Mechanism of Long-Term Potentiation of Transmitter Release Induced by Adrenaline in Bullfrog Sympathetic Ganglia

Eiichi Kumamoto and Kenji Kuba
From the Department of Physiology, Saga Medical School, Nabeshima, Saga 840-01, Japan

ABSTRACT A mechanism of the long-term potentiation of transmitter release induced by adrenaline (ALTP) was studied by recording intracellularly the fast excitatory postsynaptic potentials (fast EPSPs). The ALTP was produced during the blockade of K⁺ channels at the presynaptic terminals by tetraethylammonium (TEA). The synaptic delay, possibly reflecting a relative change in the duration of an action potential at the presynaptic terminal, was not changed during the course of the ALTP. By contrast, it was significantly lengthened by TEA and other K⁺ channel inhibitors (4-aminopyridine and Cs⁺) that markedly enhanced the evoked release of transmitter. The magnitude of facilitation of the fast EPSP, induced by a conditional stimulus to the preganglionic nerve, was decreased during the generation of the ALTP, but was unchanged during the potentiation of transmitter release caused by TEA. These results, together with theoretical considerations applying the residual Ca²⁺ hypothesis to the facilitation, suggest that the enhancement of transmitter release during the ALTP is not caused by an increased Ca²⁺ influx during a presynaptic impulse owing to the blockade of K⁺ channel or the modulation of Ca²⁺ channel, but presumably is induced by a rise in the basal level of free Ca²⁺ in the presynaptic terminal.

INTRODUCTION

Much evidence has accumulated that various transmitters and humoral agents modulate the release of transmitter from the presynaptic terminals at both vertebrate (Jenkinson et al., 1968; Kuba, 1970; Kato and Kuba, 1980; Koketsu and Yamada, 1982; Katayama and Nishi, 1984; Kato et al., 1985) and invertebrate (Klein and Kandel, 1980; Kandel and Schwartz, 1982) synapses. If these presynaptic modulations last for a long time, they provide a basis for an important mechanism of neuronal plasticity. We have recently reported (Kuba et al., 1981; Kumamoto and Kuba, 1983a; Kuba and Kumamoto, 1986) that adrenaline produces a long-lasting potentiation of transmitter (acetylcholine [ACh]) release in bullfrog sympathetic ganglia, the mechanism of which appears to involve a
rise in endogenous cyclic AMP (cAMP) and a subsequently activated metabolic process in the presynaptic terminals. However, it is not known how the activation of a cAMP-dependent process eventually results in the enhancement of transmitter release. This paper deals with the mechanism of the final step of a cascade process underlying the long-term potentiation induced by adrenaline (ALTP).

There are at least five possible mechanisms for the enhancement of impulse-induced transmitter release by adrenaline: (a) an increased Ca\textsuperscript{2+} influx caused by the broadening of an action potential as a result of the blockade of K\textsuperscript{+} conductance increase, (b) an enhancement of the Ca\textsuperscript{2+} conductance increase during a presynaptic spike, (c) an elevation of the basal level of free Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}]
, in the presynaptic terminals, (d) enhanced efficacy of the excitation-secretion coupling, and (e) an increased rate of transmitter synthesis. The results examined in the present study suggest that the ALTP is induced by an increase in basal [Ca\textsuperscript{2+}]. A preliminary account of this work has been presented elsewhere (Kumamoto et al., 1984).

**METHODS**

Isolated ninth or tenth lumbar sympathetic ganglia of bullfrogs (*Rana catesbeiana*: B-type neurons) were studied using a conventional intracellular recording technique (Nishi and Koketsu, 1960). Microelectrodes were filled with 3 M KCl (tip resistance, 30–100 M\textohm). The compositions of normal Ringer and low-Ca\textsuperscript{2+}, high-Mg\textsuperscript{2+} solutions were as described previously (Kumamoto and Kuba, 1983a). The fast excitatory postsynaptic potentials (fast EPSPs; induced by the nicotinic action of ACh; cf. Kuba and Koketsu, 1978) were recorded in either a low-Ca\textsuperscript{2+}, high-Mg\textsuperscript{2+} solution, or normal Ringer, or high-Ca\textsuperscript{2+} solution, the latter two of which contained d-tubocurarine (10–100 \mu M). The toxicity of the high-Ca\textsuperscript{2+} solution was adjusted by reducing NaCl. The quantal content and size of the fast EPSP recorded in a low-Ca\textsuperscript{2+}, high-Mg\textsuperscript{2+} solution were calculated as described previously (del Castillo and Katz, 1954; Kuba et al., 1981).

Synaptic delay was measured as the period between the positive peak of a presynaptic spike (which was recorded by an electrode inserted into the postganglionic neuron simultaneously with a postsynaptic response) and the onset of the fast EPSP (cf. Kumamoto and Kuba, 1985). Measurements were made on data obtained by electronic summation of 20–50 fast EPSPs accompanied by presynaptic spikes using an averager (ATAC-210, Nihon-Kohden, Japan). The facilitation of the fast EPSP was observed by recording paired fast EPSPs every 10 s in a low-Ca\textsuperscript{2+}, high-Mg\textsuperscript{2+} solution (Mallart and Martin, 1967; Magleby, 1973). The interval of paired stimuli ranged from 50 ms to 2 s. Since the amplitude of each fast EPSP fluctuated under this recording condition, at least 25 responses were summed electronically. Even with this procedure, the mean of the facilitations varied occasionally with time. Therefore, the “moving bin” method was employed to obtain the time course of the change in the magnitude of the facilitation (Rahamimoff and Yaari, 1973; cf. Koyano et al., 1985). Three consecutive values of the facilitation, each obtained by summing 25 responses (recorded for a 250-s period), were averaged. This “averaged” magnitude of facilitation represented a value for a 12.5-min period, which constituted a bin size, and was plotted at the midpoint of each bin. All experiments were carried out at room temperature (20–24°C). Data were examined for statistical significance by Student’s t test. The drugs used were adrenaline bitartrate (Sigma Chemical Co., St. Louis, MO), tetraethylammonium (TEA) Cl (Wako Pure Chemical Co., Japan), and d-tubocurarine (Nakarai Chemical Co., Japan).
RESULTS

