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A Novel Synaptic Transmission Mediated by a PACAP-like Neuropeptide in Drosophila

Yi Zhong and Louis A. Peña
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

Summary

Neuropeptide-mediated transmission was analyzed at Drosophila larval body-wall neuromuscular junctions. Focal application of vertebrate pituitary adenylyl cyclase-activating polypeptide (PACAP38) to the neuromuscular junction region triggered two temporally distinct muscle responses: an immediate depolarization followed by a large enhancement of K^+ current. This late enhancement occurred many minutes after the early depolarization. High frequency stimulation of motor nerve fibers evoked a postsynaptic response mimicking that induced by PACAP38. This evoked response was desensitized by preincubation of the preparation with PACAP38. PACAP38-like immunoreactivity was also found in the Drosophila CNS and at almost all larval neuromuscular junctions. Moreover, an immunoreactive band that compares well with PACAP38 in size was identified in Western blot. These results demonstrate that a PACAP-like peptide may function in invertebrates and that a neuropeptide can evoke two distinct postsynaptic responses, each separated by up to 15 min. In addition, this initial electrophysiological study provides a basis for genetic analysis of neuropeptide function in Drosophila.

Introduction

Neuropeptides comprise the largest group of neurotransmitters in nervous systems (Hokfelt, 1991), so understanding their actions will likely be a major part in the understanding of neuronal communication. In fact, peptidergic systems have been implicated in mechanisms that control functions such as stress response, sexual behavior, pain perception, and learning and memory (Terenius, 1992; Glue et al., 1993; Kovacs and Wied, 1994). A major obstacle in understanding the mechanistic role of peptidergic transmission has been the lack of specific inhibitors (Smelik, 1987; Hokfelt, 1991). The powerful genetic and molecular techniques available in Drosophila may allow mutational perturbation of specific peptide signaling pathways. However, neuropeptide transmission has yet to be analyzed electrophysiologically in Drosophila. We have begun to search for neuropeptides that function at the larval body–wall neuromuscular junctions by immunohistochemical and electrophysiological analyses.

The larval neuromuscular preparation has been extensively characterized and is suitable for quantitative analysis of synaptic transmission at identifiable synapses (Jan and Jan, 1976a, 1976b, 1978; Wu et al., 1978; Ganetzky and Wu, 1983; Johansen et al., 1989; Zhong and Wu, 1991a). Glutamate is the major excitatory transmitter at this neuromuscular junction (Jan and Jan, 1976b; Johansen et al., 1989). In addition, immunoreactivities of the neuropeptides proctolin (Anderson et al., 1988), leucokinin I (Cantera and Nassel, 1992), and insulin (Gorczyca et al., 1993) have been observed in nerve terminals innervating subsets of these muscle fibers. However, the electrophysiological function of these peptides remains to be established. It is reported in this paper that a vertebrate neuropeptide, adenylyl cyclase-activating polypeptide (PACAP), and an endogenous Drosophila factor, possibly a PACAP-like peptide released upon stimulation of motor axons, are both able to trigger a similar novel muscle response.

PACAP belongs to a neuropeptide family that includes vasoactive intestinal peptide (VIP), glucagon, and secretin (Arimura, 1992). Two bioactive forms of PACAP, PACAP38 and PACAP27, are derived from the rat precursor protein, which consists of 175 amino acid residues (Ogi et al., 1990). PACAP27 is an amidated peptide corresponding to the N-terminal 27 amino acids of PACAP38, which itself is 38 amino acids. The peptide sequences are identical in humans, rats, and sheep (Arimura, 1992). Two types of PACAP receptor have been cloned. Type 1 is coupled to both adenylyl cyclase and phospholipase C and is specific to PACAP (Spengler et al., 1993). In addition, type 1 exhibits a much higher sensitivity to PACAP38 than to PACAP27 (Spengler et al., 1993). Type 2 is coupled to only adenylyl cyclase and is sensitive to both PACAP and VIP (Hashimoto, et al., 1993). Both types of receptor are distributed widely in vertebrate peripheral tissues and in the brain, including the hypothalamus and hippocampus. PACAP38 and PACAP27 have been reported to regulate hormone or transmitter release, neurite outgrowth, and gene expression (Arimura, 1992; Deutsch and Sun, 1992; Schadlow et al., 1992; Culler and Paschall, 1991; Masuo et al., 1993).

The presence of PACAP or related neuropeptides in invertebrates has not yet been reported. We found PACAP38-like immunoreactivity in a subset of neurons in the Drosophila CNS and at motor nerve terminals that innervate larval body–wall muscle fibers. This antibody recognizes a peptide with the similar size as PACAP38 in Western blot. Furthermore, focal application of PACAP38 to the neuromuscular junction induces two distinct muscle responses at separate time windows: an early depolarization lasting many seconds and a late, hundred-fold enhancement of K^+ currents, which starts several minutes after the early depolarization and lasts for 2–5 min. A similar response can also be evoked by high frequency stimulation of motor axons, and this evoked response can be desensitized by PACAP38. Thus, the present work demonstrates that a neuropeptide can trigger two distinct postsynaptic responses, each separated by up to 15 min, and that a PACAP38-like peptide may function in invertebrates.

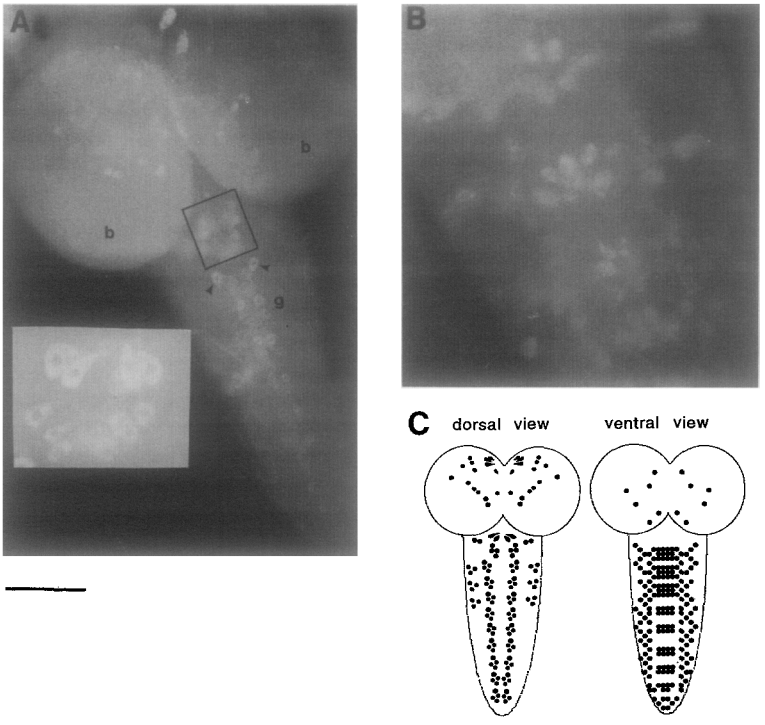

Figure 1. PACAP38-like Immunoreactivity in the Drosophila Larval CNS

(A) The dorsal view of immunoreactivity of vertebrate PACAP38 in the ganglion (g) and brain lobes (b) of a third instar larva. Arrows point to a pair of stained neuronal somas. Inset shows focused image of stained cell bodies within black square.

(B) The ventral view of PACAP38 immunoreactivity in the ventral ganglion.

