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Identification of a Peptide Specific for *Aplysia* Sensory Neurons by PCR-Based Differential Screening

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In order to identify genes specific for the sensory neurons of *Aplysia*, a miniaturized differential screening method based on the polymerase chain reaction and applicable to small amounts of tissue was used. One messenger RNA was isolated that is expressed in every mechanoreceptor sensory cluster of the *Aplysia* central nervous system. This messenger RNA encodes a peptide that seems to function as an inhibitory cotransmitter. The peptide selectively inhibits certain postsynaptic cells but not others and thereby allows the sensory neurons to achieve target-specific synaptic actions.

EACH OF THE TWO SYMMETRICAL pleural ganglia of *Aplysia californica* contains a homogeneous cluster of about 200 medium-sized neurons, which are primary mechanoreceptors and which have receptive fields that include the tail, the foot, the parapodium, and the body walls of the animal (*1*). These cells make up the afferent pathway of the monosynaptic component of the tail withdrawal reflex, which, like the gill and siphon withdrawal reflex (*2*), shows both short-term and long-term behavioral plasticity (*3*). The two homogeneous clusters have been useful for the biochemical study of presynaptic changes underlying short-term and long-term facilitation of the sensory to motor cell synapse (*4*). To explore further the characteristics of this synapse, we have sought to identify molecules specific to the sensory neurons. As a first step, we looked for transcripts differentially expressed between the pleural sensory cells and another identified neuron R2, the excitatory motor neuron to the mucous cells in the foot (*5*). Toward this end we devised a method for differential screening of cDNA libraries which matches the best documented sensitivity of conventional methods, yet requires only a small amount of tissue such as single neurons or clusters of cells.

We amplified whole populations of cDNA in vitro by using the polymerase chain reaction (PCR) methodology (*6*). The first strand of cDNA was synthesized from total RNA by priming with an oligo dT–containing primer-adapter (T primer). After removal of the T primer, a tail of dC was added and the second strand synthesis was primed with an oligo dG–containing primer-adapter (G primer). This second strand, now flanked with two distinct and known sequences, was amplified by PCR with the T and G primers (*7*). A whole population of heterogeneous sequences can thus be coamplified. This in vitro amplified cDNA is inserted into a vector to generate a library (*8*). In addition, the in vitro amplified cDNA can also be labeled and used as a total cDNA probe (*9*). Thus, we could overcome the limitation of starting material for both library and probe.

We used this technique to isolate genes differentially expressed between pleural sensory neuron clusters (positive) and the motor neuron R2 (negative). In the first round of differential screening, we selected nine clones (SCR2-A to SCR2-I) that hybridized with the sensory neuron cluster probe but not with the R2 probe. Cross-hybridization revealed that they were all copies from a single mRNA species, hereafter designated PSC1 (pleural sensory cluster 1). We next performed a Northern blot on RNA derived from two pleural sensory clusters (*10*) and obtained a band of 0.7 kb using SCR2-I as a probe. No detectable signal was apparent on an equivalent amount of RNA derived from R2, thus verifying the differential expression of PSC1 (Fig. 1). When we used SCR2-I as a probe on two other independently generated libraries, we assessed the frequency of this clone at about 1 to 3% of the library. Moreover, this method allowed us to detect much rarer messages at an abundance of 0.03%, which is 100-fold below that of PSC1.

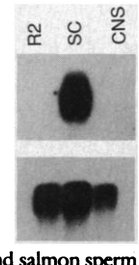

Fig. 1. Differential expression of PSC-1 in sensory cells and the motoneuron R2. (**Upper panel**) The insert from clone SCR2-I was used as a probe on 1.5 μg each of total RNA from R2 (R2), sensory clusters (SC), and the central nervous system (CNS). The hybridization was in 5× standard saline citrate (SSC), 50% formamide 5× Denhardt solution, 0.1% SDS, and salmon sperm DNA (100 μg/ml). The blot was washed for 20 min each in 2× SSC plus 0.1% SDS and 0.2× SSC plus 0.1% SDS. It was exposed for 12 hours at −70°C with an intensifying screen. (**Lower panel**) Same blot as in the upper panel rehybridized with cDNA encoding F4, a ubiquitous polyadenylated ribosomal RNA. Exposed for 3 hours.

