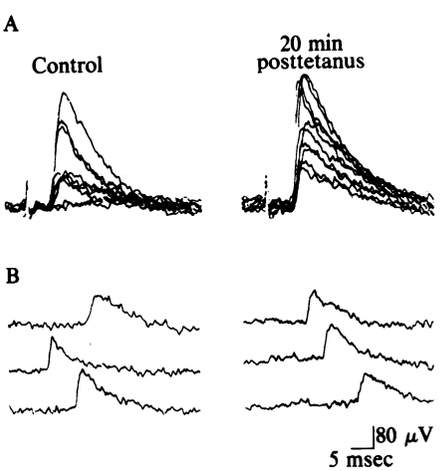

FIG. 2. Intracellular recordings of evoked EPSPs and spontaneous MEPSPs before and during LTP. (*A*) Superimposed records of evoked EPSPs showing amplitude fluctuations and the occasional occurrence of transmission failures. (*B*) Examples of spontaneous MEPSPs before and during LTP.

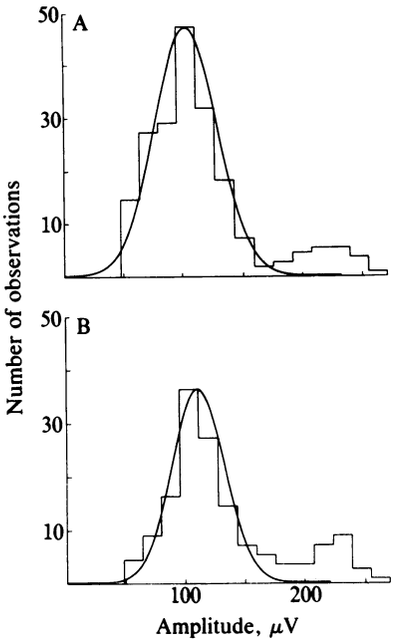

FIG. 3. Frequency distribution of MEPSP amplitudes before (*A*; control) and during (*B*; 20 min posttetanus) LTP. Data are from the cell shown in Fig. 2. Smooth curves are Gaussian density functions with the same mean and variance as the major mode of the MEPSPs.

whereas m = 4.50 and $\bar{q}$ = 96 μV during LTP. Results from this claw preparation illustrate a general result (see below)—that all three methods of quantal analysis find the induction of LTP to increase m but not $\bar{q}$.

In 6 of the 15 claw preparations that we studied it was possible to apply all three methods of quantal analysis (Table 1), whereas in 14 of the 15 claw preparations we were able to apply at least two of the three methods. The close agreement among the three m estimates is illustrated in Fig. 4, where one estimate of quantal content obtained during the control period (open symbols) and during LTP (solid symbols) is plotted against another. The data points are near the expected slope of unity (Fig. 4, broken lines). The actual slopes of the three possible regressions, obtained by the least-squares method, ranged from 0.95 to 1.02. The coefficient of determination (r^2) for all three combinations of pairs of m estimates exceeded 0.95, illustrating the excellent agreement among the three estimates (see also Table 1). The estimates of the quantal parameters were also in close agreement in those 8 preparations in which only two of the three methods were applied (data not shown). This concordance among the three methods adds confidence to the inferred quantal parameter changes (see *Materials and Methods*).

The estimated (35) standard errors of m, expressed as a coefficient of variation (see *Materials and Methods*), averaged 9% for the failures method, 11% for the direct method, and 13% for the variance method. We would therefore be unable to detect 10% changes in m and $\bar{q}$, but the resolution was more than adequate for evaluating changes of the magnitude observed during LTP. The three m estimates obtained from the same data (Table 1) differed on the average by <10%.

In all 15 neuromuscular preparations, the increase in $\bar{V}$ during LTP was accompanied by a proportional increase in m. There was never a detectable increase in $\bar{q}$. This was true whether the quantal parameter measurements were done 20 min posttetanus (as in Table 1) or hours after the tetanus (data not shown). For example, in the case presented in Fig. 1, $\bar{q}$ = 105 μV during the control period and at 1, 2, 3, and 4 hr posttetanus $\bar{q}$ = 100 μV, 102 μV, 102 μV, and 100 μV,

Table 1. Quantal analysis of LTP

$\bar{V}$, μV				Control parameter		Posttetanic parameter	
Stimulus paradigm	Control value	Posttetanic value	Quantal method	m	$\bar{q}$, μV	m	$\bar{q}$, μV
1 sec, 100 Hz	63	117	Failures	0.20	315	0.37	316
			Direct	0.22	292	0.38	304
			Variance	0.22	286	0.42	279
15 sec, 50 Hz	116	320	Failures	1.10	105	3.30	97
			Direct	0.98	118	3.30	97
			Variance	1.16	100	3.05	105
30 sec, 40 Hz	262	432	Failures	2.53	104	4.20	103
			Direct	2.73	96	4.15	104
			Variance	2.71	97	4.50	96
30 sec, 40 Hz	196	383	Failures	1.83	107	3.91	98
			Direct	1.58	124	3.19	120
			Variance	1.93	102	4.14	93
15 sec, 30 Hz	186	353	Failures	1.47	126	2.80	126
			Direct	1.46	127	2.65	133
			Variance	1.51	123	2.82	125
20 sec, 15 Hz	80	137	Failures	0.39	205	0.67	204
			Direct	0.42	188	0.67	204
			Variance	0.40	200	0.68	201