(C) The schematic representation of the distribution of stained cell bodies in the larval CNS. Note that comparing the ventral view in the (C) with the pattern of the staining in (B) indicates that the drawing only approximates the actual distribution of the immunoreactivity.

Bars, 60 μM (A); 30 μM (B) and inset in (A).

Results

PACAP-like Immunoreactivity in the Larval CNS and at the Neuromuscular Junction

Using a vertebrate PACAP38 antiserum, PACAP38-like immunoreactivity was found in a subset of larval CNS neurons as well as in motor nerve terminals. This antiserum does not cross-react with VIP, PACAP27, or several other neuropeptides tested. Figure 1 presents an example that shows the distribution of stained cell bodies in the larval ventral ganglion and brain lobes. These stained cell bodies appear to be distributed primarily in two clearly separated focal planes, each plane showing a different pattern as revealed in Figure 1A (dorsal view) and Figure 1B (ventral view). A schematic presentation of such a distribution is depicted in Figure 1C. It should be noted, however, that the schematic drawing of the distribution of the stained soma in the ventral focal plane (left panel in Figure 1C) only roughly approximates the actual situation, owing to the complexity of the distribution (Figure 1B). In addition, the decay of the labeled fluorescence limits the time for detailed observation. Nonetheless, about 320–400 soma are strongly stained in the CNS. The categories of the stained neurons remain to be determined. The pair of soma in each segment that show the strongest immunoreactivity (arrows in Figure 1A) send their axons into the motor nerve bundles (data not shown), which innervate larval body-wall muscle fibers.

PACAP38-like immunoreactivity was found in nerve terminals innervating almost all muscle fibers. Figure 2A shows examples of such immunoreactivity in motor nerve terminals arborized on muscle fibers 4, 6, 7, 12, and 13 (nomenclature of Crossley, 1978). As indicated, the staining appears to be concentrated in varicosities where synaptic vesicles are localized (Jia et al., 1993; Atwood et al., 1993). A comparison of the patterns of immunoreactivity with previous anti-horseradish peroxidase (HRP) staining, which reveals all nerve terminals arborized on muscle fibers (Johansen et al., 1989; Budnik et al., 1990; Zhong et al., 1992), suggests that PACAP38-like immunoreactivity is restricted to large-sized type 1 varicosities and is not expressed in small-sized type 2 varicosities.

PACAP38-like immunoreactivity was abolished if the antiserum was preincubated with 4 μM PACAP38 (Figure 2B) but was retained if incubated with 4 μM PACAP27. In addition, another anti-PACAP38 antiserum (IHC 8920, Peninsula) also produced a similar pattern of immunoreactivity with much weaker fluorescence (data not shown). The two antisera against PACAP27 did not stain the larval tissues. These data indicate the specificity of the staining to PACAP38.

PACAP-like Immunoreactivity on Western Blots

Western blotting was carried out to determine whether PACAP38-like immunoreactivity identifies a peptide. Faint PACAP-like immunoreactivity was detected in total protein from Drosophila larvae tissue lysates separated by Tris–Tricine polyacrylamide gel electrophoresis (PAGE), blotted onto Immobilon–P membranes and detected by chemiluminescence. When originally isolated, PACAP38 was purified from several successive ion-exchange chromatography and reverse-phase high pressure liquid chromatography fractions (Miyata et al., 1989). Thus, seeking an enrichment of PACAP-like material in Drosophila, we employed a similar but simplified crude reverse-phase organic extraction in which PACAP38 elutes from C18 high

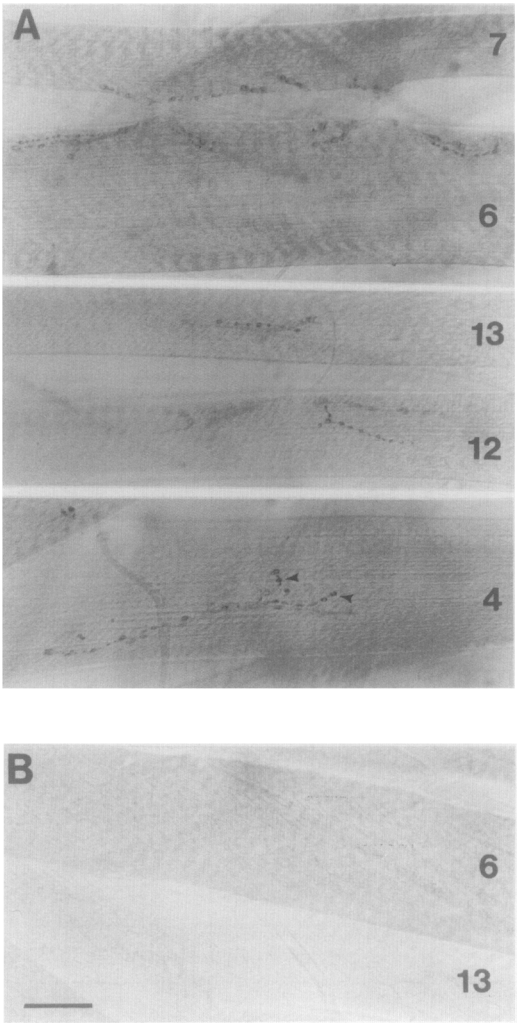

Figure 2. PACAP-like Immunoreactivity at Larval Neuromuscular Junctions

(A) PACAP38-like immunoreactivity was found in the motor nerve terminals on almost all larval body–wall muscle fibers. The representative examples of such staining in muscle fibers 4, 6, 7, 12, and 13 are shown. Arrows point to stained varicosities where synapses are formed (Atwood et al., 1993; Jia et al., 1993).

(B) PACAP38-like immunoreactivity is abolished if antiserum is preincubated with 4 μM PACAP38. Examples of muscle fibers 6 and 13 are presented.

Bar, 50 μM.

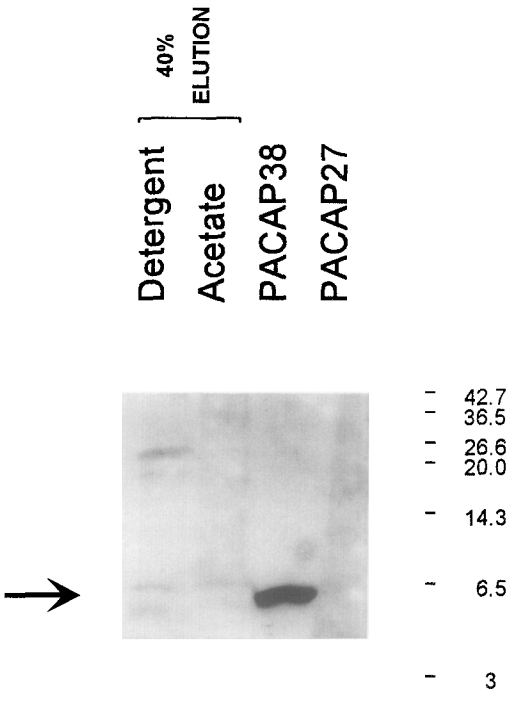

Figure 3. PACAP-like Immunoreactivity on Larval Western Blot

Chemiluminescent detection of PACAP38-like bands from Drosophila larvae by anti-PACAP38-IgG. Lanes 1 and 2 show 150 μg/lane total protein recovered in 40% CH_3CN fractions eluted from C18 Sep-Pak cartridge. Whole larvae tissue homogenized in 0.5 M acetic acid (lane 1) or in high salt, Nonidet P-40 detergent lysis buffer (lane 2). Arrow indicates presumed PACAP38-like peptide. Lanes 3 and 4 show 25 ng/lane synthetic vertebrate PACAP38 and PACAP27 peptide, respectively. At right margin, molecular weight standards in kDa.