Theoretical Basis for Testing the Increased Ca\textsuperscript{2+} Influx Hypothesis and the Elevated Basal Ca\textsuperscript{2+} Hypothesis for the Mechanism of ALTP

Characteristics of facilitation. When paired stimuli with an interval of 50–2,000 ms were applied to the presynaptic nerve, the amplitude of the second fast EPSP was enhanced (inset of Fig. 1). This facilitation of the fast EPSP amplitude must result solely from the increased release of quanta, since a difference between the quantal sizes of the first and second fast EPSPs was not seen (not shown) and there was no potentiation of ACh potentials with repetitive ionophoretic appli-
Ca\textsuperscript{2+}, (Ca)\textsubscript{n}X. The free intracellular Ca\textsuperscript{2+} concentration near the release site, [Ca\textsuperscript{2+}]\textsubscript{i}, would be composed of an increase in [Ca\textsuperscript{2+}]\textsubscript{i} caused by a nerve impulse (y\textsubscript{0}) and the resting basal [Ca\textsuperscript{2+}]\textsubscript{i} (c) (cf. Zucker and Lara-Estrella, 1983). Accordingly, if the reaction of X with Ca\textsuperscript{2+} is practically in equilibrium, the quantal content would be expressed by the following equation (cf. Dodge and Rahamimoff, 1967; Hubbard et al., 1968, for similar models with different assumptions):

\[ m = k_{es} \frac{(y_0 + c)^y}{(y_0 + c)^y + K}. \]  

where \( k_{es} \) is a constant representing the overall efficacy of the excitation-secretion coupling and \( K \) is the dissociation constant for (Ca)\textsubscript{n}X. It is assumed that \( y_0 \) during

**Figure 2.** A schematic diagram of the time course of changes in [Ca\textsuperscript{2+}]\textsubscript{i} in the terminal after a nerve impulse. It is assumed that the increased [Ca\textsuperscript{2+}]\textsubscript{i} decays at least in two phases: an initial, rapid decline, which ceases completely within a refractory period of a spike, and a late single-exponential decrease. In this diagram, the time course of the initial phase was assumed to be a simple linear function, although the exact function is not known. \( c \) is the resting basal [Ca\textsuperscript{2+}]\textsubscript{i}, \( y_0 \) is an increase in [Ca\textsuperscript{2+}]\textsubscript{i} at \( t = 0 \) caused by a nerve impulse, and \( y \) is a fraction of the increased [Ca\textsuperscript{2+}]\textsubscript{i} at \( t = 0 \), which decays single-exponentially with a rate constant, \( k_f \). Both axes are on a linear scale in an arbitrary unit. The analysis of the facilitation was made for a period of 50–2,000 ms after the spike.

Each presynaptic impulse is constant (Charlton et al., 1982) and that it declines in at least two phases, an initial, rapid phase, followed by a slow, single-exponential decay with a time constant \( 1/k_f \) (Fig. 2). The fast component would disappear completely within the refractory period of a spike (cf. Zucker and Stockbridge, 1983; Stockbridge and Moore, 1984) and contribute to the phasic release of transmitter. Only the slow exponential component of the decay phase would thus participate in the facilitation under the present experimental conditions (in the time period of 50–2,000 ms after a conditional stimulus), no matter what function
determined the initial rapid phase. Accordingly, the amount of increased $[\text{Ca}^{2+}]$, at the time of a test impulse induced at $t$ after a conditioning impulse would be the sum of $y_0$ and $y_0 \exp(-k_f t)$ ($y$ is a fraction of $y_0$ at $t = 0$, which declines single-exponentially; see Fig. 2). Consequently, the quantal content during the course of the facilitation ($m'$) would be

$$m' = k_{ex} \frac{[y_0][1 + a \cdot \exp(-k_f t)] + c']^n}{[y_0][1 + a \cdot \exp(-k_f t)] + c' + K},$$

where $a$ is equal to $y/y_0$. From Eqs. 1 and 2, the magnitude of facilitation, $F = m'/m - 1$, would be

$$F = \frac{(1 + z/x)^n - 1}{(x^n/K)(1 + z/x)^n + 1},$$

where $x = y_0 + c$ and $z = y_0a \cdot \exp(-k_f t)$. As the rise in $[\text{Ca}^{2+}]$ appears to be small in comparison with $K$ in the low-$\text{Ca}^{2+}$, high-$\text{Mg}^{2+}$ solution, where quantal contents were much smaller than those in normal Ringer (Nishi et al., 1967; see Table I), Eq. 3 is reduced to

$$F = (1 + z/x)^n - 1,$$

which can be rewritten as follows:

$$\ln[(F + 1)^{1/n} - 1] = \ln w - k_f t,$$

where $w = y/(y_0 + c)$. This demonstrates that if this particular formulation of the residual $\text{Ca}^{2+}$ hypothesis is applicable to facilitation, the left-hand side of Eq. 5 must be linear to $t$. This appears to be the case, as shown in Fig. 3, where linear relationships were obtained for time intervals between 50 and 200 ms at various values of $n$. The relationship for intervals of $>200$ ms deviated from linearity (not shown in Fig. 3; see Fig. 1). This could be due to the existence of the other $[\text{Ca}^{2+}]$ removal process, which is slower than that considered above (see Discussion). For the purpose of the present analysis, we focused only on the initial single-exponential component of facilitation, which will hereafter be called simply “facilitation.” The intercepts and slopes of straight lines in Fig. 3 gave values for $w$ and $k_f$, respectively, that are listed for various $n$ values in Table I.

As a special case for Eq. 5, the assumptions of $n = 1$ (although this is not feasible; see Discussion) and $w = 1$ yield $F = \exp(-k_f t)$. This allows a practical comparison of the time constant of facilitation in bullfrog sympathetic ganglion cells with those in other preparations. A mean value of 157 ms so calculated for time intervals of $<200$ ms was $>35$ ms at the frog neuromuscular junction (Mallart and Martin, 1967), but $<200$ ms in guinea pig superior cervical ganglion cells (McLachlan, 1975).

