Primary Afferent Depolarizations of Sensory Origin Within Contact-Sensitive Mechanoreceptive Afferents of a Crayfish Leg

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1 Centre National de la Recherche Scientifique, Laboratoire Neurobiologie et Mouvements, 13402 Marseille Cedex 20, France; and 2 Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland

Marchand, A. R., W.J.P. Barnes, and D. Cattaert. Primary afferent depolarizations of sensory origin within contact-sensitive mechanoreceptive afferents of a crayfish leg: properties, quantal analysis, and role in presynaptic integration. J. Neurophysiol. 77: 3340–3354, 1997. Recordings from the central branches of single identified dactyl sensory afferent (DSA) neurons in a crayfish in vitro preparation were performed to study modifications of the sensory message occurring before the first central synapse. These afferents comprised hairs and force-sensitive mechanoreceptors with phasic and phasotonic response characteristics in the terminal segment (dactyl) of the crayfish leg. More than one afferent spike size was often observed in intracellular recordings from these afferents, thus indicating the presence of electrical coupling between the central processes of DSA fibers. Additionally, in identified DSA fibers with large spike sizes, primary afferent depolarizations (PADs) of up to 15 mV were observed, which sometimes triggered antidromic spikes in the afferent. Nevertheless, PADs were clearly inhibitory, because they shunted the afferent spikes. They exhibited the following properties. First, each PAD was preceded by an afferent spike from a neighboring hair, indicating that the PADs had a sensory rather than central origin. Second, PADs could follow high frequencies of afferent discharges without failure, a property suggestive of monosynaptic connections, but because PAD latencies varied by ±0.5 ms it is more likely that they were mediated by a disynaptic pathway. Third, although PADs were evoked in an extremely reliable manner, their amplitude varied in a quantal manner. Most unitary PADs were the result of the release of <12 quanta, the mean quanta content lying between 4 and 5; quantal size was large, ~1 mV. Fourth, PADs showed facilitation in some fibers, whereas in others they became much smaller when occurring at brief intervals. We suggest that PADs may be an efficient and parsimonious way to limit sensory inflow in space and time, allowing the crayfish to identify precisely both weak and strong mechanical stimuli.

INTRODUCTION

There is evidence in many species that primary sensory afferents are subject to very early modulation, in particular through presynaptic inhibition. Such mechanisms have been demonstrated on both anatomic (Maxwell and Noble 1987; Ralston et al. 1984; Watson and Pflüger 1994) and physiologic grounds (Frank and Fuortes 1957; Janig et al. 1968; Nicoll and Alger 1979; Rudomin 1990a,b; Ryall 1978; Schmidt 1971). The most convincing evidence arises from intracellular recordings from primary afferent fibers, which have been obtained mostly in invertebrates such as mollusks (Shapiro et al. 1980; Small et al. 1989), insects (Blagburn and Sattelle 1987; Boyan 1988; Burrows and Laurent 1993; Burrows and Matheson 1994; Levine and Murphey 1980), and crustaceans (Cattaert et al. 1990; Kennedy et al. 1974; Kirk 1985; Marchand and Leibrock 1994; Sillar and Skorupska 1986), but also more recently in mammals (Gossard et al. 1989; Jimenez et al. 1993; Cattaert et al. 1992; Davidoff 1972; El Manira and Clarac 1991). Presynaptic inhibition of sensory afferents often manifests itself in intracellular recordings in the form of depolarizing events known as primary afferent depolarizations (PADs) (Eccles et al. 1962; Hue and Callec 1983). PADs are known to be inhibitory because they reduce the size of afferent spikes in the presynaptic neuron (Eccles et al. 1963; Kennedy et al. 1974; Levine and Murphey 1980) as well as the amplitude of synaptic potentials in the postsynaptic neuron (Blagburn and Sattelle 1987; Burrows and Laurent 1993; Cattaert et al. 1992). They thus directly affect transmission between the first- and second-order elements of the sensory system. PADs often result from an increase in membrane chloride conductance due to GABAergic synapses. Their reversal potential lies somewhat above resting potential (−77 mV) and is linked to the execution of a motor program, such as locomotion. A synaptic drive to primary afferents, which is phase-linked to the centrally generated locomotor rhythm, has been seen in mammals (Gossard et al. 1989) as well as in crustaceans (Cattaert et al. 1990; Sillar and Skorupska 1986). This is thought to adapt reflexes to the needs of the ongoing movement (El Manira et al. 1991a,b). In crustacean and insect escape systems, a similar blockade of the afferent input is thought to prevent reafferent signals from the escape movements from eliciting inappropriate reflexes. This block may be of central origin, namely associated with the motor command (Kirk and Wine 1984; Krasne and Bryan 1973), or of proprioceptive origin (Boyan 1988; Fricke and Kennedy 1983).

Of direct relevance to this study are observations that both exteroceptive and proprioceptive sensory fibers in arthropods can be presynaptically inhibited as a result of activity in the same sense organ (Burrows and Laurent 1993; Burrows and Matheson 1994; Kennedy et al. 1974; Levine and Murphey 1980). This phenomenon sometimes fits a pattern of lateral inhibition (Blagburn and Sattelle 1987; Burrows and Matheson 1994). Somewhat comparable interactions have been described in mammals, for instance between cutaneous affer-
ents (Janig et al. 1968) and between muscle afferents of groups Ia or Ib, although more complex patterns exist (Rudomin 1990a,b).