pressure liquid chromatography matrix at a specific concentration of CH_3CN/0.1% trifluoroacetic acid (TFA; Tatsuno et al., 1994; Miyata et al., 1989). Under our conditions, at a 40% CH_3CN/0.1% TFA elution, an immunoreactive band of about 5.5 kDa (Figure 3, lanes 1 and 2) appeared near the position of mouse PACAP38 positive control. This compared well with the PACAP38 peptide employed as a positive control (Figure 3, lane 3). With a calculated mass of 4,530 Da, the PACAP38 peptide exhibited an apparent migration of about 5.4 kDa in this PAGE system. In addition to the PACAP38-like immunoreactive band, a larger immunoreactive band of about 19 kDa was visible in the detergent extract (Figure 3, lane 1). This higher molecular weight band was in the range of the PACAP precursor protein having a calculated mass of 19,450 Da (Arimrua, 1992) and therefore may represent a possible PACAP precursor polypeptide in Drosophila. Only a single PACAP38-like immunoreactive band was visible in the acetate extract (Figure 3, lane 2). Employed as a negative control, PACAP27 peptide was not detectable (Figure 3, lane 4). Similar results were obtained when these experiments were done using a crude anti-PACAP38 antiserum; however, additional higher molecular weight bands were evident (data not shown). These results suggest that antibodies raised against mammalian PACAP38 indeed identifies a Drosophila polypeptide with a similar size as PACAP38 and therefore reinforce the idea about the existence of a PACAP38-like neuropeptide in Drosophila.

PACAP38-Induced Muscle Responses

To determine whether or not such immunoreactivity reflects the presence of a functional neuropeptide similar to PACAP38, the effect of focal application of PACAP38 to the neuromuscular junction was investigated. In this investigation, muscle fiber 6 ($n = 14$) and muscle fiber 12 ($n = 22$) from 24 larvae were examined. Both muscle fibers 6 and 12 exhibited a similar response. Intracellular recordings revealed a slow depolarization of 10–20 mV last-

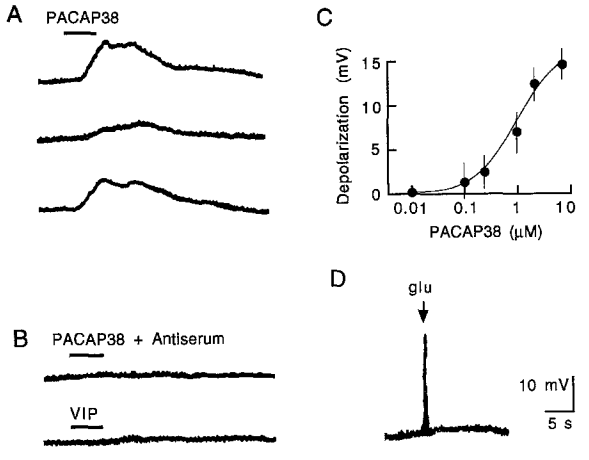

Figure 4. PACAP38-Induced Early Slow Depolarization in Larval Body-Wall Muscles

(A) Top trace shows the depolarization induced by local application of PACAP38 (4 μM). The bar indicates the time at which PACAP38 was ejected. Middle trace shows a desensitized response following the first application (50 s after). Bottom trace shows the recovery of the response 5 min after the previous application.

(B) Application of PACAP38 preincubated with antiserum (15 μl of 4 μM PACAP38 mixed with 4 μl of 1:40 antiserum) or 10 μM VIP did not induce any significant response.

(C) The dose–response curve of PACAP38-induced depolarization. Each data point represents averaged recordings from 3–5 fibers (mean ± SEM). Resting potentials for all recordings are about −65 to −80 mV.

(D) Muscle response induced by ionophoretic perfusion of Na glutamate.

ing for tens of seconds in response to a brief pressure-ejection of PACAP38 (4 μM) via a micropipette (Figure 4). Such a time course is much slower than that seen with the focal application of glutamate, which induces a response lasting for a fraction of second (see Figure 3D; also see Jan and Jan, 1976b; Johansen et al., 1989). The preparation was desensitized if a second dose of PACAP38 was applied within 1 min of a previous application (Figure 4A) or preincubation of the neuromuscular preparation with 2 μM PACAP38. Three muscle fibers were examined under Nomarski optics. We were able to localize some of large-sized varicosities (3–5 μm in diameter) on muscle fiber 6 (also see Johansen et al., 1989). Similar responses were recorded when 10 μM PACAP38 was perfused on to the top of varicosity or 30 μm away from the identified varicosity. This result suggests that the receptors that PACAP38 binds to are not restricted to the region of neuromuscular junctions.

After preincubation with its antiserum (1:200 dilution), PACAP38 lost its ability to depolarize the muscle membrane (Figure 4B), whereas mixing PACAP38 with antiserum against PACAP27 did not abolish the PACAP38-induced response. In addition, 10 μM VIP (Figure 4B) and glucagon (data not shown) did not induce any significant response. This evidence indicates the specificity of the response to PACAP38.

The two-electrode voltage-clamp analysis showed that this depolarization results from an activation of an inward current (Figure 5A; see the second current trace in the top panel). In addition, the voltage-clamp analysis also revealed a second phase of this response: modulation of K^+ currents, which was not observed until minutes after the early depolarization. Muscle membrane currents were elicited by depolarizations to −50 mV (for monitoring leakage current) and 0 mV from the holding potential of −80 mV. A step to 0 mV elicits the voltage-activated inward Ca^{2+} current and the voltage-activated and the Ca^{2+}-activated outward K^+ currents (Singh and Wu, 1989; Zhong and Wu, 1991b). However, the inward Ca^{2+} current is completely masked by the outward K^+ currents (Figure 5). Within the first 6 min after the application of PACAP38, the macroscopic membrane currents were not altered appreciably as compared with the control. Then a dramatic enhancement of K^+ currents was observed, and the increase reached its maximum after another 2 min. At its peak, the amplitude of the K^+ currents was over 100 times larger than that recorded prior to the application of PACAP38. During the next 2–3 min, the amplitude of K^+ currents gradually decreased to a magnitude similar to that in the control. The whole process, beginning from the application of PACAP38 to complete recovery, took about 12 min in this particular example. This modulation altered not only the amplitude but also the kinetics and voltage-dependence of K^+ currents (Figure 5B).

The component modulated by PACAP38 appeared to be Ca^{2+} dependent, as suggested by the following observation. The enhancement of K^+ currents was removed if the Ca^{2+} saline (1 mM Ca^{2+} and 20 mM Mg^{2+}) was replaced by the Ca^{2+}-free saline (20 mM Mg^{2+}) at the time that the enhancement of K^+ currents induced by 4 μM PACAP38 reached almost to its maximum (data not shown). This enhancement would persist for another 2–4 min in the Ca^{2+} saline. In addition to Ca^{2+}- or voltage-activated K^+ currents, the leakage current observed by a depolarization to −50 mV was also increased (comparing the current trace elicited by the −50 mV pulse in the control with that at 8' minute in Figure 5A), but the magnitude was relatively small, so that it is not apparent at the amplitude scale used in Figure 5.

