

NEUROMUSCULAR FACILITATION IN CRAYFISH: DEPENDENCE ON POTASSIUM AND STROPHANTHIDIN

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SUMMARY

1. Long-term facilitation (LTF) and short-term facilitation (STF) at excitatory neuromuscular junctions were studied under conditions of varying $[K^+]_o$ and with the addition of strophanthidin.

2. The magnitude of LTF varied inversely with $[K^+]_o$, while STF was unaffected.

3. Strophanthidin concentrations greater than 10^{-5} M greatly increased LTF, but not STF.

4. Both LTF and STF were unaffected by large decreases in excitatory junctional potential amplitude produced by the addition of GABA or Mn^{+2} .

5. The results support the hypothesis that LTF is a consequence of Na^+ accumulation in presynaptic terminals and behaves independently of STF.

INTRODUCTION

The excitatory junctional potential (e.j.p.) recorded intracellularly from crayfish muscle fibres results from the action of quanta of excitatory transmitter released from a number of terminals derived from a single motoneurone and distributed over the surface of the muscle fibre (Dudel & Kuffler, 1961*a*). Repetitive impulse activity in the motor axon leads to a progressive increase in the number of quanta of neurotransmitter released by subsequent impulses (Dudel & Kuffler, 1961*b*; Wernig, 1972, for review see Atwood, 1976). One to two seconds after a single impulse, there occurs a period of enhanced release probability to a second impulse which is termed 'short-term facilitation' (STF). One hypothesis to explain STF in this and other synapses is that it results from an accumulation of 'active Ca^{+2} ' in the nerve terminal (Katz & Miledi, 1968). Kinetic analysis of STF indicates more than one component, each independent of $[Ca^{+2}]_o$ (Linder, 1973; Zucker, 1974*b*). STF does not appear to depend on either a change in the size of the presynaptic impulse (Ortiz, 1972) or on Na^+ entry into the terminal (Zucker, 1974*a*).

With repetitive stimulation, at 5–20 Hz, the amplitude of the e.j.p. increases slowly reaching a maximum in about 20 min. On cessation of stimulation the amplitude

of test e.j.p.s declines toward normal levels in 10–20 min but a small residual facilitation may last several hours (Sherman & Atwood, 1971; Atwood, 1976). This long-term facilitation (LTF) apparently results from an increase in presynaptic transmitter release which is associated with an accumulation of sodium in the nerve terminal (Atwood, Swenarchuk, & Gruenwald, 1975) rather than calcium entry (Swenarchuk & Atwood, 1975).

The primary goal of the present study was to examine quantitatively the relation between STF and LTF measured concomitantly at the same neuromuscular junctions under conditions which presumably result in variable sodium accumulation in the nerve terminals.

METHODS

Cultured crayfish were obtained locally from *Monterey Bay Hydroculture*, Santa Cruz, and remained robust for up to one month in tap water at 15 °C. The temperature in different experiments was 18–21 °C and varied no more than ± 1 °C in any experiment.

Experiments were performed on the abductor of the dactylopodite (opener of the claw) of the first or second walking leg of the crayfish *Pacifasicus* sp. or *Procambarus clarkii*. This muscle is innervated by a single excitatory and inhibitory axon with multiple terminations along the muscle fibres. The dissection, experimental chamber, and stimulating electrodes were similar to those previously described by Dudel & Kuffler (1961*a*). Intracellular potentials were recorded with conventional 5–20 M Ω , 3 M-KCl microelectrodes. A microcalomel electrode (Beckman) served as a highly stable reference electrode. Usually 20–40 e.j.p.s were averaged electronically with a Northern Scientific NS-550 Digital Memory Oscilloscope with 1024 bit memory and 50–200 μ sec/bit temporal resolution. The averaged signals were recorded on a Brush chart recorder. The averaging procedure minimized problems due to fluctuation in the size of successive e.j.p.s.

