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Neurotransmitters of mantle and fin muscles in spear squid, *Loligo bleekeri*

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The responses to topical application of neurotransmitters to the mantle and fin muscles of the spear squid, *Loligo bleekeri*, were examined. In the mantle, the circular fibres contract in response to L-glutamate and the radial and longitudinal fibres contract in response to acetylcholine. 5-hydroxytryptamine (5-HT) did not affect contractions of any of the mantle muscle fibres.

The structure of the fin is similar to that of the mantle, with muscles arranged in three orthogonal planes. Topically applied L-glutamate causes all three muscle types to contract. Acetylcholine does not affect them. Pre-treatment with 5-HT blocks the L-glutamate response of the transverse and dorso-ventral muscles but has no effect on the longitudinal fibres. These results suggest that a secondary innervation pathway exists in musculature responsible for producing complex movements, such as the fin, but not in those with a simpler mode of action, like the mantle.

INTRODUCTION

Most squid are fast swimming active predators that require fine control over their muscles for effective prey capture and predator avoidance. The data presented here provide insight into how squid may achieve precise control over their fins. Although the mantle muscles provide powerful propulsion (Ward, 1972; Ward & Wainwright, 1972; Packard & Trueman, 1974), the fins are used for both forward and reverse swimming in both gentle and vigorous modes and for controlling pitch, yaw and roll (Clarke, 1988; Hoar et al., 1994).

Squid mantle muscles (Figure 1), which produce the escape jet response as well as the less vigorous contractions for respiration, are controlled by two different neurotransmitters: L-glutamate (L-glu) and acetylcholine (ACh) (Bone & Howarth, 1980; Bone et al., 1982). Glutamatergic nerves innervate the circular muscles, whilst the radial and longitudinal muscles are innervated by cholinergic nerves. [No previous reports alluded to a possible neuromodulatory mechanism in the mantle muscles.]

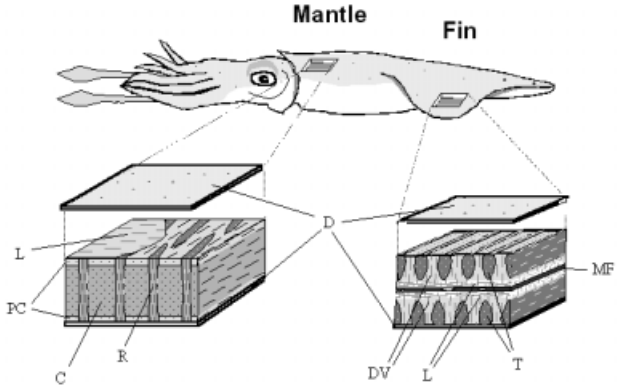

Figure 1. Arrangement of muscles in the mantle and the fin of a loliginid squid: C, central circular muscle; PC, peripheral circular muscle; L, longitudinal muscle; R, radial muscle; DV, dorso-ventral muscle; T, transverse muscle; MF, median fascia connective tissue; D, dermal and epidermal layers.

Although the organization of the fin musculature, acting as a muscular hydrostat, has been well documented by Kier (1988, 1989) the neurotransmitters have not been analysed in detail (Bone & Howarth, 1980). As in the mantle, there is a 3-D arrangement of muscle, represented in the fin by transverse, vertical (dorso-ventral) and longitudinal fibres, which lie within an extensive connective tissue framework (Johnsen & Kier, 1993) (Figure 1). In this study we show that the three fibre orientations in the fins are innervated by glutamatergic nerves, (there are no responses to ACh), and that 5-hydroxytryptamine (5-HT) may be involved in their control.