In a comparison with the response induced by perfusion of 4 μM PACAP38 as presented above, the time interval between the early depolarization and the late enhancement of K^+ current was much shorter if 10 μM PACAP38 was applied, occurring almost immediately after the early response. Conversely, the time interval became much longer if 1 μM PACAP38 was applied.

To determine whether the enhancement of K^+ currents is a result of electrical coupling between adjacent muscle fibers, depolarizing or hyperpolarizing-current pulses were injected into adjacent muscle fibers at various times following the application of PACAP38. The lack of response in muscle fibers to the injected current pulses excluded the possibility of electrical coupling. To verify whether the late response is a secondary effect due to other factors released from the motor terminals, the motor-nerve axons were pulled away from the muscle fibers. Alternatively, tetrodotoxin (TTX; 1 μg/ml), a Na^+ channel blocker, was added to the bath saline at a concentration

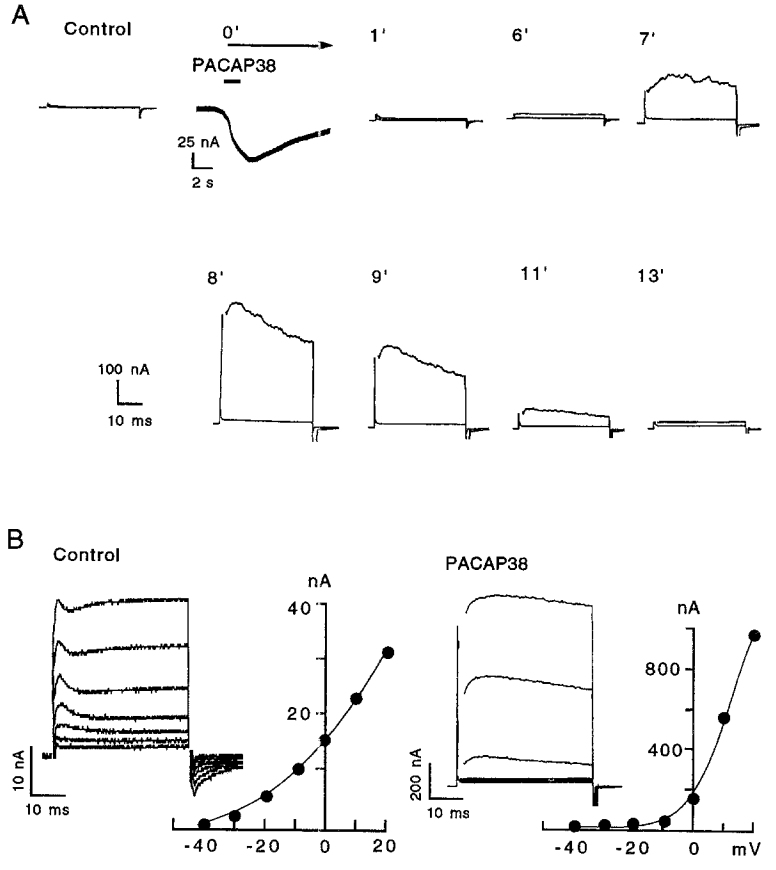

Figure 5. PACAP38 Induced Late Enhancement of Voltage-Activated K^+ Currents

(A) A typical time course of PACAP38 (4 μM)-induced modulation of K^+ currents recorded by voltage clamp. The command voltages are stepped from the holding potential of −80 to −50 and 0 mV respectively. Only a leakage current is elicited by the voltage step to −50 mV, but voltage-dependent currents are activated by depolarization to 0 mV. The bar indicates the pulse application of PACAP38, which activated an inward current when muscle membrane was clamped at −80 mV. At this voltage, the activation of any voltage-activated currents is prevented (Singh and Wu, 1989; Zhong and Wu, 1991), and it is also close to the reversal potential of K^+ channels. The inward current represents the early response, which times the beginning (0') of the PACAP38 effect. The time in minutes after pressure-ejection of PACAP38 is indicated by each trace.

(B) Current–voltage (I–V) relation of before and 11 min after application of PACAP38. The I–V curve demonstrates the voltage dependence of the modulation. Over 20 fibers were analyzed.

that abolishes any evoked transmitter release (Jan and Jan, 1976a; Wu et al., 1978; Ganetzky and Wu, 1983). In both situations, the PACAP38-induced modulation of K^+ currents was not affected. In addition, it was noted that the late response was induced almost simultaneously with the early response if 10 μM PACAP38 was applied to a region far from the nerve terminal arborization. To prevent diffusion of PACAP38 towards nerve terminals, a constant saline exchange was maintained during recordings. These results support that PACAP38 itself may induce two temporally separated muscle responses: an early depolarization and a late modulation of voltage-activated K^+ currents.

Evoked PACAP-like Response

If PACAP38-induced muscle responses reflect the function of an endogenous neuropeptide, then similar responses should be evoked by stimulation of the motor nerve axons. Recordings from muscle fiber 12 (n = 28) in 19 larvae confirmed this possibility. As mentioned earlier, glutamate is the major excitatory neurotransmitter at the larval neuromuscular junction (Jan and Jan, 1976b; Johansen et al., 1989). Each suprathreshold stimulation of the motor nerve fibers evoked glutamate-mediated excitatory junction potentials (ejps) with a fast time course (milliseconds). However, high frequency repetitive stimulation (20 Hz or higher) evoked not only fast ejps but also a 5–15 mV slow depolarization lasting 5–10 s (Figure 6). Argiotoxin (1 μM), a spider toxin that blocks open gluta-

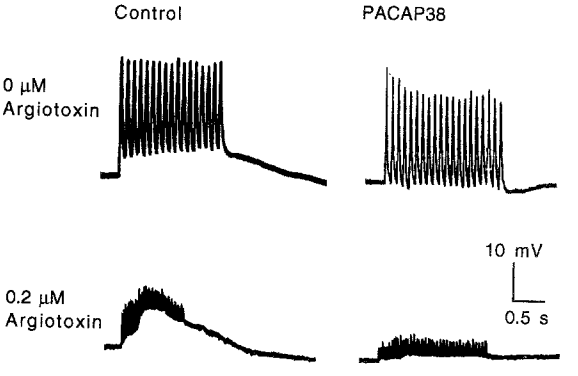

Figure 6. Evoked PACAP-like Slow Depolarization by High Frequency Stimulation of Motor Axons

Top panel shows that 10 Hz electrical stimulation of the motor axons evokes fast ejps and a slow depolarization with saline containing 2 mM Ca^{2+}. The slow depolarization is diminished by preincubation of the preparation with 2 μM PACAP38 and the antiserum (1:40 dilution) against the vertebrate PACAP38. More than four fibers in each situation have been examined. Recordings were obtained at least 10 min after the incubation. Bottom panel shows that argiotoxin, an open channel blocker of glutamate channels (Jackson and Usherwood, 1988; Broadie and Betz, 1993), diminished fast ejps evoked by 20 Hz stimulation but not the slow depolarization. Resting potentials for these recordings are within −60 to −80 mV. Since the blockade of the fast ejps prevented massive muscle contraction, higher frequency stimulation (20 Hz instead of 10 Hz) was applied to achieve larger slow response.