In the present study, intracellular recordings from the central processes of crustacean exteroceptive primary afferent fibers were obtained for the first time, in an in vitro preparation of a crayfish locomotor system. The dactyl sensory afferents (DSAs) are fibers originating from the terminal leg segment (the dactyl). They respond to mechanical stimuli such as bending of a hair or force applied to the cuticle. They mostly project through polysynaptic pathways to motorneurons of the levator group (Clarac et al. 1991) as well as to depressor motorneurons of adjacent ipsilateral legs. They are thought to intervene in locomotion by contributing a powerful synchronizing signal when the leg contacts or lifts off the ground (Müller and Clarac 1990a,b). We show here that DSA fibers present both electrical synapses and short inhibitory pathways between their central terminals.

In a few experiments, large unitary PADs were recorded for long enough to study their properties. We show that PADs recorded in DSAs can exhibit both synaptic depression and synaptic facilitation, and that their amplitude also varies spontaneously. When subjected to quantal analysis, clear amplitude peaks appear on histograms of unitary PADs. Although the data do not fit either Poisson or binomial distributions, our results do provide, for the first time, estimates of both the quantal size and quantal content of unitary PADs under physiological conditions, showing that PADs apparently arise from release sites in close spatial proximity. PADs may be produced by a restricted number of interneurons, but their short latency, high reliability, and amplitude suggest they play a crucial role in the integration of exteroceptive information.

Preliminary accounts of some of these experiments have already appeared (Barnes et al. 1993, 1995a,b).

**METHODS**

**Animals**

Both male and female crayfish (*Procambarus clarkii*), ~10 cm long from rostrum to telson, were used in the experiments (*n* = 23). They were obtained from a commercial supplier, maintained in aerated aquaria at room temperature (18–21°C), and fed once a week.

**Preparation**

An in vitro preparation of the crayfish ventral nerve cord, comprising the third, fourth, and fifth thoracic ganglia together with a fifth walking leg, was used as described in Marchand and Barnes (1992). In brief, after exposure of the nerves to the promotor, remotor, levator, and depressor leg muscles in addition to the anterior and posterior distal roots (N1AV and N1PV of Elson 1996) that go to the distal regions of the leg, the preparation was pinned down on a Sylgard-lined petri dish. The ganglion was desheathed and bathed in cool oxygenated saline composition, in mM: 195 NaCl, 5.4 KCl, 13.5 CaCl₂, 2.6 MgCl₂, and 10 tris(hydroxymethyl)aminomethane adapted from that of Van Harreveld (1936). The dissection was placed either dorsal side up or dorsal side down (to facilitate intracellular penetration of DSA afferents).

The dactyl was then restrained by means of small steel staples, and the propodite was carefully dissected until the cuticle could be detached. The opener and closer muscles were then removed to expose the DSA nerves. These consist of four thin branches that emerge from the dactyl as a ventral and a dorsal pair. En passant monopolar platinum wire electrodes insulated with Vaseline were placed alongside each pair of nerves for stimulation and recording purposes (Fig. 1A).

**Mechanical stimulation**

The responses of DSA afferents to mechanical stimuli were evaluated by a combination of extracellular and intracellular re-
cordings. An electromechanical transducer (Ling Dynamic Systems, Model V101) with a fine glass probe was used to apply pressure onto the dactyl for the stimulation of force-sensitive afferents. Hairs were stimulated selectively whenever possible by placing the transducer probe against the hair tip and deflecting it inward or outward (toward or away from the dactyl). Tufts of hairs were identified by reference to a standard diagram (Fig. 1C), individual hairs being numbered in order of decreasing size within each tuft. This allowed precise identification of individual hairs both within an experiment and across subjects.

Extracellular and intracellular recordings

In extracellular recordings from the DSA nerves, dactyl afferents were identified by spike size, shape, and pattern of discharge. Square waves, triangles, and ramp-and-hold stimuli were used, the latter to specify in more detail the dynamic characteristics of the stimulated fibers.

Intracellular recordings from DSA fibers were obtained in the fifth thoracic ganglion with the use of micropipettes filled with 3 M KCl (resistance 15--30 MΩ). The fibers were impaled at the margin of the ganglionic core, near the point of entry of the nerves (Fig. 1B). Intracellular recordings were identified as being from a single DSA fiber on the basis of two criteria. First, that intracellular depolarizing current pulses that evoked a spike in the fiber should also evoke an antidromic impulse after a fixed conduction time in the DSA sensory nerve. Second, that selective mechanical stimulation of the dactyl, or low-threshold electrical activation of the sensory nerve, should evoke a normal intracellular spike at a fixed time after each extracellular impulse in the DSA sensory nerve.

Anatomic studies

The anatomy of DSA fibers was studied by intracellular injection of Lucifer yellow. This was done by applying a ~5 nA constant current for 20--30 min, then removing the electrode. The nerve was then crushed close to the ganglion to prevent the dye from spreading to the axon. The preparation was fixated overnight in 1% paraformaldehyde after a rest of 1 h and subsequently dehydrated in a series of alcohols. It was then cleared in methyl salicylate and examined in whole mount preparation under a fluorescence microscope. The branching pattern of the fibers was drawn by projecting in turn a series of photographic slides taken of the preparation at different planes of focus.

The distribution of fiber diameters in the DSA nerves was studied from 1m sections of the nerves prepared as follows. After dissection and removal of all connective tissue, the nerves were fixed in a 0.1 M cacodylate buffer containing 2.5% glutaraldehyde and 0.5% paraformaldehyde, rinsed four times in 0.1 M cacodylate buffer (pH 7.6), and postfixed by buffered 2% osmium tetroxide. After repeated rinsing, the preparations were dehydrated in a series of alcohols and in a propylene oxide solution. They were then who derived a correction factor to apply for the calculation of the latter to specify in more detail the dynamic characteristics of the stimulated fibers.

