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# FREE-CALCIUM AND TENSION RESPONSES IN SINGLE BARNACLE MUSCLE FIBRES FOLLOWING THE APPLICATION OF L-GLUTAMATE

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## Summary

The effect of the putative transmitter, L-glutamate, on free intracellular $Ca^{2+}$, tension and membrane potential in single muscle fibres from the barnacle *Balanus nubilus* has been investigated. External application of L-glutamate (0.1—10 mM) resulted in a transient increase in free intracellular $Ca^{2+}$, monitored by the $Ca^{2+}$-activated protein aequorin. This increase in free intracellular $Ca^{2+}$ was associated with membrane depolarization and force development, and was followed by a period of 'desensitization' in which the preparation was unresponsive to L-glutamate. This could be reversed by removing L-glutamate from the external saline. External application of a number of closely related compounds, including D-glutamate and L-aspartate, were ineffective for initiating the transient light response. The L-glutamate response was virtually abolished in Na-free (Li) medium and completely abolished in Ca-free (Na) medium. The responses to L-glutamate were not reduced in Mg-free medium. The fibre's response to 1 mM L-glutamate was also inhibited by D-600 (10 $\mu$M) or by $La^{3+}$ (1 mM), suggesting that Ca was directly involved in the underlying ionic conductance changes brought about by this putative excitatory transmitter.

## Introduction

Changes in intracellular $Ca^{2+}$ and cyclic nucleotides are thought to be involved in the action of neurotransmitters and a number of hormones [1]. The study of the part played by changes in internal $Ca^{2+}$ in these events has been hampered by the lack of a suitable preparation in which free-$Ca^{2+}$ changes can be measured. By using the calcium-sensitive photo-protein aequorin [2,3], changes in free $Ca^{2+}$ have been demonstrated in single muscle fibres after direct electrical stimulation. The aim of the present study was to investigate,

in barnacle muscle, the effects on internal free $Ca^{2+}$ of the putative excitatory transmitter L-glutamate [4], and other related compounds. The single muscle fibre preparation has the distinct advantage that the initial effect of L-glutamate in depolarizing the cell membrane, as well as its secondary effect upon free calcium changes can be observed and related directly to the final event, the development of isometric tension [5].

## Materials and Methods

Single striated muscle fibres from the barnacle *Balanus nubilus* were isolated and axially injected with the photoprotein aequorin [6]. The fibres were immersed in a physiological saline and placed in front of a sensitive photomultiplier tube in order to record light emission from aequorin [6]. Tension recording was performed by employing a force transducer (RCA 5734). An axial stimulating-recording electrode was used to record changes in potential and to stimulate with constant current pulses when necessary. L-Glutamate and other amino acids were applied to the preparation by replacement of the external saline, while maintaining the temperature between 10 and 12°C. Slight movement artefacts were present with this mode of solution change and produced shifts in the baseline light emission from the muscle fibre. These were noticeable before and after each transient aequorin-light response (see Figs. 1, 2 and 3).

## Results and Discussion

*Effect of L-glutamate on excitation-contraction (E-C) coupling.* The initial experiments were carried out to investigate the effect of L-glutamate on membrane potential, aequorin-light emission and tension, and to determine the dependence on the concentration of L-glutamate of these parameters. Addition of 1 mM L-glutamate to a barnacle muscle fibre (Fig. 1) produced a rapid membrane depolarization (trace 1), a rapid increase in aequorin-light emission (trace 2) indicating an increase in the free calcium concentration within the cell, and an isometric tension response (trace 3). Associated with the membrane depolarization were a number of apparently regenerative membrane responses, which in themselves produced additional small light responses superimposed upon the overall increase in aequorin-light emission. The rapid activation of the processes of E-C coupling brought about by the action of L-glutamate, was followed by a deactivation or 'desensitization' [7,8]. This could only be reversed by removal of L-glutamate from the bathing solution. Stimulation of fibres with an axial, intracellular electrode [6] in the presence of 1 mM external L-glutamate showed that electronic membrane, aequorin-light and tension responses could be observed under conditions where the fibres were insensitive to further application of L-glutamate. This suggested that the desensitization of the L-glutamate response was not the result of general impairment of membrane properties or sarcoplasmic $Ca^{2+}$ release. In order to investigate whether desensitization involves changes in the permeability of a voltage-dependent ion channel, such as that for $Ca^{2+}$, it will be necessary to carry out voltage clamp studies under conditions where $Ca^{2+}$ spike potentials can be generated [9—11].