If this particular formulation of the residual $\text{Ca}^{2+}$ hypothesis is correct, then the magnitude of facilitation for each consecutive pair of responses during the course of repetitive stimuli would decrease, as the stimulation proceeds. Fig. 4A shows such an experiment. The ratio of the amplitude of the third EPSP ($E_3$) to the second one ($E_2$), $E_3/E_2$, was usually smaller than that of the second to the first, $E_2/E_1$ (Fig. 4B). Similarly, the ratio of the fourth EPSP amplitude ($E_4$) to
FIGURE 3. Relationships between \( \ln[F + 1]^{1/n} - 1 \) and \( t \) for various values of \( n \), using the experimental data in Fig. 1. Each point, which represents a mean ± SEM, was obtained from the pooled data shown in Fig. 1. The lines were drawn by least-squares regression for each value of \( n \). The early fast component of the facilitation caused by the initial rapid decline of the increased \([\text{Ca}^{2+}]_i\) was omitted from the graph and only the facilitation ascribed to the single-exponential decay of the \([\text{Ca}^{2+}]_i\) is shown simply for the estimation of the values for \( w \) (extrapolation by interrupted lines).

The third one, \( E_{3i}/E_{2i} \), was smaller than that of the third to the second, \( E_{2i}/E_{1i} \) (Fig. 4B and Table II). These results, which approximately fit the values expected from the theory (Table II and Appendix A), suggest that when the basal level of \([\text{Ca}^{2+}]_i\) was increased, the magnitude of facilitation would be reduced. This is further supported by the following theoretical consideration.

Changes in facilitation predicted from an elevated basal \([\text{Ca}^{2+}]_i\), or from an increased \([\text{Ca}^{2+}]_i\) influx during an impulse. If adrenaline increases the basal level of \([\text{Ca}^{2+}]_i\), the magnitude of the potentiation by adrenaline (P), defined as a

| \( n \) | \( w \) | \( k_f \) | \( a \) |
|---|---|---|---|
| 1 | 1.13 | 7.56 | 1.15–1.15* |
| 2 | 0.472 | 6.94 | 0.47–0.54 |
| 3 | 0.295 | 6.74 | 0.30–0.40 |
| 4 | 0.214 | 6.62 | 0.25–0.33 |

Values of \( w \) and \( k_f \) were calculated from the intercept (at \( t = 0 \)) and the slope of the lines in the graph of Fig. 3, respectively, and values of \( a \) were computed according to the method described in the text. The values for \( w \) and \( k_f \) that were calculated from the relationship of pooled data were almost the same as those obtained from five individual cells (unpublished observations). The \( a \) value with the asterisk contradicts the definition that \( y \) is a fraction of \( y_0 \), i.e., the \( a = y/y_0 \) value must be equal to or less than unity. This deviation apparently resulted from the experimental variation of individual data points at \( n = 1 \).
fractional increase in quantal content over the control, would be expressed by the following equation:

\[ P = \frac{(1 + \frac{u}{x})^n - 1}{(x^n/K)(1 + \frac{u}{x})^n + 1}, \]  

which at a low extracellular \( \text{Ca}^{2+} \) concentration reduces to

\[ P = (1 + \frac{u}{x})^n - 1, \]  

where \( u \) is a net increase in the basal \( \text{Ca}^{2+} \) induced by adrenaline. The value

| Table II | Changes in the Magnitude of Facilitation During the Course of Repetitive Stimuli |
|----------|---------------------------------|
| \( n \) | \( E_{2n}/E_1 \) | \( E_{2n}/E_0 \) |
|---|---|---|
| Observed | 1.45±0.07 (SEM) | 1.20±0.17 |
| Calculated | 1 | 1.30 | 1.16 |
| | 2 | 1.38 | 1.22 |
| | 3 | 1.42 | 1.26 |
| | 4 | 1.44 | 1.28 |

Values in parentheses are the number of cells. See text or the legend of Fig. 4 for the notations of \( E_{2n}/E_1 \) and \( E_{2n}/E_0 \). The theoretical values for \( E_{2n}/E_1 \) and \( E_{2n}/E_0 \) at 50-ms intervals were calculated using the values for \( h \) and \( w \) at each value of \( n \) listed in Table I (see Appendix A).
for $P$ at 30 min after wash of adrenaline was 0.62 ($\pm$ 0.10, $n = 26$, $P < 0.001$) in low-$\text{Ca}^{2+}$, high-$\text{Mg}^{2+}$ solutions (see Table III). Using this $P$ value and the values for $k_f$ and $w$ given in Table I, the ratio of the magnitude of facilitation during the ALTP ($F_{\text{adr}}$) to that in the control ($F_c$), $F_{\text{adr}}/F_c$, can be calculated for each value of $n$ from the following equation:

$$\frac{F_{\text{adr}}}{F_c} = \frac{[1 + w(P + 1)^{-1/n} \cdot \exp(-k_f t)]^n - 1}{[1 + w \cdot \exp(-k_f t)]^n - 1},$$

which is obtained from Eqs. 4 and 7. According to this hypothesis, the facilitation is expected to decrease during the ALTP for each value of $n$, as shown in Fig. 5A.

On the other hand, if adrenaline enhances $\text{Ca}^{2+}$ influx during a presynaptic impulse, $P$ at a low extracellular $\text{Ca}^{2+}$ concentration is written as

$$P = \frac{(y_0 + c)^n}{(y_0 + c)^n - 1},$$

where $y_0$ is a rise in $[\text{Ca}^{2+}]$ caused by $\text{Ca}^{2+}$ influx during an impulse after treatment with adrenaline. If adrenaline increases $\text{Ca}^{2+}$ influx by the same quantity for each impulse and does not alter the ratio $a (= y/y_0)$, $F_{\text{adr}}/F_c$ can be expressed by using Eqs. 4 and 9, as follows:

$$\frac{F_{\text{adr}}}{F_c} = \frac{[1 + [a - (a - w)(P + 1)^{-1/n}] \cdot \exp(-k_f t)]^n - 1}{[1 + w \cdot \exp(-k_f t)]^n - 1}. \tag{10}$$