The complete theoretical distribution, allowing for variations in size of individual quanta, and a Gaussian distribution, for quantal content (number of quanta released), and a Gaussian distribution, allowing for variations in size of individual quanta, as follows.

The mean $m$ of the Poisson distribution (mean quantal content of a PAD) is the average amplitude of a PAD, $E$, divided by the unitary size

$$m = E/q$$

If $N$ is the total number of events (PADs) in the histogram, the predicted number of events ($N_n$) in each peak of quantal number $x$ is then

$$N_n = N \cdot (m^n/x^x) \cdot e^{-m}$$

To predict how these events are distributed between different bins in a histogram, it is necessary to allow for variations in quantal size. To do this, the largest peaks of the histogram were fitted to a Gaussian curve scaled in width to have a variance proportional to quantal size, and scaled in height so that its area corresponded to the predicted number of events (Del Castillo and Katz 1954). From the mean ($\mu$) and standard deviation ($\sigma$) of the Gaussian curve, the content ($f$) of each bin ($y$) is given by Eq. 3

$$f(y) = \frac{1}{\sqrt{2\pi}\sigma} \exp \left[-\frac{1}{2} \left( \frac{y - \mu}{\sigma} \right)^2 \right]$$

The complete theoretical distribution, allowing for variance and peak overlap, was then obtained by pooling the Gaussians for all peaks. In this way, the theoretical distribution could be superimposed on the histogram for direct comparison with the data.

In addition to comparisons with a Poisson distribution, the data were compared with two binomial distributions (each using a different estimate of the total number of releasable quanta, $n$), calculated according to Eq. 4, where $p$ (the average probability of release) equals $m/n$

$$N_n = N \left( \frac{n!}{(n-x)!x!} \right) p^n (1-p)^{n-x}$$

Because PADs shunt the membrane, large PADs produce a smaller change in voltage than a simple multiple of the unitary quantal size. This problem has been addressed by Martin (1955), who derived a correction factor to apply for the calculation of $m$. Our calculation (see below) is a transform of voltages into steps, is simply

$$i = 0 = g_{rest}(V - V_{rest}) + g_{PAD}(V - E_{PAD})$$

Therefore $g_{PAD}/g_{rest}$, which may be assumed to vary in quantal steps, is simply

$$g_{PAD}/g_{rest} = (V - V_{rest})/(E_{PAD} - V)$$

Taking a numerical example with a very large (15 mV) PAD, we
FIG. 2. Identification of a DSA terminal and intracellular events. A: responses to selective displacement of B hairs recorded extracellularly in ventral DSA nerve (DSA) and intracellularly from central region of corresponding fiber (DSA-t). Five frames, triggered on afferent spike (1) in intracellular trace, are superimposed. Resting membrane potential of fiber: $-73 \text{ mV}$, intracellular spike was invariably associated with an impulse of particular shape in DSA nerve, with a fixed latency (here 22 ms) corresponding to conduction time. B: recording conditions as in A, triggering frames on smaller events of short duration (coupled spikes) in intracellular trace (2). In extracellular recordings, these corresponded to impulses of a shape different from normal afferent spike (1). Four superimposed frames show that coupled spikes also had a fixed latency (24 ms).

We can calculate the extra conductance that PADs produce by means of Eq. 5B

$$V_{\text{rest}} = -72 \text{ mV}$$

$$E_{\text{PAD}} = -26 \text{ mV}$$

$$V = \frac{V_{\text{rest}} + E_{\text{PAD}} + g_{\text{PAD}}}{g_{\text{rest}}}$$

Conversely, a voltage $V$ can be calculated from the ratio $g_{\text{PAD}}/g_{\text{rest}}$.

$$V = \frac{V_{\text{rest}} + E_{\text{PAD}}}{1 + \frac{g_{\text{PAD}}}{g_{\text{rest}}}}$$

We can set equally spaced bin limits in the conductance domain, then convert these into voltages with the use of Eq. 5C. The histogram constructed with this nonlinear scale allows for the diminution of PAD amplitude due to PAD conductance.

The observed numbers of events in successive peaks of the histograms were compared statistically with the theoretical distributions with the use of $\chi^2$ (ignoring peaks with a predicted number of events of $<5$) and Kolmogorov-Smirnov tests.

RESULTS

Identification of afferents and events in intracellular recordings

24 DSA fibers, identified as described in METHODS, were penetrated intracellularly in the fifth thoracic ganglion (Fig. 1B). An intracellular recording from a hair afferent is shown in Fig. 2A. An intracellular spike of large size (here $\sim 50 \text{ mV}$) can be seen to follow, at a constant latency (here 22 ms), an impulse of fixed shape in the DSA nerve. The conduction velocity of DSA afferents was found to be in the range $0.7-3.3 \text{ m/s}$, the distance from the recording point on the nerve to the ganglion being usually $\sim 40 \text{ mm}$. Figure 2B shows the existence of a second afferent spike shape, identified on the extracellular nerve recordings, which produced an intracellular depolarizing event of small amplitude but short duration ($<5 \text{ ms}$) in the same afferent fiber. The amplitude and latency of this depolarizing event (coupled spike) were constant. This differentiates coupled spikes from a third type of events in the intracellular recordings, namely PADs (see below), which had variable amplitudes and durations of $\approx 15 \text{ ms}$. On the basis of studies of other sensory organs of crayfish (El Manira et al. 1993; Marchand and Leibrock 1994) and the results of dye filling (see below), the coupled spikes are attributed to the presence of electrical synapses between the central branches of sensory afferents. They represent the image, through the junctional resistance, of a normal afferent spike in a neighboring fiber. Coupling was observed in $\approx 13$ of 24 recordings, but it has not yet been possible to compare systematically the response characteristics of coupled afferents.

