Physiological and immunocytochemical evidence that glutamatergic neurotransmission is involved in the activation of arm autotomy in the featherstar 

**Antedon mediterranea** (Echinodermata: Crinoidea)

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**SUMMARY**

The crinoid echinoderm *Antedon mediterranea* autotomises its arms at specialised skeletal joints known as syzygies that occur at regular intervals along the length of each arm. Detachment is achieved through the nervously mediated destabilisation of ligament fibres at a particular syzygy. The aim of this investigation was to identify neurotransmitters that are involved in the autotomy response. Physiological experiments were conducted on isolated preparations of syzygial joints, which can be induced to undergo autotomy-like fracture by applying stimulatory agents such as elevated $[K^+]_o$. Initial experiments with elevated $[K^+]_o$ showed that the autotomy threshold (the minimum amount of stimulation required to provoke autotomy) is lowest in syzygies at the arm base and rises distally. Of a range of neurotransmitter agonists tested, only L-glutamate invoked syzygial destabilisation, as did its analogues L-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate, but not L-(+)-2-amino-4-phosphonobutyrate (L-AP4) or N-methyl-o-aspartate (NMDA). The implication that L-glutamate stimulates syzygial fracture through AMPA/kainate-like receptors was supported by the finding that the action of L-glutamate was inhibited by the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Acetylcholine depressed the response of syzygial preparations to L-glutamate, suggesting a possible mechanism by which the autotomy threshold could be varied constitutively and facultatively. An immunocytochemical method employing a polyclonal antibody against L-glutamate conjugated to glutaraldehyde revealed L-glutamate-like immunoreactivity in all components of the putative neural pathway controlling the autotomy reflex, including the epidermis, brachial nerve, syzygial nerves and cellular elements close to the syzygial ligaments. We conclude that it is highly probable that L-glutamate acts as an excitatory neurotransmitter in the activation of arm autotomy in *A. mediterranea*.

Key words: Antedon, autotomy, crinoid, glutamate, juxtaligamental cells.

**INTRODUCTION**

The capacity for, and employment of, autotomy (self-induced defensive detachment of anatomical structures) is widespread throughout the phylum Echinodermata. All investigated examples of echinoderm autotomy have been shown to depend on the rapid destabilisation of collagenous structures, such as ligaments, tendons and dermis, that cross the autotomy plane (Wilkie, 2001). For instance, the arms of comatulid crinoids (‘featherstars’) are autotomised at a specialised type of joint – the syzygy – as a result of the sudden loss of tensile strength of the short ligament fibres that link opposing skeletal ossicles at the joint (Holland and Grimmer, 1981; Wilkie et al., 1999; Ferreri et al., 2003).

Although autotomy is practised by animals in many phyla and in some species can be a frequent occurrence with significant consequences for different aspects of their biology (Maginnis, 2006; Fleming et al., 2007), information on the nervous control of the process is patchy. Surprisingly little is known of the neural basis of tail autotomy in reptiles and other vertebrates (Clause and Capaldi, 2006). There are, on the other hand, detailed accounts of the anatomy and electrophysiology of the nervous circuitry associated with the autotomy mechanisms of decapod crustaceans (McVean, 1974; Findlay and McVean, 1977), a nudibranch (Bickell-Page, 1989) and a hydromedusa (Bickell-Page and Mackie, 1991). As far as echinoderms are concerned, knowledge of autotomy-related neurobiology is at present limited to putative motor pathways (Wilkie, 1979; Holland and Grimmer, 1981; Charlinia et al., 2009) and, electrophysiology being generally impracticable owing to the inaccessibility and/or small size of echinoderm neural elements, published physiological data have been derived from a small number of, mostly preliminary, pharmacological studies (Dobson, 1985; Wilkie et al., 1990; Wilkie et al., 1995; Wilkie et al., 1999; Byrne, 1986).

The primary aim of the investigation described here was to identify neurotransmitters that are involved in the autotomy response of the featherstar *Antedon mediterranea* (Lam.). General information on the response was obtained by inducing intact animals and detached arms to autotomise in the laboratory. Experiments were conducted on isolated arm pieces that included a syzygy, which, as shown previously (Wilkie et al., 1999), can be induced by neurostimulatory agents to destabilise and fracture, thus mimicking events at autotomy. The response of these preparations to elevated $[K^+]_o$ was examined in order to characterise their baseline behaviour. A wide range of pharmacological agonists and antagonists was then screened. Of the potential neurotransmitters tested, only L-glutamate and certain other glutamatergic agonists elicited the autotomy response in isolated preparations, whereas acetylcholine was found to inhibit the action of L-glutamate. The contention that L-glutamate acts as an excitatory neurotransmitter in the activation of featherstar
arm autotomy would be supported by evidence for its presence in cellular, particularly neuron-like, elements associated with the syzygies. The distribution of glutamate in the arm of *A. mediterranea* was therefore visualised using a specific immunocytochemical method.

**MATERIALS AND METHODS**

**Animals**

Animals were collected by scuba divers from the Tyrrhenian coast (Isle of Elba) and Ligurian coast (Bergeggi) of Italy, transported to the University of Milan and kept in artificial seawater (ASW: ‘Instant Ocean’) at 14–16°C.