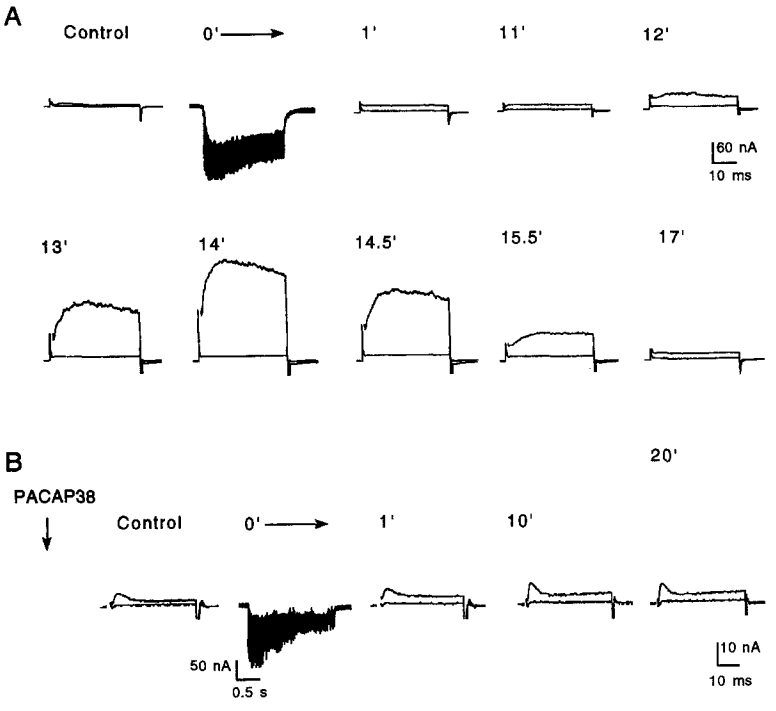

Figure 7. Evoked PACAP-like Enhancement of Voltage-Activated K^+ Currents by High Frequency Stimulation of Motor Axons

The voltage paradigm is the same as that in Figure 3, and recordings were obtained only from muscle fiber 12. The time in minutes after stimulation is indicated by each trace. At 0 min, 60 Hz stimulation of the motor axons evoked excitatory junctional currents.

(A) A typical time course of evoked modulation of K^+ currents at 1 mM Ca^{2+} concentration. The enhancement could be repeatedly induced. The interval between the early and late responses is much shorter at 2 mM Ca^{2+} concentration (data not shown). In over 20 muscle fibers examined, all were seen to respond to the 40–80 Hz stimulation.

(B) Preincubation of the muscle fibers with 0.4 μM PACAP38 abolished the evoked modulation. Incubation of PACAP38 itself induced an enhancement of K^+ currents several minutes later, after application. To see the desensitization effect, the 60 Hz stimulation was delivered after the PACAP38-induced enhancement had decayed (approximately 10 min after incubation with 0.4 μM PACAP38). The membrane currents were recorded every 1–2 min after the stimulation for up to 26 min. Since no obvious alteration was observed in any of these recordings, only current traces recorded at 1, 10, and 20 min are presented. As a control, the 2 μM VIP or glutamate appeared not to affect the evoked modulation (data not shown). Ten muscle fibers were examined. All ejps have the same scale as indicated in (B).

mate-receptor channels (Jackson and Usherwood, 1988; Broadie and Betz, 1993), diminished the evoked fast ejps but not the slow depolarization (Figure 6). To exclude the possibility that the slow response might be induced by glutamate via activation of metabotropic receptors, which are not blocked by argiotoxin, (RS)-α-methyl-4-carboxphenylglycine (MCPG; 500 μM), a metabotropic receptor blocker (Bashir et al., 1993), was added to the bath saline. MCPG did not affect the slow depolarization (data not shown). In addition, focal application of glutamate induced only a fast membrane depolarization (see Figure 5D) but no slow responses. This suggests that the evoked slow depolarization is probably not caused by glutamate release.

The time course of this evoked slow depolarization resembles the PACAP38-induced early response (see Figure 4A). Furthermore, preincubation of the preparation with 2 μM PACAP38 diminished the evoked slow depolarization (Figure 6).

High frequency stimulation evoked not only the early slow depolarization but also a delayed enhancement of K^+ current. In response to 60 Hz stimulation, K^+ currents were not altered appreciably for the first 10 min. Then, the amplitude of K^+ currents was dramatically enhanced for about 1–3 min (Figure 7A). The time interval between the evoked early and late responses varied with the external Ca^{2+} concentration in bath saline. The results presented above were obtained with a 1 mM Ca^{2+} concentration. When the Ca^{2+} concentration was increased to 2 mM, the time interval was drastically shortened to 47 ± 32 s (n = 8) instead of 9 ± 3.5 min (n = 8, at 1 mM Ca^{2+}) following the early response. It is possible that more neuropeptide may be released at a higher Ca^{2+} concentration in response to the same stimulation, thereby producing a faster response.

Again, argiotoxin and MCPG, glutamate receptor blockers, did not block the evoked enhancement of K^+ current. In addition, focal application of glutamate did not enhance muscle membrane K^+ currents up to 30 min later (data not shown). Therefore, the possibility of glutamate as a transmitter that induces the evoked slow depolarization (see above) and late modulation of K^+ current is largely excluded.

Preincubation of the preparation with 0.4 μM PACAP38 abolished the high frequency–induced modulation of K^+ currents at both 1 mM and 2 mM Ca^{2+} concentrations (Figure 7B). However, preincubation of the preparation with either 0.4 μM PACAP27 or 0.4 μM VIP did not affect high frequency–induced modulation of K^+ currents at 2 mM Ca^{2+} significantly. This evidence argues that an endogenous PACAP38-like peptide may be released from the motor nerve terminals in response to high frequency stimulation.

However, preincubation of the neuromuscular preparations with anti-PACAP38 antiserum (1:100 dilution from the original) did not exert significant effect on the evoked slow depolarization and late enhancement of K^+ currents. It has been shown in other systems that antibodies are

capable of blocking the effect of the application of a neuropeptide but are ineffective in blocking the function of the same neuropeptide released endogenously (van Oers et al., 1992). This is probably due to the brief time available for formation of an antibody–neuropeptide complex during the releasing process. Before the formation of the complex, the released neuropeptide may already have reached the receptors on the postsynaptic membrane (about tens of milliseconds).

Discussion

PACAP-like Neuropeptide in Drosophila

Existence of conserved neuropeptide families between vertebrates and invertebrates has been well documented (Josse, 1987). For instance, vasopressin and related peptides have been found in mammals, Mollusc, and insects (van Kesteren et al., 1992). In Drosophila, receptors homologous to mammalian insulin or tachykinin receptors can be activated specifically by mammalian insulin, substance P, and neuropeptide Y, respectively (Fernandez-Almonacid and Rosen, 1987; Li et al., 1991, 1992). Furthermore, insulin, substance P, and neuropeptide Y-like immunoreactivities revealed by vertebrate antibodies have also been found in Drosophila (Lundquist and Nässel, 1990). Therefore, it appears possible to use the vertebrate neuropeptide for characterizing the cellular function of its Drosophila counterpart.

PACAP is an evolutionarily conserved neuropeptide among vertebrates. The amino acid sequence of PACAP38 is identical among human, rat, and sheep, whereas only 1 amino acid residue out of 38 is replaced in the frog (Arimura, 1992; Yon et al., 1992). Such conservation might well be extended into invertebrates. Evidence presented above supports this idea.

