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Strength of synaptic transmission at neuromuscular junctions of crustaceans and insects in relation to calcium entry

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ABSTRACT Crustacean and insect neuromuscular junctions typically include numerous small synapses, each of which usually contains one or more active zones, which possess voltage-sensitive calcium channels and are specialized for release of synaptic vesicles. Strength of transmission (the number of quantal units released per synapse by a nerve impulse) varies greatly among different endings of individual neurons, and from one neuron to another. Ultrastructural features of synapses account for some of the physiological differences at endings of individual neurons. The nerve terminals that release more neurotransmitter per impulse have a higher incidence of synapses with more than one active zone, and this is correlated with more calcium build-up during stimulation. However, comparison of synaptic structure in neurons with different physiological phenotypes indicates no major differences in structure that could account for their different levels of neurotransmitter release per impulse, and release per synapse differs among neurons despite similar calcium build-up in their terminals during stimulation. The evidence indicates differences in calcium sensitivity of the release process among neurons as an aspect of physiological specialization.

KEY WORDS: neuromuscular; crustacean; *Drosophila*; synapse; quantal release; calcium

Introduction

Variation in 'strength' of transmission at individual synaptic connections has been known for many years at crustacean neuromusuclar junctions (Atwood, 1965, 1967; Bittner, 1968; Atwood and Bittner, 1971) and amphibian neuromuscular junctions (Nudell and Grinnell, 1983; Walrond and Reese, 1985). Increasingly, similar types of synaptic variation are being found throughout the mammalian central nervous system (Schikorski and Stevens, 1997). Differential transmission from a nerve cell to its targets appears to be a wide-spread general feature of nervous systems. Operationally, it permits selective spatial and temporal recruitment of parts of the population of innervated target cells.

Crustacean neuromuscular systems have provided favourable models for investigation of this feature, due to the economy of innervation of individual muscles by motor neurons: often, one or two excitatory motor neurons control large muscles containing hundreds or thousands of muscle fibres, which produce a wide variety of movements in response to different patterns of impulses in the motor neurons. Investigation at the physiological level is aided by the large size and easy identification of the individual physiologically distinct neurons, together with the large range in synaptic performance among neurons and at different endings of the same neuron. More recently, increasing attention has been attracted by the *Drosophila* larval neuromuscular junction, which has been found to be similar in structure to those of crustaceans (Atwood *et al.*, 1993), and to provide tractable (though more difficult) preparations for physiological experimentation (Jan and Jan, 1976; Kurdyak *et al.*, 1994). The *Drosophila* preparation offers the advantage of a large background of information about specific genes in the nervous system, many available mutations of these, and many reagents for detecting and localizing specific molecules. Since crustaceans and insects are in the same branch of the arthropod phylum, knowledge and reagents gained from *Drosophila* can often be used to advantage in crustaceans. Conversely, physiological and structural information gained from crustaceans can be applied towards understanding the physiology of *Drosophila* synapses.

Specific objectives of the studies reported here are: 1. To define structural features of synapses underlying their physiological performance; 2. To determine whether differences in observed calcium entry can explain variations in synaptic strength. Recent measurements of calcium signals in crustacean and *Drosophila* nerve terminals, in conjunction with ultrastructural studies, have helped to define the sites of synaptic differentiation.

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General features of synapses and neurotransmission

Synapses

Ultrastructural studies of crustacean and insect neuromuscular junctions have been carried out for many years by various authors (Govind, 1982; Atwood, 1982; Atwood *et al.*, 1993). A good general picture of the characteristics of the excitatory and inhibitory synapses has developed (Atwood and Tse, 1993). They resemble more closely synapses in the central nervous system than neuromuscular junctions of vertebrates.