**Physiological testing**

To obtain preparations for experiments, an animal was anaesthetised by immersion in 0.1% propylene phenoxetol [1-phenox propane-2-ol (see O’Neill, 1994)] in ASW, which rendered it unresponsive in seconds. All ten arms were then cut off at their junction with the second primibrachial ossicle (to each of which two arms are attached) using fine scissors, and the pinnules (lateral appendages: Fig. 1A) were removed from the basal one-third of each arm using iridectomy scissors. The main skeleton of the comatulid arm consists of a series of brachial ossicles linked at two types of joint: (i) diarthroses (usually called ‘muscular articulations’) – mobile hinge joints at which adjacent brachial ossicles are connected by paired oral (above the hinge) and single aboral (below the hinge) ligaments and by paired oral muscles; and (ii) syzygies – immobile joints at which adjacent ossicles are connected by only the syzygial ligament and which are the only joints at which arm autotomy occurs (Fig. 1A,B). Four preparations were cut with a scalpel from the basal region of an arm, each including a single syzygy in the middle with two intact diarthroses on either side (Fig. 1A,B). In most arms, the four preparations thus obtained contained the first, second, fourth and sixth basal syzygies, respectively. They were washed in ASW and then each was transferred to a separate compartment containing 2 ml ASW in a 24-well tissue-culture plate and left in a fridge at 14–15°C for at least 60 min before being used. Each experiment employed preparations from only one animal, unless stated otherwise, and was repeated at least once using preparations from a different animal.

For physiological testing, the preparations were held horizontally with the oral side uppermost, as in life, and gripped at the distal end by a spring-loaded clamp and at the proximal end by a heart-clip connected by a silver chain to one side of an isotonic lever (Fig. 1B). A weight suspended from the other side of the lever subjected the preparation to a bending load of 10 g. The application of an orally directed bending force mimicked a common pattern of loading of comatulid arms in nature when they are collecting food particles from water currents and are held in a rigid state with the aboral side facing the current (La Touche, 1978; Byrne and Fontaine, 1981). Output from a displacement transducer connected to the lever was fed to a pen-recorder. The preparations were thus subjected to two types of stimuli that had the potential to invoke the autotomy response: (i) mechanical stimuli alone; (B) syzygial failure occurred within up to 3 min after addition of the stimulatory agent; (C)
syzygial failure did not occur within 3 min after addition of the stimulatory agent.

Also measured was the ‘response time’: the interval between the time when application of a stimulatory agent began and the time when a response started.

When the effect of pretreatment with a drug at a particular concentration was being investigated, this drug was present at the same concentration in the ASW solution containing the stimulatory agent.

Statistical analysis employed Student’s two-tailed unpaired t-tests for single comparisons of mean responses. For multiple comparisons, one-way ANOVA with Tukey’s or Dunnett’s test was applied. Where variances differed significantly, the Kruskal–Wallis and Dunn’s multiple comparison tests were used. The overall responsiveness of preparations was compared using the Mann–Whitney test with responses A, B and C coded as ‘1’, ‘2’ and ‘3’. The relationship between syzygy position and responsiveness was analysed by employing an ordinal logistic regression model (see Appendix). All means are given ± one standard deviation (s.d.).

Immunocytochemistry
Arm pieces were fixed in 1% paraformaldehyde and 1% glutaraldehyde in phosphate-buffered saline (PBS) for 2 h and embedded in Durcupan epoxy resin. Sagittal sections (thickness 0.9–1 μm) were cut with a Reichert Ultracut E ultratome, mounted on slides and de-resinated. The immunocytochemical procedure used a polyclonal antibody (ab9440, purchased from ABCAM) raised in rat against L-glutamate conjugated to glutaraldehyde, which, according to the manufacturers, has no measurable cross-reactivity against glutamate in peptides or proteins. The sections were blocked in 5% goat serum in PBS containing 0.05% Tween20 (PBS-TW) for 1 h. After sections were rinsed in PBS (3×5 min), they were incubated overnight at 4°C in the primary antibody diluted 1:50 in 1% goat serum in PBS-TW. After rinsing in PBS (4×5 min), the sections were treated for 1 h with 1% bovine serum albumin in PBS, rinsed in PBS (2×10 min) and incubated overnight at 4°C in a goat anti-rabbit Alexa-Fluor-555-labelled antibody diluted 1:200 in 1% goat serum in PBS. During this and all subsequent stages, the sections were left in the dark. After rinsing in PBS (3×10 min), the sections were mounted in 80% glycerol in PBS. They were viewed in a Leica TCS-NT confocal laser scanning microscope equipped with a 75 mV multiline argon/krypton laser. Control sections, which were incubated without the primary antibody, showed no immunoreactivity. After examination for immunoreactivity, antibody-treated sections were stained with crystal violet and basic fuchsin and photographed in an Olympus BH-2 microscope using an Olympus DP-50 digital camera and analySIS software.