The estimation of this $F_{\text{adr}}/F_c$ requires the value of $a$ in addition to known quantities of $w$, $k_f$, and $P$. Assuming that the same mechanism causes both spontaneous and evoked release of transmitter, their rates can be expressed by a common term, the number of quanta per second, which represents the
probability of release and may accordingly reflect the levels of $[Ca^{2+}]$, for spontaneous and evoked release (cf. Zucker and Lara-Estrella, 1983). The probability of evoked release ($M_e$) in low-$Ca^{2+}$, high-$Mg^{2+}$ solutions (where the quantal content was 1.5; Table III) would be 300–1,500 quanta/s, assuming 1–5 ms for the duration of evoked release (cf. Kuba and Nishi, 1979), while the probability of spontaneous release ($M_s$) would be 0.01–5 quanta/s (Kumamoto and Kuba, 1985; Kuba and Kumamoto, 1986). Placing these values to the equations $M_e = k_e(y_0 + c)^n/K$ [obtained from Eq. 1 at $(y_0 + c)^n \ll K$] and $M_s = k_s c^n/K$, and giving $w$ values (Table I) to the equation $a = w(y_0 + c)/y_0$ (derived from the definition of $w$), the $a$ values for each value of $n$ were estimated (Table I, column 3). The ranges of $a$ thus calculated and the values for $w$, $k_s$, and $P$ yielded the $F_{adv}/F_c$ values according to Eq. 10. As shown in Fig. 5B, the $F_{adv}/F_c$ values, based on the increased $Ca^{2+}$ influx hypothesis, were almost equal to or slightly greater than unity for each value of $n$.

It should be noted that a change in the efficacy for excitation-secretion coupling ($k_e$) or the dissociation constant for $Ca^{2+}$ binding ($K$) would have no effect on facilitation. An increased rate of transmitter synthesis would also probably be expressed solely as an increase in $k_e$.

**Effects of Adrenaline and TEA on Facilitation**

Fig. 6A illustrates the effects of a $K^+$ channel inhibitor, TEA (100 μM), on the facilitation induced by paired stimuli. Although TEA increased the quantal content about threefold (see below), it did not affect facilitation. The lack of effect of another $K^+$ channel inhibitor, 3,4-diaminopyridine, on facilitation was also reported by Molgó and Thesleff (1984). In addition, facilitation was not influenced by increasing the $Ca^{2+}/Mg^{2+}$ ratio from 0.083 to 0.167 in low-$Ca^{2+}$, high-$Mg^{2+}$ solutions (1.02 ± 0.09 of the control $F$, $n = 7$), although the amplitude of the fast EPSP increased by a factor of 1.87 (± 0.25, $P < 0.02$). These results indicate that an increase in $Ca^{2+}$ influx during a presynaptic spike would not affect facilitation.

In contrast to the effects of TEA and the increased $Ca^{2+}/Mg^{2+}$ ratio, facilitation was depressed to 0.65 (± 0.07, $n = 7$, $P < 0.01$, at 30 min after wash) of the control $F$ during the ALTP (Fig. 6B), during which the quantal content increased by 1.62-fold. This decrease in $F$ is consistent with the assumption of an increased basal $[Ca^{2+}]$ during the ALTP (see Fig. 5A). Therefore, it is likely that adrenaline does not increase $Ca^{2+}$ influx during a presynaptic impulse, or the efficacy of excitation-secretion coupling mechanism, or the synthesis of transmitter, but raises the basal level of $[Ca^{2+}]$ in the nerve terminal.

**Dependence of ALTP on Extracellular Ca$^{2+}$ Concentrations**

Table III shows the magnitudes of an ALTP induced in low-$Ca^{2+}$, high-$Mg^{2+}$ solutions, normal Ringer, and high-$Ca^{2+}$ solution. The ALTP was almost unchanged by the variation of the $Ca^{2+}/Mg^{2+}$ ratio from 0.083 to 0.124, which altered the quantal content on average from 0.90 to 1.59, but decreased with a further rise in the ratio (0.167; averaged quantal content, 2.05), and was significantly depressed in normal or high-$Ca^{2+}$ solution. This may be accounted
for by Eq. 7 based on an increased basal $[\text{Ca}^{2+}]_i$ hypothesis, which predicts that an increased $\text{Ca}^{2+}$ influx during a presynaptic impulse reduces the ALTP.

**Evidence Against the $K^+$ Conductance Inactivation Hypothesis for the Mechanism of the ALTP**

As shown in Fig. 7, under the blockade of $K^+$ channels by TEA (100 \mu M), which enhanced the quantal content of the fast EPSP ($318 \pm 34\%$ of the control, $n = \ldots$)

| $\text{Ca}^{2+}$ | $\text{Mg}^{2+}$ | $\text{Ca}^{2+}/\text{Mg}^{2+}$ | $P$ | Control quantal content |
|------------------|------------------|-----------------------------|-----|----------------------|
| mM               | mM               |                             |     |                      |
| 0.60             | 7.2              | 0.083                       | 0.68±0.31 ($n = 6$)* | 0.90±0.10 ($n = 33$) |
| 0.72             | 6.5              | 0.111                       | 0.63±0.15 ($n = 8$)* | 1.65±0.14 ($n = 39$) |
| 0.77             | 6.2              | 0.124                       | 0.73±0.14 ($n = 8$)* | 1.59±0.09 ($n = 79$) |
| 0.90             | 5.4              | 0.167                       | 0.26±0.09 ($n = 4$)* | 2.05±0.18 ($n = 18$) |
| 1.8              | 0                | 0.10±0.17 ($n = 5$)         |     |                      |
| 7.2              | 0                | 0.20±0.07 ($n = 4$)         |     |                      |

$P$ represents the magnitude of the ALTP 30 min after treatment with adrenaline (10 \mu M). Results are shown as means ± SEM. $n$ is the number of cells. Some of the results in low-$\text{Ca}^{2+}$, high-$\text{Mg}^{2+}$ solutions indicated by asterisks were adopted from previously published data (Kumamoto and Kuba, 1985a; Kuba and Kumamoto, 1986). The values of control quantal content in low-$\text{Ca}^{2+}$ solutions were compiled from the present and earlier work (Kuba et al., 1981; Kumamoto and Kuba, 1985a, b, 1985; Kato et al., 1985; Kuba and Kumamoto, 1986).
**FIGURE 7.** Effects of TEA (100 μM) on the ALTP in two different cells. Each point is expressed as a percentage of the mean quantal content during a 15-min period in the presence of TEA, but before application of adrenaline. Horizontal bars indicate a period during which TEA or adrenaline was added to the bathing solution.