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To assess the overall distribution of PSC1 throughout the central nervous system of *Aplysia*, we synthesized a ^{35}S-labeled RNA antisense probe from the SCR2-37 plasmid (another PSC1 clone) and used it for in situ hybridization (*11*) on serial sections of each ganglion of the *Aplysia* central nervous system. The antisense probe labeled not only the pleural sensory cluster but also nine

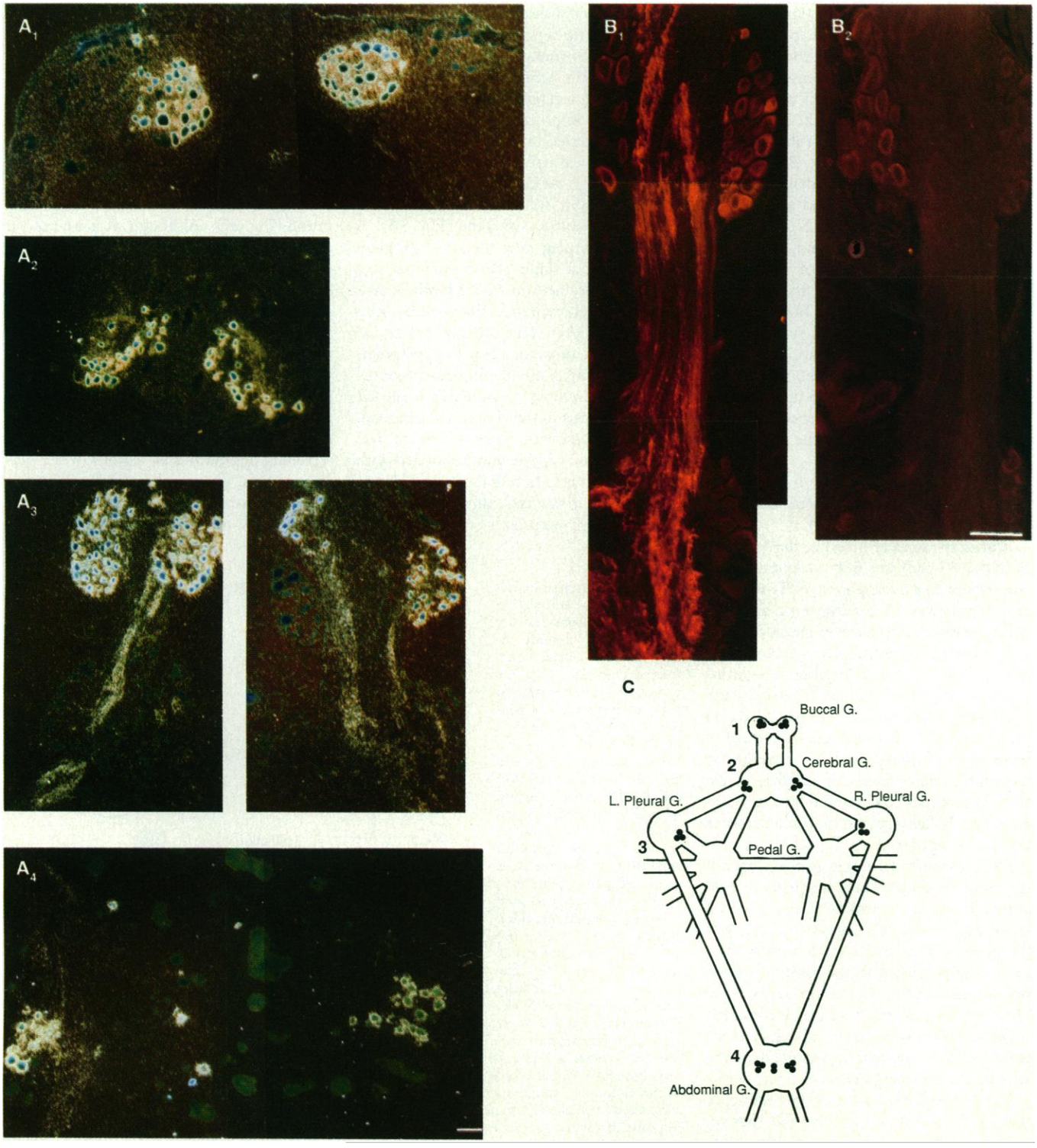

Fig. 2. (**A**) Distribution of PSC1 transcript in the central nervous system of *Aplysia*. An antisense riboprobe was synthesized from clone SCR2-37 in the presence of [^{35}S]UTP and hybridized to 10-μm-thick sections of *Aplysia* central nervous system ganglia. (A_1) Buccal ganglia. (A_2) Cerebral ganglion. (A_3) Left and right pleural and pedal ganglion. (A_4) Abdominal ganglion; the right part of the ganglion is on the left. The clustering of the putative RF cells is revealed by serial sections of the ganglion (not shown). (**B**) Distribution of PSC1 immunoreactivity by immunofluorescence in two adjacent sections of the pleural and pedal ganglion, which show pleural sensory cluster cells and bundles of their processes leading to the pedal ganglion. (B_1) Rabbit antiserum to synthetic peptide P1 at 1000-fold dilution. (B_2) Preimmune serum at 1000-fold dilution.

clusters of cells (Fig. 2A). These cells corresponded in size and location to all the mechanosensitive sensory neuron clusters previously identified physiologically in the *Aplysia* central nervous system: the two buccal clusters receiving information from the feeding organs (*12*), the two cerebral clusters (J and K) innervating the skin of the head (*13*); the two pleural sensory clusters innervating the tail, body wall, and parapodia (*1*); and the abdominal RE, LE, and RF clusters innervating the mantle shelf, siphon, and gill (*14*). Rostral to the RE and RF clusters, a few extra cells of identical size to the clustered ones were found to hybridize with an equal intensity (*14*). No other cells gave a signal above background, nor did the sense probe. The identity of the pleural sensory cells was checked by first dissecting the easily recognizable and homogeneous cluster from a ganglion and submitting it separately to in situ hybridization: each cell hybridized strongly, whereas none did in the rest of the pleural-pedal complex. Thus, PSC1 mRNA seems to be specific to the mechanosensory neurons of the central nervous system.