Response characteristics of the DSA

The characteristics of the responses of DSA neurons to mechanical stimuli were identified from extracellular recordings and classified into hair afferents and force-sensitive afferents. Hair afferents were found to possess phasic, phasotonic, or tonic characteristics of discharge, phasic units being in the majority. These different types were distinguished by means of ramp-and-hold stimuli (Fig. 3, A and B). Both the phasotonic and phasic hair afferents in Fig. 3 responded vigorously to inward movement of the appropriate hair, although afferents responding to both directions of movement were occasionally found. The afferent in Fig. 3A possessed some positional response characteristics (i.e., it was phasotonic), because it continued to discharge at a slower rate as long as the hair was not returned to its resting position. The afferent in Fig. 3B, on the other hand, discharged only as
long as movement in the appropriate direction was applied, and was thus a phasic unit.

Most force-sensitive afferents primarily encoded the rate of change of force applied to the dactyl rather than force itself, because the response did not persist during the plateau of ramp-and-hold stimuli. Each stimulated afferent was found to be selective for either increase or decrease of force, i.e., “on” or “off” (only 1 unit was found that responded to both), and relatively insensitive to the intensity of static force applied. Figure 3, C and D, is representative of this phasic type of response from force-sensitive afferents. It shows the discharge rate to be directly related to the rate of change of force. This is in keeping with the in vivo activity of DSA afferents, which primarily encode the instants of contact and of liftoff of the tip of the leg in relation to the ground (Müller and Clarac 1990a,b).

Composition of DSA nerves

To estimate the number and size distribution of DSA neurons, the branches of the DSA nerve were sectioned at the distal end of the propodite, where the dorsal pair of nerves fuses (Fig. 4). The vast majority of the total of 1,358 axons that comprise these nerve branches were very small, >55% having a diameter of ≈2 μm. There was also a small number of larger axons that appeared to belong to a different population. These larger fibers (6–11 μm diam) number ≈70 (5% of the total) and probably represent the mechanoreceptor fibers, the rest being putative chemoreceptors. There are ≈100 hairs on the dactyl. Some of them have been found to be dually innervated, but others were physiologically unresponsive. Certainly, the proportion of mechanoreceptive afferents appears lower than in crabs, where they form the upper 10% of the population (Gnatzy et al. 1984).

Branching pattern of DSA fibers and dye coupling

Morphological reconstruction of DSA fibers within the ganglion was achieved by dye filling with Lucifer yellow. Figure 5 is a drawing of the branching pattern of two fibers, each of which divides into many small branches. In this dorsoventral projection, the branches seem to cover more
unusual origin. Whereas PADs recorded in crayfish chordotonal organ afferents were of central origin (El Manira et al. 1991b), no PADs were seen in DSA fibers in the absence of mechanical or electrical stimulation. PADs in DSA fibers were linked to extracellular afferent impulses in the DSA nerve. They occurred in both hair (Fig. 6A) and force-sensitive (Fig. 7A) afferents, and could usually be evoked by electrical stimulation of the DSA nerves. On many occasions, it was possible to identify the sensory structure from which the PADs originated. In Fig. 6A, for instance, PADs followed sensory impulses resulting from mechanical stimulation of identified sensory hairs on the dactyl. In all cases, the PADs followed the sensory impulses or electrical stimuli one-to-one at a more or less constant latency (see below).

That PADs were inhibitory was confirmed by the observation that the size of an intracellularly recorded afferent spike was reduced when it occurred simultaneously with a PAD (Fig. 7A). Similarly, during a train of electrical stimuli applied to the DSA nerve, which generated both

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A major feature of intracellular recordings of DSA fibers was the presence of PADs with amplitudes of up to 15 mV and durations of $\approx 15$ ms (Fig. 6A). These PADs had an unusual origin. Whereas PADs recorded in crayfish chordotonal organ afferents were of central origin (El Manira et al. 1991b), no PADs were seen in DSA fibers in the absence of mechanical or electrical stimulation. PADs in DSA fibers were linked to extracellular afferent impulses in the DSA nerve. They occurred in both hair (Fig. 6A) and force-sensitive (Fig. 7A) afferents, and could usually be evoked by electrical stimulation of the DSA nerves. On many occasions, it was possible to identify the sensory structure from which the PADs originated. In Fig. 6A, for instance, PADs followed sensory impulses resulting from mechanical stimulation of identified sensory hairs on the dactyl. In all cases, the PADs followed the sensory impulses or electrical stimuli one-to-one at a more or less constant latency (see below).

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**Circuits involved in PADS production**

The mechanical stimuli producing PADS onto a hair afferent fiber could be systematically mapped (Fig. 6B). As Fig. 6 illustrates, the PADS tended to originate from hairs located in the same general region of the dactyl as the penetrated afferent, and to appear compatible with a circuit for generating lateral inhibition. Note additionally that coupled spikes also originate from hairs in the same region of the dactyl.

The length of the inhibitory pathway between DSA afferents responsible for PADS was investigated with the use of criteria of latency and reliability. When trains of electrical

PADs and afferent impulses, the amplitude of orthodromic spikes was inversely related to the depolarization produced by the compound PAD (Fig. 7B). This is analogous to the shunting effect observed in afferents from a crayfish chordotonal organ during centrally produced PADS (Cattaert et al. 1992).