Crustacean and insect neuromuscular junctions differ from those of amphibian and other vertebrate skeletal muscles in having numerous small contact zones with the post-synaptic apparatus of the muscle fiber, rather than a continous large contact zone. The pre- and post-synaptic membranes of the contact zone appear highly electron-dense in transmission electron micrographs, and are separated by a uniform and invariant distance of about 20 nm referred to as the synaptic cleft (Jahromi and Atwood, 1974). Sometimes tenuous fibrils can be seen connecting pre- and post-synaptic membranes across the synaptic cleft. The contact zone can also be discerned as a differentiated region in freeze-fracture replicas (Pearce *et al.*, 1986; Walrond *et al.*, 1993). This differentiated contact zone, usually accompanied by many synaptic vesicles in the presynaptic nerve terminal, has been referred to as the 'synapse' or 'synaptic contact' in previous studies.

Random sections through crustacean or *Drosophila* neuromuscular junctions often exhibit sections of synapses in which the presynaptic membrane has an attached electron-dense structure (dense body) which is selectively associated with tethered or docked synaptic vesicles (Jahromi and Atwood, 1974). Subsequent observations, especially with freeze-fracture, have indicated that exocytosis occurs at the edges of these structures (Pearce *et al.*, 1986). They appear to be homologous to the 'active zone' structures observed at various other synapses (Dreyer *et al.*, 1973; Propst *et al.*, 1986; Westrum and Gray, 1986; Schikorski and Stevens, 1994). The number of active zone structures per synapse is variable: usually only 1 occurs, but some synapses have none, and others may have several (Jahromi and Atwood, 1974). Freeze-fracture studies have revealed in addition prominent membrane-associated particles at the same location, but not all are localized at the edges of the active zone region (Govind *et al.*, 1994). Studies of other synapses have provided good evidence that many of these particles are calcium channels (Pumplin *et al.*, 1981; Roberts *et al.*, 1990). Resolution and identification of specific active zone channel structures of arthropod synapses will require further study. A summary of our current view of synaptic structure, based upon crayfish studies, is given in Figure 1. *Drosophila* and crayfish synapses are remarkably similar in general structure (Atwood *et al.*, 1993).

Structural correlates of synaptic strength

The single excitatory motor axon innervating both the 'opener' and 'stretcher' muscles in the limbs of decapod crustaceans provides a good model for studying structural correlates of synaptic strength, since in many species, endings of the same neuron produce junctional potentials (EJPs) of very different amplitude in different regions of the muscle (Bittner, 1968;