First, PACAP38 can trigger a muscle response that is also evoked by high frequency stimulation of larval motor axons (see Figures 4–7). The requirement of high frequency stimulation to evoke the PACAP-like response is consistent with the idea that the released transmitters may be neuropeptides (Jan and Jan, 1982; Hokfelt, 1991). In addition, PACAP38 can desensitize the evoked PACAP-like response (Figure 6 and Figure 7), raising the possibility that PACAP38 and the released endogenous factors act on the same population of receptors. This physiological effect of PACAP38 appears to be rather specific. None of the neuropeptides proctolin, leucokinin I, and insulin are able to induce the PACAP38-like response (Y. Z., unpublished data), although immunoreactivities of these peptides have been detected at the larval nerve terminals (Anderson et al., 1988; Cantera and Nassel, 1992; Gorczyca et al., 1993). PACAP27 (5 μM) is also unable to induce a PACAP38-like response.

Second, PACAP38-like immunoreactivity is found at the neuromuscular junction (see Figure 2), where the PACAP38-like response can be evoked. This immunoreactivity is seen along axons, in nerve terminal varicosities in the CNS, at neuromuscular junctions, and in the ring glands (data not shown), a tissue releasing various insect hormones (Riddiford, 1993). Such distribution is consistent with the idea that the PACAP38 antiserum may recognize a neuropeptide. This idea is reinforced by Western blot analysis.

Third, Western blots not only identify PACAP38-immunoreactive bands, but also a band that compares well with the PACAP38 band as the positive control (see Figure 3). In addition, it is interesting to note that the higher molecular weight PACAP38-immunoreactive band is in the range of the PACAP precursor protein, having a calculated mass of 19,450 Da (Arimura, 1992; see Figure 3). Taken together, functional, anatomical, and biochemical data corroborate that a PACAP38-like neuropeptide may function at Drosophila larval neuromuscular junctions. Further molecular or biochemical analyses are needed to determine the exact structure of the putative Drosophila PACAP. In fact, M. B. Feany has adduced genetic and molecular evidence that the *amnesiac* gene plausibly codes for a Drosophila pre-pro peptide product with homology to the PACAP neuropeptide (M. B. Feany and W. G. Quinn, personal communication).

Neuropeptide-Mediated Synaptic Transmission in Drosophila

Application of PACAP38 induces two temporally distinct muscle responses. The nature of this response exhibits interesting characteristics, some of which have not been observed previously. The early inward current depolarizes the muscle membrane (see Figure 4 and Figure 6A), which, therefore, is excitatory in nature. In contrast, the late response, a strong enhancement of voltage-activated K^+ current, is inhibitory in nature. Since this voltage-dependent K^+ current is activated at a membrane voltage of −20 mV or higher, the resting membrane potential (−60 to −85 mV) appears not to be affected directly by the enhancement of K^+ current. However, it would be expected that generation of action potential in the muscle membrane may require greater synaptic input or the duration of the action potential may be shorter, owing to the enhanced K^+ currents (Hochner et al., 1986).

Because similar responses can be evoked by a high frequency stimulation of motor axons and the evoked responses can be desensitized by PACAP38, the PACAP38-induced responses may represent a physiological mode of neuropeptide action in Drosophila. The feature of this action is that the neuropeptide-gated slow excitatory synaptic transmission can be clearly separated from the same peptide-gated modulation of K^+ currents (see Figure 6 and Figure 7). The time interval between these two responses can be as long as 15 min, and the duration appears to depend on the amount of the neuropeptide released. In addition, it is worth noting that the enhancement of voltage-activated K^+ currents can be as large as a 100-fold (see Figure 7). This K^+ current may provide a model for studying mechanisms underlying the modulation of K^+ currents.

Neurotransmitters such as acetylcholine and glutamate are capable of inducing synaptic transmission as well as modulation of K^+ currents at the same time (Charpak et al., 1990). Similar responses are also observed in a number of neuropeptides, such as FMRFamide in Aplysia (Fisher et

al., 1993; Belkin and Abrams, 1993) and luteinizing hormone–releasing hormone in frog sympathetic neurons (Jan and Jan, 1982; Bley and Tsien, 1987). However, it appears that a complete separation of the onset of the late response from the early response induced by the same neurotransmitter has not been explicitly examined in the previous studies. This initial analysis of PACAP38-induced and stimulation-evoked muscle responses in Drosophila provides a basis for genetic dissection of neuropeptide-mediated synaptic transmission.

Experimental Procedures

Electrophysiological Recordings

The larval body-wall neuromuscular preparation has been described previously. Intracellular recordings (Jan and Jan, 1976a; Wu et al., 1978; Zhong and Wu, 1991a) and voltage-clamp analysis (Singh and Wu, 1989; Haugland and Wu, 1990; Zhong and Wu, 1991b, 1993) are similar to those described previously. In brief, third instar larvae from wild-type strain Canton S (raised at room temperature) were dissected in Ca^{2+}-free saline that contained 70 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 115 mM sucrose, 20 mM $MgCl_2$, 5 mM trehalose, and 5 mM HEPES (buffered at pH 7.1). For recording, 2 mM $CaCl_2$ was added to the saline in which large postsynaptic responses were evoked. However, only 1 mM Ca^{2+} was added in some of the voltage-clamp recordings for obtaining the current–voltage curve, which requires repeated membrane depolarization. The lower Ca^{2+} concentration allows better voltage clamping because of reduced muscle contraction. The saline used in this work differs from that used previously. The work done by Stewart et al. (unpublished data) has indicated that the new saline improves stability of the larval neuromuscular preparation. Electrical stimulation of motor nerve axons was applied by a suction electrode. For intracellular recordings, data were taken by either camera directly from an oscilloscope or a chart recorder. For voltage-clamp experiments, data collection and analysis were performed using an Axoclamp 2A and a PC computer with pCLAMP software (version 5.05, Axons). Currents were filtered at 2 kHz and digitized at 4 kHz. Leakage currents were not subtracted in data presented to visualize the effects on both leakage currents and voltage-activated K^+ currents simultaneously. All data were obtained from muscle fibers 6 or 12 at room temperature (19°C–22°C).

Application of Peptides, Glutamate, and Toxin

Neuropeptides were applied by pressure ejection from a microelectrode (1–5 μm tip diameter) placed close to muscle membrane controlled by a syringe. The approximate region where the neuromuscular junctions reside on muscle fibers 6 and 12 are known from previous studies (Johansen et al., 1989; Budnik et al., 1990; Zhong et al., 1993). The amount of neuropeptide ejected for each test was so small that washing out was not required to see recovery of the peptide effect. PACAP38 and PACAP27 (Peninsula), VIP, and glucagon (Sigma) were dissolved in the bath saline (see above) at concentrations ranging from 0.1 to 40 μM. Na-glutamate (0.5 M; pH 8.0) was applied by iontophoresis (see Jan and Jan, 1976b; Johansen et al., 1989). Argiotoxin (1 μM in the saline; Accurate Chemical) and MCPG (RBI) were delivered by bath application.