PADs sometimes depolarized a cell enough to generate antidromic spikes in the DSA fiber (Fig. 8A). The reversal potential of this inhibitory event must therefore be higher than the firing threshold of the cell. Estimates of the PAD reversal potential were obtained by intracellular current injection. As expected, application of a constant hyperpolarizing current led to increases in PAD size, and vice versa (Fig. 8B). Extrapolation from a plot of PAD amplitude against membrane potential gave a value of $-26 \text{ mV}$ for the PAD reversal potential (Fig. 8C). This may be an overestimate, because the PAD may have originated at some distance from the site of current injection, but it is identical to the reversal potential of $\gamma$-aminobutyric acid (GABA)-induced depolarizations in crayfish chordotonal afferents (Cattaert et al. 1992). Such a value suggests that PADS are chloride mediated, as has been shown in other sensory afferents of crayfish (El Manira and Clarac 1991).

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With the use of twin stimuli, or when afferent spikes occurred spontaneously as pairs during mechanical stimulation (Fig. 9B), PADs were occasionally seen to occur separated by intervals $< 5$ ms. This ability to follow high frequencies of stimulation or paired stimuli at short intervals would normally suggest the existence of a monosynaptic pathway (Berry and Pentreath 1976). However, the latency between the afferent impulse and the resulting PAD showed variations over a range of $\approx 1$ ms (Fig. 9C), a characteristic of polysynaptic pathways. The most likely interpretation of this combination of properties, two associated with monosynaptic connections and a third with polysynaptic pathways, is that DSA afferents make a highly reliable synapse onto an interposed inhibitory interneuron (PADI). Indeed, in other experiments (Cattaert and Barnes, unpublished data), we have occasionally recorded from spiking interneurons whose firing could be correlated one-to-one with PADs, with a fixed latency. The pathway from DSA afferents to other DSA afferents is therefore disynaptic, rather than either monosynaptic (which would anyway not have been expected given that PADs are inhibitory) or polysynaptic.

Two types of circuits, each of which could account for some of the physiological data, are summarized in Fig. 10. In Fig. 10A, submaximal electrical stimulation of the DSA nerve evoked a coupled spike, an afferent spike, and a PAD, each at a different latency. Four different sweeps are superimposed, chosen to illustrate the variety of responses that followed the same stimulus. In two, all three events occurred; in a third, the long-latency PAD was absent; in the fourth, both afferent spike and long-latency PAD were absent. Thus none of the events were invariably linked to any of the others. This means that at least three afferent fibers were involved (Fig. 10C). One of these would be linked to the recorded fiber by an electrical synapse, whereas another one produced PADs via an interposed inhibitory neuron. The resulting response would thus depend on which afferents fired in response to each electrical stimulus. In Fig. 10B, obtained from another fiber during mechanical stimulation, PADs were found to follow one-to-one stimulating frequencies up to 90 Hz where PADs were evoked through near-threshold movements of a hair, a depletion-like phenomenon occurred (Fig. 9A). PADs indicated by open arrowheads are seen as slow depolarizations following each electrical stimulus.

Variability of PADs

One noticeable characteristic of PADs was that their size was very variable, as previously observed in other preparations (e.g., Kirk and Wine 1984). At least two sources of variability were identified. Systematic changes of either sign of the PAD average amplitudes were observed in some fibers. In one instance, when a hair afferent producing PADs was repeatedly and selectively mechanically stimulated, the average amplitude of PADs was found to increase with the frequency of stimulation (Fig. 11). This facilitation apparently occurred between 1 and 3 Hz, with no further increase seen at higher frequencies up to $\approx 10$ Hz. In a different fiber, where PADs were evoked through near-threshold movements of a hair, a depletion-like phenomenon occurred (Fig. 12): the size of PADs was directly correlated with the pre-
FIG. 9. PADs are disynaptic. A: PADs can follow 1-to-1 stimuli at a frequency of 90 Hz (Stim.). Repetitive electrical stimulation of DSA nerve (filled arrows) produced reliable PADs (open arrowheads), as well as afferent spikes and coupled spikes (small last peak), in a DSA fiber recorded intracellularly. Resting membrane potential of fiber: -72 mV. B: summation of 2 unitary PADs occurring 5 ms apart in a DSA fiber (DSA-t) from another preparation. Both PADs resulted from mechanical stimulation of a single hair and were associated with identical impulse shapes in DSA nerve (DSA). Triggering of this doublet of impulses was fortuitous. Resting membrane potential of fiber: -72 mV. C: latency of PADs in DSA fiber shown in B was not constant. Six selected frames were aligned on extracellular impulse. Range of variations in latency (over 1 ms) is too high for PADs to be monosynaptic.

**PAD quantal analysis**

Random PAD size variations were more prominent than systematic changes. To study the origin of these variations, we have made a detailed analysis of unitary PADs evoked at regular intervals by mechanical stimulation of a hair, and showed their sizes vary in a quantal manner. Our best set of data, a distribution of the amplitudes of 686 such events in the same fiber, is illustrated in Fig. 13. Instead of a normal distribution, the data clearly showed a number of peaks. A number of histograms were drawn from the same data set with the use of slightly different bin sizes (not shown). As expected, the clearest peaks occurred when the distance between peaks was an exact multiple of the sampling bin size, but all histograms with bin sizes between 0.10 and 0.26 mV did show peaks. The existence of peaks is thus not an artifact of sampling bin size. A similar conclusion was reached by Laurent and Sivaramakrishnan (1992), who showed through a simulation analysis how very unlikely it would be for the peaks and valleys of their locust inhibitory postsynaptic potential amplitude histograms to be produced by limited sampling and arbitrary binning. To show that these peaks occurred at regular intervals, a requirement of quantal release, we applied a “folding” analysis. For instance, in Fig. 13A, each peak is made up of five bins. We thus summed all the first bins for all the peaks except peak 0, i.e., the contents of bins 3, 8, 13, 18, 23, 28, 33, and 38. Then did the same for the second, third, fourth, and fifth bins of each of the peaks. The totals were significantly different from an even distribution, as would apply if the peaks were not significant or were unevenly distributed ($\chi^2$ test, $P < 0.001$). Our data were thus consistent with quantal release of transmitter.