**FIGURE 8.** Relationship between a relative change in quantal content (QC) induced by K⁺ channel blockers and that in synaptic delay (SD). Some of the data were adopted from those in an earlier paper (Kumamoto and Kuba, 1985). The quantal content was calculated from data stored on FM tape and the synaptic delay was measured from the electronically averaged records of the same data, in which the fast EPSPs were accompanied by a presynaptic spike (in 18 out of 42 cells). The solid symbols indicate data obtained by applying 25–200 μM TEA (●), 0.6–5 μM 4-AP (▲), and 5–10 mM Cs⁺ (■), respectively. The points bound by a solid line indicate changes observed in single cells by changing TEA concentrations. The insets (upper left) are the presynaptic spike (pre) and the initial part of the fast EPSP (post) averaged from 20 responses in the presence and absence of TEA. The period of synaptic delay is indicated by the horizontal bars. The dashed line drawn through the data points is the least-squares regression line (correlation coefficient, 0.778).
The ALTP could be observed, although it was significantly decreased ($P = 0.32 \pm 0.08$, at 30 min after wash in most cells [$n = 11$, except for one cell, where an unusually large potentiation [$P = 2.14$] was observed] compared with that in the absence of TEA ($P < 0.1$). Such a persistence of the ALTP in the presence of TEA suggests that adrenaline facilitates transmitter release without blocking TEA-sensitive channels, i.e., voltage-sensitive K$^+$ channels at the presynaptic terminal membrane (cf. Klein and Kandel, 1980, for the action of serotonin). The decrease in the ALTP can be explained by an increased Ca$^{2+}$ influx during a presynaptic impulse by TEA (see the preceding section). In fact, the magnitude of the ALTP in the presence of TEA was inversely correlated with that of the control quantal content (correlation coefficient, 0.69) in a similar way, as shown in Table III.

When the K$^+$ channel at the nerve terminal was blocked, the duration of a presynaptic spike was broadened. This resulted not only in an enhanced release of transmitter, but also in the prolongation of synaptic delay. Fig. 8 illustrates a good positive correlation between the increase in the quantal content of the fast EPSP and that in the synaptic delay under the effect of various K$^+$ channel inhibitors (TEA, 4-aminopyridine, and Cs$^+$) in low-Ca$^{2+}$, high-Mg$^{2+}$ solutions. If adrenaline potentiates transmitter release by broadening a presynaptic action potential as a result of blocking the K$^+$ channel, the synaptic delay would be

![Figure 9](image-url)
expected to increase by 20–30% for a twofold increase in release according to the relationship in Fig. 8. However, adrenaline had no effect on synaptic delay irrespective of the twofold potentiation of quantal content (Fig. 9). The mean synaptic delay during the ALTP was 99.5 ± 0.8% (n = 4) of the control. Consequently, it can be concluded that the ALTP does not result from the blockade of the K⁺ channel at the presynaptic terminals.

**DISCUSSION**

*K⁺ Conductance Inactivation Mechanism*

It has been reported that serotonin (or some other, unknown transmitter) elicits a long-lasting potentiation of transmitter release by a cAMP-dependent mechanism in certain molluscan neurons (Brunelli et al., 1976; Shimahara and Tauc, 1978; Abrams et al., 1984). Kandel and his co-workers (Siegelbaum et al., 1982; Shuster et al., 1985; cf. Kandel and Schwartz, 1982) analyzed the mechanism of this heterosynaptic potentiation in *Aplysia* neurons in detail and suggested that the blockade of the transmitter-sensitive K⁺ channel through the phosphorylation of either the channel itself or a membrane-associated protein by a cAMP-dependent protein kinase results in an increased Ca²⁺ influx during a presynaptic impulse and causes the potentiation. However, the observations in the present study are apparently inconsistent with this “K⁺ conductance inactivation mechanism” as an explanation for the enhanced release of transmitter during the ALTP. First, the facilitation of the fast EPSP was not affected by TEA, which is thought to block voltage-dependent K⁺ channels and raise Ca²⁺ influx, but is significantly depressed during the ALTP. Second, the magnitude of the ALTP was smaller at relatively high rates of evoked release in normal Ringer or in the presence of TEA than it was at low rates of release in low-Ca²⁺ solutions, whereas the treatment with K⁺ channel blockers (TEA or 4-aminopyridine) caused a significant potentiation of transmitter release even at a high level of release (in normal Ringer: Heuser et al., 1979; Akasu and Yamada, 1980; Maeno, 1980; Enomoto and Maeno, 1981). Third, synaptic delay was not altered during the ALTP, although it was significantly prolonged by various K⁺ channel inhibitors. Fourth, the ALTP was observed when transmitter liberation was augmented by blocking the K⁺ channel with TEA. The latter two reasons are also inconsistent with the possibility that a prolongation of presynaptic spike duration occurs by a blockade of TEA-insensitive, voltage-dependent K⁺ channels during the ALTP (cf. Kandel and Schwartz, 1982).

*Ca²⁺ Conductance Increase Mechanism*

Adrenaline is known to increase the Ca²⁺ current at the myocardial cell membranes through the modulation of Ca²⁺ channel by the activation of a cAMP-dependent protein kinase (Reuter and Scholz, 1977; Cachelin et al., 1983; cf. Reuter, 1983). It is therefore possible that the enhancement of transmitter release during the ALTP results from an increase in Ca²⁺ conductance during a presynaptic impulse. However, the present observations also rule out this mechanism. Procedures to increase Ca²⁺ influx during a presynaptic spike such as an increase in extracellular Ca²⁺ or the application of TEA had no effect on
facilitation, whereas the magnitude of facilitation was significantly suppressed during the ALTP.

Increased Basal [Ca$^{2+}$], Hypothesis

The depression of facilitation during the ALTP is best explained by an increase in the basal level of Ca$^{2+}$ in the presynaptic terminal, applying the residual Ca$^{2+}$ hypothesis (Katz and Miledi, 1968; Rahamimoff, 1968; Barrett and Stevens, 1972) to the mechanism of facilitation. This conforms to the previous finding that spontaneous release of transmitter was also enhanced during the ALTP (Kuba et al., 1981; Kuba and Kumamoto, 1986). However, this conclusion rests in large part on the validity of the residual Ca$^{2+}$ hypothesis as the mechanism of facilitation.