RESULTS
General aspects of the autotomy response of A. mediterranea
A total of 105 autotomy events was induced in nine intact animals by squeezing their arms with forceps. In all cases, autotomy occurred at the nearest syzygy on the proximal side of the forceps, with up to four diarthroses being present between the forceps and the autotomizing syzygy. Detached arms could also undergo autotomy. Another 35 autotomy events were induced in seven isolated arms that had been amputated from three animals. In 34 of these, detachment occurred at the syzygy closest to the proximal edge of the forceps, and, in one, both the nearest and the next syzygy were destabilised. After autotomy induced in both intact animals and isolated arms, the detached distal portion of the arm showed rhythmic cycles of flexion and extension in the oro–aboral plane.

Physiology of isolated syzygial preparations
Effects of elevated [K+]o
Elevation of [K+]o to 20–100 mmol l−1 invoked syzygial fracture. In 100 syzygial fractures induced by 100 mmol l−1 K+, the response time was 0–163 s (mean ± s.d. 38.0±30.6 s). In 43 of these, syzygial fracture was preceded by diarthrial flexion with a response time of 0–42 s (mean ± s.d. 6.1±9.2 s). In 38 (27.5%) out of a total of 138 responses, 100 mmol l−1 K+ caused diarthrial flexion without subsequent syzygial fracture (Fig. 3A,B,C).

The responsiveness of preparations to mechanical stimulation and elevated [K+]o depended on their position in the arm. As shown in Fig. 4A, the readiness of the preparations to destabilise declined significantly from the base of the arm. In all subsequent experiments, care was taken to exclude position as a variable.

Responsiveness to mechanical stimulation and elevated [K+]o was inhibited by pretreatment for 30–60 min with the local anaesthetic procaine hydrochloride (1 mmol l−1; Fig. 4B). Responsiveness was restored by immersion for at least 2 h in ASW.

The response to elevated [K+]o, was blocked by pretreatment for 30 min with the broad-spectrum receptor antagonist chlorpromazine. None of nine preparations treated with 1 mmol l−1 chlorpromazine responded to 100 mmol l−1 K+ within 3 min, whereas all nine controls underwent syzygial fracture (mean response time ± s.d. 43.0±14.2 s). Similar tests were conducted using pretreatment with pharmacological antagonists that would be expected to inhibit specifically adrenergic (100 μmol l−1 propranolol; 100 μmol l−1 phenotolamine), cholinergic (1 mmol l−1 atropine; 1 mmol l−1 mecaminime), serotonergic (10 μmol l−1 parachlorophenylalanine; 10 μmol l−1 methiothepin) or nitric oxide-mediated (100 μmol l−1 Nω-nitro-l-arginine methyl ester plus 200 μmol l−1 hydroxocobalamin) mechanisms. None of these affected the responsiveness to 100 mmol l−1 K+ or mechanical stimulation (data not shown).

Effects of L-glutamate
L-Glutamate invoked syzygial fracture at concentrations of 500 μmol l−1 and above. In 58 syzygial fractures caused by 1 mmol l−1 L-glutamate, the response time was 26–286 s (mean ± s.d. 124.7±69.0 s). In 20 cases, syzygial fracture was preceded by diarthrial flexion (response time 0–188 s, mean ± s.d. 53.5±59.5 s) (Fig. 3D,E). L-Glutamate never (in 200 tests using concentrations of 0.5–5 mmol l−1) induced diarthrial flexion that was not followed
by syzygial fracture. Because the sensitivity of preparations to l-glutamate varied between animals, subsequent experiments comparing its action with that of other agents were preceded by preliminary tests to establish the consistently effective l-glutamate concentration, which varied from 1–5 mmol l⁻¹.

l-Aspartate (500 μmol l⁻¹ and above) induced syzygial fracture. In 11 syzygial fractures caused by 1 mmol l⁻¹ l-aspartate, the response time was 49–270 s (mean ± s.d. 140.6±69.8 s). Only one of these syzygial fractures was preceded by diarthrial flexion.

Several other glutamate receptor agonists were tested. Syzygial fracture was not induced within 5 min by the metabotropic agonist L-(+)-2-amino-4-phosphonobutyrate (L-AP4; 1 mmol l⁻¹; N=9) or by the ionotropic agonist N-methyl-D-aspartate (NMDA; 1 mmol l⁻¹; N=8). The ionotropic agonist α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA; 1 mmol l⁻¹) induced fracture within 5 min in three preparations out of seven tested (response times 14, 257 and 273 s, respectively). The ionotropic agonist kainate (1 mmol l⁻¹) induced fracture within 5 min in all 12 preparations tested (response time 26–195 s, mean ± s.d. 97.0±41.5 s), one of these fractures being preceded by diarthrial flexion (response times 0, 45 and 65 s, respectively). In another series of tests using preparations from a single animal, which were unresponsive to 1 mmol l⁻¹ l-glutamate, kainate induced syzygial fracture at concentrations as low as 0.1 μmol l⁻¹.

The response to l-glutamate was depressed by chlorpromazine and by the glutamate receptor antagonists ketamine and 6-cyano-7-nitroquinoline-2,3-dione (CNQX). Kynurenate did not have a statistically significant effect (Fig. 4C).

The following pharmacological agents were also tested on isolated syzygial preparations, and none was found to activate the autotomy response at concentrations up to 1 mmol l⁻¹: adrenaline, isoprenaline, β-adrenergic acid, dopamine, octopamine, glycine, hydroxylamine and serotonin (data not shown). Acetylcholine and other cholinergic agonists had a modulatory effect, as described below.