The transcripts were not restricted to the cell body but were distributed along the length of the axon (Fig. $2A_3$).

Within the sensory neurons, the sequence of SCR2-37 (*15*) was 588 nucleotides long and contained a hydrophobic NH_2-terminal region that was 32 residues long and that could correspond to a leader peptide. There was a potential N-linked glycosylation site at position 31. There were several arginine residues (Arg^{41}-Arg^{42}, Arg^{45}, Arg^{47}, and Arg^{49}) and a Lys^{56}-Arg^{57} pair preceded by a glycine. These features, often found in propeptides, usually lead to cleavage by trypsin-like endopeptidases, removal of the COOH-terminal basic residue by a carboxypeptidase B–like activity, and amidation at the glycine residue.

Using the inferred amino acid sequence of the open reading frame, we generated an antibody against a peptide (P1) extending from Phe^{43} to Phe^{54}-amide. There was immunoreactivity to P1 in all sensory clusters, in agreement with the result of in situ hybridization (*16*). Immunoreactivity was observed not only in the cell bodies and the processes, but also at the varicosities characteristic of the presynaptic terminals of the sensory neurons (Fig. 2B).

To identify the structure of the product of the PSC1 mRNA, we purified the endogenous peptide by immunoprecipitation with the antiserum to P1 and then by reversed-phase high-pressure liquid chromatography (HPLC) (*17*). The single major peak yielded a mixture of two peptide sequences with a common COOH-terminal, compatible with an equimolar mixture of peptide A (A^{46}RYRVGYMF^{54}-NH_2) and peptide B (T^{33}RSKNNVPRRFPRARYRVGYMF^{54}-NH_2) (*18*). It is likely that the canonic sequence Gly^{55}-Lys^{56}-Arg^{57} serves as a COOH-terminal cleavage site for both peptides. Synthetic versions of these two amidated peptides comigrated with the endogenous ones. We verified that peptide A was not a degradation product generated during the purification process (*19*).