One of the main premises of quantal transmitter release is that the probability of the release of a quantum is uniform at all the sites forming a functional synapse with a particular neuron (McLachlan 1978). The distribution should thus match a Poisson distribution if $m$ (the mean number of quanta released) is small compared with $n$ (the total number of available quanta), whereas a comparison with a binomial distribution would be more appropriate if the probability of release was higher. Table 1 shows the observed number of events in successive peaks of the histogram compared with the predicted numbers for both Poisson and binomial distributions, calculated according to the equations given in METHODS. Calculations of the binomial distributions used estimates of $n$ that either assumed that the largest PADs represented release of the total number of releasable quanta ($n = 12$) or assumed that $n$ had a value somewhat larger than the number of quanta released by the largest PADs ($n = 15$). Although the data were significantly different ($P < 0.01$) from each of these three theoretical distributions whether we applied $\chi^2$ or Kolmogorov-Smirnov tests, the Poisson distribution is clearly closer to the data than either of the binomial distributions. From this we infer that even the largest PADs probably represent release of only a small number of the available quanta.

A possible reason for the poor fit with any of the theoretical distributions is the shunting effects of PADs on the membrane, for this results in nonlinear summation of unit conductance changes. Correcting for these effects as described in METHODS resulted in the distribution shown in Fig. 13B. Visually, the data peaks appear to coincide more closely with the theoretical (Poisson convolved with Gaussian) dis-


**Distribution.** But, even though the peaks occur at more regular intervals, the fit to a Poisson distribution or binomial distributions with estimates for \( n \) of 16 and 20 is no better than before \( (P < 0.001, \chi^2 \text{ and Kolmogorov-Smirnov tests}) \). It appears that all these theoretical distributions underestimate the height of peaks 0–II as well as of the last peaks (peaks IX and above). The actual data thus show more variance in quantal content than theory allows. Probably this is because the data do not fit the criterion of stationarity \( (\text{McLachlan 1978}) \). As described in the previous section, PADs could show both synaptic depression and facilitation, so that it is inevitable that the quantal content of a PAD would be influenced by the time elapsed since the previous one.

Nevertheless, the small mean value of the histogram and the number of spontaneous failures \( \text{(events near 0 amplitude)} \) are consistent with the idea that few quanta of neurotransmitter are released during each unitary event. From Fig. 13, we obtain the following figures: that the mean quantal content of a PAD lies between 4 and 5 \( (m = 4.55 \text{ or } 4.77 \text{ depending on whether or not a correction factor is applied}) \); that the largest PADs represent the release of no more than 12–16 quanta; and that the quantal size is \( \sim 1 \text{ mV} (0.96 \pm 0.15 \text{ mV, mean } \pm \text{ SD}) \).

**Discussion**

**Responses of DSAs to mechanical stimuli**

The DSA fibers described here in crayfish may be compared with those in rock lobsters and crabs. DSA fibers in rock lobsters are known to discharge during the stance phase of locomotion \( (\text{Müller and Clarac 1990a,b}) \), but hairs may well be sensitive to water motion \( (\text{Wiese 1976}) \). In crabs, the force-sensitive mechanoreceptors are located within the funnel canal organs \( (\text{Gnatzy et al. 1984; Schmidt and Gnatzy 1984}) \). A proximal population of receptors encoding forces and loads at the tip of the leg can be distinguished from a distal population of receptors with less specific response characteristics that mostly signal contact or vibrations \( (\text{Liberst et al. 1987}) \). The DSA fibers in our study had characteristics intermediate between those of the two populations described in crabs. Most of the hair afferents and the force-sensitive afferents were selective with respect to the direction of imposed effort. A large majority of hair afferents had phasic or phasotonic characteristics of response to bending, purely tonic units being uncommon. Force-sensitive afferents did not respond to constant force, but discharged proportionally to the rate of change of force applied to the dactyl tip. This lends support to the idea that the regulation of static leg loading in crayfish is not primarily allocated to the DSAs, but rather to proprioceptors such as cuticular stress detectors \( (\text{Klärner and Barnes 1986; Leibrock et al. 1996; Marchand et al. 1995}) \). DSAs may be mostly involved in coordinating locomotion \( (\text{Müller and Clarac 1990a,b}) \) and encoding external events \( (\text{Marchand and Barnes 1992}) \).

**Central projections of the DSAs**

The central region of the hair afferent shown in Fig. 5 has a morphology quite distinct from the terminals of crayfish proprioceptors such as the coxobasipodite chordotonal organ \( (\text{El Manira et al. 1991a,b}) \) and the cuticular stress detectors \( (\text{Marchand et al. 1995}) \). Both of the latter possess large anterior and posterior branches with ramifications. DSA fibers branch more extensively when they enter the ganglion and their arborizations are distributed over a much larger area of the ganglion. They appear to project many diffuse thin processes. Indeed, the small diameter of most branches \( (2–10 \mu m \text{ at most}) \) posed considerable difficulties when trying to penetrate or keep an afferent for intracellular recordings. With respect to the neuroanatomic analysis of Elson \( (1996) \), the ventral position of DSA branches makes it likely that they contact the ventral neuropil \( (\text{named VN by Elson 1996}) \). This idea is reinforced by the fact that, in the periphery, DSA axons travel in nerve N1PV (data not available).
Origin and inhibitory properties of PADs

We have shown PADs in DSA fibers to be linked to extracellular afferent impulses in the DSA nerve. In our experimental conditions (preparation at rest without rhythmic motor activity) no PADs were recorded in the absence of mechanical stimulation. In contrast, PADs recorded in crayfish chordotonal organ afferents are thought to result from the activity of locomotor centers, but can be recorded even in the absence of rhythmic motor activity (El Manira et al. 1991b). Together, these two pieces of evidence suggest that PADs in DSA fibers are exclusively of peripheral origin.