MATERIALS AND METHODS

Loligo bleekeri (Keferstien, 1866) were captured in fixed nets and maintained at the Ine Marine Laboratory of the National Institute of Physiological Sciences (NIPS), Okazaki, Japan in a seawater circulation system (approximately 14°C). Squid were sacrificed, the skin and dermis were removed and slices of muscle were dissected from different regions of the mantle and fin. A vibratome (Camden Instruments) was used to cut slices of tissue from specific regions of muscle. All dissections were conducted in oxygenated artificial seawater (ASW) which was nominally calcium-free, chilled to a slush (in mM: NaCl, 450; KCl, 9; $MgCl_2$, 60; HEPES, 20. pH 7.8, NaOH). Slices were then transferred to oxygenated glucose-enriched ASW to recover for up to one hour (in mM: NaCl, 470; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; HEPES,

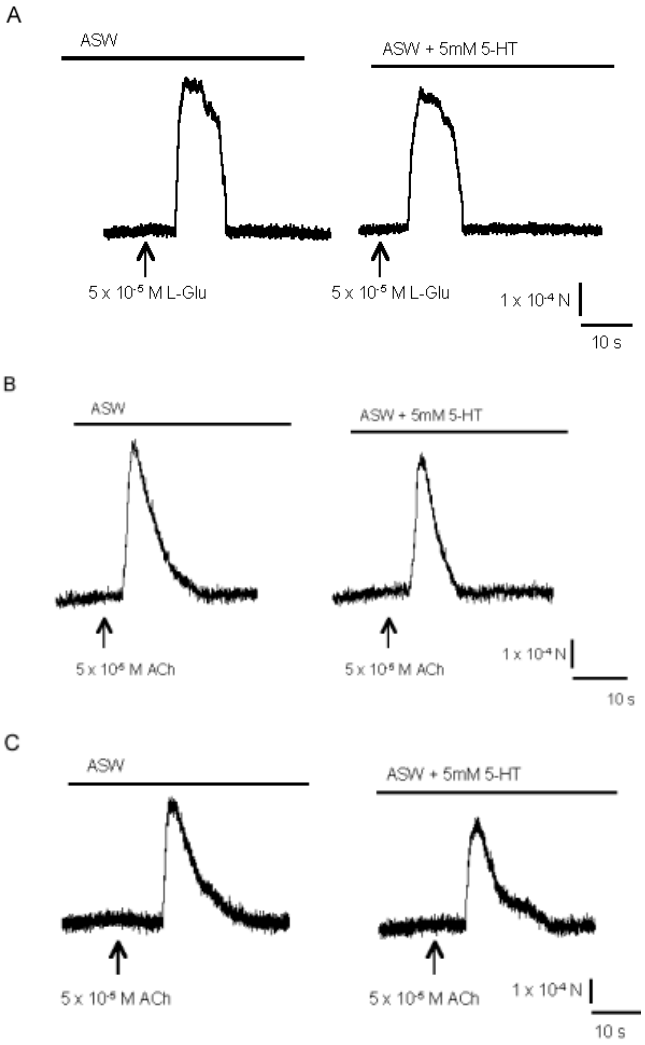

Figure 2. Mantle muscle responses to putative neurotransmitters in ASW. Following a 5 min recovery period in ASW the same slices were subjected to a 5 min treatment of up to 5mM 5-HT in ASW. Their responses to the same neurotransmitters were then tested. (A) Circular fibre contraction in response to 5×10^{-5} M L-glut (N=5); (B) radial fibre contraction evoked by 5×10^{-5} M ACh (N=4); (C) longitudinal fibre contraction due to 5×10^{-5} ACh (N=4).