Is facilitation caused by residual free Ca$^{2+}$? There are several experimental findings that support the residual Ca$^{2+}$ hypothesis for the facilitation. First, the time course of facilitated release by paired stimuli and a progressive reduction in the magnitude of facilitation during repetitive stimulation were well described by this hypothesis (Figs. 3 and 4 and Table II). Second, the lack of a change in the magnitude of facilitation by the procedures that raised Ca$^{2+}$ influx during a presynaptic impulse could be accounted for by this hypothesis (Figs. 5B and 6A). The tail part of the facilitation, however, deviated from the values expected from the present residual Ca$^{2+}$ hypothesis (Fig. 1). It is possible that the deviation results from the simple assumptions of the initial fast and subsequent single-exponential decay of the increased [Ca$^{2+}$] in the terminal (presumably by diffusion of Ca$^{2+}$ away from the internal surface of the membrane) after an impulse, as shown in Fig. 2 (Zucker and Stockbridge, 1983; Stockbridge and Moore, 1984). There must be much slower removal processes of intraterminal Ca$^{2+}$, e.g., active Ca$^{2+}$ extrusion at the plasma membrane or Ca$^{2+}$ uptake by Ca$^{2+}$-storing organelles, such as mitochondria and endoplasmic reticulum. In any case, however, it is quite clear that the deviation of the tail part of the facilitation from the theory does not seriously affect the conclusion, since the present analysis was limited to a period during which these slow processes would be negligible.

The role of cooperative actions of Ca$^{2+}$ in facilitation. If the present residual Ca$^{2+}$ hypothesis formulated for the facilitation of evoked release is correct, it should also hold for a change in spontaneous release after a presynaptic spike. In fact, there was a slight rise in the frequency of miniature EPSPs (MEPSPs) after the generation of a single fast EPSP, which was estimated to be 1.24 (± 0.07, n = 5) of the control (M = 4.1 ± 1.0 quanta/s) for the period of 500 ms, beginning at 100 ms after a spike. The residual Ca$^{2+}$ hypothesis predicts increases in the MEPSP frequency of 11.0–51.2, 2.71–6.45, 1.74–2.56, and 1.48–1.82 for n values of 1, 2, 3, and 4, respectively (see Appendix B). A comparison between experimental and theoretical values for changes in MEPSP frequency after a single fast EPSP would give a restriction to the n value in the present hypothesis, which is >4. This value of n in the bullfrog sympathetic ganglia is consistent with those in other preparations, e.g., 2 or 3 for the squid giant synapse (Charlton et al., 1982; Zucker and Stockbridge, 1983; Smith et al., 1985), 4 for the frog endplate (Dodge and Rahamimoff, 1967; Stockbridge and Moore, 1984), and 5 for the crayfish neuromuscular junction (Zucker and Lara-
Thus, the existence of cooperative action of Ca\(^{2+}\) at the presynaptic terminal is obviously essential for the residual Ca\(^{2+}\) hypothesis to be valid for the facilitation of both spontaneous and evoked release. The precise value of \(n\) in the bullfrog sympathetic ganglia, however, must be determined in a more explicit way. Nevertheless, it is quite clear that this ambiguity does not affect the conclusion of the increased basal [Ca\(^{2+}\)], for the mechanism of the ALTP.

The origin of Ca\(^{2+}\) for the ALTP. An increase in the basal [Ca\(^{2+}\)], in the terminal could be caused by either a mobilization of Ca\(^{2+}\) from the internal store or an increase in the resting Ca\(^{2+}\) influx in the terminal membrane. Although we cannot decide between these possibilities, there is a finding that may be worthwhile to discuss. In regard to the first possibility, it might be assumed that the amount of Ca\(^{2+}\) to be mobilized in the terminal would be relatively independent of the extracellular Ca\(^{2+}\). If this were the case, raising the extracellular Ca\(^{2+}\) would not change the magnitude of adrenaline-induced rise in basal [Ca\(^{2+}\)], but would increase the Ca\(^{2+}\) influx during a presynaptic impulse. This would decrease the magnitude of the ALTP according to Eq. 7. Although this was indeed observed at a relatively higher value of the Ca\(^{2+}/\text{Mg}\(^{2+}\) ratio (another reason for this will be given later), the magnitude of the ALTP was constant in a low range of the ratio (Table III), which is inconsistent with the first possibility. On the other hand, if the elevation of basal [Ca\(^{2+}\)], is due to a rise in resting Ca\(^{2+}\) influx, raising the extracellular Ca\(^{2+}\) would enhance a rise in the basal [Ca\(^{2+}\)] in proportion to an increase in Ca\(^{2+}\) influx during a presynaptic impulse (\(y_0\)). Under this condition, the magnitude of the ALTP would not be changed considerably, as expected from Eq. 7. This was the case in a certain range of Ca\(^{2+}\) concentration. However, when the extracellular Ca\(^{2+}\) was raised further, the ALTP eventually decreased because of the nonlinear dependence of release mechanism on Ca\(^{2+}\) according to Eq. 6.

Other Possible Mechanisms for the ALTP

There is no direct evidence to support or rule out the possibilities that the synthesis of transmitter in the preganglionic terminals and the efficacy of the excitation-secretion coupling mechanism, presumably including exocytosis of the terminal membrane, were increased during the ALTP. However, the reduction of facilitation during the ALTP and the dependence of the ALTP on the external Ca\(^{2+}\) concentration may not be accounted for by these two mechanisms. Accordingly, it can be concluded that an increase in the basal level of Ca\(^{2+}\) in the terminal is the best explanation for the enhancement of transmitter release during the ALTP.

APPENDIX A

Estimation of Facilitations During a Train of Fast EPSPs from the Facilitation Induced by Paired Stimuli

We assume that \(y_0\), \(\gamma\), and \(k_f\) are the same for every nerve impulse and that the individual \(y_0\) and \(\gamma\) during a train sum linearly with \(c\). At time \(t\) after the arrival of \(j\) impulses with a time interval \((t)\), an increment in [Ca\(^{2+}\)], is expressed as

\[
y(1 - \exp(-jk_f t))\exp(-kt) \quad \frac{1 - \exp(-kt)}{1 - \exp(-jk_f t)}.
\]
Accordingly, the ratio of the amplitude of the \((j + 1)\text{th} \) fast EPSP \((E_{j+1})\) to the \(j\text{th} \) one \([E_{(j-1)\text{th}}]\) is
\[
\frac{E_{j+1}}{E_{(j-1)\text{th}}} = \left[ \frac{1 + (w - 1)H - wH^{j+1}}{1 + (w - 1)H - wH^j} \right]^n,
\]
where \(H = \exp(-kft)\). From this equation, it is obvious that as the number of impulses \((j)\) increases, the amplitude of the fast EPSP becomes constant.