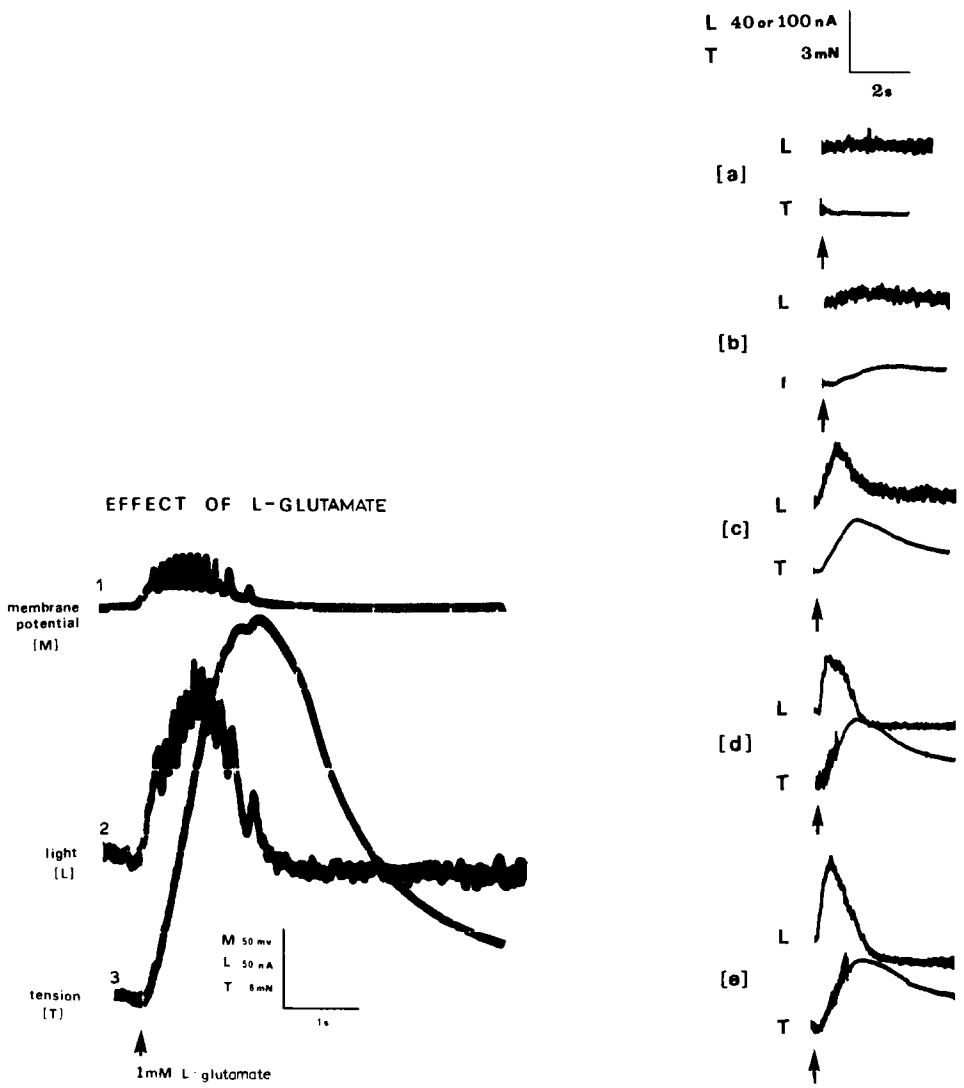


Fig. 1. The effect of 1 mM L-glutamate applied externally at (↑) upon the membrane potential (M), aequorin light response (L) and isometric tension responses (T) of a single barnacle muscle fibre. Temperature, 10—12°C.

Fig. 2. The effect of increasing external concentrations of L-glutamate applied externally at (↑) upon the aequorin-light emission (L) and isometric tension response (T) of a single barnacle muscle fibre. L-glutamate (mM): a, 0; b, 0.1; c, 0.3; d, 1.0; e, 3.0. Temperature, 10—12°C. d, e, 100 nA.