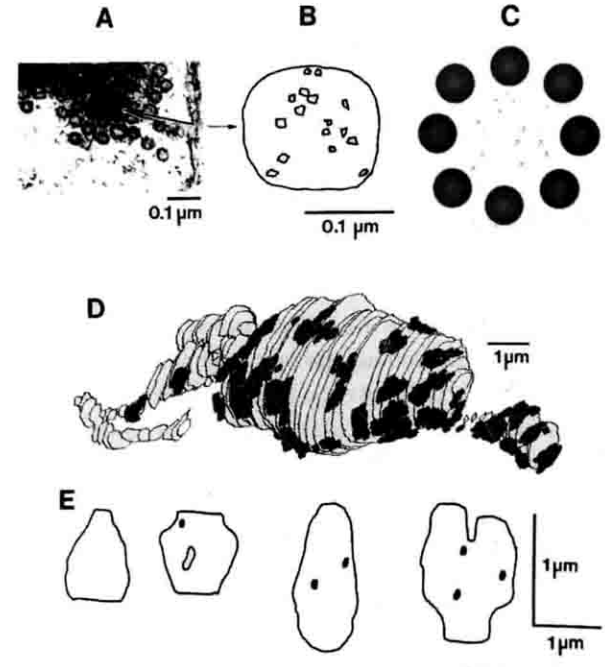

Fig. 1. Synaptic structure in the crayfish opener excitor motor axon. *Each varicosity of the nerve terminal possesses numerous synapses with variable active zone complement. The individual active zones* **(A)** *typically show a ring of tethered vesicles with a dense structure in the centre, as shown in electron micrographs of synapses in tangential sections near the surface of the presynaptic membrane. Within the active zone, freeze-fracture micrographs of the presynaptic nerve terminal membrane exhibit a variable number (typically 10-20) of membrane-associated large particles (putative calcium channels) as illustrated in* **B**. *These channel particles are at various distances from the tethered vesicles, as illustrated in the composite diagram of the active zone shown in* **C**. *Reconstructions of nerve terminal varicosities from serial electron micrographs* **(D)** *show many individual synapses (dark structures) on each varicosity. The individual synapses, seen in face view in reconstructions* **(E)**, *possess different numbers of active zones; those with more than one are termed 'complex' synapses (from Cooper et al., 1996b; and Kennedy, 1996).*

Atwood and Bittner, 1971; Cooper *et al.*, 1995). As illustrated in Figure 2, the endings of the single axon are more numerous on some fibers (in the central region) yet generate a small EJP at low frequency, while the endings on other fibers (in the proximal region) are less extensive, but generate a larger EJP. These differences are due in large measure to more quanta of transmitter released per impulse by the endings producing the larger EJPs (Cooper *et al.*, 1996a).

Several electron microscopic studies of this neuron, and of the single excitatory neuron to the accessory flexor muscle of the American lobster (Govind *et al.*, 1980; Walrond *et al.*, 1993), have consistently shown that synapses generating the large EJP have more active zones per synapse, and in some cases, larger active zones. As shown in Table 1, based upon work with the crayfish

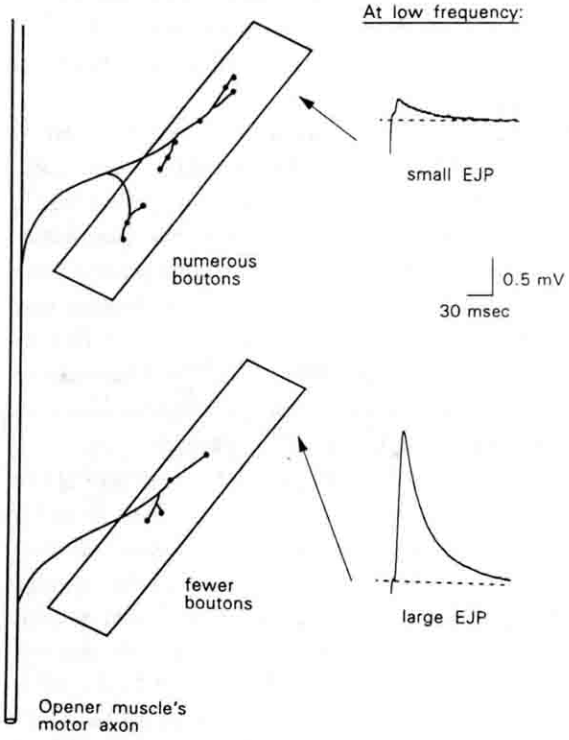

Fig. 2. Differential properties of individual nerve endings. *The crayfish opener excitor motor axon supplies endings of different gross morphology and physiology to different muscle fibers (after Cooper* et al., *1996a). The endings on proximal fibers (lower) generate a larger EJP than those on central fibers (upper).*

opener motor axon (Cooper *et al.*, 1996a), there is good general agreement between the ratios of EPSP amplitude and the proportion of 'complex' synapses (those with more than one active zone) in the central (low-output) and proximal (high-output) regions. Furthermore, stimulation-induced calcium signals from high-output terminals are larger than those from low-output terminals, indicating more calcium entry per synapse in the former (Cooper *et al.*, 1995). The hypothesis that emerges from these observations is that 'complex' synapses have a higher probability of transmission, and probably admit more calcium during stimulation, and thus endow the nerve ending that has more of them with a greater low-frequency transmitter-releasing capability (Cooper *et al.*, 1996b). Thus, structural features contribute to functional performance. Local trophic factors, possibly derived from the postsynaptic targets, are hypothesized to play a major role in establishing and maintaining the transmitter-releasing characteristics of the endings of a single axon (Frank, 1973; Davis and Murphey, 1994).