The normal bathing solution consisted of (mM): NaCl, 195; CaCl₂, 15; KCl, 5; MgCl₂, 3; HEPES buffer, 10 (pH 7.0–7.2). Solutions with varied $[K^+]_o$ were obtained by corresponding increase or decrease in $[Na^+]_o$. Strophanthidin was obtained from Sigma Chemical Co., St Louis; a stock solution (10⁻² M in 95% ETOH) was stored for up to one month at -20 °C. Appropriate dilutions were made immediately before use.

The bathing solution was changed by superfusing the chamber with 30 ml of test solution (bath volume 1.5 ml). The preparation was equilibrated at least 15 min following changes in ionic composition and at least 30 min when strophanthidin was employed.

Determination of STF and LTF

In most experiments, suprathreshold, paired stimuli (20 ms interval) were delivered to the excitatory axon at overall frequencies of 4.8–7.1 Hz. Control e.j.p. amplitudes were measured after 10 s stimulation. Since STF decays with a time-constant of less than 1 s (Linder, 1973, 1974), it increases to a stable plateau in less than 10 s at the

overall frequency employed. STF for the 20 ms interpulse interval was determined from the relation: $STF = v_2 - v_1/v_1$ where v_1 is the mean amplitude of the first e.j.p. and v_2 the amplitude of the second of the pair (Mallart and Martin, 1967). This allowed us to evaluate STF at any time during continuous stimulation. In some experiments the magnitude and time course of STF under various experimental conditions was obtained by varying the interpulse interval from 10–150 ms at an overall frequency of 0.5 Hz.

The growth of LTF was determined from the relation:

$$LTF = \frac{v_1(t) - v_1(10s)}{v_1(10s)},$$

where $v_1(10s)$ is the amplitude of the first of the paired e.j.p.s (see STF above) taken after 10 s of continuous stimulation and $v_1(t)$ is the amplitude of the first of the paired e.j.p.s measured at intervals from 10 s to 10 min during continuous stimulation. The decay of LTF was determined at intervals from 2 to 20 min following the cessation of continuous stimulation, by averaging 40 e.j.p.s following 10 s of preliminary stimulation exactly as with the original control. This procedure allowed us to evaluate STF (as the 20 msec incremental increase in e.j.p. amplitude) at rest or superimposed on LTF during its growth and decay.

In those cases where the second e.j.p. occurred before the complete decay of the first, the baseline for the second e.j.p. was determined by graphically extrapolating the falling phase of the first e.j.p. (Mallart & Martin, 1967).

RESULTS

LTF, STF and $[K^+]_o$

The effects of different $[K^+]_o$'s on the averaged responses to paired stimuli before, during and following prolonged repetitive stimulation are depicted in Fig. 1. The amplitude of the e.j.p.s increases with repeated stimulation in all $[K^+]_o$ and was greatest at 1.0 mM $[K^+]_o$. By contrast, the ratio of the amplitude of the first e.j.p. to the second of the pair remained essentially constant under all conditions.

The calculated values for STF and LTF as a function of $[K^+]_o$ and LTF as a function of time are graphed in Figs. 1A and B. In Fig. 1A, the difference in the sensitivity of LTF and STF to $[K^+]_o$ is clear. From Fig. 1B it can be seen that LTF has not reached a maximum at the end of 10 min for all $[K^+]_o$; the 10 min continuous stimulation period was used for convenience in order to test a variety of reversible treatments on the same cell during the 4–8 h during which preparations remained stable.

It is known (Swenarchuk, 1975) that the amplitude and time-course of LTF depends on the frequency of stimulation and can vary over a wide range; it may require 30 min to develop fully and many hours for complete recovery. Under the conditions of our experiments, LTF was generally not observed with the $[K^+]_o$'s employed when the overall stimulation frequency was < 10 Hz. In some experiments, especially at increased $[K^+]_o$, e.j.p.s actually decreased in amplitude during 10 min of stimulation at 5 Hz.