**APPENDIX B**

**Facilitation of Spontaneous Transmitter Release Predicted from the Residual \(Ca^{2+}\) Hypothesis**

The \([Ca^{2+}]\), during facilitation at \(t\) (greater than the refractory period of a presynaptic spike) after a conditioning impulse is larger by \(y \cdot \exp(-kft)\) than the basal level, \(c\), as shown in Fig. 2. Like the evoked release, the frequency of spontaneous transmitter release is assumed to be proportional to the \(n\)th power of \(Ca^{2+}\) concentrations in the nerve terminal. Then, integrating release probability from \(t = t_1\) to \(t_2\) after the conditional stimulus yields the ratio of the number of quanta released spontaneously during a post-stimulus period \((t_2 - t_1)\) to that during an equivalent period before the conditional one:
\[
\frac{N_t}{N_c} = \frac{\int_{t_1}^{t_2} [y \cdot \exp(-kft) + c]^n dt}{c^n(t_2 - t_1)},
\]
which is
\[
L_1 + 1, \\
L_2 + 2L_1 + 1, \\
L_3 + 3L_2 + 3L_1 + 1, \quad \text{and} \\
L_4 + 4L_3 + 6L_2 + 4L_1 + 1, \quad \text{for} \quad n = 1, 2, 3, \text{and} 4, \text{respectively. Here,} \ L_j \text{ is defined as}
\]
\[
\left( \frac{2}{c} \right)^j \frac{\exp(-jkft_2) - \exp(-jkft_1)}{(t_2 - t_1)jk^j}.
\]

The value for \(y/c\) in Eq. B1 can be estimated from the equation \(y/c = w(M_\text{evoked} / M_\text{spon})^{1/n}\), which is obtained from the ratio of the probabilities of evoked \((M_\text{evoked})\) to spontaneous \((M_\text{spon})\) release and from the definition of \(w\). Eq. B1 determines a reasonable value of \(n\) under the restriction of the observed values of \(N_t/N_c\).

This work was supported by Grants-in-Aid for Scientific Research and Special Project Research from the Ministry of Education, Science, and Culture of Japan.

*Original version received 19 June 1985 and accepted version received 21 January 1986.*

**REFERENCES**

Abrams, T. W., V. F. Castellucci, J. S. Camardo, E. R. Kandel, and P. E. Lloyd. 1984. Two endogenous neuropeptides modulate the gill and siphon withdrawal reflex in *Aplysia* by
presynaptic facilitation involving cAMP-dependent closure of a serotonin-sensitive potassium channel. *Proceedings of the National Academy of Sciences.* 81:7956–7960.

Akasu, T., and M. Yamada. 1980. The effect of 4-aminopyridine on the nicotinic transmission of bullfrog sympathetic ganglion cells. *Kurume Medical Journal.* 27:143–148.

Barrett, E. F., and C. F. Stevens. 1972. The kinetics of transmitter release at the frog neuromuscular junction. *Journal of Physiology.* 227:691–708.

Brunelli, M., V. Castellucci, and E. R. Kandel. 1976. Synaptic facilitation and behavioral sensitization in *Aplysia*: possible role of serotonin and cyclic AMP. *Science.* 194:1178–1181.

Cachelin, A. B., J. E. de Peyer, S. Kokubun, and H. Reuter. 1983. Ca$^{2+}$ channel modulation by 8-bromocyclic AMP in cultured heart cells. *Nature.* 304:462–464.

Charlton, M. P., S. J. Smith, and R. S. Zucker. 1982. Role of presynaptic calcium ions and channels in synaptic facilitation and depression at the squid giant synapse. *Journal of Physiology.* 323:173–193.

del Castillo, J., and B. Katz. 1954. Quantal components of the end-plate potential. *Journal of Physiology.* 124:560–578.

Dodge, F. A., Jr., and R. Rahamimoff. 1967. Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *Journal of Physiology.* 193:419–432.

Enomoto, K., and T. Maeno. 1981. Presynaptic effects of 4-aminopyridine and streptomycin on the neuromuscular junction. *European Journal of Pharmacology.* 76:1–8.

Heuser, J. E., T. S. Reese, M. J. Dennis, Y. Jan, L. Jan, and L. Evans. 1979. Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *Journal of Cell Biology.* 81:275–300.

Hubbard, J. I., S. F. Jones, and E. M. Landau. 1968. On the mechanism by which calcium and magnesium affect the spontaneous release of transmitter from mammalian motor nerve terminals. *Journal of Physiology.* 194:355–380.

Jenkinson, D. H., B. A. Stamenović, and B. D. L. Whitaker. 1968. The effect of noradrenaline on the end-plate potential in twitch fibres of the frog. *Journal of Physiology.* 195:743–754.

Kandel, E. R., and J. H. Schwartz. 1982. Molecular biology of learning: modulation of transmitter release. *Science.* 218:433–443.

Katayama, Y., and S. Nishi. 1984. Sites and mechanisms of actions of enkephalin in the feline parasympathetic ganglion. *Journal of Physiology.* 351:111–121.

Kato, E., K. Koketsu, K. Kuba, and E. Kumamoto. 1985. The mechanism of the inhibitory action of adrenaline on transmitter release in bullfrog sympathetic ganglia: independence of cyclic AMP and calcium ions. *British Journal of Pharmacology.* 84:435–443.

Kato, E., and K. Kuba. 1980. Inhibition of transmitter release in bullfrog sympathetic ganglia induced by gamma-aminobutyric acid. *Journal of Physiology.* 298:271–283.

Katz, B. 1969. The Release of Neural Transmitter Substances. Charles C Thomas Co., Springfield, MA. 33 pp.