The lowest concentration of L-glutamate causing a detectable increase in aequorin-light emission and tension was 0.1 mM (Fig. 2). The light and tension responses increased as the concentration of L-glutamate was increased, up to a concentration of 10 mM (Fig. 2). In all cases the light and tension responses were also associated with a membrane depolarization. At concentrations of L-glutamate in the range 0.1—10 mM the desensitization could be reversed within 30 s of washing the fibre with fresh medium. However, the

desensitization produced at higher concentrations (50 mM) could only be reversed by incubating the fibres in glutamate-free saline for several minutes. In some fibres, as long as 30 min was required before the preparation would respond again to a standard challlenge of 1 mM L-glutamate. Further investigation of the desensitization after addition of 0.1 mM L-glutamate, a concentration which produced only a very small stimulation in aequorin-light and tension (Fig. 2), revealed that fibres were insensitive to the further addition of 1 mM L-glutamate.

*Effects of other putative transmitters.* The concentration of L-glutamate required to initiate the characteristic membrane potential, calcium transient and tension responses was relatively high, but was nevertheless similar to that required for other preparations in which L-glutamate is effective [4]. It was possible that other agents, similar in structure to L-glutamate, might also be effective but at substantially lower concentrations. External application of other mono- and dicarboxylic amino acids [4], including L-pyroglutamate, applied over a wide range of concentrations were ineffective in initiating E-C coupling responses (Table I). In addition, these amino acids were unable to inhibit the effect of 1 mM L-glutamate at the concentration investigated.

*External ionic requirement for L-glutamate response.* It has been reported that the action of L-glutamate on some invertebrate muscles may require the presence of extracellular $Ca^{2+}$ and $Na^{2+}$ [4]. In order to investigate the requirement for extracellular $Ca^{2+}$, $Mg^{2+}$ and $Na^{+}$ in the effect of L-glutamate on barnacle muscle these ions were removed individually from the medium bathing the muscle fibres. L-Glutamate (1 mM) was then added as soon as possible (within about 30 s) and the aequorin-light and tension responses recorded (Fig. 3). Removal of external $Ca^{2+}$, but not $Mg^{2+}$, abolished the responses of the fibres to 1 mM L-glutamate. There was also no detectable membrane potential response to L-glutamate in the absence of external $Ca^{2+}$. On

TABLE I

AMINO-ACIDS AND AMINES INEFFECTIVE ON E-C COUPLING IN SINGLE MUSCLE FIBRES

| Amino acids | Concentration † |
|---|---|
| L-Glutamate | 10 μM, 30 μM |
| D-Glutamate | 1 mM *, 3 mM * |
| D-L amino adipic | 0.2 mM *, 2 mM * |
| L-Aspartate | 1 mM *, 3 mM, 10 mM, 50 mM * |
| L-Glutamate $NH_2$ | 1 mM * |
| L-Pyroglutamate | 10 μM, 1 mM |
| GABA | 1 mM *, 10 mM * |
| L-Alanine | 3 mM |
| Glycine | 3 mM |
| **Amines** | **Concentration** |
| 5-Hydroxy tryptamine | 10 μM, 0.1 mM, 1 mM |
| Adrenaline | 1 mM |
| D-L Octopamine | 10 μM, 0.1 mM, 1 mM |
| Phenylephrine | 10 μM |
| Isoprenaline | 10 μM, 0.1 mM |

* No inhibitory effect upon 1 mM L-glutamate response.

† All were dissolved in physiological saline, pH 7.2—7.3.