PADs were found to be mediated by a short pathway, involving only one intercalated inhibitory neuron (PADI), as already postulated in insect studies (Burrows and Mathe-son 1994). The variability found in the latencies of PADs is an indication that PADIs are indeed spiking neurons, and is attributed to fluctuations in the PADI’s potential and thus in the time needed for the afferent synapse to drive it above firing threshold. Nevertheless, the synapse between the afferent and the PADI is extremely reliable, because PADs fail to follow the appropriate afferent spike in only a few percent of cases. PADIs still remain to be identified anatomicly, as has been done in the case of command-derived presynaptic inhibition (Kirk and Wine 1984), but some candidate PADIs (spiking interneurons whose impulses are strictly linked with PADs in afferent fibers) have occasionally been recorded (Cattaert and Barnes, unpublished observations).

The inhibitory nature of PADs is confirmed by the fact that they decrease the amplitude of afferent spikes recorded in DSA terminals. Furthermore, their reversal potential seems similar to that of PADs in the coxobasipodite chordotonal organ (Cattaert et al. 1992) or cuticular stress detectors (Marchand and Leibrock 1994) that are chloride-mediated.

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**Fig. 11.** Facilitation of PADs. A and C: detailed distribution of PAD amplitudes seen at 0.91 and 8.77 Hz, respectively. At higher frequencies, peak around 5 mV appeared displaced to right, and values >10 mV became common (24 instead of 4). n, number of PADs. B: mean amplitude of PADs evoked by repetitive mechanical stimulation of a single hair at different frequencies.

shown), which makes massive projections within VN through nerves R1PViii and R1PViv (Elson 1996). In contrast to the presence of monosynaptic connections between chordotonal afferents and motoneurons (El Manira et al. 1991a,b), there appear to be no direct projections of DSAs onto proximal motoneuronal groups. Instead, there are indirect projections to the levator motoneurons (Clarac et al. 1991; Marchand and Barnes 1992). Whether the apparently diffuse arborizations of the DSA form topographic projections remains to be determined.

**Fig. 12.** Depression of PADs. Plot of amplitude of PADs evoked by afferent spikes in DSA nerve during mechanical stimulation of a single hair, as a function of interspike intervals. It shows 2 types of relationships depending on range of interspike intervals. For intervals <0.200 ms, amplitude was highly significantly correlated to time elapsed since last spike (r = 0.785, n = 130), indicating synaptic depression. For higher intervals, no correlation appeared (n = 104).
Presynaptic inhibition is likely to result, at least in part, from the large increase in membrane conductance (up to 50%, see METHODS) when chloride channels open, rather than from the changes in membrane potential (Burrows and Laurent 1993). It is also probable that PADs will inhibit by inactivating the sodium channels that carry the afferent impulses (Graham and Redman 1994). Because of their huge size, which may exceed 15 mV, unitary PADs may sometimes exceed the threshold for spike initiation and thus give rise to impulses that travel antidromically (i.e., toward the periphery) along the sensory neurons. Efferent sensory impulses have been occasionally reported in other preparations (e.g., Dubuc et al. 1988; Horridge 1963). Whether such antidromic impulses have any function is unclear. Certainly they will block incoming sensory spikes by collision. However, to really represent an inhibitory process, such antidromic spikes should not be propagated centrally. In crayfish chordotonal afferents, such impulses failed to elicit excitatory postsynaptic potentials in postsynaptic cells (Cattaert et al. 1992), presumably because of the shunting effect of the PADs that gave rise to them. Moreover, antidromic discharges are capable of reducing the mechanical sensitivity of coxobasipodite chordotonal organ sensory neurons (Cattaert, unpublished data).

**Quantal properties of PADs**

Because of the huge size of the PADs, the possibility that “quantal” PADs might actually represent the probabilistic firing of several individual PADIs rather than the quantal variation of a single PADI has to be considered. Because no direct recordings of PADIs are available for analysis, this question cannot be answered directly. But it would have been very unlikely for several PADIs ($\geq 8$) to discharge independently with a similar probability (required for a near-Poisson distribution) and to produce depolarizations of the same amplitude (needed for distinct peaks). Even if this were the case, the
TABLE 1. Comparison of the contents of each peak of the PAD amplitude histogram illustrated in Fig. 13A with Poisson and binomial distributions

<table>
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<tr>
<th>Peak Number</th>
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</table>

$\chi^2$ test $P < 0.001$ $P < 0.001$ $P < 0.001$

Kolmogorov-Smirnov test $P < 0.001$ $P < 0.001$ $P < 0.001$

PAD, primary afferent depolarization; $N$, total number of events; $n$, total number of releasable quanta; $m$, mean of distribution.

variable latency at which PADIs fire (inferred from the data of Fig. 9C) would result in such compound PDAs being recognizable compound, because they would be a product of temporal as well as spatial summation. Less than 1% of PDAs had such a compound form (e.g., Fig. 9C), so this explanation can be dismissed. Indeed, quantal fluctuations have already been found at other presynaptic inhibitory sites (Kirk and Wine 1984).