Parts of the structural hypothesis have been tested in *Drosophila* by examining structure and function of an identified neuron (RP3 neuron) innervating ventral abdominal longitudinal muscle 6. A hypomorphic mutant which reduces expression of the adhesion molecule FasII reduces the number of varicosities at the neuromuscular junction, but synaptic transmission is maintained at the normal level (Stewart *et al.*, 1996). The reason for this is that individual synapses are larger and have more active zones per synapse in the mutant; correspondingly, the quantal emission per varicosity is enhanced. In another study (Renger *et al.*, 1997), it has been found that the *rutabaga* (rut^1) mutation, which reduces the level of cyclic AMP in the nervous system, exhibits lower than normal transmission (Zhong and Wu, 1991), possibly because the number of docked vesicles is reduced. Individual synapses of this mutant are larger than normal and have more active zones. In this case, the synaptic structure responds adaptively to genetically imposed handicaps to neurotransmission: generation of larger, more complex synapses would help to maintain transmission at a viable level despite the cyclic AMP deficit, which restrains the effectiveness of transmission.

The structural hypothesis for synaptic function, developed to explain differences in performance among endings of one neuron (Fig. 2), cannot adequately account for differences in transmission observed in distinctive 'phasic' and 'tonic' motor neurons innervating the same target (Fig. 3). In the leg extensor muscle of the crayfish, phasic EJPs are typically much larger than tonic EJPs (Fig. 3). Focal recordings obtained with macro-patch electrodes during a low frequency of stimulation (1 Hz) at a single site where both terminals are present typically show differences in quantal content of at least 50-fold (Bradacs *et al.*, 1997), and sometimes more than 1000-fold (Msghina *et al.*, 1998). However, the individual synapses of the phasic axon are not larger or more numerous at such locations (Table 1; King *et al.*, 1996). Limited freeze-fracture data from phasic synapses suggest that the number of prominent membrane particles is also not greater. This raises the question: Do the individual active zones of the phasic nerve

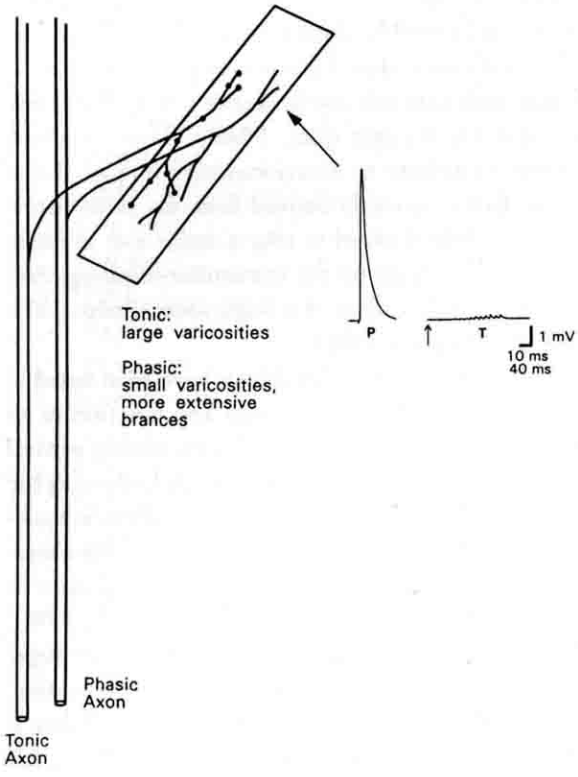

Fig. 3. Differential properties of different neurons. *The crayfish leg extensor muscle is supplied by phasic and tonic neurons which innervate the muscle fibers with nerve endings differing greatly in morphology and physiology (after Bradacs* et al., *1997). Phasic endings are more extensive but thinner than tonic endings, and generate a much larger EJP. P, phasic EJP, single stimulus (10 ms time scale); T, tonic EJPs, train of stimuli (40 ms time scale).*

terminals admit more calcium (due to higher probability of calcium channel opening), and is this sufficient to account for the large differences in transmitter release?