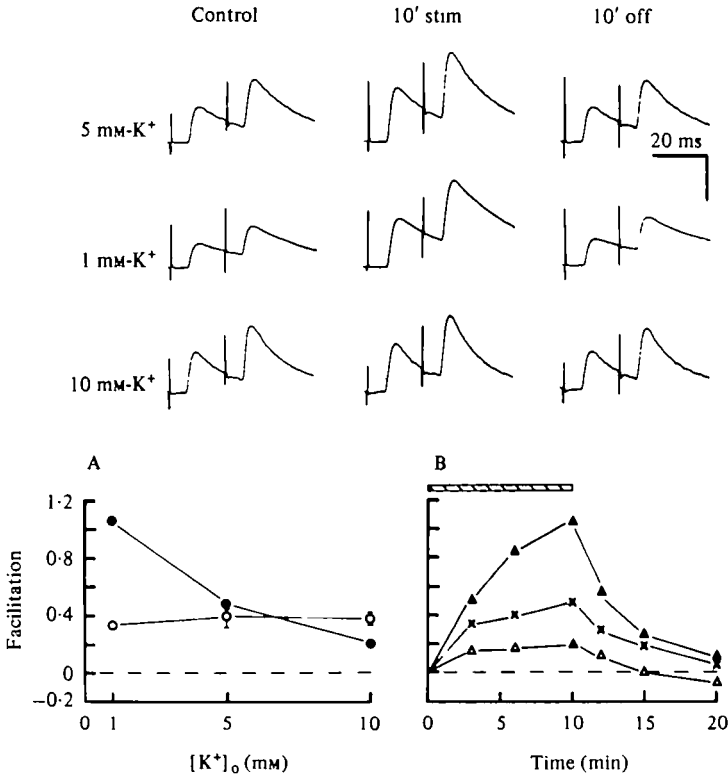


Fig. 1. Effect of varying $[K^+]_o$ on facilitation. Top: Records of averaged pairs of e.j.p.s obtained in normal (5 mM), increased (10 mM), and decreased (1 mM) $[K^+]_o$. Paired stimuli delivered every 140 msec. E.j.p.s averaged at the beginning (control), after 10 min of continuous stimulation (10' stim) and 10 min after cessation of stimulation (10' off). Bottom: A, graph of relation between STF (○) from above records (\pm S.D.; bars are shown when S.D. exceeds size of the symbol) and LTF (●) after 10 min continuous stimulation. B, time-course of development and recovery of LTF in the same cell. 5 mM- $[K^+]_o$ (×), 10 mM- $[K^+]_o$ (△), 1 mM- $[K^+]_o$ (▲). Hatched bar indicates period of stimulation. Membrane potentials: 5 mM- $[K^+]_o$ 67 mV; 10 mM- $[K^+]_o$ 55 mV; 1 mM- $[K^+]_o$ 86 mV. Temperature, 18.5 °C. Vertical bar equals 2 mV for 5 mM and 10 mM- $[K^+]_o$ and 5 mV for 1 mM- $[K^+]_o$.

LTF, STF and Strophanthidin

A dose-effect curve for the action of the cardiac aglycone strophanthidin on STF and LTF is illustrated in Fig. 2. As with decreasing $[K^+]_o$, strophanthidin increases LTF but not STF. In the experiment illustrated, STF appeared to decrease slightly as LTF increased but in other experiments no obvious change in STF was noted. This decrease may have arisen from non-linearities in the relation between membrane potential and e.j.p. amplitude (Taraskevich, 1971) or from a superimposed depression of release due to depletion of transmitter at high release rates (Mallart & Martin, 1968; Birks & Cohen, 1968). With cessation of stimulation the e.j.p. amplitude returned to near control levels within 10 min at strophanthidin concentrations less than 10^{-4} M. At higher concentrations, the e.j.p. amplitude remained elevated or even increased during the recovery period. After 5×10^{-4} M strophanthidin, washing for 100 min with normal Ringer's reduced LTF to 1.1. As the 5×10^{-4} M strophanthidin solution contained