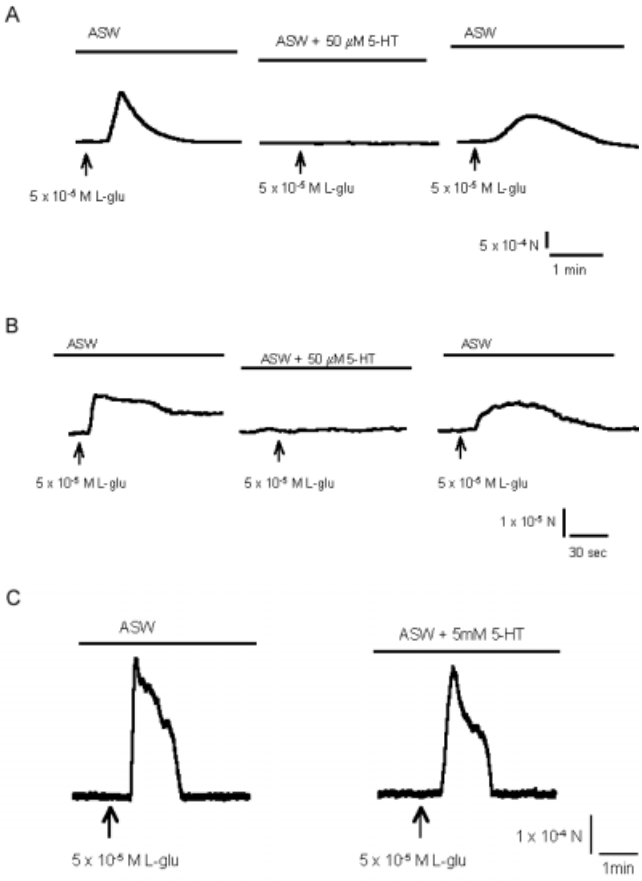

Figure 3. Fin muscle contractions in response to 5×10^{-5} M L-glu in ASW. A 5 min recovery period was given before the same slices were treated for 5 min in ASW with up to 5mM 5-HT. Where 50 μM 5-HT prevented contraction a recovery was seen after 5 min of washing in ASW. (A) Transverse muscle (N=5); (B) dorso-ventral muscle (N=5); (C) longitudinal muscle (N = 5).

10. pH 7.8, NaOH). Slices remained viable for up to five hours after dissection.

One end of a 0.1 cm^2 muscle slice, approximately 100 μm thick, was pinned onto the Sylgard base of a perfusion chamber. A dissecting pin, mounted on an isometric strain gauge (SensoNor AE801, Norway), impaled the free end of the slice and tension was adjusted until a slight transient force was apparent. Recordings were transferred to a PC via a Labmaster DMA interface (Scientific Solutions). Data were stored using Axotape 2.0 software (Axon Instruments) and traces generated using SigmaPlot 5 (SPSS Inc.). The dimensions of the slices and the method of slicing and recording enabled specific types of muscle to be independently tested. It was possible to determine if an additional muscle type was present in a slice because any contractions caused a thickening of the slice pushing the strain gauge in the opposite direction. No attempts were made to differentiate between the aerobic and anaerobic muscle fibres of the circular mantle muscles or the transverse fin muscles.

To investigate the effects of different neurotransmitters on the preparations, the muscles were perfused with ASW (in mM; NaCl, 450; KCl, 9; $CaCl_2$, 10; $MgCl_2$, 50; HEPES, 15. pH 7.8, NaOH). The flow was stopped to add neurotransmitters by topical application. Set concentrations of putative neurotransmitters in ASW were also perfused across the slices. The neurotransmitters used were acetylcholine chloride (Katayama Chemicals, Japan), L-glutamate Na salt and 5-HT creatinine sulphate (Sigma).

RESULTS

Mantle muscles

The circular fibres of the mantle contracted in response to 50 μM L-glu (Figure 2A) while the radial and longitudinal fibres contracted when 50 μM ACh was applied (Figure 2B,C), confirming previous reports. These concentrations produced near maximal contraction responses in each muscle type. Preliminary data, not included here, show that the radial and longitudinal mantle muscles contract in response to applications of nicotine, but not

muscarine, suggesting that the cholinergic receptors of the radial and longitudinal muscles are nicotinic.

Muscle contractions in response to the aforementioned neurotransmitters were not prevented in mantle muscle slices that were pre-treated for 5 min with up to 5 mM 5-HT in ASW, (Figure 2A–C). When 5-HT was applied to contracted muscles, following excitatory neurotransmitter application in ASW, there was no evidence of more rapid relaxation (data not shown).