Katz, B., and R. Miledi. 1968. The role of calcium in neuromuscular facilitation. *Journal of Physiology.* 195:481–492.

Klein, M., and E. R. Kandel. 1980. Mechanism of calcium current modulation underlying presynaptic facilitation and behavioral sensitization in *Aplysia*. *Proceedings of the National Academy of Sciences.* 77:6912–6916.

Koketsu, K., and M. Yamada. 1982. Presynaptic muscarinic receptors inhibiting active acetylcholine release in the bullfrog sympathetic ganglion. *British Journal of Pharmacology.* 77:75–82.

Koyano, K., K. Kuba, and S. Minota. 1985. Long-term potentiation of transmitter release
induced by repetitive presynaptic activities in bull-frog sympathetic ganglia. Journal of Physiology. 359:219–233.

Kuba, K. 1970. Effects of catecholamines on the neuromuscular junction in the rat diaphragm. Journal of Physiology. 211:551–570.

Kuba, K., E. Kato, E. Kumamoto, K. Koketsu, and K. Hirai. 1981. Sustained potentiation of transmitter release by adrenaline and dibutyryl cyclic AMP in sympathetic ganglia. Nature. 291:654–656.

Kuba, K., and K. Koketsu. 1978. Synaptic events in sympathetic ganglia. Progress in Neurobiology. 11:77–169.

Kuba, K., and E. Kumamoto. 1986. Long-term potentiation of transmitter release induced by adrenaline in bull-frog sympathetic ganglia. Journal of Physiology. In press.

Kuba, K., and S. Nishi. 1979. Characteristics of fast excitatory postsynaptic current in bullfrog sympathetic ganglion cells. Effects of membrane potential, temperature and Ca ions. Pflügers Archiv European Journal of Physiology. 378:205–212.

Kumamoto, E., and K. Kuba. 1983a. Independence of presynaptic bimodal actions of adrenaline in sympathetic ganglia. Brain Research. 265:344–347.

Kumamoto, E., and K. Kuba. 1983b. Sustained rise in ACh sensitivity of a sympathetic ganglion cell induced by postsynaptic electrical activities. Nature. 305:145–146.

Kumamoto, E., and K. Kuba. 1985. Effects of K+-channel blockers on transmitter release in bullfrog sympathetic ganglia. Journal of Pharmacology and Experimental Therapeutics. 235:241–247.

Kumamoto, E., K. Kuba, S. Minota, and M. Nohmi. 1984. Mechanism of the potentiation of transmitter release by adrenaline in bullfrog sympathetic ganglia. Journal of the Physiological Society of Japan. 46:366. (Abstr.)

Maeno, T. 1980. Kinetic analysis of a large facilitatory action of 4-aminopyridine on the motor nerve terminal of the neuromuscular junction. Proceedings of the Japan Academy (Series B). 56:241–245.

Magleby, K. L. 1973. The effect of repetitive stimulation on facilitation of transmitter release at the frog neuromuscular junction. Journal of Physiology. 234:327–352.

Mallart, A., and A. R. Martin. 1967. An analysis of facilitation of transmitter release at the neuromuscular junction of the frog. Journal of Physiology. 193:679–694.

McLachlan, E. M. 1975. An analysis of the release of acetylcholine from preganglionic nerve terminals. Journal of Physiology. 245:447–466.

Molgó, J., and S. Thesleff. 1984. Studies on the mode of action of botulinum toxin type A at the frog neuromuscular junction. Brain Research. 297:309–316.

Nishi, S., and K. Koketsu. 1960. Electrical properties and activities of single sympathetic neurons in frogs. Journal of Cellular and Comparative Physiology. 55:15–30.

Nishi, S., H. Soeda, and K. Koketsu. 1967. Release of acetylcholine from sympathetic preganglionic nerve terminals. Journal of Neurophysiology. 30:114–134.

Otsuka, M., M. Endo, and Y. Nonomura. 1962. Presynaptic nature of neuromuscular depression. Japanese Journal of Physiology. 12:573–584.

Rahamimoff, R. 1968. A dual effect of calcium ions on neuromuscular facilitation. Journal of Physiology. 195:471–480.

Rahamimoff, R., and Y. Yaari. 1973. Delayed release of transmitter at the frog neuromuscular junction. Journal of Physiology. 228:241–257.

Reuter, H. 1983. Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature. 301:569–574.
Reuter, H., and H. Scholz. 1977. The regulation of the calcium conductance of cardiac muscle by adrenaline. *Journal of Physiology*. 264:49–62.

Shimahara, T., and L. Tauc. 1978. The role of cyclic AMP in the modulation of synaptic efficacy. *Journal de Physiologie*. 74:515–519.

Shuster, M. J., J. S. Camardo, S. A. Siegelbaum, and E. R. Kandel. 1985. Cyclic AMP-dependent protein kinase closes the serotonin-sensitive K⁺ channels of *Aplysia* sensory neurones in cell-free membrane patches. *Nature*. 313:392–395.

Siegelbaum, S. A., J. S. Camardo, and E. R. Kandel. 1982. Serotonin and cyclic AMP close single K⁺ channels in *Aplysia* sensory neurones. *Nature*. 299:413–417.

Smith, S. J., G. J. Augustine, and M. P. Charlton. 1985. Transmission at voltage-clamped giant synapse of the squid: evidence for cooperativity of presynaptic calcium action. *Proceedings of the National Academy of Sciences*. 82:622–625.

Stockbridge, N., and J. W. Moore. 1984. Dynamics of intracellular calcium and its possible relationship to phasic transmitter release and facilitation at the frog neuromuscular junction. *Journal of Neuroscience*. 4:803–811.

Tashiro, N., J. P. Gallagher, and S. Nishi. 1976. Facilitation and depression of synaptic transmission in amphibian sympathetic ganglia. *Brain Research*. 118:45–62.

Zucker, R. S., and L. O. Lara-Estrella. 1983. Post-tetanic decay of evoked and spontaneous transmitter release and a residual-calcium model of synaptic facilitation at crayfish neuromuscular junctions. *Journal of General Physiology*. 81:355–372.

Zucker, R. S., and N. Stockbridge. 1983. Presynaptic calcium diffusion and the time courses of transmitter release and synaptic facilitation at the squid giant synapse. *Journal of Neuroscience*. 3:1263–1269.