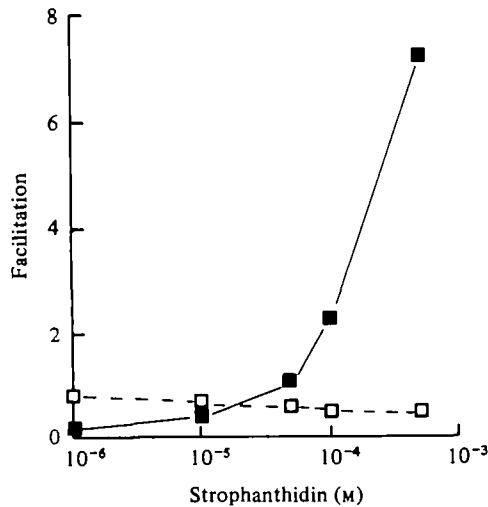


Fig. 2. Effect of strophanthidin on facilitation. LTF (■) and STF (□) at varying concentrations of strophanthidin. Test interval for STF was 20 msec. LTF determined after 10 min with pairs of stimuli delivered every 210 msec. Preparation was bathed in test solutions for 20 min before testing. Control LTF (0.2) was equal to that in 10^{-6} strophanthidin.

5% ethanol (see Methods), a separate control run was made in normal Ringer's solution containing 5% ethanol. This solution decreased the amplitude of the e.j.p. to about one half but had no significant effect on either LTF or STF.

Combined Effects of $[K^+]_o$ and Strophanthidin on LTF

The increase in LTF produced by simultaneously lowering $[K^+]_o$ from 10 mM and adding strophanthidin was greater than expected from simple addition of the effects produced by either alone. This 'super additive' effect on LTF measured after 10 min of stimulation is depicted as a three-dimensional projection in Fig. 3. In addition, treatments which produced greatest LTF also retarded its decay in the recovery period. Indeed, in several cases LTF produced in 5×10^{-5} M strophanthidin and 1.0 mM- $[K^+]_o$ continued to increase even during the post stimulation recovery period. After the preparation was returned to normal (5 mM) $[K^+]_o$ and zero strophanthidin the e.j.p. amplitude returned to near pretreatment levels.

E.J.P. Amplitude and Facilitation

The question arises whether LTF or STF are related to the amount of transmitter released from the presynaptic terminal, the amplitude of the nerve terminal action potential, or the response of the postsynaptic membrane to the transmitter. Therefore, we measured both STF and LTF in the presence of GABA or Mn^{+2} .

GABA and Mn^{+2} both decrease the e.j.p., but by different mechanisms. GABA increases the chloride permeability of both the presynaptic terminal and the postsynaptic membrane (Takeuchi and Takeuchi, 1966), thereby decreasing presynaptic depolarization, transmitter release and postsynaptic efficacy of the excitatory neurotransmitter. The presynaptic effect is quantitatively more important (Dudel, 1965).

Mn^{+2} inhibits the entry of Ca^{+2} into the nerve terminal, thereby reducing transmitter