The same issue arises for *Drosophila* neurons: quantal release per synapse differs among neurons, but the structural features of the synapses are not different (Atwood *et al.*, 1993). The question of whether differences in calcium entry can account for the diversity of transmitter release seen for different neurons is considered next, taking *Drosophila* observations as an example.

Calcium entry and strength of neurotransmission

Differences in release at *Drosophila* motor nerve terminals

The longitudinal muscles (muscles 6 and 7) of *Drosophila* larvae are innervated by two excitatory motor axons which generate EJPs of different amplitude: motor neuron RP3 generates a smaller EJP than motor axon 6/7b, despite its larger terminal varicosities (Kurdyak *et al.*, 1994). Motor axon 6/7b supplies type Is ('small') boutons to these muscles; these are smaller in diameter than those of the motor axon of the RP3 neuron, which supplies type Ib ('big') boutons (Atwood *et al.*, 1993).

Three explanations have been put forward to explain the differences in EJP amplitude. First, the greater number of Is than Ib boutons innervating muscle 6 (Kurdyak *et al.*, 1994) collectively release more transmitter onto postsynaptic receptors and generate the larger EJP. Second, and non-exclusively, Is boutons may also release more transmitter per impulse than Ib boutons. Third, postsynaptic effects, such as differences in the density of synaptic postsynaptic receptors, may contribute to the differences in EJP amplitudes.

We tested the second possibility by measuring the number of quanta released at individual Type Ib and Is boutons in a newly developed physiological solution (Stewart *et al.*, 1994). Determination of the average number of quanta released per impulse (mean quantal content,) revealed no significant differences between the two bouton types (Ib: 2.7 ± 0.5, n = 19; Is: 2.0 ± 0.3, n = 15). However, if we divide the values for for the Ib and Is bouton, respectively, by the average number of synapses each type possesses (Ib = 40 synapses, Is = 7

	Quantal content per varicosity, 1 Hz	Quantal content per synapse	Mean synapse contact area (μm^2)	Active zones per 100 synapse profiles	% complex synapses
(a) Opener motor axon: high- and low-output terminals					
Central (Low output)	0.15	0.006	0.30	15	8
Proximal (High output)	0.75	0.028	0.36	36	40
Ratio High/Low	5	4.7	102	204	5
(b) Leg extensor motor axons: phasic and tonic					
Phasic	9.5	0.2	0.2	-	50
Tonic	0.06	0.006	0.25	-	16
Ratio Phasic/Tonic	210	333	0.8	-	3.1

(Data from: Cooper *et al.*, 1996a; King *et al.*, 1996; Msghina *et al.*, 1998).

Table 1. Comparison of physiological and structural features for crayfish synapses.

synapses; Atwood *et al.*, 1993), we obtain a different release efficacy per synapse for the two bouton types. The release efficacy of the Is synapse, (estimated as 0.29 quanta per synapse per impulse) is approximately 4 times greater than that of the Ib synapse (0.07), suggesting more effective transmission at individual Is synapses.

Calcium build-up in *Drosophila* nerve terminals

The question as to whether greater effectiveness of transmission for Is synapses results from a larger Ca^{2+} signal was addressed in *Drosophila* boutons. Using fluo-3 as the indicator, we obtained Ca^{2+} signals (monochromatic fluorescence changes, expressed as increase in calcium concentration during stimulation relative to the resting level) in experiments in which both bouton types innervating muscle 6 were visible in the same field of view (Karunanithi *et al.*, 1997; Fig. 4A). The average time courses of the Ca^{2+} signals are shown in Figure 4B for the two bouton types at three frequencies of stimulation (5, 10 and 20 Hz). The Ca^{2+} signals increased in amplitude significantly with increasing frequency for both bouton types, but there were no significant differences in the average Ca^{2+} signal between the two bouton types at 5 and 20 Hz, although a significant difference emerged at 10 Hz.