We next tested whether peptide A had pharmacological activity in the pedal ganglia (*20*). Although the peptide did not produce effects on most follower cells, it reliably produced inhibition on some (Fig. 3A). A large follower neuron in the pedal ganglion responded to a single action potential in a pleural sensory neuron with a biphasic synaptic potential, excitation followed by inhibition (Fig. $3A_2$). This cell also responded to application of peptide A with hyperpolarization (Fig. $3A_1$). Similar biphasic responses were seen in some follower cells to the LE sensory neurons in the abdominal ganglion. In four experiments, such cells were also tested with the peptide and responded with hyperpolarization. In cells that responded to the peptide, a synaptic hyperpolarization was often only seen after stimulation with trains of action potentials and not with single spikes (Fig. 3B). By contrast, the identified motor neuron L7 and a number of unidentified followers only had excitatory synaptic responses. These cells failed to respond to the peptide.

Thus, this peptide, which we call sensorin-A, produces actions that are consistent with it being an inhibitory co-transmitter (*21*) that acts in conjunction with the fast excitatory transmitter released by sensory neurons. Whereas the fast excitatory transmitter affects all follower cells, the peptide inhibits only some, and it is in those follower cells that sensory neurons produce inhibition (Fig. 3C). Although we have not yet established that sensorin-A is released from the sensory neuron, our data suggest that the sensory neuron can have different effects on different follower neurons by means of sensorin-A release, presumably because only certain follower cells have an adequate density of receptors for the inhibitory peptide (Fig. 3C).

The technique we used to clone PSC1 extends the applicability of differential screening approaches to regions of the nervous system, where only a small amount of starting material is available. This method allowed us to clone a fairly abundant

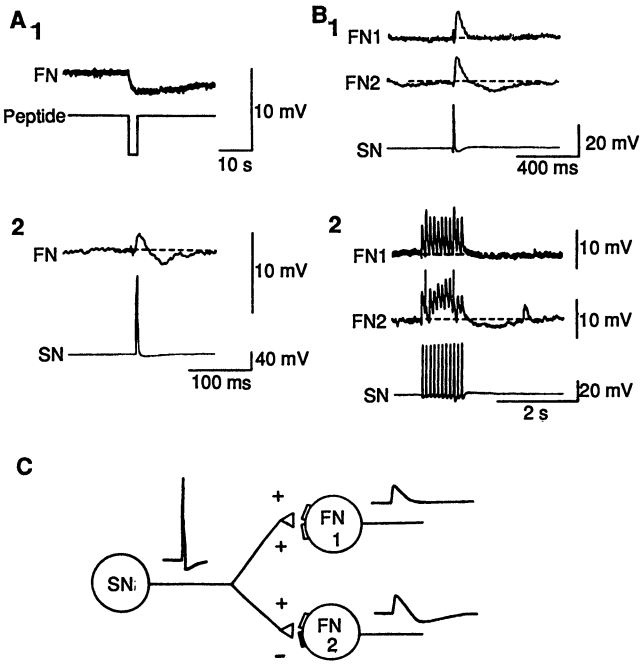

Fig. 3. Electrophysiological responses to the stimulation of sensory neurons and the application of peptide A. Experiments were performed as in (*20*). (A_1) Response of a follower neuron (FN) to application of peptide A. Voltage recording in NSW plus 60 mM Ca^{2+} from a pleural sensory cell and an identified follower cell in the pedal ganglion located on the pedal pleural commissure. The follower neuron hyperpolarizes after application of peptide A (3 μl of 100 μM peptide A in puffer pipette, applied with a 3-s pressure puff (peptide). Similar results were observed in three experiments. (A_2) Response of the same cell as in (A_1) to sensory neuron stimulation in NSW. The cell responds to a single spike in pleural sensory neuron (SN) with a biphasic response (FN). Application of peptide to this cell also caused it to hyperpolarize. (**B**) Response of cells in the abdominal ganglion to stimulation of LE sensory neurons. (B_1) An LE sensory neuron was stimulated intracellularly to produce one spike (SN) (the spike amplitude was clipped by the frequency response of the chart recorder). One follower cell, the ink motor cell L14 (FN1), responds with a pure excitatory potential, while a second follower cell (unidentified) (FN2) responds with a biphasic excitatory-inhibitory response. (2) The same sensory neuron as in (B_1) stimulated to fire a train of action potentials (10 Hz for 1 s). L14 displays a small slow inhibitory response (FN1), whereas the other follower cell displays a larger hyperpolarization (FN2). (**C**) Diagram of one possible mechanism of follower cell response specificity. Peptide A may be released as a cotransmitter from all branches of stimulated sensory neurons (SN). The effectiveness of the peptide in producing synaptic inhibition is regulated by the density of receptors to the peptide on specific follower neurons.