Fin muscles

The transverse muscles contracted in response to 50 μM L-glu but not to ACh. By pre-treating the transverse muscles with 50–100 μM 5-HT for 5 min, contractions in response to L-glu were prevented (Figure 3A), but this inhibition disappeared after a 5 min wash in ASW. The ASW rinse was standardized at 5 min but the L-glu response typically returned after approximately 1 min of washing. There was no evidence that application of 5-HT to contracted transverse fibres induces more rapid relaxation.

The dorso-ventral muscles contracted in response to application of 50 μM L-glu, (Figure 3B) but not by up to 150 μM ACh. These fibres did not contract in response to L-glu when pre-treated for 5 min with 50 μM 5-HT (Figure 3B). The L-glu response returned after washing in ASW. Again, there was no evidence to suggest that 5-HT induced a more rapid relaxation in contracted dorso-ventral fibres. A threshold concentration for the 5-HT blockages has yet to be established.

The longitudinal fibres were observed to contract in response to 50 μM L-glu but not to 100–150 μM ACh. The response to L-glu was still present following a 5-min pre-treatment with up to 5mM 5-HT (Figure 3C). No difference in the characteristics of the L-glu contractions of the longitudinal fibres was seen in ASW compared with ASW with 5-HT.

DISCUSSION

Our results are consistent with previous reports of responses of mantle muscles to topically applied neurotransmitters (Bone & Howarth, 1980; Bone et al., 1982). The three distinct layers of circular muscle contract in response to L-glutamate (Figure 1), whereas the radial and longitudinal muscles are activated by acetylcholine. It has been suggested that the close proximity of the circular muscles, responsible for expelling water from the mantle, to the radial and longitudinal fibres, which are activated during refilling, requires different neurotransmitters. This remains unclear but it is assumed that from the arrangement and alternating contractions of the mantle muscles there is no need for a very close control over the degree of contraction. This presumption is supported by the data shown here that each mantle muscle type has a single excitatory neurotransmitter and that 5-HT did not have any modulatory effects.

In contrast, all three fin muscle types showed glutamatergic activation, with no recorded responses to acetylcholine. In addition, 5-HT has an effect on the transverse and dorso-ventral muscles, but not on the longitudinal muscles. It is clear when observing live squid that modulation of the pattern of movement of the fins contrasts with the stereotyped movements of the mantle. The three-dimensional arrangement of the fin muscles is similar to that of the mantle but the intricate movements of the fins suggest that their muscles may require a greater degree of control. Our data suggests that 5-HT may provide a mechanism for modulation of contraction in the fin muscles.

The muscles of the fin are orthogonally arranged and function as a muscular hydrostat and the different muscle types are activated simultaneously to provide antagonism (Kier, 1988, 1989; Kier et al., 1989). According to previous descriptions of fin movements the transverse and dorso-ventral muscles work together to cause the fin to bend (Kier, 1989). The longitudinal muscles appear to be in an almost constant state of contraction to control fin length, which is probably not altered during the course of a single fin beat cycle.

Other muscle systems in cephalopods that have 5-HT modulation include the aorta of *Sepia* (Schipp et al., 1991) and the muscles of the chromatophores. The chromatophores are expanded by radially arranged muscles activated by glutamatergic nerves (Bone & Howarth, 1980), but have 5-HT modulation (Florey, 1966; Florey & Kriebel, 1969; Messenger et al., 1997). 5-hydroxytryptamine affects presynaptic activity at the neuromuscular junction (Florey, 1969) and postsynaptically as shown by work on isolated chromatophore muscles, in which calcium release from internal stores was prevented by blocking ryanodine-sensitive channels by 5-HT (Lima et al., 1998; Lima, 2000).

It is not clear how 5-HT affects the transverse and dorso-ventral muscles of the fin. Further investigations are required on enzymatically isolated muscle fibres to determine if the action is postsynaptic and if, like the chromatophore system, 5-HT blocks the ryanodine-sensitive release channels of the internal calcium stores. This is the first study to offer a putative candidate for a neuromodulatory mechanism in squid locomotor muscles and more work is being conducted to see if 5-HT has similar effects in other areas of muscle that may also require close control, like those of the arms and tentacles.

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