Because of the similarity of the gross cytoplasmic Ca^{2+} signals for the two bouton types, differences in synaptic efficacy between them may arise due to other intraterminal factors. If we examine the plot of the peak Ca^{2+} signal versus bouton diameter (Fig. 5) there is a linear relationship between the two for both bouton types. There is no indication of a marked difference in calcium signals in the two neurons, when bouton size is taken into account. Type Ib boutons are larger in average diameter and possess 5 times more synapses on average than Is boutons (Atwood *et al.*, 1993). Thus, although the largest Ib boutons (Fig. 5, open circles) give rise to larger Ca^{2+} signals than Is boutons (Fig. 5, filled circles), indicating calcium entry through available synaptic active zone calcium channels, a smaller proportion of Ib synapses release transmitter for each nerve impulse. This suggests that much of the Ca^{2+} entering may not acutely actuate transmitter release, and that the probability of release for a given amount of Ca^{2+} entry is lower for Type Ib boutons. Factors such as calcium sensitivity of the release process, possibly linked to qualitative or quantitative differences in synaptic proteins, may differ between the two bouton types, yielding the observed difference in synaptic efficacy.

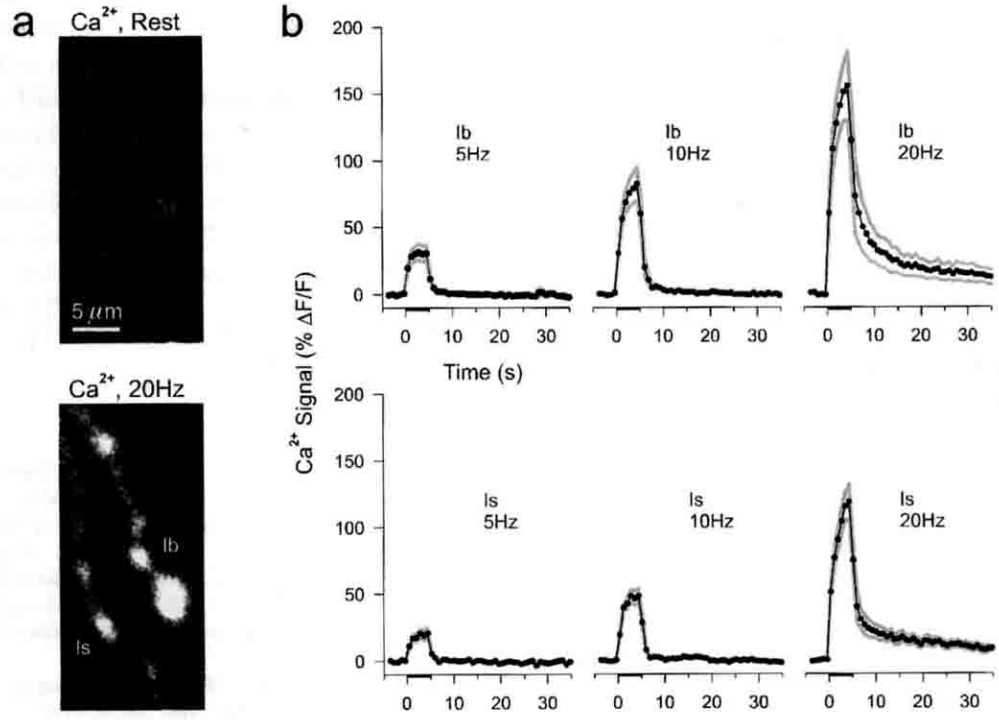

Fig. 4. Comparison of Ca^{2+} signals between Type Ib and Is boutons in *Drosophila*. **A)** *Ca^{2+} fluorescence at rest (top image) and during a 20 Hz stimulus (bottom image) for type Ib and Is boutons. Images taken with confocal microscopy.* **B)** *Effects of stimulation frequency on Ca^{2+} signals averaged for four experiments in which both type Ib (n = 9) and Is (n = 10) boutons were visible and responded in the same field of view, including the experiment imaged in (A). Trains lasting 5 s were delivered at time zero (indicated by bar) at stimulation frequencies of 5, 10, and 20 Hz. The Ca^{2+} responses were averaged and plotted by bouton type (black). The upper and lower boundaries for the standard error of the mean are plotted in grey (from Karunanithi* et al., *1997).*