Immunohistochemistry

Dissected larvae (Budnik et al., 1990; Zhong et al., 1992) were fixed in nonalcoholic Bouin's solution (25 ml formalin, 5 ml glacial acetic acid, 75 ml saturated picric acid) for 1–2 hr. Then, the CNS and body-wall musculature were separated. The samples were incubated overnight at 4°C with anti-PACAP38 antiserum (RIN 8920, Peninsula) in 0.1 M phosphate buffer (pH 7.2), 0.2% Triton X-100 (PBT) at 1:200 for the neuromuscular preparation and at 1:400 for the CNS. After washing out the first antibody with PBT (3 times, 30 min each), the musculature was incubated with 1:20 HRP-conjugated goat anti-rabbit IgG and the CNS with 1:400 fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel). Staining of the neuromuscular preparation was revealed by the HRP-catalyzed diaminobenzidine reaction, and staining of the CNS was observed by fluorescent microscopy. Another PACAP38 antiserum (IHC 8920, Peninsula) only weakly stained the Drosophila tissues. Antiserum against PACAP38 does not cross-react with VIP or even PACAP27. The same procedure was applied for anti-PACAP27 antisera, (RIN 8922 and IHC 8922, Peninsula), but even when the concentration was raised to 10 times higher (1:40 dilution), the specific staining was not observed. For preabsorption experiments, anti-PACAP38 antiserum was preabsorbed in 4 μM PACAP38 and 4 μM PACAP27, respectively, in PBT at 4°C overnight and centrifuged at 16,000 × g. Then, the supernatant was incubated with the tissues.

PACAP Extraction and Western Blot

PACAP-like peptides were extracted from whole Drosophila larvae by two methods, essentially as described by Tatsuno et al. (1994), and by a standard nonionic detergent cell lysis. Whole larvae (0.4–0.6 g) were homogenized in an A-type Dounce homogenizer with 1.5 ml of 0.5 M acetic acid on ice. The homogenate was transferred to a microfuge tube and subsequently boiled for 15 min, quenched on ice, then centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was injected onto a C18 Sep-Pak cartridge (Waters Associates), washed with water, and eluted stepwise with 1.5 ml each of 5%, 40%, and 80% CH_3CN/0.1% TFA. The eluates were frozen on a liquid N_2 bath and lyophilized by a Speed-Vac Concentrator (Savant) until the volumes reached 50–100 ml. Alternatively, larvae were homogenized as above, but with a high salt, nonionic detergent lysis buffer instead. The lysis buffer (Harlow and Lane, 1988) was prepared with 50 mM Tris (pH 7.4), 500 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40, 100 mg/ml Pefabloc–SC, 10 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor, 10 mg/ml aprotinin, and 10 mg/ml TPCK (protease inhibitors from Boehringer-Mannheim). Aliquots of the homogenates and fractions were assayed for protein content using the BCA Assay (Pierce Chemical). To resolve low molecular weight proteins and peptides, samples were subjected to polyacrylamide gel electrophoresis using the Tris–Tricine gel and buffer system of Shäggar and Von Jagow (1987), but with reduced cross-linker (3%) for the separating gel. Purified synthetic PACAP27 and PACAP38 were loaded onto separate lanes as negative and positive controls, respectively.

For Western blotting, wet transfer to polyvinylidene difluoride Immobilon–P membranes (Millipore) was done overnight at 60 V with Towbin Buffer (25 mM Tris, 192 mM glycine, 10% methanol) in a Hoefer Transphor tank (Hoefer Scientific Instruments) cooled with a Lauda refrigerated, circulating water bath (Brinkman Instruments). Blots were stained with Ponceau–S to confirm transfer of the smallest of broad-range molecular weight standards (New England Biolabs), blocked by incubating 1 hr in 5% nonfat dry milk, and rinsed 4 times with 20 mM Tris (pH 7.6), 137 mM NaCl, 2.6 mM KCl, and 0.1% Tween–20 between all steps. Blots were incubated 1 hr with anti–PACAP38 antiserum (RIN 8920, Peninsula) at a 1:20,000 dilution or with IgG purified fraction anti–PACAP38–IgG (IgG 8920, Peninsula) at a 1:7,500 dilution. Primary antibody was detected by chemiluminescence with the ECL Kit (Amersham) according to the directions of the manufacturer.

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References

Anderson, M. S., Halpern, M. E., and Keshishian, H. (1988). Identification of the neuropeptide transmitter proctolin in Drosophila larvae: characterization of fiber-specific neuromuscular endings. J. Neurosci. *8*, 242–255.

Arimura, A. (1992). Pituitary adenylate cyclase activating polypeptide (PACAP): discovery and current status of research. Regul. Pept. *37*, 287–303.

Atwood, H. L., Govind, C. K., and Wu, C.-F. (1993). Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in Drosophila larvae. J. Neurobiol. *24*, 1008–1024.

Bashir, Z. I., Bortolotto, Z. A., Davies, C. H., Berretta, N., Irving, A. J., Seal, A. J., Henley, J. M., Jane, D. E., Watkins, J. C., and Collingridge, G. L. (1993). Introduction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. Nature *363*, 347–350.

Belkin, J. K., and Abrams, W. T. (1993). FMRFamide produces biphasic modulation of the LFS motor neurons in the neural circuit of the siphon withdrawal reflex of Aplysia by activating Na^+ and K^+ currents. J. Neurosci. *13*, 5139–5152.

Bley, K. R., and Tsien, R. W. (1987). Inhibition of Ca^{2+} and K^+ channels in sympathetic neurons by neuropeptides and other ganglionic transmitters. Neuron *2*, 379–390.

Broadie, K. S., and Betz, M. (1993). Development of the embryonic neuromuscular synapse of Drosophila melanogaster. J. Neurosci. *13*, 144–166.

Budnik, V., Zhong, Y., and Wu, C.-F. (1990). Morphological plasticity of motor axons in Drosophila mutants with altered excitability. J. Neurosci. *10*, 3754–3768.

Cantera, R., and Nassel, D. R. (1992). Segmental peptidergic innervation of abdominal targets in larval and adult dipteran insects revealed with an antiserum against leucokinin I. Cell Tissue Res. *269*, 459–471.

Charpak, S., Gahwiler, B. H., Do, K. Q., and Knopfel, T. (1990). Potassium conductances in hippocampal neurons blocked by excitatory amino-acid transmitters. Nature *347*, 765–767.

Crossley, C. A. (1978). The morphology and development of the Drosophila muscular system. In The Genetics and Biology of Drosophila, Volume 2B, M. Ashburner and T. R. F. Wright, eds. (New York: Academic Press), pp. 449–559.

Culler, M. D., and Paschall, C. S. (1991). Pituitary adenylate cyclase-activating polypeptide (PACAP) potentiates the gonadotropin-releasing activity of luteinizing hormone-releasing hormone. Endocrinology *129*, 2260–2262.

Deutsch, P. J., and Sun, Y. (1992). The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. J. Biol. Chem. *265*, 14008–14015.

Fernandez-Almonacid, R., and Rosen, O. M. (1987). Structure and ligand specificity of the *Drosophila melanogaster* insulin receptor. Mol. Cell. Biol. *7*, 2718–2727.

Fisher, T., Lin, C.-H., and Kaczmarek, L. K. (1993). The peptide FMRFa terminates a discharge in Aplysia bag cell neurons by modulating calcium, potassium, and chloride conductances. J. Neurophysiol. *69*, 2164–2173.

Ganetzky, B., and Wu, C.-F. (1983). Neurogenetic analysis of potassium currents in Drosophila: synergistic effects on neuromuscular transmission in double mutants. J. Neurogenet. *1*, 17–28.

Glue, P., Nutt, D., and Coupland, N. (1993). Stress and psychiatric disorder: reconciling social and biological approaches. In Stress: From Synapse to Syndrome, S. C. Stanford and P. Salmon, eds. (London: Academic Press), pp. 53–75.

Gorczyca, M., Augart, C., and Budnik, V. (1993). Insulin-like receptors and insulin-like peptide are localized around neuromuscular junctions in Drosophila. J. Neurosci. *13*, 3692–3704.