Our quantal analysis, to our knowledge the first to be undertaken on PADs, shows that PADs have a small quantal content, much smaller than the 200–300 quanta released at the frog neuromuscular junction (Del Castillo and Katz 1954; Martin 1955) but equivalent to many central synapses (see, e.g., Edwards et al. 1990; Redman 1990). The figure (range 0–16; mean between 4 and 5) is very similar to that for GABAergic inhibitory interneurons in insect ganglia (Laurent and Sivaramakrishnan 1992). However, the mean quantal size is substantially greater (1,000 $\mu$V) than reported in other preparations (e.g., 150–400 $\mu$V) (Laurent and Sivaramakrishnan 1992; Nicholls and Wallace 1978).

The maximum number of quanta released per impulse is a measure of the number of vesicle release sites, whereas the existence of distinct peaks on the PAD amplitude histograms indicates that these release sites are at similar electrotonic distances from the recording site. Given the complex geometry of DSA fibers, and the probability that, as in other crayfish mechanoreceptive afferents (Cattaert et al. 1992), conduction within the ganglion is by electrotonic spread, this means that these release sites must be clustered, perhaps being located within a single synaptic contact. There is of course the possibility that other release sites exist, electrotonically too distant to be detected from our electrode sites. This would be consistent with electron microscopic data of Lee and Krasne (1993), which suggest that presynaptic inhibitory synapses are located near primary afferent release sites in crayfish terminal abdominal ganglia.

That the data did not quite follow a Poisson distribution, and were a poor fit to two binomial distributions, could have a number of explanations. Most probably, this is because the influences of synaptic depression and/or facilitation contradict the criterion of data stationarity (McLachlan 1978), although other possibilities such as the presence of more than one synaptic bouton (Korn et al. 1993) or nonuniform release probabilities between different release sites (Walmsey et al. 1988) cannot be excluded. The one-to-one characteristics of the first-order synapse make it unlikely that the PADIs receive useful postsynaptic inhibition, but quantal fluctuations and the existence of facilitation suggest the possibility that some regulation of afferent sensitivity might be exerted through presynaptic modulatory influences on PADI transmitter release.

Functional interpretation

The existence of a short and reliable inhibitory pathway between DSA afferents suggests that PADs play an important role in shaping the afferent message. PADs will reliably occur within a few milliseconds of the first afferent impulses reaching the ganglion. Because analyses of presynaptic inhibitory mechanisms in crayfish ganglia (Kirk and Wine 1984; Cattaert, unpublished data) suggest that relatively few PADIs synapse onto many afferent terminals, the large size of PADs and of their quantal components means that these PADIs are used as effectively as possible. DSA afferents are very sensitive to mechanical stimuli and should thus easily be subject to saturation of the afferent message. Presynaptic inhibitory drive associated with the PADs could, however, tune down synaptic transmission in several ways, depending on the connectivity pattern of PADIs and afferent fibers. Three important questions remain open, namely the number of PADIs, the number of fibers impinging onto each PADI, and the extent of inhibition from each PADI to the DSA fibers. Our limited data, showing that not all hairs may elicit PADs in a given terminal, indicate that there may be a selective distribution of the input to the PADIs, as expected in a system involved in the control of walking (Fricke and Kennedy 1983).

The present data on crustacean leg exteroceptors bear some similarities with the lateral inhibitory pattern between cercal hair afferents in the first instar cockroach (Blagburn and Sattelle 1987). It appears, for instance, that the afferent from a given hair of the dactyl may receive inhibitory input from other hairs in the same general region. We have shown that both hair afferents and force-sensitive afferents may be subjected to presynaptic inhibition, i.e., receive PADs when neighboring hairs are stimulated. Lateral presynaptic inhibition thus appears to be a widespread feature of arthropod cutaneous mechanoreceptors that subserve an exteroceptive function. Because PADs in sensory fibers can be reliably evoked by mechanical stimulation of single afferents, a condition that was seldom obtained in earlier studies (Burrows and Matheson 1994), this preparation is particularly suitable for a systematic investigation of the pattern of inhibition and its significance.

At least three possible functions of PADs may be proposed. First, to prevent saturation, a widespread activation of DSA fibers could be accompanied by a strong presynaptic inhibitory drive, as shown during high-frequency electrical stimulation. This would constitute an “automatic gain control” mechanism, as discussed...
by Burrows and Matheson (1994) and Hatsopoulos et al. (1995). Whereas localized stimuli would strongly activate some fibers, more widespread stimulation would activate more fibers less strongly because of inhibition. This might also limit habituation (Krasne and Bryan 1973), which has been shown to occur less readily with intense stimuli (Marchand and Barnes 1992).

Second, to encode specific stimuli while retaining high sensitivity, presynaptic inhibition may act in the spatial domain. In other sensory systems such as cutaneous afferents in vertebrates (Schmidt 1971) and cercal hair afferents in the cockroach (Blagburn and Satelle 1987), lateral presynaptic interactions may enhance contrast or directional selectivity, thus improving the localization of a stimulus. Third, because of its short pathway, presynaptic inhibition may also act in the temporal domain, either to enhance the phasic characteristics of the sensory message or to select input according to time, as has been proposed by Celebrini et al. (1993) in the primate visual system. The earliestafferent impulse arriving at the center might block most of the effects of subsequent afferent discharges through presynaptic inhibition. Assuming that fibers with the best selectivity with respect to the mechanical stimulus will be the first to discharge, this “winner takes all” mechanism could improve the identification of intense stimuli.

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