Discussion

The current information from crustacean and insect neuromuscular junctions indicates conservation of the basic synaptic 'plan' of these systems, but considerable refinement in detail, resulting in wide differences in physiology. For a single 'tonic' neuron, substantial differences in transmitter output are associated with synaptic structural differences. There is a good correlation between the relative occurrence of 'complex' synapses and the differences in quantal content (Table 1). The higher incidence of 'complex' synapses correlates also with a larger calcium entry as detected by the relative build-up of calcium in varicosities during stimulation (Cooper *et al.*, 1995). For the case of high- and low-output terminals of one axon, it is reasonable to hypothesize that synaptic structure is an important variable related to synaptic efficacy.

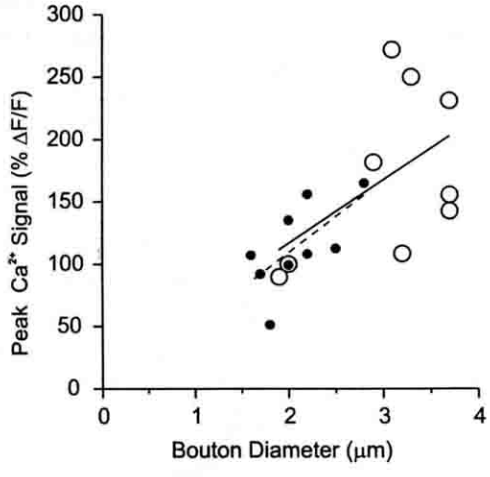

Fig. 5. Peak Ca^{2+} signal versus bouton size in *Drosophila*. *The peak Ca^{2+} signal during 20 Hz stimulation is plotted against the bouton diameter for both Ib (open circles) and Is (filled circles) boutons in four experiments. Regression lines have been fitted to the points representing the two bouton types (Ib: open circles and solid line, slope = 50.75, r = 0.26; Is: closed circles and dotted line, slope = 58.09, r = 0.42). The two types show similar calcium signals for boutons of the equivalent size, but the largest signals are from the larger Ib boutons.*

However, when we compare different neurons innervating the same target, such as those supplying the ventral longitudinal muscles of larval *Drosophila*, and the phasic and tonic neurons of crustacean limb muscles, a simple structural explanation is inadequate. For crayfish phasic and tonic neurons, the output of quantal units per synapse for one impulse at low frequencies of stimulation is typically 2 to 3 orders of magnitude greater for the phasic neuron, and this does not correlate with the general ultrastructural features of the synapses (Table 1). In *Drosophila*, the two neurons examined in detail were found to differ in their quantal output per synapse, but the calcium build-up during stimulation is similar (Fig. 5). The observed calcium signals represent the equilibrated residual calcium in the boutons following the dissipation of high-intensity local calcium domains (Llinas *et al.*, 1992) formed at synapses during opening of calcium channels in the active zone; this equilibration is thought to occur very rapidly (Delaney and Tank, 1994). Since the kinetics of build-up and subsidence of the calcium signals are similar in the two bouton types (Fig. 4), it is likely that there are not major differences in processes determining equilibration and removal of calcium. Thus, the lower efficacy of quantal release per synapse in Ib boutons is most likely releated to differences in calcium sensitivity of the release process, rather than to differences in entry of calcium at active zones and its subsequent build-up and removal from the terminal. The working hypothesis for synaptic differentiation is that calcium-dependent components of the release machinery at the active zone have different sensitivity to calcium in physiologically distinctive neurons.

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