**Effects of acetylcholine**

Acetylcholine (1 mmol l⁻¹) invoked diarthrial flexion that was rarely followed by syzygial fracture. In 44 tests, the response time was 0–52 s, mean ± s.d. 4.2±9.5 s. Syzygial fracture was observed in only one of these tests and occurred in a preparation that underwent extreme diarthrial flexion both before and after the addition of acetylcholine (Fig. 3F,G). The cholinergic agonists carbamoylcholine, acetyl-β-methylcholine and nicotine (all at 1 mmol l⁻¹) also caused diarthrial flexion, usually without subsequent syzygial fracture. Syzygial fracture occurred in two out of 11 responses to carbamoylcholine, two out of 18 responses to acetyl-β-methylcholine and four out of 14 responses to nicotine. These syzygial fractures usually followed large-magnitude diarthrial flexions.

Pretreatment of preparations with 1 mmol l⁻¹ acetylcholine for 3 min inhibited the response to l-glutamate, although the degree of inhibition varied between animals. In one experiment, all 13 acetylcholine-treated preparations failed to respond to 5 mmol l⁻¹ l-glutamate within 3 min, whereas all 11 untreated controls showed syzygial fracture (response time 39–162 s, mean ± s.d. 88.9±38.1 s). In another experiment using preparations from a different animal in which the controls, in contrast to those of the previous experiment, were responsive to mechanical stimulation, acetylcholine pretreatment strongly inhibited their mechanical sensitivity, and 2 mmol l⁻¹ l-glutamate caused most preparations to fracture within 3 min, although with a significantly prolonged mean response time (ACh-treated: 124.9±35.8 s; controls 57.2±11.9 s; P<0.001; Fig. 4D). Pretreatment for 3 min with nicotine (1 mmol l⁻¹) or acetyl-β-methylcholine (1 mmol l⁻¹) also depressed the response to l-glutamate, their inhibitory effect being generally weaker than that of acetylcholine. In an experiment in which the mean response time of control preparations to 5 mmol l⁻¹ l-glutamate was 59.1±23.3 s (N=5), that for nicotine-treated preparations was 100.8±29.9 s (N=5; in comparison with controls, P<0.05: ANOVA plus Dunnnett’s test) and for acetyl-β-methylcholine-treated preparations was 119.0±19.2 s (N=5; in comparison with controls, P<0.01).

If cholinergic agonists inhibit the response to l-glutamate, cholinergic antagonists might be expected to augment it. However, pretreatment with neither atropine (1 mmol l⁻¹) nor hexamethonium (1 mmol l⁻¹) had any consistent effect on the sensitivity of preparations to l-glutamate or mechanical stimulation (data not shown).
**Immunocytochemistry**

Glutamate-like immunoreactivity was detected mainly in the epidermis, brachial nerve and structures near or within the arm ligaments and muscles. In some locations, identifiable cell bodies were immunolabelled; in most locations, the dominant immunoreactive components were granules ranging in diameter from under 0.4 μm to 3 μm, with the smaller granules often aligned in single rows. There were no apparent differences between the size distributions of these granules in different anatomical locations.

In the aboral epidermis, immunoreactivity was localised mainly to cell bodies within the epidermis and possible unipolar neurons adjacent to it (Fig. 5C). The epithelial cells of the oral food groove epidermis were unlabelled, apart from sparse isolated granules, although, near the basal lamina, there were intensely immunoreactive cell bodies and granules where the basiepithelial nerve plexus is located (Fig. 5E). The brachial nerve was strongly labelled, the immunoreactivity taking the form mainly of linear arrangements of granules parallel to the longitudinal axis of the nerve. Presumed cell bodies within the nerve were also immunostained (Fig. 6B). Numerous immunoreactive granules, some forming short alignments, were present on the surface of the brachial muscle fibres (not illustrated). Immunoreactive structures associated with the diarthral ligaments consisted of granules within cell bodies located in the ossicles close to the insertions of the ligaments and, inside the ligaments, rows of fine granules parallel to the collagen fibres and larger, isolated and more strongly labelled granules that sometimes formed clusters (Fig. 6D). Immunoreactive perikarya were seen in the ossicles near the insertions of the syzygial ligament, and there was labelling of lines of granules and other irregular structures at the location of the syzygial nerves, particularly where they branched from the brachial nerve (Fig. 6F,G). Fine granules within the syzygial ligament were also labelled (Fig. 6G). There was no obvious difference between the syzygial and diarthral ligaments regarding the density or size of their immunoreactive granules, taking
Activation of featherstar arm autotomy into account the much shorter length of the syzygial ligament fibres (see Fig. 6D,G). The only other structures that were found to exhibit immunoreactivity were a few isolated cells and granule alignments within the brachial ossicles (not illustrated).