mRNA, PSC1, that is a specific marker of mechanoreceptors in *Aplysia*. This message codes for a peptide, sensorin-A, that seems to function as an inhibitory co-transmitter on a subpopulation of follower cells. In addition to its physiological role, this specific and abundant RNA and its peptide should also prove useful for tracing the origins and developmental fate of various classes of sensory neurons

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7. First strand cDNA synthesis was carried out from about 1 μg of total RNA (*10*) with a T primer (GGCGAATTCTGCAGTTTTTTTTTTTTTTT). The first strand was isolated by ion exchange chromatography on tips 5 (Qiagen) by the manufacturer's protocol. The RNA was then hydrolyzed by alkali treatment. After neutralization and phenol-chloroform extraction, the cDNA was precipitated with 0.8 volumes of isopropanol. We added a polyC tail in 5 μM dCTP for 30 min at 14°C, after which the sample was extracted with phenol-chloroform and precipitated. Amplification was carried out with the T primer and a G primer (GGCGACGTGGATCCGGGGGGGGGGGGGGG). The first cycle was a 2-min plateau at 95°C, a 2-min slope to 45°C, a 2-min plateau at 45°C, a stepwise shift to 72°C, and a 4-min plateau at 72°C. The 20 to 25 subsequent cycles were as follows: a 2-min plateau at 95°C, a 1-min slope to 50°C, a 1-min plateau at 50°C, a stepwise shift to 72°C, and a 4-min plateau at 72°C. The extent of amplification was monitored by trichloroacetic acid precipitation of 6-μl aliquots, and the reaction was stopped when we obtained about 200 to 500 ng of DNA.
8. A. Belyavsky, T. Vinogradova, K. Rajedwsky, *Nucleic Acids Res.* **17**, 2919 (1989). The amplified cDNA was digested with Bam H1 and Pst 1, separated on a 2% low melting point agarose, and the entire smear was excised, extracted with phenol, and precipitated. Approximately 1 ng of insert was ligated to pKSM13^{+} digested with Pst 1–Bam HI and used to transform B72 strain of *Escherichia coli* by electroporation, yielding 10^6 colonies.
9. To label the amplified cDNA, we submitted 40 ng of amplified cDNA to an additional cycle of amplification. Unlabeled dCTP was replaced by 200 μCi[^{32}P] dCTP. The cycle was a 2-min plateau at 95°C, a 1-min slope to 50°C, a 1-min plateau at 50°C, a stepwise increase to 72°C, and a 2-hour plateau at 72°C. For differential screening of the cDNA library, we transferred samples of the library (2000 to 5000 colonies per 13.5-cm^2 plate) to nitrocellulose. Replicas of each filter were then hybridized with labeled cDNA (10^6 dpm/ml) for 24 hours [T. Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)]. We determined that the relative abundances of individual mRNA species were conserved by the amplification process by using both an amplified cDNA population and a nonamplified cDNA population as probes on a cDNA library. This operational assessment of the techniques does not rule out that the representation of clones rarer than the threshold of detection (around 0.04%) is affected by amplification. We tested the reproducibility of the process by comparing the patterns of hybridization of two independently amplified cDNA probes on a library. The patterns were identical (not shown). To reproduce the hybridization patterns reliably, we found it essential not to size select the cDNA after or in the course of amplification. As a consequence the inserts are very short (150 to 800 bp, with a mean of about 250 bp).
10. *Aplysia* (75 to 125 g) were obtained from the Howard Hughes Facility in Woods Hole and anesthetized before the central nervous system was removed, and the sensory clusters (SC) from the pleural ganglia and the giant motoneurons R2 from the abdominal ganglia were dissected. After incubation in culture medium for 1 hour, the cells were extracted by vortexing in a 1:1 (v/v) heated (65°C) mixture of ACE/SDS (10 mM NaOAc, pH 5.1, 50 mM NaCl, 3 mM EDTA, 0.5% SDS) and ACE-saturated phenol with 20 μg of glycogen. The aqueous phase was re-extracted twice at room temperature by 1:1 (v/v) phenol-chloroform, once with chloroform, and precipitated with ethanol. One sensory cluster yielded 200 ng of total RNA.