These results are from the six claw preparations in which all three methods of quantal analysis were applied. Values of $\bar{V}$ and the quantal parameters m and $\bar{q}$ were obtained during the control period and again during LTP beginning 20 min posttetanus.

respectively (failures method). In contrast to the constant value of $\bar{q}$ throughout the experiment, there was a posttetanic increase in the value of m. During the control period m = 1.10, whereas at 1, 2, 3, and 4 hr posttetanus, m = 3.04, 2.97, 2.42, and 2.31, respectively (failures method).

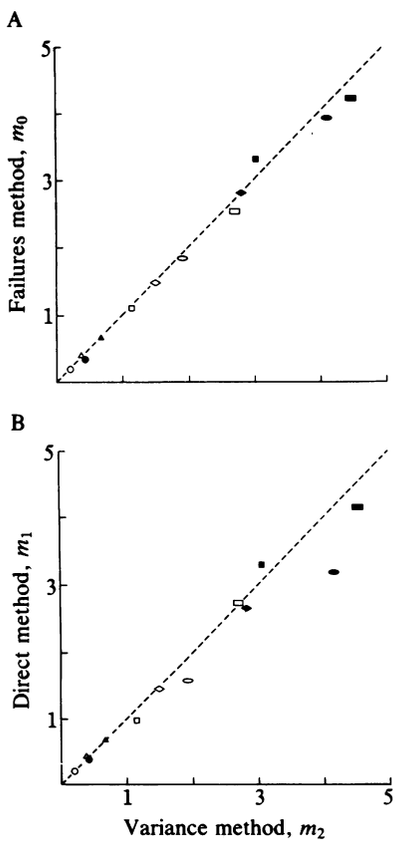

FIG. 4. Comparison of m before (open symbols) and during (solid symbols) LTP, calculated by using the three different methods of quantal analysis. Results from the method of variance (abscissa) are plotted against those of the failures (*A*) and direct (*B*) methods (ordinate). Different symbol shapes distinguish data from different claw preparations. The broken lines have unity slope.

DISCUSSION

The present study demonstrates that LTP occurs at the crayfish opener-excitor neuromuscular synapse. Using intracellular recordings, we were able to observe LTP for durations as long as 9 hr following seconds of repetitive stimulation. This extreme asymmetry between the duration of the posttetanic synaptic enhancement and the duration of the synaptic activity required to produce the change is what distinguishes LTP from certain other persistent posttetanic increases in synaptic efficacy such as have long been known to occur in the spinal cord. In the latter case, posttetanic increases in synaptic efficacy have occasionally been reported to last for >1 hr—but only if the synapses are tetanically stimulated for almost the same amount of time (refs. 41–43; cf. refs. 44 and 45).

As part of our effort to understand the mechanisms responsible for LTP, we applied three traditional methods of quantal analysis. All three methods yielded quantal parameter estimates that were in excellent agreement with one another and all three methods indicated that the induction of LTP had no detectable effect on the value of $\bar{q}$, while the value of m increased to the same extent that the EPSP amplitudes increased (see Fig. 4, Table 1, and accompanying text). This finding supports predictions of the hypothesis (cf. refs. 1 and 2) that the enhanced synaptic efficacy results from a presynaptic mechanism that increases neurotransmitter release (see *Materials and Methods*).

Results of neurochemical studies performed on two other systems are also consistent with a presynaptic change. *In vivo* studies of the hippocampal formation report that LTP is accompanied by a prolonged increase in the evoked release of radiolabeled glutamate, a putative neurotransmitter substance in these synapses (8, 9). Similarly, *in vitro* studies of the superior cervical sympathetic ganglion have shown that LTP is accompanied by an increase in the amount of evoked release of acetylcholine, which is known to be the neurotransmitter conveying the enhanced synaptic efficacy (4, 7, 46). The preceding evidence does not, however, rule out the possibility that processes initiated on the postsynaptic side of

the cleft may contribute to some aspect of the induction or expression of LTP. This possibility warrants serious investigation, especially in synaptic systems that display associative LTP (37, 47), a phenomenon that could conceivably involve different or supplementary control mechanisms.

We thank Harold Atwood, Larry Keenan, and Robert Zucker for useful comments on the manuscript. This research was made possible by National Institutes of Health Grants NS18861 and NS21561, McKnight Foundation Scholar's and Development Awards, and Air Force Office of Scientific Research Contract 2312/A1.

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