**DISCUSSION**

**Autotomy reflex**

The initial experiments showed that autotomy occurs always at the nearest syzygy proximal to the point of stimulation in both intact animals and isolated arms. The simplest explanation for this invariable relationship between the sites of stimulation and fracture is that the autotomy response is a reflex that involves only a local nervous pathway connecting the site of damage to the nearest proximal syzygy. This was confirmed by the incidental observation that mechanical stimulation of the epidermis resulting from the handling of isolated arm pieces sometimes invoked syzygial fracture even before the preparations were installed in the test apparatus, which implies that such arm pieces contain the complete neural apparatus required for the autotomy response, including a sensory pathway from the epidermis to the brachial nerve, and a motor pathway from the brachial nerve to the juxtaligamental cells whose processes permeate the syzygial ligament and are likely to be directly responsible for its destabilisation at autotomy (Holland and Grimmer, 1981; Heinzeller and Welsch, 1994; Ferreri et al., 2003; Wilkie, 2005).

**Significance of syzygial and diarthrial responses in isolated preparations**

Syzygial fracture results from the drastic loss of tensile strength of the syzygial ligament (Holland and Grimmer, 1981; Wilkie et al., 1999; Ferreri et al., 2003). In the set-up used for these experiments, in which the initial resistance of preparations to a bending force acting in the oral direction could have been due to only the diarthrial ligaments and not the brachial muscles [as these are located on the oral side of the interbrachial hinge joint (Fig. 1B)], diarthrial flexion theoretically could have resulted from either: (i) a reduction in the mechanical resistance of the diarthrial ligaments that was sufficient to allow the joint to be flexed by the 10 g bending load alone or with the assistance of muscle contraction, or (ii) a contraction of the brachial muscles that was strong enough to overcome the (unaltered) resistance of the ligaments, thereby extending the aboral ligament and/or compressing the oral ligaments. That only muscle contraction was involved is highly unlikely as estimates of the tensile strength of...
stiffened mutable echinoderm ligaments [e.g. 6–38 MPa (see Wilkie, 1984)] exceed the maximal contractile tension recorded for any muscle [1.4 MPa for bivalve ABRM (see Sugi et al., 1999)]. Furthermore, with regard specifically to diarthrial flexion in response to elevated \([K^+]_o\) and acetylcholine, as these agents stiffen the aboral diarthrial ligament of another crinoid (Motokawa et al., 2004), it is reasonable to infer that they had the same effect on the equivalent ligament of *A. mediterranea*. Experimental investigation of the possible contribution of muscle contraction to the diarthrial flexions recorded by our set-up was not attempted as it would have required a different methodology and was beyond the scope of the present enquiry.

Another issue raised by our experimental approach is the possibility that mechanical stimulation, which was caused in vitro by the heart-clip, clamp and externally imposed bending force and might be caused in vivo by an attacking predator, is a prerequisite for the induction of syzygial failure. The results of pilot experiments provided no evidence for this. Both whole specimens of *A. mediterranea* and isolated arms autotomised repeatedly when immersed in seawater containing 100 mmol\(^{-1}\) \(K^+\) without any mechanical stimulation. Furthermore, unattached syzygial preparations that were left in the same medium and subjected to no mechanical stimulation also underwent syzygial destabilisation. Externally applied mechanical stress thus appears not to be a prerequisite for activation of the autotomy reflex in either intact arms or isolated syzygial preparations.

**Effects of elevated \([K^+]_o\)**

Elevated \([K^+]_o\) caused both syzygial fracture and diarthrial flexion. Elevated \([K^+]_o\) is presumed to cause the depolarisation of excitable membranes, which in the present context could belong to any of the neuronal elements, including juxtaligamental cells, that control syzygial ligament tensility. However, the reversible inhibition of the response by procaine might indicate that \(K^+\) ions affect primarily nervous conduction rather than the juxtaligamental cells themselves. Of the several pharmacological antagonists tested, only chlorpromazine was found to inhibit the action of elevated \([K^+]_o\). Chlorpromazine is an inhibitor of nitric oxide synthase and blocks dopaminergic, cholinergic, \(\alpha\)-adrenergic, serotonergic and non-NMDA ionotropic glutamatergic receptors (Sepúlveda et al., 1994; Sakihara et al., 1996; Zarnowska and Mozrzymas, 2001; Pechenik et al., 2002). This result, and the failure to detect any inhibitory effect when preparations were treated with nitric-oxide-depleting agents or antagonists of adrenergic, cholinergic and serotonergic receptors, left dopamine and glutamate receptors as potential targets for chlorpromazine.

In this investigation, preparations were subjected first to mechanical stimulation while being installed into the test apparatus and then to chemical stimulation in the form of elevated \([K^+]_o\) or other agents. Analysis using an ordinal logistic regression model (expounded fully in the Appendix) revealed that there was a statistically significant correlation \((P<0.001)\) between the longitudinal location of a syzygial preparation in the intact arm and
its responsiveness to stimulation, such that the responsiveness was greatest near the base and decreased distally, at least within the range of syzygies investigated (usually numbers 1, 2, 4 and 6). Thus, for example, the proportion of preparations fracturing in response to mechanical stimulation alone (category A) was greatest at the base. This suggests that the autotomy threshold for mechanical stimulation – that is, the minimal amount of stimulation required to provoke the autotomy reflex (McVean, 1974; Arnold, 1988) – rises progressively from the base of the arm. A constitutive gradation in the autotomy threshold in segmented appendages with multiple autotomy planes appears not to have been described before. It would be interesting to know whether any progressive variation in autotomy threshold occurs in comparable structures such as amphibian and reptile tails (Wake and Dresner, 1967; Arnold, 1988) or the arms of brittlestars and luidiid starfish (Wilkie, 2001) and, if it does, whether it has the same directionality – with the threshold lowest at the basal end. This might be adaptive, in that the nearer a predator is to the base of such structures, the nearer it is to the main body of the animal (the central calyx in the case of comatulids), the greater the risk of the whole animal being consumed, and therefore the greater the urgency to jettison the appendage in order to distract the predator while the targeted animal escapes.