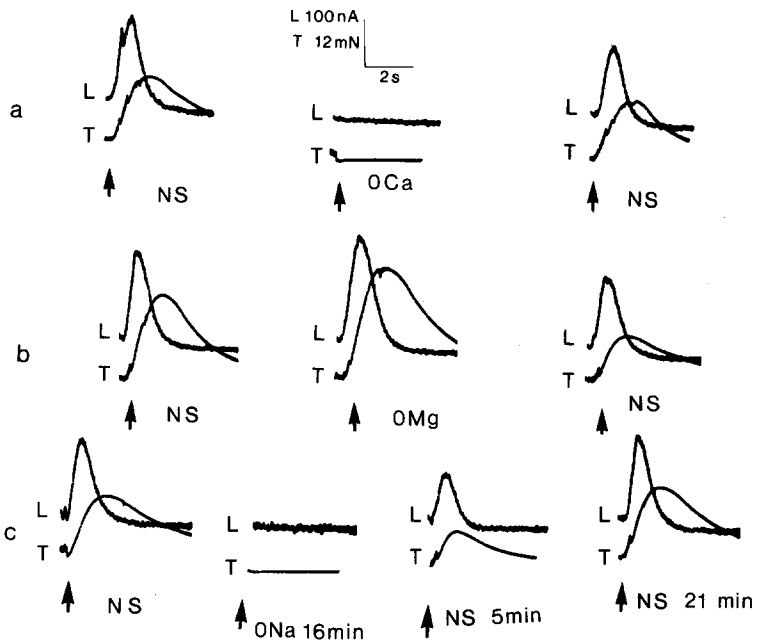


Fig. 3. The effect of replacing external monovalent and divalent cations upon the aequorin light (L) and isometric tension (T) responses produced by the external application of 1 mM L-glutamate (↑). Normal saline (NS) represents the control response in all cases. a, Effect of removing $[Ca]_o$ (replaced by Na); b, effect of removing $[Mg]_o$ (replaced by Na); c, effect of removing $[Na]_o$ (replaced by Li). Control responses performed 5 and 21 min subsequent to Na-free challenge. Temperature, 10—12° C.

replacing the Ca-free medium by normal, $Ca^{2+}$-containing medium, the addition of L-glutamate (1 mM) showed that the aequorin-light and tension responses were restored (Fig. 3). The replacement of normal medium by Na-free (Li) medium markedly reduced the response of the fibre to L-glutamate. However, an incubation of some 15—20 min in the presence of Na-free (Li) medium was required to abolish the effect of 1 mM L-glutamate on the aequorin-light and tension response (Fig. 3). Under these conditions the membrane response to L-glutamate was also abolished. In some fibres, however, even after a 30 min incubation in Na-free (Li) medium, small membrane, aequorin-light and tension responses could be detected on addition of 1 mM L-glutamate. The inhibition by Na-free (Li) medium could be reversed after incubation in normal medium (Fig. 3). In these experiments a concentration of 1 mM L-glutamate was employed as it produced an easily detectable, yet not maximal, response in free intracellular $Ca^{2+}$ and tension (Fig. 2).

An external ionic requirement for transmitter action could involve both effects on transmitter binding to its receptor and effects on ionic current flow through the receptor-mediated ion channel. Furthermore, since both $Ca^{2+}$ and $Mg^{2+}$ bind to L-glutamate [12] the question arose as to what was the active molecular species of L-glutamate which binds to the receptor. The fact that the fibres responded to L-glutamate in Mg-free medium implied that Mg-glutamate was not the active component. In fact, the fibre responses appeared to be potentiated in Mg-free medium. However, in view of the rapid

loss of response to L-glutamate in Ca-free medium, the possibility that Ca-glutamate was the active species could not be ruled out. The mechanism of the inhibition by Na-free medium is unknown. The time dependency of the effect of $Na^+$ may be related to the time required to completely equilibrate the solution in the extracellular cleft system in these fibres [11,13—15] with the external medium.

Under some conditions it was possible to induce 'spike' potentials by electrical stimulation of these muscle fibres. These spike potentials appeared to be due to the 'opening' of voltage-dependent calcium channels in the cell membrane [9,10]. The verapamil derivative D-600 (10—100 $\mu$M) and $La^{3+}$ (1 mM), both of which have been shown to block substantially these voltage-dependent calcium channels in these and other cells [9,16], also inhibited the membrane, aequorin-light and tension responses induced by L-glutamate application. In the barnacle muscle fibres, in order to obtain maximum inhibition by these agents, it was necessary to incubate the fibres with media containing D-600 or $La^{3+}$ for at least 30 min before addition of L-glutamate. This may have been necessary to allow time for diffusion into the extracellular space or 'cleft' systems of these muscle fibres [11,13—15]. Since $Ca^{2+}$ appeared to be directly involved in the ionic conductance change on stimulation of the fibres with L-glutamate it was not possible to ascertain whether free glutamate or Ca-glutamate was the active species of L-glutamate causing stimulation of the muscle fibres. Voltage-clamp experiments may provide some important further information concerning the role of extracellular $Ca^{2+}$ in the effect of L-glutamate on these fibres.

The results presented here strongly suggest that L-glutamate can act as an excitatory transmitter in these striated crustacean muscle fibres. In these experiments the whole fibre was exposed to L-glutamate in order to observe maximum light and tension responses, hence the relative role of junctional and extra-junctional glutamate receptors [17] has not been determined. It has recently been reported that the concentration of cyclic GMP is elevated in the fibres by an increase in free $Ca^{2+}$ following neural stimulation [18]. This preparation, therefore, has a number of important applications. These include the investigation of effects of ions on transmitter action and on the process of transmitter desensitization, as well as in determining the effects of other putative agonists or antagonists on intracellular $Ca^{2+}$ and the relationship of these effects to changes in cyclic nucleotide concentration [18].

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