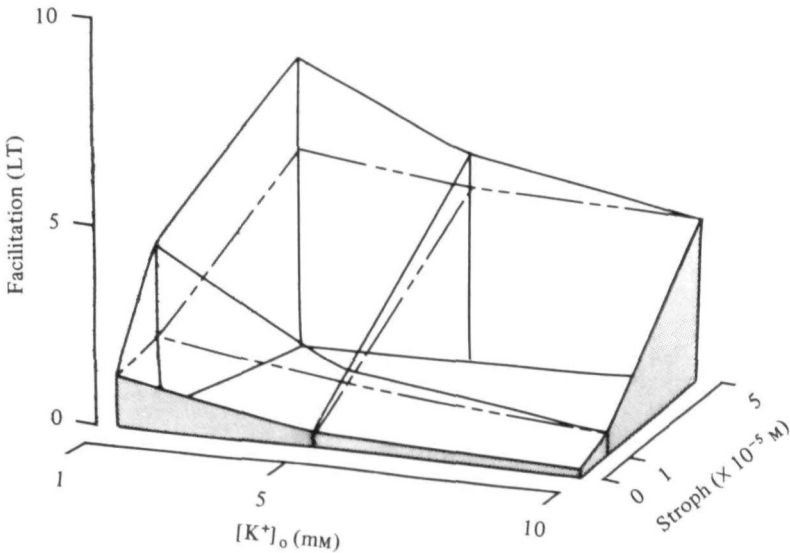


Fig. 3. Interaction of $[K^+]_o$ and strophanthidin on LTF. Computer graphic 3-dimensional projection of LTF at various combinations of $[K^+]_o$ and strophanthidin. Points of intersection of solid lines on surface of upper solid are observed LTF in a single cell in the nine different solutions. Points of intersection of dashed lines on the surface of the embedded solid were calculated assuming the increase in LTF produced by decreasing $[K^+]_o$ from 10 mM at any level of strophanthidin added linearly to LTF produced by increasing strophanthidin at 10 mM- $[K^+]_o$. Note that observed LTF was always greater than that predicted from simple addition of the effects of $[K^+]_o$ or strophanthidin alone.

output (Swerarchuk, 1975). The effects of GABA and Mn^{+2} are shown in Table 1. In the experiment using 2×10^{-4} M GABA the e.j.p. amplitude was decreased 10-fold with little effect on either LTF or STF. Similar results were obtained in the presence of 4 mM Mn^{+2} . The inhibitory effect of both compounds was readily reversed after 5 to 10 min in normal bathing medium. The lack of effect of Mn^{+2} on STF are concordant with the results obtained by Zucker (1974b) showing that STF was unaffected varying $[Ca^{+2}]_o$.

Decay of STF

To test further the apparent independence of STF and LTF as suggested by the results above we measured the time course of decay of STF at various $[K^+]_o$'s with and without the addition of strophanthidin and before, during and after LTF. Representative results are shown in Fig. 4. Comparable decay curves were obtained in solutions, containing 1 mM $[K^+]_o$, 5 mM $[K^+]_o$ plus 5×10^{-5} M strophanthidin and

Table 1. *Effects of GABA and Mn^{+2} on e.j.p. amplitude and facilitation*

(Separate experiments for GABA and Mn^{+2} . Control, and means \pm S.D. of values obtained before and after drug exposure. STF measured before and after LTF.)

	Control	GABA (2×10^{-4} M)	Control	Mn^{+2} (4 mM)
e.j.p. (mV)	2.1 ± 0.2	0.2	2.8 ± 1.1	0.16
LTF (10 min)	0.62 ± 0.11	0.73	0.25 ± 0.04	0.30
STF (20 s)	0.33 ± 0.06	0.40 ± 0.12	0.41 ± 0.12	0.52 ± 0.09