It is also relevant to point out here that the effects of elevated $\left[K^+\right]_o$ (and other stimulatory agents) could of necessity be observed only in preparations that had not fractured in response to the initial mechanical stimulation resulting from handling and compression by the heart-clip and clamp. In the light of our evidence that the autotomy threshold for mechanical stimulation can vary, for example with position in the arm, as discussed above, or with seasonal factors, as discussed below under ‘Effects of acetylcholine’, it is parsimonious to assume that these represented preparations whose autotomy threshold was at the upper end of the range of normal variation and that they were not in any abnormal physiological state that might complicate interpretation of our experimental results.

**Effects of L-glutamate**

A range of pharmacological agonists, including dopamine, was tested on isolated preparations. Only L-glutamate invoked syzygial fracture. That L-glutamate and glutamate receptors are involved in stimulatory effects of L-aspartate and other glutamate analogues. The activation of the autotomy reflex was supported by the tested on isolated preparations. Only L-glutamate invoked syzygial response times for L-glutamate, L-aspartate and kainate all differed significantly from that for 100 mmol l$^{-1}$ K$^+$ (and other stimulatory agents) could of necessity be of 58 responses to 1 mmol l$^{-1}$ t-glutamate (Fisher’s exact test, $P=0.0028$). Furthermore, 38% of responses to 100 mmol l$^{-1}$ K$^+$ consisted of diarthrial flexion alone, but t-glutamate, even at a concentration of 5 mmol l$^{-1}$, never induced diarthrial flexion alone. Elevated $\left[K^+\right]_o$, therefore appears to excite motor pathways to both the syzygies and the diarthroses. t-Glutamate might directly excite only the pathway to the syzygial ligament, but, once this is activated, and perhaps if an excitatory threshold is exceeded, there is collateral stimulation of pathways to the diarthroses. There are also differences in the response times to K$^+$ and t-glutamate that might be due to the indiscriminate action of K$^+$. Table 1 shows that K$^+$ produced significantly shorter response times for both diarthrial flexion and syzygial fracture than glutamatergic agonists. K$^+$ is likely to act indiscriminately on excitable membranes at multiple locations in the nervous pathways, whereas the sites of action of t-glutamate and its analogues should be specific, restricted in number and distribution, and less accessible to drugs.

**Table 1. Comparison of the responses of syzygial preparations to chemical agents**

<table>
<thead>
<tr>
<th>Chemical Agent</th>
<th>Range (mmol l$^{-1}$)</th>
<th>Mean ± s.d. (s)</th>
<th>N</th>
<th>Range (mmol l$^{-1}$)</th>
<th>Mean ± s.d. (s)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$^+$ (100 mmol l$^{-1}$)</td>
<td>0–163</td>
<td>38.0±30.6</td>
<td>100</td>
<td>0–42</td>
<td>6.1±9.2</td>
<td>43</td>
</tr>
<tr>
<td>L-Glutamate (1 mmol l$^{-1}$)</td>
<td>26–286</td>
<td>124.7±69.0</td>
<td>58</td>
<td>0–188</td>
<td>53.5±59.5</td>
<td>20</td>
</tr>
<tr>
<td>L-Aspartate (1 mmol l$^{-1}$)</td>
<td>49–270</td>
<td>140.6±69.8</td>
<td>11</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1</td>
</tr>
<tr>
<td>Kainate (1 mmol l$^{-1}$)</td>
<td>26–195</td>
<td>97.0±41.5</td>
<td>12</td>
<td>0–65</td>
<td>36.8±33.4</td>
<td>3</td>
</tr>
<tr>
<td>Acetylcholine (1 mmol l$^{-1}$)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0–52</td>
<td>4.2±9.5</td>
<td>44</td>
</tr>
</tbody>
</table>

The mean syzygial fracture response times for L-glutamate, L-aspartate and kainate all differed significantly from that for 100 mmol l$^{-1}$ K$^+$ (Kruskal–Wallis and Dunn’s Multiple Comparison tests; $P<0.001$). The mean diarthrial flexion response time for L-glutamate differed significantly from that for 100 mmol l$^{-1}$ K$^+$ ($P<0.001$); there was no significant difference between the latter and those for kainate and acetylcholine ($P>0.05$). n.a., not applicable.
Effects of acetylcholine

Further differentiation between the motor pathways to the syzygies and diarthroses was afforded by the action of cholinerigic agonists. These caused diarthrial flexion that was succeeded by syzygial fracture only rarely (particularly after large-magnitude diarthrial flexions that might have caused tissue damage and secondarily initiated the autotomy response). Both a muscarinic agonist (acetyl-β-methylcholine) and a nicotinic agonist (nicotine) produced diarthrial flexion, which is compatible with reports that acetylcholine, muscarinic agonists and nicotinic agonists cause de-stiffening of the aboral arm ligaments and cirral ligaments of a stalked crinoid (Birenheide et al., 2000; Motokawa et al., 2004) and suggests that both muscarinic and nicotinic acetylcholine receptors are involved. This would not be peculiar to echinoderm ligaments as muscarinic and nicotinic cholinceptors co-exist in echinoderm muscles (Devlin, 2001).