Harlow E., and Lane, D. (1988). Antibodies: A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Press), pp. 447–450.

Hashimoto, H., Ishihara, T., Shigemoto, R., Mori, K., and Nagata, S. (1993). Molecular cloning and tissue distribution of a receptor for pituitary adenylate cyclase-activating polypeptide. Neuron *11*, 333–342.

Haugland, F. N., and Wu, C.-F. (1990). A voltage-clamp analysis of gene dosage effects of the *Shaker* locus on larval muscle potassium currents in Drosophila. J. Neurosci. *10*, 1357–1371.

Hochner, B., Klein, M., Schacher, S., and Kandel, E. (1986). Action-potential duration and the modulation of transmitter release from the sensory neurons of Aplysia in presynaptic facilitation and behavioral sensitization. Proc. Natl. Acad. Sci. USA *83*, 8410–8414.

Hokfelt, T. (1991). Neuropeptides in perspective: the last ten years. Neuron *7*, 867–879.

Jackson, H., and Usherwood, P. (1988). Spider toxins as tools for dissecting elements of excitatory amino acid transmission. Trends Neurosci. *11*, 278–283.

Jan, L. Y., and Jan, Y. N. (1976a). Properties of the larval neuromuscular junction in Drosophila melanogaster. J. Physiol. *262*, 189–214.

Jan, L. Y., and Jan, Y. N. (1976b). L-glutamate as an excitatory transmitter at the Drosophila larval neuromuscular junction. J. Physiol. *262*, 215–236.

Jan, Y. N., and Jan, L. Y. (1978). Genetic dissection of short-term and long-term facilitation at the Drosophila neuromuscular junction. Proc. Natl. Acad. Sci. USA *75*, 515–519.

Jan, L. Y., and Jan, Y. N. (1982). Peptidergic transmission in sympathetic ganglia of the frog. J. Physiol. *327*, 219–246.

Jia, X.-X., Gorczyca, M., and Budnik, V. (1993). Ultrastructure of neuromuscular junctions in Drosophila: comparison of wild type and mutants with increasing excitability. J. Neurobiol. *24*, 1025–1044.

Johansen, J., Halpern, M. E., Johansen, K. M., and Keshishian, H. (1989). Stereotypic morphology of glutamatergic synapses on identified muscle cells of Drosophila larvae. J. Neurosci. *9*, 710–725.

Josse, J. (1987). Evolution aspects of neuropeptides. Prog. Brain Res. *72*, 35–45.

Kovacs, G. L., and De Wied, D. (1994). Peptidergic modulation of learning and memory processes. Pharmacol. Rev. *46*, 269–291.

Li, X.-J., Wolfgang, W., Wu, Y.-N., North, R. A., and Forte, M. (1991). Cloning, heterologous expression and developmental regulation of a Drosophila receptor for tachykinin-like peptides. EMBO J. *10*, 3221–3229.

Li, X.-J., Wu, Y.-N., North, R. A., and Forte, M. (1992). Cloning, functional expression, and developmental regulation of a neuropeptide Y receptor from Drosophila melanogaster. J. Biol. Chem. *267*, 9–12.

Lundquist, T., and Nässel, D. R. (1990). Substance P-, FMRFamide-, and gastrin/cholecystokinin-like immunoreactive neurons in the thoraco-abdominal ganglia of the flies Drosophila and Calliphora. J. Comp. Neurol. *294*, 161–178.

Masuo, Y., Matsumota, Y., Tokito, F., Tsuda, M., and Fugino, M. (1993). Effects of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) on the spontaneous release of acetylcholine from the rat hippocampus by brain microdialysis. Brain Res. *611*, 207–215.

Miyata, A., Arimura, A., Dahl, R. R., Minamino, N., Uehara, A., Jiang, L., Culler, M. D., and Coy, D. H. (1989). Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem. Biophys. Res. Commun. *164*, 567–574.

Ogi, K., Kimura, C., Onda, H., Arimura, A., and Fujino, M. (1990). Molecular cloning and characterization of cDNA for the precursor of rat pituitary adenylate cyclase activating polypeptide (PACAP). Biochem. Biophys. Res. Comm. *173*, 1271–1279.

Riddiford, L. (1993). Hormones and Drosophila development. In The Development of Drosophila melanogaster, M. Bate and A. Martinez Arias, eds. (Plainview, New York: CSHL Press), pp. 899–940.

Schadlow, V. C., Barzilai, N., and Deutsch, P. J. (1992). Regulation of gene expression in PC12 cells via an activator of dual second messengers: pituitary adenylate cyclase activating polypeptide. Mol. Cell Biol. *3*, 941–951.

Shäggar, H., and von Jagow, G. (1987). Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. *166*, 368–379.

Singh, S., and Wu, C. F. (1989). Complete separation of four K^+ currents in Drosophila. Neuron *2*, 1325–1329.

Smelik, P. G. (1987). Adaptation and brain function. Prog. Brain Res. *72*, 3–9.

Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H., and Journot, L. (1993). Differential signal transduction by five splice variants of the PACAP receptor. Nature *365*, 170–175.

Tatsuno, I., Somogyvari-Vigh, A., and Arimura, A. (1994). Developmental changes of pituitary adenylate cyclase activating polypeptide (PACAP) and its receptor in the rat brain. Peptides *15*, 55–60.

Terenius, L. (1992). Opioid peptides, pain and stress. Prog. Brain Res. *92*, 375–384.

van Kesteren, E. R., Smit, A. B., de With, N. D., van Minnen, J., Dirks, R. W., van der Schors, R. C., and Josse, J. (1992). A vasopressin-related peptide in the mollusc Lymnaea stagnalis: peptide structure, prohormone organization, evolutionary and functional aspects of Lymnaea conopressin. Prog. Brain Res. *92*, 47–57.

van Oers, J. W. A. M., van Bree, C., White, A., and Tilders, F. J. H. (1992). Antibodies to neuropeptides as alternatives for peptide receptor antagonists in studies on the physiological actions of neuropeptides. Prog. Brain Res. *92*, 225–234.

Wu, C.-F., Ganetzky, B., Jan, L. Y., Jan, Y.-N., and Benzer, S. (1978). A Drosophila mutant with a temperature-sensitive block in nerve conduction. Proc. Natl. Acad. Sci. USA *75*, 4047–4051.

Yon, L., Feuiloley, M., Chartrel, N., Arimura, A., Conlon, J. M., Fournier, A., and Vaudry, H. (1992). Immunohistochemical distribution and biological activity of pituitary adenylate cyclase-activating polipeptide (PACAP) in the central nerve system of the frog rana ridibunda. J. Comp. Neurol. *324*, 485–499.

Zhong, Y., and Wu, C.-F. (1991a). Altered synaptic plasticity in Drosophila memory mutant with altered cAMP cascade. Science *251*, 198–201.

Zhong, Y., and Wu, C.-F. (1991b). Alteration of four identified K^+ currents in Drosophila muscle by mutations in *eag*. Science *252*, 1562–1564.

Zhong, Y., and Wu, C.-F. (1993). Modulation of K^+ currents in Drosophila: a hypothetical role for the Eag subunit in multimeric K^+ channels. J. Neurosci. *13*, 4669–4679.

Zhong, Y., Budnik, V., and Wu, C.-F. (1992). Synaptic plasticity in Drosophila memory and hyperexcitability mutants: role of cAMP cascade. J. Neurosci. *12*, 644–651.