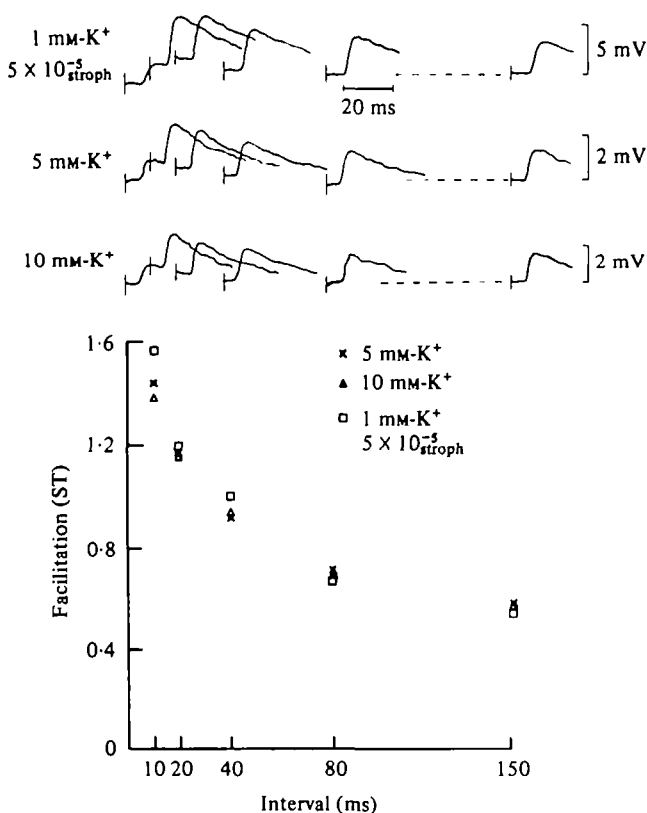


Fig. 4. Timecourse of decay of STF. Top: Superimposed tracings of averaged e.j.p.s (20 trials at 0.5 Hz) at varying intervals in 5 mM-[K⁺]_o, 10 mM-[K⁺]_o and 1 mM-[K⁺]_o + 5 × 10⁻⁵ strophanthidin. Records in 1 mM-[K⁺]_o + 5 × 10⁻⁵ M strophanthidin were obtained while LTF was still present, i.e. 3–10 mins after 10 min of continuous stimulation at 9.6 Hz. Note that the e.j.p. amplitude due to residual LTF is about 2 × control. Bottom: Graph of STF decay under various conditions tested.

1 mM [K⁺]_o plus 5 × 10⁻⁵ M strophanthidin prior to repetitive stimulation. It thus appears that the magnitude and decay of STF are unaffected not only by solutions in which greatly different degrees of LTF can be produced but also whether or not LTF is present.

DISCUSSION

The present results suggest that LTF and STF lead to increased transmitter release by differently affecting excitation-secretion coupling. The hypothesis that LTF results from Na⁺ accumulation in the presynaptic terminal (Sherman & Atwood, 1971; Atwood *et al.* 1975) is supported by our results with changes in [K⁺]_o and the addition of strophanthidin. Reduced [K⁺]_o and strophanthidin are both known to decrease Na⁺ pump activity, accentuating the increase in [Na⁺]_i during repeated stimulation. The super-additive effect of decreasing [K⁺]_o and adding strophanthidin as seen in Fig. 3 is consistent with the observation that cardiac glycoside binding to membrane ATPase increases at low [K⁺]_o (Baker & Willis, 1972). The

increased $[Na^+]_i$ could increase transmitter release by: (1) enhancing Ca^{+2} influx during the nerve impulse (Niedergerke, 1963; Baker, Blaustein, Hodgkin & Steinhardt, 1969), (2) Na^+ induced release of Ca^{+2} from intracellular storage compartments such as mitochondria (Carafoli, 1973); or (3) alteration of the membrane Ca^{+2} conductance channels (Llinas, Steinberg & Walton, 1976). Alterations in the synthesis, storage or availability of transmitter (Vaca & Pilar, 1979) with increased $[Na^+]_i$ may also be involved.

In *Drosophila* nerve muscle junctions, Jan & Jan (1978) have recently described LTF with properties somewhat analogous to those described here. They found that Na^+ accumulation leads to a prolonged Ca^{+2} sensitivity of the presynaptic terminal after a nerve impulse and thus a protracted period of transmitter release. Although some prolongation of the e.j.p. currents occurs in crayfish (C. L. Ortiz & R. K. Orkand, unpublished observation) it is much less marked than that found in *Drosophila*.

By contrast, STF is unaffected by increases in $[Na^+]_i$, changes in $[K^+]_o$, strophanthidin (Zucker, 1974c), GABA, Mn^{+2} or Ca^{+2} (Linder, 1974). It possibly results from increased transient calcium accumulation in the terminal (Zucker, 1974b, c), but other hypotheses have been presented (Atwood, 1976).

These results suggest a possible mechanism for long term modulation of synaptic transmission within the nervous system. The slow fluctuations in $[K^+]_o$ with activity (Orkand, Nicholls & Kuffler, 1968) will modify Na^+ pump activity in nerve terminals thereby altering synaptic efficacy. In addition, some drug effects on the central nervous system may also be mediated via changes in presynaptic Na^+ transport (Woodbury, 1971).

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