11. In situ hybridizations were performed according to R. T. Fremeau and co-workers [*Science* **234**, 1265 (1986)] as modified by S. Beushausen and coworkers [*Neuron* **1**, 853 (1988)].
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15. Cloned plasmids were purified with CsCl density gradient centrifugation and sequenced by the dideoxy chain termination method, with Sequenase DNA polymerase (USB). The sequence is deposited in the EMBL database, accession number X56770.
16. Immunocytochemical staining was performed as described [H. B. Kistler *et al.*, *J. Neurosci.* **5**, 72 (1985)] except that tissue was fixed at room temperature. The 16-μm cryostat sections were incubated with a 1000-fold dilution of rabbit antiserum to peptide P1 and a 40-fold dilution of rhodamine-conjugated goat antibody to rabbit immunoglobulin G (Cappell).
17. Dissected pleural sensory clusters and R2 cells were labeled with [^{35}S]methionine and extracted in 0.1 M acetic acid. The extracts were applied to an analytical HPLC column (Dynamax-300Å) and eluted with a linear gradient from 4% acetonitrile plus 0.1% TFA to 32% acetonitrile plus 0.1% TFA in 28 min at 1 ml/min. Four major peaks of radioactivity were obtained. Only the peak migrating at 25% acetonitrile was missing in the R2 extract and could be precipitated with antiserum to peptide P1. To purify this peptide, the central nervous system from 90 *Aplysia* was extracted in 0.1 M acetic acid [E. C. Cropper, R. Tenenbaum, M. A. Gawinowicz-Kolds, I. Kupfermann, K. R. Weiss, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5483 (1987)]. Labeled peptide obtained from the HPLC peak as above was added as a tracer to the supernatant. The supernatant was applied to C18 cartridges (Sep-Paks, Waters Associates), washed with 10% acetonitrile plus 0.1% TFA and eluted with 40% acetonitrile plus 0.1% TFA. One-third of the resulting material was buffered with 50 mM Hepes (pH 7.6) and immunoprecipitated with antiserum to P1. This material was separated by HPLC as above. A single major peak eluted at 25% acetonitrile. This peak was sequenced with gas-phase sequencer 470A (Applied Biosystems).
18. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
19. Two pleural sensory clusters were labeled with [^{35}S]methionine overnight and extracted in 0.1 M acetic acid after addition of synthetic peptides A and B. The supernatant was applied to a Suplex pkb 100 column (Supelco) and eluted with a linear gradient from 5% acetonitrile plus 0.01 M HFBA to 45% acetonitrile plus 0.01 M HFBA in 40 min at 1 ml/min. Peptide A eluted at 37% acetonitrile and peptide B at 40% acetonitrile. Peaks of radioactivity comigrated with each of these synthetic peptides. Each peak was collected and applied to a Dynamax A300 column as in (*17*). In each case, radioactivity comigrated with the synthetic peptide, eluting at 25% acetonitrile.
20. Abdominal ganglion and pleuro-pedal ganglia from *Aplysia* (50 to 100 g) were perfused at 2 ml/min in 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM tris, pH 7.6 (NSW). Experiments were performed at 20° to 23°C. For electrophysiological recording, sensory cells were identified by position, pigmentation, size, spike discharge upon impalement, and ability to produce synaptic potentials in follower cells. After identifying follower cells of sensory neurons, synaptic potentials elicited by single spikes (0.1 to 0.03 Hz) and by trains of spikes in sensory neurons (10 Hz for 1 to 2 s) were examined. Peptide A was then applied to sensory neuron follower cells from micropipets (10-μm tip diameter) positioned 100 to 200 μm above the cells of interest. Pressure pulses and times were controlled by a pressure delivery system (WPI, New Haven, CT). Pressure of 1 to 10 psi for 1 to 5 s delivered 1 to 5 μl of peptide-containing solution. The concentration of peptide A in the puffer pipettes was 10 μM to 100 μM in the appropriate seawater carrier. There was no response of cells to NSW. Results are from experiments on 17 abdominal ganglia and 5 pleuro-pedal ganglia.
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