Despite its role as an important excitatory neurotransmitter in echinoderm motor responses (Devlin, 2001), including the de-stiffening of diarthrial ligaments in crinoid arms, acetylcholine is clearly not involved in the destabilisation of syzygial ligaments in A. mediterranea. On the contrary, it depressed the response of syzygial preparations to L-glutamate, as did acetyl-β-methylcholine and nicotine. These results are of interest because: (i) they raise the possibility that the position-dependent variation in autotomy threshold might be due to graded cholinerigic inhibition, and (ii) they suggest a possible mechanism by which the autotomy threshold could be adjusted facultatively. There is evidence from other animals that, although autotomy might be brought about by an automatic reflex, the level of stimulation required to trigger the reflex can fluctuate temporally in individuals, thus indicating that there is ‘central’ control of the autotomy threshold, presumably to ensure that anatomical loss is instigated only in circumstances where there is a high probability that the benefits will outweigh the costs (Arnold, 1988). The readiness to autotomise might be influenced by factors such as the number of previous autotomies (McVean, 1974), nutritional state (Arnold, 1988) and reproductive condition (Bateman and Fleming, 2006). Evidence for this phenomenon in A. mediterranea is as yet incidental. It was noted in the experiment examining the relationship between location in the arm and autotomy threshold that there could be marked differences in the responsiveness of preparations from the same level in different arms of one animal. It was also mentioned above that the effective L-glutamate concentration varied between animals. This applied to animals collected at the same time from the same location, but was even more pronounced between animals from batches collected at different times and might be related to reproductive state as preparations from a batch of animals with ripe gonads (which are concentrated in the proximal and middle part of the arm) showed less sensitivity to L-glutamate than those from a batch without ripe gonads collected from the same population a few weeks previously. This might be an indication that seasonal changes in reproductive hormone levels influence the autotomy threshold. Seasonal changes in sex steroid levels occur in certain echinoderms (Hines et al., 1992) but have yet to be demonstrated clearly in crinoids (Lavado et al., 2006).

In the light of results from earlier experiments, we hypothesised that syzygial destabilisation is inhibited by tonically firing neurons that are switched off during the autotomy response (Wilkie et al., 1999). We now amplify this hypothesis by proposing that the inhibitory neurons are cholinerigic. A possible precedent for tonic cholinerigic inhibition of connective tissue de-stiffening in echinoderms has been identified in asteriid starfish, in which atropine by itself causes de-stiffening of isolated preparations of oral and aboral body wall (Wilkie et al., 1990; Wilkie et al., 1995). In the current investigation, neither atropine (a muscarinic antagonist) nor hexamethonium (a nicotinic antagonist) alone had any consistent effect. Further work is therefore needed to characterise fully the depressive action of cholinerigic agonists on syzygial destabilisation in A. mediterranea.

Immunocytochemistry

Ours appears to be the first demonstration of glutamate-like immunoreactivity (GLI) in an echinoderm. Immunoreactive structures were widespread throughout the arm of A. mediterranea and took the form mainly of discrete granules either obviously associated with cell bodies or, more commonly, remote from cell bodies and often aligned in single rows resembling L-glutamate-immunoreactive varicose axons occurring in other phyla (Sakurai et al., 1998; Hatakeyama et al., 2007). The granules are presumably intracellular L-glutamate storage sites, the smallest perhaps representing presynaptic terminals.

The crinoid nervous system is usually regarded as consisting of three subsystems: the aboral, ectoneural and hyponeural, although Heinzeller and Welsch (Heinzeller and Welsch, 1994) proposed a simpler anatomical division into ecto/entodermal (which includes the ectoneural) and mesodermal (which includes the aboral and hyponeural). In contrast to the other four echinoderms of which the ectoneural is the most developed subsystem, in crinoids the aboral subsystem is dominant and the ectoneural is greatly reduced (Heinzeller and Welsch, 2001). In A. mediterranea, however, prominent GLI was present in both the ectoneural subsystem – that is, in the basiepithelial plexus of the oral food groove, which is thought to have a primarily sensory function – and in the aboral subsystem, represented mainly by the brachial nerve, which includes sensory and motor elements (Heinzeller and Welsch, 2001). In the aboral epidermis, which lacks a basiepithelial plexus, GLI was localised to certain cells resembling in their shape and distribution the columnar ciliated cells that are thought to have a sensory function (Heinzeller and Welsch, 1994) and to adjacent monopolar neuron-like cells that might be part of an afferent pathway to the brachial nerve.

Branches of the brachial nerve penetrate the brachial ossicles near each insertion region of the syzygial and diarthrial ligaments and innervate the juxtaligamental cell bodies at these locations. Welsch and colleagues (Welsch et al., 1995) found four types of neuronal varicosities making close contact with the juxtaligamental perikarya of the diarthrial ligaments, although functional contact between neurons and the juxtaligamental cells of the syzygial ligaments has still to be demonstrated (Holland and Grimmer, 1981). In this investigation, GLI was observed in the brachial nerve branches and associated cell bodies of both the syzygial and diarthrial ligaments of A. mediterranea. As both juxtaligamental and neuronal perikarya are present at the ligament insertions, electron cytochemistry will be required to determine which cell types are labelled. Immunoreactive granules were also present within both the syzygial and diarthrial ligaments. Although aligned longitudinally, as are the juxtaligamental cell processes, these granules were much sparser than the intracellular granules of juxtaligamental processes (e.g. Heinzeller and Welsch, 1994; Ferreri et al., 2003) and might therefore represent glutamaterigic terminals of neuronal axons directly innervating the juxtaligamental processes. However, glutamate storage vesicles are typically clear and small (diameter 30–40 nm) (Crivellato et al., 2005) and, although possibly present in the vicinity of juxtaligamental cell bodies (Welsch et al., 1995),
cell processes containing such vesicles have not been reported as occurring within the ligaments. The only other structures showing strong GLI were the brachial muscles. The immunoreactive granules on the surface of individual muscle fibres is clear evidence for their glutamatergic innervation. Heinzeller and Welsch (Heinzeller and Welsch, 1994) found three populations of nerve fibres within comatulid brachial muscles, one of which contained clear vesicles with a maximal diameter of 50 nm and, although more likely to be cholinergic, could include a glutamatergic subpopulation.

The immunocytochemical investigation demonstrated that L-glutamate is likely to be present in most anatomical components of the putative reflex that initiates syzygial destabilisation—that is, the epidermis, which contains sensory cells; the basiepithelial plexus; the brachial nerve; and the syzygial nerves. It has therefore provided further evidence that L-glutamate has a neurotransmitter role in the autotomy reflex. However, as GLI was involved solely in the autotomy response and that it might have a widespread role as a neurotransmitter in the crinoid nervous system. It is not known at present whether this is peculiar to crinoids and therefore possibly an ancestral feature, with the further implication that the prevalence of glutamatergic mechanisms declined during the evolution of the other surviving echinoderm classes, or whether it is a potent that intensive investigation of non-crinoid echinoderms will reveal L-glutamate to be as important a signalling molecule in the Echinodermata as it is in other phyla.

**CONCLUSIONS**

We have provided: (i) physiological evidence for the presence in the nervous system of *A. mediterranea* of glutamate receptors of the AMPA/kainate type, activation of which initiates the autotomy reflex, and (ii) immunocytochemical evidence for the presence of localised intracellular accumulations of L-glutamate at various sites in the neural pathway that is likely to mediate the autotomy reflex. We therefore conclude that it is highly probable that L-glutamate participates in the activation of arm autotomy. L-Glutamate is widely acknowledged to be the major excitatory neurotransmitter in vertebrate and invertebrate nervous systems (Pierobon et al., 2004; Kew and Kemp, 2005; Hatakeyama et al., 2007). There are even putative non-NMDA-like glutamate receptors in plants, which indicate that L-glutamate was utilized as a signalling molecule before plants and animals diverged (Chiu et al., 1999). However, the published literature provides only sparse evidence for L-glutamate having a neurotransmitter (or any) role in echinoderms. In addition to an assortment of physiological actions of uncertain significance and/or restricted taxonomic distribution (Ikegami et al., 1967; Gosselin and Jangoux, 1999; Janecki and Rakusus-Suszczewski, 2004), L-glutamate has been found to have an excitatory effect on a few neuromuscular preparations (Hill, 1970; Protas and Muske, 1980; Peters and Shelton, 1981) and directly triggers light emission in one ophiuroid species (although not in others that have been tested) (De Bremaeker et al., 1999; Mallefet et al., 2004).

In all these cases, the potency of L-glutamate is comparatively low—for example, in comparison with that of acetylcholine. L-Glutamate also appears not to have a phylum-wide role in the control of echinoderm autotomy as it exerts no influence on ophiuroid arm or disc autotomy mechanisms (Wilkie, 1978; Dobson, 1985).

The Crinoidea is the sister taxon to the other four living echinoderm classes and is the first of the extant classes to appear in the fossil record, its earliest representatives occurring in early orдовoisian deposits (Janies, 2001; Guensburg and Sprinkle, 2003). There is little doubt that palaeozoic crinoids could autotomise their arms at syzygial joints (Baumiller, 2008). It is therefore intriguing that the strongest evidence to date for L-glutamate-mediated neurotransmission in an echinoderm should relate to a neural reflex that might have been conserved for 500 million years. However, our immunocytochemical results indicate that L-glutamate is not involved solely in the autotomy response and that it might have a widespread role as a neurotransmitter in the crinoid nervous system.
Acknowledgements

References

I. C. Wilkie and others

REFERENCES

